Safety Assessment of Transgenic Organisms

OECD CONSENSUS DOCUMENTS

Volume 4

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Foreword

Genetically engineered crops (also known as transgenic crops) have been approved for commercial release in an increasing number of countries, for planting or for use as commodities. Genetically engineered varieties of over a dozen different plant species have received regulatory approval in several OECD and non-OECD countries from all regions of the world, the large majority of plantings being for soybean, maize, cotton and rapeseed (canola), as outlined in *The Bioeconomy to 2030: Designing a Policy Agenda* (OECD, 2009). During the period from 1996 to 2009, for example, there was an almost eighty-fold increase in the area grown with transgenic crops worldwide, reaching 134 million hectares in 2009, as mentioned in *Global Status of Commercialized Biotech/GM Crops* (James, 2009). Such approvals usually follow a science-based risk/safety assessment.

The environmental safety/risks of transgenic organisms are normally based on the information on the characteristics of the host organism, the introduced traits, the environment into which the organism is introduced, the interaction between these, and the intended application. The OECD's Working Group on Harmonisation of Regulatory Oversight in Biotechnology decided at its first session, in June 1995, to focus its work on identifying parts of this information, which could be commonly used in countries for environmental safety/risk assessment to encourage information sharing and prevent duplication of effort among countries. Biosafety Consensus Documents are one of the major outputs of its work.

Biosafety Consensus Documents are intended to be a "snapshot" of current information on a specific host organism or trait, for use during regulatory assessments. They are not intended to be a comprehensive source of information on everything that is known about a specific host or trait, but they do address the key or core set of issues that member countries believe are relevant to risk/safety assessment. Several non-member economies, as well as other international organisations, are associated with the work and share their expertise. The information collated in the Consensus Documents is said to be mutually acceptable among member countries and also other countries wishing to use them for their assessment process.

To date, 38 Biosafety Consensus Documents have been published. They include documents which address the biology of crops, trees and micro-organisms as well as those which address specific traits which are used in transgenic crops. In addition, documents of broader nature aiming to facilitate harmonisation have been developed: *Designation of a Unique Identifier for Transgenic Plants* (2002, revised in 2006); and *Molecular Characterisation of Plants Derived from Modern Biotechnology* (2010).

Volumes 3 and 4 of this publication contain a compilation of those Biosafety Consensus Documents published between September 2006 and September 2010. These volumes also include two previously published presentation texts (slightly updated since Volumes 1 and 2):

- *An Introduction to the Biosafety Consensus Documents of OECD's Working Group for Harmonisation in Biotechnology* explains the purpose of the documents and how they are relevant to risk/safety assessment. It also describes the process by which the documents are drafted, using a "lead country" approach.

- Then, the *Points to Consider for Consensus Documents on the Biology of Cultivated Plants* offer a structured checklist of points for authors to consider when drafting, or to experts evaluating a Consensus Document. Each point is described for its relevance to risk/safety assessment.

Along with Volumes 1 and 2, the present publication offers ready access to those Consensus Documents which have been published thus far. As such, it should be of value to applicants for commercial uses of transgenic crops, regulators in national authorities as well as the wider scientific community.

As each of the Consensus Documents may be updated in the future as new knowledge becomes available, users of this book are encouraged to provide any information or opinions regarding the contents of the Consensus Documents or indeed, OECD's other harmonisation activities. Comments can be provided at: *biosafety@oecd.org*.

The published Consensus Documents are also available individually from the OECD's Biotrack website, at no cost (www.oecd.org/biotrack).

Acknowledgements

This book is the result of the common effort of the participants in the OECD's Working Group on Harmonisation of Regulatory Oversight in Biotechnology. Each section is composed of a "Consensus Document" which was prepared under the leadership of a participating country or countries, as listed at the end of this volume. During their successive draftings, valuable inputs and suggestions for the documents were provided by a number of delegates and experts in the Working Group, being from OECD Members, non member economies and observer organisations.

Each Consensus Document was issued individually, as soon as finalised and agreed for declassification, by the OECD Environment, Health and Safety Division in the *Series on Harmonisation of Regulatory Oversight in Biotechnology.* Volumes 3 and 4 of this publication, containing the 2006-2010 Consensus Documents, were prepared and edited by Bertrand Dagallier and Carina Arambula, under the supervision of Peter Kearns, at the EHS Division, OECD Environment Directorate.

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Introduction to the biosafety consensus documents

1. About OECD's Working Group for biosafety

The OECD's *Working Group on Harmonisation of Regulatory Oversight in Biotechnology* (the Working Group) comprises delegates from the 33 member countries of OECD and the European Commission. Typically, delegates are from those government ministries and agencies, which have responsibility for the environmental risk/safety assessment of products of modern biotechnology. The Working Group also includes a number of observer delegations and invited experts who participate in its work, such as Argentina; the Russian Federation; the United Nations Environment Programme (UNEP) and; the Secretariat of the Convention on Biological Diversity (SCBD); the Food and Agriculture Organization of the United Nations (FAO), the United Nations Industrial Development Organisation (UNIDO); and the Business and Industry Advisory Committee to the OECD (BIAC). In recent years, with the increasing use of biotech products in many regions of the world together with the development of activities relating to tropical and subtropical species, there has been increased participation of non-member economies including Brazil, Cameroon, China, Estonia, India, the Philippines and South Africa.

2. Regulatory harmonisation

The Working Group was established in 1995[1] at a time when the first commercial transgenic crops were being considered for regulatory approval in a number of OECD member countries. From the beginning, one of the group's primary goals was to promote international regulatory harmonisation in biotechnology among members. Regulatory harmonisation is the attempt to ensure that the information used in risk/safety assessments, as well as the methods used to collect such information, are as similar as possible. It could lead to countries recognising or even accepting information from one anothers' assessments. The benefits of harmonisation are clear. It increases mutual understanding among countries, which avoids duplication, saves on scarce resources and increases the efficiency of the risk/safety assessment process. This in turn improves safety, while reducing unnecessary barriers to trade (OECD, 2000).

3. The need for harmonisation activities at OECD

The establishment of the Working Group and its programme of work followed a detailed analysis by member countries of whether there was a need to continue work on harmonisation in biotechnology at OECD, and if so, what it should entail. This analysis was undertaken by the *Ad Hoc* Group for Environmental Aspects of Biotechnology (established by the Joint Meeting[2]), in 1994 mainly.

The *Ad Hoc* Group took into consideration, and built upon, the earlier work at OECD which began in the mid-1980s. Initially, these OECD activities focused on the environmental and agricultural implications of field trials of transgenic organisms, but this was soon followed by a consideration of their large-scale use and commercialisation. (A summary of this extensive body of work is found in Appendix I.)

4. Key background concepts and principles

The *Ad Hoc* Group took into account previous work on risk analysis that is summarised in *Safety Considerations for Biotechnology: Scale-up of Crop Plants* (OECD, 1993a). The following quote gives the flavour: "Risk/safety analysis is based on the characteristics of the organism, the introduced trait,

1 . The original title of the Working Group was the "Expert Group for the Harmonisation of Regulatory Oversight in Biotechnology". It became an OECD Working Group in 1998.

2 . The Joint Meeting was the supervisory body of the *Ad Hoc* Group and, as a result of its findings, established the Working Group as a subsidiary body. Today, its full title is the Joint Meeting of the Chemicals Committee and the Working Party on Chemical, Pesticides and Biotechnology.

the environment into which the organism is introduced, the interaction between these, and the intended application." This body of work has formed the basis for environmental risk/safety assessment that is now globally accepted. In considering the possibilities for harmonisation, the *Ad Hoc* Group paid attention to these characteristics and the information used by risk/safety assessors to address them.

This was reinforced by the concept of familiarity, also elaborated in the above-mentioned document (OECD, 1993a). This concept "is based on the fact that most genetically engineered organisms are developed from organisms such as crop plants whose biology is well understood". "Familiarity allows the risk assessor to draw on previous knowledge and experience with the introduction of plants and micro-organisms into the environment." For plants, familiarity takes account of a wide-range of attributes including, for example, knowledge and experience with "the crop plant, including its flowering/reproductive characteristics, ecological requirements, and past breeding experiences" (OECD, 1993a – see also Appendix I for a more detailed description). This illustrates the role of information related to the biology of the host organism as a part of an environmental risk/safety assessment.

The *Ad Hoc* Group also considered the document *Traditional Crop Breeding Practices: An Historical Review to Serve as a Baseline for Assessing the Role of Modern Biotechnology* (OECD, 1993b) which focuses on host organisms. It presents information on 17 different crop plants, which are used (or are likely to be used) in modern biotechnology. It includes sections on phytosanitary considerations in the movement of germplasm and on current uses of these crop plants. There is also a detailed section on current breeding practices.

5. A common approach to risk/safety assessment

An important aspect for the *Ad Hoc* Group was to identify the extent to which member countries address the same questions and issues during risk/safety assessment. Big differences would mean difficulties in working towards harmonisation, while a high level of similarity would suggest it more feasible.

This point was resolved by two studies considered by the *Ad Hoc* Group: one covered crop plants (OECD, 1995a; 1995b) while the other concerned micro-organisms (OECD, 1995c; 1996). Both studies involved a survey with national authorities responsible for risk/safety assessment. The aim was to identify the questions they address during the assessment process (as outlined in national laws/regulations/guidancetexts) in order to establish the extent of similarity among national authorities. The studies used the information provided in the OECD's *Blue Book* on Recombinant DNA Safety Considerations (OECD, 1986) as a reference point, in particular, the sections covering: i) General Scientific Considerations; ii) Human Health Considerations; and iii) Environmental and Agricultural Considerations (appendices b, c and d). Both studies showed a remarkably high degree of similarity among countries in the questions/issues addressed in risk/safety assessment.

6. The emergence of the concept of consensus documents

The Working Group was therefore established in the knowledge that national authorities have much in common, in terms of the questions/issues addressed, when undertaking risk/safety assessment. It also took into account those characteristics identified as part of the assessment (*i.e. the organism*, *the introduced trait* and *the environment*) around which harmonisation activities could focus.

It was further recognised that much of the information used in risk/safety assessment relating to the biology of host organisms (crop plants, trees, animals or micro-organisms) would be similar or virtually the same in all assessments involving the same organism. In other words, the questions addressed during risk/safety assessment which relate to the biology of the organism --for example, the potential for gene transfer within the crop plant species, and among related species, as well as the potential for weediness--

remain the same for each application involving the same host species. This also applies to some extent to information related to introduced traits.

Consequently, the Working Group evolved the idea of compiling information common to the risk/safety assessment of a number of transgenic products, and decided to focus on two specific categories: the biology of the host species; and traits used in genetic modifications. The aim was to encourage information sharing and prevent duplication of effort among countries by avoiding the need to address the same common issues in applications involving the same organism or trait. It was recognized that biology and trait consensus documents could be agreed upon relatively quickly by the member countries (within a few years). This compilation process was quickly formalised in the drafting of Consensus Documents.

7. The purpose of consensus documents

The Consensus Documents are not intended to be a substitute for a risk/safety assessment, because they address only a part of the necessary information. Nevertheless, they should make an important contribution to environmental risk/safety assessment.

Consensus Documents are intended to be a "snapshot" of current information, for use during the regulatory assessment of products of biotechnology. They are not intended to be a comprehensive source of information covering the full knowledge about a specific host organism or trait; but they address – on a consensus basis – the key or core set of issues that countries believe to be relevant to risk/safety assessment.

The aim of the documents is to share information on these key components of an environmental safety review in order to prevent duplication of effort among countries. The documents are envisaged to be used: a) by applicants as information to be given in applications to regulatory authorities; b) by regulators as a general guide and reference source in their reviews; and c) by governments for information sharing, research reference and public information.

Originally, it was said that the information in the Consensus Documents is intended to be *mutually recognised* or *mutually acceptable* among OECD Members, though the precise meaning of these terms is still open for discussion. During the period of the *Ad Hoc* Group and the early days of the Working Group (1993-1995), the phrase *Mutual Acceptance of Data* was discussed. This concept, borrowed from OECD's Chemicals Programme, involves OECD Council Decisions that have legally binding implications for member countries. In the case of the Consensus Documents there has never been legally binding commitment to use the information they contain, though the Working Group is interested in enhancing the commitment of countries to make use of the documents. Participation in the development of documents, and the intention by countries to use the information, is done in "good faith." It is expected, therefore, that reference will be made to relevant Consensus Documents during risk/safety assessments. As these documents are publicly-available tools, they can be of interest for any country wishing to use them in national assesments.

8. The process through which consensus documents are initiated and brought to publication

There are a number of steps in the drafting of a specific Consensus Document. The first step occurs when a delegation, in a formal meeting of the Working Group, makes a proposal to draft a document on a new topic, typically a crop species or a trait. If the Working Group agrees to the proposal, a provisional draft is prepared by either a single country or two or more countries working together. ("lead country approach"). Typically, the lead country(ies) has had experience with the concerned crop or trait and is able to draw on experts to prepare a provisional draft.

The provisional draft is first reviewed by the Bureau of the Working Group [3] to ensure that the document addresses the range of issues normally covered by Consensus Documents and is of sufficiently high quality to merit consideration by the Working Group as a whole.

Based on the comments of the Bureau, a first draft is prepared for consideration by the full Working Group. This is the opportunity for each delegation to review the text and provide comments based on their national experiences. Inputs are incorporated in a second draft, which is again circulated to the Working Group. At this point, the Working Group may be asked to recommend that the document be declassified. Such a recommendation is only forthcoming when all delegations have come to a consensus that the document is complete and ready for publication. Sometimes, however, the text may need a third or even more discussions in the Working Group before a declassification could be contemplated.

When the Working Group has agreed to recommend a document for declassification, it is forwarded to the supervisory Committee, the Joint Meeting, which is invited to declassify the document. Following the agreement of the Joint Meeting, the document is then published.

It is important to note that the review of Consensus Documents is not limited to formal meetings of the Working Group. Much discussion also occurs through electronic means, especially *via* the protected website dedicated to the Working Group. This enables a range of experts to have input into drafts.

For a number of documents, it has also been necessary to include information from non-member countries. This wider share of expertise has become increasingly important in recent years with the development of of activities relating to tropical and subtropical species. And this has been particularly true in the case of crop plants where the centre of origin and diversity occurs in a non-member country(ies). In these cases, UNEP, UNIDO and FAO have assisted in the preparation of documents by identifying experts from concerned countries. For example, this occurred with the Consensus Document on the Biology of Rice.

9. Current and future trends in the Working Group

The Working Group continues its work on the preparation of specific Consensus Documents, and on the efficiency of the process by which they are developed. An increasingly large number of crops and other host species (trees, animals, micro-organisms) are being modified, for an increasing number of traits, and the Working Group aims to fulfil the current needs and be prepared for emerging topics.

At the OECD Workshop on Consensus Documents and Future Work in Harmonisation, held in Washington DC in October 2003, the Working Group considered how to set priorities for drafting future Consensus Documents among the large number of possibilities. The Workshop also recognised that published Consensus Documents may be in need of review and updating from time to time, to ensure that they include the most recent information. The Working Group is considering these aspects on a regular basis when planning future work. For the preparation of future documents, the Workshop identified the usefulness of developing a standardised structure of Consensus Documents. The Working Group contemplated to develop, firstly, a "*Points to Consider*" document for the biology documents and then that of the trait documents. The text on biology documents, published in 2006, is reproduced in the following section of this publication.

3 . The Bureau comprises the Chair and vice-Chairs of the Working Group. The Bureau is elected by the Working Group once per year. At the time of preparing this publication - Vol. 3 and 4, the Chair is from the United States, and the vice-Chairs from Canada, Finland, Japan and the Netherlands.

10. The OECD Task Force for the Safety of Novel Foods and Feeds

The *OECD Task Force for the Safety of Novel Foods and Feeds* (Task Force), established in 1999, addresses aspects of the assessment of human food and animal feed derived from genetically engineered crops. As with the Working Group, the main focus of the Task Force work is to ensure that the types of information used in risk/safety assessement, as well as the methods to collect such information, are as similar as possible amongst countries. The approach is to compare transgenic crops and derived products with similar conventional ones that are already known and considered safe because of recognised experience in their use. Harmonised methods and the sharing of information are facilitated through the Task Force activities.

Similarly to the biosafety programme, the main outcome of the foods and feeds programme is the set of Consensus Documents on compositional considerations of new varieties of specific crops. The Task Force documents compile a common base of scientific information on the major components of crop plants, such as key nutrients, toxicants, anti-nutrients and allergens. These documents constitute practical tools for regulators and risk/safety assessors dealing with these new varieties, with respect to foods and feeds. To date, 20 Consensus Documents have been published on major crops and on general considerations for facilitating harmonisation. They constitute the Series on the Safety of Novel Foods and Feeds which is also available on the OECD's website at no cost (www.oecd.org/biotrack).

The publication of the full Foods and Feeds Series in a single document is contemplated for 2011.

The Working Group and the Task Force are implementing closely-related and complementary programmes, focused on environmental aspects for the first one, on food and feed aspects for the second. Their cooperation on issues of common interest resulted recently in the first Consensus Document developed jointly by the two bodies, the *Consensus Document on Molecular Characterisation of Plants Derived from Modern Biotechnology*, published in September 2010. The document is included in Volume 3 of this publication.

Appendix I

OECD biosafety principles and concepts developed prior to the Working Group (1986-1994)

Since the mid-1980s the OECD has been developing harmonised approaches to the risk/safety assessment of products of modern biotechnology. Prior to the establishment of the Working Group, OECD published a number of reports on safety considerations, concepts and principles for risk/safety assessment as well as information on field releases of transgenic crops, and a consideration of traditional crop breeding practices. This Appendix notes some of the highlights of these achievements that were background considerations in the establishment of the Working Group and its development of Consensus Documents.

Underlying scientific principles

In 1986, OECD published its first safety considerations for genetically engineered organisms (OECD, 1986). These included the issues relevant to human health, the environment and agriculture that might be considered in a risk/safety assessment. In its recommendations for agricultural and environmental applications, it suggested that risk/safety assessors:

- "Use the considerable data on the environmental and human health effects of living organisms to guide risk assessments;
- Ensure that recombinant DNA organisms are evaluated for potential risk, prior to application in agriculture and the environment by means of an independent review of potential risks on a case-by-case basis;
- Conduct the development of recombinant DNA organisms for agricultural and environmental applications in a stepwise fashion, moving, where appropriate, from the laboratory to the growth chamber and greenhouse, to limited field testing and finally to large-scale field testing; and
- Encourage further research to improve the prediction, evaluation, and monitoring of the outcome of applications of recombinant DNA organisms."

The role of confinement in small scale testing

In 1992, OECD published its Good Developmental Principles (GDP) (OECD, 1992) for the design of small-scale field research involving transgenic plants and micro-organisms. This document describes the use of *confinement* in field tests. Confinement includes measures, to avoid the dissemination or establishment of organisms from a field trial, for example, the use of physical, temporal, or biological isolation (such as the use of sterility).

Scale-up of crop-plants – "risk/safety analysis"

By 1993, the focus of attention had switched to the *scale-up* of crop plants as plant breeders began to move to larger-scale production and commercialisation of transgenic plants. OECD published general principles for, *scale-up* (OECD, 1993a), which re-affirmed that, "safety in biotechnology is achieved by the appropriate application of risk/safety analysis and risk management. Risk/safety analysis comprises hazard identification and, if a hazard has been identified, risk assessment. Risk/safety analysis is based on

the characteristics of the organism, the introduced trait, the environment into which the organism is introduced, the interaction between these, and the intended application. Risk/safety analysis is conducted prior to an intended action and is typically a routine component of research, development and testing of new organisms, whether performed in a laboratory or a field setting. Risk/safety analysis is a scientific procedure which does not imply or exclude regulatory oversight or imply that every case will necessarily be reviewed by a national or other authority" (OECD, 1993a).

The role of familiarity in risk/safety assessment

The issue of *scale-up* also led to an important concept, *familiarity*, which is one key approach that has been used subsequently to address the environmental safety of transgenic plants.

The concept of familiarity is based on the fact that most genetically engineered organisms are developed from organisms such as crop plants whose biology is well understood. It is not a risk/safety assessment in itself (U.S.-NAS, 1989). However, the concept facilitates risk/safety assessments, because to be familiar, means having enough information to be able to make a judgement of safety or risk (U.S.-NAS, 1989). Familiarity can also be used to indicate appropriate management practices including whether standard agricultural practices are adequate or whether other management practices are needed to manage the risk (OECD, 1993a). Familiarity allows the risk assessor to draw on previous knowledge and experience with the introduction of plants and micro-organisms into the environment and this indicates appropriate management practices. As familiarity depends also on the knowledge about the environment and its interaction with introduced organisms, the risk/safety assessment in one country may not be applicable in another country. However, as field tests are performed, information will accumulate about the organisms involved, and their interactions with a number of environments.

Familiarity comes from the knowledge and experience available for conducting a risk/safety analysis prior to scale-up of any new plant line or crop cultivar in a particular environment. For plants, for example, familiarity takes account of, but need not be restricted to, knowledge and experience with the following (OECD, 1993a):

- "The crop plant, including its flowering/reproductive characteristics, ecological requirements, and past breeding experiences;
- The agricultural and surrounding environment of the trial site;
- Specific trait(s) transferred to the plant line(s);
- Results from previous basic research including greenhouse/glasshouse and small-scale field research with the new plant line or with other plant lines having the same trait;
- The scale-up of lines of the plant crop varieties developed by more traditional techniques of plant breeding;
- The scale-up of other plant lines developed by the same technique;
- The presence of related (and sexually compatible) plants in the surrounding natural environment, and knowledge of the potential for gene transfer between crop plant and the relative; and
- Interactions between/among the crop plant, environment and trait.".

Risk/safety assessment and risk management

Risk/safety assessment involves the identification of potential environmental adverse effects or hazards, and determining, when a hazard is identified, the probability of it occurring. If a potential hazard or adverse affect is identified, measures may be taken to minimise or mitigate it. This is risk management. Absolute certainty or "zero risk" in a safety assessment is not achievable, so uncertainty is an inescapable aspect of all risk assessment and risk management (OECD, 1993a). For example, there is uncertainty in extrapolating the results of testing in one species to identify potential effects in another. Risk assessors and risk managers thus spend considerable effort to address uncertainty. Many of the activities in intergovernmental organisations, such as the OECD, address ways to handle uncertainty (OECD, 2000).

Appendix II

References cited in chronological order

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Présentation des documents de consensus sur la sécurité biologique

1. A propos du Sous-groupe de l'OCDE pour la sécurité biologique

Le *Sous-groupe de l'OCDE sur l'harmonisation de la surveillance réglementaire en biotechnologie* (le Sous-groupe) comprend des délégués des 33 pays Membres de l'OCDE et de la Commission européenne. Généralement, les délégués sont des fonctionnaires des ministères et organismes gouvernementaux chargés de l'évaluation des risques pour l'environnement et de la sécurité des produits issus de la biotechnologie moderne. Le Sous-groupe comprend aussi plusieurs délégations et experts invités qui participent à ses travaux en qualité d'observateurs, notamment l'Argentine, la Fédération de Russie, le Programme des Nations Unies pour l'environnent (PNUE), le Secrétariat de la Convention sur la diversité biologique (SCDB), les Organisation des Nations Unies pour l'alimentation et l'agriculture (FAO) et pour le développement industriel (ONUDI) et le Comité consultatif économique et industriel auprès de l'OCDE (BIAC). Ces dernières années, du fait de l'utilisation croissante des produits issus de biotechnologie dans plusieurs régions du monde et le développement d'activités portant sur les espèces tropicales et sub-tropicales, la participation au Sous-groupe des économies non membres s'est intensifiée, avec notamment le Brésil, le Cameroun, la Chine, l'Estonie, l'Inde, les Philippines et l'Afrique du Sud.

2. Harmonisation de la réglementation

Le Sous-groupe a été créé en 1995[1] à l'époque des premières demandes d'autorisation réglementaire de cultures commerciales transgéniques dans plusieurs pays Membres de l'OCDE. Dès le départ, un des objectifs-clés du Sous-groupe a été de promouvoir l'harmonisation internationale de la réglementation en matière de biotechnologie entre les pays Membres. L'harmonisation réglementaire vise à assurer que les éléments d'information utilisés pour l'évaluation des risques et de la sécurité, ainsi que les méthodes pour les collecter, soient aussi uniformes que possible. Elle peut conduire les pays à reconnaître, voire à accepter les informations provenant d'évaluations réalisées par d'autres. Les avantages de l'harmonisation sont évidents. Elle accroît la compréhension mutuelle entre pays, ce qui évite la duplication des efforts, économise les ressources limitées et accroît l'efficacité des procédures d'évaluation des risques et de la sécurité. Tout cela a pour effet d'améliorer la sécurité, tout en réduisant les obstacles inutiles au commerce (OCDE, 2000).

3. Pourquoi mener des activités d'harmonisation à l'OCDE ?

La création du Sous-groupe et de son programme de travail résulte d'une réflexion approfondie menée par les pays Membres pour déterminer s'il convenait de poursuivre les travaux sur l'harmonisation en biotechnologie dans le cadre de l'OCDE et, dans l'affirmative, ce que ces travaux impliqueraient. Cette analyse a été conduite par le Groupe *ad hoc* sur les aspects environnementaux de la biotechnologie (créé par la Réunion conjointe[2]), principalement en1994.

Le Groupe *ad hoc* a pris en considération et mis à profit les précédents travaux menés à l'OCDEdès le milieu des années 80. Ces activités antérieures de l'OCDE étaient initialement centrées sur les conséquences pour l'environnement et l'agriculture des essais au champ d'organismes transgéniques, mais ont vite porté ensuite sur leur utilisation à grande échelle et leur commercialisation. (Un résumé de ces travaux figure en Appendice I.)

1 . Le Sous-groupe s'appelait à l'origine le « Groupe d'experts pour l'harmonisation de la surveillance réglementaire en biotechnologie ». Il est devenu un Sous-groupe de l'OCDE en 1998.

2 . La Réunion conjointe était l'organe de tutelle du Groupe *ad hoc* et qui a, au vu de ses résultats, établi le Sous-groupe comme un organe subsidiaire. Aujourd'hui, son nom officiel est la Réunion conjointe du Comité des produits chimiques et du Groupe de travail sur les produits chimiques, les pesticides et la biotechnologie.

4. Principaux concepts et principes fondamentaux

Le Groupe *ad hoc* a pris en compte les précédents travaux sur l'analyse des risques qui sont présentés dans le document intitulé *Considérations de Sécurité Relatives à la Biotechnologie : Passage à l'Échelle Supérieure des Plantes Cultivées* (OCDE 1993a). L'extrait suivant en donne un aperçu : « L'analyse de risque/de sécurité s'appuie sur les caractéristiques de l'organisme, le caractère introduit, l'environnement dans lequel l'organisme est libéré, les interactions de ces facteurs entre eux et l'utilisation prévue. » Ces travaux ont servi de point de départ à l'évaluation environnementale des risques et de la sécurité, aujourd'hui acceptée mondialement. En examinant les possibilités d'harmonisation, le Groupe *ad hoc* s'est intéressé à ces caractéristiques ainsi qu'aux informations utilisées par les évaluateurs des risques et de la sécurité pour les examiner.

A cela s'ajoute le concept de familiarité, aussi décrit dans le document mentionné ci-dessus (OCDE 1993a). Ce concept «… est basé sur le fait que la plupart des organismes transformés génétiquement sont développés à partir d'organismes comme les végétaux cultivés, dont la biologie est bien comprise ». « La familiarité permet à l'évaluateur de risques d'appliquer ses connaissances et son expérience de l'introduction des végétaux et des micro-organismes dans l'environnement. » S'agissant des végétaux, la familiarité tient compte d'un grand nombre d'éléments, par exemple, des connaissances et de l'expérience concernant « les végétaux cultivés, y compris leurs caractéristiques de floraison et de reproduction, leurs exigences écologiques et les expériences passées en matière de sélection » (OCDE 1993a – voir Appendice 1 pour une description plus détaillée). Cela montre bien le rôle des informations concernant la biologie de l'organisme hôte lors de l'évaluation des risques pour l'environnement et de la sécurité.

Le Groupe *ad hoc* a également pris en compte le document décrivant les «*Méthodes Traditionnelles de Sélection des Plantes : un Aperçu Historique Destiné à Servir de Référence pour l'Évaluation du Rôle de la Biotechnologie Moderne* » (OCDE 1993b), qui met l'accent sur les organismes hôtes. On y trouve des informations sur 17 espèces végétales cultivées qui sont (ou pourraient être) utilisées en biotechnologie moderne. Ce document comprend des sections consacrées aux aspects phytosanitaires du transfert de matériel génétique, et aux utilisations actuelles de ces plantes cultivées. Une section détaillée est également consacrée aux pratiques actuelles de sélection.

5. Une approche commune de l'évaluation des risques et de la sécurité

L'une des missions importantes du Groupe *ad hoc* était d'évaluer dans quelle mesure les pays Membres étudient les mêmes éléments et mêmes problèmes lors de l'évaluation des risques et de la sécurité. Des différences importantes indiqueraient des difficultés à rechercher une harmonisation ; à l'inverse, de nombreuses similitudes indiqueraient un travail d'harmonisation plus aisé.

Deux études ont permis au Groupe *ad hoc* de répondre à cette question. La première concernait les plantes cultivées (OCDE, 1995a ; 1995b) et la seconde les micro-organismes (OCDE, 1995c ; 1996). Ces deux études étaient basées sur une enquête auprès des autorités nationales chargées de l'évaluation des risques et de la sécurité. L'objectif était d'identifier les questions couvertes par le processus d'évaluation (selon la législation nationale, la réglementation ou les documents d'orientation) afin d'établir le degré de similitude entre les autorités nationales. Ces études ont pris pour référence les éléments contenu dans « *Le Livre bleu* » de l'OCDE sur les considérations de sécurité relatives à l'ADN recombiné (OCDE 1986), en particulier les sections relatives aux : i) Considérations scientifiques générales ; ii) Considérations relatives à la santé humaine ; et iii) Considérations relatives à l'environnement et l'agriculture (appendices b, c et d). Les deux études ont permis de constater que les questions et problèmes traités par les pays Membres pour évaluer les risques et la sécurité présentaient un remarquable degré de similitude.

6. Emergence du concept de « document de consensus »

Le Sous-groupe a donc été établi en sachant que les questions et problèmes traités par les autorités nationales aux fins de l'évaluation des risques et de la sécurité présentaient de nombreux points communs. Il a également pris en compte les caractéristiques identifiées dans le cadre de cette évaluation (*l'organisme*, *le caractère introduit*, et *l'environnement*), sur lesquels pouvaient se concentrer les activités d'harmonisation.

Il a été ensuite reconnu qu'une majeure partie des informations utilisées dans l'évaluation des risques et de la sécurité concernant la biologie des organismes hôtes (plantes cultivées, arbres, animaux ou micro-organismes), étaient identiques ou presque dans toutes les évaluations portant sur une même organisme. En d'autres termes, les questions relatives à la biologie de l'organisme examinées dans le cadre de l'évaluation des risques et de la sécurité --par exemple, le potentiel de transfert de gènes au sein d'une espèce cultivée, et entre espèces apparentées, de même que le caractère adventice potentiel-- sont les mêmes pour chaque demande impliquant une même espèce hôte. C'est aussi le cas, dans une certaine mesure, pour les informations relatives aux caractères introduits.

En conséquence, le Sous-groupe a souhaité regrouper les informations communes utilisées dans l'évaluation des risques et de la sécurité d'un certain nombre de produits transgéniques, et a décidé de se concentrer sur deux catégories particulières : la biologie des espèces hôtes ; et les caractères utilisés dans les modifications génétiques. L'objectif était d'encourager le partage de l'information et d'éviter la duplication d'efforts en permettant aux pays de ne pas traiter plusieurs fois des mêmes questions communes pour les demandes concernant le même organisme ou le même caractère. Il a été souligné que des documents de consensus sur la biologie ou sur les caractères pouvaient être adoptés relativement vite par les pays Membres (en quelques années). Ce processus de compilation a rapidement débouché sur la rédaction de documents de consensus.

7. Objet des documents de consensus

Les documents de consensus ne prétendent pas se substituer à l'évaluation des risques et de la sécurité, car ils ne concernent qu'une partie de l'information nécessaire. Cependant, ils devraient faciliter grandement l'évaluation environnementale des risques et de la sécurité.

Les documents de consensus visent à fournir un aperçu des données courantes pouvant être utilisées dans le processus d'évaluation réglementaire des produits issus de la biotechnologie. Ils ne prétendent pas offrir une source d'informations exhaustive sur l'ensemble des connaissances concernant un organisme hôte ou un caractère particulier ; mais ils traitent, sur la base d'un consensus, des questions centrales jugées pertinentes par les pays pour l'évaluation des risques et de la sécurité.

Ces documents visent à faciliter l'échange d'informations sur ces composantes clés des évaluations de la sécurité environnementale afin d'éviter que les activités menées dans les pays ne fassent double emploi. Ils sont destinés : a) aux demandeurs d'autorisation pour indiquer le type d'information à fournir aux autorités de réglementation ; b) aux autorités chargées de la réglementation comme guide général et source de référence pour leurs examens ; et c) aux gouvernements aux fins de l'échange d'information, comme références de recherche et pour l'information du public.

Il a été déclaré initialement que les informations contenues dans les documents de consensus devaient être *mutuellement reconnues ou mutuellement acceptées* par les pays Membres de l'OCDE, bien que le sens de ces expressions reste encore à préciser. L'expression *acceptation mutuelle des données* a été étudiée pendant la période de mandat du Groupe *ad hoc* et le début du Sous-groupe (1993-1995). Cette notion est empruntée au Programme des produits chimiques de l'OCDE pour désigner un ensemble de Décisions du Conseil de l'OCDE qui ont un caractère contraignant pour les pays Membres. Dans le cas des documents de consensus, il n'a jamais été obligatoire d'utiliser les informations y figurant, même si le Sous-groupe est intéressé à impliquer davantage les pays dans l'utilisation de ces documents.

La participation des pays à l'élaboration des documents, et leur intention d'utiliser les informations qu'ils contiennent, sont présumées de bonne foi. On attend donc des documents de consensus qu'ils puissent servir de référence dans les évaluations des risques et de la sécurité. Ces documents étant des outils d'accès public, ils peuvent intéresser tout pays qui souhaite les utiliser dans des évaluations nationales.

8. Processus de développement des documents de consensus jusqu'à publication

La rédaction d'un document de consensus se fait en plusieurs étapes. Cela commence lorsqu'une délégation, à l'occasion d'une réunion officielle du Sous-groupe, propose d'établir un document sur un nouveau sujet, le plus souvent une espèce cultivée ou un caractère. Si le Sous-groupe approuve la proposition, un premier projet est préparé par un, ou deux ou plusieurs pays en collaboration (« approche par pays pilote »). En général, le ou les pays pilote(s) possèdent une expérience de la plante ou du caractère visé et peuvent identifier des experts pour préparer une première version.

Cette version préliminaire est d'abord examinée par le Bureau du Sous-groupe[3] qui vérifie que le document couvre bien tous les aspects généralement pris en compte par les documents de consensus, et que sa qualité est suffisante pour le soumettre à l'ensemble du Sous-groupe.

Un premier projet est établi à la lumière des commentaires du Bureau, puis présenté à l'ensemble du Sous-groupe. Chaque délégation peut ainsi étudier le texte et formuler des commentaires en fonction de l'expérience de son pays. Ces commentaires sont pris en compte dans une deuxième version qui est à nouveau diffusée au Sous-groupe. A ce stade, le Sous-groupe peut être invité à recommander la déclassification du document. Cette demande intervient uniquement lorsque toutes les délégations ont décidé d'un commun accord (consensus) que le document était complet et prêt pour publication. Il arrive cependant que le texte nécessite un troisième examen, voire plus, par le Sous-groupe avant qu'une déclassification ne puisse être envisagée.

Lorsque le Sous-groupe est convenu de recommander le document pour déclassification, il est transmis à l'organe de tutelle, la Réunion conjointe, qui est invitée à le déclassifier. Une fois approuvé par la Réunion conjointe, le document est publié.

Il importe de noter que l'examen des documents de consensus dépasse le cadre des réunions officielles du Sous-groupe. De nombreux échanges de vues se font aussi par voie électronique, notamment dans le cadre du site Web protégé dédié au Sous-groupe. Ceci permet à divers experts de compléter les projets.

Pour plusieurs des documents, il s'est révélé nécessaire d'inclure aussi des informations provenant de pays non membres. Cet échange élargi d'expertise est devenu de plus en plus important ces dernières années avec le développement d'activités portant sur les espèces tropicales et sub-tropicales. Cela s'est produit notamment pour les plantes cultivées dont les centres d'origine et de diversité se trouvent dans un ou des pays non membre(s). Le PNUE, l'ONUDI et la FAO ont alors contribué à la préparation des documents en identifiant les experts appropriés des pays concernés. C'était le cas, par exemple, lors de l'élaboration du document de consensus sur la biologie du riz.

9. Évolutions actuelles et futures pour le Sous-groupe

Le Sous-groupe poursuit ses travaux de préparation des documents de consensus, ainsi que l'amélioration de l'efficacité de leur processus d'élaboration. Un nombre croissant de plantes cultivées et autres espèces hôtes (arbres, animaux, micro-organismes) fait l'objet de modifications, pour un nombre

3 . Le Bureau comprend le Président et les Vice-présidents du Sous-groupe. Le Bureau est élu par le Sous-groupe une fois par an. Au moment où de la préparation de la présente publication - Vol. 3 et Vol. 4, le Président est un délégué des Etats-Unis, les Vice-présidents sont du Canada, de la Finlande, du Japon et des Pays-Bas.

croissant de caractères transférés, et le Sous-groupe vise à satisfaire les besoins actuels tout en préparant les sujets émergeants.

Lors de l'atelier de l'OCDE consacré aux documents de consensus et aux travaux futurs d'harmonisation, tenu à Washington D.C. en octobre 2003, le Sous-groupe a examiné comment établir un ordre de priorité pour la préparation des documents de consensus parmi les nombreux classements possibles. Les participants ont également reconnu qu'il pourrait être nécessaire de périodiquement revoir ou de mettre à jour les documents de consensus, pour veiller à ce qu'ils contiennent les informations les plus récentes. Le Sous-groupe examine régulièrement ces aspects lorsqu'il planifie son futur travail. Pour faciliter l'élaboration des prochains documents, les participants à l'atelier ont jugé utile d'établir une structure type de document de consensus. Le Sous-groupe a donc décidé de préparer des documents guides listant les « points à examiner », tout d'abord pour les documents de consensus relatifs à la biologie, puis pour ceux portant sur les caractères. Le texte sur les documents de biologie, publié en 2006, figure en section suivante de la présente publication.

10. Le Groupe d'étude de l'OCDE sur la sécurité des nouveaux aliments destinés à la consommation humaine et animale

Le *Groupe d'étude de l'OCDE sur la sécurité des nouveaux aliments destinés à la consommation humaine et animale* (Groupe d'Etude), établi en1999, traite des aspects de l'évaluation des aliments issus des cultures transformées génétiquement et destinés à la consommation humaine et animale. L'objet principal du travail du Groupe d'étude, similaire à celui du Sous-groupe, est d'assurer que les types d'information utilisés lors de l'évaluation des risques et de la sécurité, ainsi que les méthodes utilisées pour collecter cette information, sont aussi similaires que possibles entre pays. L'approche consiste à comparer les cultures transgéniques et les produits qui en dérivent avec leurs homologues conventionnels qui sont déjà connus et considérés comme sûrs du fait de l'expérience reconnue de leur utilisation. Les activités du Groupe d'étude facilitent les méthodes harmonisées et un échange d'information.

Comme pour le programme sur la sécurité biologique, le produit principal du programme sur les aliments est la Série de documents de consensus sur la composition des nouvelles variétés d'espèces cultivées. Les documents du Groupe d'étude compilent une base commune d'information scientifique sur les composants essentiels des plantes cultivées tels que les principaux nutriments, toxiques, anti-nutriments et allergènes. Ces documents constituent des outils pratiques pour les responsables de la réglementation et les autorités en charge de l'évaluation des risques et de la sécurité de ces nouvelles variétés, en ce qui concerne les aliments destinés à la consommation humaine et animale. A ce jour, 20 documents de consensus on été publiés sur les espèces cultivées majeures, et sur des aspects généraux visant à faciliter l'harmonisation. Ils constitutent la Série sur la Sécurité des Nouveaux Aliments Destinés à l'Alimentation Humaine et Animale, également accessible gratuitement en ligne sur le site de l'OCDE (www.oecd.org/biotrack).

La publication de l'intégralité de la Série sur les Nouveaux Aliments, rassemblée en un seul document, est envisagée au cours de l'année 2011.

Le Sous-groupe et le Groupe d'étude mettent en oeuvre des programmes à la fois proches et complémentaires, centré sur les aspects environnementaux pour le premier, sur les aspects d'alimentation humaine et animale pour le second. Leur coopération sur des sujets d'intérêt commun a résulté récemment dans la publication du premier document de consensus élaboré conjointement par les deux instances, intitulé « *Consensus Document on Molecular Characterisation of Plants Derived from Modern Biotechnology* » (*Document de Consensus sur la Caractérisation Moléculaire des Plantes issues de la Biotechnologie Moderne*)", édité en septembre 2010. Ce document est reproduit dans le Volume III de la présente publication.

Appendice I

Principes et concepts de l'OCDE relatifs à la sécurité biologique, établis antérieurement à la création du Sous-groupe (1986-1994)

Depuis le milieu des années 1980, l'OCDE a développé des approches harmonisées pour l'évaluation des risques et de la sécurité des produits de biotechnologie moderne. Avant la création du Sous-groupe, l'OCDE a publié plusieurs rapports d'experts portant sur des questions de sécurité, les concepts et principes relatifs à l'évaluation des risques et de la sécurité, ainsi que sur la dissémination des plantes transgéniques en champ, et sur la question des pratiques traditionnelles de sélection des espèces cultivées. Le présent appendice récapitule les éléments essentiels de ces travaux qui ont servi de point de départ à la création du Sous-groupe et à l'élaboration des documents de consensus.

Principes scientifiques sous-jacents

En 1986, l'OCDE a publié ses premières études sur la sécurité des organismes transgéniques (OCDE, 1986). Celles-ci comprenaient des questions intéressant la santé humaine, l'environnement et l'agriculture qui pourraient être prises en compte dans l'évaluation des risques et de la sécurité. Dans les recommandations pour les applications agricoles et environnementales, il était suggéré que les évaluateurs des risques et de la sécurité :

- « Utilisent des données nombreuses sur les effets au niveau de l'environnement et de la santé humaine des organismes vivants afin de guider les évaluations des risques ;

- Assurent que les organismes formés de molécules d'ADN recombiné sont évalués pour déterminer les risques possibles, préalablement à leur application dans l'agriculture et dans l'environnement par un examen distinct des risques potentiels de façon ponctuelle ;

- Dirigent le développement d'organismes formés d'ADN recombiné pour des applications agricoles et environnementales d'une manière progressive, allant si approprié, du laboratoire à la chambre de culture et à la serre, puis à des essais limités en conditions réelles, et finalement à des essais au champ à grande échelle ;

- Encouragent la recherche pour améliorer les prédictions, l'évaluation et le suivi des résultats des applications d'organismes formés d'ADN recombiné. »

Rôle du confinement dans les essais à échelle réduite

En 1992, l'OCDE a publié ses Bons Principes de Developpement (OCDE, 1992) pour la conception de recherche sur le terrain à échelle réduite impliquant des végétaux et micro-organismes transgéniques. Ce document décrit l'utilisation du *confinement* des essais de terrain. Le confinement comprend des mesures pour éviter la dissémination ou l'établissement d'organismes à partir d'un essai de terrain, par exemple les mesures d'isolement physique, temporel ou biologique (comme l'utilisation de la stérilité).

Mise à l'échelle des végétaux cultivés – « analyse des risques et de la sécurité »

À partir de 1993, l'attention s'est dirigée vers la *mise à l'échelle* des végétaux cultivés du fait que les sélectionneurs commençaient à envisager la production à grande échelle et la commercialisation de plantes transformées génétiquement. L'OCDE a publié les principes généraux pour la *mise à l'échelle* (OCDE,

1993a), lesquels réaffirmaient que, « La sécurité en biotechnologie est réalisée par l'application appropriée de l'analyse des risques et de la sécurité et de la gestion des risques. L'analyse des risques et de la sécurité comprend l'identification des dangers et, si un danger a été identifié, la gestion du risque. L'analyse des risques et de la sécurité est fondée sur les caractéristiques de l'organisme, le trait caractéristique introduit, l'environnement dans lequel l'organisme est introduit, les interactions entre l'environnement et l'organisme de même que l'application prévue. L'analyse des risques et de la sécurité est menée préalablement à une action visée et est en général une composante de routine de la recherche, du développement et des essais de nouveaux organismes, que ces actions soient effectuées en laboratoire ou sur le terrain. L'analyse des risques et de la sécurité est une procédure scientifique qui n'implique ni n'exclut une surveillance au niveau de la réglementation, et qui n'exige pas que chaque cas soit nécessairement examiné par une autorité nationale ou autre » (OCDE, 1993a).

Rôle de la familiarité dans l'évaluation des risques et de la sécurité

La question de la *mise à l'échelle* a également mené à un concept important, la *familiarité*, qui est l'une des approches stratégiques utilisées par la suite pour traiter de la sécurité environnementale des végétaux transgéniques.

Le concept de familiarité est basé sur le fait que la plupart des organismes transformés génétiquement sont développés à partir d'organismes comme les plantes cultivées dont la biologie est bien comprise. Il ne constitue pas une évaluation des risques et de la sécurité en tant que tel (U.S.-NAS, 1989). Toutefois, le concept facilite les évaluations des risques et de la sécurité parce que la familiarité suppose que l'on dispose de suffisamment d'éléments pour être en mesure de poser un jugement sur la sécurité ou sur le risque (U.S.-NAS, 1989). La familiarité peut aussi servir à identifier les pratiques de gestion appropriées, comme par exemple déterminer si les pratiques agricoles usuelles sont adéquates ou si d'autres conduites des cultures sont nécessaires pour gérer le risque (OCDE, 1993a). La familiarité permet à l'évaluateur de risques d'appliquer ses connaissances et son expérience de l'introduction des végétaux et des micro-organismes dans l'environnement, ce qui lui indique les pratiques de gestion appropriées. Comme la familiarité dépend aussi de la connaissance de l'environnement et de ses interactions avec les organismes introduits, l'évaluation des risques et de la sécurité effectuée dans un pays peut ne pas s'appliquer à un autre pays, Toutefois, au fur et à mesure des essais en champ, l'information grandit sur les organismes impliqués et sur leurs interactions avec divers environnements.

La familiarité provient des connaissances et de l'expérience disponibles pour analyser les risques et la sécurité préalablement à la mise à l'échelle de toute nouvelle lignée végétale ou variété cultivée dans un environnement particulier. Pour les végétaux par exemple, la familiarité tient compte, sans y être limitée, des connaissances et de l'expérience au niveau (OCDE, 1993a) :

- « des végétaux cultivés, y compris leurs caractéristiques de floraison et de reproduction, leurs exigences écologiques et les expériences passées en matière de sélection des végétaux ;
- de l'environnement agricole et environnant du site d'essais ;
- du ou des trait(s) caractéristique(s) spécifique(s) transféré(s) à la ou les lignée(s) de végétaux ;
- des résultats des précédents travaux de recherche fondamentale, notamment la recherche en serre et à échelle réduite sur la nouvelle lignée de végétaux ou sur d'autres lignées présentant les mêmes traits caractéristiques ;
- de la mise à l'échelle de lignées de végétaux cultivés développés par des techniques plus traditionnelles de sélection des végétaux ;

- de la mise à l'échelle d'autres lignées de végétaux développées par la même technique ;

- de la présence de végétaux apparentés (et sexuellement compatibles) dans l'environnement naturel et des connaissances au niveau de la possibilité de transfert génique entre la plante cultivée et la plante apparenté ;

- des interactions entre la plante cultivée, l'environnement et les traits caractéristiques et des interactions au sein de la plante cultivée. »

Évaluation des risques et de la sécurité, et gestion des risques

L'évaluation des risques et de la sécurité suppose l'identification des effets nocifs ou des dangers possibles pour l'environnement et la détermination, lorsqu'un danger est identifié, de la probabilité qu'il se produise. Si un danger ou un effet nocif sur la santé est identifié, des mesures doivent être entreprises pour le minimiser ou l'atténuer. C'est ce que l'on appelle la gestion des risques. La certitude absolue, ou l'absence totale de risques, est impossible à obtenir en matière d'évaluation de la sécurité. L'incertitude est donc un aspect inévitable de toutes les évaluations des risques et de toute gestion des risques (OCDE, 1993a). Par exemple, l'on retrouve de l'incertitude en extrapolant les résultats des tests effectués sur une espèce pour identifier les effets possibles chez une autre espèce. Les évaluateurs et les gestionnaires de risques déploient donc des efforts considérables à traiter les incertitudes. Plusieurs des activités des organisations gouvernementales, comme l'OCDE, tentent de déterminer des façons de gérer ces incertitudes (OCDE, 2000).

Appendice II

Références (par ordre chronologique)

OCDE (1986), *Considérations de sécurité relatives à l'ADN recombiné. Considérations de sécurité relatives à l'utilisation d'organismes obtenus par des techniques de recombinaison de l'ADN dans l'industrie, dans l'agriculture et dans l'environnement* («Le Livre Bleu »), OCDE, Paris.

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OCDE (1993a), *Considérations de sécurité relatives à la biotechnologie : Passage à l'échelle supérieure des plantes cultivées*, OCDE, Paris.

OCDE (1993b), *Méthodes traditionnelles de sélection des plantes : Un aperçu historique destiné à servir de référence pour l'évaluation du rôle de la biotechnologie moderne*, OCDE, Paris.

OCDE (1995a), *Commercialisation of Agricultural Products Derived through Modern Biotechnology: Survey Results*, OCDE, Paris.

OCDE (1995b), *Report of the OECD Workshop on the Commercialisation of Agricultural Products Derived through Modern Biotechnology*, OCDE, Paris.

OCDE (1995c), *Analysis of Information Elements Used in the Assessment of Certain Products of Modern Biotechnology*, OCDE, Paris.

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OCDE (2000), *Rapport du Sous-groupe sur l'harmonisation de la surveillance réglementaire en biotechnologie au Sommet du G8 d'Okinawa*, OCDE, Paris.

Points to consider for consensus documents on the biology of cultivated plants

Introduction

Most environmental risk/safety assessments of transformed (genetically modified or engineered) plants are based upon a broad body of knowledge and experience with the untransformed species based on familiarity with the crop plant. The intent of the biology consensus documents is to describe portions of this body of knowledge directly relevant to risk/safety assessment in a format readily accessible to regulators. The document is not an environmental risk/safety assessment of the species. Rather, the consensus document provides an overview of pertinent biological information on the untransformed species to help define the baseline and scope (the comparator against which transformed organisms will be compared), in the risk/safety assessment of the transformed organism. Consensus documents are not detailed crop handbooks or manuals of agricultural or silvicultural practice or economic botany, but rather focus on the biological information and data that may be clearly relevant to the assessment of newly transformed plants.

This *Points to Consider* document is meant as a structured explanatory checklist, regarding both order and contents, of relevant points to consider in preparing or evaluating a consensus document on the biology of a cultivated vascular plant species or other taxonomic group of interest, in relation to biotechnology and environmental risk/safety assessment. The general approach laid out in this document may also be pertinent to non-vascular plants (for example mosses) as well as fungi and micro-organisms; however, these groups are biologically and ecologically so different that further adaptation and refinement of the general approach will be necessary.

The biology consensus documents that have been published to date, as well as most in preparation, deal with crops, timber trees, and fruit trees [excepting the one on *Pleurotus* spp. (oyster mushrooms) and several on micro-organisms]. The plants of interest that have been the subject of the documents are primarily row crops, or trees managed silviculturally or grown in plantations or orchards. They are vascular plants, either flowering plants (angiosperms) or conifers (gymnosperms).

The points to consider as covered in the present document create a basic format and scope to be used for writing or reviewing new consensus documents and updating the earlier documents. While this *Points to Consider* document is meant to provide a basic format and scope, it is not intended to be rigid or inflexible. Of the biology consensus documents to date, some have addressed a particular point in depth, others lightly, and some not at all, depending on the relevance of the point to the plant species or other group of interest. Should additional points beyond those covered in this document be needed for a particular plant, the additional information can be included in the body of the consensus document, or in appendices. If a particular point is not covered in a consensus document, the text may briefly explain why the point, in the particular case, is not relevant.

Authors of the draft of a plant biology consensus document should be familiar with this *Points to Consider* document as well as existing consensus documents in the OECD Series on Harmonisation of Regulatory Oversight in Biotechnology (SHROB), in order to develop the appropriate scoping and presentation of information and data and for general editorial style. Existing consensus documents, particularly more recent ones, may provide detailed examples (some noted below) that are helpful models or thought-provoking for particular cases.

An understanding of the biology of the species or other group of interest will aid in determining the kinds of information pertinent to the environmental risk/safety assessment. This *Points to Consider* document provides an explanation of why the point (as enumerated below) is important in risk/safety assessment of the transformed plant, and presents a rationale for how the information in the point relates to risk/safety assessment. For a particular environmental risk/safety assessment, biological or ecological information in addition to that presented in the consensus document may be needed to address the regional environments into which the genetically engineered plant is proposed to be released.

1. Species or taxonomic group

The focus of each biology consensus document has usually been a species, but in some cases the focus has been a group of species or a genus, or just a subspecies or a cultivar group (examples are below). The primary focus of this *Points to Consider* document also is the species of interest, so appropriate adjustments will be necessary if the focus of the consensus document is more broad or narrow.

1.1. Classification and nomenclature

Give the scientific name of the cultivated species of interest, with its authors, and pertinent synonyms (*i.e.* actively used alternative scientific names, if any). If necessary to delimit the plant, also give the horticultural name, *e.g.* the cultivar group (*e.g. Beta vulgaris* subsp. *vulgaris* Sugar Beet Group). Provide main international common name(s) at least in English for the species of interest. Give the taxonomic context of the species (family always, perhaps the order, and perhaps the subfamily, tribe, subgenus or section). If the taxonomy is not settled, be relatively conservative in choosing the taxonomy, and briefly explain the alternative(s). The latest taxonomic or nomenclatural study is not necessarily definitive, and may need time for scientific consensus before it becomes adopted. A common name for the crop species of interest can be introduced here, to be used in much of the document as a more familiar name (aide-memoire).

Describe the taxonomic relationships of the cultivated species: related species, and related genera particularly if there is good potential for spontaneous hybridisation or the generic limits are unsettled. A list of related species (with brief ranges) should be given and include all the relatives with a potential for hybidization (*i.e.* crossable relatives). This topic is dealt with in detail in Section IV. The listing here may provide brief information on chromosome numbers and ploidy if these data are pertinent to the taxonomic differentiation of the species, whereas a more complete coverage of the relevant details is provided in Section III or IV.

> ***Rationale***: The scientific name enables an unequivocal understanding (*i.e.* a circumscription) of the plant of interest, at the appropriate level, such as the species or the subspecies. This addresses what the species (or other group) is and what it is called (*i.e.* circumscription and name). The list of close relatives could help in subsequent analysis to form an idea of the kinds of pertinent traits such as disease resistance or stress tolerance that may already occur in these direct relatives of the cultivated plant, and may help elucidate how genes/traits are shared and may move *via* gene flow amongst related populations. The list of close relatives may aid in understanding the range of diversity and variability between the species and its natually crossable relatives.
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> ***Examples***: OECD Series on Harmonisation of Regulatory Oversight in Biotechnology (SHROB) No. 14 (rice, Section II, pp. 12-14); No. 16 (poplars, Section II, pp. 15-18); No. 18 (sugar beet, Section I, pp. 11-12); No. 22 (eastern white pine, Section II, p. 12); and No. 31 (sunflower, Section I, pp. 11-13).

1.2. Description

Give a brief non-technical description of the species of interest, understandable to the non-specialist. Provide the habit and general characteristics of the plant, for example that it is an annual, a long-lived tree, or a biennial cultivated as an annual crop, and that it is, for instance, grown for fibre, fruit, or seeds. Also provide a concise technical (taxonomic) description sufficient to make a positive identification of the plant (or part). Illustration (a line drawing or black-and-white photo) may be useful. To clarify distinctiveness, emphasise the practical diagnostic or distinguishing morphological or other characters. Limit jargon, by the precise use of phrases and familiar words. A table of main differences or taxonomic

key may be instructive (*e.g*. *Oryza sativa* and *O. glaberrima* in SHROB No. 14). If necessary, for example when based on recent information or a new approach, present or reference the analytical methods by which a differential identification of the similar plants (*e.g*. species) is now made.

> ***Rationale***: These descriptions provide broad orientation, and as well accurate identification. They briefly explain how the species of interest is actually identified in relation to others. Additionally, the description may give particular characteristics of the plant to aid in defining the scope of a risk/safety assessment. Although an exact identification often is based on experience (*i.e*. recognition) or on regional publications, rigorous or subtle analysis using specialist resources sometimes is required.
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> ***Examples***: OECD SHROB No. 8 (potato, Section IV, pp. 14-15) and No. 28 (European white birch, Section I, pp. 12-13).

1.3. Geographic distribution, natural and managed ecosystems and habitats, cultivation and management practices, and centres of origin and diversity

This subsection covers the primary or crop species of interest, including the plants that are wild or free-living (whether native or naturalised) or weedy, and as cultivated or managed in the field. Crossable relatives with the relevant information and data on their intraspecific and interspecific crossing are discussed in Sections III and IV.

1.3.1. Geographic distribution

Describe the overall geographic distribution (if helpful including altitudinal range or climatic region), indicating broadly where the species of interest is native (*i.e.* indigenous), where it has been naturalised (introduced but free-living), and where it is in cultivation. A general map may be useful.

> ***Rationale***: Knowledge of the geographic distribution sets the context for understanding the potential interaction of the species with its relatives and with the surrounding ecosystems. For example, it is important to make a distinction between the species' native and naturalised occurrence when assessing the potential effects and the importance of gene flow.
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> ***Examples***: OECD SHROB No. 8 (potato, Sections II & III, pp. 12-13); No. 13 (white spruce, Section III, pp. 15-16); and No. 16 (poplars, Section II, pp. 15-18).

1.3.2. Ecosystems and habitats where the species occurs natively, and where it has naturalised

Indicate the natural and non-cultivated or non-managed ecosystems where populations of the species of interest are native (indigenous) and where introduced and now naturalised (free-living) components of the vegetation. Designated natural areas (*e.g.* protected reserves, parks) where the species may be an invasive problem would be noted here. A species weedy in disturbed waste (*e.g.* abandoned) areas would be included here, whereas the species weedy in intensively managed areas would be discussed in the following subsection. Those ecosystems and habitats in which the species of interest occurs and its abundance are indicated here, whereas its ecological interactions with biotic components of the ecosystems and habitats are developed in Section V.

> ***Rationale***: The focus of this subsection is the relatively natural, self-sustaining context, rather than the land areas strongly managed for plant production. Knowledge of where the species occurs indigenously or is free-living provides baseline information for understanding the range of habitats in which the species exists, the range of behaviours exhibited in those habitats, and how characteristics of the species determine the range of habitats where it occurs. This information provides an understanding of the species' potential for interaction with its relatives and surrounding habitats.

Example: OECD SHROB No. 28 (European white birch, Section III, pp. 19-20).

1.3.3. Agronomic, silvicultural, and other intensively managed ecosystems where the species is grown or occurs on its own, including management practices

Describe where the species is dependent on management for survival or persistence over several years of usual conditions. Areas where the plant may be a weed problem would be discussed here. Areas to be discussed could include habitats such as annual row crops or bordering areas, tree plantations, orchards and vineyards, along regularly managed roadsides, rights-of-way, irrigation ditches, etc. Identify the pertinent general agronomic or other practices, and if relevant, regional differences in practices (including various practices within a region). Information might briefly encompass site preparation after clear-cutting, tillage, sowing or planting, weed control, control of volunteers, harvesting, plant protection practices during crop growth and after harvest, transport practices, and the use of harvested materials (*e.g.* for silage). The relevant ecological interactions of the species with particular organisms in these managed ecosystems are discussed in Section V.

> ***Rationale***: The focus of this subsection is on the plant's survival in agro-ecological, silvicultural, and other such managed areas, to provide the baseline environmental information on how the plant responds to or is managed by accepted agronomic, silvicultural or similar intensive practices. Identification of significant cultivation or management practices provides an understanding of measures available to manage or control the plant.
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> ***Examples***: OECD SHROB No. 7 (oilseed rape, Section III, p. 13); No. 14 (rice, Section VII, pp. 26-27); No. 15 (soybean, Sections II & V, pp. 13 & 14); and No. 18 (sugar beet, Sections I & II, pp. 16-17).

1.3.4. Centres of origin and diversity

Describe the known or probable primary centre(s) of origin, as well as secondary centres where additional important variability or biodiversity may occur, whether naturally (*e.g. Beta*) or through the process of domestication (*e.g. Zea mays*, *Solanum tuberosum* subsp. *tuberosum*). The evolutionary centres important for natural biodiversity should be mentioned, and the central areas of domestication and landrace diversity, with indication of the centres' relative importance. Genetic diversity is covered in Section III. Provide a brief sketch of the history or extent of domestication including mention of relevant domestication traits (*e.g.* non-shattering, loss of seed dormancy).

> ***Rationale***: The interaction of the cultivated plant with close relatives especially in a centre of origin is an important consideration because gene flow, varietal competition, or a change in cultivation practices may alter this especially rich and valuable diversity. If the plant is not expected to be grown near a center of diversity, the absence of such relatives would also be important. A brief review of domestication may provide insight showing the continuity of modification of the species and the degree of the crop plant's adaptation to or dependence on the managed environment.
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> ***Examples***: OECD SHROB No. 9 (bread wheat, Section III, pp. 13-16); No. 27 (maize, Section IV, pp. 18-20); and No. 31 (sunflower, Section I, pp. 14-15.

2. Reproductive biology

2.1. Generation time and duration under natural circumstances, and where grown or managed

Important aspects of generation time and duration include the time to first flowering and total life cycle of the plant, and time from planting to plow-down. Include the effects of agronomic, silvicultural, and similar practices when describing generation time and duration of the cultivated plant. Important differences within both the natural and the cultivated regions should be noted.

> ***Rationale***: The generation time and duration are indications of the terms in which environmental effects may occur. Precocious generation times and shorter durations in agriculture affect the likelihood of outcrossing with free-living (wild) relatives, and give a general indication of when outcrossing may first occur.
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> ***Examples***: OECD SHROB No. 14 (rice, Sections V & VII, pp. 21 & 26-27) and No. 18 (sugar beet, Section I, pp. 13-14).

2.2. Reproduction (production of flowers or cones, fruits, seeds, and vegetative propagules)

Include a characterisation of the key stages in the life cycle necessary for the plant to survive, reproduce, and disperse. Particular attention is given to any uncommon survival structures or strategies and their importance under natural and cultivation conditions, and to the dependence of survival and reproduction on ecological and geographical factors.

> ***Rationale***: The reproductive capabilities of a plant determine the means by which the plant can produce progeny and spread or disperse. Both the plant and its progeny may affect the environment, including other organisms, and thus the time frame and geographic area over which effects might occur.

2.2.1. Floral biology

Describe the general floral dynamics (*e.g*. flowering season, flowering time, anthesis, selfing and/or outcrossing). Relevant genetic details of the outcrossing and/or selfing are addressed in Section III.

> ***Rationale***: This information will assist in understanding some of the factors that affect the potential for gene flow, and in assessing particular management strategies for reducing gene flow when outcrossing may occur. Such management strategies may include induced male sterility or asynchronous flowering times.
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> ***Examples***: OECD SHROB No. 8 (potato, Section VI, p. 17); No. 14 (rice, Section V, p. 21); and No. 21 (Sitka spruce, Section III, p. 15).

2.2.2. Pollination (wind, insects, both, etc.), pollen dispersal, pollen viability

Describe observed modes of pollen dispersal, indicating the most prevalent way. Important insect or other animal pollinators should be indicated. Give data on the range of pollen dispersal through the air and/or by the animal vectors, if known. Note how climatic or regional (*e.g.* geographic) differences can affect pollination. Provide available information or data on the influence of pollen quantity, movement, viability, load and competition on outcrossing, which is discussed in Sections III and IV. The details on pollination as they pertain to the plant are covered here, whereas details particularly pertinent to the pollinator are covered in Section V.

Rationale: Pollen biology is an important component in the assessment of potential for gene flow, and in the evaluation of a need for and the type(s) of pollen confinement strategies such as buffer rows or isolation distances.

Examples: OECD SHROB No. 8 (potato, Section VI, p. 17) and No. 18 (sugar beet, Section IV, pp. 22-23).

2.2.3. Seed production, and natural dispersal of fruits, cones, and/or seeds

Briefly describe the sexual reproductive structures, including relevant morphological characteristics of fruits (or cones) and seeds, and note any inherent means of dispersal (*e.g.* shattering, fruit splitting, ballistic). Note the quantity of seeds produced by a plant (*e.g.* seeds per fruit and number of fruits). Provide information on the means and range of dispersal (*e.g.* by gravity, wind, water, on and/or in animals), and if there are several means indicate their relative importance. Cover apomixis below, in Subsection 2.2.5.

Rationale: The number of seeds and seed/fruit dispersal mechanisms are factors to consider in understanding the potential for establishment of free-living plants or populations, and thus the time and geographic area over which environmental effects might occur. The range of variability of these factors is also an important consideration.

Examples: OECD SHROB No. 15 (soybean, Section IV, p. 14) and No. 28 (European white birch, Section IV, p. 23).

2.2.4. Seed viability, longevity and dormancy, natural seed bank; germination, and seedling viability and establishment

Discuss factors in the establishment of any seed bank, including its transience or persistence, and the viability, longevity and dormancy of seeds under natural conditions. Note any special conditions that affect dormancy and/or germination (*e.g.* depth of burial, light and/or temperature, passage through an animal's digestive tract, or need for fire) that might be particularly relevant. Note any special requirements for the establishment and survival of seedlings (*e.g.* soil qualities or regime), as the organism's fitness may be revealed at this challenging phase in the life cycle.

Rationale: Seed viability is a key factor to consider in assessing the likelihood of survival of non-cultivated plants. Natural seed banks are often the main source of weeds in cultivated fields, whether they are previous-crop volunteers or non-crop weedy relatives. Whether seedlings can establish usually is a primary limiting factor in continuing the life cycle.

Example: OECD SHROB No. 7 (oilseed rape, Section VI, p. 17).

2.2.5. Asexual propagation (apomixis, vegetative reproduction)

Take into account natural vegetative cloning (*e.g.* in grasses and poplars), the kinds of propagules (special structures, and/or fragmented plant pieces), dispersal of the propagules, and their viability. Discuss the relative importance of asexual reproduction for the plant, including any differences dependent on habitat or region. For apomixis (non-sexual production of seeds), similarly consider its relative importance and effectiveness.

Rationale: If a plant has a strategy that includes asexual propagation, this could be a means for considerable or quite different dispersal or spread, and consequently may also affect the time frame and geographic area over which environmental effects might occur.

Example: OECD SHROB No. 16 (poplars, Section IV, p. 23).

3. Genetics

3.1. Relevant detailed genetic information on the species

Give a basic overview of the relevant genetic constitution and genetic dynamics of the species. If more appropriate in a particular case, some basic genetic information (*e.g.* ploidy, ancestral/progenitor genomes) may be more fully or instead discussed in Section IV. In this Section III (including subsections as needed), cover for example and if appropriate cytogenetics (*e.g.* karyology, meiotic behavior), nuclear genome size, possible extent of repetitive or non-coding DNA sequences, main genetic diversity or variability (*e.g.* among or within populations or varieties, and of alleles at a locus), evidence of heterosis or inbreeding depression, maternal and/or paternal inheritance of organellar genomes, and methods of classical breeding (*e.g.* utility from employing mutagenesis with the species). The relevance of the information to the species' variability and the potential effects of transformation are paramount in deciding what to include, as the focus is not to provide this genetic characterisation for plant development.

Intraspecific crossing with both non-cultivated strains (*e.g.* weedy races) and among non-transformed cultivars is appropriately covered here (perhaps with a table or diagram), including any genetic or cytoplasmic constraints or limitations to crossing (*e.g.* cytoplasmic or nuclear sterility, incompatibility systems). Interspecific crosses are addressed in the following section.

> ***Rationale***: The information in this section includes genetic and breeding data, such as details of genomic or genetic stability (including gene silencing) and intraspecific outcrossing behaviour and potential, only to the extent that such information describes parameters that influence how genetic material (including new material) behaves in particular genetic backgrounds, and in outcrossing. Interspecific hybridisation is in a separate section (which follows) because intraspecific crossing is more likely (and familiar), and interspecific hybrids may bring in broader or more extensive concerns.
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> ***Examples***: OECD SHROB No. 9 (bread wheat, Sections III & V, pp. 13-17 & 20-24); No. 12 (Norway spruce, Section VI, pp. 21-23); No. 13 (white spruce, Section V, pp. 22-24); No. 14 (rice, Section VI, pp. 23-25); No. 24 (*Prunus* spp. – stone fruits, Section II, pp. 15-20); and No. 31 (sunflower, Section IV, pp. 27-28).

4. Hybridisation and introgression

4.1. Natural facility of interspecific crossing (extent, sterility/fertility)

Describe interspecific (including intergeneric) crosses observed under natural conditions. Provide a list and perhaps a diagram of the documented hybrids, *i.e.* the crossings that may occur unaided under usual environmental conditions — if the crossable relatives (other species) might be present. The information could include a discussion of ploidy (and ancestral/progenitor genomes). Provide an indication or review of the likelihood of first-generation (F_1) hybrids and later generations of these F_1 hybrids, and as well whether the F_1 hybrids may be bridges for genes to cross into other (non-parental) species. Rare plant species are considered here and in the following subsection. Indicate naturally hybridising species that are weedy (including invasive) in the list of hybridising species (detailed discussion of their weediness in a local environment would be covered in an environmental risk/safety assessment).

> ***Rationale***: The ability of a cultivated species to hybridise with other cultivated or wild species is a significant factor in determining whether genes or traits could be transferred to other species.
>
> ***Examples***: OECD SHROB No. 7 (oilseed rape, Section VII, pp. 18-21); No. 9 (bread wheat, Section V, pp. 20-24); and No. 16 (poplars, Sections III & VI, pp. 20 & 28-29).

4.2. Experimental crosses

Discuss the experimental data available on outcrossing under controlled conditions, and theoretical possibilities for and barriers to outcrossing. This information is in contrast to that in the previous subsection, which indicates the outcrossing to readily crossable relatives. Experimental data that is the result of forced crosses employing special techniques (*e.g.* embryo rescue) would be relevant only if such studies help to clarify degree of relatedness and likelihood of natural crossing. Theoretical considerations or experimental information might be, for example, on cytogenetic data and meiotic behaviour, or sexual incompatibility systems.

> ***Rationale***: Experimental data and theoretical considerations may broaden the understanding of potential (or as yet unknown) unaided (natural) gene transfer. The information and data are only relevant if unaided crossing in the field can occur.
>
> ***Examples***: OECD SHROB No. 8 (potato, Section VII, pp. 19-21); No. 13 (white spruce, Section VI, pp. 25-26); No. 16 (poplars, Section VI, pp. 28-29); and No. 22 (eastern white pine, Section IV, p. 17).

4.3. Information and data on introgression

Provide an indication or review of the likelihood of F_1 hybrids backcrossing into one or both parents. Provide information on both natural and experimental introgression (extensive backcrossing), and on the (types of) genes or the traits for which introgression has been demonstrated. For example, extensive backcrossing and introgression may be only in one direction, rather than into both parental lines or species' populations. Information should include the extent of likely natural (*i.e.* unaided) introgression or generations of experimental backcrossing, and the fertility and fecundity of the resultant plants.

> ***Rationale***: Of primary consideration is whether interspecific crossing will lead to the introgression of genes. Interspecific crossing is a necessary but typically not a sufficient step for considerable introgression to occur. Even if introgression occurs, it is not the presence but the expression of the gene or trait that may be of primary importance.
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> ***Examples***: OECD SHROB No. 7 (oilseed rape, Section VII, pp. 20-21); No. 24 (*Prunus* spp. – stone fruits, Section II, p. 30); and No. 31 (sunflower, Section IV, pp. 28-29).

5. General interactions with other organisms (Ecology)

5.1. Interactions in natural ecosystems, and in agronomic, silvicultural or other ecosystems where the species is cultivated or managed

Provide a general overview (including subsections as needed) of main functional ecological interactions of the species of interest within these natural and managed ecosystems and habitats, for example symbiotic relationships, food webs (*e.g.* fruit and seed consumers or predators), noxious/toxic or other important interactions with insects (*e.g.* chemical defense) and other animals, and with plants (*e.g.* allelopathy). Tritrophic interactions may also be considered. Subsections 1.3.2 and 1.3.3 list and briefly characterise the natural (unmanaged) and managed ecosystems and habitats in which the species of interest occurs. The importance of a pollination system to the animal pollinator is detailed here, whereas the importance to the plant is addressed in Subsection 2.2.2. A listing of pertinent pests and pathogens (and diseases) may be presented as an appendix, with only those that are critically relevant discussed here.

> ***Rationale***: The description of the basic general ecology of the species of interest is useful when determining the scope of interactions that may be used as a baseline for understanding the influences the cultivated plant may have on organisms that are in usual close contact.

A general understanding of the interactions of the species with other organisms will aid in determining whether any concerns may arise with a change in the genetics of the species.

Examples: OECD SHROB No. 7 (oilseed rape, Section VII & Appendix, pp. 21 & 29) and No. 13 (white spruce, Section VII, pp. 28-31).

6. Human health and biosafety

6.1. Plant characteristics relevant for human health

Provide brief information on major natural toxicants and common allergenic or medicinal properties of the plant. In some cases, it may be relevant to mention similar information from related species (*e.g.* glycoalkaloids in crossable wild relatives of *Solanum tuberosum* subsp. *tuberosum*, potato).

Rationale: This theme can be regarded as human ecology, a subset of Section V that warrants coverage separately. Baseline information is briefly described, relating to human health as it might be affected by cultivation of the plant (*e.g.* levels of latex or psoralen). Potential effects on human health would be thoroughly treated elsewhere, such as in an OECD plant compositional consensus document for dietary issues.

Example: OECD SHROB No. 8 (potato, Section IV, p. 14).

7. Additional information

The possibility is expressly left open for topics of additional information that is pertinent to environmental risk/safety assessment, as a section in the main text of the document, and/or as appendices.

8. References

As much as possible, the references should be peer-reviewed literature available internationally. After the references directly cited in the text, this section could include a subsection on additional useful references ‘for further reading’.

Example: OECD SHROB No. 7 (oilseed rape, Section IX, pp. 27-28).

Appendix I – Common pests and pathogens

Provide a list of causative organisms for diseases (pathogens) and pests that commonly occur in the crop under agronomic, silvicultural, or equivalent conditions.

Rationale: Provide as considered useful for risk/safety assessment rather than usual production management. Critically important organisms and ecological relationships (*e.g.* a virus disease that is a principal management issue) are covered in Section V. The risk/safety assessment would then consider whether the transformation in the crop would be of environmental concern.

Examples: OECD SHROB No. 18 (sugar beet, Appendix, pp. 32-37 and No. 31 (sunflower, Section V & Appendices 1 & 2, pp. 31 & 37-47).

Appendix II – Biotechnological developments

General information on the kinds of traits being introduced into the species may be included. Provide information directly necessary for defining the scope or detail of biological information that would be useful. For example, transgenes under experimental development for a crop might result in a change in environmental fitness or range and habitats of the plant or its relatives (*e.g.* disease resistance, and

drought, frost or salinity tolerance). Other biotechnological developments (*e.g.* to assist in marketing) may not be pertinent to address here.

> ***Rationale***: An overview of biotechnological developments may help to assure that the biological information included in a consensus document is pertinent to the environmental risk/safety assessments anticipated. Consensus documents that include the biotechnological developments to bring traits into the crop can be quite useful in explaining the relevance of assessing certain kinds of biosafety information.
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> ***Examples***: OECD SHROB No. 14 (rice, Appendix III, pp. 42-45) and No. 27 (maize, Appendix A, pp. 39-41).

Part 1.

Consensus documents on the biology of crops

Section 1.
Cotton (*Gossypium* spp.)

1. Introduction: Description and uses

Generally cotton refers to four species of the genus *Gossypium* L. apparently domesticated independently in four separate regions, in both the Old World and the New World (Sauer, 1993; Brubaker *et al*., 1999c). The word is derived from the Arabic "quotn", "kutum" or "gutum" and refers to the crop that produces spinnable fibres on the seed coat (Lee, 1984; Smith, 1995). *Gossypium* (cotton) comprises approximately 50 species worldwide in the arid to semi-arid tropics and subtropics (Fryxell, 1992; Wendel and Cronn, 2003) (Appendix 1). The cultivated species are grouped according to their level of ploidy:

- Diploids (AA) ($2n = 2x = 26$): *Gossypium herbaceum* L. and *Gossypium arboreum* L.;
- Tetraploids (AADD) ($2n = 4x = 52$): *Gossypium barbadense* L. and *Gossypium hirsutum* L.

Of these four cultivated species, *Gossypium hirsutum* and *G. barbadense* account for 95% or more of world cotton production (Jenkins, 1993; May and Lege, 1999; Zhang *et al*., 2008). Throughout this document the word cotton often is used to include both of these dominant crop species, but where the differences are significant and relevant, individual species are differentiated. *Gossypium hirsutum*, widely known as upland cotton or sometimes American, Mexican or Acala cotton, accounts for over 90% of the production. *Gossypium barbadense*, which accounts for some 5%, is commonly known as extra long-staple cotton or Pima or Egyptian cotton.

1.1. Description

A full description of the cotton plant is provided by Oosterhuis and Jernstedt (1999). Potentially perennial but typically grown commercially as annual crops, both species include plants that can grow into a bush or small tree. *Gossypium hirsutum* grows to 1.5-2 (-5) m tall and *G. barbadense* to 3 m. However, both are typically cultivated as plants approximately 1-1.5 m high, with destruction after harvesting the fruits for lint and seed. The plants have a taproot which can reach a depth of 1-3 m depending on the variety's age, soil characteristics and the management regime, and many lateral roots. Cotton plants have a prominent upright main stem, monopodial and indeterminate in growth, which bears the branches and leaves (Hanan and Hearn, 2003; Marur and Ruano, 2001, 2004; Ritchie *et al*., 2007). The number and length of axillary branches vary depending on the variety and environmental conditions.

The leaves are arranged alternately in a spiral around the axis of the main stem or branch. Phyllotaxis is 3/8 back over the last leaf. Leaves vary in size, shape, texture and hairiness. Most lamina are palmate, with several sinuses and lobes more or less defined, varying in shape from rounded to acute. The leaves are usually large and relatively hairy (Hu and Zhao, 1992; Susín *et al*., 1988; Bourland *et al*., 2003), although there are hairless, smooth-leaved varieties (Delattre, 1992); there are a large number of stomata mostly on the abaxial surface. The petiole is normally as long as the leaf lamina, and flanked by two stipules (persistent or falling early) at its juncture with the stem. Leaf characteristics differ considerably between *G. hirsutum* and *G. barbadense* (Wise *et al*., 2000).

Generally the leaves of *G. barbadense* are 3- to 7-lobed (Fryxell, 1984, 1992) and mature leaves are larger and thinner than in *G. hirsutum*. The generally 3- to 5-lobed leaves of *G. hirsutum* are mostly flat throughout development and diaheliotropic, tracking the sun to maximise light absorption. *Gossypium barbadense* leaves exhibit significant cupping or curling which reduces photoinhibition and allows for more light penetration into the plant's canopy over the course of the day. Although *G. barbadense* leaves have higher stomatal density than in *G. hirsutum*, the stomata are smaller, so there is less stomatal surface area per leaf (Lu *et al*., 1997; Wise *et al*., 2000).

Two types of branches are produced: vegetative or monopodial (continuing growth from terminal bud), and fruiting or sympodial (continuing growth from lateral bud). In terms of structure, the vegetative branches are much like the main stem. Flowers are produced only after secondary or tertiary branching. The fruiting branches develop mainly from the first axillary bud of the upper nodes of the plant. They are smaller in diameter and more horizontal than vegetative branches. The sympodial development of fruiting branches gives them a slightly zigzag appearance in contrast to the fairly straight vegetative branches.

Each fruiting branch produces six to eight solitary flower buds (called "squares") (Hutmacher, 2004). The bud is a pyramidal structure, and has three large laciniate triangular bracts surrounding the flower (as an epicalyx or involucre). Just within these bracts is the true calyx, which consists of five short sepals fused together into a cup at the lowest, widest part of the flower (McGregor, 1976). Inside the calyx are five petals separated except at the base, which form the corolla. Inside the corolla (and fused to its base) is a staminal column with many (50 to 125 or more) shortly stalked unilocular anthers distributed along it; this tubular column surrounds the elongated style. The style terminates in a club-shaped lobed stigma exserted somewhat beyond the end of the staminal column and positioned sometimes below but usually beyond the distal-most anthers. Unlike *G. hirsutum*, the *G. barbadense* stigma extends well beyond the anthers (McGregor, 1976), which increases the potential for cross-pollination.

Gossypium hirsutum flowers are of a uniformly creamy white to pale yellow colour, with cream pollen, and secrete a low volume of nectar; *G. barbadense* flowers are yellow with maroon blotches at the inner base (that serve as a nectar guide), have orange pollen, and produce more nectar of a lower sugar concentration (McGregor, 1976; Moffett, 1983).

The pistil's basal rather conical portion is the superior ovary, consisting of three to five carpels or locules. The ovary of *G. hirsutum* often has four or five carpels, each with 8 to 12 ovules, which are aligned in two parallel vertical grooves along the axile placenta (the central column where the carpels join). The capsular fruit, which splits open at maturity, is referred to as the "boll"; it is spherical or ovoid with a beak at the top, and differs among species in shape, size and colour. *Gossypium hirsutum* bolls are usually of a pale-green colour and relatively smooth with few punctate gossypol glands; those of *G. barbadense* are of darker green and conspicuously pitted with numerous glands.

The fertilised ovule develops into a seed, the epidermis of which gives rise to many single-celled seed hairs (epidermal trichomes) of two kinds: long hairs referred to as "lint" and coarser short hairs referred to as "fuzz" or "linters" (Applequist *et al*., 2001; Zhang *et al*., 2007). Differentiation between the hair types has been maximised to produce the white lint in elite cultivars. Cottonseed oil accumulates in the cytoplasm of the seed embryo cells (Gotmare *et al*., 2004); it is not associated with the gossypol glands. Descriptions of the seed are provided by Hopper and McDaniel (1999) and Ritchie *et al*. (2007), and an overview of advances in knowledge of the development of seed and fibre is provided by Ruan (2005).

The cotton plant is characterised by the presence of small lysigenous cavities known as "gossypol glands", which are found in most tissues except xylem. They contain terpenoid aldehydes in an oily water-soluble matrix, which forms an essential oil known as "gossypol" (Khan *et al*., 1999). Gossypol is toxic to non-ruminant mammals, birds, and many insects and microbes, thus providing a constitutive as

well as inducible defense against herbivory and microbial attack. Glandless cotton has been developed for food purposes, but the plants are more susceptible to damage (Lusas and Jividen, 1987; Delattre, 1992). Cotton plants also have usually a single extrafloral nectary on the midvein of the underside of the leaf blade (somewhat outward from its base), and nectaries as well at the base of each involucral bract (on the outer and the inner sides). Their sugary nectar can draw insects that may provide defense against herbivory or increase it, depending on the overall management or ecological context (Adjei-Maafo and Wilson, 1983a, 1983b; Wäckers and Bezemer, 2003; Wäckers and Bonifay, 2004; Röse *et al*., 2006).

1.2. Uses

The main product of the cotton plant is fibres — their qualitative characteristics have been valued and analysed over many centuries (Vreeland, 1999; Wakelyn *et al*., 2007a, 2007b). Cotton crops provide the world's premier source for natural fibres, which are mainly used in the manufacture of a large number of textiles. Low-quality fibre can be used for manufacturing felt, mattress filling and special paper, and the processed celluose is used for various consumer products such as toothpaste, lipstick, ice cream and mayonnaise. There also is a range of applications in the chemical industry.

The seeds, even though extensively and intensively used worldwide as well, tend to be regarded as a secondary product or byproduct. The seeds are used to obtain edible oil, which is considered to be of very good quality within the range of vegetable oils (O'Brien *et al*., 2005); as chaff for livestock feed; and as high-protein cake and flour, which are used mainly for livestock feed (Section VII). The flour is sometimes used for human consumption (in low amounts, or after extraction of the gossypol or from gossypol-free varieties). Gossypol has been used as a male contraceptive (Coutinho, 2002). Cottonseed oil is of interest as a lubricant and a biofuel (Karaosmano lu *et al*., 1999).

The nations producing the most cotton lint and cottonseed in 2006 were China, USA, India and Pakistan (Table 1). *Gossypium hirsutum* is called long-staple cotton, and the characteristic length of its fibres is 22-36 mm. *Gossypium barbadense*, extra-long-staple cotton, has fibres usually over 35 mm in length; it is cultivated mainly in Egypt, Peru, Sudan, USA and some Central Asian countries. The combination of best agronomic practices, an increasing level of qualification of farmers, and application of technological advances has boosted unit yields. The highest yields in 2006 were 1861 kg/ha of lint, and 2793 kg/ha of cottonseed (Table 1).

Table 1. Countries with the highest yields and/or the most production of cotton lint and/or cottonseed in the 2006 growing season (FAO, 2007)

2006	Yield: kg/ha		Production: tonnes	
	lint	cottonseed	lint	cottonseed
Australia	1861	2631		
Brazil			1,210,000	1,784,672
Cambodia		2712		
China	1243	2485	6,730,000	13,460,000
India			3,563,880	7,127,760
Israel	1717	2732		
Pakistan			2,186,800	4,065,200
Syria	1409	2793		
Turkey	1646	2469	900,000	1,350,000
USA			4,498,000	6,665,900
Uzbekistan			1,171,000	2,376,200

2. Taxonomy and centers of origin, diversity and domestication

2.1. Taxonomy

The genus *Gossypium* L. is a member of the family Malvaceae, subfamily Malvoideae and tribe Gossypieae, which has about nine genera (*cf.* Seelanan *et al*., 1997). The genus emerged as a separate evolutionary lineage some 11-14 million years ago (Senchina *et al*., 2003; Wendel and Cronn, 2003). *Gossypium* has three main centres of biological diversity: Africa and the Arabian Peninsula, Australia, and Mexico (see Appendix 1); and three centres of domestication: Africa and Asia, Mesoamerica (*i.e.* Mexico and Central America), and South America. The two diploid cultivated species (*G. herbaceum* and *G. arboreum*) are from the Old World (Africa-Asia). The two tetraploid cultivated species are from the New World — Mesoamerica (*G. hirsutum*) and South America (*G. barbadense*). Although the record is less clear for Asia, each of the four cultivated species may have been domesticated independently (Sauer, 1993; Brubaker *et al*., 1999c).

About 50 species of *Gossypium* are generally recognised (see Appendix 1) (Fryxell, 1992; Percival *et al*., 1999), although some taxonomic study is still needed. About 45 of the species are diploids, which are divided into three geographical groups and corresponding subgenera; 5 species are tetraploids, which are included in one subgenus (Fryxell, 1984, 1992; Wendel and Cronn, 2003; Cronn and Wendel, 2004):

- African-Arabian group (subgenus *Gossypium*): about 14 species (possibly fewer); naturally distributed principally in Africa, also on the Arabian Peninsula, and reaching Pakistan and perhaps farther eastward (Vollesen, 1987; Fryxell, 1992; Stanton *et al*., 1994; Wendel and Cronn, 2003). One species is endemic to the Cape Verde Islands;

- Australian group (subgenus *Sturtia*): about 17 species (16 taxonomically described), naturally distributed mostly in the northwestern Kimberley region, but also in the northern tropics and in the central arid zone, with one species reaching the eastern warm-temperate zone (Fryxell *et al*., 1992; Seelanan *et al*., 1999; Brown and Brubaker, 2000);

- American group (subgenus *Houzingenia*): about 14 species (13 taxonomically described), 12 occurring naturally in western Mexico (one reaching northward into Arizona, USA) and one each in the Galapagos Islands and in Peru (Fryxell, 1988; Small and Wendel, 2000; Cronn *et al*., 2003; Álvarez *et al*., 2005; Álvarez and Wendel, 2006; Ulloa *et al*., 2006);

- American and Pacific group (subgenus *Karpas*): 5 tetraploid species; 3 naturally distributed in the Americas (one in Mesoamerica, 2 in South America), and one each in the Galapagos Islands and in the Hawaiian Islands (Small *et al*., 1998; Wendell and Cronn, 2003).

The diploid species are placed into eight cytogenetic genome groups, and the tetraploids in one group (Endrizzi *et al.*, 1985; Stewart, 1995; Wendel and Cronn, 2003), shown in Table 2.

The species of the genus usually recognised taxonomically are given in Appendix 1 along with their natural geographic distributions, designated genomes, and general groupings phylogenetically (Endrizzi *et al.*, 1985; Fryxell, 1992; Percival *et al.*, 1999; Wendel and Cronn, 2003).

Table 2. Genome groups of *Gossypium*

Genome group	Number of species	Native distribution
A	**2**	Africa, possibly Asia
B	**3**	Africa (including Cape Verde Islands)
E	**7+**	NE Africa, Arabian Peninsula, SW Asia
F	**1**	East Africa
C	**2**	Australia
G	**3**	Australia
K	**11 (or 12)**	NW & N Australia
D	**13 (or 14)**	Americas (primarily Mexico, also Peru), Galapagos Islands
AD	**5**	Americas, Galapagos Islands, Hawaiian Islands

2.2. Major evolutionary events

DNA-sequence phylogenetic data suggest that 6-7 million years ago, following a trans-oceanic dispersal event, a D genome diverged from the African lineage that eventually gave rise to the A genome, and became a separate lineage in the Americas (primarily Mexico) (Senchina *et al*., 2003; Wendel and Cronn, 2003; Cronn and Wendel, 2004; *cf.* Graham, 2006). From another long-distance dispersal event 1-2 million years ago, a tetraploid originated through hybridisation of an African plant of the A-genome group, perhaps most closely related to the present-day species *G. herbaceum*, with a resident plant of the D-genome group, most closely related to the present-day species *G. raimondii* (Wendel *et al.*, 1992; Senchina *et al*., 2003; Wendel and Cronn, 2003; Kebede *et al.*, 2007). The nascent disomic AD allotetraploid from that single polyploidisation event evolved into the five present-day tetraploid species (Endrizzi *et al.*, 1985; Cronn *et al.*, 1999).

Gossypium raimondii, a rare species of northwestern Peru, is considered to be the diploid with the genome that has retained the most similarity to this ancestral D-genome species (Liu *et al*., 2001; Guo *et al*., 2007); it is one of the more recently evolved of the DD species, having diverged in isolation as a result of a long-distance dispersal event from Mexico (Wendel and Cronn, 2003; Álvarez *et al*., 2005). *Gossypium raimondii* has genetic similarities with *G. gossypioides*, which is a local species in southern Mexico (Oaxaca) and considered evolutionarily basal within the New World diploids. Nonetheless, *G. gossypioides* has a strikingly unusual history, involving several natural interspecific hybridisations — apparently including introgression from yet another African immigrant (evolutionarily prior to divergence of the African B, F and A genomes) (Cronn *et al*., 2003; Cronn and Wendel, 2004; Álvarez *et al*., 2005; Guo *et al*., 2007).

Soon after separation of the D-genome lineage, African *Gossypium* further diverged with a long-distance dispersal event and establishment of an Australian lineage (which evolved into the three genome groups C, G and K). The lineage in Africa evolved further into four genome groups, first with divergence

of the E-genome lineage, subsequently the B-genome lineage, and most recently the F- and A-genome lineages (Cronn *et al*., 2002; Cronn and Wendel, 2004).

Chloroplast and mitochondrial DNA are inherited maternally in *Gossypium* (Small and Wendel, 1999). The means and route of the relatively recent long-distance dispersal of the A-genome fruit/seed(s) and place of origin of the progenitor allotetraploid continue to be researched (Wendel and Cronn, 2003). The A and D genomes of the South American tetraploid *Gossypium mustelinum* (northeastern Brazil) are genetically most similar to the ancestral type that differentiated into the five present-day widely dispersed tetraploids (Wendel *et al*., 1994). The disseminule of an AA species may have travelled *via* sea currents from Africa to the Americas (Stephens, 1966; *cf.* Renner, 2004). Then, pollen from an American diploid (DD) species fertilised the immigrant, and chromosome doubling produced the original AADD tetraploid; the AA coloniser either did not persist or possibly established a small population that went extinct.

2.3. Domestication and early cultivation

2.3.1. Old World diploids

The cultivated AA diploids of the Old World are typically short-staple cottons, with a fibre length of less than 23 mm. These cottons can be important regionally, and still may be preferred especially in harsh or dry growing conditions (Basu, 1996; Rajendran *et al*., 2005). The two species (with *G. arboreum* as the larger crop) now provide only about 4% of world production, however, and are largely displaced in much of the Old World by the New World tetraploids.

Both AA species have been studied thoroughly using many methodologies (*e.g.* agronomic, morphological, cytogenetic, genetic, molecular) and are definitely biologically distinct, although their differences are observable in divergent suites of shared characters rather than by obvious diagnostic characters (Wendel *et al*., 1989; Stanton *et al*., 1994; Rana and Bhat, 2004; Gao *et al*., 2005; Desai *et al*., 2006; Kebede *et al*., 2007). *Gossypium herbaceum* typically has less anthocyanin (so becomes less "sun-red"), shorter leaves with shallower sinuses and broader lobes, epicalyx bracts broader and with twice as many apical teeth or lobes, smaller flowers, more rounded bolls, larger seeds and finer lint than *G. arboreum* (Abedin, 1979; Stanton *et al*., 1994). *Gossypium arboreum* has an interchromosomal translocation in comparison to the generically typical arrangement in its sister species *G. herbaceum* (Song *et al*., 1991; Desai *et al*., 2006).

Wild (non-feral) *Gossypium herbaceum* subsp. *africanum* occurs naturally in the savanna biome across southern Africa (Vollesen, 1987; Wendel *et al*., 1989; *cf*. Jürgens, 1997), whereas the domesticated plant *G. herbaceum* subsp. *herbaceum* is found disjunctly farther to the northeast, being grown mainly from Ethiopia to Central Asia, northwestern China and India (Wendel *et al*., 1989; Guo *et al*., 2006). *Gossypium arboreum* is grown primarily across Asia farther to the east, from India (where it is cultivated more than *G. herbaceum*) to Korea (Wendel *et al*., 1989; Basu, 1996; Guo *et al*., 2006). The original ranges or centres of domestication of *G. arboreum* and *G. herbaceum* subsp. *herbaceum* are unclear (Wendel *et al*., 1989; Brubaker *et al*., 1999c).

The archaeological evidence of early cotton use in the Old World is not at the species level. Circumstantially, *G. herbaceum* subsp. *herbaceum* might be from Southwest Asia (*e.g.* Abedin, 1979; Fuller, 2006) and *G. arboreum* possibly from India (Santhanam and Hutchinson, 1974). Early utilisation and probable cultivation of cotton have been reported from Pakistan before 5000 BC (Moulherat *et al*., 2002); North Arabia (Jordan) about 4450-3000 BC, but perhaps present by trade according to Betts *et al*. (1994); South India in 1500 BC (Fuller *et al*., 2004); and southern Libya in 900 BC – 500 AD (Pelling, 2005). Both species may have reached North Africa and Greater Mesopotamia before earliest historic times (Watson, 1983; Potts, 1997). Cotton and weaving are mentioned in early texts in Asia (*e.g.* India and China) and the Mediterranean region.

2.3.2. New World tetraploids

2.3.2.1. *Gossypium barbadense*

Originally wild (*i.e.* non-feral) *Gossypium barbadense* is considered to occur naturally in the dry coastal region of northern Peru and southern Ecuador (Schwendiman *et al*., 1985; Percy and Wendel, 1990; Westengen *et al*., 2005). The earliest archaeological evidence of the cultivation of *G. barbadense* dates to 5500 BC in northwestern Peru (Dillehay *et al*., 2007). This cotton species was apparently domesticated and grown extensively in the northwestern Peruvian and southwestern Ecuadorian region, and was spread into the Andes and farther eastward in South America, and onward to the Caribbean and southern Mesoamerica (Brubaker *et al*., 1999c; Vreeland, 1999; Pearsall, 2003; Westengen *et al*., 2005; Johnston *et al*., 2006; Dillehay *et al*., 2007).

2.3.2.2. *Gossypium hirsutum*

Gossypium hirsutum is native in Mesoamerica, but its natural range as well as its centres of domestication and development are obscured by millennia of use (Stephens, 1967; Lee, 1984; Jones *et al*., 1989; Wendel *et al*., 1992; Brubaker and Wendel, 1994; Stark *et al*., 1998; Brubaker *et al*., 1999c; Whitmore and Turner, 2002). Collections from this large and diverse region, even of free-living plants, generally have varying characteristics of domestication rather than of a genuinely wild species — for example, having larger and more flaring capsules, larger seeds, loss of seed dormancy and of day-length sensitivity, and more and finer lint which is also more easily detachable (Hutchinson, 1951; Stephens, 1958; Fryxell, 1979). The oldest archaeological remains of *G. hirsutum*, dating to 3500-2300 BC, seem to be domesticated forms and were found in the Tehuacan Valley of central Mexico (Smith and Stephens, 1971; WWF and IUCN, 1997; *cf*. Pope *et al*., 2001).

Through intensive study of germplasm collections from the widespread complex in the region, Hutchinson (1951) distinguished six domesticated races (not botanical varieties) and one wild race based mainly on their habit and morphology, and found that these races had generally distinct geographic distributions, with the most differentiation of the domesticated types in southern Mexico:

- *morrilli* — inland montane, southern Mexican plateau and northward
- *palmeri* — Pacific slope, southern Mexico west of Isthmus of Tehuantepec
- *richmondi* — Pacific slope in Gulf of Tehuantepec region
- *punctatum* — Yucatan Peninsula, and northward on Atlantic slope, to Florida (USA) and Bahamas
- *yucatanense* — wild, northwestern coast of Yucatan Peninsula
- *latifolium* — Guatemala (both slopes) and southernmost Mexico (Chiapas), nearby areas
- marie-galante— northern Central America (Guatemala) southward to Colombia on both coasts, Caribbean region (Antilles) and northeastern Brazil

Research using isozymes (allozymes) only confirmed the distinction of Caribbean marie-galante (Wendel *et al*., 1992); RFLP and SSR analyses have supported recognition of additional landraces (Brubaker and Wendel, 1993, 1994; Lacape *et al*., 2007). The next most distinct lineage is *punctatum*, and then *latifolium*. Of these three major domesticated lineages (Iqbal *et al.*, 2001), marie-galante is a perennial, from which mocó cotton is still cultivated in Brazil (Freire and Moreira, 1991; Moreira *et al*., 1995; Johnston *et al*., 2006); punctatum and latifolium are annualised. The original Amerindian Hopi Moencopi cotton (Arizona, southwestern USA) is considered to belong to *punctatum* (Lee, 1984). Race *palmeri* is closely related to race *latifolium*, and the SSR research found *morrilli* and *richmondi* to

be distinct lineages that are close to *palmeri*. *Gossypium lanceolatum* is not a distinct species but a local Mexican landrace, in the domesticated race *palmeri* (Brubaker and Wendel, 1993).

The modern studies have maintained yucatanense as a truly wild ecotype, sprawling plants which are isolated in populations along the northwestern coastal strand of Mexico's Yucatan Peninsula (Hutchinson, 1951), but perhaps occur naturally eastward even as far as Guadeloupe island in the Lesser Antilles (Ano *et al*., 1982; Lacape *et al*., 2007). Wild-like or wild populations of *G. hirsutum* are widely scattered and rare, growing near beaches or confined on small islands; such populations do not occur inland from the coast, but feral plants are found inland (Brubaker and Wendel, 1994).

2.3.2.3. Origin of upland cotton

The upland type of *Gossypium hirsutum* and derived varieties are the mainstay of the worldwide industry (May and Lege, 1999). Upland cotton is thought to have its centres of origin and diversity near the border of Mexico with Guatemala (Hutchinson *et al.*, 1947; Hutchinson, 1951; Brubaker and Wendel, 1994), apparently within *G. hirsutum* race latifolium. This type appears to have become prevalent in southeastern USA around the middle of the 18th century (Phillips, 1976; Smith *et al*., 1999). Somewhat later (about 1785), Sea Island cotton (*G. barbadense*) from the Bahamas was widely grown in the U.S. Atlantic coastal regions of Georgia and South Carolina (Brown and Ware, 1958; Smith *et al*., 1999).

The first seed stocks of the cotton arriving in USA were called Georgia green seed (Hutchinson *et al.*, 1947). This type normally grew from a ginned seed with persisting green-coloured fuzz; the Sea Island-type had hairless or bare ginned seed, placing it among the "black-seed" cottons. Sea Island cotton was cultivated in the lowlands, whereas green-seed cotton was more inland and consequently became known as upland cotton (Smith *et al*., 1999). In the 18th century similar stocks also were taken to Southeast Asia (Lee, 1984). In the 19th century further Mexican green-seed cultigens were introduced into the USA and came to be known as varieties of upland cotton (Brown and Ware, 1958; Smith *et al.*, 1999). Cotton was introduced into many tropical and subtropical countries during the U.S. civil war period (1861-1865), including Australia (Constable *et al*., 2001). Crosses between many varieties of introduced cottons have caused the worldwide expansion of upland cotton (Lee, 1984; Smith *et al.*, 1999; Iqbal *et al.*, 2001). The intensive modern cotton industry only became established in Australia in the 1960s (Hearn and Fitt, 1992).

3. Agronomic requirements and practices

3.1. Abiotic environment

Although originating in the tropics and subtropics, cotton has come to be cultivated mostly in subtropical and warm-temperate zones — regions which provide more than half of world production (*cf.* Table 1). For this geographical shift to be possible as a crop, the species' photoperiod needed to change — the naturally short-day plant became a day-neutral plant that could be cultivated as an annual crop in the longer summers (Smith *et al.*, 1999).

3.1.1. Climate

The geographical distribution of the cotton crop reaches 43-45º N (Central Asia, China) but is primarily grown between 37º N and 32º S (*e.g.* Australia, northern Argentina). Temperature is the main climatic factor determining the geographic range in which cotton can be grown (Freeland *et al*., 2006). Generally the plant is highly sensitive to temperature (Reddy *et al.*, 2006). Seeds do not germinate, nor seedlings begin their activity, until the temperature rises to 15ºC; they are delayed above 38ºC. *Gossypium barbadense* seedling development in the first 2 weeks is generally not sensitive to temperatures between 15ºC and 40ºC, but 3 weeks after emergence the young plants are generally more sensitive than *G. hirsutum* (*e.g.* having fewer fruiting branches at 35º/27ºC than at 30º/22ºC, and

no fruiting branches at 40º/32ºC) (Reddy *et al*., 1992b). Nonetheless, there are *G. barbadense* cultivars with heat tolerance close to that of *G. hirsutum* (Cornish *et al*., 1991; Radin *et al*., 1994; Srivastava *et al.*, 1995). The optimum daytime temperature range for *G. hirsutum* is 30-35ºC, with a loss of fruit above 35ºC, and with a 50% yield reduction at 25ºC (Reddy *et al*., 1992a).

After planting *G. hirsutum*, 180-200 frost-free days are needed for normal development, with an average of 150 days of suitable temperatures (*i.e.* 1200 heat units above 15.5ºC accumulated) (Duke, 1983); for *G. barbadense*, 200-250 days are needed (Unruh and Silvertooth, 1997). Although the values differ among varieties, from the planting of cotton to 60% boll opening about 2050 heat units (degree-days or day-degrees) are the required minimum (Ritchie *et al*., 2007; OGTR, 2008).

3.1.2. Soil and water

Cotton plants are cultivated in a wide variety of soils, but the crop develops best in deep arable soils with good drainage, filled with organic matter and with a high moisture-retention capacity. Yet cotton is grown in cracking clays in some countries. Cotton is a salt-tolerant plant, with *G. barbadense* more salt tolerant than *G. hirsutum* (Ashour and Abd-El'Hamid, 1970). Salinity stress nonetheless has adverse effects on germination and emergence (Ashraf, 2002); the most common stress effect is general stunting of the plant's growth (Cothren, 1999).

Irrigation allows cultivation in poor-quality soils, with necessary moisture and nutrients provided in a controlled way. Irrigation is carried out mainly at ground level, flooding the furrows, which requires adequate leveling of the field.

At least 500 mm of rainfall is required during the growing season for dryland (non-irrigated) cotton crops. Cotton is also grown as an irrigated crop, and it is still common to use sprinklers with fixed or mobile outlets, with total coverage. The use of drip irrigation has increased, which allows a saving in water and use of soil that is less than optimum (due to its sloping surface, lack of fertility, or an excessively high salt content). Generally *G. barbadense* has similar water requirements to *G. hirsutum*; the longer growing season of *G. barbadense* may however require additional irrigation to mature its later-set bolls (Silvertooth, 2001). Carefully timing the application of water optimises the plant's vegetative growth, flowering and boll production (McWilliams, 2003). Flower and boll formation in *G. barbadense* are enhanced by a short duration of sunshine, high minimum humidity and low evaporation rate (Sawan *et al*., 2004, 2005).

3.2. Cultivation

3.2.1. Sowing

Sowing cotton is an operation that requires careful soil preparation, in order to achieve sufficient moisture, to allow favourable germination and rapid development of roots. Pre-prepared ridges are recommended, to obtain adequate drainage of water and maintain optimum temperature. The optimal sowing date is determined by temperature. Temperature is the dominant factor affecting the cotton plant's development and yield (ACCRC, 2001; Robertson *et al*., 2007). The sowing can commence when the minimum soil temperature at a depth of 10 cm exceeds 14ºC for at least 3 consecutive days. Lint yield is adversely affected if *G. hirsutum* is planted too early (due to cold temperatures) or too late (due to a shortened growing season) (Kittock *et al*., 1987). Since *G. barbadense* prefers a longer growing season (> 200 days) for yield increase it is more sensitive to delays in planting (Kittock *et al*., 1981, 1985; Silvertooth, 2001).

Acid-delinted seeds are treated with fungicides against seedling disease complexes and with insecticides to protect seedlings from sucking insect pests and wireworm soil insects (beetle larvae), and are sown at a rate of 20-25 kg/ha. Sowing is carried out with precision machines, in 4 or 6 rows with

spacing of 95-105 cm to adapt to mechanical collection of bolls. A proportion of the crop is sometimes planted in an Ultra Narrow Row configuration, for example with row spacing of 30 cm and a target population of 25 to 30 plants per m^2.

Germination takes place under favourable conditions of temperature and humidity. Cotton remains in a seedling stage longer than some other crop species. Sometimes the seeds are sown under plastic, a technique that ensures high germination and enhances floral development. In Spain this practice is totally mechanised, and (depending on the year) may be applied on more than two thirds of the sown surface area.

3.2.2. Fertilisers

Phosphorus (P) and potassium (K) are applied according to soil content, at the base of the plants. Nitrogen (N) is distributed between the plant's base and top with an application rate of up to 200 or 250 units of N, depending on the environmental and crop conditions. *Gossypium barbadense* requires slightly more N, P and K per unit of lint produced (Unruh and Silvertooth, 1996). However it is more sensitive to a slight excess of N, which can stimulate higher vegetative growth and delay maturity (Silvertooth *et al*., 1995).

3.2.3. Growth regulators

Growth regulators or herbicides may be applied to control vegetative growth and development of the crop, and to assist in its harvest. Examples include a growth regulator applied early in bud/flower production to reduce internode length, increase boll retention, promote early flowering and/or produce a more open canopy, and a growth regulator applied to the crop close to harvest time to stimulate opening of mature bolls and to defoliate (Cothren, 1999; Ritchie *et al*., 2007).

3.3. Biotic environment

3.3.1. Vesicular arbuscular mycorrhizae (VAM)

In most soils successful growth of cotton crops depends on the interaction with mycorrhizal fungi (Youssef and Mankarios, 1974; ACCRC, 2002; Nehl and Allen, 2004). The fungi (*e.g. Glomus mosseae*) grow intercellularly in the root cortex. They form vesicular arbuscules with the plasma membrane in the cortical cells, which are the sites of mineral exchange from the fungus to the plant and carbohydrate exchange from the plant to the fungus. Improvement in phosphate uptake is the main advantage for the cotton plants. VAM fungi also can reduce incidence and severity of diseases of the plants (Hu and Gui, 1991; Liu, 1995).

3.3.2. Pests

Pest and disease control is a highly significant cost (Oerke, 2006), and repeated applications of insecticides and fungicides may be employed. Numerous insect pests feed on cotton (Matthews, 1989; Delattre, 1992). Insects that are natural enemies of the pests are encouraged as part of integrated pest management systems. Cultivation of varieties with genetically engineered resistance to some insects has been a major advance in management of the crop against some major pests.

Arthropod pests may affect boll production or fibre quality. Aphids (*Aphis gossypii*, *A. craccivora*, *Myzus persicae*) and the silverleaf whitefly *Bemisia tabaci* are the usual pests which most affect fibre quality, producing sticky cotton with dark stains if not controlled late in the season. The pink bollworm *Platyedra gossypiella*, various Hemiptera such as *Lygus* bugs, and various mites such as the two-spotted spider mite *Tetranychus urticae* also diminish fibre yield and quality. Important pests affecting boll production include cotton bollworms (*Helicoverpa armigera*, *H. punctigera*), and the spiny bollworm

Earias insulana mainly reduces fibre production. Lepidoptera such as the beet armyworm *Spodoptera exigua* and Egyptian cotton leafworm *Spodoptera littoralis* are less common. The cotton boll weevil *Anthonomus grandis* is a highly aggressive pest in some areas. Other important pests include the leafhopper *Empoasca lybica* (the cotton jassid).

Gossypium barbadense has some resistance to *Earias* spp. (Reed, 1994), jassids (Matthews, 1994) and spider mites, possibly due to its higher content of gossypol than *G. hirsutum* (engonca *et al*., 1986; Matthews and Tunstall, 1994).

Nematodes that may be damaging in some regions or areas include particularly the root-knot nematodes *Meloidogyne incognita* (as well as *M. acronea*), reniform nematode *Rotylenchulus reniformis*, lance nematodes *Hoplolaimus columbus* (and several other spp.) and sting nematode *Belonolaimus longicaudatus* (Robinson, 1999), and as well associated ring nematodes *Criconemella* spp., spiral nematodes *Helicotylenchus* spp., needle nematode *Longidorus africanus*, stunt nematodes *Merlinius* spp. and *Tylenchorhynchus* spp., stubby-root nematodes *Paratrichodorus* spp., pin nematode *Paratylenchus hamatus*, lesion nematodes *Pratylenchus* spp., spiral nematodes *Scutellonema* spp., and American dagger nematodes — the *Xiphinema americanum* group.

3.3.3. Diseases

Among cotton diseases (Kirkpatrick and Rothrock, 2001), the most prominent is Verticillium wilt, which is caused by *Verticillium dahliae*. This fungal disease is extensively distributed in areas where *G. hirsutum* is cultivated; conventionally bred resistant varieties are available in Australia (OGTR, 2008). Other diseases, such as damping off, are caused by a complex of pathogens that have a major effect on the crop. The main causative agents are *Rhizoctonia solani*, *Pythium ultimum*, *Thielaviopsis basicola* and *Fusarium* spp.

Many other fungi have been associated with diseases of cotton, either as the primary agents or secondary invaders: *Alternaria* spp., *Ascochyta gossypii*, *Aspergillus flavus*, *Brasilomyces malachrae*, *Cladosporium herbarum*, *Fusarium* spp. (*e.g. F. oxysporum* f. sp. *vasinfectum*), *Glomerella gossypii* (anamorph *Colletotrichum gossypii*), *Lasiodiplodia theobromae* (synonym *Diplodia gossypina*), *Leveillula taurica* (anamorph *Oidiopsis haplophylli* [synonyms *O. gossypii*, *O. sicula*]), *Macrophomina phaseolina*, *Mycosphaerella* spp., *Nematospora* spp., *Phakopsora gossypii*, *Phymatotrichopsis omnivora*, *Phytophthora* spp., *Puccinia cacabata* and *P. schedonnardi*, *Pythium* spp. and *Sclerotium rolfsii*.

Boll rot caused by these diseases leads to serious production losses. Damage is more severe in crops cultivated with high humidity and low light intensity, and it increases if the bolls have mechanical lesions. Mainly, the damage these fungi cause is the contamination of fibres, especially if open bolls remain exposed to rain or high humidity for a long period. In addition to causing undesired discolouring of the fibre, these agents may give rise to enzyme degradation in some basic components, as frequently occurs in cellulose.

Other diseases of cotton are caused by bacteria, for example *Xanthomonas campestris* pv. *malvacearum*, and by viruses, for example abutilon mosaic geminivirus, cotton leaf crumple geminivirus, cotton leaf curl geminiviruses, cotton yellow mosaic geminiviruses and cotton anthocyanosis virus. Cotton bunchy top, cotton leaf mottle and cotton leaf roll diseases are of unknown etiology.

3.3.4. Weeds

Weed control in cotton fields is of considerable importance, and is carried out with mechanical methods by passing through the crop rows, and by chemical methods. Many different herbicides are employed in the cultivation of cotton, with their application during pre-sowing and/or pre-emergence of seedlings or less frequently in post-emergence (Table 3). Integrated weed management measures reduce

reliance on single herbicide groups, and include crop rotations and farm hygiene to prevent weed seed spreading (Charles, 2002; Roberts and Charles, 2002). The cultivation of varieties with herbicide tolerance developed by genetic engineering has also significantly improved weed management of the crop.

The commonly occurring and the most troublesome weeds vary considerably by region and management practices. Genera often having species of notable concern in areas are listed in Appendix 2.

Table 3. Timing of application of various herbicides

Land inclusion and pre-sowing
Pre-sowing
Pre- and post-sowing
Immediately post-sowing
Pre-sowing and pre-emergence
Pre-emergence
Pre- and post-emergence
Immediately post-sowing, and post-emergence
Post-emergence

3.4. Harvest and processing (ginning, crushing)

To facilitate harvest and subsequent ginning (freeing up of fibres from seed to obtain the lint), the plant is defoliated by means of a chemical treatment. This improves cleanliness and the quality of the fibres. Mechanised harvest is done by means of spindle picker machines in two or four rows.

A final step is ginning the cotton in saw gins, to make bales classified according to grade and length of fibre. The separated cottonseed is further processed, first by separating the hulls from the kernels. The kernels are crushed, and the oil extracted and processed for use in human food or other products. The hulls are used for livestock feed or industrial products, and the remainder of the kernel (which is high in protein) is converted into cottonseed meal for livestock. In the case of *G. hirsutum*, the fuzzy seed (*i.e.* seed with linters) is delinted, *i.e.* processed mechanically or chemically to remove the linters. These residual short fibres are used for a variety of purposes, such as a cellulose base for food or other consumer products. To maintain its superior fibre quality, the picking and ginning techniques for *G. barbadense* cotton are different than those used for *G. hirsutum*. As *G. barbadense* does not produce linters, its seed exists either as the unprocessed "seed cotton" or processed black seed.

3.5. Crop rotation

Cotton crop rotation is usually carried out by alternating with other traditional crops in the area. However, in contrast to best agricultural practices, sometimes cotton is planted in the same field again, for 2 years or longer. The number of repetitions is hindered by the damage to the crop caused by diseases, especially *Verticillium* wilt.

4. Reproductive Biology, Dispersal and Establishment

4.1. Floral biology, pollination and development of seeds

The sequence of flowering is from the lower to the upper part of the plant, and from the centre to the outside. Anthesis takes place 25-30 days after the appearance of the floral bud. Secretion of bracteal (extrafloral) nectar starts 5-6 days before flowering and initially peaks on the day of anthesis (Adjei-Maafo and Wilson, 1983a; Wäckers and Bonifay, 2004). On the day preceding anthesis the corolla extends well above the bracts, and early the following morning the large flower opens and secretion of floral nectar begins (Waller *et al*., 1981); the petals turn dark pinkish and wilt by evening of the same day (Fryxell, 1979; Waller *et al*., 1981; Eisikowitch and Loper, 1984; Sanchez and Malerbo-Souza, 2004). The anthers open soon after the flower and shed their pollen grains, some 900-350 per anther; the grains are spheroidal and very large (100-140 μm diameter), with *G. barbadense* having larger grains than *G. hirsutum* (Srivastava, 1982; Wetzel and Jensen, 1992; Kakani *et al*., 1999; Sava kan, 2002; Watanabe *et al*., 2006). The stigma generally is receptive at anthesis (McGregor, 1976).

Self-pollination usually takes place. As the pollen grains are large, heavy and somewhat sticky, dissemination by wind is absent or negligible (McGregor, 1976; Umbeck *et al*., 1991; Borém *et al*., 2003). Under humid laboratory conditions, Richards *et al*. (2005) found that about 90% of the pollen grains were viable after 8 hrs, nearly 31% still viable after 16 hrs and about 7.5% viable after 32 hrs, but after 8 hrs on the proboscis of *Helicoverpa armigera* moths, pollen grains were about 81% non-viable.

Although cotton is mostly self-pollinating, in the presence of suitable insect pollinators it is also cross-pollinating at generally low levels, which improves yields (McGregor, 1976; Tanda, 1984; Mamood *et al*., 1990; Rhodes, 2002; Sanchez and Malerbo-Souza, 2004; Llewellyn *et al*., 2007). The species pool and concentration of pollinators vary according to region, location, season and timing. The extent of spontaneous (unaided) or natural outcrossing thus depends greatly upon local insect populations, including introduced and native species (Moffett *et al*., 1976; Berger *et al*., 1988; Freire *et al*., 2002; Rhodes, 2002; Sanchez and Malerbo-Souza, 2004; Danka, 2005; Van Deynze *et al*., 2005; Llewellyn *et al*., 2007). Nectar from the extrafloral bracteal nectaries (epicalyx) is more accessible than nectar from the floral nectaries inside the calyx, so flower visitors are not always potential pollinators (Moffett *et al*., 1975; McGregor, 1976; Tsigouri *et al*., 2004; Danka, 2005). Bumble bees (*Bombus*), honey bees (*Apis*), *Anthophora*, *Melissodes* and *Halictus* bees and *Scolia* wasps are important pollinators in some areas (McGregor, 1976; Free, 1993; Delaplane and Mayer, 2000). *Apis mellifera* can be an important pollinator, but it does not prefer *Gossypium* pollen (McGregor, 1976; Eisikowitch and Loper, 1984; Vaissière *et al*., 1984; Waller *et al*., 1985; Loper, 1986; Vaissière, 1991; Vaissière and Vinson, 1994; Danka, 2005; Van Deynze *et al*., 2005). In using insecticides to manage the crop, mitigation measures are taken to preserve the pollinator populations, for example by not applying insecticides during the effective period of flowering (Delattre, 1992; Bourland *et al*., 2001; Sekloka *et al*., 2007).

Isolation of test plots or the crop thus depends on the presence and flight distances of the insect pollinators, and the result desired (Llewellyn *et al*., 2007). Conditions and objectives vary tremendously. Pollen-mediated gene flow declines steeply, typically being below 1% beyond 10 m from the source (Van Deynze *et al*., 2005). From experiments, isolation distances suggested for field tests include 8-10 m (Turkey) (Sen *et al*., 2004); 10 m (Greece) (Xanthopoulos and Kechagia, 2000); 20 m (Brazil) (Freire, 2002a); 33 m (India) (Singh and Singh, 1991); and 60 m (China) (Zhang *et al*., 2005). Barriers composed of other cotton can be effective (Simpson and Duncan, 1956); in Australia the accepted practice has been a distance of 20 m with a cotton buffer or 50 m of bare ground, but somewhat larger distances may be preferable sometimes (Llewellyn *et al*., 2007). Border rows of *Zea mays* 4 m wide reduced cotton cross-pollination in Brazil from 15% to 5%, and an isolation distance of 100 m has been recommended when maize barriers are used (Castro *et al*., 1982; Freire, 2005).

As the field area under cultivation for cotton increases or the goal in separation becomes more strict, the recommended isolation distance increases, or there are large regions of exclusion. The OECD Seed Schemes recommend separation distances of 200 m for production of Certified commercial seed of *G. hirsutum* and 600 m for *G. barbadense*, and separation distances of 600 m and 800 m respectively for Basic (*i.e.* Foundation) seed (OECD, 2008). Suggestions for isolation of cotton crop fields in Brazil, depending on the objective, are a distance of 250 m or 800 m (Freire, 2005). In some situations an isolation distance of 1000 m (1 km) or more may be necessary (Australia) (Llewellyn *et al*., 2007). Van Deynze *et al*. (2005) found 0.04% pollen-mediated gene flow at 1625 m (California, USA). In Hawaii, large-scale production of Bt cotton is prohibited to avoid crossing with the endemic *Gossypium tomentosum* (Hawkins *et al*., 2005). In Northeast Brazil, to safeguard the few extant wild populations of the endemic *Gossypium mustelinum*, a mapped zone of exclusion of cotton cultivation of at least 3 km has been proposed (Barroso *et al*., 2005; Freire, 2005).

Suitable pollen grains that have been deposited on the surface of the large sticky stigma *via* self-pollination or cross-pollination germinate within 30 min (Pundir, 1972). There is some sensitivity to the genotypic origin of the pollen, with effects ranging from positive to negative, even to incompatibility between some strains of *G. hirsutum* (McGregor, 1976; Gawel and Robacker, 1986; Pahlavani and Abolhasani, 2006). The pollen tube typically grows through the style for 12-30 hrs to the ovary and ovule, after which fertilisation is completed. Cell division in the zygote takes place 4-5 days after anthesis.

The young seed commences with fertilisation. Normal development follows a sigmoid curve, with the most rapid growth of the seeds and boll (fruit) occurring from about the 7th day to 18th day after anthesis (Oosterhuis and Jernstedt, 1999). Definitive size of the ovoid seed is reached about 25 days after anthesis. Boll development is characterised by three phases: enlargement, filling and maturation. Initially as the seeds grow the cotton fibres elongate; both seeds and fibres give maximum volume to the boll. Each fibre develops from a single epidermal cell of the seed coat. After 3 weeks, the boll-filling phase begins, with cellulose deposited inside the lumen of the elongated fibres. The filling phase continues into the 6th week, then the boll maturation phase begins and the boll dries out (Ritchie *et al*., 2007).

Each mature boll has three to five locules or "locks", within which are the seeds surrounded by their fibres. The average number of seeds in a boll depends on many factors, including genotype, location of the boll on the plant, and stresses during plant development and growth; roughly 20 to 35 (even 45) seeds per boll can be typical. Post-fertilisation failure can result in the development of "motes", embryos that do not ripen into mature seeds but develop partially, including growth of immature fibres of various lengths that complicate lint production (Bolek, 2006).

4.2. Dispersal

The dispersal ability of the genus *Gossypium* is apparent from its unusual evolutionary history, including various trans-continental dispersal events and several interspecific hybridisations resulting in new lineages. For example, within the last 1-2 million years, there have been long-distance dispersals of the progenitor of *Gossypium darwinii* from South America to the Galapagos Islands, and the progenitor of *Gossypium tomentosum* from Mesoamerica to the Hawaiian Islands, and in each case, the tetraploid coloniser evolved into an endemic species that became well established, dispersing to various islands within its archipelago (Wendel and Percy, 1990; Sherwood and Morden, 2004).

Over the several millennia that early peoples achieved domestication and expansion in cultivation of *Gossypium*, the four utilised species were spread beyond their natural centres of origin and diversity to new regions (Brubaker *et al*., 1999c), and sometimes have become established and free-living or naturalised to varying degrees. In this way the genuinely wild distributions of the domesticated species were obscured (Stephens, 1958). The region where wild (non-feral) *Gossypium barbadense* occurs in South America is rather clear (Westengen *et al*., 2005), but the original range of *G. hirsutum* in Mesoamerica (and perhaps the Caribbean) is quite unclear (Stephens, 1958; Brubaker and Wendel,

1994). Similarly, the original centres or ranges of *G. arboreum* and *G. herbaceum* subsp. *herbaceum* are obscure.

The dispersal of seeds varies in different areas or settings and situations and from one season to another (OGTR, 2008). In a natural setting, wind, water and birds may serve as dispersal agents (Stephens, 1958, 1966; HEPX, 2007). In an agricultural setting, greater dispersal of cottonseed generally may occur during transport (Addison *et al*., 2007), stock-feeding (Coppock *et al*., 1985; Sullivan *et al*., 1993a, 1993b) or adverse weather conditions, and rarely by animals (Smith, 1995).

4.3. Seed dormancy and germination

Although *Gossypium* seeds can have a natural capability of 2-3 months of innate or induced dormancy, "hard" seeds are undesirable for crop production, and the trait has been minimised or completely eliminated in modern cultivars through domestication and selective breeding (Stephens, 1958; Hopper and McDaniel, 1999; Paiziev and Krakhmalev, 2006; OGTR, 2008).

The quality or vigour (potential for rapid, uniform emergence of seedlings) of *G. hirsutum* seeds can vary between seed lots (Hopper and McDaniel, 1999). Factors such as chemical composition of the mature seed and pre-harvest environmental conditions contribute to the relative quality of cottonseed. Selection to improve seedling vigour has been incorporated into *G. hirsutum* breeding programs (Bourland, 1996).

Germination depends largely on the type of cottonseed (Eastick and Hearnden, 2006). The *G. hirsutum* black seed used for planting (*i.e.* ginned and acid-delinted seed) has the highest germination rate. New seed has a low germination rate, attributed to mechanical hindrance of cotyledon emergence by the surrounding fibres. Fuzzy seed (*G. hirsutum*) has an intermediate germination rate.

The type of habitat that the seed is dispersed into affects germination. An experimental study on spread and persistence of *G. hirsutum* (Eastick and Hearnden, 2006) found germination highest in disturbed habitats such as stockyards and the edges of waterways, especially if the seed had been buried, and much less likely in undisturbed habitats and roadside sites. The experiments aimed to maximise germination and initial establishment of seedlings by sowing seeds into cleared ground, lightly burying the seeds and then hand-watering. Subsequent persistence and recruitment at a site were solely dependent on the habitat.

4.4. Weediness and naturalisation

Gossypium hirsutum and *G. barbadense* can occur as escapes from agriculture. Cotton can become feral and naturalise locally in suitable areas in many regions. Nonetheless cotton has been grown as a crop for decades to centuries in many countries without being reported as strongly invasive or a serious weed (*e.g.* Holm *et al*., 1979, 1997; Randall, 2002; Weber, 2003). Abiotic and biotic factors determine whether introduced *Gossypium* will establish in the particular environment, including the length of the growing season and severity of a cold or a dry season, rainfall, soil type, competition from other plants, herbivory (by insects and other animals), and physical destruction such as stock trampling or fire (Eastick and Hearnden, 2006).

Recently the weediness and naturalisation potentials of cotton were thoroughly reviewed for cotton-growing regions in Australia (OGTR, 2008). The crop species were not considered to threaten agricultural productivity, or native biodiversity (Tothill *et al*., 1982; Lazarides *et al*., 1997). *Gossypium hirsutum* has been grown since the 1960s or 1970s in a number of places in northern Australia. Isolated naturalised populations of *G. hirsutum* and *G. barbadense* occur, including within conservation areas (Sindel, 1997; Eastick, 2002).

Cotton volunteers are found in all Australian cotton-growing areas and are relatively common where cottonseed is used as livestock feed (Eastick and Hearnden, 2006). Typically such volunteers are grazed by livestock and/or killed by roadside management practices, limiting their potential to persist and reproduce (Eastick and Hearnden, 2006; Addison *et al*., 2007).

Surveys in 2002, 2004 and 2005 along Australian routes for transporting ginned *G. hirsutum* seed for stockfeed indicated that plants infrequently established in the roadside environment, mostly as transient populations despite more than 12 years of using the routes (Addison *et al*., 2007). *Gossypium hirsutum* volunteers tended to establish in highly and regularly disturbed environments.

In another study (Eastick, 2002; Eastick and Hearnden, 2006), persistence of *G. hirsutum* plants for more than 1–2 years was found only in habitats having increased availability of water and/or nutrients, such as cattle yards. Although the cotton plants in cattle yards might grow to reproductive maturity, persistence and seed dispersal were limited by trampling and grazing; no volunteers were found in undisturbed bush habitats surrounding the areas.

A rigorous model has been developed to predict the regions in Australia that are climatically suitable for long-term survival of feral cotton (Rogers *et al*., 2007; OGTR, 2008). The modelling program predicted that the winter temperatures in current Australian cotton-growing areas are too cold to support the establishment of permanent populations. The model indicated that dry stress is the major limiting factor in northern Australia, and predicted potential naturalisation of cotton with matching climates on the northeastern coast. Soil fertility, plant competition and fire were identified as factors that could reduce the probability of permanent populations establishing.

5. Genetics and Hybridisation

Germplasm resources of *Gossypium* have been described in detail (Percival *et al*., 1999). The various objectives followed in breeding cotton and the technology used are dependent on factors such as biological constraints and abiotic stress resistances, and other factors such as market demands (Niles, 1980; Calhoun and Bowman, 1999; Mergeai, 2006a). A survey of breeders in 2000 showed that most of the breeding work in *G. hirsutum* involved crossing closely related parents followed by backcrossing or reselecting from existing crosses, with less than 3% of the breeding material coming from non-*G. hirsutum* sources (Bowman, 2000). In Australia breeding has contributed about 45% to the improvements in yield since 1983 (Constable *et al*., 2001).

5.1. Genomes

Gossypium species are classified into eight diploid genomic groups and one tetraploid group (Section II, Table 2) based on cytogenetics, along with their capability to form viable or fertile interspecific hybrids experimentally (Edwards & Mirza 1979; Endrizzi *et al*. 1985; Stewart 1995). Generally species within a group can form hybrids with normal meiotic pairing and at least some F_1 fertility, whereas crosses between groups rarely form hybrids, and if so they have meiotic abnormalities and are infertile.

The D genome is the smallest, with a mean 2C nuclear DNA content of 1.81 picograms, and the A genome is almost twice as large — 3.47 pg; the mean DNA content of the AD-genome tetraploids is nearly additive, with 4.91 pg, and suggests a small loss of DNA subsequent to polyploidisation (Hendrix and Stewart, 2005; Grover *et al*., 2007, 2008). The size difference in diploid genomes (and the tetraploid subgenomes) is primarily a result of differential amplification of repetitive DNA transposable elements (Hawkins *et al*., 2006).

In the allotetraploid crop species ($2n = 4x = 52$), the A group's generally larger 13-chromosome set can be distinguished from the D group's generally smaller 13-chromosome set and the individual

chromosomes in each subgenome identified (Muravenko *et al*., 1998; Rong *et al*., 2004; Wang *et al*., 2006). In *G. barbadense*, the A-genome chromosomes average 4.20 μm in length, with the largest seven being 4.34 μm or more (the full range is 2.23 to 5.81 μm), whereas the D-genome chromosomes average 3.29 μm long (and range from 1.76 to 4.25 μm) (Muravenko *et al*., 1998). The 13 individual chromosomes of the A-genome diploid ($2n = 2x = 26$) species *G. arboreum* have been identified and correlated with their counterparts in the A subgenome of *G. hirsutum* (Wang *et al*., 2008).

Comprehensive overviews of the results from recent genomic investigations of *Gossypium* have been provided (Preetha and Raveendren, 2008; Zhang *et al*., 2008). The most complete tetraploid genetic map so far (from *G. hirsutum* × *G. barbadense* F_2s) comprises 2584 loci, at an average inter-marker distance of 1.72 cM (~ 606 kbp), in 26 linkage groups — thus covering all 13 individual chromosomes of each subgenome (Rong *et al*., 2004). Genetic linkage maps of the *G. arboreum* genome have been made and correlated with the A subgenome of *G. hirsutum* (Desai *et al*., 2006; Ma *et al*., 2008).

Based on a mean 2C nuclear DNA content of 4.93 pg for *G. hirsutum*, the haploid DNA (1C-value) is estimated to be 2410 Mbp (Hendrix and Stewart, 2005). An international coalition of researchers has plans underway to completely sequence the nuclear genome of *G. hirsutum* (Chen *et al*., 2007), first by sequencing the ancestrally close D-genome relative *G. raimondii*, which has a much smaller genome (1C of 880 Mbp) (Hendrix and Stewart, 2005). The complete nucleotide sequences of the chloroplast genomes of *G. hirsutum* (Lee *et al*., 2006) and *G. barbadense* (Ibrahim *et al*., 2006) have been determined.

The complexity in the *Gossypium* genome occurs in a multitude of diverse dimensions. The diploid genus itself is considered a paleopolyploid (as is possibly the case for most angiosperms). An ancient polyploidisation event (whole genome duplication) (perhaps $2n = 14$ to $2n = 28$) appears to have occurred 13-15 (-30) million years ago in the Malvaceae lineage that evolved into what is treated as the emergent diploid genus *Gossypium* ($2n = 26$) (Muravenko *et al*., 1998; Brubaker *et al*., 1999a; Wendel and Cronn, 2003; Blanc and Wolfe, 2004; Rong *et al*., 2004, 2005; Ma *et al*., 2008). Consequently, genes were duplicated in that ancient originating event, the species continued genomic and genic evolution (Small *et al*., 2004), and genes were duplicated again in the rather recent formation of the allotetraploid lineage that has provided the two predominant crop species.

The functioning and evolutionary fortune of the plethora of counterpart genes (homoeologs) subsequent to the *Gossypium* allopolyploidisation event have been receiving substantial investigation. A broad array of divergent outcomes can occur, in some cases immediately with the onset of the genome doubling (and gene duplicating) event, in other cases during the long course of evolutionary time (Wendel and Cronn, 2003; Adams and Wendel, 2004; Adams, 2007; Liu and Adams, 2007; Wang *et al*., 2007; Flagel *et al*., 2008). Paterson (2005) has sketched how some cotton QTLs for crop improvement relate to such homoeologs.

5.2. Intraspecific crossing

Typically, the profitability of production mostly depends on lint yield, so the ultimate objective of many breeding programs is to increase it. Using intraspecific hybrid vigour to increase the yield has long been an objective (Zhang and Pan, 1999). Lint yield is a complex trait under complex genetic and environmental interactions, requiring a good balance among yield components. Heterosis has not been easy to employ due to the lack of an efficient crossing system. Many male-sterile systems have been explored (Percy and Turcotte, 1991; Basu, 1996), but male steriles and their restorer factors often have not been stable in different environments. Heterosis is not used commercially except where a large labor force can make emasculations and crosses by hand. At least 40% of cotton production in India has been derived from intraspecific hybrids of *G. hirsutum* (Chaudhry, 1997). Meredith (1999) reported an average useful heterosis of 21.4% (or 276 kg/ha) for F_1 hybrids and 10.7% for F_2 hybrids, although heterosis for fibre properties averaged only 0-2% for most characteristics.

5.3. Interspecific crossing

Under intensive experimental conditions, species in a few other genera of Malvaceae have been reported to form fertile hybrids with *Gossypium* (Mehetre *et al*., 1980), but spontaneous intergeneric hybridisation is highly improbable. Within the genus *Gossypium*, the sexual transmission of genetic material of cultivated cottons *via* pollen has been possible to certain of the species. For improvement of the main crops, *Gossypium* species can be grouped into three gene pools based on their ability to generate fertile hybrids and homoeologous recombination (Stewart, 1995; Percival *et al*., 1999).

Most cultivated cotton is tetraploid (primarily *G. hirsutum*), and thus relatively incompatible with the diploid species — normally plants from these two groups do not hybridise spontaneously and produce fertile offspring, and experimental crosses are difficult and require complex breeding schemes (Mergeai, 2006b). Moreover, experimental F_1 hybrids between the genome groups of diploids are nearly always sterile, or are weak (Endrizzi *et al*., 1984, 1985; Brown and Brubaker, 2000; Cronn and Wendel, 2004).

5.3.1. Primary gene pool — the tetraploids

The tetraploid (AADD) species are sexually compatible, which accords with their differentiation following a hybridisation event only 1-2 million years ago; since then they have diverged into three evolutionary lineages (Wendel and Cronn, 2003). The primary gene pool comprises the subgenus *Karpas*: the three wild tetraploid species (*G. mustelinum*, *G. darwinii*, *G. tomentosum*) and the wild, commensal, landrace, cultigen and feral *G. barbadense* and *G. hirsutum*. Experimental crosses among these entities are rather easy and genetic recombination frequency is high; favourable traits have been incorporated from this gene pool (particularly from *G. hirsutum* and *G. barbadense*) into the modern crops (Endrizzi *et al.*, 1984, 1985; Meredith, 1991; Stewart, 1995; Percival *et al*., 1999).

5.3.1.1. Spontaneous hybridisation of tetraploids in the New World

Native populations of the three completely wild tetraploid species are widely separated biogeographically (Appendix 1). Moreover, the clearly genuinely wild native populations of *G. barbadense* and *G. hirsutum* are also completely separated from the other three species, and from each other (Brubaker and Wendel, 1994; Westengen *et al*., 2005; Johnston *et al*., 2006). Thus, fully natural hybridisations do not occur among the tetraploids; however, spontaneous (unaided) hybridisation might occur when cultivated plants are brought within range of these wild species or primordially wild populations. Spontaneous hybridisation between various other populations or plants of the cultivated species also may occur.

5.3.1.2. Gossypium mustelinum

Gossypium mustelinum is a local, very rare endemic in semi-arid northeastern Brazil (Freire *et al*., 1998; Batista *et al*., 2005; Barroso *et al*., 2006; WWF and IUCN, 1997), and is considered most similar to the original allotetraploid progenitor (Wendel *et al*., 1994). Experimentally, *G. mustelinum* can form fertile F_1 as well as F_2 hybrids and backcrosses with *G. hirsutum*, and to some extent with *G. barbadense* (Freire, 2002b; Freire *et al*., 2002; Gardunia *et al*., 2007). The evidence of spontaneous introgression in Brazil is uncertain, and relates particularly to *G. hirsutum* (Wendel *et al*., 1994; Freire, 2002a; Freire *et al*., 2002; Borém *et al*., 2003; Johnston *et al*., 2006).

5.3.1.3. Gossypium darwinii

Gossypium darwinii is a widespread endemic in the Galapagos Islands, and is considered most closely related to *G. barbadense* (Wendel and Percy, 1990; Lacape *et al*., 2007). The *Gossypium barbadense* that settlers brought to the Galapagos apparently included plants that earlier had introgressed with *G. hirsutum*. The introduced domesticated cotton has not become widely naturalised, but

spontaneous gene flow has occurred into *G. darwinii* (Wendel and Percy, 1990). Experimentally, F_2 hybrids produced from crosses of *G. barbadense* and *G. darwinii* are fertile and vigorous.

5.3.1.4. Gossypium barbadense and *Gossypium hirsutum*

The original native habitat of *G. barbadense* is considered to be the dry coastal region of northern Peru and southern Ecuador (Schwendiman *et al*., 1985; Percy and Wendel, 1990; Westengen *et al*., 2005). The original native habitat of *Gossypium hirsutum* is considered to involve central Mesoamerica (Hutchinson, 1951; Stephens, 1958; Brubaker and Wendel, 1994). The natural distribution of *G. hirsutum* as a wild species is particularly obscure because of millennia of early use, domestication and expanded cultivation.

Many of the advanced stocks of commercial *G. barbadense* have benefited from the introgression of *G. hirsutum* characteristics by plant breeding (Wang *et al*., 1995). Reciprocally, introgression of *G. barbadense* into *G. hirsutum* has exploited (for example) the excellent fibre qualities of the former. However, the hybrid vigour resulting is associated with excessive vegetative growth and late maturity, which make adaptation of such hybrids limited to those areas with a long growing season. This problem was reduced by obtaining precocious, short-growing season *G. barbadense*-types that may be used to produce the F_1 hybrids with *G. hirsutum* (Feaster and Turcotte, 1980).

Despite these commercial breeding successes, introgression of *G. hirsutum* into *G. barbadense* is conspicuously low in germplasm collections from Mesoamerica and the Caribbean where *G. barbadense* has been grown with the predominant *G. hirsutum* since prehistoric times (Brubaker *et al*., 1993; Westengen *et al*., 2005). The relative absence of introgression into *G. barbadense* may result from various isolating mechanisms, involving plant reproductive biology, agroecology and ecology (Percy and Wendel, 1990; Brubaker *et al*., 1993; Jiang *et al*., 2000; Freire *et al*., 2002; Borém *et al*., 2003; OGTR, 2008). In contrast, introgression of *G. barbadense* into *G. hirsutum* under such conditions is relatively common (Wendel *et al*., 1992; Brubaker *et al*., 1993; Brubaker and Wendel, 1994). Isozyme analysis found the most introgression into *Gossypium hirsutum* race marie-galante (Brubaker and Wendel, 1994; Brubaker *et al*., 1999c), but SSR analysis of several samples of marie-galante from the Caribbean and mocó from Brazil did not detect such introgression (Lacape *et al*., 2007; *cf*. Freire *et al*., 2002; Borém *et al*., 2003).

5.3.1.5. Gossypium tomentosum

Gossypium tomentosum is endemic in the Hawaiian Islands, and apparently the closest relative to *G. hirsutum* (DeJoode and Wendel, 1992; Hawkins *et al*., 2005; Waghmare *et al*., 2005; *cf.* Westengen *et al*., 2005). The flowers of *G. tomentosum* reportedly are receptive at night rather than during the day and pollinated by moths, not bees (Stephens, 1964; Fryxell, 1979). Feral *Aethina concolor* beetles enhance its self-pollination, and perhaps effect cross-pollination (Burraston *et al*., 2005; Burraston and Booth, 2006). Spontaneous movement of genetic material from cultivated *G. hirsutum* to the wild endemic remains speculative (*cf.* Waghmare *et al*., 2005). Also, despite morphological suggestion of spontaneous hybrids between naturalised *G. barbadense* and the wild endemic (Stephens, 1964; Münster and Wieczorek, 2007), no allozyme evidence of introgression has been found (DeJoode and Wendel, 1992).

5.3.2. Secondary and tertiary gene pools — the diploids

All species in these gene pools are diploids. In addition to cytological barriers to hybridisation, varied physiological barriers exist between the diploids and *G. hirsutum*, the usual focus for improvement. *In vitro* culture of ovules partly solves the problem (Stewart and Hsu, 1978). Three main breeding strategies (Mergeai, 2006b) have been devised to overcome sterility barriers and can lead to successful introgression of desirable traits (Endrizzi *et al.*, 1985; Meredith, 1991; Stewart, 1995). In two schemes,

crossing a diploid and *G. hirsutum* results in sterile triploids (3*x*), with few rare exceptions (Brown, 1951; Meyer, 1974). Hexaploids (6*x*) are then made (using colchicine) by chromosome doubling of the triploid genome. The hexaploid can then be crossed with a different diploid and result in a tri-species tetraploid hybrid. Or, *G. hirsutum* can be crossed with the hexaploid; the resultant pentaploids (5*x*) can be self-crossed, or crossed again with *G. hirsutum*, resulting in a tetraploid.

The secondary gene pool includes the evolutionarily closer diploids, thus comprising the D-genome species (subgenus *Houzingenia*) and the A-genome species, as well as the African B- and F-genome species (Appendix 1) (Phillips, 1966; Phillips and Strickland, 1966; Wendel and Cronn, 2003).

Bridge-crosses between two diploid species, induced genome doubling, and then crossing with *G. hirsutum* are another useful strategy for gene transfer (Mergeai, 2006b). Such an approach using the A-genome and D-genome species produces synthetic AD tetraploids, which may be readily crossed with *G. hirsutum*. Genes from the A or D genome may thus be transferred to the upland cotton crop (Stewart and Stanton, 1988; Saravanan *et al*., 2007). For example, the ATH tri-species hybrid (*G. arboreum* × *G. thurberi*) × *G. hirsutum* has been used to introduce fibre strength.

The tertiary gene pool includes the evolutionarily distant diploids, thus comprising the African-Arabian E-genome species, and the Australian C-, G- and K-genome species (Appendix 1) (Wendel and Cronn, 2003). Potential exploitation of desirable traits/genes in this gene pool (as well as evaluation of transgene diffusion potential) have stimulated considerable research in the Australian subgenus *Sturtia* (Brown *et al.*, 1997; Zhang and Stewart, 1997; Brubaker *et al.*, 1999b; Brown and Brubaker, 2000). Desirable traits include gossypol-free seeds, which occur in both the C- and G-genome species.

It has not been possible to obtain hybrids of *G. hirsutum* with the G-genome species, whereas hybrids can be obtained readily with the C-genome species; the situation is intermediate and variable with the K-genome species (Brown and Brubaker, 2000). The experimental hybrids among the species of subgenus *Sturtia* range from being totally infertile, to having some meiotic fertility in backcrosses but the plants are weak (Brown *et al*., 1997; Brown and Brubaker, 2000). The first gene from the tertiary gene pool introgressed into *G. hirsutum* came from the C genome's *G. sturtianum*, and involved control of terpenoid aldehyde methylation (to reduce gossypol formation) (Bell *et al*., 1994). Although *G. sturtianum* is the species in subgenus *Sturtia* that crosses most readily with *G. hirsutum*, the F_1 is completely infertile.

6. Biotechnology and genetic transformation

The efforts to domesticate and improve cotton span millennia, from selection and conventional breeding, to chemical and radiation mutagenesis, to advanced biotechnological techniques. Some of the traits of continuing interest to incorporate into cultivated cottons include disease and insect resistances and drought and salt tolerances for the crop, whereas other traits are focused on improving the crop's products (Basu, 1996; Paterson and Smith, 1999; Wilkins *et al*., 2000; Jenkins and Saha, 2001; Hake, 2004). Embryo rescue is employed to obtain plants from interspecific hybridisations that will abort (Mehetre and Aher, 2004). A research focus in the 1960s and 1970s was development of new cell culture methods. Callus cultures were the starting point to isolate protoplasts, with a view to making wide crosses *via* protoplast fusion with sexually incompatible germplasm (Carlson *et al.*, 1972). Price *et al.* (1977) first defined the conditions for establishment of callus cultures, from six cotton species. The first report of a cell culture system to obtain somatic embryos from cotton callus cultures was by Price and Smith (1979), and improvements have continued (Kumar and Tuli, 2004; Sakhanokho *et al*., 2004; Sun *et al*., 2006).

The main vector used for introducing particular genes into cotton is *Agrobacterium tumefaciens*, with the first reports of transformations in the nuclear genome by Umbeck *et al.* (1987) and Firoozabady *et al.* (1987). A gene-transfer system was developed culturing *Agrobacterium* with sections of 6- to 7-day-old

cotton-seedling hypocotyls (Fillatti *et al.*, 1989). The *Agrobacterium*-mediated approach continues to be of major utility (Wilkins *et al.*, 2004). The first report of cotton plants transformed using projectile bombardment was by Finer and McMullen (1990), which was followed by genotype-independent particle bombardment of four cultivars (McCabe and Martinell, 1993). The bombarded cells are grown in tissue culture to differentiate and develop into shoots or whole plants. A major problem has been achieving regeneration — only a limited number of cotton varieties (*i.e.* genotypes) regenerate relatively easily. Transformation has also been achieved in the chloroplast genome (Kumar *et al.*, 2004).

Insect-resistant and herbicide-tolerant cotton varieties have been developed by means of genetic engineering and are commercially grown in a number of countries. The first agronomically important gene inserted into cotton was for insect resistance, *cry1Ab* from *Bacillus thuringiensis* (Bt) (Perlak *et al.*, 1990). Other insecticidal genes from Bt have been introduced (especially *cry1Ac* and *cry2Ab*, and more recently *vip3A*), which encode particular proteins selectively toxic to various Lepidopteran pests. An insecticidal gene *AaHIT* from the scorpion *Androctonus australis* is also being explored in transgenic cotton against some lepidopterans (Wu *et al.*, 2008).

Cotton lines have been genetically engineered to tolerate the herbicides bromoxynil, glufosinate ammonium, glyphosate or sulfonylurea. Transgenic cottons in commercial production include plants having stacked transgenes, for example a Bt (Cry1Ac + Cry2Ab) + glyphosate-tolerant cotton.

With continuing development, there are likely to be transgenic cottons improved in other ways, for example with fibres that are stronger (Zhu *et al.*, 2006; Shang-Guan *et al.*, 2007) or have non-crease characteristics similar to polyester (John and Keller, 1996); or with seeds that are gossypol-free (Sunilkumar *et al.*, 2006) or with improved oil composition (Chapman *et al.*, 2001; Liu *et al.*, 2002).

7. Human health and biosafety

Cottonseed oil has been in common use since at least the middle of the 19th century (Jones and King, 1993). Cottonseed meal or flour is also sometimes used for human consumption when derived from gossypol-free varieties, or if the gossypol has been extracted or is present in the food at low levels. Information on processing of cottonseed (of both *G. hirsutum* and *G. barbadense*) and its major products (oil, meal, hulls and linters) and their composition including the key food and feed nutrients, toxins and anti-nutrients have been summarised by the OECD (2004).

Cottonseed is a valuable foodstuff for cattle, combining high energy, high fibre and high protein (Ensminger *et al.*, 1990b), and is used as whole seed, hulls, flour and cake. The whole seed of *G. hirsutum* also includes linter fibers (~ 10% of seed weight), which are nearly pure cellulose and highly digestible. The seed oil gives it high energy value (Coppock *et al.*, 1985). Cattle and sheep are fed cottonseed hulls as an important source of roughage. The hulls are removed from whole seed, and composed mainly of hemicellulose and lignin, with the linters remaining attached. Gin trash is also fed to ruminants, and has 90% of the food value of the hulls (Ensminger *et al.*, 1990a).

Extracts of cotton plants have been used medicinally (*e.g.* Sawyer, 1955; Hasrat *et al.*, 2004). Because of its several physiological effects, the medical potential of gossypol is being investigated (Dodou *et al.*, 2005), and it has been used as a male contraceptive (Coutinho, 2002).

7.1. Toxins

Cotton plants contain compounds that can have adverse effects on human and animal health (OGTR, 2008). Cotton tissue, particularly the seeds, can be toxic when ingested in large quantities because of the anti-nutritional and toxic compounds (Abou-Donia, 1976; Tumbelaka *et al.*, 1994; Smith, 1995). Most important with respect to human health and biosafety are gossypol, which is a terpenoid aldehyde, and cyclopropenoid fatty acids (CPFAs), as well as tannins.

The gossypol and CPFAs in cottonseed limit its use as a protein supplement in animal feed. Ruminants are less affected because these compounds are detoxified by digestion in the rumen (Kandylis *et al*., 1998). Cottonseed as a stockfeed is limited to a relatively small proportion of the diet, and must be introduced gradually to avoid the potentially toxic effects (Blasi and Drouillard, 2002).

Because *Gossypium barbadense* cottonseed possesses almost no linters, it is digested differently by cattle than *G. hirsutum*. The nearly naked seeds are thought to sink in the rumen and so be less masticated and digested (Coppock *et al*., 1985; Sullivan *et al*., 1993a, 1993b; Zinn, 1995; Solomon *et al*., 2005). To improve digestibility of *G. barbadense* seed it is often cracked prior to feeding even though this increases the exposure to gossypol. Cows that consumed cracked *G. barbadense* seed at ~ 7.5% of their diet had reduced fertility (Santos *et al*., 2003).

7.1.1. Gossypol

Gossypol is found primarily in the pigment glands of the roots, leaves, flower buds and seeds (Smith, 1961, 1967). It is toxic to non-ruminant mammals, birds, and many insects and microbes; in mammals the toxic effects can include reduced appetite, body weight loss and dyspnea (Berardi and Goldblatt, 1980). Gossypol can render lysine metabolically unavailable and impact on the normal functioning of mitochondria (Yannai and Bensal, 1983; Cuellar and Ramirez, 1993; Risco *et al*., 1993).

Although glandless cotton has been developed for food purposes, the plants are more susceptible to pests (Delattre, 1992). Inactivation or removal of gossypol and CPFAs during processing has enabled use of cottonseed meal for catfish, poultry and swine (Jones and Wedegaertner, 1986; Lusas and Jividen, 1987).

Gossypol exists as two different isomers (mirror-image forms of the same compound), which are in different proportions in *G. barbadense* and *G. hirsutum* (Stipanovic *et al*., 2005). *Gossypium barbadense* has more of the (–)-gossypol form (Sullivan *et al*., 1993b), which has greater biological activity. The isomers have different toxicity levels and the toxicity varies in different animals (Wang *et al*., 1987; Bailey *et al*., 2000; Lordelo *et al*., 2005, 2007).

Both isomers exist in free and bound forms. In intact whole cottonseed, gossypol is in the free form. The free form is more biologically active, whereas the bound form is generally not released in the rumen. In ruminants, with well-developed rumen microflora, free gossypol is converted to bound gossypol, thus preventing its entering the bloodstream (Santos *et al*., 2002). During the processing of whole cottonseed, gossypol partitions into meal and oil components. Most of the gossypol in meal becomes bound to proteins, thus becoming less toxic.

The levels of gossypol and related terpenoids in cottonseed vary (0.4–2.0 %) by species, variety, fertiliser application, and environmental conditions including biotic pressure from insects and diseases (Bell, 1986). The amount of gossypol in *G. barbadense* is generally higher than in *G. hirsutum* and with more of the gossypol in the free form, which reduces the amount of cottonseed of *G. barbadense* that can be recommended for cattle feed (Kirk and Higginbotham, 1999).

7.1.2. Cyclopropenoid fatty acids

Cyclopropenoid fatty acids (CPFAs) are present in the cotton seeds, and tannins in the leaves and flower buds (Chan *et al*., 1978; Lane and Schuster, 1981; Mansour *et al*., 1997); both are thought to act as deterrents to insects. CPFAs such as malvalic, sterculic and dihydrosterculic acids constitute approximately 0.5–1.0% of the total lipid content of the seed (Schneider *et al*., 1968). The level of CPFAs is generally higher in *G. hirsutum* than *G. barbadense* (Frank, 1987).

CPFAs are anti-nutritional compounds, which interfere with the metabolism of saturated fats (Rolph *et al*., 1990; Cao *et al*., 1993). They are destroyed by the processing of cottonseed oil for use

in margarine or salad oil for humans, but in less-processed animal feed CPFAs can cause unwanted effects (Goodnight and Kemmerer, 1967; Hendricks *et al*., 1980; Tumbelaka *et al*., 1994).

7.2. Allergens

Processed cotton fibre contains over 99% cellulose (Wakelyn *et al*., 2007a, 2007b), and is used widely in pharmaceutical and medical applications because of its low capacity to cause irritation. Inhalation of cotton dust by mill workers can cause an asthma-like condition called byssinosis (Nicholls, 1992), which may be complicated by fungal contamination of the cotton dust (Salvaggio *et al*., 1986).

Appendix 1. *Gossypium* species[1]

Species	Genome	Distribution
G. arboreum L.	A_2	Asian cultigen
G. herbaceum L. subsp. *herbaceum*	A_{1-1}	NE African - Central Asian cultigen
G. herbaceum subsp. *africanum* (G. Watt) Vollesen [synonym *G. herbaceum* var. *africanum* (G. Watt) J.B. Hutch. ex S.C. Harland]	A_{1-2}	southern Africa
G. longicalyx J.B. Hutch. & B.J.S. Lee	F_1	C-E Africa
G. triphyllum (Harv.) Hochr.	B_2	SW Africa
G. anomalum Wawra ex Wawra & Peyr.	B_1	SW & N sub-Saharan Africa
G. capitis-viridis Mauer	B_3	Cape Verde Islands
G. trifurcatum Vollesen[2]	?	NE Africa
G. stocksii Masters	E_1	Somalia to Pakistan
G. areysianum Deflers	E_3	Arabia
G. incanum (O. Schwartz) Hillcoat	E_4	Arabia
G. somalense (Gürke) J.B. Hutch.	E_2	NE Africa
G. benadirense Mattei	E	NE Africa
G. bricchettii (Ulbrich) Vollesen	E	NE Africa
G. vollesenii Fryxell	E	NE Africa
G. robinsonii F. Muell.	C_2	W Australia
G. sturtianum J.H. Willis var. *sturtianum*	C_1	C to E Australia
G. sturtianum var. *nandewarense* (Derera) Fryxell	C_{1-n}	E Australia
G. bickii Prokh.	G_1	N-C Australia
G. australe F. Muell.	G_2	NW Australia
G. nelsonii Fryxell	G	N-NE Australia
G. cunninghamii Todaro	K	N Australia
G. anapoides J.M. Stewart, Craven & Wendel, ined.? [3]	K	NW Australia
G. costulatum Todaro	K	NW Australia
G. enthyle Fryxell, Craven & J.M. Stewart	K	NW Australia
G. exiguum Fryxell, Craven & J.M. Stewart	K	NW Australia
G. londonderriense Fryxell, Craven & J.M. Stewart	K	NW Australia
G. marchantii Fryxell, Craven & J.M. Stewart	K	NW Australia
G. nobile Fryxell, Craven & J.M. Stewart	K	NW Australia
G. pilosum Fryxell	K	NW Australia
G. populifolium (Bentham) F. Muell. ex Todaro	K	NW Australia
G. pulchellum (C.A. Gardner) Fryxell	K	NW Australia

Species	Genome	Distribution
G. rotundifolium Fryxell, Craven & J.M. Stewart	K	NW Australia
G. gossypioides (Ulbrich) Standley	D_6	W Mexico
G. armourianum Kearney	$D_{2\text{-}1}$	NW Mexico (Baja California)
G. harknessii Brandegee	$D_{2\text{-}2}$	NW Mexico (Baja California)
G. turneri Fryxell	D_{10}	NW Mexico
G. laxum L.L. Phillips	D_8	CW Mexico
G. aridum (Rose & Standley) Skovsted	D_4	NW-SW Mexico
G. lobatum Gentry	D_7	CW Mexico
G. schwendimanii Fryxell & S.D. Koch	D_{11}	CW Mexico
G. thurberi Todaro	D_1	NW Mexico, Arizona
G. trilobum (Sessé & Moc. ex DC.) Skovsted	D_9	W Mexico
G. davidsonii Kellogg	$D_{3\text{-}d}$	NW Mexico (Baja California)
G. klotzschianum Andersson	$D_{3\text{-}k}$	Galapagos Islands
G. raimondii Ulbrich	D_5	NW Peru
G. hirsutum L.[4]	$(AD)_1$	Mesoamerica
G. tomentosum Nuttall ex Seemann	$(AD)_3$	Hawaii
G. barbadense L.	$(AD)_2$	W South America
G. darwinii G. Watt	$(AD)_5$	Galapagos Islands
G. mustelinum Miers ex G. Watt	$(AD)_4$	NE Brazil

[1] Mostly after Endrizzi *et al.* (1984), Fryxell (1992), Fryxell *et al.* (1992) and Percival *et al*. (1999).

[2] *Gossypium trifurcatum* was described by Vollesen (1987) and is accepted by Fryxell (1992). Percival *et al*. (1999) indicated that it might belong in the genus *Cienfuegosia*, but cpDNA analysis by Rapp *et al*. (2005) supports its placement in *Gossypium*.

[3] *Gossypium anapoides* has been noted in recent literature (Stewart *et al*., 1997; Zhang and Stewart, 1997; Brubaker *et al*., 1999b; Percival *et al*., 1999; Brown and Brubaker, 2000; Cronn and Wendel, 2004), but it may (as yet) not have been published as a new species in accord with the *International Code of Botanical Nomenclature*.

[4] *Gossypium lanceolatum* Todaro is not a distinct species, but instead considered to be a local Mexican landrace — in domesticated *Gossypium hirsutum* race palmeri (Brubaker and Wendel, 1993).

Appendix 2. Genera of weeds regionally common in cotton

Dicotyledons	Monocotyledons
Abutilon	*Alopecurus*
Achyranthes	*Cenchrus*
Alternanthera	*Commelina*
Amaranthus	*Cynodon*
Boerhavia	*Cyperus*
Capsella	*Dactyloctenium*
Celosia	*Digitaria*
Chamaesyce (*Euphorbia*)	*Echinochloa*
Chenopodium	*Eleusine*
Convolvulus	*Leptochloa*
Croton	*Lolium*
Datura	*Panicum*
Desmodium	*Paspalum*
Diplotaxis	*Poa*
Fumaria	*Rottboellia*
Geranium	*Setaria*
Heliotropium	*Sorghum*
Hibiscus	
Ipomoea	
Matricaria	
Merremia	
Oxalis	
Papaver	
Parthenium	
Pavonia	
Plantago	
Polygonum	
Portulaca	
Raphanus	
Ridolfia	
Senna (*Cassia*)	
Sesbania	
Sida	
Sinapis	
Solanum	
Stellaria	
Trianthema	
Tribulus	
Urtica	
Xanthium	

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Section 2.
Bananas and plantains (*Musa* spp.)

1. Taxonomy and genomic groups

1.1. Background

Edible bananas and plantains belong primarily to *Musa* section *Musa* (traditionally *Eumusa*). Within this section, the originally Asian species *Musa acuminata* and *Musa balbisiana* have provided the sources for domestication and development of the great majority of edible fruit. Dessert and cooking bananas and plantains are major foods worldwide, cultivated in over 130 countries throughout tropical regions and in some subtropical regions, in Asia, Africa, Latin America and the Caribbean, and the Pacific. Global production is difficult to determine because the plants are so often grown locally in small plots (*e.g.* household gardens) and consumed locally. The reported area cultivated worldwide in 2006 was 4.2 million hectares of bananas and 5.4 Mha of plantains, with a world production of 70.8 millon metric tonnes of bananas and 34.0 Mmt of plantains (FAO, 2008). The international trade, which involves just a few varieties of fruit, accounts for 15% of production. In addition to the edible species, *Musa textilis* (abacá, Manila hemp) is important for fibre production, and there are several ornamental species (Häkkinen, 2007).

The English word plantain apparently was derived from the Spanish plátano (Simmonds, 1966/1973; Smole, 2002), a name that is used throughout the Spanish-speaking world although its scope changes regionally (and the word's origin is unknown). (Plantain is also employed for the genus *Plantago*, unrelated plants in the Plantaginaceae.) In much of Central America and South America the word plátano is reserved for the starchy fruit and banana is used as in English for the sweet fruit, whereas plátano is used for both fruit types in Mexico and Spain — including the Canary Islands, to which the plants were brought by early Portuguese explorers and from which they were carried to the New World (Ferrão, 1992; Galán Saúco, 1992). The word banana, which probably originated in West Africa and was put into wider use by the Portuguese, also has narrow to broad usages, which frequently can include all the edible *Musa*, or routinely all species of the genus *Musa*. Thus one can speak of a plantain banana and a dessert banana (*e.g.* Tezenas du Montcel, 1985/1987) to maintain the typical distinction between a starchy fruit and a sweet fruit.

In their centres of domestication in Southeast Asia and South Asia there is as well a broad crop concept, with single vernacular names that do not differentiate between dessert and cooking bananas (including plantains): kalpatharu in India, kera in Nepal, chiao in China, chuối in Vietnam, kluai in Thailand, pisang in Malaysia and Indonesia and saging in the Philippines. The numerous cultivated varieties in these regions are typically distinguished by adjectives (*cf.* Table 1) (*e.g.* Valmayor *et al.*, 2000a, 2000b; Uma and Sathiamoorthy, 2002). In Africa, where *Musa* was introduced several thousand years ago (Mbida *et al.*, 2001), the vernacular names also reflect the considerable diversity of cultivated varieties and their uses, and plantain has varied meanings (Swennen and Vuylsteke, 1987; Swennen, 1990a).

Plantains and bananas when distinguised for the respectively starchy and sweet fruits, correspond roughly to two supposed species (but actually hybrids) described by Linnaeus in 1753 and 1759,

to which he gave the respective names *Musa paradisiaca* and *Musa sapientum*. *Musa paradisiaca* referred to cultivated plants (similar to the modern 'French Plantain') with bracts and male flowers usually persistent, and horn-shaped fruits that are rather starchy and cooked to become palatable (*cf*. Linnaeus, 1736/1967, 2007). *Musa sapientum* referred to plants (similar to one of the popular dessert bananas of the tropics — 'Silk') with dehiscent bracts and male flowers, and slightly acidic sweet fruits eaten fresh. The distinction was adopted widely, referring to plantain types as *M. paradisiaca* and dessert types as *M. sapientum*; this "species" nomenclature is now outdated, but is still found in use occasionally. Although recognition of the two main types worked fairly well with the commonly cultivated plants from tropical America and West Africa that were then becoming familiar to Europeans, the plants being grown in Southeast Asia (as well as eastern Africa) were much more diverse — for example with dual-purpose plants having fruits consumed either fresh or cooked, and plants with dehiscent bracts and male flowers but starchy fruits used for cooking (Karamura, 1999; Valmayor *et al*., 2000a, 2000b).

Nomenclaturally, as a generality for a limited purpose, the name *Musa* ×*paradisiaca* L. can be correctly used just to indicate any bispecific hybrid of *M. acuminata* and *M. balbisiana* (*M.* ×*sapientum* L. is simply a synonym) — as both of the plants Linnaeus described are now considered to be AAB triploids (Figure 1) (Argent, 1984; Karamura, 1999). However, for exact work, a more discriminative advanced classificatory system is required, and the genomic groups of the hybrids and species need to be given (*e.g.* Table 1) (Stover and Simmonds, 1987; Valmayor *et al*., 2000b; Pillay *et al*., 2004).

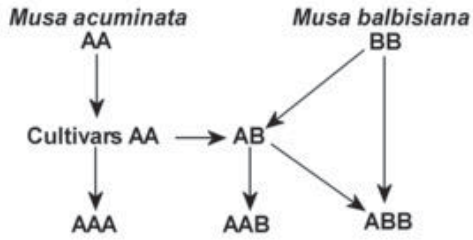
1.2. Taxonomic and genomic classifications

Bananas and plantains are in the genus *Musa* L. of the small family Musaceae (which also includes the genera *Ensete* and *Musella*), in the monocot order Zingiberales (formerly Scitamineae) (Kress *et al*., 2001; Kress and Specht, 2005, 2006). *Musa* has over 60 known wild species (see Appendices 1 & 2), with known diploid (2*n*) chromosome numbers of 22, 20, or rarely 18 or 14 (Simmonds, 1962; Shepherd, 1999). The diploid chromosome number together with phenotypic characteristics of the inflorescence and seeds have been the basis for conventional division of the genus into four, or sometimes five, sections (Appendices 1 & 2): section *Musa* (nomenclaturally correct but often traditionally still called *Eumusa*), *Rhodochlamys*, *Callimusa* and *Australimusa*, and sometimes also *Ingentimusa* (Simmonds, 1962; Stover and Simmonds, 1987; Simmonds and Weatherup, 1990b; Häkkinen, 2004b; De Langhe, 2009). However, researchers are finding some sections ill-defined, and that fewer sections may be more accurate (Jarret and Gawel, 1995; Shepherd, 1999; De Langhe, 2000b; Wong *et al*., 2002, 2003; Nwakanma *et al*., 2003; Bartoš *et al*., 2005; *cf*. Häkkinen *et al*., 2007c; De Langhe *et al*., 2009).

Musa section *Musa* as commonly circumscribed includes over a dozen wild species. Among these are *Musa acuminata* Colla and *Musa balbisiana* Colla, which have provided the great majority of edible bananas and plantains through selection and crossing both intraspecifically (within and between *M. acuminata* subspecies and cultigens), and interspecifically (between these two species) (Figure 1).

Cheesman (1947, 1948a, 1948b) pioneered the modern scientific classification of *Musa* (Simmonds, 1966/1973). Most edible bananas (in the broad sense) derive either from one diploid species, or from its diploid or triploid (or rarely tetraploid) hybrids with one other diploid species. The *Musa acuminata* haploid genome is designated with the letter A, the *M. balbisiana* genome with B. The dessert or sweet bananas often (but not always) are triploids and diploids of *M. acuminata*, and the plantains and other starchy cooking bananas generally (but not always) are triploids derived from hybridisations between *M. acuminata* and *M. balbisiana* (see Figure 1).

Figure 1. Crossing relationships of most cultivated edible *Musa*



A few edible cultigens of interest locally are derived from section *Australimusa* (TT) plants crossed with *Musa acuminata* (resulting in AT or AAT genomes) or also with *M. balbisiana* (resulting in ABBT), and in addition there are a few diploids (AS) from crosses of *M. acuminata* with the section *Musa* species *M. schizocarpa* (SS) (Table 1). Furthermore, the Fe'i bananas, a completely different edible group of cultigens, were domesticated separately from within *Australimusa*; they have not been characterised further genomically (Table 1). *Musa lolodensis* and/or *M. maclayi* may have provided the wild source species for this domestication (Sharrock, 2001).

1.3. Centres of origin of wild Musa species

The genus *Musa*, which is over 50 million years old (Kress and Specht, 2006), has diversified throughout much of the greater Southeast Asian region (*cf.* WWF and IUCN, 1995). *Musa acuminata* and *Musa balbisiana* diverged about 4.6 million years ago (Lescot *et al*., 2008). Appendices 1 & 2 provide a provisional classification of the wild species of the genus placed in the five sections as conventionally used (*cf.* Daniells *et al.*, 2001; Pollefeys *et al*., 2004; Häkkinen and Väre, 2008c), and as well their known natural wild distributions. However, the taxonomy of *Musa* is far from settled at the sectional, species, and infraspecific levels, although it has been receiving considerable attention in recent years. The research and explorations are resulting in discovery and description of many distinctive new species as well as many botanical varieties, and in reclassifications of taxa. However some taxa remain little known. The natural geographical range of some taxa is obscure because of limited exploration, and in some cases the difficulty in distinguishing between natural populations and naturalised or adventive (*i.e.* free-living but non-native) plants (*e.g.* De Langhe, 2009). The nomenclature of some taxa is also unsettled (*cf.* Häkkinen and Väre, 2008c; Väre and Häkkinen, 2009a, 2009b). Therefore, Appendices 1 & 2 are a comprehensive sketch of the work actively in progress, but consequently incomplete and not definitive.

The apparently natural range of genuinely wild *Musa acuminata* is the most extensive in the genus, from northeastern India across continental Southeast Asia (including southern China) and the Philippines, and through the Malay Peninsula and Malay Archipelago to northeastern Australia. This species also has the greatest taxonomic diversity. Rigorous taxonomic study of this polymorphic species throughout its range in the wild is needed to evaluate the evolutionary significance of the many named wild subspecies and botanical varieties, some of which are little known. Most of its many subspecies and/or botanical varieties (Appendix 1) are native only west of Wallace's line (between Borneo and Sulawesi), which demarcates the biogeographic discontinuity between Asia and Australasia (Ploetz and Pegg, 1997). In the past 2 million years the region's climate and sea level have undergone considerable fluctuation (Woodruff, 2003; Bird *et al*., 2005). The climate was drier and more seasonal and the sea 100-120 m lower during glacial maxima 250,000 years ago and again roughly 20,000 years ago (Voris, 2000; Woodruff, 2003). Consequently, a single continental landmass (called Sundaland) then included Indochina, Thailand and the Malay Peninsula integrally with Sumatra, Java and Borneo (and associated smaller islands of the Sunda shelf). The Philippines Archipelago was then largely consolidated. New Guinea and Australia were then part of a separate single landmass, Sahul (which included the now-

submerged Sahul shelf). The many subspecies and/or botanical varieties of *Musa acuminata* occurring in various areas within this large and diverse region are presented geographically in Appendix 1 to clarify their natural patterns of distribution.

The natural range of *Musa balbisiana* extends from India including the Andaman Archipelago (Sarma *et al*., 1995; Uma and Sathiamoorthy, 2002; Uma *et al*., 2005; Uma and Buddenhagen, 2006; Fuller and Madella, 2009) across northern Myanmar (Burma), Thailand (Swangpol *et al*., 2007; *cf*. De Langhe *et al*., 2000) and Indochina to southern China (Ge *et al*., 2005; Wang *et al*., 2007) and the Philippines (Sotto and Rabara, 2000; Valmayor *et al*., 2002). Farther southward, it may be naturally rare or instead only be introduced and naturalised on the southern Malay Peninsula and in the Malay Archipelago, as well as on New Guinea and New Britain (*cf*. Simmonds, 1962; Argent, 1976; Sharrock, 1990; De Langhe and de Maret, 1999; Häkkinen and Väre, 2008c; De Langhe, 2009). Essentially, *M. balbisiana* may be among the species that originated in the Indochina biogeographic province, which transitions in the mid-portion of the Malay Peninsula to the Sundaland biogeographic province. These two regions are biogeographically distinct (both in floras and faunas) possibly because seaways separated the northern and southern portions of the present-day peninsula (in the Isthmus of Kra area) with the fluctuations in oceanic height at various times in the past (Woodruff, 2003).

2. Domestications, crop difusion, cultigen classification

2.1. Domestications and diversification

There is evidence of utilisation of bananas in the highlands of New Guinea 10,000 years ago and cultivation by 7,000 years ago (Denham *et al*., 2003, 2004; Denham, 2005). Some wild bananas in Southeast Asia were no doubt used for non-food purposes such as shelter, fibre and dyes, and their starchy corms may initially have been the main focus for human food as the wild seedy fruits are not very palatable (De Langhe, 1996; Sharrock, 1997; Kennedy, 2009). Edibility of the fruits of diploid *Musa acuminata* likely developed through selection. The increasing interest in them was probably linked to the occurrence of parthenocarpy (asexual fruit development) and seed decline in the primitive types of *M. acuminata* consequent to their vegetative propagation. Crossing of the seeded edible diploids (AA) — over time involving several of the many subspecies of *M. acuminata* in diverse regions (Appendix 1) — resulted in the formation of generally sterile hybrid progeny with the genome AAA, also propagated vegetatively. Diverse hybridisations with *M. balbisiana* resulted in mostly sterile hybrid progeny mainly with the genomes AB (dessert bananas), AAB (plantains) and ABB (cooking bananas) (Figure 1) (De Langhe and de Maret, 1999; Kagy and Carreel, 2004). Rarely other combinations arose such as bispecific tetraploid AABB cultigens, and quite infrequently a few additional species were involved (subsection 1.2, and Table 1).

The spectrum of starchy and/or sweet fruits found entirely within some of these main genomic groups shows that the A genome itself is quite diverse — several subspecies of *M. acuminata* (Appendix 1) were involved in varied ways (inter-subspecific hybridisations) in the emergence of varying crop lineages (Horry and Jay, 1990; Lebot, 1999; Carreel *et al*., 2002; Kagy and Carreel, 2004; Swangpol *et al*., 2007; Boonruangrod *et al.*, 2008, 2009; Perrier *et al*., 2009). The Australasian (Sahul) *Musa acuminata* ssp. *banksii* appears to be the primary source of most of the domesticated plants, which may have arisen first in New Guinea (Lebot, 1999; Denham *et al*., 2003, 2004; Perrier *et al*., 2009). Some bananas and plantains apparently also variously involve the more western (Sundaland) *M. acuminata* ssp. *errans* (a Philippines endemic), *M. acuminata* ssp. *malaccensis* (ranging from Java to Thailand) or *M. acuminata* ssp. *zebrina* (a Java endemic) (Appendix 1) (*cf*. Donohue and Denham, 2009).

Traditional domesticated varieties of diploid and triploid dessert and cooking bananas are still abundant throughout Southeast Asia (Valmayor *et al*., 2000a, 2000b), with their primary areas of

diversity in Malaysia and New Guinea. Somaclonal variation, significant because of their vegetative propagation, has increased the genetic diversity (Section VII). Most of the plantain (AAB) types apparently originated in the Philippines and India, the only Asian regions where there is still a great diversity of plantain cultigens (De Langhe, 1996; De Langhe and de Maret, 1999; *cf.* Ball *et al.*, 2006; De Langhe, 2009; Perrier *et al.*, 2009) — the wealth of plantain varieties is found in remote areas of Luzon (Philippines) (Valmayor *et al.*, 2002) and in some parts of South India (Uma and Sathiamoorthy, 2002). West Africa to Central Africa became a secondary centre of diversification, where more than 120 distinct plantain varieties developed (Swennen and Vuylsteke, 1987; Swennen, 1990a; De Langhe *et al.*, 1996; Blench, 2009).

A completely separate edible group, the Fe'i bananas, was domesticated and developed from within the *Australimusa* section of the genus. *Musa lolodensis* and/or *M. maclayi* (Appendix 2) may have provided the source(s) for domestication in New Guinea (Jarret *et al.*, 1992; De Langhe and de Maret, 1999; Sharrock, 2001). The Fe'i group is composed of cultigens characterised by the reddish sap of the plant, shiny bright green bracts, and carotenoid-rich orange-fleshed fruit (Englberger *et al.*, 2003) produced in erect bunches rather than the hanging bunches typical of eumusa types. These crop plants were grown throughout the Pacific region, and notably dominant on Tahiti (Sharrock, 2001; Kennedy, 2008; *e.g.* Englberger and Lorens, 2004).

2.2. Diffusion and migration of crops

Austronesian-speaking people (perhaps from Borneo) may have been the first humans to colonise Madagascar, between 200-500 AD. However much earlier Melanesians apparently reached the East African coast, and brought along several kinds of edible *Musa* for planting (Shigeta, 2002; Perrier *et al.*, 2009). There is suggestive evidence of contact between India and East Africa 4000 years ago, although the earliest definite historical traces of cultural contact date from 2000 years ago (Lejju *et al.*, 2006). Apparent *Musa* leaf phytoliths have recently been found in Uganda dating to 4100-4500 years ago (Lejju *et al.*, 2006; but *cf.* Neumann and Hildebrand, 2009). Based on botanical and ethnographic evidence, De Langhe *et al.* (1996) considered that plantains (AAB) probably reached Africa over 3000 years ago. Apparent *Musa* phytoliths found in Cameroon date to approximately 2500 years ago (Mbida *et al.*, 2001, 2004, 2005, 2006; Vansina, 2003; Vrydaghs *et al.*, 2003; *cf.* Neumann and Hildebrand, 2009). After plantains reached humid forest climates in lowland western Africa, the crop underwent intensive diversification (De Langhe and de Maret, 1999; Karamura, 1999; De Langhe *et al.*, 2005; Blench, 2009).

Edible *Musa* seem to have been distributed throughout Polynesia (remote Oceania) by approximately 200 AD (Kagy and Carreel, 2004; Kennedy, 2008). And between 1000 BC and 500 AD (during Sabean civilisation) possibly some edible *Musa* (ABB cooking bananas, non-plantain AABs) from India reached southwestern Arabia and nearby northeastern Africa through Arab and/or Persian traders, but there is no certain evidence (De Langhe, 2000a). There is inconclusive linguistic speculation on the Arabic words mauz and banana and their association with *Musa* plants (De Langhe, 2000a).

Edible *Musa* probably arrived to the Canary Islands from West Africa during the 15th century (Ferrão, 1992; Galán Saúco, 1992). They were introduced from these islands to the Americas by at least 1516 but likely earlier (the first recorded introduction was to "La Española" island in the Caribbean). *Musa* spread rapidly throughout the tropical regions of the New World (*cf.* Johannessen, 1970). Their considerable distribution soon after Columbus' first voyage is well documented, leading to speculation on a presence even prior to 1492 (Moreira, 1999; Smole, 2002; *cf.* Perrier *et al.*, 2009). However the usual explanation is that the rapid foothold and spread ran parallel to the slave trade, for which banana and plantain were considered a staple food (Simmonds, 1966/1973). The relative durability of propagation material and the speed with which the plant produces fruit favour this hypothesis (Stover and Simmonds, 1987). By the early 1800s the cultivars 'Dwarf Cavendish' and 'Gros Michel' had been introduced to the New World from Southeast Asia.

The modern international trade began in the late 19th century, and with improving transportation and storage, banana increasingly became a major food item in temperate-zone markets of the Western World as well as the Far East. Select cultivars were introduced into the Canary Islands by the end of that century and increasingly exported to other European countries (mainly England and France), and are now grown commercially in Portugal, Spain and Turkey.

2.3. Classification of cultivated Musa

The vast majority of plantains, cooking bananas and dessert bananas are sterile seedless triploids (Figure 1) (INIBAP, 1995; Zeller, 2005), but some diploid or rarely tetraploid cultigens are grown, nearly all derived either from *Musa acuminata* or from hybridisation between this diverse species and *M. balbisiana*. Plantains and cooking bananas usually are bispecific triploid hybrids, AAB or ABB respectively, with the exception of the East African Highland bananas, which are starchy AAA triploids used in that region either for beer production or as a cooked vegetable (Karamura, 1999; Karamura and Pickersgill, 1999; Carreel *et al*., 2002).

The dessert bananas are more diverse genomically. The most important to commerce are those of the Cavendish subgroup, which supply 95% of the world export trade. They are AAA triploids, the best known being the cultivars 'Grande Naine', 'Williams', 'Valery, 'Robusta', Poyo' and 'Dwarf Cavendish'; among the other dessert AAA triploids is 'Gros Michel', at one time the world's leading commercial cultivar but now nearly absent due to its high susceptibility to Fusarium wilt disease. Other dessert bananas of smaller economic importance include AA diploids, such as the fruit called 'Pisang Mas' in Southeast Asia and 'Bocadillo' or 'Orito' in parts of Latin America (which is well known due to its excellent taste, and prised by European gourmet fruit retailers); some AB diploids; AAB triploids like 'Silk' (*e.g*. 'Manzano') and 'Pome'; and the modern AAAB tetraploid 'Goldfinger'.

There have been many efforts to inventory and classify the wealth of cultigens and cultivars of bananas and plantains in different *Musa* germplasm collections. Evaluation based on morphology had been the standard (Simmonds and Shepherd, 1955; Simmonds and Weatherup, 1990a; IPGRI-INIBAP and CIRAD, 1996; Ortiz, 1997a). Modern techniques to detect differences have employed isozyme polymorphism (Bhat *et al.*, 1992a, 1992b); and various DNA molecular markers, such as RAPD (Howell *et al.*, 1994; Bhat and Jarret, 1995; Bhat *et al.*, 1995; Damasco *et al.*, 1996); RFLP (Gawel *et al.*, 1992; Jarret *et al.*, 1992; Carreel *et al.*, 1993; Fauré *et al.*, 1993; Bhat *et al.*, 1994); AFLP (Ude *et al*., 2002a, 2002b; Noyer *et al*., 2005); and microsatellites (SSRs) (Lagoda *et al.*, 1996; Dussart, 2001; Creste *et al.*, 2004; Buhariwalla *et al*., 2005). Chloroplast DNAs of A and B cytoplasms have been differentiated (Umali and Nakamura, 2003; Boonruangrod *et al.*, 2008), and as well their mitochondrial DNAs (Boonruangrod *et al*., 2008), and the genomic distribution of nuclear ribosomal DNA has been studied in various species (Bartoš *et al*., 2005; Boonruangrod *et al*., 2009).

Table 1 provides a summary of some familiar or otherwise notable cultivated varieties (cultigens, landraces, cultivars) of edible *Musa* species and hybrids classified by cross and the level of ploidy (*cf*. Stover and Simmonds, 1987; Daniells *et al.*, 2001; Carreel *et al.*, 2002; Zeller, 2005). Molecular methods have provided a breakthrough, which is confirming the ploidy and genomic group of most accessions, but also resulting in reclassification of several varieties (*e.g*. Pillay *et al*., 2006).

Table 1. Some cultivated varieties of edible *Musa*

CROSSED SPECIES	GENOME	GROUP/ VARIETY/ CULTIVAR
Section *Musa* (*Eumusa*)		
M. acuminata (AA) × *M. acuminata* (AA)		
	AA	Sucrier: Pisang Mas / Bocadillo Pisang Lilin Pisang Jari Buaya Inarnibal
	AAA	Cavendish: Robusta, Valery Grande Naine (Grand Nain) Pisang Masak Hijau / Lacatan Gros Michel Ambon Putih Red Ibota Mutika/Lujugira (Highland bananas)
	AAAA	Pisang Ustrali Golden Beauty
M. acuminata (AA) × *M. balbisiana* (BB)		
	AB	Ney Poovan: Kisubi Kunnan Kamaramasenge
	AAB (ABA, BAA)	Mysore: Poovan Pisang Raja Silk: Silk Fig, Rasthali, Maçã Pome/Prata Pacovan, Pacha Naadan Nendra Padaththi French Plantain Nendran, Obino l'Ewai False Horn Plantain Agbagba Horn Plantain Three Hand Planty Pisang Nangka Pisang Kelat Laknao (Laknau) Iholena (Pacific plantains) P p 'ulu / Mai'a Maoli (Pacific plantains)
	ABB (BAB)	Bluggoe Ney Mannan Monthan Saba

CROSSED SPECIES	GENOME	GROUP/ VARIETY/ CULTIVAR
		Cardaba Kalapua Peyan Pelipita[1] Pisang Awak/Pisang Klutuk[2] Klue Teparod (Kluai Tiparot)[3]
	AAAB	Goldfinger
	AABB	Laknau Der
M. acuminata (AA) × *M. schizocarpa*		
	AS, SA	Ato Ungota Vunamami Kokor Wompa
Section *Australimusa* (TT)		
M. lolodensis, *M. maclayi*		
	Fe'i group[4]	Karat Kole Karat Pako Karat Pwehu Menei Rimina Utafan Utimwas Utin Iap Wain
Section *Musa* (*Eumusa*) (AA & BB) × Section *Australimusa* (TT)		
	AT (TA)	Umbubu
	AAT (TAA)	Karoina Mayalopa Sar
	ABBT	Yawa 2 Giant Kalapua

1 8 A chromosomes, 25 B chromosomes (D'Hont *et al.*, 2000; Heslop-Harrison and Schwarzacher, 2007)

2 Pisang Awak was reclassified as AABB by Pillay *et al.* (2006)

3 Earlier considered to be ABBB (Horry *et al.*, 1998)

4 Genome not characterised further

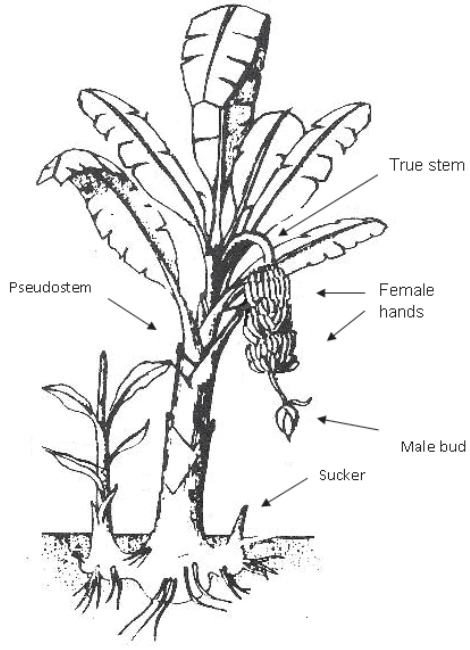
3. Plant cycle and agronomic systems

Bananas and plantains are cultivated in tropical and subtropical regions worldwide within latitudes 30º North and 30º South of the equator and in some additional subtropical regions. In suitable climates, fruits are harvested year-round (Tixier *et al*., 2004; Zeller, 2005).

3.1. Description

Bananas and plantains are large herbaceous plants which can confer the aspect of a tree (Figure 2). One wild species (*Musa ingens*) can reach 16 m tall, although most commercial types grow to between 2 m and 5 m. The main trunk is a pseudostem formed by the concentric assembly of the leaf sheaths (modified petioles), which is crowned by a rosette of very large oblong to elliptic leaves. The leaf blade (up to 2 m^2) normally is transversely split (by wind) between parallel veins, which assists cooling and photosynthesis (Karamura and Karamura, 1995). Leaves are produced successively until the single inflorescence is cast (Allen *et al*., 1988), and are present in variable number (10 to 20 under healthy conditions) depending on the variety, the climate and cultivation practices. Each leaf takes 7-14 days to emerge (Turner *et al*., 2007).

Figure 2. Schematic representation of a cultivated banana plant



The true stem is a subterranean organ (corm) which extends upwards at the core of the pseudostem until culminating in the inflorescence which emerges from the top. The meristem of the true stem produces all other parts of the plant (Swennen and Rosales, 1994). The many main roots emerging from the rootstock are rather straight, adventitious cords (2-10 mm in diameter) that extend up to 2-3 (-5) m outwards, from which (with a density of 8 to 10 per cm of cord root) branch lateral roots (0.3-4 mm in diam) that extend up to 1 m, and from which tertiary rootlets extend for several cm. Under usual commercial growing conditions, most of the root system is within the first 60-100 cm radius from the plant and reaches to 20-40 cm in depth (though roots have been found to 150-180 cm deep in exceptional soils) (Blomme *et al*., 2000; Belalcázar *et al*., 2005; Draye *et al*., 2005; Turner *et al*., 2007).

There are two conspicuous main phases in the development of the banana plant: a vegetative phase characterised by the appearance of leaves, and a reproductive phase during which the inflorescence emerges (Stover and Simmonds, 1987; Swennen and Rosales, 1994; Karamura and Karamura, 1995; Turner *et al*., 2007). This is a simplification of the actual developmental process, as the apical meristem has already differentiated from its vegetative phase into a reproductive meristem before the appearance of the last new leaves. At the transition from the vegetative to the floral stage the plant's growing point shifts mitotic activity, with the whole dome contributing to the aerial stem and inflorescence development. Six months or more after planting, the inflorescence tip first appears at the top of a pseudostem, and the inflorescence continues to elongate and mature, in crop plants typically turning downward. Floral clusterings appear, enlarge, and differentiate into flowers. This elongating aerial stem is totally dependent for its mechanical support upon the surrounding mass of leaf sheaths (the pseudostem).

The large inflorescence is composed of flowering clusters which occur helicoidally along its axis (reproducing the phyllotaxy pattern of the leaf system) and are each covered by a large bract typically ranging from yellow over reddish to purplish. Each nodal cluster has 12-20 flowers, which are aligned in two tightly adjacent rows. The flowers in a cluster usually are either functionally female or male (see Section V), but sometimes are hermaphroditic or neuter (Kwa and Tomekpé, 2000). The first (*i.e.* basal) 3-9 (to 18) clusters, or hands, are female and give rise to the edible fruits — often known as fingers. Subsequent (*i.e.* more distal) clusters are of an intermediate or usually a male character and do not produce edible fruit (in most cultivars, rudimentary fruitlets fall before the edible fingers mature). The distal portion of the inflorescence may continue to elongate and produce many clusters of male flowers from its large terminal bud (which however is absent in some varieties). The group of commonly 5-15 fruiting hands which constitutes the basal portion of the inflorescence is the harvestable unit, called a bunch.

Commercial fruits typically develop by vegetative parthenocarpy (*i.e.* with neither fertilisation nor pollination required), and are completely seedless and sterile. The maturing fruits typically bend upwards in some cultivars, whereas in other cultivars the fruits splay outwards from the inflorescence stalk. Normal commercial bunch weight is 15-30 kg although bunches over 45 kg are not unusual when plants are properly cultivated (and exceptional cases over 125 kg have been recorded). A medium-sized dessert banana finger weighs around 160 g (whereas most wild *Musa* fruits are about 30 g to rarely 80 g).

Up to 2 years are required in a fruiting cycle. Depending on climate, cultivation conditions and the cultivar, vegetative growth lasts 6 to 12 months, and the period between emergence of the inflorescence and harvesting of the bunch can be 2.5 to 10 months (Stover and Simmonds, 1987; *cf.* Turner *et al*., 2007). The time from planting to shooting (inflorescence emergence) generally varies from about 180 to 250 days. Shoot to harvest time in most tropical areas below 500 m elevation varies from about 75 to 125 days, and from about 110 to 250 days in most subtropical areas.

Although the principal plant dies after producing fruit, the whole plant is perennial in as much as suckers successively replace the senescent aerial part without need for replanting. Several suckers emerge consecutively from buds located at leaf axils on the rhizomatous rootstock (Kurien *et al*., 2002). Under commercial cultivation most suckers are regularly eliminated — leaving to replace the mother plant either the firstly emitted vigorous sucker, or the sucker capable of producing a bunch when better prices can be obtained. Sucker growth is in competition with bunch development, so rigorous sucker pruning is needed (Dens *et al.*, 2008). During the dependent or juvenile phase, the sucker develops short narrow (lanceolate) leaves that increase in size sequentially until the appearance of the well-developed adult leaves. The subsequent independent phase (easily distinguished in the Cavendish subgroup of cultivars) is signaled by the first adult leaf, which has an orthogonal angle at the base of the blade's left half (as viewed from beneath) (Galán Saúco, 1992).

3.2. Abiotic environment

3.2.1. Edaphic adaptation

Bananas can be cultivated in soils of varied textures, from very sandy to very clayey, although the former soil extreme may require more frequent watering or a mulching system to maintain water content, and the latter soil extreme causes problems of aeration. Good soil drainage is necessary to cultivate the crop efficiently (Simmonds, 1966/1973). Soil depth should be at least 80 cm and preferably reach 120 cm, but can be as shallow as 40-60 cm for single-cycle high-density plantations from *in vitro* plant material (Stover and Simmonds, 1987).

A high content of organic material (> 2.5%) and pH range between 6 and 7 are optimal, but values between 4.2 and 8.1 are found in established plantations (Soto, 1985). A good soil must have large amounts of potassium (K) and magnesium (Mg). Generally 200-350 mg of potassium per kg of soil is considered enough (unless the soil has large quantities of calcium). The K/Mg ratio must be around 0.25 in sandy soils and close to 0.50 in heavier soils. Soils with a high content of soluble potassium render banana plants more tolerant to salts. Under these conditions the development of the plant is not affected in soils with conductivity up to 7 dS/m (Stover and Simmonds, 1987). Average annual fertiliser applications to the commercial crop are often in the range of 200-450 kg/ha nitrogen (N_2); 400-1200 kg/ha potassium (K_2O); and 40-96 kg/ha phosphorus (P_2O_5) (Lahav and Turner, 1983; Soto, 1985). Banana plants are accumulators of silicon (Henriet *et al.*, 2006).

3.2.2. Light

Light intensity under normal plantation densities and crop conditions is not a serious concern. But a lack of light, for example from excessive plantation density, can cause a prolonged plant cycle, and mild sun exposure together with excess water reduce growth and development. Photosynthetic activity increases rapidly at light intensities between 2,000 and 10,000 lux, and increases further between 10,000 and 30,000 lux. Except under extreme conditions, day-length variations do not have a substantial influence (Champion, 1963/1968; *cf.* Turner *et al*., 2007). Exposure to high light intensities combined with high temperatures causes scorching in the plant.

3.2.3. Temperature

The rate of plant growth and time to fruit maturity are chiefly determined by temperature. Optimum temperature for foliar growth is 26-28ºC (Ganry, 1980), and for fruit development is slightly higher at 29-30ºC. Temperatures of 37ºC or more cause leaf scorch, and growth stops at 38-40ºC. As for low temperatures, foliar emergence for the tallest Cavendish clones stops below 16ºC and all growth stops at about 10ºC (Aubert, 1971; Stover and Simmonds, 1987). Flower development is strongly affected by temperature (Turner *et al*., 2007). If low temperatures (10-15ºC) occur prior to flowering, the emergence of the inflorescence must overcome the pressure imposed by leaves that remain blocked. Consequently, the true stem can be twisted and the pseudostem broken (Stover and Simmonds, 1987). When emergence of the inflorescence is difficult, it is necessary to help by making a "caesarean" cut. Even in cases free of blockage, floral differentiation during subtropical winter may result in the generation of bunch malformations (typically in May in the northern hemisphere, November in the southern hemisphere). Low temperatures — even close to the normal of 12ºC for fruit transport — cause fruit damage, such as uneven softening of the pulp during ripening and shortening of shelf life. Below 9ºC, latex coagulation occurs in the pericarp and the fruit's ripening capacity is lost (Stover and Simmonds, 1987).

3.2.4. Water requirements, humidity and wind

The banana plant's hydric state is generally considered the second main factor responsible for growth and development and fruit production, being particularly important above 20ºC. The plant has high water

requirements due to its fast development and large foliar area, but it has some tolerance to drought apparently by closing stomata and maintaining strong root pressure (Turner *et al*., 2007). A plant's typical daily water transpiration has been estimated to vary between 5.6 mm under direct sun exposure and 1.9 mm when totally cloudy (Champion, 1963/1968; Turner *et al*., 2007). The amount needed increases as the temperature rises, and above 30ºC the water consumption can double. Between 60% and 80% relative humidity is ideal (*cf*. Lu *et al*., 2002).

In regions of limited rainfall intensive modern plantations are equipped with drip irrigation systems, but sprinkling or flood irrigation systems are still frequent in many plantations. Bananas can sometimes tolerate up to 48 hrs of flooding with flowing water (*e.g.* after a storm), but stagnant water with full sunshine kills adult plants.

In general, cultivars better adapted to subtropical conditions also better tolerate wind speeds up to 40 km/h. But at the higher speeds severe crop damages occur, and wind above 55 km/h can cause total destruction of the plant.

3.3. Cultivation practices

The main commercially important *Musa* cultivars are grown alone, commonly in open-air plantations in the tropics. Cultivar selection, planting design and culture practices play important roles in the plant's development. The most frequent designs in commercial plantations have 2000 to 2400 plants per hectare, with one plant per planting hole, aligned in rows that are separated by wide aisles. Particular cultivars can be difficult to manage depending on the planting distances. Cutting off several leaves after flowering is often recommended to favour light penetration for plant development. Modern planting designs use drip irrigation and mulching, which facilitates mechanical and chemical weed control. Lists of weeds commonly associated with banana cultivation, and herbicides commonly employed, are provided by Belalcázar *et al*. (1991).

In subtropical regions where climatic requirements may not be sufficiently fulfilled, greenhouse cultivation offers the possibility of increasing fruit quality and yields. Spain (Canary Islands) and Morocco are the major countries producing banana under greenhouse, both with about 3000 ha. Commercial greenhouse cultivation is also carried out in Greece (Crete) (400 ha) (Galán Saúco, 1992), South Africa (3 ha) (Eckstein *et al.*, 1998), and several other countries including Portugal, Tunisia, Turkey, Korea and Argentina. Israel has been interested in increasing greenhouse plantation (beyond about 50 ha) mainly due to the reduction in water consumption.

In many parts of the humid tropics *Musa* is grown traditionally with other crops (Swennen, 1990b; Rodrigo *et al*., 1997; Norgrove and Hauser, 2002; Achard *et al*., 2004). As the *Musa* plants require a large interrow, other crops may be successfully grown in between. For example *Dioscorea* (yams), *Alocasia macrorrhizos* (giant taro) and *Piper methysticum* (kava) are intercropped in Micronesia, and *Colocasia esculenta* (taro, cocoyam) in Papua New Guinea. In southern Nigeria intercropping with *Manihot esculenta* (cassava) was found to reduce yield but also the incidence of black Sigatoka disease. In southern Cameroon the fields are prepared by clearing secondary forest manually and intercropping plantain with *M. esculenta*, *Cucumeropsis mannii* (white-seed melon, egusi-itoo) and *Xanthosoma sagittifolium* (yautia, tannia). *Musa* is also grown as an intercrop in the early stage of establishing plantations of coconut (*Cocos nucifera*), betelnut (*Areca catechu*), coffee (*Coffea* spp.), black pepper (*Piper nigrum*), cocoa (*Theobroma cacao*) and rubber (*Hevea brasiliensis*). *Musa* is sometimes grown in combination with timber production, for example under *Casuarina* trees in the Papua New Guinea highlands and *Ficus natalensis* (Natal fig) in southwestern Uganda.

3.3.1. Propagation

Most edible bananas and plantains are vegetatively parthenocarpic, producing fruits without needing any stimulus from pollination or seed development. Because the fruits of most cultivars are either always or normally seedless, agricultural propagation is principally asexual by means of suckers and other vegetative multiplication techniques, including micropropagation. Plants are generally propagated from traditional planting material (which may be obtained from commercial plantations), either emerged suckers (of different sizes) or sucker corms originating from axillary buds, or from the lateral buds of corms or rhizomes of decapitated grandmother plants (which may have or lack suckers). Modern plantations are being established with plants propagated through *in vitro* culture (Section VII), which provides a large quantity of uniform, disease-free (including virus-free) plants. This approach however can generate somaclonal variants that result in off-type plants if not properly managed.

3.3.2. Sucker selection and desuckering

As a general rule, the more advanced the development of the mother plant, the taller must be the sucker selected to replace it in the plant's following cycle. In the tropics it is generally recommended to preserve the axial sucker, which is normally the most vigorous and was first emerging from the mother plant (Champion, 1963/1968; Simmonds, 1966/1973). In South Africa and Israel deep-emerging suckers are preferable (particularly the axial sucker). However, given these subtropical climates, the period of flower differentiation is also matched with the warmest months in summer. This is done to achieve better yields and direct the harvest period to wintertime when prices are traditionally higher. In the Canary Islands the usual desuckering practice is elimination of new shoots emerging during wintertime (before April). The sucker that will replace the mother plant is chosen in June or July from among those that emerged in the springtime.

Desuckering by mechanical or chemical means eliminates undesired suckers and so enhances the plant's yield (*cf.* Kurien *et al*., 2006; Dens *et al.,* 2008); only those suckers that will support continuity of the plantation are kept. Desuckering can be accomplished by repeatedly cutting off the emerging plant at ground level, the mechanical removal of the apical meristem, or application of 2-5 ml of kerosene; 2.5 ml per plant can be injected directly through the leaf bases of intact, 10-40 cm high suckers. For plants started from *in vitro* culture, which have high sucker proliferation during their first cycle, desuckering must be done before new shoots are over 40 cm high to avoid competition and toppling of the mother plant.

3.3.3. Practices enhancing fruit development

The developing commercial fruit is protected by carrying out several operations to prevent the bunch from falling to the ground and appearance of skin blemishes. The first practice is deleafing — leaves that may be touching the newly developed hands are cut off, and collapsed and leaf-spot infected leaves are removed. Next are bagging, debudding, and dehanding. This operation consists of bagging the bunch, removal of the male-flowers bud (the distal portion of the inflorescence), and breaking off the false hand plus one or two of the smallest apical hands. Bunch-bagging is a common practice to reduce the time between flowering and harvest, and improve fruit yield and quality. The bags are cylindrical (open-ended), perforated, and made of transparent or blue polyethylene; the ends are left open but secured to avoid wind damage. Bags are normally placed after bunch deflowering and application of insecticide, about 3 weeks after bunch emergence.

3.4. Harvest and storage

Most of the fruit's pulp develops from the outer part of the trilocular ovary (Stover and Simmonds, 1987). Fruit is age-graded in order to prevent the occurrence of ripe fruits during storage and transport. Determination of the ripening stage is normally done by observation of the bunch-filling degree (which

varies depending on the time after emergence of the inflorescence or flowering and the month of bunch formation), the size of the fruit and fruit angularity, its specific gravity, and pulp-to-skin ratio. Quality rules have establishd that the minimal finger thickness must reach 27 mm.

Bunch cut-off at harvest time must be done with extreme care. One worker partially cuts the pseudostem halfway up its length (not necessary for Dwarf Cavendish) while the other puts the bunch on his shoulders and holds it firmly until the first worker cuts the rachis across just above the first hand.

Banana is a highly perishable fruit, with post-harvest losses of 30-40% (Salunkhe and Desai, 1984). Methods for extending the shelf life of fruits include the harvesting of bunches a few days before they reach full maturity, storage at reduced temperatures (about 15ºC), storage in a modified atmosphere of enriched CO_2 with low O_2 (3-4%), packing in film bags to prevent weight loss, pre-treatment with fungicides, and skin coating (Waskar and Roy, 1996). For cultivars susceptible to high levels of CO_2 an alternative storage method is vacuum packing (Mary and Sathiamoorthy, 2003).

4. Diseases and pests

The most relevant interactions between cultivated bananas and plantains and other organisms are the effects of agricultural diseases and pests, and as well weeds. Bananas and plantains are affected by many diseases and pests, some of which are of profound importance for these major food crops. The main diseases have been summarised by Jones (2002a) and Ploetz *et al*. (2003), the main pests by Gold *et al*. (2002) and Jones (2002a). Some commonly serious or critical diseases and pests (Jones, 2002b) are discussed below (also note Table 2, in Section VI).

4.1. Fungi

Musa plants are known to provide substrate for over 200 species of fungi (a zygomycete, 60 ascomycetes, 23 basidiomycetes, and 120 mitosporic fungi) (Photita *et al.*, 2002).

Sigatoka leaf spot diseases of banana involve two related ascomycetous fungi: ***Mycosphaerella fijiensis***, which causes black leaf streak disease, commonly known as **black Sigatoka**; and *M. musicola*, which causes Sigatoka disease, now usually known as **yellow Sigatoka** (Mourichon *et al*., 1997; Marín *et al*., 2003). Symptoms include respectively dark brown or pale yellow streaks on lower leaf surfaces and large areas of leaf destruction, which causes premature fruit ripening and reduces yields. Black Sigatoka is the more serious because younger leaves show symptoms and more damage is caused to photosynthetic tissue, and it affects many cultivars such as plantains and cooking bananas that have resistance to yellow Sigatoka (Mobambo *et al.*, 1993, 1996; Manzo-Sánchez *et al*., 2005). **Eumusae leaf spot disease** (formerly Septoria leaf spot disease), caused by *M. eumusae*, has been recognised relatively recently, occurring in several countries across eastern Asia as well as Mauritius and Nigeria (Carlier *et al*., 2000; Crous and Mourichon, 2002).

Black Sigatoka has been reported throughout the Pacific, Asia, Africa, Latin America and the Caribbean. Yield losses from 30-50% have been reported (Mobambo *et al.*, 1993). As much as 27% of production costs in tropical America can go to its control (Stover and Simmonds, 1987), and the disease can be responsible for 15-20% of exported fruit's retail price. Yellow Sigatoka is one of the classic global plant epidemics from the first half of the 20th century, and has now been reported in most banana-growing areas. Devastation of commercially grown bananas by the Sigatoka diseases and the danger to a major food staple led to creation in 1984 of the International Network for the Improvement of Banana and Plantain (INIBAP). Perhaps no other diseases or pests have so seriously jeopardised banana production throughout the world (Marín *et al*., 2003).

In commercial plantations systemic fungicides are used to control black Sigatoka, but resistance to some fungicides has developed in strains of both *M. fijiensis* and *M. musicola*. Incorporating genetic resistance into the crops has become the long-term goal (see Section VI).

Fusarium wilt or Panama disease of banana is caused by several soil-inhabiting fungi called ***Fusarium*** *oxysporum* f. sp. *cubense* (Ploetz, 2006). The disease has been reported from all banana-growing regions except Melanesia, some South Pacific islands and some countries bordering the Mediterranean; it is regarded as one of the most destructive plant diseases of modern times (Moore *et al*., 1995; Ploetz, 2000, 2005a, 2005b). Some clones in the AAA Cavendish subgroup are susceptible, including 'Valery', 'Grande Naine' and 'Williams', as well as some clones in the AAB group (*e.g*. 'Silk', 'Pome') and the ABB Bluggoe subgroup. The decline of commercial plantations of 'Gros Michel' resulted in a shift in the mid-20th century to cultivars in the Cavendish subgroup as the main export banana types (Ploetz, 2000, 2005a, 2005b). However, in the early 1990s Cavendish plantations in Southeast Asia and northern Australia began to experience significant losses (Hwang and Ko, 2004).

External symptoms include yellowing of leaf margins which spreads from the oldest to youngest leaves, and lengthwise splitting of the lower portion of the outer leaf sheaths. The leaves gradually collapse and die (forming a skirt around the pseudostem), and new leaves are pale and small. Many infected suckers may be produced before the clump dies.

Several factors influence the development of this disease — the fungus strain and crop cultivar, drainage, environmental conditions and soil type. Four races of *Fusarium oxysporum* f. sp. *cubense* are typically recognised. Three races are pathogenic to particular sets of *Musa* cultivars. Race 1 caused the collapse of the 'Gros Michel' export trade. The now-emerging tropical race 4 affects cultivars that produce 80% of the world's bananas and plantains (Ploetz, 2005b, 2006). These pathogens also colonise alternative hosts, such as relatives of banana and several weed species. The fungi can survive in the soil for up to 30 years as chlamydospores, in infested plant debris, and in the roots of alternative hosts. Spread is most commonly by infected rhizomes or suckers and adherent soil. The disease can spread very rapidly if spores are carried in surface runoff water or contaminate an irrigation reservoir.

Soils in which microbial populations suppress the pathogens are found in Central America, the Canary Islands, South Africa and Australia. Chemical control, flood-fallowing, crop rotation and use of organic amendments have not been very effective. The best means of control is host resistance (*cf*. Javed *et al*., 2004). Useful AAB and ABB hybrids have been bred (Table 2), but no Cavendish replacement. Biotechnology, mutation breeding and somaclonal variation are being employed to produce resistant genotypes (Ploetz, 2005b).

4.2. Unknown agents

False Panama disorder (de Beer *et al*., 2001), which can easily be confused with Fusarium wilt, was first described by Deacon *et al.* (1985). Cavendish bananas in South Africa and the Canary Islands are affected, but other dessert bananas and plantains are affected in Panama, Colombia, Surinam and Grenada. The incidence is usually low, but as much as 60% (perhaps more) of a plantation can be impacted.

External symptoms include yellowing of leaves, necrosis, leaf death, and small bunches. The discoloured vascular strands are usually discontinuous. Examination of a rhizome that has been split open characteristically shows brown spots and white strands.

False Panama disorder seems not to appear in old plantations, nor to be transmitted from one plant to another. Although it has not been possible to isolate a pathogen from affected plants, it is accepted that a fungal pathogen is not the primary cause. Factors influencing the appearance of the disorder may include low temperatures, nematodes, drought, nutritional imbalances and waterlogging (Rabie, 1991; de Beer

et al., 2001). The incidence of the disorder can be kept down or prevented by reducing stress to newly planted material.

Another serious disorder that can be confused with Fusarium wilt has been called **matooke wilt**, although it is of unknown aetiology. At first thought to be limited to East African Highland bananas (in Uganda matooke refers to traditional bananas), it was later found in Uganda on more recently introduced types as well (Kangire and Rutherford, 2001). Leaves may appear healthy or be smaller with marginal necrosis. Pseudostems are thin, dry and weaker, and fruits small and less developed. Internally the pseudostem has conspicuous brown to purple vascular strands. Affected plants are found in areas where household or animal wastes have been discarded.

4.3. Bacteria

Moko disease is a vascular wilt of bananas and plantains caused by strains of phylotype II ("race 2") in the ***Ralstonia*** *solanacearum* (*Pseudomonas solanacearum*) complex (Eyres *et al*., 2005; Fegan and Prior, 2006); it was first reported in the 1890s in Trinidad. In the Caribbean, Central America and South America this bacterial wilt has caused severe losses in smallholder plantations; it is nearly absent in the eastern hemisphere, but has been found in the Philippines. The Cavendish subgroup (AAA) and some ABB cultivars (*e.g*. 'Bluggoe') are susceptible, whereas 'Pelipita' (ABB) is highly resistant, and the Horn plantain (AAB) is seldom infected. **Bugtok disease**, widespread on cooking bananas (*e.g*. 'Saba', 'Cardaba') in the Philippines, is similar and apparently caused by the same bacterium complex (Soguilon *et al*., 1995; Hayward, 2006).

Another bacterial vascular wilt, **banana blood disease**, is caused by strains of phylotype IV ("race 4") of the species complex (Eden-Green, 1994; Fegan and Prior, 2006). This disease was first reported in about 1914 from South Sulawesi in central Indonesia, causing the abandonment of dessert banana plantations being developed on a nearby island. Although its distribution was very limited, it has recently been spreading in Java and elsewhere in Indonesia and poses a serious threat.

Its symptoms are similar to those of Moko disease, and vary according to the growth stage of the plant and route of infection. They include a conspicuous transient yellowing, loss of turgor, desiccation and necrosis. The youngest leaves cease emerging and develop whitish and then necrotic panels in the lamina. Blackening and shrivelling of male flowers is frequently found in mature plants. Daughter suckers may show general wilting, but sometimes healthy suckers are produced. Internally, vascular bundles exhibit a reddish-brown discolouration, and the fruits are usually uniformly discoloured reddish-brown and rotten.

Infection occurs *via* inflorescences, and the disease is transmitted by insects or infected planting material; the pathogen can probably persist in soil or plant debris. Fruits from infected plants may be a source of infection, as affected bunches can appear normal. Sanitation measures include disinfection of cutting tools, field sanitation and selection of disease-free planting materials.

Banana Xanthomonas wilt (or banana bacterial wilt) is a devastating disease emerging in East Africa to which most cultivars appear vulnerable; it is caused by ***Xanthomonas*** *vasicola* pv. *musacearum* (Biruma *et al*., 2007; Aritua *et al.*, 2008). Symptoms are somewhat cultivar-specific and determined by route and stage of infection. Foliar symptoms often resemble those of Fusarium wilt, but excretion of a yellowish bacterial ooze from cut tissues is characteristic of this new disease. Yield losses, which can reach 100%, vary with cultivar susceptibility, crop growth stage at infection and prevailing climatic conditions, with effects being worse during seasons of high rainfall. Disease transmission and sanitation measures appear similar to those given above for banana blood disease.

4.4. Viruses

Banana bunchy top disease, the most important widespread viral disease of banana, is caused by a babuvirus (Nanoviridae) transmitted by the banana aphid *Pentalonia nigronervosa* (Thomas *et al*., 1994; Ferreira *et al*., 1997; Su *et al*., 2003). The disease is native to Southeast Asia, and occurs in many countries of the eastern hemisphere (including Pacific islands); in Africa it has been found in Gabon, D.R. Congo (formerly Zaire) and Burundi. It has also been detected in the southern Caribbean on the island of Curaçao. The disease can cause devastating plantation losses, which have reached 100% in some districts of Pakistan (Pietersen and Thomas, 2003). Infected plants have a rosetted appearance with narrow, upright and progressively shorter leaves. Leaf edges roll upwards and show marginal yellowing. Dark green streaks are found on midrib and petiole, extending downward into the pseudostem. The symptoms only appear on the leaves forming after infection — most diagnostic are short dark green dots and dashes along the minor leaf veins, which form hooks as they enter the edge of the midrib.

All tested *Musa* species and cultivars appear susceptible to the banana bunchy top virus (BBTV), but the incubation period may vary. BBTV is controlled by eradication of diseased plants, and use of virus-free planting material. Severe attacks may require strict programmes with the goal of regional eradication.

Banana streak disease was first recognised in Côte d'Ivoire in the mid-20th century and later reported in Morocco, where the incidence of infection exceeded 50% in many 'Dwarf Cavendish' plantations. Banana streak badnaviruses (BSVs) (Caulimoviridae) have been found worldwide, infecting a wide range of Musa genotypes, with yield losses of 7-90% (Lockhart, 1995; Lockhart and Jones, 1999; Agindotan *et al*., 2006). Co-infection with banana mild mosaic virus (BanMMV) (Flexiviridae) can increase the disease's severity (Pietersen and Thomas, 2003). Although some clones are asymptomatic or tolerant of field infection, the broad range of cultivars susceptible to BSVs include the Cavendish subgroup, 'Gros Michel', Highland bananas, plantains and cooking bananas. The modern increase and movement of Musa germplasm, including the tissue cultures of improved hybrids, have been significantly affected by the risk of spreading these badnaviruses.

Several *Badnavirus* species are recognised to be involved in this disease complex (Lheureux *et al*., 2007; Gayral *et al*., 2008). Disease can result from a BSV species transmitted exogenously, but can arise endogenously as well. Varied DNA sequences from BSVs have integrated into both the B and A genomes. Viral sequences (integrant virus species) in the B genome can become activated and cause disease, for example as a result of stress from tissue-culturing plants (micropropagation), sexually creating interspecific hybrids for breeding purposes, or temperature shifts (Ndowora *et al*., 1999a, 1999b; Geering *et al*., 2005; Fargette *et al*., 2006; Gayral *et al*., 2008). Practical molecular methods are being devised to detect the DNA of episomal BSV infecting *Musa* separately from concomitant BSV endogenous pararetroviral sequences (Agindotan *et al*., 2006; Le Provost *et al*., 2006).

Symptoms of banana streak disease vary considerably — depending on the pathogen species and strain, host cultivar, environment and season — from inconspicuous chlorotic flecking to lethal chlorosis. The most common symptoms are chlorotic streaks and blotched leaves. Other symptoms include streaks in the pseudostem, narrow leaves, constriction and reduction of the bunch, fruit-peel streaks and peel splitting. Transmission by citrus mealybugs (especially *Planococcus citri*) occurs, but spread is primarily by multiplication and dissemination of infected planting material. Control consists mainly in use of starting materials (in the lab and the field) free of both non-integrant BSVs and B-genome integrated virus sequences, and the prompt elimination of infected plants.

Banana bract mosaic disease is widespread in the Philippines and has been found as well in Vietnam, Thailand, India, Sri Lanka and Western Samoa. A potyvirus, banana bract mosaic virus (BBrMV), has been isolated from infected plants (Thomas and Magnaye, 1996; Thomas *et al*., 1999). BBrMV occurs in a wide range of edible banana genotypes. It is common in the ABB cultivars 'Cardaba'

and 'Saba' popular in the Philippines — up to 40% yield losses have been recorded there in 'Cardaba' and the also-popular 'Lakatan'. BBrMV and another potyvirus, abacá mosaic virus (AbaMV), also infect *Musa textilis* in the Philippines (Pietersen and Thomas, 2003).

The diagnostic symptom of BBrMV disease is a dark reddish-brown mosaic pattern on the bracts subtending male flowers in the inflorescence. Initial symptoms (depending on cultivar) include green or reddish-brown streaks or spindle-shaped lesions on the petioles and a congested leaf arrangement. Lamina symptoms, spindle-shaped chlorotic streaks parallel to the veins, are most prominent on young leaves in recent infections. When dead leaf sheaths are pulled away from the pseudostem, distinctive dark-coloured mosaic patterns, stripes or spindle-shaped streaks are visible. There are no reports of mechanical transmission, but BBrMV is transmitted by several species of aphids. The virus is also transmitted in vegetative planting material including "bits", suckers and tissue-cultured plantlets.

Strains (in subgroup I but as well II) of **cucumber mosaic cucumovirus** (CMV) (Bromoviridae) are increasingly being found on bananas, and may cause no symptoms or mild to in some cases severe disease — chlorosis, mosaic and heart rot (Pietersen and Thomas, 2003). CMV occurs worldwide, has the largest host range of any plant virus, and is transmitted by many species of aphids. It is particularly important to use CMV-free source material for *in vitro* mass propagation. Agronomic practices include co-operation regionally to avoid growing other host crops nearby and eliminate weed hosts.

4.5. Insects

The **banana weevil or banana borer *Cosmopolites*** *sordidus* (Coleoptera: Curculionoidea, Dryophthoridae) generally is the most important insect pest of banana and plantain and also impacts the related Musaceae crop enset (genus *Ensete*) (Gold and Messiaen, 2000; Gold *et al*., 2001). Losses of more than 40% to even 100% of the planting have been recorded, as the weevil populations increase in successive cropping cycles. Problems are most severe in plantains and Highland cooking bananas, contributing to disappearance of the latter crop in parts of East Africa (Kiggundu *et al*., 2007). In commercial Cavendish plantations, this weevil has been reported to be relatively unimportant.

The species evolved in Southeast Asia (Malaysia-Indonesia) and has been spread to all banana and plantain production regions. Adults are attracted by volatiles emanating from the host plants. Cut corms and young suckers are especially susceptible. Larvae bore into the corm and interfere with root initiation, kill existing roots, limit nutrient uptake, reduce plant vigour, delay flowering, and increase susceptibility to diseases and other pests, and to toppling by wind.

Control in commercial banana plantations is mainly chemical, using nematicides with insecticidal activity and specific insecticides. However, the banana weevil has shown the ability to develop resistance to most classes of chemicals. Botanical compounds may serve as deterents. Hot-water treatment has been widely promoted for control. Significant biological control may sometimes be achieved *via* foraging ants (*e.g. Pheidole* and *Tetramorium*, Myrmicinae), some species of which will even forage in the weevil's tunnels (Abera-Kalibata *et al*., 2007).

Primary sources of resistance are found in some wild accessions or clones, such as Calcutta 4 (group AA), 'Yangambi-Km 5' (AAA), FHIA-03 (AABB) (or its parents), Kayinja (ABB) and the IITA diploid hybrids TMB2x 7197-2 and TMB2x 8075-7 (Pavis and Lemaire, 1996; Kiggundu *et al*., 2003, 2007).

The **banana stem weevil or banana pseudostem borer *Odoiporus*** *longicollis* (Coleoptera: Curculionoidea, Dryophthoridae) is among the most important pests of banana and plantain (Padmanaban and Sathiamoorthy, 2001). The species is believed to have originated in the region of South Asia and Southeast Asia. It is a key pest in India, China, Thailand, Malaysia and Indonesia (Valmayor *et al.*, 1994). Plantains and Highland bananas are preferred. The weevil can cause 10-90% yield loss depending on crop growth stage and management efficiency.

Adults are attracted by volatiles released by the host plants. Infestation normally starts in 5-month-old plants, retarding growth and development, and increases susceptibility to wind lodging. Early symptoms include pinhead-sized holes in the pseudostem, exudation of a gummy substance, and fibrous extrusions from the base of petioles. The pseudostem shows extensive tunnelling in the leaf sheaths. Rotting occurs due to secondary infection. The fruits are dehydrated, with premature ripening.

Chemical control involves injection of a systemic organophosphorus compound into the pseudostem or swabbing with either surfactants or mud slurry containing an insecticide, or fumigation. After harvest the banana stumps must be removed and destroyed. Natural enemies, including arthropods, entomopathogenic nematodes and entomopathogens have great potential as biological control agents to reduce the weevil population in severely infested home gardens.

Host plant resistance may offer a long-term solution. Charles *et al.* (1996) identified 27 *Musa* accessions that exhibited tolerance to the pest. A high degree of resistance can be found in some *Musa balbisiana* clones.

4.6. Nematodes

The **burrowing nematode *Radopholus similis*** is one of the most important root pathogens attacking bananas in the tropical zone (Sarah *et al*., 1996), especially in Cavendish types. Vegetative propagation using infested corms or suckers has disseminated this pest worldwide (Marín *et al*., 1998). It is common on plantains and cooking bananas, except in Central America, West Africa, the highlands of central-eastern Africa and subtropical zones of production. The distribution is mainly due to the species' preference for a temperature range from 32º to 24ºC.

Radopholus similis is a migratory endoparasite, completing its life cycle in 20-25 days in root and corm tissues. Their destruction results in reduction of host growth and development, even to uprooting and toppling. Yield losses can be reduced through propping or guying pseudostems. There can be severe losses in bunch weight, and a longer time between harvests. Crop losses depend on soil fertility, drainage and nematode pathogenicity, and vary from 15 to 30 to 75% in three cycles of production.

Nematode populations may be reduced to an undetectable level by a 1-year fallow with non-host plants, 6-7 weeks of complete flooding, or soil fumigation. Recontamination of soil is avoided through use of nematode-free plants and treated corms or suckers.

Some physical and chemical barriers to infection have been detected in roots (Wuyts *et al*., 2006, 2007). Sources of resistance are found in several genome groups in *Musa acuminata* and *M. balbisiana* wild and cultivated diploids (Moens *et al*., 2005; Dochez *et al*., 2006). Pisang Jari Buaya (AA) cultivar types are the source of resistance in the modern AAAB cultivar 'Goldfinger'. 'Yangambi-Km 5' (AAA) also shows resistance. Endophytes of *Fusarium oxysporum* can induce systemic resistance to *R. similis* (Vu *et al*., 2006).

The **root-lesion nematodes *Pratylenchus coffeae*** and *P. goodeyi* are major pests of *Musa* wherever they occur (Bridge *et al*., 1997). *Pratylenchus coffeae* is probably a native of the Pacific and Pacific Rim countries but now has a worldwide distribution, especially affecting Southeast Asia. In Central America and South America it is the most important nematode species affecting Cavendish cultivars, whereas in Africa it affects plantains. This species has an extremely wide host range and is a major pest on other crops. In contrast, *Pratylenchus goodeyi* has a much more restricted distribution. It is considered indigenous to Africa, and is an important pest of Highland bananas and plantains. It is the main nematode affecting Cavendish bananas in the Canary Islands and has the potential to become an important pest where bananas are grown in cooler climatic zones of the Mediterranean and Middle East.

Pratylenchus coffeae and *P. goodeyi* are both migratory endoparasites of the root cortex and corm of banana, plantain and abacá (*Musa textilis*). Their presence in banana root lesions is generally highly

correlated with infection by fungi. Nematode populations are reduced by a bush fallow period exceeding one year, removal of roots, paring of corms to remove the lesions and exposure to direct sunlight. Hot water treatment eradicates almost all nematodes from planting material. The nematicides for control of *Radopholus similis* are expected to be equally effective for the root-lesion nematodes.

Root-knot nematodes, especially ***Meloidogyne*** *incognita* and *M. javanica,* occur on banana and plantain roots wherever the crops are grown (De Waele and Davide, 1998). These nematodes are often found with *Radopholus similis* and *Pratylenchus* spp. (Moens *et al*., 2006), which tend to outnumber and eventually replace the root-knot nematode populations but provide feeding sites for *Meloidogyne*. *Meloidogyne* spp. can become abundant in areas where *R. similis* has not been introduced or the climate is too cold for it, or *P. goodeyi* is not present. In Asia, especially Southeast Asia, *Meloidogyne* spp. are often the most common and abundant nematode species on cooking and dessert bananas.

Root-knot nematodes often occur together with soil-borne fungi; there are indications that synergistic effects exist between these groups of pathogens, but they have not been documented. In roots of 'Grande Naine' infected with *M. incognita*, the endomycorrhiza *Glomus mosseae* isolates suppressed root galling and nematode build-up. *Glomus intraradices* increases plant growth by enhancing nutrition.

Meloidogyne spp. are sedentary endoparasites with a wide host range, especially on dicots. On banana and plantain the most obvious symptoms of infection are swollen, galled primary and secondary roots. Root-tip growth ceases and new roots proliferate just above the infected tissues, giving rise to a lower number of secondary and tertiary roots. Aboveground symptoms include yellowing and narrowing of leaves, stunted plants and reduced fruit production. Losses may range from 25 to 60%.

Meloidogyne spp. may spread with infected planting material. Corms can be disinfected by peeling followed by treatment with hot water, a nematicide, or 5-10 min of 1% sodium hypochlorite. Restraining methods include maintenance of weed-free fallows and cover crops or rotation systems. Limited fallowing may be ineffective, however, since *Meloidogyne* spp. can persist in the soil in the absence of bananas for up to 29 months.

Root extracts from the plants Tagetes erecta, Leucaena leucocephala, Cynodon dactylon and Mimosa pudica, bulb extracts from Allium sativa and A. alia, and leaf extracts from Anthocephalus chinensis and Eichhornia crassipes are highly effective against M. incognita egg hatching and infestation. Purified extracts of several Penicillium spp. (P. oxalicum, P. anatolicum), Aspergillus niger and liquid and powder formulations containing Paecilomyces lilacinus and Penicillium oxalicum have shown high nematicidal activity.

5. Reproductive biology and ecology

The male and female flowers of edible *Musa* differ in several respects (Simmonds, 1966/1973; Stover and Simmonds, 1987). The female flowers are larger (*e.g.* 10 cm long), with a well-developed inferior trilocular ovary that exceeds the somewhat tube-like toothed perianth in length, a stout style and stigma, but stamens reduced to staminodes. In male flowers the exserted stamens have well-developed anthers, but the ovary is rudimentary, with a slender style and small stigma. Beginning at the base of the inflorescence (Section III), each hand of female flowers is exposed by the opening of its large bract; as pollination is not necessitated in the crop plants, the ovaries continue to develop parthenocarpically into the persistent fruits (which lack an abscission layer). The male flowers of a cluster, after being exposed by its large bract for about 24 hrs from dusk to the subsequent night, abscise from the abortive ovary in most cultivars and are shed whole.

5.1. Pollination

Pollination is essential for development of fruits in wild species of *Musa*. Bats and sunbirds (Nectariniidae) as well as honeybees play main roles in the pollination ecology of *Musa*. The sticky pollen is transported by bats and/or birds seeking the copious nectar (in some cases also pollen), and/or by insects (Nur, 1976; Start and Marshall, 1976; Gould, 1978). In regions where suitable bats or sunbirds are not present, insects (*e.g.* honeybees) may play the key role in pollination — which then tends to be more local. *Musa* species with pendulous inflorescences are considered to be especially pollinated by bats, those with erect inflorescences by sunbirds (Itino *et al*., 1991). *Musa itinerans* flowers produce nectar in two peaks, attracting both bats and birds, each at their respective time period during the night or morning, with both groups having similar pollination effectiveness (Liu *et al*., 2002a). Production of scent and nectar by bananas and plantains suggests that pollination may occur from dusk to nearly mid-morning (Gould, 1978; Mutsaers, 1993). In South India, the production of nectar by *Musa* ×*paradisiaca* peaked at 20:00 hr (Elangovan *et al*., 2000).

In wild populations of *Musa balbisiana* within its natural range in China, populations were pollinated by the long-tongued fruit bat *Macroglossus sobrinus* and insects as well as the sunbird *Arachnothera longirostris* (little spiderhunter); no gene flow was detected without animal vectors (Ge *et al*., 2005). Throughout Southeast Asia the least blossom bat *Macroglossus minimus* is an important *Musa* pollinator. In lowland Papua New Guinea these bats are ubiquitous; in a study there the bats regularly visited both wild and cultivated *Musa* and had home ranges of 0.6-15.1 ha (averaging 5.8 ha) (Winkelmann *et al*., 2003). In northeastern Papua New Guinea's Bismarck Archipelago, black-bellied fruit bats *Melonycteris melanops* are common (and endemic). Their home ranges of 0.5-9.2 ha (averaging 2.3 ha with a long axis of 371 m) had a core area that was based on one to six flowering cultivated banana plants, which were utilised for nighttime nectar feeding as well as daytime roosting (Bonaccorso *et al*., 2005).

Populations of the pollinating long-tongued fruit bat (*M. sobrinus*) are diminishing in parts of southern China because of increasing forest use, thus genetically isolating wild populations of *Musa balbisiana* (Ge *et al.*, 2005). In northeastern Australia where the tropical forest is reduced and fragmented, southern blossom bats *Syconycteris australis* now utilise the floral resources provided by cultivated bananas (Law and Lean, 1999). In Papua New Guinea these common bats can have a home range of 2.7-13.6 ha, with a long axis of 263-725 m (Winkelmann *et al*., 2000).

In the most primitive edible *Musa* cultivars in Southeast Asia seeds are of frequent occurrence (Stover and Simmonds, 1987). However, among cultivated bananas and plantains generally, pollination never or seldom results in seeds (*e.g.* Ortiz and Crouch, 1997; Ssebuliba *et al*., 2006a). Seedlessness is due to a complex of causes involving sterility genes, polyploidy, and chromosomal structural changes in varying degrees depending on the cultivar or particular clone (Section VI). Most of the edible *Musa* are triploids and almost completely sterile (Vuylsteke *et al*., 1993d). Much of the characteristic sterility in commercial clones for the export trade (*i.e.* the AAA Cavendish subgroup) is due to high inherent female sterility. Male gametic sterility does not always occur along with female sterility. Fertility can depend not only on maternal conditions (*cf.* Fortescue and Turner, 2005) but also pollen availability and viability. The 'Gros Michel' dessert banana can produce many seeds if planted in areas where suitable pollen and pollinators are around. In screening germplasm of the International Institute of Tropical Agriculture (IITA) for viable pollen, nearly 40% of the *Musa* accessions (67 of 168) were sufficiently fertile for use as the male parent in breeding (Dumpe and Ortiz, 1996).

In a study of pollen viability in the anthers just before anthesis along with a detailed review (Fortescue and Turner, 2004), some viable pollen was reported even in "male-sterile" triploids. Viability was calculated from the total quantity of pollen produced — between 11 and 1495 grains. Pollen of seeded diploid species was three times more viable than pollen of edible fertile tetraploids, which had three to four times more viable pollen than edible triploids. Among triploids cultivated in Australia, 'Gros Michel' had 13% viable pollen, and triploids from India could reach 29% viable pollen. Triploids

used in the genetic improvement programme in Cuba had 40-43% fertile pollen (Landa *et al*., 1999). The size of the spherical pollen grain increases with the ploidy level; in diploids the diameter is about 100 µ, in triploids 112 µ and in tetraploids 135 µ (Tenkouano *et al*., 1998).

5.2. Seeds

Although the production of seeds in most cultivated varieties is either not possible or difficult and rare, in specialised breeding work some varieties can be forced to produce seeds (Section VI) (Stover and Simmonds, 1987; Tomekpé *et al*., 2004; Ssebuliba *et al*., 2006b). For example, 0-183 and 0-219 seeds per bunch have resulted from pollinating triploid plantains (AAB) with the pollen from diploid bananas (AA) (respectively Tomekpé *et al*., 2000, and Swennen and Vuylsteke, 1993). Furthermore seed set can fluctuate substantially within the year (Swennen and Vuylsteke, 1990). Pollen viability in plantains and cooking bananas is increased by high relative humidity, low temperature and low solar irradiance (Ortiz and Vuylsteke, 1995; Ortiz *et al.*, 1998a). These researchers found that seed set was highest in ABB cooking bananas, followed by AAB French Plantains, and lowest in AAA dessert bananas. Cultivars with two rather than three sets of homologous chromosomes thus had an increased frequency of viable eggs.

Musa fruits are consumed by many species such as bats, birds, rodents and primates, which in some cases can be considered pests of the crop (*e.g.* Naughton-Treves *et al*., 1998; Duncan and Chapman, 1999; Liu *et al*., 2002a; Tang *et al*., 2005). Mature fruits of wild *Musa* have many seeds (*e.g.* 30-400). In the wild, utilisation of fruits by such mammals and birds can result in seed dispersal (*e.g.* Liu *et al*.2002a; Tang *et al*., 2005; Meng *et al*., 2008). *Musa balbisiana* was introduced and has now become naturalised in Taiwan (Chiu *et al*., 2007). In Belize (Central America), this seedy species has become invasive locally in several relatively open disturbed areas (Meerman, 2003; Meerman *et al*., 2003).

6. Genetics and hybridisation

6.1. Genetic diversity and genomic architecture

There is quite limited information about most wild *Musa* species' ranges and habitats (De Langhe, 2000b). Wild populations apparently tend to be isolated, maintaining significant genetic diversity among the dispersed populations rather than within each population (Asif Javed *et al*., 2002; Ge *et al*., 2005; Wang *et al*., 2007). In Southeast Asia where wild bananas occur naturally, they are often in open disturbed areas such as on steep slopes, and they are often among the first colonisers when natural regeneration follows forest clearance (*e.g.* Tang *et al*., 2003) — thus showing pioneer characteristics. Semi-wild or free-living populations occur in human-made habitats such as the edges of cleared land and on roadsides. *Musa* genetic resources are now found in an array of habitats such as forest edges and openings, fallow land, and home gardens and farms. The human communities living around such habitats maintain variability through cultivation of traditional cultigens and landraces, and by conservation of natural areas and ecosystems (Sharrock and Engels, 1997; Uma and Buddenhagen, 2006). Both of the wild diploid progenitor species are still valuable genetic resources for banana improvement (De Langhe, 2000b). Their populations contain important traits such as resistance to various diseases, and the ability to thrive in relatively dry and cool environments (Ude *et al*., 2002b; Häkkinen and Wang, 2007; Wang *et al*., 2007).

Knowledge of *Musa* had been limited due to the complex and varied genomes between sections, but with modern molecular methods great progress is being made. For example the traditional cooking bananas 'Kluai Ngoen' and 'Pisang Awak' have been reclassified and considered to have the genome AABB (Horry *et al*., 1998; Pillay *et al*., 2006). In recent years there have been studies on genome identification (Pillay *et al*., 2000, 2006) and genome structure (D'Hont *et al*., 2000). Genomic *in situ* hybridisation (GISH) can differentiate the chromosomes of the four designated genomes of the diploid species — A (*M. acuminata*), B (*M. balbisiana*), S (*M. schizocarpa*) and T (section *Australimusa* spp.)

— and determine the genome constitution of interspecific cultivated clones (D'Hont *et al*., 2000). The molecular characterisation of genomes in *Musa* and applications have been reviewed by Pillay *et al*. (2004).

The cytogenetics of the nuclear genome has been reviewed by Doležel (2004). The karyotypes of the *Musa* A and B chromosome sets appear similar (Osuji *et al*., 2006). The diploid genome is rather small — Kamaté *et al*. (2001) measured the 2C nuclear DNA content of *M. balbisiana* as 1.16 pg, and *M. acuminata* averaged 1.27 pg with a variation of 11% between subspecies, whereas triploid 2C values ranged from 1.61 to 2.23 pg (*cf*. Bartoš *et al*., 2005). These diploids therefore have some 560 to 610 million bp of DNA (Kamaté *et al*., 2001), of which about half has been estimated to be non-coding DNA (H ibová *et al*., 2007).

6.2. Natural or spontaneous hybridisation, genetic behaviour and genetic mapping

There are reports of probable natural hybridisation between wild species of *Musa* in their native ranges, and between wild subspecies of *Musa acuminata*, and of probable introgression in the wild. The following are representative observations (not a complete review, given the extent of incomplete botanical and genetic knowledge). For example, the native range of *Musa balbisiana* likely does not go as far south as Java (*cf*. Häkkinen and Väre, 2008c) so the hybrid with *M. acuminata* found there (Simmonds, 1962) would be a spontaneous cross instead of a truly natural cross between indigenous species.

- *Musa acuminata* ssp. *halabanensis* × *Musa acuminata* var. *sumatrana*
  Indonesia (Sumatra) (Nasution, 1991)
- *Musa acuminata* ssp. *malaccensis* × *Musa acuminata* ssp. *truncata*, including introgression
  Peninsular Malaysia (Simmonds, 1962, but *cf.* Wong *et al*., 2001a; Asif Javed *et al*., 2002)
- *Musa acuminata* ssp. *malaccensis* × *Musa acuminata* ssp. *siamea*
  Thailand (De Langhe *et al*., 2000)
- *Musa acuminata* ssp. *banksii* × *Musa schizocarpa*
  Papua New Guinea (Argent, 1976; Arnaud and Horry, 1997; Shepherd, 1999; Carreel *et al*., 2002)
- *Musa acuminata* ssp. *siamea* × *Musa laterita*
  Thailand (*cf*. Simmonds, 1962; De Langhe *et al*., 2000)
- *Musa acuminata* × *Musa balbisiana*
  Indonesia (Java) (Simmonds, 1962) — spontaneous
- *Musa balbisiana* × *Musa nagensium*
  Myanmar (Simmonds, 1962)
- *Musa balbisiana* × *Musa sikkimensis*
  Northeast India (Sikkim) (Simmonds, 1962)
- *Musa balbisiana* × *Musa textilis*
  Philippines (Simmonds, 1962; Carreel *et al*., 2002)
- *Musa borneensis* × *Musa textilis*
  Borneo (Wong *et al*., 2002; Häkkinen and Meekiong, 2005)— spontaneous (rather than natural), as *M. textilis* was introduced to Borneo and has naturalised
- *Musa boman* × *Musa lolodensis*
  Papua New Guinea (Argent, 1976; Nasution, 1993)

- *Musa flaviflora* × *Musa velutina*, including introgression
  Northeast India (Simmonds, 1962)

The great majority of domesticated bananas are products of an array of prehistoric crosses between diploid *Musa acuminata* plants of several subspecies (A genomes) and further crosses of such cultigens with the less diverse *Musa balbisiana* (B genome), which has resulted in several ploidy levels (diploids, triploids, a few tetraploids) and an array of varying progeny (*e.g.* Table 1). A spontaneous doubling of gamete chromosomes from *n* to 2*n* apparently facilitated such crossings (Ortiz, 1997b; Raboin *et al.*, 2005; Ssebuliba *et al*., 2008; Perrier *et al*., 2009). Experimental crosses in early cytogenetic studies also made hybrids between *M. acuminata* and *M. balbisiana*. Experimental interspecific crosses have been carried out between various species within section *Musa* ("*Eumusa*"), between eumusa species and species in other sections, and between non-eumusa species in the other sections (Dodds, 1943, 1945; Dodds and Pittendrigh, 1946; Dodds and Simmonds, 1946, 1948a, 1948b; Simmonds, 1962; Shepherd, 1999; Choy and Teoh, 2001).

Relatively little research on inheritance has been undertaken in *Musa* spp. due to the several characteristics of domesticated bananas and plantain that make inheritance analysis difficult. Since the first studies on inheritance of fruit parthenocarpy (Cheesman, 1932; Dodds, 1943, 1945), half a century passed to the next such study, on inheritance of whole-plant albinism (Ortiz and Vuylsteke, 1994a). A number of studies followed (*cf.* Ortiz and Vuylsteke, 1996, 1998b; Ortiz, 2000), for example on inheritance of black Sigatoka resistance by the wild diploid Calcutta 4 (Ortiz and Vuylsteke, 1994b).

Some genetic maps have been developed that help to further understand the genetics of traits of interest. Fauré *et al.* (1993) developed the first map by using isozymes and RFLPs. A global programme for *Musa* improvement, PRO*MUSA*, was formed in 1997. Research that includes a large consortium for genomics and genetic improvement of *Musa* is making available useful information for breeding (GMGC, 2002; Roux *et al*., 2008). A genetic map developed through a joint effort characterised more than 300 markers (Lagoda *et al.*, 1998a). Another map was based on single sequence repeats (SSRs) (Lagoda *et al.*, 1996). CIRAD published 45 sequence-tagged microsatellite site (STMS) markers in the EMBL database (Lagoda *et al.*, 1998b). Fluorescent *in situ* hybridisation (FISH) has been used to determine the number and distribution of the 18S-25S and 5S rDNA sites on mitotic chromosomes of edible diploid bananas (Doleželová *et al.*, 1998). Bacterial artificial chromosome (BAC) libraries have been constructed of *Musa acuminata* composed of 55,152 clones (Vilarinhos *et al.*, 2003), and *Musa balbisiana* composed of 36,864 clones (Šafá *et al*., 2004), as well as a BIBAC (binary BAC) library of a diploid *M. acuminata* cultivar (Ortiz-Vázquez *et al*., 2005).

6.3. Experimental breeding

Breeding improvement for crops in which most of the accessions and the cultivated varieties are highly sterile is challenging. Most cultigens that are commercially acceptable are parthenocarpic triploids producing few seeds if any — and yet seedlessness is a characteristic that the breeder must retain in the crop (Hamill *et al.*, 1992). Experimental crossing of banana and plantain is hampered by polyploidy, the differences between their sets of subgenomes, and the plants being generally sterile, with seedless fruit. Banana improvement programmes rely on development of new hybrid genotypes obtained from the fertilisation of female-fertile plants with pollen of male-fertile plants. Even when viable hybrids can be obtained experimentally, most are sterile. In plantain some crosses have yielded a very few seeds, with great variability. Asexual *in vitro* multiplication and other biotechnological methods have been developed to obtain viable progeny (see Section VII).

6.4. Breeding schemes

Different breeding schemes are based on crosses between plants of different ploidy, from which improved tetraploids or triploids can be selected (Bakry *et al*., 2001; Escalant *et al*., 2002; Tenkouano

and Swennen, 2004; Oselebe *et al*., 2006). The breeding approach produces tetraploid progeny *via* crosses of triploid females and diploid males, choosing the female parents from the few triploids that show some female fertility. This strategy also includes producing secondary triploid hybrids from tetraploid × diploid crosses, achieved by hybridisation of African plantain landraces with wild or cultigen Asian diploid banana accessions (Vuylsteke *et al*., 1993d). The key intermediate step is to obtain improved diploids with novel traits of interest (Tenkouano *et al*., 2003).

Crossing to generate new hybrids has proven efficient in plantain improvement when genetic variability can be included through use of female-fertile parents (Swennen and Vuylsteke, 1993; Ortiz, 1997c), but obtaining new commercial cultivars is difficult because most clones lack female fertility (Rosales and Pocasangre, 2002). Ortiz *et al.* (1998a) suggested optimising apparent male fertility by higher solar irradiation and temperature. Often the level of fertility of accessions available to a breeding programme still needs to be determined.

Table 2. Some important *Musa* hybrids produced by agricultural breeding programmes

HYBRID NAME	PLOIDY	CHARACTERISTICS & COMMENTS	REFERENCE
DESSERT BANANAS:			
FHIA-01, Goldfinger	tetraploid	black Sigatoka resistant, Fusarium wilt races 1 and 4 resistant Grown in Latin America, Africa and Australia	Rowe and Rosales, 1993; Orjeda and Moore, 2001; Gallez *et al.*, 2004
FHIA-02	tetraploid	black Sigatoka resistant	Ortiz and Vuylsteke, 1996
FHIA-17	tetraploid	Fusarium wilt race 1 resistant	Orjeda and Moore, 2001
FHIA-18	tetraploid	black Sigatoka resistant, Fusarium wilt resistant Grown in Latin America and Australia	Rowe and Rosales, 1993
BITA-3	tetraploid	black Sigatoka resistant	Ortiz *et al.*, 1995b
IRFA 909	aneuploid	yellow and black Sigatoka resistant, Fusarium wilt resistant	Ortiz and Vuylsteke, 1996
TM3x series	secondary triploids	black Sigatoka resistant	Ortiz *et al.*, 1998b
PLANTAINS:			
TMPx series	tetraploids	black Sigatoka resistant	Vuylsteke *et al.*, 1993c
TMP2x series	diploids	black Sigatoka resistant	Vuylsteke and Ortiz, 1995
PITA-9	tetraploid	black Sigatoka resistant	Vuylsteke *et al.*, 1995
FHIA-21	tetraploid	black Sigatoka resistant	Ortiz and Vuylsteke, 1996
PITA-14	tetraploid	black Sigatoka resistant, BSV tolerant	Ortiz and Vuylsteke, 1998a
TM3x series	secondary triploids	black Sigatoka resistant	Ortiz *et al.*, 1998b
PITA-15	secondary triploid	best fruit quality amongst TM3x series	Ortiz and Vuylsteke, 1998a
PITA-16	secondary triploid	BSV and CMV tolerant	Ortiz and Vuylsteke, 1998a
PITA-20	secondary triploid	higher yield	Ortiz and Vuylsteke, 1998a
COOKING BANANAS/			
TMBx series	tetraploids	black Sigatoka resistant	Vuylsteke *et al.*, 1993a
FHIA-03	tetraploid	black Sigatoka resistant Grown in Latin America and Africa	Ortiz and Vuylsteke, 1996; Gallez *et al.*, 2004

The different general breeding schemes of the major *Musa* research centres, and their main achievements, have been reviewed by Ortiz *et al.* (1995a) and Rosales and Pocasangre (2002). Modern breeding started with a banana improvement programme active from 1922 to 1980 in the Caribbean region (Trinidad and Jamaica). The programme's strategy was based on crosses between the triploid (AAA) cultivar 'Gros Michel' and its mutants with a range of resistant male diploid parents. The programme did not produce any commercially acceptable hybrid, but set the basis for future breeding strategies by using improved male diploids (Cheesman, 1948b; Simmonds, 1966/1973; Shepherd, 1974; Ortiz *et al.*, 1995a). In 1959 an intensive breeding programme was established in Honduras, initially supported by the United Fruit Company. The programme was later donated to the Fundación Hondureña de Investigación Agrícola (FHIA) which started its activities (with international support) in 1984. This research focused on developing a superior diploid population to be used for production of primary tetraploids. Selected accessions were crossed to 'Gros Michel' and its dwarf mutants to produce tetraploids combining specific resistances and good agronomic characteristics (Stover and Buddenhagen, 1986). In 1989 FHIA released the tetraploid (AAAB) FHIA-01 or 'Goldfinger', the first multi-resistant dessert hybrid that is grown commercially (Rowe and Rosales, 1993; Ortiz and Vuylsteke, 1996). Other variously broad improvement programmes are carried out in Brazil, Nigeria, Cameroon, Uganda, India, Australia and the French West Indies.

6.5. Targets and results

The main targets in breeding are resistance to major diseases and pests (Section IV) caused by (1) the Sigatoka complex (*Mycosphaerella* spp.) and the different races of the Fusarium wilt pathogen; (2) Moko disease; (3) viruses; (4) nematodes; and (5) the weevil borer. Among agronomic and quality traits of primary breeding attention are (1) yield; (2) fruit quality regarding finger shape, fruit tastes for local consumption and for export, culinary traits, and ripening and shipping characteristics; and (3) traits such as dwarfism, toppling resistance, short ratoon cycles, regular suckering, and foliage and bunch peduncle strengths (Persley and De Langhe, 1987; Ortiz *et al.*, 1995a).

Sources of resistance to the Sigatoka leaf spot diseases are available in subspecies of *Musa acuminata* such as ssp. *malaccensis*, ssp. *burmannica* and ssp. *truncata* (Appendix 1) (Vuylsteke *et al.*, 1993c). Resistance to Fusarium wilt has been detected in *M. acuminata* ssp. *burmannica* and ssp. *malaccensis*, and the fertile diploid hybrid SH-3142 (Rowe and Rosales, 1989; Javed *et al*., 2004). The AA diploid Pisang Jari Buaya group of accessions is resistant to the burrowing nematode, but the parent has given problems with sterility and inviability of seeds. Nonetheless the hybrid SH-3142 has such resistance and is being utilised in further development (Rowe and Rosales, 1989; Viaene *et al.*, 2003).

In spite of difficulty in breeding and the few commercially acceptable improved cultivars, significant advances are being made. Several tetraploid plantain hybrids with potential for use in disease-devastated areas of tropical Africa have been bred by the International Institute of Tropical Agriculture (IITA) (Vuylsteke *et al.*, 1993b, 1993c). Ortiz *et al.* (1998b) developed triploid black Sigatoka-resistant banana and plantain germplasm (designated TM3x) by crosses between resistant diploids and triploids. Orjeda and Moore (2001) have reported on the performance of various cultivars resistant to *Fusarium*.

A selection of important hybrids obtained in several breeding programmes follows in Table 2.

7. Genetic improvement and biotechnology

Genetic improvement of *Musa* is difficult for a variety of reasons (Section VI) (Ortiz *et al.*, 1995a; Ortiz and Vuylsteke, 1996). Breeding is hampered because so few viable seeds and seedlings are obtained, but as well because up to 2 years are required in a seed-to-seed crop cycle. The different approaches to improve banana and plantain are mainly *via* crossing, mutation, somatic embryogenesis, somaclonal variation, and genetic engineering (genetic modification, genetic transformation) (Jain and

Swennen, 2004). Colchicine and oryzalin are used to induce chromosome duplication as a step in *Musa* breeding schemes (Hamill *et al.*, 1992; Tezenas du Montcel *et al.*, 1994; Van Duren *et al.*, 1996; Ganga and Chezhiyan, 2002; Bakry *et al*., 2007). Vegetative propagation techniques are widely used, because of the low levels particularly of female fertility, and the relative lack of useful genetic variability and the limited number of landraces and accessions selected from natural germplasm. Breeding schemes utilise *in vitro* culture extensively, and in some cases employ mutagenesis and genetic engineering.

7.1. Mutagenesis

The appearance of spontaneous mutants at a higher rate than in many other crops makes physical mutagenesis an attractive technique for banana and plantain improvement (Novak *et al.*, 1993; Domingues *et al.*, 1994; Roux, 2004; Kulkarni *et al*., 2007). For example, with FAO/IAEA (Food and Agriculture Organization of the United Nations / International Atomic Energy Agency) co-operation, by irradiation researchers were able to obtain aluminium-tolerant mutants of the AAA banana cv. Nanicão (Matsumoto and Yamaguchi, 1990, 1991); early-flowering mutants GN-60 Gy/A (Novak *et al*., 1990) and FATOM-1 (GN-60/A) (Tan *et al*., 1993) from cv. Grande Naine; and improved agronomic characteristics of cv. Dwarf Parfitt, a Cavendish cultivar resistant to *Fusarium oxysporum* f. sp. *cubense* race 4 (Smith *et al*., 1993; OGTR, 2008).

7.2. **In vitro** *culture, somatic embryogenesis and somaclonal variation*

In vitro culture of meristems, embryos, and cell suspensions has been used for micropropagation, including major *Musa* germplasm conservation (Van den houwe *et al*., 1995, 2003) and exchange (Krikorian *et al*., 1995; Van den houwe *et al.*, 2006). Micropropagation is extensively used for the commercial production of planting material, and for germplasm conservation in the International *Musa* Germplasm Collection at the INIBAP Transit Centre in Belgium, which is under the auspices of FAO. At this global banana collection facility, all banana accessions are kept either as meristem cultures under low light and temperature conditions (*i.e.* medium-term conditions) (Van den houwe *et al.*, 2006) or as cryopreserved meristems (Agrawal *et al.*, 2004; Panis *et al.*, 2005a, 2005b, 2007).

Meristem culture of *Musa* began in 1959 (Baker, 1959; Baker and Steward, 1962), and much of the development work was accomplished in the 1980s. Micropropagation was achieved using several tissues: apical meristems (Álvarez *et al.*, 1982; Mante and Tepper, 1983; Tulmann Neto *et al.*, 1989), shoot tips (Ma and Shii, 1972; Cronauer Mitra and Krikorian, 1984b; Stover, 1987; Vuylsteke, 1989; Vuylsteke *et al.*, 1991; Bhagwat and Duncan, 1998), and floral explants (Balakrishnamurthy and Sree Rangaswamy, 1988; Cronauer Mitra and Krikorian, 1988; Dore Swamy and Sahijram, 1989).

Cronauer Mitra and Krikorian (1984a) first reported accomplishing somatic embryogenesis in *Musa*. Subsequently regeneration *via* somatic embryogenesis in diploid and triploid bananas has been reported by various researchers (*e.g.* Banerjee *et al.*, 1987), using for explants rhizome tissues (Novak *et al.*, 1989; Sannasgala *et al.*, 1995; Mendes *et al.*, 1996; Lee *et al.*, 1997); meristematic buds (Schoofs *et al.*, 1998; Sales *et al.*, 2001; Strosse *et al*., 2003); leaf bases (Novak *et al.*, 1989; Shchukin *et al.*, 1997; Conceição *et al.*, 1998); immature zygotic embryos (Escalant and Teisson, 1989); and young male flowers (Escalant *et al.*, 1994; Grapin *et al.*, 1996, 2000; Ganapathi *et al.*, 1999; Gómez Kosky *et al.*, 2002; Jalil *et al.*, 2003). Gómez Kosky *et al.* (2002) report having achieved 89% regeneration of plantlets from somatic embryos of the AAAB cultivar FHIA-18. Somatic embryogenesis is only successful in some cultivars, and has different varietal responses (Strosse *et al.*, 2006).

Although somaclonal variation can be a problem in micropropagation (Vuylsteke *et al.*, 1988, 1991, 1996; Sahijram *et al*., 2003; Oh *et al*., 2007), some researchers have focused on using it as a source of genetic variability (Reuveni *et al.*, 1986; Stover, 1987; Israeli *et al.*, 1991) where not enough is otherwise available, as may be the case for Cavendish cultivars (Hwang *et al.*, 1996). For example, a dwarf somaclonal mutant of the Cavendish banana 'Thai Chiao No. 1' resistant to *Fusarium oxysporum* f. sp.

cubense has been found (Tang and Hwang, 1998). The frequency of off-types from shoot-tip culture has varied from 0 to 70%, depending upon the originating genotype (Vuylsteke *et al*., 1991).

Embryogenic cell suspension cultures can be obtained from a great variety of source materials, such as leaf sheaths or rhizome fragments of plants produced *in vitro* (Novak *et al.*, 1989); thin sections of a highly proliferating bud culture (Gómez Kosky *et al.*, 2002); immature zygotic embryos (Escalant and Teisson, 1993; Marroquin *et al.*, 1993); triploid somatic embryos (Escalant and Teisson, 1993); scalps (Dhed'a, 1992; Schoofs *et al.*, 1998, 1999; Strosse *et al*., 2003, 2006); and male flower buds (Escalant *et al.*, 1994; Côte *et al.*, 1996). Plant regeneration in cell suspension cultures has been achieved in the cooking banana cv. Bluggoe (ABB group) (Dhed'a *et al.*, 1991) and in cv. Pisang Mas (AA group) (Jalil *et al*., 2003).

Protoplasts can easily be isolated but callus formation has only been obtained when starting material was established under quite specific conditions (Novak *et al*., 1989; Megia *et al*., 1992, 1993). The production of protoplasts and plant regeneration from them is straightforward when embryogenic cell suspensions are used as the starting material (Panis *et al*., 1993). Somatic hybridisation has been obtained through electrofusion of banana protoplasts (Matsumoto *et al.*, 2002b; Haïcour *et al.*, 2004).

7.3. Genetic engineering

Direct genetic transformation (*i.e.* genetic engineering) of *Musa* is likely to have a major impact on banana and plantain improvement (Sági *et al.*, 1998; Rout *et al*., 2000; Escalant and Panis, 2002; Viljoen *et al*., 2004). The first genetic modification by microprojectile bombardment was achieved using embryogenic cell suspensions from meristematic buds of the ABB cooking banana 'Bluggoe' (Sági *et al.*, 1995c). The efficiency of one to four transgenic plants regenerated per bombardment plate made this technique practical (Remy *et al.*, 1998b; Dale, 1999). The first *Agrobacterium tumefaciens*-mediated transformation was achieved using meristems from tissue-cultured plants of the AAA Cavendish clone 'Grande Naine' (May *et al.*, 1995). Transformation by electroporation has been achieved using protoplasts of the ABB 'Bluggoe' and meristems from the AAB plantain 'Harton' (Sági *et al.*, 1994, 1995a, 1995b; de García and Villarroel, 2002).

Thus plants in the three most economically important genomic groups have been genetically engineered, and by a variety of techniques. 'Grande Naine' has been stably transformed and regenerated *via* microprojectile bombardment of embryogenic suspension cells derived from immature male flowers (Becker *et al.*, 2000). Stable transformation and regeneration have been achieved in the cultivars 'Williams' (AAA), 'Three Hand Planty' and 'Maçã' (AAB), and 'Bluggoe' (ABB) (Remy *et al.*, 1998a, 1998b; Swennen *et al.*, 1998, 2003; Sági *et al.*, 2000; Matsumoto *et al.*, 2002a), and in the tetraploid cultivar FHIA-21 (AAAB) (Daniels *et al.*, 2002).

Research *via* genetic engineering of banana and plantain is focused on fungal, insect, nematode, and virus resistances, as well as gene discovery. The three main strategies employ non-specific non-banana genes such as those encoding antimicrobial proteins (AMPs) (Broekaert *et al.*, 1995, 1997; Sági *et al.*, 1998; Remy *et al.*, 1999; Chakrabarti *et al.*, 2003; Pei *et al*., 2005); systemic acquired resistance (Malek and Dietrich, 1999); and specific resistance genes (R genes) (Hammond Kosack and Jones, 1997). Enhanced *in vitro* resistance in transgenic bananas to both Fusarium wilt and black Sigatoka has been reported (Remy *et al.*, 1998b). Genetic engineering has also lead to the identification of novel banana promoter sequences (Santos *et al.*, 2007).

In the case of nematodes, other strategies include use of anti-feeding proteins (Von Mende *et al.*, 1993) or other proteins that interfere in the nematode-plant interaction. A Cavendish banana has been transformed (using *Agrobacterium tumefaciens*) to express a bioengineered rice cystatin that confers resistance to the burrowing nematode (Atkinson *et al.*, 2004).

The major antiviral strategies are coat protein-mediated resistance (Dale *et al.*, 1993; Fauquet and Beachy, 1993) and RNA-mediated resistance. A large number of Cavendish and Bluggoe lines have been transformed with BBrMV-derived sequences designed to trigger RNA-mediated resistance (Dale, 1999). Banana streak viruses are a special case that may prove most difficult to control because BSV dsDNA has integrated into the *Musa* genome (see Section IV). The badnaviral sequences in the A genome are incomplete and have not caused infection, but the complete BSV genome has been found in the B genome — in fact four badnavirus species have been found as separate integrants (Geering *et al*., 2005; Gayral *et al*., 2008). *Musa balbisiana* (BB) harbouring integrated BSVs are still resistant, but when the haploid B subgenome occurs in an AAB triploid or AAAB tetraploid, stress can trigger BSV activation, infection and disease (Gayral *et al*., 2008).

The present aim in *Musa* breeding is to develop a wide range of improved varieties, bringing together conventional breeding and biotechnology, including the rapidly developing genomics and proteomics technologies (Crouch *et al*., 1998; Dale, 1999; Rout *et al*., 2000; Escalant and Panis, 2002; Escalant *et al*., 2002; Carpentier *et al.*, 2005, 2007, 2008a, 2008b; Heslop-Harrison and Schwarzacher, 2007; Samyn *et al*., 2007). The development of molecular markers and marked-assisted selection methods is improving the efficiency of selection of improved cultivars. The Global *Musa* Genomics Consortium was created in 2001 to support such endeavours (GMGC, 2002; Frison *et al*., 2004).

8. Human health and biosafety

People are the main consumers of banana and plantain fruits and derived products. The fruits are utilised in a multitude of ways in the human diet. They are also used in animal feed, and for starch and alcohol production. The plants can be a source of fertiliser, fibre, wrapping for food, plates and umbrellas, and for making clothes, shoes, furniture and paper (Morton, 1987; Sharrock, 1997). Bananas and plantains constitute a major food staple for many millions of people, and a main source of carbohydrates, vitamins and minerals. The vast majority of producers are small-scale farmers growing these diverse tropical crops either for home consumption, or local markets. Bananas and plantains are often the cheapest food to produce, as well as providing a valued source of income *via* local trade and international trade. Through increasing sales the plants sometimes become an important cash crop, in some cases providing the sole source of income for rural populations.

8.1. Medicinal and therapeutic value

Easy digestibility and good nutritional content make ripe banana an excellent food, particularly suitable for young children and the elderly. In the green stage, the liquified fruit is used in Brazil to treat dehydration in infants, as its tannins tend to protect the lining of the intestinal tract against further loss of liquids (Moreira, 1999). In general banana is appropriate for consumption when a low-fat, low-sodium and/or cholesterol-free diet is required, making banana recommended particularly for people with cardiovascular or kidney problems, arthritis, gout or gastrointestinal ulcers (Robinson, 1996).

As the fruit is easy to carry and peel, it is a quick healthy method of replenishing energy, due to its high energetic value of 75-115 kcal/100 mg of pulp (lower for banana, higher for plantain). Both bananas and plantains contain complex carbohydrates capable of replacing glycogen (*cf*. Nascimento *et al*., 1999), and important vitamins (particularly B_6 and C) and minerals (potassium, calcium, magnesium, iron). Some varieties are very rich in provitamin A (Davey *et al.*, 2009). Ripe fruit has been used to treat asthma and bronchitis and control diarrhoea. Boiled and mashed ripe fruit (especially mixed with other plant substances) is cited as a good remedy against constipation.

Many purported remedies are not well documented and would require further investigation (*cf*. Orhan, 2001). The banana pseudostem is cooked in India as the dish khich khach, taken monthly to prevent constipation (Gopinath, 1995). The juice extracted from the male bud is considered good for stomach

problems. Fresh leaves have been used medicinally for a range of disorders from headaches to urinary tract infections, and stem juice was considered a remedy for gonorrhoea. The peel of ripe bananas has antiseptic properties and can be used to prepare a poultice for wounds or applied directly in an emergency (Frison and Sharrock, 1999).

Bananas are considered an ideal vehicle for edible vaccines as the dessert fruits are widely appealing, palatable and can be eaten raw (Mor *et al.*, 1998). There is extensive research aiming to express different vaccines in the fruit (*e.g.* Brown, 1996; Bapat *et al.*, 2000; Sala *et al.*, 2003; Arntzen *et al.*, 2004; Sunil Kumar *et al.*, 2005).

8.2. Allergenicity

Allergy to banana and plantain is increasingly identified, especially in some people adversely affected by latex, but as well in some people allergic to some tree or weed pollens (Magera *et al.*, 1997; Mikkola *et al.*, 1998; Grob *et al.*, 2002). Allergens are present in fresh fruit and refrigerated bananas, and in extracts — where the allergens may be concentrated (Magera *et al.*, 1997).

Appendix 1. Provisional classification of the wild species of *Musa* L. section *Musa*

Taxa (sections, species, subspecies, botanical varieties) (in some cases, a taxon's recognition or placement is tentative)	Natural distribution (approximate, sometimes incomplete)	References
♦ **Section *Musa* (*Eumusa*) (2*n* = 22)**		
Musa acuminata Colla [1]	Sri Lanka?, India to China, Indochina, Malesia (*i.e.* Malay Archipelago) and Northeast Australia	Cheesman, 1948b; De Langhe and Devreux, 1960; Nasution, 1991; Silva, 2000; Nasution and Yamada, 2001; Wong *et al*., 2001a; Wattanachaiyingcharoen *et al*., 2004; Boonruangrod *et al*., 2008, 2009
●● *M. acuminata* ssp. *burmannica* N.W. Simmonds synonym *M. acuminata* ssp. *burmannicoides* De Langhe [2]	India, Myanmar (formerly Burma), Thailand	De Langhe and Devreux, 1960; Horry and Jay, 1990; Shepherd, 1990; Carreel *et al*., 1994, 2002; Ude *et al*., 2002b; Uma and Buddenhagen, 2006; Boonruangrod *et al*., 2008; De Langhe, 2009
M. acuminata var. *chinensis* Häkkinen & H. Wang	China	Häkkinen and Wang, 2007; Häkkinen, 2008
● *M. acuminata* ssp. *siamea* N.W. Simmonds	China (Yunnan, Guangxi)?, Indochina, Thailand; Peninsular Malaysia?	Shepherd, 1990, 1999; Carreel *et al*., 1994, 2002; De Langhe *et al*., 2000; Asif Javed *et al*., 2001b, 2002; Wong *et al*., 2002
●● *M. acuminata* ssp. *malaccensis* (Ridley) N.W. Simmonds	Thailand, Peninsular Malaysia (lowlands), Indonesia (Sumatra, Java)	Shepherd, 1990; Nasution, 1991; De Langhe *et al*., 2000; Wong *et al*., 2001a; Asif Javed *et al*., 2002 Wattanachaiyingcharoen *et al*., 2004
M. acuminata var. *flava* (Ridley) Nasution	Peninsular Malaysia, Borneo (Kalimantan)	Simmonds, 1956; Nasution, 1991; Asif Javed *et al*., 2001b; Häkkinen and Väre, 2008c
● *M. acuminata* ssp. *truncata* (Ridley) Kiew	Peninsular Malaysia (highlands)	Shepherd, 1990; Asif Javed *et al*., 2001a, 2001b, 2002; Häkkinen and De Langhe, 2001; Wong *et al*., 2001a; Carreel *et al*., 2002; Ude *et al*., 2002b
M. acuminata var. *alasensis* Nasution	Indonesia (Sumatra)	Nasution, 1991
M. acuminata ssp. *halabanensis* (Meijer) M. Hotta	Indonesia (Sumatra)	Meijer, 1961; Nasution, 1991
M. acuminata var. *sumatrana* (Beccari ex André) Nasution	Indonesia (Sumatra)	Meijer, 1961; Nasution, 1991; Häkkinen and Väre, 2008c
M. acuminata var. *longepetiolata* Nasution	Indonesia (Sumatra)	Nasution, 1991
M. acuminata var. *bantamensis* Nasution	Indonesia (Java)	Nasution, 1991
M. acuminata var. *breviformis*	Indonesia (Java)	Nasution, 1991

Nasution		
●● *M. acuminata* ssp. *zebrina* (Van Houtte ex Planchon), comb. nov. ined.? synonym *M. acuminata* var. *zebrina* (Van Houtte ex Planchon) Nasution	Indonesia (Java)	Horry and Jay, 1990; Nasution, 1991; Carreel *et al*., 2002
M. acuminata var. *nakaii* Nasution	Indonesia (Java)	Nasution, 1991
M. acuminata var. *cerifera* (Backer) Nasution	Indonesia (Java)	Nasution, 1991
M. acuminata var. *rutilipes* (Backer) Nasution	Indonesia (Java, Bali)	Nasution, 1991; Häkkinen and Väre, 2008c
● *M. acuminata* ssp. *microcarpa* (Beccari) N.W. Simmonds	Borneo	Shepherd, 1990; Nasution, 1991; Häkkinen and De Langhe, 2001; Wong *et al*., 2001a; Carreel *et al*., 2002; Perrier *et al*., 2009
● *M. acuminata* ssp. *errans* (Blanco) R.V. Valmayor	Philippines	Shepherd, 1990; Valmayor, 2001; Carreel *et al*., 2002; Valmayor *et al*., 2004
M. acuminata var. *tomentosa* (Warburg ex K. Schumann) Nasution	Indonesia (Sulawesi)	Nasution, 1991; Häkkinen and Väre, 2008c
M. acuminata ssp. *acuminata*	Indonesia (Moluccas, New Guinea: Papua [formerly Irian Jaya])	Nasution, 1991
●● *M. acuminata* ssp. *banksii* (F. Mueller) N.W. Simmonds synonym *M. banksii* F. Mueller	New Guinea, Australia (Queensland)	Argent, 1976; Ross, 1987; Shepherd, 1990; Tezenas du Montcel, 1990; Wong *et al*., 2003; *cf.* Häkkinen and Väre, 2008c
Musa balbisiana Colla [3]	Sri Lanka?, India, Nepal, Myanmar, Thailand, Indochina, China, Philippines	Argent, 1976; Sharrock, 1990; Silva, 2000; Sotto and Rabara, 2000; Wu and Kress, 2000; Ge *et al*., 2005; Uma *et al*., 2005, 2006b; Uma and Buddenhagen, 2006; Wang *et al*., 2007; Boonruangrod *et al*., 2008; De Langhe, 2009; Väre and Häkkinen, 2009a; *cf.* Häkkinen and Väre, 2008c; Fuller and Madella, 2009
M. balbisiana var. *andamanica* D.B. Singh, Sreekumar, T.V.R.S. Sharma & A.K. Bandyopadhyay	India (Andaman Islands)	Singh *et al*., 1998; Uma and Buddenhagen, 2006
M. balbisiana var. *balbisiana*	(see distribution of species)	
Musa basjoo Siebold ex Iinuma	China [4]	Liu *et al*., 2002b; Turner *et al*., 2002; Häkkinen and Väre, 2008c, 2009
Musa cheesmanii N.W. Simmonds	India (Northeast); Nepal?	Simmonds, 1956; Häkkinen, 2008

Musa formosana (Warburg) Hayata synonym *M. basjoo* var. *formosana* (Warburg) S.S. Ying	Taiwan	Wu and Kress, 2000; Ying, 2000; Chiu *et al*., 2004; *cf.* Häkkinen and Väre, 2008c
Musa griersonii Noltie	Bhutan	Noltie, 1994a, 1994b
Musa itinerans Cheesman — with 6+ varieties	India (Northeast), Myanmar, Thailand, Indochina, China	Wu and Kress, 2000; Liu *et al*., 2002b; Valmayor *et al*., 2005; Uma and Buddenhagen, 2006; Häkkinen *et al*., 2008
Musa nagensium Prain — with 2 varieties	India (Northeast), Myanmar, China (Yunnan), Thailand	Liu *et al*., 2002b; Uma and Buddenhagen, 2006; Häkkinen, 2008
Musa ochracea Shepherd	India (Northeast?)	Shepherd, 1999; Häkkinen and Väre, 2008c
Musa schizocarpa N.W. Simmonds	New Guinea	Argent, 1976; Tezenas du Montcel, 1990
Musa sikkimensis Kurz	India (Northeast), Bhutan; Myanmar, Thailand?	Simmonds, 1956; Noltie, 1994a; De Langhe *et al*., 2000
Musa thomsonii (King ex Baker) A.M. Cowan & J.M. Cowan synonym *M. flaviflora* N.W. Simmonds	India (Northeast), Bhutan	Noltie, 1994a; Häkkinen and Wang, 2007; Häkkinen and Väre, 2008c
Musa yunnanensis Häkkinen & H. Wang — with 4 varieties	China (Yunnan)	Häkkinen and Wang, 2007, 2009; Häkkinen, 2008

[1] No comprehensive, modern study of *Musa acuminata* taxonomic diversity in the wild has been done. The listed botanical subspecies and varieties thus do not represent different degrees of evolutionary divergence (as has been discerned for example in *M. maclayi* — see Appendix 2), but simply reflect the unintegrated recognition of biological entities (taxa) by different researchers. The agronomically most well-known subspecies (noted with the symbol ● or ●●) and relatively more accepted subspecies (noted with the symbol ●●) (*cf.* Boonruangrod *et al*., 2008, 2009; Perrier *et al*., 2009) have been studied the most extensively, although primarily from a limited sample of germplasm accessions.

[2] *Musa acuminata* ssp. *burmannicoides*, which was presented by De Langhe and Devreux (1960), is not considered distinct from *M. acuminata* ssp. *burmannica* (Shepherd, 1990; Carreel *et al*., 1994, 2002; Ude *et al*., 2002b; Boonruangrod *et al*., 2008, 2009). The name is based on accession Calcutta 4 from the Calcutta Botanic Garden, and considered to be a wild-type plant of unknown provenance in India or Myanmar; this germplasm has been propagated and utilised extensively in breeding and research.

[3] Two additional botanical taxa have been recognised by Häkkinen and Väre (2008c): *Musa balbisiana* var. *brachycarpa* (Backer) Häkkinen (Indonesia: Java); and *M. balbisiana* var. *liukiuensis* (Matsumura) Häkkinen (Japan: Ryukyu Islands: Okinawa). The genuinely natural range of *M. balbisiana* is unclear (*e.g*. De Langhe, 2009).

[4] Erroneously considered native in Japan (Liu *et al*., 2002b; *cf.* Turner *et al*., 2002).

Appendix 2. Provisional classification of the wild species of *Musa* L. sections *Ingentimusa*, *Rhodochlamys*, *Callimusa* and *Australimusa*, as well as *Incertae sedis* (section unknown)

Taxa (sections, species, subspecies, botanical varieties) (in some cases, a taxon's recognition or placement is tentative)	Natural distribution (approximate, sometimes incomplete)	References
♦ Section *Ingentimusa* Argent (2*n* = 14)		Argent, 1976; Wong *et al*., 2003
Musa ingens N.W. Simmonds (2*n* = 14)	Papua New Guinea	Argent, 1976
♦ Section *Rhodochlamys* Baker (2*n* = 22)		Häkkinen and Sharrock, 2002; Häkkinen, 2003b, 2005b, 2007, 2009a; Uma *et al*., 2006a; Häkkinen *et al*., 2007c
Musa aurantiaca G. Mann ex Baker	India (Northeast), Myanmar, China (Tibet)	Uma *et al*., 2006a; Häkkinen, 2007; Häkkinen and Väre, 2008a
Musa chunii Häkkinen	Myanmar, China (Yunnan)	Häkkinen, 2009b
Musa laterita Cheesman	India (Northeast), Myanmar, Thailand	Häkkinen, 2001, 2003b, 2007; Uma *et al*., 2006a
Musa mannii H. Wendland ex Baker	India (Assam)	Häkkinen, 2007; Häkkinen and Teo, 2008
Musa ornata Roxburgh synonym *M. rosacea* Hort., non Jacquin	India, Bangladesh; Myanmar?	Sundararaj and Balasubramanyam, 1971; Argent, 1984; Alquini, 1987, 1988; Hore *et al*., 1992; Uma and Buddenhagen, 2006; Uma *et al*., 2006a; Häkkinen, 2007; Väre and Häkkinen, 2009a
Musa rosea Baker synonym *M. angcorensis* Gagnepain	Cambodia, Vietnam	Häkkinen, 2006c, 2007
Musa rubinea Häkkinen & Teo	China (Yunnan)	Häkkinen and Teo, 2008
Musa rubra Wallich ex Kurz	India (Mizoram), Myanmar	Häkkinen, 2003b, 2007, 2009b
Musa sanguinea Hooker f.	India (Northeast), China (Yunnan)	Liu *et al*., 2002b; Uma *et al*., 2006a; Häkkinen, 2007
Musa siamensis Häkkinen & R. H. Wallace	Thailand, Cambodia	Häkkinen, 2007; Häkkinen and Wallace, 2007, Häkkinen *et al*., 2007c
Musa velutina H. Wendland & Drude or *Musa dasycarp*a Kurz [1]	India (Northeast), Myanmar	Uma *et al*., 2006a; Häkkinen, 2007; Häkkinen and Väre, 2008b; Väre and Häkkinen, 2009b
Musa zaifui Häkkinen & H. Wang	China (Yunnan)	Häkkinen and Wang, 2008
♦ Section *Callimusa* Cheesman (usually 2*n* = 20)		Wong *et al*., 2002; Häkkinen, 2004b, 2009a; Häkkinen *et al*., 2007b
Musa azizii Häkkinen	Borneo (East Malaysia)	Häkkinen, 2005a

Musa barioensis Häkkinen	Borneo (East Malaysia)	Häkkinen, 2006a
Musa bauensis Häkkinen & Meekiong	Borneo (East Malaysia)	Häkkinen and Meekiong, 2004
Musa beccarii N.W. Simmonds (2*n* = 18) — with 2 varieties	Borneo (East Malaysia)	Wong *et al*., 2001b; Häkkinen, 2004b; Häkkinen *et al*., 2005, 2007a, 2007b
Musa borneensis Beccari — with 6 varieties; includes *M. flavida* M. Hotta	Borneo (East Malaysia, Brunei, Kalimantan)	Häkkinen and Meekiong, 2005
Musa campestris Beccari — with *ca*. 6 varieties	Borneo (East Malaysia, Brunei, Kalimantan)	Häkkinen, 2003a, 2004a, 2004c
Musa coccinea Andrews synonym *M. uranoscopos* sensu Loureiro, non Rumphius ?	China, Indochina	Wu and Kress, 2000; Argent and Kiew, 2002; Liu *et al*., 2002b; Häkkinen and Väre, 2008c
Musa exotica R.V. Valmayor	Vietnam	Danh *et al*., 1998; Valmayor, 2001; Valmayor *et al*., 2004
Musa gracilis Holttum ex Cheesman	Peninsular Malaysia	Kiew, 1987
Musa hirta Beccari	Borneo (East Malaysia)	Häkkinen, 2004b
Musa lawitiensis Nasution & Supardiyono — with 4 varieties; includes *M. suratii* Argent	Borneo (East Malaysia, Brunei, Kalimantan)	Wong *et al*., 2001b; Häkkinen, 2006b
Musa lokok Geri & Ng	Borneo (East Malaysia)	Geri and Ng, 2005
Musa monticola M. Hotta ex Argent	Borneo (East Malaysia)	Wong *et al*., 2001b
Musa muluensis M. Hotta	Borneo (East Malaysia)	Häkkinen, 2004b
Musa paracoccinea A.Z. Liu & D.Z. Li	China, Vietnam	Liu *et al*., 2002b
Musa pigmaea M. Hotta, nom. nud.	Borneo (East Malaysia, Kalimantan)	Häkkinen, 2004b; Häkkinen *et al*., 2005
Musa salaccensis Zollinger ex Backer	Indonesia (Sumatra, Java)	Meijer, 1961; Nasution, 1993; Häkkinen and Väre, 2008c, 2009
Musa splendida A. Chevalier	Vietnam	Valmayor *et al*., 2004
Musa tuberculata M. Hotta	Borneo (Brunei)	Häkkinen, 2004b
Musa violascens Ridley	Peninsular Malaysia	Kiew, 1987
Musa voonii Häkkinen	Borneo (East Malaysia)	Häkkinen, 2004b
♦ Section *Australimusa* Cheesman (2*n* = 20)		Sharrock, 2001; Wong *et al*., 2002, 2003
Musa alinsanaya R.V. Valmayor	Philippines	Valmayor, 2001; Valmayor *et al*., 2004

Musa angustigemma N.W. Simmonds synonym *M. peekelii* ssp. *angustigemma* (N.W. Simmonds) Argent	Papua New Guinea	Jarret *et al*., 1992
Musa boman Argent	Papua New Guinea	Argent, 1976
Musa bukensis Argent	Papua New Guinea	Argent, 1976
Musa fitzalanii F. Mueller	Australia (Queensland)	Ross, 1987
Musa insularimontana Hayata	Taiwan	Wu and Kress, 2000; Ying, 2000; Chiu *et al*., 2007
Musa jackeyi W. Hill	Australia (Queensland)	Ross, 1987
Musa johnsii Argent	Indonesia (New Guinea: Papua [formerly Irian Jaya])	Argent, 2001
Musa lolodensis Cheesman	Indonesia (Moluccas), New Guinea	Argent, 1976; Nasution, 1993
Musa maclayi F. Mueller ***ex*** Miklouho-Maclay	Papua New Guinea	Argent, 1976
M. maclayi ssp. *ailuluai* Argent		
M. maclayi ssp. *maclayi*		
M. maclayi ssp. *maclayi* var. *erecta* (N.W. Simmonds) Argent		
M. maclayi ssp. *maclayi* var. *maclayi*		
M. maclayi ssp. *maclayi* var. *namatani* Argent		
Musa peekelii Lauterbach	Papua New Guinea	Argent, 1976
Musa textilis Née	Philippines	Valmayor *et al*., 2002
♦ ***Incertae sedis***		
Musa lutea R.V. Valmayor, L.D. Danh & Häkkinen	Vietnam	Valmayor *et al*., 2004
Musa sakaiana Meekiong, Ipor & Tawan	Borneo (East Malaysia)	Meekiong *et al*., 2006
Musa tonkinensis R.V. Valmayor, L.D. Danh & Häkkinen	Vietnam	Valmayor *et al*., 2005
Musa viridis R.V. Valmayor, L.D. Danh & Häkkinen	Vietnam	Valmayor *et al*., 2004

[1] *Musa velutina* is not the earliest name for this species, but the earlier name *Musa dasycarpa* has seldom been used, and there is a recent formal nomenclatural proposal to conserve *Musa velutina* as the accepted name (Väre and Häkkinen, 2009b).

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Part 2.

Documents on micro-organisms

Section 1.
Information used in the assessment of environmental applications of *Acinetobacter*

1. Introduction

This document represents a snapshot of current information that may be relevant to risk assessments of micro-organisms in the genus *Acinetobacter*. This document presents information in the scientific literature and other publicly-available literature about the known characteristics of *Acinetobacter* species encountered in various environments (including clinical settings) and with diverse potential applications (environmental, industrial, agricultural, and medical).

In considering information that should be presented on this taxonomic grouping, the Task Group has discussed the list of topics presented in "The Blue Book" (*i.e*. *Recombinant DNA Safety Considerations* (OECD, 1986) and attempted to pare down that list to eliminate duplications as well as those topics whose meaning is unclear, and to rearrange the presentation of the topics covered to be more easily understood.

2. General considerations

Members of the genus *Acinetobacter* have been known for many years, often under other generic names. Detailed accounts of their history and nomenclature are found in reviews (Grimont and Bouvet, 1991; Dijkshoorn, 1996; Towner, 1996), or as chapters in books whose appearance testifies to the growing interest and importance of this group of bacteria (Towner, 1991b; Bergogne-Bérézin *et al.*, 1996).

The genus *Acinetobacter* includes Gram-negative coccobacilli, with a DNA G + C content of 39-47 mol %. Physiologically, they are strict aerobes, non-motile, catalase positive and oxidase negative. They grow well on complex media and can grow on simple mineral medium with a single carbon source, including acetate, fatty acids, and sometimes hydrocarbons. Some are readily transformable by extracted DNA, which makes them attractive objects for genetic manipulation and studies of gene organization and regulation.

Acinetobacter spp. are considered ubiquitous, having been found in many environments including soil, fresh and salt water, some extreme environments (in particular waste streams and polluted environments), in association with plants and animals (including humans), on vegetables and other foodstuffs, and, increasingly, in clinical settings. They can form biofilms and survive on dry surfaces for extended periods.

Their metabolic versatility (*e.g*. ability to degrade a wide variety of organic compounds or detoxify heavy metals), ability to synthesize various biological products with potential commercial uses (*e.g*. bioemulsans, biodispersants, enzymes, antifungal antibiotics), and capacity to promote plant growth and antagonize plant pathogens, has led to their use or proposed use for a number of environmental applications (*e.g.* bioremediation of sites contaminated with hydrocarbons and heavy metals) and

agricultural applications (*e.g.* as plant growth promoting bacteria or biocontrol agents for fungal and bacterial pathogens of plants).

Possibly the greatest current interest in this genus arises from the ease with which clinically-relevant *Acinetobacter* spp. have developed resistance to antibiotics. Though most environmental strains, in particular those with biotechnological applications, are not considered virulent pathogens, some strains are closely related to opportunistic multi-drug resistant pathogens like *A. baumannii* which cause serious and sometimes fatal epidemics in intensive care units of hospitals. The need to treat *Acinetobacter* infections and to understand their epidemiology has added impetus to the search for technologies which allow precise and rapid identification of pathogenic strains.

2.1 Subject of document; species included and taxonomic considerations

This document covers a group of at least 32 species (genomic species), each comprising a group of strains which are at least 70% related by DNA hybridization (Grimont and Bouvet, 1991; Dijkshoorn, 1996) (see Table 1). Not all of these species have received a formal name, as indicated in Table 1. The definition of the genus is clear enough to allow unambiguous identification of strains at the generic level. However, the different species are often too diverse in phenotypes to be clearly assigned to one of the known species using traditional metabolic tests, which explains the confusion in the names used in the literature. Genotypic methods and particularly polyphasic approaches have had a great impact on the development of a coherent taxonomy for the *Acinetobacter* genus, as discussed in 2.3. In addition, because most of the sampling for taxonomic studies was performed in the clinical environment, the *Acinetobacter* strains from the natural environment are often difficult to classify. Therefore taxonomy within the *Acinetobacter* genus is not completely elucidated. Unclassified strains still remain in clinical (Nemec *et al.*, 2000) and environmental (Carr *et al.*, 2001a, 2001b) samples. More extensive sampling is likely to reveal the presence of new species. To keep up-to-date with the species nomenclature within the *Acinetobacter* genus, the reader is referred to the official publication for prokaryotic taxonomy, the *International Journal of Systematic and Evolutionary Microbiology* at http://ijs.sgmjournals.org/. Summaries and specifics of current official taxonomy can also be found either at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, German Collection of Micro-organisms and Cell Cultures) Bacterial Nomenclature Up-to-Date site at www.dsmz.de/microorganisms/bacterial_nomenclature.php, the Taxonomy Browser of the National Center for Biotechnology Information (NCBI) at www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html, or the List of Prokaryotic Names with Standing in Nomenclature (LPSN) maintained by Prof. Euzeby at http://bacterio.cict.fr.

2.2 Characteristics of the organism which permit identification, and the methods used

2.2.1. Characterization of the Genus Acinetobacter

Brisou and Prévot (1954) and Juni (1984) described the morphological and physiological characters as follows: Gram-negative, asporogenous, non-motile, catalase-positive, oxidase-negative bacilli that had been previously placed in *Achromobacter*, (0.9 - 1.6 μm in diameter and 1.5 - 2.5 μm in length), straight, becoming spherical in the stationary phase of growth.

The unusual morphology can be a help in diagnosis: in smears prepared from clinical specimens or from a one-day old blood-agar plate, cells of *Acinetobacter* appear quite spherical and to be grouped in pairs (*i.e.* a coccobacillary diplobacillus). When grown in the presence of penicillin or in repeated subcultures, elongated club-shaped cells are seen. Although considered to be Gram-negative, cells are frequently difficult to de-stain, resulting in dark, Gram-positive areas. The Gram reaction may be clarified, however, by the use of the modern Gram-sure technology (Leonard *et al.*, 1995) and by

electron microscopic examination of thin sections, since the Gram-positive or -negative designation is now more a matter of fine structure than of colour.

On Mueller-Hinton agar medium, the colonies are smooth, non-pigmented, and generally irridescent. Cells are mucoid when they are encapsulated. Strains of *Acinetobacter* are not inhibited by penicillin, unlike those organisms now belonging to the genus *Moraxella*, which were previously included in *Acinetobacter.* Resistance to penicillin seems to be an intrinsic characteristic, since it was observed in 1951 in bacteria presumably not previously exposed to the antibiotic (Bergogne-Bérézin *et al.*, 1996).

Acinetobacter species are obligatory aerobes and, in general, unable to reduce nitrate to nitrite (Juni, 1984). Most species grow in defined media containing a single carbon and energy source and grow well on common complex media; they use ammonium or nitrate salts as the source of nitrogen and display no growth factor requirements. A simple mineral medium such as the mineral base medium, MBM acetate, will suffice for most strains.

The molecular G+C content of the DNA (mol.%) ranges from 39 to 47 (Bergogne-Bérézin and Towner, 1996). The average values for *Acinetobacter* species, calculated from figures given by Bouvet and Grimont (1986), range from 40.0 to 45.6 and are too close to distinguish separate species.

Because of the lack of any striking phenotypic character, Juni (1972) devised a genetic test for *Acinetobacter*, based on the ability of a competent *Acinetobacter* tryptophan auxotroph (strain Ivl-10 or strain trpE27) to be transformed to prototrophy by a crude DNA preparation from a test species.

2.2.2. Differentiation of Acinetobacter from Related Taxa

Acinetobacter species have been assigned to at least fifteen different genera and species, including *Bacterium anitratum*, *Herellea vaginocola*, *Mima polymorpha*, *Achromobacter, Alcaligenes, Micrococcus calcoaceticus* and 'B5W', *Moraxella glucidolytica* and *M. lwoffii* (Towner, 1996). The genus *Acinetobacter* was originally placed by Juni (1984) in the *Neisseriaceae*, but Rossau *et al.* (1991) grouped it, together with a small number of related genera, based on DNA-rRNA hybridization studies in a separate family, the *Moraxellaceae*. The *Moraxellaceae* cluster belongs to the class *Protobacteria* and is a member of Superfamily II, which includes the authentic pseudomonads and related organisms. Diaminopropane was determined to be the characteristic polyamine in all the species of the genus *Acinetobacter*, in general accounting for about 90% of the total polyamines present (Kampfer *et al.*, 1992). Fatty acid analysis also gives a profile that distinguishes *Acinetobacter* from *Moraxella* and *Neisseria* (Veron *et al.*, 1993). *Acinetobacter* is the only oxidase-negative genus within the *Moraxellaceae*.

2.2.3. The Species of Acinetobacter

2.2.3.1. Development of a system of classification

Brisou and Prévot (1954) recognized two species, *A. calcoaceticus*, and *A. lwoffii*, separated only by the ability of the former to oxidise glucose. Division on one phenotypic character only, however, was not accepted by many bacteriologists, and a single species, *A. calcoaceticus*, was recognized as late as the 1984 publication of Bergey's Manual. However, by that time the original genus, as proposed, had been found to be biochemically and genetically heterogeneous (Baumann *et al.*, 1968; Johnson *et al.*, 1970), although the lack of any clear-cut divisions based on the phenotypic characters used at that time precluded criteria for establishing unique species.

At the time the present document was written, thirty-two genomic species were currently recognized within the *Acinetobacter* genus, of which 20 have a valid species name (Table 1). The basis for the present classification was established by Bouvet and Grimont (1986), with the description of 12 DNA-DNA hybridization groups (genomic species) within the genus, including six that were given valid names.

Five additional proteolytic genomic species were subsequently delineated (Bouvet and Jeanjean, 1989), none of these being named. Tjernberg and Ursing (1989) described three more genomic species from their clinical isolates, one of these identical to genomic species 13 described by Bouvet and Jeanjean (1989). Gerner-Smidt and Tjernberg (1993) described two additional genomic species from clinical isolates. They were very similar, but not identical to species of the *A. calcoaceticus/A. baumannii* complex (*Acb* complex), genomic species 3, and Tjernberg and Ursing's species 13 (13TU). Four more species from human clinical specimen (Nemec *et al.*, 2001, 2003; Kilic *et al*., 2008), seven more from activated sludge (Carr *et al.*, 2003), and two from the Korean Yellow sea (Yoon *et al*., 2007) were described and named.

One other species, *A. thermotoleranticus* was isolated from waste waters of a match factory (Stepanyuk *et al.*, 1992). This species' properties have not been rigorously determined. The bacterial cells are characterized by polymorphism, some variability in Gram reaction though basically Gram-negative, cell division by constriction and the formation of what are described as prospore-like bodies. The range of temperature tolerance, 4-47^{o}C, is extremely wide. Further work is required to establish the identity of this species before it can be fully accepted as an *Acinetobacter* species.

The reader is referred to sections 8.7 and 18.2 for an overview of the clinical characteristics of each *Acinetobacter* species (including mechanisms for pathogencity, virulence and invasiveness in 8.7), and an overview of environmental characteristics of each species (including natural habitats, geographic distribution, and association with wild and domestic animals in 18.2).

Table 1 *Acinetobacter* species and type strain

Species	Type Strain	Source of Type strain	Risk group (ATCC)	Reference
Species with standing nomenclature :				
* *A. baumannii* (Genomic species 2)	ATCC 19606T	Urine	2	1
A. baylyi (strain ADP1)	B2T	Activated sludge		10
A. bouvetii	4B02T	Activated sludge		10
* *A. calcoaceticus* (Genomic sp. 1)	ATCC 23055T	Human clinical specimen	2	1
A. gerneri	9A01T	Activated sludge		10
A. grimontii	17A04T	Activated sludge		10
A. haemolyticus (Genomic species 4)	ATCC 17906T	Sputum	2	1
A. johnsonii (Genomic species 7)	ATCC 17909T	Duodenum	2	1
A. junii (Genomic species 5)	ATCC17908T	Urine	2	1
A. lwoffii (Genomic species 8-9)	NCTC 5866T	Human clinical specimen		1
A. parvus	LGM 21765	Ear		9
A. radioresistens (Genomic sp. 12)	IAM 13186T	Cotton tampon		1,2
A. schindleri	LUH 5832T	Urine (patient with cystitis)		6
A. tandoii	4N13T	Activated sludge		10
A. tjernbergiae	7N16T	Activated sludge		10
A. towneri	AB1110T	Activated sludge		10
A. ursingii	LUH 3792T	Blood (patient with endocarditis)	2	6
A. septicus	AK001	Blood and catheter (patients bacteremia)		11
A.. marinus and *A. seohaensis*	SW-3T and SW-100T	Yellow Sea in Korea		12
Other species:				
*Genomic species 3	ATCC 19004	Cerebrospinal fluid	2	1
Genomic species 6	ATCC 17979	Throat	2	1
Genomic species 10	ATCC 17924	Human clinical specimen	2	1
Genomic species 11	ATCC 11171	Sewage containing gas works effluent	1	1
*Genomic species 13TU	ATCC 17903	Human clinical specimen	2	4
Genomic species 14BJ	382	Human clinical specimen		3
Genomic species 13BJ/14TU	ATCC 17905	Conjunctiva	2	3, 4
Genomic species 15BJ	79	Human clinical specimen	2	3
Genomic species 15TU	ATCC 11748	Conjunctiva	2	4
Genomic species 16BJ	ATCC 17988	Urine	2	3
Genomic species 17BJ	943	Human clinical specimen		3
*Genomic species “close to 13TU”	10090	Human clinical specimen		5
*Genomic species “between 1 and 3”	10095	Human clinical specimen		5
Acinetobacter sp. RAG-1[13]	ATCC 31012T	Tar on beach, Venice Lagoon	1	7, 8

* A. calcoaceticus/A. baumannii (Acb) complex. 1. Bouvet and Grimont (1986); 2. Nishimura *et al.* (1988); 3. Bouvet and Jeanjean (1989); 4. Tjernberg and Ursing (1989); 5. Gerner-Smidt and Tjernberg (1993); 6. Nemec *et al.* (2001); 7. Di *et al.* (1997); 8. Vaneechoutte *et al.* (1999a); 9. Nemec *et al.* (2003); 10. Carr *et al.* (2003) 11. Kilic *et al.* (2008). 12. Yoon *et al.* (2007). 13. *Acinetobacter sp.* RAG-1 has been associated with different species in the past as explained in more detail in section 2.3.5.

2.2.3.2. Phenotypic, biochemical, physico-chemical, and spectroscopic methods of identification and differentiation

Phenotypic methods include phenotypic description of the cells and colonies, panels of metabolic tests particularly based on carbon usage, as well as antibiotic resistance tests. The use of phenotypic characters in identification has been criticized by several authors, including Vaneechoutte *et al*. (1995) and Dijkshoorn (1996), because of the length of incubation time needed to obtain a test result (up to 7 days) and because strains placed in the same genomic group by DNA hybridization were not always alike phenotypically. Such inconsistency prevented the use of phenotypic characters as the sole criterion for the definition of the later genospecies discovered by Tjernberg and Ursing (1989) and by Bouvet and Jeanjean (1989). Examples of this inconsistency can also be seen in the results obtained by Bouvet and Grimont (1986, Table 8) and by Gerner-Smidt *et al*. (1991) (Table 3). The latter authors were only able to correctly identify 78% of the 198 strains assigned previously to 14 genospecies, using the phenotypic tests devised by Bouvet and Grimont (1987) and shown in Table 2. Moreover, the use of phenotypic characters has often failed to discriminate adequately between closely related species. A gross similarity in phenotypic profile within the first three genospecies, as shown in Table 2, has led to them, together with *Acinetobacter* species 13, being referred to collectively as the *Acinetobacter calcoaceticus-A. baumannii* complex (Gerner-Smidt *et al.*, 1991; Bergogne-Bérézin and Towner, 1996). Kampfer *et al*. (1993) used numerical taxonomic methods to classify 211 strains of *Acinetobacter* on the basis of 145 biochemical tests, and showed that a phenotypic profile is taxonomically useful only when a large number of tests are used. However, when species have been sub-divided by another method, one or two phenotypic tests can be used to discriminate successfully between just a few species (Vaneechoutte *et al.*, 1995).

A number of phenotypic identification schemes have been set up for identifying *Acinetobacter* species. That of Bouvet and Grimont (1987) is shown in Table 2. Other authors proposed simplified phenotypic identification schemes (Kampfer *et al.*, 1993; Kenchappa and Sreenivasmurthy, 2003). Also, commercial systems, such as API 2ONE (bioMérieux), Biolog GN (Biolog, Inc.), and others, which have been applied especially to clinical isolates of *Acinetobacter* species and those of other genera, are discussed by Dijkshoorn (1996). In spite of their deficiencies, carbon source utilization tests and other phenotypic tests are still used for identification and extensive characterization of strains or species of *Acinetobacter*, as a complement to more discriminative genotypic methods. For example, the carbon source assimilation patterns used as part of the polyphasic analysis that lead to the delineation of seven novel species of *Acinetobacter* from activated sludge were determined using the Biolog GN Identification system (Biolog, Inc.) (Carr *et al.*, 2003). The role played by phenotypic characterization in the species definition in bacteriology is discussed in Stackebrandt *et al*. (2002). Metabolic tests and other visual characterization of the colonies is no longer used for routine identification of bacterial isolates, except in less endowed diagnostic laboratories worldwide were they are considered as an alternative method to the expensive molecular methods (Kenchappa and Sreenivasmurthy, 2003) discussed in section 2.3.4.

Biochemical and physico-chemical methods have also been developed. The different cell constituents that have been used in identifying *Acinetobacter* species include cell and cell envelope proteins, fatty acids and polyamine. Biochemical composition, however, are characteristic of groups of *Acinetobacter* species, but are not always suitable for fine-tuned discrimination between species according to Kampfer *et al*. (1993) and Dijkshoorn *et al*. (1996).

There is a growing interest in the use of physico-chemical spectroscopic methods for prokaryotic systematics. Four spectroscopic methods allowing whole organism biochemical profiling were used recently for *Acinetobacter* species identification: Pyrolysis Mass Spectrometry (PyMS) was used for the screening of large numbers of *Acinetobacter* spp. from activated sludge systems (Carr *et al.*, 2001b). Fourrier-transform infrared (FT-IR) is a rapid, whole organism fingerprinting method that gave results

similar to those published with polyphasic methods for the identification of *Acinetobacter* species from environmental samples (Winder *et al.*, 2004). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) was also rapid, and also foreseen as a powerful tool for environmental monitoring (Ruelle *et al.*, 2004). Finally, Raman spectroscopy generated data similar to those obtained by amplified fragment length polymorphism (AFLP) analysis in less time for *Acinetobacter* species identification in clinical samples (Maquelin *et al.*, 2005).

Table 2 Simplified phenotypic identification scheme for the first twelve *Acinetobacter* spp.
(based on Bouvet and Grimont, 1987, Tables I & II)

	Genospecies.										
	1	2	3	4	5	6	7	8/9	10	11	12
Growth at : 44ºC	-	+	-	-	-	-	-	-	-	-	-
41ºC	-	+	+	-	+	-	-	-	-	-	-
37ºC	+	+	+	+	+	+	-	+	+	+	+
Acid from D-glucose	-	+	+	V	-	V	-	V	+	-	V
Gelatin Hydrolysis	-	-	-	+	-	+	-	-	-	-	-
Utilization of:											
DL-lactate	+	+	+	-	+	-	+	+	+	+	+
DL-4-amino butyrate	+	+	+	+	V	-	V	V	+	+	+
trans-Aconitate	+	+	+	V	-	-	-	-	-	-	-
Citrate	+	+	+	+	V	+	+	-	+	+	-
Glutarate	+	+	+	-	-	-	-	-	+	+	+
Aspartate	+	+	+	V	V	V	V	-	+	V	-
Azelate	+	+	+	-	-	-	-	+	V	V	+
B-Alanine	+	+	+	-	-	-	-	-	+	+	-
L-histidine	+	+	+	+	+	+	-	-	+	+	-
D-malate	-	+	+	+	+	V	V	V	+	+	-
Malonate	+	+	V	-	-	-	V	-	-	-	+
Histamine-	-	-	-	-	-	-	-	-	V	+	-
L-phenylalanine	+	V	V	-	-	-	-	-	-	-	+
Phenylacetate	+	V	V	-	-	-	-	+	V	V	+

Notes: + positive, - negative, V variable

2.2.3.3. Genotypic methods

The bulk of the literature in the taxonomy of the *Acinetobacter* genus involves molecular techniques. These are all more rapid and more discriminative than phenotypic and biochemical methods discussed above (Tang *et al.*, 1998). The most common techniques used for species-level identification are listed in Table 3, along with their discriminatory power and application. The DNA-DNA hybridization method was originally used for the delineation of 12 DNA homology groups among *Acinetobacter* from human clinical specimen, each group being recognized as a genomic species, or genospecies (Bouvet and Grimont, 1986). This method could not discriminate species from an important, unidentified group and is therefore no longer used by itself, except in complement to other techniques in polyphasic studies (Nemec *et al.*, 2001, 2003; Carr *et al.*, 2003). DNA-DNA hybridization data should be included in the definition of new species (Stackebrandt *et al.*, 2002).

More rapid and more discriminative methods were developed, including partial sequencing and several DNA typing methods involving PCR and restriction enzyme profiling. Partial sequencing of the genome is used to differentiate species in routine examination of samples and to establish phylogenetic trees within the *Acinetobacter* genus. The sequences that were used include the highly variable sequences of the 16S rDNA (Ibrahim *et al.*, 1997; Misbah *et al.*, 2005), the16S-23S intergenic spacer region (ISR) (Chang *et al.*, 2005), and the *gyr*B gene (Yamamoto and Harayama, 1996).

16S rDNA sequence analysis can also be combined with other techniques in polyphasic studies in order to delineate a new species (Nemec *et al.*, 2001, 2003; Carr *et al.*, 2003).

DNA typing methods are rapid and useful to establish inter- and intraspecies relatedness. Methods that target the whole genome have a large degree of intraspecific variability that make them more useful for strain differentiation (Vaneechoutte *et al.*, 1999b) as discussed in section 2.4, than for species identification. Fingerprinting methods that target a single gene, such as ARDRA (amplified ribosomal DNA restriction analysis) of 16S rDNA have conserved patterns and are therefore widely used for species differentiation as part of polyphasic studies (Nemec *et al.*, 2001, 2003; Carr *et al.*, 2003). ARDRA and other methods that involve fragment restriction after PCR amplification should be preferred over methods where restriction digest occurs before PCR, because the former option is less prone to contamination and more reproducible (Stackebrandt *et al.*, 2002).

Table 3 Common molecular methods used for species identification, with their target gene, level of resolution, discriminatory power and examples of application in the *Acinetobacter* genus

Method	Target gene	Discriminatory power	Application(s)
DNA-DNA hybridization	Whole genome	Low	Species delineation (Bouvet and Grimont, 1986).
ARDRA	16S rDNA	Better than recA RFLP (Jawad *et al.*, 1998b). Low (Koeleman *et al.*, 1998) to acceptable (Vaneechoutte *et al.*, 1995)	Species identification in clinical isolates (Vaneechoutte *et al.*, 1995; Dijkshoorn *et al.*, 1998; Chandra *et al.*, 2002).
tRNA spacer fingerprinting	tRNA spacer	Low	Species identification (Ehrenstein *et al.*, 1996). Combined with PCR-based fingerprinting, assessment of intra-species diversity in *A. baumannii* (Sarma *et al.*, 2004).
Sequencing of conserved markers:			
	16S rDNA		Phylogenetic studies (Ibrahim *et al.*, 1997). Species identification (Misbah *et al.*, 2005).
	16S-23S intergenic spacer region		Species identification (Chang *et al.*, 2005). Study subspecific diversity (Carr *et al.*, 2004).
	gyrB		Phylogenetic studies (Yamamoto *et al.* 1999).
	rpoB and flanking sequences (rplL-rpoB and rpoB-rpoC)		Species identification and differentiation (La Scola *et al.*, 2006)
Polyphasic		High	Species delineation (Carr *et al.* 2003; Nemec *et al.*, 2003). Typing of hospital strains of the Acb complex (Nemec *et al.*, 1996)

The proposed standard for the delineation of new species is a polyphasic analysis that integrates phenotypic and genotypic data from DNA-DNA hybridization, sequencing of highly conserved genes (*e.g.* 16S rDNA and rpoB) and other genotyping data (Stackebrandt *et al.*, 2002) such as those from ARDRA and AFLP (Nemec *et al.*, 2001, 2003; Carr *et al.*, 2003). This approach is labour-intensive, and is not likely applied for the routine examination of samples and identification of isolates.

DNA arrays are seen as tools of great potential for applications in the field of microbial identification (Stackebrandt *et al.*, 2002). Examples in the literature describing their use for detection of *Acinetobacter* species in environmental and clinical samples are provided in section 24.4.

2.2.3.5. The use of species names in the literature

The comparatively recent differentiation of a large number of species of *Acinetobacter* often makes it difficult to be precise about their habitats and biological activity. The large amount of literature on *Acinetobacter* published before the first paper by Bouvet and Grimont (1986), refers only to *A. calcoaceticus*, *A. lwoffii,* or the ill-defined biotypes of *A. calcoaceticus* which have since been synonymised with other species of *Acinetobacter.* For example, *A. baumannii* was formerly known as *Acinetobacter calcoaceticus* var. *anitratus*, and *A. johnsonii* as a subset of *A. calcoaceticus* var. *lwoffii*. References to these taxa are consequently difficult to interpret (Weaver, 1994; Weaver and Actis, 1994). In the remainder of this document, the term *Acinetobacter* or *Acinetobacter* sp. has been used in referring to the earlier literature, unless the identification is clear.

Two strains from environmental sources appeared regularly in the literature under different species names and merit particular attention: the oil-degrading and bioemulsan-producing strain RAG-1 and the naturally competent strain ADP1. Strain RAG-1 isolated from Israeli sea water near a beach was originally described as *Arthrobacter* RAG-1. It was later referred to as *A. calcoaceticus* RAG-1 (Bayer *et al.*, 1981; Rosenberg and Rosenberg, 1981; Rosenberg *et al.*, 1981; Shabtai and Gutnick, 1985, 1986; Minas *et al.*, 1988; Reddy *et al.*, 1989; Leahy *et al.*, 1993; Zhang *et al.*, 1997; Sullivan *et al.*, 1999; Johri *et al.*, 2002) or *A. lwoffii* RAG-1 (Alon and Gutnick, 1993; Nakar and Gutnick, 2001, 2003), *A. junii* (Menezes *et al.*, 2005), and *A. radioresistens* (Pessione *et al.*, 1996, 2003; Pessione and Giunta, 1997). In parallel, *Acinetobacter venetianus* strain VE-C3 was isolated from a Venice lagoon and described by Di Cello *et al*. (1997). Vaneechoutte *et al*. (1999a) established that the strain previously recognized as RAG-1 and *A. venetianus* VE-C3 belonged to the same genomic species. The phenotypic characters of each strain were compared in Baldi *et al.* (1999).

Strain ADP1 is a soil bacterium exceptionally competent for natural transformation. The strain is a mutant obtained after the ultraviolet irradiation of strain BD413 isolated by Taylor and Juni (1961a, 1961b, 1961c). Beside strains BD413 and ADP1, only one other strain was reported to be highly competent for natural transformation, which is strain 93A2 (Baumann *et al.*, 1968). These three strains were recently grouped within one newly delineated species, *A. baylyi* (Young *et al.*, 2005). Strain ADP1 was also referred to as *A. calcoaceticus* ADP1 (Geissdorfer *et al.*, 1995, 1997; Parche *et al.*, 1997; Williams and Shaw, 1997; Kalscheuer and Steinbuchel, 2003; Krehenbrinkand Steinbuchel, 2004; Elbahloul *et al.*, 2005), or more often as *Acinetobacter* sp. ADP1 (Barbe *et al.*, 2004).

2.2.4. Biotyping of strains within species

Typing methods are particularly important tools for establishing sources and mode(s) of transmission for epidemic strains of the pathological species of *Acinetobacter*. Biotypes have been identified in several species of *Acinetobacter*, of which the most important is *A. baumannii.* Some strains of *A. baumannii* which cause clinical outbreaks are more aggressive than those which cause sporadic infection, but can be tracked and controlled once they have been assigned to a specific biotype. The epidemiological importance of recognizing and tracking biotypes of this species is discussed further under section 16. Strain typing has also been performed in *Acinetobacter* isolates from terrestrial and

aquatic environments. A better comprehension of the genotypic and phenotypic diversity within the *Acinetobacter* genus and species will allow a better comprehension of the ecology of that taxon, and better risk management. Because of the exponential growth of data in the field of strain differentiation in both clinical and environmental samples, the present review will not list all the strains that have been reported to date. However, the most common typing techniques will be discussed.

Strain differentiation is now usually performed with genotyping methods that target the whole genome or a cluster of genes (Table 4). These methods generate complex patterns with a high intraspecific variability that ensures proper strain differentiation. Among the clusters of genes selected for strain differentiation, the most common are the *rrn* operon targeted by the ribotyping method. Large-scale projects of strain differentiation typically involve a combination high-throughput/low resolution method followed by a higher resolution method. For example, the SENTRY Antimicrobial Surveillance Program initiated in 1997 as a global network for the longitudinal tracking of antimicrobial resistance has incorporated a molecular typing protocol involving: First, automated ribotyping and second, PFGE (pulsed-field gel electrophoresis), a higher resolution method for the typing of isolates with identical ribopatterns (Deshpande *et al.*, 2004; Gales *et al.*, 2004). A standard procedure for PFGE of macrorestriction fragments of *A. baumannii* was set up and validated for its interlaboratory reproducibility and its potential for use in the construction of an Internet-based database for international monitoring of epidemic strains (Seifert *et al.*, 2005).

The use of ribotyping as a high-throughput method is widespread especially for the strain typing of clinical samples. However, it does not preclude the use of other molecular techniques based on PCR such as RAPD, AFLP, REP-PCR, ERIC-PCR and MLST, listed in Table 4. Random (as in RAPD, random amplified polymorphic DNA) or specific (as in AFLP) amplification of a highly variable region of the genome are two methods with lower discriminatory power than PFGE (Silbert *et al.*, 2004) that can be used to differentiate strains within species from both clinical and environmental samples (Carr *et al.*, 2001a; Spence *et al.*, 2002, 2004). REP-PCR (repetitive extragenic palindromic sequence-based PCR) and ERIC-PCR (Enterobacterial repetitive intergenic consensus-based) are two methods with a discriminatory power that is similar to each other and to ribotyping (Liu and Wu, 1997) but lower than that of PFGE (Silbert *et al.*, 2004). They were mostly used to type strains from the Acb complex (see for example Martin-Lozano *et al.*, 2002) and other Gram-negative bacilli from hospitals (Silbert *et al.*, 2004).

Among the methods of great promise for strain differentiation are three techniques that are not widely used yet: MLST (multilocus sequence typing), DNA arrays (section 24.4), and spectroscopic methods (section 2.3.2). MLST targets housekeeping genes subjected to stabilizing selection, and the current consensus is that an informative level of phylogenetic data would be obtained from the determination of a minimum of five genes under stabilizing selection for encoded metabolic functions (housekeeping genes) (Stackebrandt *et al.*, 2002). This approach has been used recently for the typing of strains of *A. baumannii* (Bartual *et al.*, 2005) and in combination with electrospray ionization mass spectrometry can be used to quickly identify and genotype *Acinetobacter* isolates to determine epidemiology and clonality during infectious outbreaks (Ecker *et al*., 2006).

Strain differentiation can be done by methods based on biochemical constituents of the cells instead of DNA-based. Serotyping has been widely used, though the results are not always accurate. Monoclonal antibodies were raised against the O antigens of the lipopolysaccharides (section 3.2.2) from strains belonging to the *A. calcoaceticus-A. baumannii* complex, and shown to be useful for the rapid identification of strains belonging to that complex (Pantophlet *et al.*, 2002). Serotype-based approach is still being developed in order to provide a complete O-serotyping scheme for those clinically important *Acinetobacter* groups, as it has been successfully established for other clinically significant Gram-negative bacteria.

Fatty acid profiling is still used in diagnostic laboratories to establish relatedness of different isolates (Glucksman *et al.*, 2000; Kaiser *et al.*, 2002; Hinton, Jr. *et al.*, 2004), often in combination with

other methods such as DNA typing methods (Turpeinen *et al.*, 2004). Spectroscopic methods (Ruelle *et al.*, 2004; Maquelin *et al.*, 2005) discussed in section 2.3.4 were shown by some groups to have a very high power of resolution, that would make them suitable for strain differentiation and extremely promising in terms of scale-up.

Table 4 Molecular methods of strain typing, with their target gene, level of resolution, discriminatory power and examples of application in the *Acinetobacter* genus

Method	Target gene	Discriminatory power	Application
Automated ribotyping	*Rrn* operon	Similar to ERIC-PCR, slightly lower than PFGE (Seifert and Gerner-Smidt, 1995; Silbert *et al.*, 2004)	High-throughput, rapid epidemiologic analysis (Brisse *et al.*, 2000; Gales *et al.*, 2004)
RAPD	Whole genome	Compares with AFLP (Koeleman *et al.*, 1998)	Typing strains in clinical isolates (Spence *et al.*, 2002) combined with RFLP (Wroblewska *et al.*, 2004). Typing of strains from activated sludge (Carr *et al.*, 2001a)
AFLP	Whole genome	Comparable to PFGE for *A. baumannii* (D'Agata *et al.*, 2001) Compares with RAPD (Koeleman *et al.*, 1998)	Molecular typing of clinical isolates (Spence *et al.*, 2004) Identification of genomic species (Janssen *et al.*, 1997; Koeleman *et al.*, 1998)
REP-PCR	Whole genome	Higher than clinical and microbiological methods including antibiotyping (Martin-Lozano *et al.*, 2002). equivalent (Bou *et al.*, 2000) or lower (Liu and Wu, 1997) than PFGE.	Typing strains of the Acb complex (Liu and Wu, 1997; Bou *et al.*, 2000; Martin-Lozano *et al.*, 2002)
ERIC-PCR	Whole genome	Similar to REP-PCR and ribotyping, slightly lower than PFGE (Liu and Wu, 1997; Silbert *et al.*, 2004)	Typing Gram-negative bacilli from hospital (Silbert *et al.*, 2004)
MLST	Housekeeping genes	Comparable to PFGE and AFLP (Bartual *et al.*, 2005)	Strain typing in *A. baumannii* (Bartual *et al.*, 2005)
PFGE	Whole genome	Highest (Silbert *et al.*, 2004)	Fine discrimination of strains grouped by automated ribotyping (Brisse *et al.*, 2000; Gales *et al.*, 2004)

Tests for antibiotic susceptibility of *Acinetobacter* strains have been developed (Joly-Guillou *et al.*, 1987; Gerner-Smidt and Frederiksen, 1993). Since antibiotic resistance is often encoded on mobile genetic elements that are prone to horizontal transfer, antibiotic resistance patterns are more useful for epidemiological typing than for taxonomic identification. Epidemiological typing using antibiotic resistance patterns is discussed in section 3.7.3.

2.3. Biological features

2.3.1. Growth requirements

Most strains of *Acinetobacter* can grow on a simple mineral medium containing a single carbon and energy source (Towner, 1991b). The vast majority of isolates resemble saprophytic pseudomonads in that they are able to use any one of a large range of organic compounds. Some *Acinetobacter* strains, characterized by a relatively narrow nutritional spectrum, were found in nosocomial infections, whereas others with a rich genetic repertoire are found in various environments (Barbe *et al.*, 2004). Compounds metabolized by members of the *Acinetobacter* genus include aliphatic alcohols, some amino acids,

decarboxylic and fatty acids, unbranched hydrocarbons, some sugars, and many relatively recalcitrant compounds such as 2,3-butanediol, benzoate, mandelate, n-hexadecane, cyclohexanol, cresol, and chlorinated phenol compounds (Juni, 1978; Towner, 1996). These versatile characteristics are based on a number of unusual biochemical pathways.

Although most strains are unable to utilize glucose as a carbon source, many species are able to partially oxidize several monosaccharides (glucose, galactose, mannose, xylose arabinose and ribose). For example, *Acinetobacter sp.* ADP1 can grow on glucose as sole carbon source despite its lack of important genes for glucose assimilation (glucokinase, hexokinase and the phosphotransferase system). The only apparent way this organism can utilize glucose is by a periplasmic oxidation, *via* a membrane-bound glucose dehydrogenase (Barbe *et al.*, 2004). Smith *et al.* (2004) hypothesized that *Acinetobacter* sp. may have developed a synergistic relationship with yeasts to facilitate glucose utilization.

Most strains of *Acinetobacter* cannot reduce nitrate to nitrite, but both nitrate and nitrite can be used as nitrogen sources by means of an assimilatory nitrate reductase. The ability to ammonify organic nitrogen has been reported. The complete genome analysis of *Acinetobacter* ADP1 revealed that genes coding for proteins involved in nitrate assimilation formed a cluster (Barbe *et al.*, 2004).

2.3.2. Main physiological attributes

2.3.2.1. Properties of the outer membranes and periplasm of Acinetobacter species

Acinetobacter spp. are Gram-negative bacteria, and therefore possess an outer membrane that protects the cell and mediate all the bacteria-environment interactions. The main constituent of the outer membrane are lipopolysaccharides (LPS), a complex molecule composed of three parts: 1) a lipid A embedded in the outer membrane, 2) a central polysaccharide, 3) a lateral O chain, the O-antigen. The lipopolysaccharide (LPS) of the outer membrane of *Acinetobacter* species differs in subtle but important ways from that of the enterobacteria. Their composition makes them resistant to acid hydrolysis, which may be suitable for organisms that form organic acids from glucose. Furthermore, their LPS is freely liberated into the external medium, especially when cells are grown on hydrophobic substrates (Borneleit and Kleber, 1991). LPS are major determinants of virulence in pathogenic species, principally from their lipid A part. In an infected host, LPS is better known as endotoxin where it acts as a potent stimulator of the inflammatory response. These aspects are discussed in sections 16 and 17. Structures of a number of O-specific polysaccharides and their antigenic characterization have been published (Vinogradov *et al.*, 2003) making them available as useful chemotaxonomic and antigenic markers for the identification and differentiation of *Acinetobacter* strains, as discussed in section 2.4. Noteworthy, *Acinetobacter* sp. ADP1 does not have O-antigen (Barbe *et al.*, 2004).

The outer membrane contains proteins that regulate trans-membrane transport of molecules. Small molecules such as monosaccharides can enter into the bacterial cell *via* porins. However, the intake of larger molecules necessitates specific transporters. Of significant clinical importance, an altered expression of outer membrane proteins and efflux pumps contributes to antibiotic multiresistance of Gram-negative bacilli (Thomson and Bonomo, 2005). Antibiotic resistance is extensively discussed in section 16. Other proteins of the outer membranes have been associated with virulence. The outer membrane protein 38 (Omp38) may act as a potential virulence factor to induce apoptosis of epithelial cells in the early stage of *A. baumannii* infection (Choi *et al.*, 2005). Other outer membrane proteins related to virulence are the iron-regulated outer membrane proteins. OM73-like and FatA-like proteins have been found in nosocomial strains of *A. baumannii.* They contribute to the efficiency of the bacteria to utilize iron, a host resources that is limiting, and therefore contribute to the virulence of a particular strain (Dorsey *et al.*, 2003). SDS-polyacrylamide gel systems have been used for the study of outer membrane protein profiles of clinical strains of *A. baumannii* (Cuenca *et al.*, 2003)

The outer membrane of non-clinical *Acinetobacter* strains also contains proteins of great importance for the interaction of the bacteria with its environment. Membrane proteome of *A. radioresistens* S13 during aromatic exposure has been studied with two-dimensional gel electrophoresis. Among the membrane proteins found only expressed or overexpressed in the presence of aromatic substrate were: 1) a Na(+)/H(+) antiporter, whose function is likely to be regulation of intracellular pH, 2) an ABC type sugar transport system, probably involved in capsular polysaccharide translocation, 3) an outer membrane protein ascribable to an OmpA-like protein, known as "alasan", a bioemulsifying agent involved in solubilizing and enhancing bioavailability of hydrocarbons, 4) a trimeric porin of the PhoE family also belonging to the outer membrane and involved in facilitating the transport of anions (especially phosphate), and 5) two glycosyl transferases probably involved in capsules and/or lipopolysaccharide biosynthesis (Pessione *et al.*, 2003). Also among membrane transport proteins of importance in environmental strains are VanK, PcaK, BenK, and MucK, four members of the ubiquitous major facilitator superfamily of transport proteins that contribute to aromatic catabolism in *Acinetobacter* sp. strain ADP1 (D'Argenio *et al*., 1999).

Cell adhesion depends on cell hydrophobicity and seems to proceed mainly *via* proteins of the outer membrane. This aspect is discussed in section 3.6.

Proteins essential for natural competence in *Acinetobacter* spp. are also found in the outer membrane or periplasmic space, as discussed in section 7.1.

The space between the outer membrane and the plasma membrane of Gram-negative bacteria is known as the periplasm. The enzymes in the *Acinetobacter* spp. periplasm are mainly hydrolytic, and enable the cell to digest macromolecules or to confer protection against certain antibacterial agents. A number of species can produce true exoenzymes, including lipases, esterases, phospholipases, ß-lactamases, peptidases, and others (Borneleit and Kleber, 1991).

2.3.2.2. Metabolism of hydrocarbons and aromatic compounds

Like *Pseudomonas* species, *Acinetobacter* spp. are able to modify the diverse structures of many aromatic compounds to common intermediates that can feed into central pathways. In strain ADP1 (genome completely sequenced; section 6.1), about 20% of the genes are associated with catabolic functions. Almost all of these genes are located in five major "islands of catabolic diversity", within one quarter of the overall genome (Barbe *et al.*, 2004). Some of the substrates that can be degraded by *Acinetobacter* sp. ADP1 are listed in Table 5. *Acinetobacter* is among the bacterial genera most often found in petroleum-contaminated habitats. The ability of members of this genus to metabolize hydrocarbons, including alkanes and cyclohexane, has been reviewed in detail elsewhere (Asperger and Kleber, 1991; Trudgill, 1991). Other examples of compounds that can be degraded by *Acinetobacter* are lignin-related compounds, polychlorinated biphenyls (PCBs) and various pesticides (Bergogne-Bérézin *et al.*, 1996), and several compounds produced by plants in response to stress, such as the hydroxycinnamates, caffeate, coumarate, and ferulate (Barbe *et al.*, 2004). Potential applications of biodegrading strains are discussed in 5.2. .

Table 5 Some substrates metabolized by *Acinetobacter sp.* strain ADP1. Valérie Barbe *et al*.Unique features revealed by the genome sequence of Acinetobacter sp. ADP1, a versatile and naturally transformation competent bacterium. Nucleic Acids Research. 2004 Vol. 32. Issue 19. Pp. 5766-5779. By permission of Oxford University Press

Substrate	Metabolic product(s) or intermediates
Alkanesulfonates	Sulfite + Aldehyde
Vanillate	Protocatechuate
Betaine	Betaine aldehyde
Acetoin	Acetate
Urea	Ammonia
Salicylate esters	Catechol
Aryl esters	Catechol
Benzoate	Catechol
Catechol	Succinate + Acetyl CoA
Alkanesulfonates	Sulfite + Aldehyde
Dibenzothiophene	Hydroxybiphenyl + Sulfite
Sulfuric esters	Sulfate + Phenol/Alcohol
Nitriles	Aliphatic amides
Amidase	Ammoniac + Acide
Protocatechuate	(transporter)
Dicarboxylic acids	Succinate + Acetyl CoA
Protocatechuate	Succinate + Acetyl CoA
Quinate	Protocatechuate
p-hydroxybenzoate	Protocatechuate
Chlorogenate	Quinate + Caffeate
Caffeate	Protocatechuate
Malonate	Acetate
Nitrate/Nitrite	Ammonia
Sarcosine	Glycine + Formaldehyde

2.3.3. Temperature

Members of the *Acinetobacter* genus have various temperature ranges for growth. There are many references in the literature to strains of *Acinetobacter* as being psychrophilic, since they are often isolated from refrigerated foodstuffs at 4-6^{o}C, including poultry (Hinton, Jr. *et al.*, 2004), fish (Gonzalez *et al.*, 2000; Gonzalez-Rodriguez *et al.*, 2002), beef (Sakala *et al.*, 2002), and milk (Uraz and Citak, 1998). *Acinetobacter* spp. have also been isolated from soil or river sediment in Nordic countries and from antarctic seawater (Breuil *et al.*, 1975; Bruni *et al.*, 1999; Tendeng *et al.*, 2003). However, the growth optima for *A. calcoaceticus* in sludge has been determined as 29-35^{o}C (Du Preez and Toerien, 1978), and some other environmental isolates of *Acinetobacter* prefer incubation temperatures from 20 to 30^{o}C (Towner, 1991b). Most strains of *Acinetobacter* will grow at 33^{o}C, but the upper limit differs among the species (Grimont and Bouvet, 1991). *A. johnsonii* strains cannot grow at 37^{o}C whereas strains of *A. baumannii* and some strains of *Acinetobacter* species 13 can grow up to 44^{o}C (Weaver and Actis, 1994). The bacterium called *A. thermotoleranticus* (not validated) was reported to grow from 4-47^{o}C, with an optimum at 36-37^{o}C (Stepanyuk *et al.*, 1992). *Acinetobacter* spp. were also detected in microbial assemblages associated with high-temperature (60-90^{o}C) petroleum reservoirs (Orphan *et al.*, 2000)

2.3.4. pH and salt

Acinetobacter is reported to have a slightly acid pH optimum for growth, and vigorous aeration at pH 5.5-6.0 also favours their enrichment (Towner, 1991b) (see also section 24.1). The *Acinetobacter*

genus is generally considered as moderately halophilic as species grow optimally in media containing 0.5-2.5M salt (Kushner, 1985). A number of *Acinetobacter* strains have been isolated from marine environments (Puchenkova, 1988; Bruni *et al.*, 1999; Xie *et al.*, 2005), a salt marsh (Buchan *et al.*, 2001), and an inland saltern (del Moral *et al.*, 1987).

2.3.5. Survival

Some strains of *Acinetobacter* are clearly able to survive and grow at cold temperatures (see section 3.3). When the effect of temperature on survival was studied at 4, 15 and 25°C, however, survival of *Acinetobacter* strains was shortest at 25°C and, in most cases, longest at 4°C (McEldowney and Fletcher, 1988). *A. baumannii* survives for several days on inanimate objects and surfaces found normally in medical environments, even in dry conditions on dust particles. The potential ability of *A. baumannii* to form biofilms could explain its outstanding survival properties, bacterial biofilms being arrangements in which the cells are morphologically, metabolically and physiologically different from their planktonic counterparts (Tomaras *et al.*, 2003), as discussed in section 3.6. Survival properties play a significant role in *Acinetobacter* dissemination and outbreaks caused by *A. baumannii,* as discussed in sections 3.6, 9, 12, and 13. The survival of *Acinetobacter* strains used for bioremediation has been monitored on contamination sites, as discussed in section 5.2.

2.3.6. Dissemination and adhesion

One of the striking features of *Acinetobacter* is its ability to adhere to surfaces. Microbial adhesion is detrimental to both human life and industrial and medical processes, causing infection and contamination by pathogens and biofilm formation. However, adhesion can also be beneficial in some environmental bioprocesses such as oil degradation. Therefore, microbial adhesion has attracted much attention from researchers in various fields and *Acinetobacter*-based biofilms are often used as model systems when investigating behaviour in clinical and non-clinical environments of attached bacteria (sections 4, 9, 14.2, and 20.4).

Most studies of this ability have been directed to adherence to oil droplets and other hydrophobic surfaces (Asperger and Kleber, 1991; Gutnick *et al*., 1991). Cell adhesion to hydrocarbons depends on cell hydrophobicity and seems to proceed mainly *via* proteins. It occurs differently on various strains of *Acinetobacter*. For example, in *Acinetobacter* sp. strain MJT/F5/199A it occurs *via* an acidic protein of 65 kDa, probably a glycoprotein, in *A. venetianus* RAG-1 it occurs *via* fimbriae, and in *Acinetobacter* sp. strain A3 it occurs *via* two proteins of 26.5 kDa and 56 kDa. Adhesion of cells to oil droplets and cell hydrophobicity can be determined by the microbial adhesion to hydrocarbon (MATH) test or by other quantitative tests such as those involving measurement of zeta potential and water contact angles (Baldi *et al.*, 1999).

Adherence to solid supports has been described for various strains on various types of supports including human skin, human epithelial cells (Braun and Vidotto, 2004), red blood cells (Gospodarek *et al.*, 1998), plastic and glass surfaces (Tomaras *et al.*, 2003), medical devices such as intubation tubes, catheters, artificial heart valves, water lines and cleaning instruments (Donlan and Costerton, 2002). Adherence occurs with various mechanisms including general hydrophobicity, production of extracellular polysaccharides and presence of specialized adherence structures. For example, in *Acinetobacter*, at least two appendages have been observed (thin and thick pili), and in *A.baylyi* BD413 thin pili appear to be involved in adherence to biotic and abiotic surfaces (Gohl *et al*., 2006). In *Acinetobacter* sp. strain Tol 5, two morphological types of appendages, an anchor-like appendage and a peritrichate fibril-type appendage, have been observed on cells of an adhesive bacterium by use of electron microscopic techniques, and both are involved in adherence by this Tol 4 (Ishii *et al.*, 2004). In *A. baumannii*, the presence of pili-like structures on the surface of *A. baumannii* ATCC 19606 cells was also shown to be essential in the early steps of the process that leads to the formation of biofilm

structures on plastic surfaces (Tomaras *et al.*, 2003), and E3, an 854 kDa surface-expressed protein homologous to the staphylococcal biofilm-associated protein, is directly involved in biofilm formation by an *A. baumanni* isolated from bloodstream and may be involved in intercellular adhesion within the mature biofilm (Loehfelm *et al.*, 2008).

Acinetobacter spp. also likely promote biofilm formation by other micro-organisms, as demonstrated by the ability of *A. calcoaceticus* to act as a bridge during co-aggregation of bacteria from different genera (Chaves-Simões *et al.*, 2008).

Long survival and adhesion properties favour the dissemination of *Acinetobacter* spp. in hospitals, as discussed in sections 13 and 14.

2.3.7. Bioemulsans

High-molecular-mass bioemulsifiers, referred to as bioemulsans, are amphipathic polysaccharides, proteins, lipopolysaccharides, lipoproteins, or complex mixtures of these biopolymers that stabilize oil-in-water emulsions. A large number of bacteria produce bioemulsans, and *Acinetobacter* spp. are among the best studied.

The best studied bioemulsan is emulsan, produced by the oil-degrading micro-organism *A. venetianus* RAG-1. Emulsan forms and stabilizes oil-water emulsions with a variety of hydrophobic substrates. It is a protein-polysaccharide complex composed of an unbranched polysaccharide backbone with fatty acid side chains complexed to proteins, among which the most important functionally is an esterase (Bach *et al.*, 2003). During growth on minimal medium, emulsan accumulates on the cell surface as a minicapsule and is released into the medium as the cells approach stationary phase (Pines *et al.*, 1983). The release of emulsan is promoted by higher concentrations of hexadecane and inorganic nutrients in *A. venetianus* RAG-1 and *Acinetobacter* sp. strain HO1-N (Leahy *et al.*, 2003).

Another bioemulsifier is produced by *A. radioresistens* KA53 (isolated from oil-polluted soil) and is referred to as alasan (Navon-Venezia *et al.*, 1995). It is a high molecular weight complex of a polysaccharide and three proteins (AlnA, AlnB and AlnC). AlnA has been shown to be an OmpA-like protein that is largely responsible for the emulsifying activity of alasan (Toren *et al.*, 2002a). AlnB is part of the ubiquitous family of thiol-specific antioxidant enzymes known as peroxiredoxins. Recombinant AlnB had no emulsifying activity but stabilized oil-in-water emulsion generated by AlnA (Bekerman *et al.*, 2005). During exponential growth alasan is primarily bound to the cells, and during stationary phase it is released into the extracellular fluid, which is also the case with emulsan (Navon-Venezia *et al.*, 1995).

Among the other bioemulsans, *Acinetobacter* sp. BD4 produces a large polysaccharide capsule. Under certain growth conditions, the capsule is released together with the bound protein, producing a highly active emulsifier complex. The purified polysaccharide and protein components have no emulsifying activity by themselves. However, mixing the polysaccharide and protein led to the reconstitution of the emulsifying activity. Other *Acinetobacter* surfactants that have been reported include biodispersan from *Acinetobacter* sp. A2, an emulsifier effective on heating oil, and whole cells of *Acinetobacter* sp. A2 (Rosenberg *et al.*, 1988a, 1998b).

The biological functions of bioemulsan are reviewed in Ron and Rosenberg (2001). Briefly, and importantly for the use of *Acinetobacter* spp. in bioremediation, they increase the bioavailability of hydrophobic water-insoluble substrates such as polyaromatic hydrocarbons, and they bind heavy metals. More important from a clinical point of view, bioemulsans have a role in attachment-detachment to and from surfaces and a role in the formation of biofilms (reviewed in Ron and Rosenberg, 2001). The presence of a bioemulsan on a surface prevents the attachment of other micro-organisms or higher organisms such as Zebra mussels. Horizontal transfer of alasan has been shown from *A. radioresistens*

KA53 to heterologous bacteria, where it bound their surface and changed their surface properties (Osterreicher-Ravid *et al.*, 2000).

The potential clinical, industrial and environmental applications of the *Acinetobacter* bioemulsans are reviewed in section 5.3.

2.4. Simulated systems used to study behaviour of Acinetobacter in clinical and natural environments

Microcosms have been used to investigate gene transfer between *Acinetobacter* and other bacteria (Daane *et al.*, 1996; Nielsen *et al.*, 1997; Ray and Nielsen, 2005). The transfer of plasmids governing antibiotic resistance between *A. calcoaceticus* and other soil bacteria was determined in microcosms containing different types of soil. Microcosms consisting of chemically pure sea sand, and water and material sampled from a groundwater aquifer were compared in studying natural transformation of *A. calcoaceticus* by mineral-associated DNA (Naik *et al*., 1994). See also section 7.

Soil microcosms have also been used to assess the efficiency of *Acinetobacter* to degrade various substrates including hydrocarbons (Mishra *et al.*, 2004) .

In vitro biofilms have been used, for example, to study metabolic interactions (for example, between *Acinetobacter* sp. C6 and a *P. putida* P1 (Moller *et al.*, 1998; Christensen *et al.*, 2002; Andrews *et al.*, 2005), microbial interactions amongst and rate of substrate removal by attached and suspended members of a three-member consortium (*Acinetobacter* sp., *Enterobacter* sp., and *Candida* sp.) degrading synthetic brewery wastewater (Tam *et al.*, 2005a) and how different bacterial species interact in multispecies biofilms (in this case, harbouring an *A. iwoffii* strain) and how they react when exposed to antibiotics or invasive bacterial species (Burmølle *et al*., 2006)).

In vitro biofilms have also been used to study adhesion to plastic and glass surfaces by *A. baumannii* ATCC 19606 (Tomaras *et al.*, 2003) and the effect of the age of *A. baumannii* biofilms on the activity of the antibiotics sulbactam and imipenem (Vidal *et al.*, 1997).

Acinetobacter-based biofilms have also been used to study horizontal exchange of genetic information in the environment (for example, natural transformation of *Acinetobacter* sp. strain BD413 (Ray and Nielsen, 2005); see also section 20.4).

2.5. History of use (examples of environmental and industrial applications)

The main environmental and industrial applications of *Acinetobacter* species (suspected roles as well as proposed and actual uses) have been removal of phophates (section 5.1), bioremediation of sites contaminated with hydrocarbons, heavy metals, and pesticides (section 5.2) and production of bio-surfactants like emulsan and biodispersan (section 5.3). Other potential applications have been described (section 5.4).

2.5.1. Removal of Phosphates

Acinetobacter spp. were once thought to play an important role in the biological removal of phosphate from wastewater on the basis that they were regularly isolated in pure culture from activated sludges. However, recent data obtained from several molecular studies show that *Acinetobacter* spp. are not present in significant or important numbers in the phosphate-accumulating bacterial populations, casting some doubt on their role in phosphorus-removing systems (Blackall *et al.*, 2002; Kong *et al.*, 2002; Liu *et al.*, 2005). See also section 24.4.

2.5.2. Bioremediation of industrial pollutants

2.5.2.1. Aromatic compounds and other hydrocarbons

One of the prime uses of *Acinetobacter* has been the bioremediation of hydrocarbon-polluted waters and land sites. The remarkable catabolic versatility of *Acinetobacter* (section 3.2.3) is exploited for restoration of soil contaminated with hydrocarbon residues, as reviewed in Salleh *et al*. (2003). Strains of *Acinetobacter* and other bacteria employed in such restoration are often isolated from the damaged site itself. They are usually isolated as part of a consortium where *Acinetobacter* is identified at the generic level only (Al-Awadhi *et al.*, 2002; Olaniran *et al.*, 2004; Menezes *et al.*, 2005). *Acinetobacter* strains with a wide degree of metabolic versatility have been isolated and used in the biodegradation of a wide range of compounds, including phenols, cresols, toluene, and cyclohexane (Towner, 1991b), acrylic oligomers and polymers (Kawai, 1993), acetonitrile (Xie and Yang, 1990), 4-chlorobenzoic acid (Kobayashi *et al.*, 1997, 1998), lignin and furan and phenolic compounds containing lignocellulosic hydrolysate (Vasudevan and Mahadevan, 1990, 1992; Jain *et al.*, 1997; Lopez *et al.*, 2004), polychlorinated biphenyls (PCBs) (Rojas-Avelizapa *et al.*, 1999), dichloroethenes (Olaniran *et al.*, 2004) and polycyclic aromatic hydrocarbons (Yu *et al.*, 2005).

Biodegradation of aromatic compounds can occur in contaminated soils, sea or river water (Al-Awadhi *et al.*, 2002; Hashizume *et al.*, 2002; Ruzicka *et al.*, 2002), or in the air (Juteau *et al.*, 1999; Zilli *et al.*, 2000).

Attempts were made to identify species and strains of *Acinetobacter* that are the most efficient at degrading specific compounds. In one study, a total of 96 crude oil-degrading bacterial strains were isolated from sites contaminated with different types of petroleum hydrocarbons. The strains were identified by 16S rDNA sequencing. Out of the 96 isolates, 25 strains were identified as *A. baumannii*. Strains with differential degradation capacities for different fractions of crude oil were identified (Sarma *et al.*, 2004). Other species that were originally found in contaminated sites include *A.venetianus* ATCC 31012, also known as *Acinetobacter* strain RAG-1 (see section 2.3.5).

Acinetobacter strains found in contaminated sites are likely associated with other bacteria or part of biodegrading consortia (see also paragraphs 68 and 71). Synergistic relationships with algae have even been observed for the treatment of aromatic pollutants (Borde *et al.*, 2003).

Parameters important for *in situ* bioremediation (including key environmental parameters and survival of introduced strains at a bioremediation site) have been investigated (Gallego *et al.*, 2001; Mishra *et al.*, 2001a, 2004). For example, full-scale bioremediation studies showed that introduced strains of *A. baumannii* were stable after one year at the contaminated site (Mishra *et al.*, 2001b).

2.5.2.2. Heavy metals

A study of metal tolerance in moderately halophilic eubacteria revealed that *Acinetobacter* sp. strains were the most heavy-metal tolerant, with the majority of them showing tolerance towards eight different metal ions (Nieto *et al.*, 1989). High levels of multiple metal resistance is often correlated to antibiotic resistance in isolates of *Acinetobacter* (Deshpande *et al.*, 1993; Dhakephalkar and Chopade, 1994). Horizontal transfer of mercury resistance genes in natural populations of bacteria was observed and attributed to two circumstances, which are the frequent location of *mer* operons on plasmids and their association with transposons (Kholodii *et al.*, 2004), as discussed in sections 6 and 7.

The capability of *Acinetobacter* strains to transform or accumulate heavy metals could potentially be exploited in the bioremediation of metal-contaminated soil or water. Examples are listed in Table 6. In addition to the metal detoxification systems, *Acinetobacter* strains produce bioemulsans that bind metal ions and enable their subsequent recovery, a process known as biosorption (Gutnick, 1997; Gutnick and Bach, 2000). Application of the *Acinetobacter* bioemulsans were described in sections 3.7 and 5.3.

Table 6 Examples of the use of *Acinetobacter* sp. for bioremediation of soils and effluents contaminated with heavy metals

Contaminated environment	Species/strains used	Reference
Textile or tannery industrial effluent containing heavy metals	*Acinetobacter* sp.	Ugoji and Aboaba, 2004 Srivastava and Thakur, 2007
Lead from digested sewage sludge	*Acinetobacter calcoaceticus* var. anitraus	Mak *et al.*, 1990
Chromium-contaminated activated sludge or wastewater	*Acinetobacter* sp. *A. haemolyticus*	Francisco *et al.*, 2002 Zakaria *et al.*, 2007
Silver contaminated photographic wastewater	*Acinetobacter baumannii* BL54	Shakibaie *et al.*, 1999

2.5.2.3. Pesticides

Some *Acinetobacter* strains have been used for the bioremediation of soil and water contaminated by diverse pesticides (Table 7). Pesticide degradation can be encoded by a plasmid (Don and Pemberton, 1981) as discussed in section 6.2. Lamb *et al.* (2000) reported the genetic engineering of *Acinetobacter* sp. strain BD413 to express the cytochrome P450 xenobiotic-metabolising enzyme CYP105D1 from *Streptomyces griseus*. The engineered strain could degrade several organic pollutants including chlortoluron, which is seen as a promising avenue for bioremediation.

Table 7 Examples of the use of *Acinetobacter* sp. for bioremediation of soils and effluents contaminated with pesticides

Pesticide	Species/strains used	Reference
Propanil	*Acinetobacter calcoaceticus*	Correa and Steen, 1995
Propachlor (2-chloro-*N*-isopropylacetanilide)	*Acinetobacter* strain BEM2	Martin *et al.*, 1999
Chlortoluron	*Acinetobacter* sp.strain BD413[1]	Lamb *et al.*, 2000
Teflubenzuron, the active component in the insecticide commercialized as Nomolt	*Acinetobacter* sp.	Finkelstein *et al.*, 2001
Soil contaminated with atrazine, and other triazine pesticides, viz., simazine, terbutryn, cyanazine, and prometon.	*Acinetobacter* sp.	Singh *et al.*, 2004
Pentachlorophenol (PCP)	*Acinetobacter* sp.	Martins *et al.*, 1997
Herbicide diclofop-methyl	*Acinetobacter baumannii*	Smith-Greeier and Adkins, 1996
Herbicides 2,4-dichlorophenoxyacetic acid and 4-chloro-2-methylphenoxyacetic acid	*Acinetobacter* sp. [2]	Don and Pemberton, 1981
Glyphosate	*A. lwoffii* HN401	Chung *et al.*, 1996
Soil contaminated by Diuron	*A. johnsonii*	Dellamatrice and Rossim Monteiro, 2004

[1] genetically engineered to express the cytochrome P450 xenobiotic-metabolising enzyme CYP105D1 from *Streptomyces griseus*

[2] horizontal transfer of plasmids isolated from bacteria identified at the time as *Alcaligenes paradoxus* and *Alcaligenes eutrophus*

2.5.3. Stabilization of oil-water emulsions, biosorption, and bioemulsans

Hydrophobic bacteria all have the potential to stabilize oil-water emulsions. The combination of strong cell-cell interactions and the strong adherence between the cells and oil droplets was likely responsible for the emulsion gel structure observed for *A. venetianus* RAG-1 (Dorobantu *et al.*, 2004).

Also, a large part of the emulsifying and biosorption capabilities of *Acinetobacter* spp. rely on the production of bioemulsans (section 3.7). Bioemulsans increase the bioavailability of hydrophobic substrates such as polyaromatic hydrocarbons and bind heavy metals. These two features improve the efficiency of microbial bioremediation of contaminated sites and/or facilitate pollutant recovery. The potential commercial applications of bioemulsans produced by *Acinetobacter* are discussed below:

2.5.3.1. Emulsan

The structure and properties of emulsan are described in section 3.7 and a method for producing different emulsans from low-cost agriculture-based feedstocks like soy molasses and tallow oil has been described (Panilaitis *et al*., 2007).

Inhibitory and stimulatory effects have been reported on oil biodegradation after substrates were pretreated with purified emulsan. When the biodegradation of emulsan-treated and untreated crude oil by *Acinetobacter* was compared (Foght *et al.*, 1989), the treatment stimulated aromatic mineralization but reduced mineralization of linear alkanes and other saturated hydrocarbons, both by pure cultures and by a mixed bacterial population. The inhibitory effect may be due to a requirement for a direct physical interaction of the cells with the hydrophobic substrate.

Other potential applications in the petroleum industry are reviewed in Bach and Gutnick (2004) including viscosity reduction during pipeline transport following formation of heavy oil/water emulsions, and production of fuel oil/water emulsions for direct combustion (Gutnick *et al.*, 1991). The emulsan produced by *Acinetobacter* sp. strain RAG- 1 was highly efficient in removing hydrophobic compounds such as hexachlorobiphenyl from soil slurries (van Dyke *et al.*, 1993).

One can take advantage of the biosorption capabilities of *Acinetobacter* in bioremediation of metal-contaminated sites, as discussed in section 5.2.2. Gold biosorption by *A. calcoaceticus* from a solution containing hydrogen tetrachloroaurate (III) has been reported (Tsuruta, 2004).

Emulsan may also have a potential use as an emulsifer in the food industry (Shepherd *et al.*, 1995). It has been suggested that incorporation of emulsan in mouthwash or toothpaste could significantly reduce dental plaque formation, and a patent has been filed for this process (Bergogne-Bérézin *et al.*, 1996).

Emulsan activates macrophages in a dose-dependent manner so it could be used as an adjuvant to enhance the immune response to a vaccine (Panilaitis *et al.*, 2002).

Zhang *et al*. (1997) found that growing *Acinetobacter* RAG-1 on different fatty acids could change the structure and the emulsifier characteristics of the emulsans formed. Several strategies were investigated to modulate the side chain structure, and hence the functional properties of emulsans (Gorkovenko *et al.*, 1997; Gutnick, 1997; Gutnick and Bach, 2000; Johri *et al.*, 2002).

U.S. Patent No. 4,395,353 discloses uses for emulsan from *Acinetobacter* sp. RAG-1. Other disclosed applications include the method to produce a highly purified bioemulsifier that does not contain any contaminant LPS, an endotoxin (section 16) that is often present in crude emulsan preparations (U.S. Patent No. 6,512,014), the use of emulsans in soap, shampoo and body cream (U.S. Patent No. 4,999,195), halogenated emulsans useful as biosurfactant, antimicrobial agent, imaging probe, diagnostic and contrast agent, and the methods for making and using them (U.S. Patent No. 60,450,653).

2.5.3.2. Alasan

Purified alasan enhanced the aqueous solubility and biodegradation rates of polyaromatic hydrocarbons, probably through a hydrophobic reaction with these substances (Barkay *et al.*, 1999). To date, it has mainly been used for research: The production of a recombinant surface-active protein

(emulsification and solubilization of hydrocarbons in water) from a defined gene makes it possible for the first time to conduct structure-function studies of a bioemulsan (Toren *et al.*, 2002b).

U.S. Patent No. 5,840,547 discloses alasan and its production from *A. radioresistens* KA53.

2.5.3.3. Biodispersan

This surfactant differs from emulsan in that it adheres to the surface of and disperses inorganic minerals (Rosenberg *et al.*, 1988b). Two strains of *Acinetobacter* sp., A2 and HE5, were found to produce extracellular polymers that were capable of dispersing limestone particles in water. The active component of these biodispersans was purified and shown to be an anionic polysaccharide with the relatively low average molecular weight of 51,400 Da in comparison with the emulsans from other strains of *Acinetobacter* sp. producing hydrocarbon-in-water emulsions. The latter emulsans have molecular weights of about 1,000,000 Da (Rosenberg *et al.*, 1988b). Limestone is widely used in manufacturing such common products as paints, ceramics and paper, so that purified biodispersan has potential application in these industries too. The addition of biodispersan when limestone was ground into particles increased efficiency by decreasing the time required for grinding by more than 50% and also gave a more uniformly ground product. Further refinement of the biodispersan was achieved by isolating protein-secretion defective mutants of strain A2, since proteins secreted by the bacterium in extracellular fluid created problems in the purification and application of biodispersan. The mutants also produced equal or even higher levels of total biodispersan than the original strain (Elkeles *et al.*, 1994).

Whereas the production of bioemulsan, which acts on suspensions of hydrocarbon in water, appears to be widespread among *Acinetobacter*, the production of mineral dispersants is apparently restricted to a relatively small number of *Acinetobacter* strains (Rosenberg *et al.*, 1988a). Emulsifying and dispersing activities are due to different types of surface-active materials, and the production of either appears to be strain-specific.

2.5.4. Other applications

Several other potential applications have been explored. A few examples are listed here.

Acinetobacter spp. have been proposed for production of carnitine, single-cell protein, immune adjuvants, and glutaminase-asparaginase (used in cancer treatment). They have also been proposed for leaching manganese from ores (Bergogne-Bérézin *et al.*, 1996). *Acinetobacter* spp. or their products have also been proposed for use as plant growth promoters and bio-control agents against bacterial and fungal pathogens of plants (section 20). *A. iwoffi* has been proposed for use as an allergy-protective sensitizer (section 16). *Acinetobacter* sp. ST-550 was used experimentally to produce high levels of indigo in a water-organic solvent two-phase system containing high levels of indole (Doukyu *et al.*, 2002). Cellulolytic enzymes of an *A. anitratus* sp. were suggested to improve xanthophyll extraction from marigold flower (Navarrete-Bolanos *et al.*, 2003, 2004). Alkaline lipase was produced and recovered from *A. radioresistens* (Liu and Tsai, 2003).

Acinetobacter spp. have been used extensively as a biosensor. For example, ADP1 was used as microbial sensor for the detection of the pesticides metaphos, sumithion, and PNP in aqueous media (Guliy *et al.*, 2003) and as a non-destructive *in planta* bioluminescent indicator of production of salicylate and methylsalicylate, which is part of the plant response to pathogens and integral to systemic acquired resistance in plants (Huang *et al*., 2006). *Acinetobacter* sp. DF4 was used as whole-cell-based bioluminescent biosensor to monitor toxicity of heavy metals in water and wastewater (Abd-El-Haleem *et al*., 2006).

The natural transformation competency of *Acinetobacter* and its ubiquity in the environment make it an ideal sensor/model system for detecting horizontal gene transfer from plants, animal or other micro-organisms (discussed in section 20.3).

The main application of *Acinetobacter* is probably in the field of experimental research. For example, *Acinetobacter* sp. ADP1 has been used as a model organism in genetic and genomics studies (section 6) and microbiology and molecular biology laboratories because of its versatile metabolism (section 3.2.2) and its remarkable propensity to undergo natural transformation (section 7.1). Strain ADP1 is generally considered as a non-toxic and non-pathogenic strain by scientists in the field (section 15), and therefore used liberally even in undergraduate laboratory training (Metzgar *et al.*, 2004; Young *et al.*, 2005).

2.6. Chromosome and plasmid genetics and genomics

2.6.1. Whole genome sequencing

The complete genome of *Acinetobacter* sp. ADP1 was the fist *Acinetobacter* genome sequenced (Barbe *et al.*, 2004). ADP1 strain was chosen because of its robust physiological properties and capacity for genetic manipulations (Barbe *et al.*, 2004). Strain ADP1 belongs to the species *A. baylyi* (Young *et al.*, 2005), as discussed in section 2.3.4, and was derived from strain BD413 following UV irradiation, as discussed in paragraph 36. Whole genome sequencing revealed that ADP1 has a single, circular chromosome of 3 598 621 base pairs (bp) with an average G+C content of 40.3%. The chromosome of strain ADP1 contains 3325 coding sequences (CDSs), on average 930 bp in size. These CDSs cover 88.8% of the chromosome. A probable biological function has been assigned for more than 62.6% of the 3325 identified protein coding genes. The ADP1 genome has a small fraction of repetitive sequences (1.6%) ranging from a few short repeats to several complex ones. The longest repeated sequence is known as transposon Tn5613 (Gerischer *et al.*, 1996; Barbe *et al.*, 2004). Genome analysis has also revealed two principal prophage regions. Sixty-four genes have been identified in the longest region (54 kb), of which 45 are unique to *Acinetobacter*. The others resemble the phage sequences found in *Xyllela fastidiosa* and *Pseudomonas putida*. Studies are in progress to determine whether this region still corresponds to a functional prophage. The sequence of the second prophage region, which is 9kb in length, is similar to that of a filamentous phage of *Pseudomonas aeruginosa* (Pf3) (Barbe *et al.*, 2004).

The complete genomes of various *A. baumanni* strains have also been sequenced. *A. baumanni* is a major cause of hospital-acquired infection throughout the world (section 8.7). Whole genome sequencing of *A. baumannii* ATCC 17978 showed that it has single, circular chromosome (3 976 746 bp, 3830 predicted open reading frames [ORFs]) and two plasmids (pAB1, 13 404 bp and pAB2, 11 520 bp; no resistance genes reported on the plasmid). Of the 3830 predicted chromosomal gene products, 2137 (55.79%) share homology with gene products from non-pathogenic ADP1 and 17.2% of ORFs are located in 28 putative 'alien islands', indicating that the genome has acquired a large amount of foreign DNA from other bacteria (see also below regarding *A. baumannii* strains AYE and SDF). Consistent with its role in pathogenesis, 16 of the 'alien islands' contain genes implicated in virulence, indicating *A. baumannii* devotes a considerable portion of its genes to pathogenesis. Based on virulence assays with insertional mutants and *Caenorhabditis elegans* (which consumes bacteria by crushing lysis, enzymatic digestion, and subsequent absorption of nutrients by the intestine) and *Dictyostelium discoideum* (a unicellular amoebae which consumes bacteria by phagocytosis), six of the islands were shown to contain virulence genes, including two novel islands containing genes that lacked homology with others in the databases (Smith *et al*., 2007).

The complete genomes of *A. baumannii* strains AYE (multidrug-resistant, epidemic in France, and associated with a mortality of 26% of infected patients) and SDF (fully susceptible to many antibiotics and associated with human body lice) were sequenced so as to investigate the mechanisms of antibiotic resistance gene acquisition. Strain AYE has a circular chromosome of ~3.9 Mb and harbours three plasmids (5, 9, and 94 kb) while strain SDF has a circular chromosome of ~3.2 Mb and two plasmids (6 and 25 kb). None of the plasmids carries any known resistance markers. Strain AYE exhibits an 86-kb genomic region termed a 'resistance island' in which 45 resistance genes are clustered. This island had a G+C content that is markedly different from the rest of the chromosome (52.8% *versus*

38.8%), contains genes associated with genome instability (*e.g.* integrases, transposases, and insertion sequences), and genes with diverse phylogenetic origins (indicating recent acquisition from other bacteria). At the homologous location in strain SDF, a ~20 kb genomic island was identified with a G+C content of 31.3% (suggesting it is native to the strain) and devoid of resistance markers but which encoded 25 putative ORFs, some of which are involved in mobilizing genetic material (*e.g.* transposases, a transposition helper). The ~20 kb island is likely a hotspot for insertion of resistance markers that is in an 'empty state', which could explain the rapid acquisition of resistance markers under antimicrobial pressure. Based on sequence similarity and phylogenetic analyses, most of the resistance genes found in strain AYE appear to have been recently acquired from other genera of bacteria, in particular *Pseudomonas*, *Salmonella*, and *Escherichia*. In addition, 19 new putative resistance genes were discovered in AYE (Fournier *et al*., 2006).

To remain updated on progress in eubacterial whole genome sequencing projects, the reader is referred to the Entrez Genome site of the National Center for Biotechnology Information (NCBI) at www.ncbi.nlm.nih.gov/genomes/static/eub_g.html .

2.6.2. Plasmids

Plasmids are relatively small, circular, extra chromosomal DNA molecules that replicate independently of the host chromosome. Plasmids encode genes important for adaptability and survival in a broad range of environments, such as antibiotic or heavy metal resistance genes or genes encoding proteins involved the catabolism of various compounds. Bacteria may carry several plasmids or none. Several studies reported that more than 80% of *Acinetobacter* spp. isolates carry multiple indigenous plasmids (Gerner-Smidt, 1989; Seifert *et al.*, 1994a; Pardesi *et al*., 2007). Plasmids may carry metal or antibiotic resistance genes and/or metabolic genes organized in functional cassettes that may be bracketed by conserved elements, forming a transposon and/or an integron (section 6.3).

Acinetobacter easily acquire plasmids from the Enterobacteriacea by conjugation, but have more difficulty in transferring them back to *Escherichia coli*, which has limited some of the studies of their genetic properties (Bergogne-Bérézin *et al.*, 1996). Plasmids can also be transferred to other organisms by transformation (section 7.1).

Plasmids from *Acinetobacter* spp. that have been fully sequenced and deposited in the NCBI database are listed in Table 8. An updated list of sequenced plasmids can be found at http://www.ncbi.nlm.nih.gov/genomes/static/eub_p.html.

Table 8 *Acinetobacter* sp. plasmids that have been fully sequenced

Species	Plasmid	NCBI Accession Number	Size (base pair)	Resistance or catabolic Function	Reference
A. baumannii 19606	pMAC	NC_006877	9540 bp	Organic peroxide resistance	Dorsey *et al*., 2006
Acinetobacter sp. SUN	pRAY	NC_000923	6076 bp	aminoglycoside resistance gene, aadB	Segal and Elisha, 1997, 1999
Acinetobacter sp. EB104	pAC450	NC_002760	4379 bp	Cytochrome p450 alkane hydrolase	Unpublished
A. baumannii ATCC 17978	pAB1	NC_009083	13408 bp	None described	Smith *et al*., 2007
	pAB2	NC_009084	11302 bp	None described	Smith *et al*., 2007
A. venetianus VE-C3	pAV1	NC_010309	10820 bp	None described	Mengoni *et al*., 2007
	pAV2	NC_010310	15135 bp	None described	Mengoni *et al*., 2007

2.6.2.1. Noteworthy plasmids

- Antibiotic resistance:

  Several plasmid–encoded antibiotic resistance genes have been found in *Acinetobacter*. No attempt is made here to list all here, but as an example, a self-conjugative plasmid belonging to incompatibility group 6-C was detected in A. *calcoaceticus* BM2500 (Goldstein *et al*., 1983) and it could be transferred to *Escherichia coli* K12 and back, though the latter transfer took place at extremely low frequency. The genetics of resistance and significance of plasmids in transfer of antibiotic resistance genes in human health are discussed further in section 14. The possibilities and mechanisms of gene transfer, including antibiotic resistance gene transfer, from other living organisms to micro-organisms in natural ecosystems are also discussed in section 20.4.

- Heavy metal resistance:

  Another group of plasmids confer heavy metal resistance to strains of *Acinetobacter*, particularly in polluted environments. Several plasmids seem to contribute to mercury resistance. For example, mercury resistance in *Acinetobacter* strain W45 was attributed to three plasmids which governed the production of mercuric reductase, which converts Hg++ to Hgo (Towner, 1991a). Resistance to silver in *A. baumannii* BL88 was shown to be mediated by a 54-kb plasmid (pUPI199). Transfer of this plasmid to *Escherichia coli* DH5a conferred silver resistance to the latter bacterium and the ability to accumulate up to 13 ppm of silver in its cells (Deshpande and Chopade, 1994). This result is consistent with these workers' hypothesis that *Acinetobacter* serves as a reservoir of naturally occurring metal resistance plasmids. Antibiotic and heavy metal resistance genes can be localized on the same plasmid (Shakibaie *et al*., 1998).

- Metabolic traits:

  A group of metabolic plasmids encode degradative steps in the metabolism of aromatic hydrocarbon and aliphatic compounds as a source of energy. For example, plasmid pKF1 (80 kb) assists in the degradation of polychlorinated biphenyls (PCBs), whose highly chlorinated forms are normally non-degradable. Plasmid pSS50, a 53-kilobase self-transmissible plasmid of broad host range that has been isolated from several *Alcaligenes* and *Acinetobacter* species, has been shown to mediate the mineralization of 4-chlorobiphenyl to carbon dioxide and water (Shields *et al.*, 1985). *A. venetianus* strains harbour plasmids containing sequences homologous to the *Pseudomonas oleovorans* alkBFGH genes (Di *et al.*, 1997). Plasmid-encoded genes specifying aniline oxidation were found in *Acinetobacter* sp. strain YAA (Fujii *et al.*, 1997). Plasmid pUPI126-mediated indole-3-acetic acid production was studied in *Acinetobacter* strains from the rhizosphere of wheat (section 20.1). Plasmid pUPI126 also encoded resistance to selenium, tellurium, and lead (Huddedar *et al.*, 2002). Don and Pemberton (1981) described the biophysical and genetic properties of six independently isolated plasmids encoding the degradation of the herbicides 2,4-dichlorophenoxyacetic acid and 4-chloro-2-methylphenoxyacetic acid.

2.6.3. Transposons and integrons

Transposons (transposable genetic elements) are discrete DNA sequences that are capable of genetic rearrangement, and may be made up of one or more insertion sequences. The transposon Tn*5* was found to encode streptomycin resistance in non-enteric bacteria (O'Neill *et al.*, 1984). Strains of *A. calcoaceticus* and four bacteria in different genera which carried the kanamycin resistance-encoding transposon Tn*5*, were 15 to 500 times more resistant to streptomycin than transposon-free strains. Antibiotic resistance genes found in transposons also include PER-1, identified as part of a composite transposon bracketed by two novel insertion elements, ISPa12 and ISPa13, belonging to the IS4 family (Poirel *et al.*, 2005), and the tet(A) determinant contained in a transposon closely related to those of Tn*1721*(Ribera *et al.*, 2003). A defective mercury-resistance transposon (Tn(d)PKLH2) that has lost its

transposition genes was described in several environmental *Acinetobacter* strains. It was proposed that this transposition-deficient transposon could be translocated *via* recombination events at the nearby res (resolution) site and IS element (Kholodii *et al.*, 1993, 2004).

Transposons can be found on plasmids (Doi *et al.*, 2004) or they can be integrated in the chromosome (Poirel *et al.*, 2005). As example; the same expanded-spectrum beta-lactamase PER-1 gene was found in a chromosomally-integrated transposon in some *A. baumannii* isolates, and on a conjugative plasmid in others (Poirel *et al.*, 2005); transposon Tn*5613* of unknown function (but closely linked to various aromatic catabolic genes) is integrated into the chromosome of *Acinetobacter* sp. ADP1 (section 6.1.1) (Gerischer *et al.*, 1996; Barbe *et al.*, 2004); and conjugative transposon Tn*2009* of *A. junii* 329 is located on the chromosome, is indistinguishable from a *Streptococcus pneumoniae* element (indicationg recent horizontal exchange), and can be transferred to various other Gram negative bacteria (Ojo *et al*., 2006).

A major role in the dissemination and evolution of antimicrobial resistance in many Gram-negative organisms has been attributed to integrons. Integrons are conserved, transposon-like DNA elements which have the ability to capture and mobilize gene cassettes. Insertion and excision of these cassettes occur *via* a site-specific recombinase that belongs to the integrase family. Integrons have three components within their conserved 5′ region: (i) an integrase gene (*intI*) encoding the IntI integrase, (ii) a gene (*attI*) encoding the cassette integration site, and (iii) one or more promoters responsible for the expression of gene cassettes if present. Mobile gene cassettes, mostly containing antibiotic-resistance determinants, can be inserted or excised by a site-specific recombination mechanism catalyzed by the integrase (Gombac *et al.*, 2002).

Different integron types have been recognized on the basis of the sequence of the integrase gene. Class 1 integrons were the most common integrons found in Gram-negative bacteria such as *Acinetobacter*, especially in *A. baumannii* isolates (Gallego and Towner, 2001; Da Silva *et al.*, 2002; Houang *et al.*, 2003; Poirel *et al.*, 2003; Segal *et al.*, 2003; Nemec *et al.*, 2004; Zarrilli *et al.*, 2004; Abbott *et al.*, 2005). Class 1 integrons have also been found in *Acinetobacter* strains of environmental origin (Petersen *et al.*, 2000).

Integrons of class 2 include transposon Tn*7* and relatives. They were found in *Acinetobacter* in a much lesser extent than the class 1 integrons (Gonzalez *et al.*, 1998; Seward *et al.*, 1998; Koeleman *et al.*, 2001). A hybrid class 2 integron composed of *intI2* and the 3′ conserved segment of class 1 integrons was reported in *A. baumannii* (Ploy *et al.*, 2000). Class 3 integrons-encoded resistance were reported in *A. baumanni* (Seward, 1999; Shibata *et al.*, 2003).

Integrons can be found on plasmids or chromosomally integrated, where they are very stable.

The important role played by integrons in antibiotic resistance and in the epidemic behavior of *A. baumannii* was emphasized in many studies. Koeleman *et al*. (2001) report that integrons are present in 50% of the *A. baumannii* strains analyzed. Epidemic strains of *A. baumannii* were found to contain significantly more integrons than nonepidemic strains. Also, the presence of integrons was significantly correlated with simultaneous resistance to several antibiotics. Plasmids were detected in 42% of the strains. However, there was no significant correlation between the presence of plasmids and antibiotic resistance. Hence, it is likely that integrons play an important role in antibiotic resistance and epidemic behavior of *A. baumannii* (section 14.2).

Integrons are useful markers for epidemic strains of *A. baumannii* as discussed in section 14.3 (Severino and Magalhaes, 2004; Turton *et al.*, 2005).

2.6.4. Plasmid and chromosome stability

Stability of plasmids and other mobile elements in *Acinetobacter* appears to be variable (Towner, 1991a). Plasmids that carry biotechnologically-valuable metabolic properties may be easily lost, so that it can be advantageous to insert constructs of interest into the chromosome. For example, Jeong *et al.* (1996) used the pobA gene of *Acinetobacter* sp. strain ADP1, the structural gene for 4-hydroxybenzoate-3-hydroxylase, as a cloning site for a series of genes for the catabolic degradation of catechol. Stability of this property depended on the direction of insertion, and the authors suggested that this could help in constructing hybrid bacteria with improved metabolic stability.

Amplification of large chromosomal segments occurred during the evolution of *Acinetobacter*, as evidenced by the extraordinary clustering of the strain ADP1 catabolic islands (Barbe *et al.*, 2004). Gene amplification in *Acinetobacter* sp. strain ADP1 involves site-specific short homology-independent illegitimate recombination (Reams and Neidle, 2004). Reams and Neidle (2003) have shown that large genomic supra-operonic clusters in *Acinetobacter* undergo high level amplification to confer new catabolic traits. They demonstrated that a relatively high number of benzoate-degrading mutants emerged when grown on benzoate, and that the mutation consisted of an approximate 20 fold amplification of a chromosomal region containing the cat genes. In the absence of benzoate as selection pressure, the copy number went back to its original number (Reams and Neidle, 2003).

Certain regions of the *A. baumannii* chromosome act as antibiotic resistance gene insertion 'hot spots', as discussed in section 6.1.

2.7. Capability to horizontally transfer genetic information

2.7.1. Transformation

The active uptake of naked, extracellular DNA and successful incorporation into the genome is termed transformation. DNA can be in the form of a plasmid or linear fragements. Transformation is considered a major horizontal gene transfer mechanism contributing to genetic adaptation and evolution of prokaryotic cells (Ray and Nielsen, 2005). Bacterial genetic competence for natural transformation has been defined as a physiological state that permits the uptake of exogenous DNA. This process can be dissected into the discrete, sequential steps of DNA binding, DNA translocation across the inner and outer membranes, and subsequent recombination with homologous counterparts in the genome or plasmid amplification. A broad range of bacterial species have been reported to undergo natural transformation (Busch *et al.*, 1999).

The transfer of genetic material by transformation in a strain of *Acinetobacter* was first demonstrated in 1969 and formed the basis of the genetic test for the identification of members of the genus (Juni, 1972) (section 2.1). Competence for quantitative transformation in *Acinetobacter* strain BD413 occurs throughout the life cycle but with a peak early in the exponential growth phase (Cruze *et al.*, 1979). A strain with higher competency than BD413 for natural transformation was isolated and named ADP1 (section 6.1). *Acinetobacter* sp. ADP1 became a model system to study natural transformation and horizontal gene transfer in the environment (section 20.4), as well as a convenient tool for other genetic investigation studies (Young *et al.*, 2005).

Mechanisms of DNA transfer have been reviewed (Averhoff and Friedrich, 2003; Averhoff, 2004).

DNA transport machinery mediating uptake of naked DNA in *Acinetobacter* spp. is composed of pilin-like proteins including comP (Porstendorfer *et al*., 1997, 2000), comC (Link *et al*., 1998), comA (Friedrich *et al.*, 2001), comB (Herzberg *et al*., 2000), comE and comF (Busch *et al.*, 1999), and several others that were identified in the chromosome of *Acinetobacter* sp. ADP1 based on homology to competence-related proteins from other bacteria (Barbe *et al.*, 2004). The broad distribution of pilin-like factors among different bacteria independent of their phylogenetic relationships and their natural

environments indicates that these proteins may play a central role in the transformation mechanisms of these bacteria and that these components are highly conserved.

Once translocated into the cell, the exogenous DNA can be maintained independently of the chromosome (for example, if it is a plasmid), or can be integrated in the chromosome (if it is linear DNA). Demanèche *et al.* (2002) suggested that genetic transformation proceeds more frequently *via* integration of DNA with sufficient sequence similarity into the host chromosome than by the autonomous replication of plasmid molecules. *Acinetobacter* spp. do not discriminate between their own and foreign DNA (Dubnau, 1999). Chromosomal integration can occur by homologous recombination leading to a replacement of alleles, or to integration of heterologous sequences when bracketed by homologous regions. This only works when sequences have a high degree of homology. A study investigated the relationship between the length of inserts (434, 733, 2228, and 2400 bp) and flanking sequence homology (100 bp to ~11 000 bp) on transformation frequency in *A. baylyi* strain BD413. A minimum of 500 bp on each flank was required for transformation to be affected by flanking homology. Furthermore, it was shown that the ratio of flanking homology to insert size and not the total size of donor DNA is the most important variable determining transformation frequency. A multiple regression equation was developed to predict transformation frequency from homology, insert size, and total fragment size for gene insertions (Simpson *et al*., 2007a). For the integration of DNA with low homology to its own, two mechanisms were identified: 1) recognition of short specific sequences by transposases or integrases that can cut and paste sequences at these sites; or 2) illegitimate (nonhomolgous) recombination events that join DNA pieces with low homology (de Vries and Wackernagel, 2002; Hülter and Wackernagel, 2008). Non-homologous DNA fragments have a very low probability to be integrated by illegitimate recombination events during transformation (de Vries *et al.*, 2001). However, *Acinetobacter* sp. BD413 was transformed effectively by heterologous DNA fragments if the fragments contained a single homology region to the recipient genome (de Vries and Wackernagel, 2002). Such a homology-facilitated illegitimate recombination could explain the horizontal gene transfer of non-mobile gene cassettes. Horizontal gene transfer in the environment by transformation is discussed in section 20.4.

2.7.2. Transduction

Transduction is the transfer of DNA sequences from one bacterium to another *via* lysogenic infection by a bacteriophage (transducing phage). The occurrence of bacteriophages, as potential mediators of gene transfer, has been well documented for *Acinetobacter* (Vivian, 1991; Ackermann *et al.*, 1994). Strains of *Acinetobacter* differ in their susceptibility to bacteriophages, which are often readily obtained from sludge effluent. Most phages were lytic, but one temperate phage (P78) was capable of mediating generalized transduction in one specific host strain (Herman and Juni, 1974). The complete nucleotide sequence of ssRNA phage AP205 propagating in *Acinetobacter* species and classified as a member of the genus *Levivirus* was reported (Klovins *et al.*, 2002).

2.7.3. Conjugation

Conjugation is the joining of two bacterial cells when genetic material is transferred from one bacterium to another. Conjugation in *Acinetobacter* was first reported by Towner and Vivian (1976), using strain EBF 65/65, and the broad-host-range plasmid RP4 as a mobilizing vector. This plasmid, originally isolated from *Pseudomonas aeruginosa*, is capable of mobilizing the *Acinetobacter* chromosome and transferring chromosomal genes between different mutant auxotrophic derivatives of strain EBF 65/65. Another plasmid, pAV1 (distinct from pAV1 described in Table 8), transferred genes at frequencies up to 10%, *i.e.*, at a rate about 1000-fold higher than RP4 (Vivian, 1991). A variety of plasmids can be transferred by conjugation to *Acinetobacter* from enteric bacteria, although not all are stably maintained (Towner, 1991a). Antibiotic resistance is often acquired through conjugative plasmids (sections 6.2 and 15).

3. Human health considerations

3.1. Diseases caused and mechanism of pathogenicity, including invasiveness and virulence

3.1.1. General overview

For many years it has been difficult to assess the clinical significance of *Acinetobacter* infections as the taxonomy of the genus has been frequently changed. *Acinetobacter* has increased in importance during the last decade, especially in its role as a nosocomial pathogen (*i.e.* a pathogen peculiar to clinical environments).

A. baumannii is now one of the most frequently encountered nosocomial pathogens in intensive therapy units, and is renowned for being difficult to treat because of resistance to most antibiotics. Carbapenems are the remaining drugs of choice in many centres, but carbapenem resistance is now emerging in strains worldwide.

Acinetobacter causes significant infections in intensive care units (ICUs) specializing in respiratory, neurosurgical, neonatal care and in treatment of burns, where patients are particularly prone to infection with multi-resistant isolates of *A. baumannii*, as reviewed in Towner (2000). Principal sites and types of infection include the respiratory tract, blood (septicaemia), peritoneum, urinary tract, surgical wounds, meningitis, and skin or eye infections. The most common species involved is *A. baumannii*. Other species are sometimes reported in the literature, as detailed in section 8.6. As discussed in section 2.3.5 however, the species names found in the literature are not always accurate, especially for early studies based on phenotypic identification.

According to the National Nosocomial Infections Surveillance system, *Acinetobacter* spp. were isolated in 1% of all nosocomial infections from 1990 to 1992 (Emori and Gayne, 1993). The true frequency of infections caused by *Acinetobacter* spp. is difficult to assess with accuracy because *Acinetobacter* spp. are ubiquitous and readily colonize several body sites (section 12).

Bergogne-Bérézin (1997) stated that "As ubiquitous organisms (fortunately of low virulence), with few requirements for growth and survival, *Acinetobacter* spp. are prone to persist indefinitely in the hospital environment and to cause infections periodically when iatrogenic factors are present -- *i.e.*, overuse of broad-spectrum antibiotics, high-risk patients and cross-infection." According to Landman *et al*. (2002), multiresistant hospital-acquired bacteria represent a serious public health issue rather than an individual hospital's problem, and it would require an intensive coordinated effort to be effectively addressed.

3.1.2. Respiratory infections

Acinetobacter respiratory infection is usually acquired *via* artificial respiration devices (Levi and Rubinstein, 1996); following colonization of the oropharyngeal mucosa, the stage is set for contamination of the airways. Ventilator-associated pneumonia are reviewed in Shaw (2005). *Acinetobacter* pneumonia has a more serious prognosis than other types of pneumonia: a mortality rate of 71.4% was observed in these patients compared to one of 54.2% in those where the disease was caused by other organisms (Fagon *et al.*, 1993). Severe community-acquired pneumonia has been reviewed (Ewig and Torres, 2002).

3.1.3. Bacteraemia

Immuno-compromised patients comprise the largest group of patients with bacteraemia, followed by those with malignant disease, trauma and burns. A substantial number of *A. baumannii* bacteraemias represent catheter-related infections that usually carry a favourable prognosis (Seifert, 1995). The percent of *Acinetobacter* infections in blood cultures in different European hospitals was about 8% from 1974 to

1991, with a much higher percentage (26.6%) reported from Germany in 1993 (Bergogne-Bérézin, 1997). Bacteraemia caused by *A. baumannii* has been reviewed (Cisneros and Rodriguez-Bano, 2002).

3.1.4. Peritonitis

Acinetobacter spp. are important causes of peritonitis in patients (including children) undergoing continuous ambulatory peritoneal dialysis, including amongst children, and *A. baumannii* is prominent among these (Zurowska *et al*., 2008). Technique failure and diabetes mellitus are the most common risk factors (Bergogne-Bérézin *et al.*, 1996; Levin *et al.*, 1999).

3.1.5. Meningitis

A. baumannii has caused meningitis secondary to invasive procedures (Siegman-Igra *et al.*, 1993; Seifert *et al.*, 1995; Filka *et al.*, 2000; Rodriguez *et al.*, 2001; Pandian *et al.*, 2004; Wroblewska *et al.*, 2004), or following a head trauma (Venkataraman *et al.*, 1999). Nosocomial (Kralinsky *et al.*, 2000; Wang *et al.*, 2005) and community-acquired (Chang *et al.*, 2000; Lu *et al.*, 2002) meningitis have been reviewed.

3.1.6. Possible link with transmissible spongiform encephalopathies

Transmissible spongiform encephalopathies (TSE) are fatal neurodegenerative diseases that include "scrapie" in sheep, bovine spongiform encephalopathy (BSE) in cattle, Creutzfeldt-Jakob disease (CJD) and kuru in humans, and chronic wasting disease in deer. BSE-affected animals suffer from "hindquarters" paralysis, which is also one of the main features of "experimental allergic encephalomyelitis" (EAE), the animal model for Multiple Sclerosis. The presence of clinical and histopathological similarities in these diseases suggests a common pathology. Specific brain peptides, which produce EAE, were shown to have "molecular mimicry" with *Acinetobacter.* BSE-affected animals and patients suffering from MS have been found to have elevated levels of antibodies to both *Acinetobacter* and *Pseudomonas* bacteria, as well as autoantibodies to both white and gray matter brain components. The hypothesis is proposed that *Acinetobacter/Pseudomonas* bacteria may have evoked both BSE and MS through the mechanism of "molecular mimicry" and autoimmunity in a similar way to *Streptococcus* microbes producing rheumatic fever and Sydenham's chorea (Hughes *et al.*, 2001, 2003; Ebringer *et al.*, 2005b, 2005c). A protein responsible for molecular mimicry has been proposed to be the *Acinetobacter calcoaceticus* enzyme uridine-diphosphate-N-acetyl glucosamine-1-carboxy-vinyl-transferase which contains an amino acid sequence homolog to the bovine prion sequence RPVDQ (Wilson *et al.*, 2004). Whether *Acinetobacter* is the triggering agent of MS and BSA remains to be determined, but according to Ebringer *et al*. (2005a) and Wilson *et al*. (2003) the presence of antibodies to *Acinetobacter* species in MS patients or cattle with BSE opens the possibility of developing a laboratory diagnostic marker of disease activity, the myelin-*Acinetobacter*-neurofilament index, or MAN assay. Wilson *et al*. (2003) reported that *A. radioresistens*, *Acinetobacter* (sp3), *A. haemolyticus* (sp4), *A. johnsonii* (sp7), *A. lwoffii* (sp8) and *Acinetobacter* (sp9) gave 100% sensitivity and 100% specificity for detecting BSE. The highest anti-bacterial antibody level compared to controls was obtained with *A. johnsonii*.

However, there is no consensus in the scientific community about the involvement of *Acinetobacter* in the pathogenesis of TSE, or about the usefulness of antibodies toward *Acinetobacter* as diagnostic tool; Chapman *et al*. (2005) found no greater incidence of high-affinity antibodies against the organisms studied in MS vs. other neurological diseases, and so conclude that *A. calcoaceticus* and *P. aeruginosa* are unlikely to be implicated in the pathogenesis of MS. Nielsen *et al*. (2002) found that antibody levels in normal and affected animals overlapped considerably, thus casting doubt on the usefulness of these antigens as diagnostic tools for TSEs and on the hypothesis of *A. calcoaceticus* being a cause of TSEs.

3.1.7. Comments on each species

A. baumannii is understood to be, by far, the most important nosocomial pathogen within the genus. For example, a literature search of reports published between 2000 and 2005 in Scopus (www.scopus.com) using "*Acinetobacter*" and "outbreak" as keywords returned at least 26 reports of outbreaks caused by *A. baumannii*, but only one caused by *Acinetobacter* sp. 13TU and one by *A. junii.* Out of the 26 outbreaks caused by *A. baumannii*, 22 reported multi-drug resistance, among them 10 specified that drug-resistance included carbapenem resistance. Infections due to *A. baumannii* in the intensive care units have been reviewed (Chastre, 2003).

Acinetobacter genomic species 3 and 13 sensu Tjernberg and Ursing (13TU) are the most relevant clinically after *A. baumannii* according to van Looveren and Goossens (2004). These three species are closely related members of the so-called Acb complex (Table 1), hence the importance of using high resolution typing methods to analyze clinical strains. Multiresistant strains of these species causing bacteraemia, pneumonia, meningitis, urinary tract infections and surgical wound infections have been isolated from hospitalised patients worldwide (Van Looveren and Goossens, 2004). *Acinetobacter* species 13 was responsible for a case of bacteraemia following heart surgery (Mesnard *et al.*, 1994). Population structure and antibiotic resistance of *A. baumannii* and 13TU isolates from hospitals in the UK have been studied by RAPD (Spence *et al.*, 2002). The persistence and clonal spread of a single strain of *Acinetobacter* 13TU in a large Scottish teaching hospital was analyzed by PFGE (McDonald *et al.*, 1999). *Acinetobacter* species 3 was the chief species among four *Acinetobacter* causing various infections within a neonatal intensive care unit (Horrevorts *et al.*, 1995). It was also diagnosed as the cause of catheter-related bacteremia (Seifert, 1995).

A. calcoaceticus. This species has been recorded as the cause of community acquired pneumonia (Bilgic *et al.*, 1995), endocarditis in children with congenital heart disease (Malik, 1995), and burn patients (Ziolkowski *et al.*, 1993). However, in most cases the identification was doubtful and, as stated by Villegas and Hartstein (2003), most clinical laboratories would now categorize them as *A. baumannii*.

A. lwoffii has been involved in a case of community-acquired pneumonia (Domingo *et al.*, 1995), a pneumonia following bone-marrow transplantation (Lossos *et al.*, 1995). However, the identification may not be accurate in those cases. *A. lwoffii* could be involved in non-*H-pylori* induced gastritis (Rathinavelu *et al.*, 2003). *A. lwoffii* also causes diseases in several animals, as discussed in section 21.2.

A. junii caused serious sepsis in six preterm infants. The outbreak was investigated by ARDRA and four other typing methods, so the identification is reliable. All infections were thought due to bacterial contamination of an intravenously administered fat emulsion, which was shown to be an excellent growth medium for the bacteria (Bernards *et al.*, 1997; de Beaufort *et al.*, 1999). An outbreak of bacteraemia in paediatric oncology patients was also caused by *A. junii.* Environmental sampling showed the water system to be contaminated. Molecular typing using automatic laser fluorescence analysis of randomly amplified polymorphic DNA (RAPD-ALFA) revealed two distinct strains (Kappstein *et al.*, 2000).

A. haemolyticus. A case of infective endocarditis due to this species was reported (Castellanos *et al.*, 1995). The disease was linked to previous cardiovascular surgery and was cured by administration of imipenem and gentamicin. *A. haemolyticus* was isolated from febrile neutropenic children with neoplastic disease (Wojak and Gospodarek, 2004). According to Astal (2005), this species and others are increasingly resistant to ciprofloxacin and responsible for an increasing number of hospital infections.

A. johnsonii. Mixed bacterial meningitis due to this species and *Streptococcus faecium* was recorded (Sarma and Mohanty, 1995). Following a discussion of some catheter-related infections (Seifert *et al.*, 1993c, 1994b), *A. johnsonii* was regarded as an organism that could cause rare cases of infections of the bloodstream.

A. radioresistens was involved in only one case of community-acquired bacteremia, where the patient was HIV-positive (Visca *et al.*, 2001). *A. radioresistens* can therefore be considered as a cause of opportunistic infection in immuno-deficient patients.It may also be the reservoir for some clinically-relevant antibiotic resistance genes of *A. baumannii* (section 14.4).

Four novel *Acinetobacter* species were delineated from isolates of human clinical specimens: *A. parvus, A. schindlerii, A. ursingii*, and *A. septicus* (Nemec *et al.*, 2001, 2003; Kilic *et al*., 2008). Both *A. parvus* and *A. schindlerii* are normally isolated from blood or, more often, from non-sterile body sites of outpatients. In contrast, *A. ursingii* mainly comprises isolates from seriously ill, hospitalized patients. The identification in *A. ursingii* of typing characters similar to those found in two epidemiologically related isolates indicates that *A. ursingii* has the potential to spread among patients (Nemec *et al.* 2001). *A. septicus* was isolated from the blood and catheters of bacteriemic patients of a neo-natal intensive care unit and appears to be closely related to *A. ursingii*, although some notable genetic and phenotypic differences are apparent (Kilic *et al*., 2008).

Information is lacking for other *Acinetobacter* species largely associated with the human environment.

3.2. Communicability and means of dissemination of medically-relevant strains in community-acquired and clinically-acquired infections

Dissemination mechanisms in non-clinical environments have not been extensively studied. However, Achar *et al.* (1993) and Joly-Guillou and Brun-Buisson (1996) considered community-acquired infections to be rare, estimating that only 5-10% of cases of bacteraemia due to *Acinetobacter* were acquired in this way. Such infections are very active and have a high mortality rate. Chronic pulmonary disease, diabetes mellitus, and tobacco and alcohol consumption appear to be major predisposing factors. Zeana *et al.* (2003) reported that *Acinetobacter* isolates from the community were characterized by a large variety of unrelated strains (83.3%), and were distinct from the hospital isolates, of which 58.3% were closely related. Moreover, there were no multi-drug-resistant strains in the community compared with 36.8% among hospital isolates. A community-acquired infection amongst foundry workers resulting in pneumonia was traced to contamination of the air within the foundry and was associated with 15% of the workers there (Cordes *et al.*, 1981).

Communicability *via* lice has been suggested as a possible mechanism for community-acquired *A. baumannii*, in particular amongst homeless individuals with poor hygiene (La and Raoult, 2004; Houhamdi *et al.*, 2005). In rabbits, lice did not spread *A. baumannii via* feeding, nor is *A. baumannii* transmitted to progeny (eggs and larvae). However, lice excreted living *Acinetobacter* spp. within their feces (Houhamdi and Raoult, 2006)

A case of *Acinetobacter* sp. infection following dog bite was reported (Auerbach and Morris, Jr., 1987).

In the clinical environment, there is good circumstantial evidence that patients may be infected by an airborne route (Noble, 1991; Joly-Guillou and Brun-Buisson, 1996) though proof of direct aerial transmission from patient to patient is lacking. Room humidifiers were reported to be the source of some hospital outbreaks (Smith and Massanari, 1977; Gervich and Grout, 1985).

Contact spread and air-borne spread are both possible modes of transmission of *Acinetobacter* in hospitals (Gerner-Smidt, 1994), including *via* contaminated examination gloves (Diaz *et al*., 2008) and case notes (Panhotra *et al*. 2005). Reservoirs of *Acinetobacter* in the hospital environment have been well reviewed by Joly-Guillou and Brun-Buisson (1996) and contaminated materials have been identified as the source of infections. Extensive contamination of the environment occurs in the vicinity of infected or colonized patients, although air contamination in their absence is relatively rare. Hospital reservoirs include virtually any surface, especially components of mechanical ventilators (Villegas and Hartstein,

2003). *Acinetobacter*, together with other bacteria, have also been detected in topical and oral medicaments (Delarosa *et al.*, 1993).

Another method of dissemination may be within biofilms in water supply lines. A faucet aerator contaminated with *A. junii* was the common source of an outbreak of bacteraemia in paediatric oncology patients (Kappstein *et al.*, 2000). *Acinetobacter* was part of the biofilm flora inside dental air-water syringes forming individual micro colonies embedded in extracellular polymeric material (Tall *et al.*, 1995).

Other modes of dissemination in hospital environment also include airborne dissemination *via* contaminated aerosols and air conditioners (McDonald *et al.*, 1998), room humidifiers (Smith and Massanari, 1977; Gervich and Grout, 1985) or *via* the dissemination of respiratory secretions during tracheal tube suctioning in an intensive care unit (Ng *et al.*, 1999).

A. baumannii has been detected in foodstuffs and the implications of this finding for hospital-acquired infections have been discussed (Berlau *et al.*, 1999b). The role of insects in the spread of *Acinetobacter* spp. in hospitals has also been evaluated (Sramova *et al.*, 1992).

3.3. Infective dose

This infective dose for human infections caused by *Acinetobacter* apparently has not been determined. However, when mice were injected intraperitoneally with 40 clinical isolates of *Acinetobacter*, the LD50 values ranged from 10^3 to 10^6 viable cells per mouse (Avril and Mesnard, 1991).

3.4. Host range

Acinetobacter has a wide range of animal hosts (see Table 9, below), including humans, in which it may apparently live saprophytically or cause disease, as discussed in sections 18 and 19.2.

3.5. Capacity for colonization

An epidemiological study was performed to investigate the colonization with *Acinetobacter* spp. of the skin and mucous membranes of 40 patients hospitalized in a cardiology ward and 40 healthy controls. Thirty patients (75%) and 17 controls (42.5%) were found to be colonized with *Acinetobacter* spp., and the colonization rates of patients increased during their hospital stay. The most frequently isolated species were *A. lwoffii* (47%), *A. johnsonii* (21%), *A. radioresistens* (12%), and DNA group 3 (11%). The most important nosocomial *Acinetobacter* spp., *A. baumannii and* DNA group 13TU, were found only rarely (0.5 and 1%, respectively) on human skin (Seifert *et al.*, 1997). Comparable results were obtained by Berlau *et al.* (1999a). Patil and Chopade (2001) analyzed the antimicrobial susceptibility of *Acinetobacter* species on the skin of healthy humans. They found 7 *Acinetobacter* species, *A. lwoffii* being the dominant isolate, and they all displayed susceptibility to most of the commonly used antimicrobials.

Seifert *et al*. (1993b) analyzed the distribution of *Acinetobacter* species in clinical culture materials, and found that most isolates were recovered from respiratory tract specimens (n = 251; 42.9%), blood cultures (n = 116; 19.9%), wound swabs (n = 90; 15.4%), catheter tips (n = 75; 12.8%), and urinary tract specimens (n = 20; 3.4%). *A. baumannii* strains were isolated most frequently (n = 426; 72.9%), followed by A. species 3 (n = 55), *A. johnsonii* (n = 29), and *A. lwoffii* (n = 21). In addition, DNA from an *Acinetobacter* sp. has been detected in human sperm. The bacteria detected in the sperm were concluded not to be commensal but the result of male genitourinary tract infections (Kiessling *et al*., 2008).

A number of factors have been identified as increasing the risk of pneumonia or colonization of the lower respiratory tract by *Acinetobacter* in intensive care units, including advanced age, chronic lung disease, immuno-suppression, surgery, use of antimicrobial agents, presence of invasive devices such as

endotracheal and gastric tubes, and type of respiratory equipment. A high rate of colonization can be found in debilitated hospital patients, especially during outbreak situations. The predominant site of colonization is the skin, but other sites, such as the respiratory or digestive tract, may also be involved and may occasionally predominate (Bergogne-Bérézin and Towner, 1996). One important problem in diagnosis is the difficulty to differentiate infections caused by *Acinetobacter* spp. from colonization. Studies that use clear diagnostic criteria and strict microbiological documentation are needed (Levin, 2003).

3.6. Possibility for survival outside of human host

The general survival mechanisms of *Acinetobacter* spp. in the environment were presented in section 3.5. The ability to survive desiccation is probably unique to *Acinetobacter* among clinically relevant Gram-negative rods (Gerner-Smidt, 1994). An endemic strain from an intensive care unit could survive with only a slight decrease in numbers over a period of 14 days when applied to a steel table in saline, and for more than two months when applied in a serum supplemented broth. Other studies have confirmed the long survival of *Acinetobacter* under dry conditions (*e.g.*, on lint and on plastic foils), especially in the presence of proteins (Hirai, 1991). A. *calcoaceticus* and *A. lwoffii* were found to persist for means of 8.2 and 10.2 days respectively, *i.e.* for a longer period than could *Staphylococcus aureus*. Jawad *et al*. (1998a) found that ten clinical isolates of *A. radioresistens* could survive for more than 5 months on a glass surface, while strains of *A. lwoffii* and *A. baumannii* survived for only 3 and 20 days respectively. Survival of *A. baumannii* on dry surfaces was analyzed by Wendt *et al*. (1997), who conclude that if resistance to dry conditions may promote the transmissibility of a strain, it is not sufficient to make a strain an epidemic one. However, in the case of an outbreak, sources of *Acinetobacter* must be expected in the dry environment.

3.7. Antibiotic resistance

3.7.1. Resistance to antibiotics and other anti-microbials

A particular concern in recent years has been the frequent multiple antibiotic resistance exhibited by nosocomial species of *Acinetobacter*, and the resulting therapeutic problems involved in treating patients with nosocomial infections in intensive care units (ICUs). Resistance genes can be transferred between different bacterial species, as reviewed by Naiemi *et al*. (2005) (see sections 6.2 and 6.3), and between *Acinetobacter* species (see section 14.4), in particular those in clinical and non-clinical environments (section 20.4). Susceptibility data compiled by Bergogne-Bérézin (1996) comparing the reaction of four species of *Acinetobacter* to twenty-two antibiotics over the period 1964-1982 indicate that a significant shift in the resistance of *Acinetobacter calcoaceticus*, *A. baumannii* (*A. anitratus)*, *A. johnsonii* and *A. lwoffii* occurred after 1980. *A. baumannii* is still the most resistant species (Van Looveren and Goossens, 2004).

Many clinically-relevant *Acinetobacter* strains are now resistant to most commonly used antibacterial drugs, including amino penicillins, ureido penicillins, cephalosporins, cephamycins such as cefoxitin, most aminoglycosides-aminocyclitols, chloramphenicol and tetracyclines, ß-lactams and fluoroquinolones (Bergogne-Bérézin, 1996). Carbapenems, especially imipenem (Seifert *et al.*, 1993a), first showed good inhibition of isolates of *Acinetobacter*, including *A. baumannii.* For a time, imipenem was one of the few drugs used to treat *Acinetobacter* infections successfully (Bergogne-Bérézin, 1996). Since then, imipenem resistance has become more common. For example, in Spain, Ruiz *et al*. (1999) studied changes in susceptibility in 1532 clinical isolates of *Acinetobacter* from the *A. calcoaceticus* - *A. baumannii* complex, taken from 1991 to 1996. The percent of imipenem-resistant isolates rose steadily from 1.3 to 80%. New avenues for treatment are presented in section 17.

Treatment options for multi-drug resistant *Acinetobacter* spp. have been reviewed (Hartzell *et al*., 2007; Peleg, 2007; Gilad and Carmeli, 2008) and the potential of using other types of anti-microbials exists, for example, peptides (Knoetze *et al*., 2008) or copper complexed with organic compounds (Rosu *et al*., 2006).

In addition to antibiotic resistance, the importance of resistance to disinfectants or enhanced virulence due to specific disinfectants must also be considered, as discussed in section 23.

Members of the *Acinetobacter* genus develop antibiotic resistance extremely rapidly, which has been proposed as a consequence of long-term evolutionary exposure to antibiotic-producing organisms in soil (Van Looveren and Goossens, 2004). Several intrinsic resistance genes were described in environmental strains (Agerso and Guardabassi, 2005; Messi *et al.*, 2005) and human commensal strains (section 14.4).

3.7.2. Mechanisms and genetics of antibiotic resistance

This subject has been reviewed (Van Looveren and Goossens, 2004; Vila *et al*., 2007) Briefly, resistance against ß-lactam antibiotics such as penicillin, cephalosporins and carbapenems is mediated in most cases by ß-lactamases. ß-lactamases can be encoded on plasmids or on the main chromosome. Several ß-lactamase genes have been described, including TEM-1, TEM-2, OXA-21, OXA-37, PER-1, VEB-1 (Van Looveren and Goossens, 2004) and VIM-4 (Figueiredo *et al*., 2008). Carbapenem resistance is generally mediated by class D ß-lactamase such as the OXA-23, -24, -25, -26, -27 and -40 (Van Looveren and Goossens, 2004). Resistance to both imipenem and meropenem in multidrug-resistant clinical strains of *A. baumannii* can also be associated with changes in the outer membrane composition, such as the loss of a heat-modifiable 29-kDa outer membrane protein designated CarO (Limansky *et al.*, 2002; Mussi *et al.*, 2005), which mediates uptake of carbapenems as well as L-ornithine and other basic amino acids (Mussi *et al*., 2007). Carbapenem used to be the antibiotic of choice for the treatment of *Acinetobacter* infections. However, carbapenem resistance is observed increasingly, especially in isolates recovered from intensive care units. This resistance phenotype is often associated with multidrug resistance, leading to limited choices for treating *A. baumannii* infections, as discussed in section 17.

Resistance to aminoglycosides such as streptomycin and gentamicin is common in clinical isolates. It is generally due to plasmid- or transposon-encoded modifying enzymes which inactivate the antibiotics by adding phosphate, acetyl or nucleoside groups. Other mechanisms include an alteration of the target ribosomal protein, ineffective transportation of the antibiotic inside the bacteria (Van Looveren and Goossens, 2004), and active efflux out of the cell, like that mediated by the AdeABC system on netilmicin (Nemec *et al*., 2007).

Quinolones, such as ciprofloxacin, nalidixic acid, and fluorinated quinolones had a good activity against *Acinetobacter* strains until 1988. Since then, resistance emerged rapidly in clinical isolates. Resistance has been attributed to mutations in genes encoding the bacterial gyrase or topoisomerase, or by mutations in the chromosomally encoded drug influx-efflux system (Van Looveren and Goossens, 2004). In addition, fluoroquinolone resistance in an *A. baumannii* isolate is apparently mediated by QnrA, which protects DNA gyrase and topoisomeraseIV from the inhibitory activity of quinolones (Touati *et al*., 2008).

Acinetobacter also developed resistance against other types of antibiotics, such as tetracycline, *via* an efflux pump or a ribosomal protection system found on transposon or on plasmid, chloramphenicol, *via* an unknown mechanism (Van Looveren and Goossens, 2004), and tigecycline, likely *via* an efflux-based mechanism (Peleg *et al*., 2007; Ruzin *et al*., 2007). Tigecycline is a tetracycline-like antibiotic that was ‘fast-track’ approved in 2005 specifically for treatment of multiple antibiotic resistant bacteria like *A. baumannii*, and it is noteworthy that tigecycline resistance in *Acinetobacter* was reported shortly after it was approved.

Antibiotic resistance is encoded on one or more genes located on a plasmid or on the chromosome, and can be bracketed by conserved elements forming a transposon or an integron (section 6). Resistance profiles can change by a combination of plasmid- and integron-associated acquisition, especially in a unit with high antibiotic selective pressures. Clinical *Acinetobacter* isolates with different epidemic behavior were investigated for the presence of integrons and plasmids and for antibiotic susceptibility. Integrons were demonstrated in 50% of the strains. Epidemic strains of *A. baumannii* were found to contain significantly more integrons than nonepidemic strains. Also, the presence of integrons was significantly correlated with simultaneous resistance to several antibiotics. The role of 'silent' carriers of resistance genes acting as a reservoir for resistance determinants was demonstrated in a survey of 87 imipenem-sensitive clinical isolates of *A. baumannii*; two were shown to harbour poorly expressed VIM-1 (Ikonomidis *et al*., 2008).

The role of plasmids in the development of antibiotic resistance and in the epidemiologic behavior of *A. baumannii* is probably less prominent than that of the integrons since plasmids were detected in only 42% of the strains, and the presence of plasmids was not correlated with antibiotic resistance (Koeleman *et al.*, 2001). However, plasmids have been implicated in the horizontal dissemination of 'silent' chromosomally-encoded *A. radioresistans* antibiotic reisistance genes to *A. baumannii* (section 14.4).

The ability to adhere to medical surfaces and cells is a well recognized mechanism by which *Acinetobacter* exerts detrimental clinical effects (section 3.6), and some studies have investigated the relationship between biofilm formation and antibiotic resistance. For example, the capacity to form biofilms and resist antibiotics in 92 unrelated *A. baumannii* strains was investigated using a microtitre plate assay. Fifty-six of the isolates formed biofilm, and these isolates were less frequently resistant to imipenem or ciprofloxacin than were non-biofilm-forming isolates (25% vs. 47%; and 66% vs. 94%, respectively). Non-biofilm-forming isolates were associated with treatment in an intensive care unit, ciprofloxacin resistance, and isolation from a respiratory sample, while biofilm-forming isolates were associated with previous use of aminoglycoside (Rodríguez-Baño *et al*., 2008). The topic of biofilms and antibiotic resistance in biofilms harbouring *A. baumanni* or *A. iwoffi* was also discussed in section 4.

3.7.3. Use in differentiation and epidemiology

Because resistance genes are often localized on mobile genetic elements (MGEs) such as transposons or plasmids, and because they can be horizontally acquired under selection pressure, two identical or clonally related strains may harbour different antibiotic patterns. Infectious control personnel should constantly monitor changes in resistance profiles (Wu *et al.*, 2004). To achieve this, susceptibility tests can be performed by disc diffusion and agar dilution methods. Molecular methods of epidemiological typing that do not require bacterial culture have also been used, including plasmid profiling (Perilli *et al.*, 1996), repetitive-DNA-element PCR fingerprinting using the (GTG)5 primer (Huys *et al.* 2005), integrase gene PCR (integron typing) (Turton *et al.*, 2005), and multiplex PCR targeting various clinically-relevant markers (Dillon *et al.*, 2005; Evans *et al*., 2008). It should be emphasized that these epidemiological typing methods are not equivalent to the species or strain typing methods already described in section 2, since one single clone may harbour different resistance patterns, or alternatively different strains or even different species may harbour identical resistance patterns that were horizontally acquired. Strain genotyping methods described in section 2.4 must be performed in order to investigate if one or more strains are involved in an outbreak, and to identify the common source of contamination.

3.7.4. Horizontal dissemination of Antibiotic resistant amongst Acinetobacter in the clinical and non-clinical environment

Acinetobacter spp. with intrinsic resistance to antibiotics occur naturally in the environment. Such organisms may acquire additional resistance genes from bacteria introduced into soil or water, and the resident bacteria may be the reservoir or source of widespread resistant organisms found in many

environments. Ash *et al*. (2002) isolated antibiotic-resistant bacteria in freshwater samples from 16 U.S. rivers and measured the prevalence of antibiotic resistant bacteria. *Acinetobacter* spp. were among the most common resistant organisms isolated.

Antibiotic resistant *Acinetobacter* spp. were isolated in higher amounts from environments where antibiotics were present and exerted a selective pressure on the bacterial community, such as in pharmaceutical plant effluent (Guardabassi *et al.*, 1999) or in a fish farming pond (Petersen *et al.*, 2002). The latter study concluded that integrated fish farming seems to favour antimicrobial-resistant bacteria in the pond environment and attributed it to the selective pressure of antimicrobials in the pond environment and/or to the introduction of antimicrobial-resistant bacteria from animal manure. Potential risks to human health were not addressed in this study and remain to be elucidated (Petersen *et al.*, 2002). Guardabassi *et al*. (2000), who analyzed for tetracycline resistance determinants in aquatic *versus* clinical *Acinetobacter* isolates, concluded that they were different and that these differences, together with the inability of clinical strains to transfer tetracycline resistance *in vitro* to aquatic strains, contra-indicate any important flow of tetracycline resistance genes between clinical and aquatic *Acinetobacter* populations.

A. radioresistans strains were found to harbour chromosomally-encoded silent OXA-23-like genes (code for carbapenem-hydrolysing oxacillinases; see section 14.2) and plasmids with a similar backbone to those in several OXA-23-positive *A. baumannii*. Because *A. radioresistans* and *A. baumannii* are commonly-encountered human commensals found on the skin of healthy humans, this strongly suggested that *A. radioresistens* is the source of the OXA-23 gene, and that the plamids are the vectors for horizontal exchange of the OXA-23-like genes (Poirel *et al*., 2008). Similarly, insertion sequences like ISApa1 (which is often adjacent ot OXA-23 and is thought to provide the promoter required for expression of OXA-23 and other linked antibiotic resistance genes) is implicated in the horizontal dissemination of OXA-23 amongst *A. baumannii* (Valenzuela *et al*., 2007).

3.8. Toxigenicity and pathogenicity

Gram-negative bacteria produce LPS components in their cell walls that can function as endotoxin, as discussed in section 3.2.1. The active principle of the endotoxin seems to be the lipid A. *Acinetobacter* LPS was shown to cause lethal toxicity in mice, pyrogenicity in rabbits, complement fixation *in vitro*, and other reactions (Avril and Mesnard, 1991). Also, Garcia *et al*. (1999) showed that LPS of *A. baumannii* could induce a mitogenic response and elicits the formation of a tumor necrosis factor in mouse spleen cells. The authors concluded that *Acinetobacter* LPS is probably responsible for the characteristic signs of disease and death following septicaemia.

Other potential toxins include the phospholipase C from *Acinetobacter* sp. that had effects on whole red cells and red cell membranes according to early reports from Lehmann (1973). *A. baumannii* was shown to induce apoptotic cell death in epithelial cells through caspase-3 activation. The outer membrane protein Omp38 was proposed as a potential virulence factor inducing apoptosis (Lee *et al.* 2001; Choi *et al.*, 2005).

Braun and Vidotto (2004) analyzed several strains of *A. baumannii* isolated from urine, and found that none of them harboured genes identical to those coding for the virulence factors previously described in uropathogenic *Escherichia coli* (*e.g.* adhesins such as P fimbriae, S and F1C, Dr antigen family, type 1 fimbriae, and curlifibers; fibronectin receptor; toxins such as cytotoxic necrotizing factor; siderophores, such as yersiniabactin and aerobactin; invasins such as IbeA; polysaccharide coatings such as group II and III capsules; serum resistance; and colicin V production).

Barbe *et al.* (2004) claimed that *Acinetobacter* sp. strain ADP1 is non-pathogenic based on the fact they could not find any virulence-related genes such as those coding for known toxins, invasins and proteins of the secretory system following analysis of the whole genome sequence (see section 6.1).

However, 10 ORFs encoding hemolysin-like proteins of unknown function were found (Barbe *et al.*, 2004).

3.9. Allergenicity

The LPS of Gram-negative bacilli acts as a potent stimulator of the inflammatory response and can elicit allergenic response. Hypersensitivity pneumonitis, occupational asthma, industrial bronchitis or occupational lung disease are often associated with working environments with high levels of LPS and bacteria such as members of the ubiquitous genus *Acinetobacter*. Workers of an automobile parts engine manufacturing plant, a potato processing plant, a purebred horse farm, and a poultry hatchery exhibited serum precipitins to *A.Iwoffii, A. baumannii,* and *A. calcoaceticus* (Mackiewicz *et al.*, 1996; Zacharisen *et al.*, 1998; Dutkiewicz *et al.*, 2002; Skórska *et al*., 2007). It has been suggested that bacteria like *A. lwoffii* F78 (originally isolated from a cowshed) can be used as an allergy-protective because of its ability to reduce allergic reactions (Debarry *et al*., 2007).

3.10. Availability of appropriate prophylaxis and therapies

Acinetobacter infections are difficult to treat owing to their frequent multiple resistance to the antibiotics currently available for the treatment of nosocomial infections. Combination therapy including imipenem, ceftazidime, amikacin and the newer fluoroquinolones is usually recommended although strains are increasingly multi-resistant to these too. In the failure of these, colistin or sulbactam, two old agents that generate important side effects, may be appropriate, as reviewed (Jain and Danziger, 2004; Pasquale and Tan, 2005). As discussed by Levin (2003) however, complete randomized and controlled trials are lacking to support the ample use of these therapeutic options. The only data available are from *in vitro* susceptibility tests, experiments with mouse model or case studies.

Because of the tendency of *Acinetobacter* spp. to persist and spread in the hospital environment, compliance with good infection control practices, such as improved compliance with hand hygiene, strict patient isolation, meticulous environmental cleaning, and temporary closure of the unit to new admissions, is essential in preventing outbreaks.

Several studies have identified that previous antibiotic treatment was the only risk factor for *A. baumannii* acquisition (Zarrilli *et al.*, 2004; Carbonne *et al.*, 2005). Hence an appropriate use of antibiotic is critical to reduce the emergence of new multi-drug resistant strains (Coelho *et al.*, 2004; Jain and Danziger, 2004).

Several authors underline the importance of clear diagnostic and strict microbiological documentation during and after hospital outbreak. Ideally, genospecies determination should be performed with an appropriate method (sections 2.3 and 2.4) in order to suggest a treatment that is specific to the species and strain. This is not performed routinely in most clinical laboratories according to Levin (2003).

Several reviews specifically address the prevention (Kollef, 2004) and treatment (Rello *et al*., 2005; Vidaur *et al*., 2005) of ventilator-associated pneumonia. Emerging treatments for skin-associated infections and for treating people with burns, ulcers, and injuries include 1) chitosan acetate-based films [improved skin recovery times in patients, showed good anti-microbial effects against various pathogens including *A. baumanni*, and biodegrades *in situ* (Cárdenas *et al*., 2008)] and 2) polyethylene glycol/dopa polymer-based silver-catalysed gel (promoted healing of wounds in mice infected with various pathogens including *A. baumanni*, and is incorporated within the healing wound (Yates *et al*., 2007)).

4. Environmental and Agricultural Considerations

4.1. Natural habitat and geographic distribution, climatic characteristics of original habitats

4.1.1. General overview

The genus *Acinetobacter* is ubiquitous, being present in soil, water, and sewage (Towner, 1991b), as well as in association with humans and a variety of animals (Table 9), plants (section 20), and foodstuffs (*e.g.* raw milk and other uncooked food) (see also sections 3.3, 3.4, and 9). It has been estimated that *Acinetobacter* may constitute as much as 0.001% of the total heterotrophic aerobic population of soil and water (Baumann, 1968) and been found at densities exceeding 10^4 organisms per 100 ml in raw sewage (LaCroix and Cabelli, 1982). *Acinetobacter* has also been isolated from other heavily polluted waters, including, for example, the floating macroscopic filaments found at the surface of extremely acidic waters in Río Tinto Spain (García-Moyano *et al*., 2007) and leachate from automobile tire disposal sites (Leff *et al*., 2007). However, *Acinetobacter* is found more frequently near the surface of fresh water and where fresh water flows into the sea (Droop and Janasch, 1977). *Acinetobacter* is also isolated from other diverse and sometimes extreme environments, including, for example, as part of bacterial association oxidising sulphur in deep-sea hydrothermal vents in the Pacific Ocean (Durand *et al.*, 1994), upwelling water column off the coast of Namibia (Nathan *et al.*, 1993), communities inhabiting the rhizospheres of plants colonizing mine tailings (Zhang *et al*., 2007), amongst culturable bacteria isolated from ancient salt deposits of the Yipinglang Salt Mine, Yunnan Province, China (Chen *et al*., 2007) or Miocene lacustrine clays of the cypris formation excavated 200-m below the surface during open-cast brown coal mining (Sokolov Brown Coal Basin, North-Western Bohemia, Czech Republic) (Elhottová *et al*., 2006), and in a water-flooded petroleum reservoir of an offshore oilfield in China, although mesophiles like the *Acinetobacter* detected may have been introducted during exploitation of the reservoir (Li *et al*., 2007).

A further striking feature of *Acinetobacter* is its association with the exterior and internal organs of diverse species within the animal kingdom, the range of which is summarized below in Table 9. The wide distribution of *Acinetobacter* spp. in the environment, in particular healthy humans, presents a possible health hazard to persons with low resistance to potentially pathogenic micro-organisms. However, the risk is difficult to assess since most publications only report the presence of *Acinetobacter* sp., with no indication of the genomic species. The new molecular techniques described in section 24.4 are likely going to bring more data to help elucidate the ecology of *Acinetobacter* species.

Table 9 Occurrence of *Acinetobacter* in the Animal Kingdom

Animal	Major Taxonomic Group	Note	Reference
Rotifers	Rotiferae		Tanasomwang and Muroga, 1989
Coral (*Oculina patagonica*)	Fungi/Metazoa	Analyzed healthy, bleached, and cave corals, and *Acinetobacter* spp., dominated bleached corals	Koren and Rosenberg, 2008
Ostreopsis lenticularis (toxic benthic dinoflagellate)	Dinoflagellate	Symbiont bacterial flora essential for growth and toxicity development	Ashton *et al.*, 2003
Caenorhabditis elegans	Nematoda	No symptom	Grewal, 1991
Vestimentiferan tubeworm (*Lamellibrachia* sp.) from a bathyal methane-seep	Vestimentifera	Endosymbiotic flora in trophosome	Kimura *et al.*, 2003
Shrimps (various spp.)	Arthropoda	General Ice-stored or frozen for food Diseased (Syndrome 93)	Lee and Pfeifer, 1977 Guardabassi *et al.*, 1999; Lakshmanan *et al.*, 2002 Costa *et al.*, 1998
Crab (various spp.)	Arthropoda	As food In haemolymph of normal crab	Lee and Preifer, 1975 Sizemore *et al.*, 1975
Termites	Arthropoda (insect)	Symbiotic intestinal flora (cellulose degradation)	Schafer *et al.*, 1996
Cockroaches, Flies, Beetles	Arthropoda (insects)	As bacterial reservoir in hospitals	Sramova *et al.*, 1992
Oryctes rhinoceros (coconut pest)	Arthropoda (insect)	Pathogen	Kannan *et al.*, 1980
Triatoma infestans	Arthropoda (insect)	Faeces	Rondinone *et al.*, 1978
Ants (various spp)	Arthropoda (insect)	As bacterial reservoir in urban environment and hospitals	Sramova *et al.*, 1992; Fowler *et al.*, 1993; De Zarzuela *et al.*, 2005
Mosquito (*Culex* spp.)	Arthropoda (insect)	From external surface and alimentary canal. As potential candidates for genetic manipul. to control the disease transmission capabilities of the host. Increase susceptibility to viral disease.	Zayed and Bream, 2004 Pidiyar *et al.*, 2004 Mourya *et al.*, 2002

Human body louse, ticks, lice and fleas	Arthropoda (insect)	As bacterial reservoir. Isolated in gut.	La *et al.*, 2001; Murrell *et al.*, 2003; La and Raoult, 2004
House dust mites (*Dermatophagoides farinae* and *D. pteronyssinus*)	Arthropoda (arachnida)	As a minor constituent of bacterial commensals	Valerio *et al*., 2005
House fly (*Musca domestica*)	Arthropoda (insect)	Isolated from feaces, vomitus, external surfaces and internal organs	Nazni *et al*., 2005
Ectoparasitic chewing lice of pocket gophers	Arthropoda (insect)		Reed and Hafner, 2002
Oil fly, *Helaeomyia petrolei* (from asphalt seeps)	Arthropoda (insect)	In insect gut	Kadavy *et al.*, 1999, 2000
Diamond-back moth (*Plutella xylostella*)	Arthropoda (lepitoptera)	In larval guts of prothiofos (insecticide)-resistant and susceptible but not field specimens	Indiragandhi *et al*., 2007, 2008
Chinese scorpion (*Buthus martensii Karsch*)	Arthropoda (arachnida)	In intestine	Wang *et al*., 2007
Cotton bollworm (*Helicoverpa armigera*)	Arthropoda (lepitoptera)	In larval midgut of field specimans	Xiang *et al*., 2006
Charonia sauliae (trumpet shell)	Mollusca	Tetrodotoxin productivity of bacteria isolated from gut	Narita *et al.*, 1989
Mollusca (various spp.)	Mollusca		Puchenkova, 1988
Alligator mississippiensis, Crocodilus niloticus	Reptilia	Skin, meat	Oblinger *et al.*, 1981; Madsen, 1993
Fish (various spp.)		In eel, salmon, trout In diseased fish In fish meat Antibiotic resistance	Horsley, 1973; Esteve and Garay, 1991; Huber *et al.*, 2004 D'Souza *et al.*, 2000 Acuff *et al.*, 1984; Gennari and Tomaselli, 1988; Middlebrooks *et al.*,1988; Gonzalez *et al.*, 2000 Petersen *et al.*, 2002; Miranda *et al.*, 2003
Chicken	Aves	Meat, carcass From septicaemic hens With antibiotic resistance	Grimont and Bouvet, 1991; Olivier *et al*., 1996 Erganis *et al*., 1988; Kaya *et al*., 1989 Hofacre *et al*., 2001
Turkey (wild and domestic)	Aves	From coecum of healthy individuals	Scupham *et al*., 2008

Seabirds (various spp.)	Aves	From cloacae of rehabilitated birds	Steele *et al*., 2005
Lovebird (*Agapornis roseicollis*)	Aves	From lungs of animal with severe respiratory symptoms	Robino *et al.*, 2005
Mouse, rat	Mammalia	Mouse pneumonia model Rat thigh abscess model Mice with gastritis and hypergastrinemia Large intestines (tissue and luminal contents) of restricted-flora and specific-pathogen-free mice	Montero *et al*., 2002, 2004 Fetiye *et al.*, 2004 Zavros *et al.,* 2002 Scupham *et al*., 2006
Cats	Mammalia	Skin, vagina, uterus of healthy animal Nosocomial disease Cat suffering from necrotizing fasciitis with septic shock	Krogh and Kristensen, 1976 ; Clemetson and Ward, 1990 Francey et al., 2000; Boerlin *et al.*, 2001 Brachelente *et al*., 2007
Dog	Mammalia	Mouth, large airways of diseased animal Normal or infected skin Nosocomial disease	Johnson and Fales, 2001; Kasempimolporn *et al.,* 2003 Krogh and Kristensen, 1976, 1981; Kristensen & Krogh, 1978\ Francey *et al*. 2000; Boerlin *et al*. 2001
Pigs	Mammalia	Bacteriosperma in porcine semen	Althouse and Lu, 2005
Horses	Mammalia	Normal nose, conjunctiva Animal with chronic haematuria. Animal with respiratory symptoms. Nosocomial strain.	Cabassi *et al*., 1975; Cattabiani *et al*., 1976; Moore *et al*., 1988; Gemensky-Metzler *et a*l., 2005 Rajasekhar *et al.,* 1978 Boguta *et al*., 2002; Newton *et al*., 2003 Vaneechoutte *et al.*, 2000; Boerlin et al., 2001
Goats	Mammalia	Udder infection	Ndegwa *et al*., 2001
Cows	Mammalia	Connection with BSE	Tiwana *et al.*, 1999; Wilson *et al*., 2003, 2004

Llamas	Mammalia	Transtracheal aspirates and pleural fluid of normal animal	Gerros and Andreasen, 1999
Balaena mysticetus (bowhead whale)	Mammalia	Skin	Shotts, Jr. *et al*., 1990
California sea lion pups (*Zalophus californianus*)	Mammalia	Nasal cavity of healthy animals	Hernández-Castro *et al*., 2005
Lemurs (*Propithecus verreauxi deckeni* and *Eulemur fulvus rufus*)	Mammalia	Enteric bacterial flora	Junge and Louis, 2005

4.1.2. Comments on each species

A. calcoaceticus. *A. calcoaceticus* is the only species from the Acb complex (section 2.3.1) that is not found predominantly in the clinical environment, although it was sometimes isolated from human clinical specimens (section 8.7). *A. calcoaceticus* is the most widely distributed species according to literature data, occurring in marine (Al-Awadhi *et al.*, 2002) and terrestrial waters (Olapade *et al.*, 2005; Xu and Leff, 2004), thermal springs (Mosso *et al.*, 1994), soil and stone monuments (Turtura *et al.*, 2000), sludge plants (Muyima and Cloete, 1995), in several animals and in a variety of foodstuffs including milk, meat products and vegetables (Handschur *et al.*, 2005), on decomposing leaf surfaces (McNamara and Leff, 2004), in the airborne microflora (Prazmo *et al.*, 2003), and with plant-associated micro-organisms (Kuklinsky-Sobral *et al.*, 2004). Antibodies against *A. calcoaceticus* and other *Acinetobacter* species were found in brains of bovines suffering from BSE, as discussed in section 8.6 (Tiwana *et al.*, 1999).

The reports cited here and several others referring to the presence of *A. calcoaceticus* in the environment should be taken with care since molecular methods of identification were only developed in recent years and were not always applied in environmental laboratories. This remark is true for all *Acinetobacter* species, but particularly for those of the Acb complex that are very similar phenotypically. In addition, the identification scheme within the *Acinetobacter* genus was originally developed from human clinical samples, and until very recently, species isolated from natural environments were referred to as *Acinetobacter* sp., or incorrectly associated to another species such as *A. calcoaceticus* (see section 2.3.5).

A. baumannii. *A. baumannii* is the most frequent species of *Acinetobacter* in a clinical environment (section 8.7). This species is by no means restricted to a clinical environment. For example, it has also been found in Manitoba soils following enrichment with diclofop-methyl as a carbon source (Smith-Greeier and Adkins, 1996), or selected from crude-oil contaminated soil for further use in soil bioremediation (Mishra *et al.*, 2001a, 2001b, 2004). Merican *et al*. (2002) reported the identification of a bacterium from petroleum contaminated soil that is, based on 16S rRNA gene sequence, closely related *A. baumannii* but that may possibly be a new *Acinetobacter* sp. *A. baumannii* was also found in industrial waste-waters or used for industrial waste-water decontamination (Pauli and Kaitala, 1995; Shakibaie *et al.*, 1999) and in foodstuffs (Berlau *et al.*, 1999b). *A. baumannii* was found in several animals, including pets and insects such as lice and flies, as listed in Table 9. Human health considerations in term of communicability and dissemination were discussed in section 9.

Acinetobacter sp. 3. Bouvet and Jeanjean (1995) considered that *A. baumannii* and *Acinetobacter* species 3 and 13 were the most prevalent *Acinetobacter* species in clinical specimens but that their identity may have been masked through confusion with *A. calcoaceticus*. Species 3 has also been found in soil (Grimont and Bouvet, 1991).

A. haemolyticus. This species has been isolated from activated sludge and occasionally from patients and the hospital environment (Grimont and Bouvet, 1991). Several surveys have indicated its presence at a low level in clinical specimens (Gospodarek and Kania, 1992; Gerner-Smidt and Frederiksen, 1993).

A. junii. This species was originally isolated from human clinical specimen. *A. junii* is present in hospital environments, and was involved in a few hospital outbreaks (section 8.7). Its presence has also been reported among plant-endophyte bacteria (Kuklinsky-Sobral *et al.*, 2004), in foodstuff (Saha and Chopade, 2005), and in diesel-contaminated soils (Menezes *et al.*, 2005).

A. johnsonii. A. johnsonii is common on normal human skin, as discussed in section 12. It is also found in clinical samples (section 8.7). Most of the reports however are related to aqueous environments such as freshwater (Miranda *et al.*, 2003), freshwater fish (Gonzalez *et al.*, 2000) and wastewater (Boswell *et al.*, 1999, 2001; Oerther *et al.*, 2002). Its phosphate metabolism and presence in activated sludge has been extensively studied (Bonting *et al.*, 1993, 1999; van Veen *et al.*, 1993a, 1993b, 1994; Boswell *et al.*, 2001; Itoh and Shiba, 2004; Shiba *et al.*, 2005). *A. johnsonii* was found airborne in a fibreglass insulation manufacturing facility (Walters *et al.*, 1994). *A. johnsonnii* and *A. lwoffii* predominated in spoiled meat and milk (Gennari *et al.*, 1992; Gennari and Lombardi, 1993).

A. lwoffii . This species is the most frequently isolated *Acinetobacter* species on normal human skin (section 12). It has also been found on several animals, were it was sometimes associated with disease (section 20.2). It was found in a freshwater fish farming pond (Miranda *et al.*, 2003), freshwater and tapwater (Hashizume *et al.*, 2002), bottled water (Nsanze *et al.*, 1999), industrial waste-waters (Pauli and Kaitala, 1995) and phosphate removal systems (Grimont and Bouvet, 1991). *A. lwoffii* and *A. johnsonnii* predominated in spoiled meat and milk (Gennari *et al.*, 1992; Gennari and Lombardi 1993).

A. radioresistens. The original report of this species was from soil and cotton plants (Nishimura *et al.*, 1988). It has also been isolated from activated sludge (Knight *et al*., 1995), a petroleum-contaminated site (Nadarajah *et al.*, 2002), and a fish farming pond (Miranda *et al.*, 2003). *A. radioresistens* was recovered from the water supply aboard the International Space Station (Baker and Leff, 2005) and from the Mars Odyssey spacecraft (La Duc *et al.*, 2003). *A. radioresistens* is found on the skin of healthy humans (section 12) but only occurs sporadically in a clinical environment (Gerner-Smidt and Frederiksen, 1993), but may be clinically-relevant because it appears to be a reservoir for antibiotic resistance genes (section 14.4).

Acinetobacter sp.13. This species may have been confused with *A. baumannii*, since surveys have only recently detected it in a clinical environment (Ratto *et al.*, 1995; Weernink *et al.*, 1995). Bouvet and Jeanjean (1995) also considered that this species may have been confused with *A. calcoaceticus. Acinetobacter* sp. 13 has not been reported outside hospitals.

Other *Acinetobacter* species. Other species of *Acinetobacter* have been isolated less frequently, so that little is known of their role in the environment, clinical or otherwise (Grimont and Bouvet, 1991). The original isolates of *Acinetobacter* species 6BG, 10BG, 11BG, 14TU, 15TU, 14BJ, 15BJ, 16BJ and 17BJ were all of human origin (Bouvet and Grimont, 1986; Bouvet and Jeanjean, 1989; Tjernberg and Ursing, 1989). One sample of genospecies 11BG was also isolated from a cow's udder (Bouvet and Grimont, 1986). Genospecies 6, 10 and 14 have since been isolated from clinical samples (Gerner-Smidt and Frederiksen, 1993; Horrevorts *et al*., 1995).

Seven novel species were delineated among isolates from activated sludge (Carr *et al.*, 2003), four novel species were isolated from human clinical samples (Nemec *et al*., 2001, 2003; Kilic *et al*., 2008) (section 8.7), and two novel species were isolated from the Korean Yellow sea (Yoon *et al*. 2007). To date, no other habitats were reported for these species.

The range of habitat of the genospecies of *Acinetobacter* may be more extensive than has been indicated to date, since, as Vaneechoutte *et al*. (1995) have commented, the lack of practical and rapid

methods to identify isolates has limited our knowledge of their ecology and epidemiology in the past. However, molecular methods of identification that do not require bacterial plating and cultivation have been developed during the past decade. These methods described in section 25.3 enabled a better comprehension of the taxonomy, ecology and epidemiology in the *Acinetobacter* group. In spite of these technical advances, uncertainties persist because the natural environments have been much less sampled and analyzed that the clinical environment, and literature data are not always consistent.

4.2. Non-human pathogenicity

4.2.1. Plant pathogenicity

Acinetobacter has not been reported to be a plant pathogen.

4.2.2. Non-Human animal pathogenicity

The extensive association of *Acinetobacter* with animals has been reviewed above under section 19.1. The information in many of the references cited in Table 9 suggests that this association may change easily from commensalism to parasitism. In addition, *Acinetobacter* has been clearly implicated in several cases of infection, including an epizootic ulcerative syndrome in fishes (Singh *et al.*, 1994), mucoid enteritis of rabbits (McLeod and Katz, 1986), epididymitis in rams (Lozano, 1986), udder infections in goats (Ndegwa *et al.*, 2001), septicaemia, respiratory symptoms and other symptoms in horses (Boguta *et al.*, 2002; Newton *et al.*, 2003), skin diseases in dogs (Kristensen and Krogh, 1978; Krogh and Kristensen, 1981), pneumonia in an orangutan (Iverson and Connelly, 1981). Species clearly identified in animal infections are also those commonly involved in human pathology. However, *A. lwoffii* seems involved more often in non-human infections than in human infections. This species caused severe respiratory symptoms in lovebird (Robino *et al.*, 2005), septicaemia in hens (Kaya *et al.*, 1989), bacteriospermia in pigs (Althouse and Lu, 2005) and arthritis in a racing pigeon (Duchatel *et al.*, 2000). The role of *A. baumannii* as a nosocomial pathogen has also been recognized for dogs, cats and horses in intensive care units (Francey *et al.*, 2000; Boerlin *et al.*, 2001), and as the cause of necrotizing fasciitis and septic shock in a cat (Brachelente *et al*., 2007).

Mice infected with multi-resistant strains of *A. baumanni* are often used as model systems to evaluate new drugs and antibiotics (Braunstein *et al.*, 2004; Dijkshoorn *et al.*, 2004; Montero *et al.*, 2004) and *Caenorhabditis elegans* and *Dictyostelium discoideum* infected with insertional-mutants are used as virulence assays for assessing the roles played by specific genes in pathogenicity (section 6.1).

The presence of antibodies to *A. calcoaceticus*, but not other bacteria, was found in brains of animals suffering from bovine spongiform encephalopathy (BSE) (Tiwana *et al.*, 1999). This was discussed in section 8.6.

4.3. Interactions with and effects on other organisms in the environment

4.3.1. Enhancement of plant growth and other effects

Acinetobacter appears to play a part, though not a major one, in the growth of certain plants such as soybean, wheat, and canola. For example, when soybeans were inoculated with *Bradyrhizobium japonicum*, it was observed that the resulting nodules, which contained *Acinetobacter*, produced hydrogen. *Acinetobacter* strains isolated from the nodules were also able to oxidise hydrogen, a property not otherwise known for this genus (Wong *et al.*, 1986). *Acinetobacter* spp. closely related to *A. calcoaceticus* and *A. junii* were identified in a study of soybean-associated bacteria showing characteristics related to plant growth promotion (Kuklinsky-Sobral *et al.*, 2004). *Acinetobacter* associated with wheat roots was shown to produce indole-acetic acid that exerted a beneficial effect on growth (Leinhos and Vacek, 1994; Lippmann *et al.*, 1995). Also, pot experiments with wheat showed

a significant increase in plant growth in plants inoculated with *Acinetobacter* spp. identified as *A. junii*, *A. baumannii*, *Acinetobacter* genospecies 3, and *A. haemolyticus*. The increase was correlated with the presence of plasmid-encoded gene for indole-acetic acid (IAA) production (Huddedar *et al.*, 2002). An *Acinetobacter* sp. capable of promoting canola growth was isolated from the gut of diamondback moth larval guts. The plant growth promoting features were likely due in part to its ablity to produce indole acetic acid (Indiragandhi *et al*., 2008). Plant-growth promoting *Acinetobacter* spp. have also been found in association with wheat from salinified soils (Egamberdieva *et al*., 2008).

A. baumannii and a strain identified as Alcaligenes eutrophus were observed to colonise the outer root cells of seedlings of canola and wheat (vanZwieten et al., 1995). When 2,4-D was added to the hydroponic medium supporting the growth of the seedlings, the concentration of this herbicide decreased rapidly. Plants inoculated with the bacteria were subsequently found to be less susceptible to damage by the herbicide under these experimental conditions.

Xylanolytic Acinetobacter spp. were isolated on the bract phyllosphere of the date palm (Phoenix dactylifera) and may be involved in the early degradation steps of dry palm tree bracts (Rivas *et al*., 2007). Phosphate solubilizing Acinetobacter spp. were isolated from the rhizoplane of rice (Oryza sativa) and the strongest solubilizer was shown to densely colonize rice root surfaces, suggesting a role for bacteria like these in increasing soil phosphate bioavailability (Islama et al., 2007).

4.3.2. Antagonism to plant pathogens

Acinetobacter as well as other bacteria and fungi, were shown to be antagonists of *Rhizoctonia solani*, the cause of rice sheath blight (Gokulapalan and Nair, 1991) and of common plant pathogens such as *Sclerotinia sclerotiorum*, *S. minor* and *Gaeumannomyces graminis* (Oedjijono *et al.*, 1993). As a larval pathogen of *Oryctes rhinoceros* (Kannan *et al.*, 1980), a strain of *Acinetobacter* could be used to control this pest of coconuts. *A. calcoaceticus* and other micro-organisms *in vitro* inhibited *Xanthomonas campestris* pv *vignicola* on soybean (Jindal and Thind, 1990), *Pyrenophora tritici-repentis* on wheat (Li and Sutton, 1995), and *Fusarium moniliforme* on maize (Hebbard *et al.*, 1992). Antibiotic production seems to be responsible for the antifungal activity of these antagonistic *Acinetobacter* spp. For example, *A baumannii* LCH001 (isolated from the healthy stems of *Cinnamomum camphor*, the camphor tree) inhibits the growth of several phytopathogenic fungi such as *Cryphonectria parasitica*, *Glomerella glycines*, *Phytophthora capsici*, *Fusarium graminearum*, *Botrytis cinerea*, and *Rhizoctonia solani*. Inhibition is likely *via* the cyclic peptide iturin A and some isomers (Liu *et al*., 2007). These results indicate a potential application of *Acinetobacter* spp. or their products as biocontrol agent for plant diseases caused by fungal pathogens.

Acinetobacter sp. strain OM-H10 (closely related to *Acinetobacter* genospecies 11 and isolated from wild *Agaricales* fungi) degrades tolaasin, the inducer of brown blotch disease of cultivated mushrooms produced by *Pseudomonas tolaasii* (Tsukamoto *et al.*, 2002). Similarly, *Acinetobacter* sp. strain C1010 (closely related *A. parvus* and isolated from cucumber rhizospheres) degrades quorum-sensing acyl-homoserine lactones involved in induction of plant pathogenicity in *Burkholderia glumae* and *Erwinia carotovora* ssp. *carotovora* (Kang *et al.*, 2004). These results suggest possible applications of *Acinetobacter* spp. as bio-control agents for various pathogenic Gram-negative bacteria.

4.3.3. Cooperative and symbiotic interactions

Acinetobacter spp. engage in several cooperative or symbiotic interactions with other micro-organisms or higher organisms (Table 9 and section 5.2).

4.3.4. Horizontal gene transfer in the environment, on plants, and in animals

Horizontal gene transfer is defined as a genetic transfer between different species and is recognized to ocurr by at least three mechanism: transformation (section 7.1), transduction (section 7.2), and conjugation (section 7.3). *Acinetobacter* is capable of receiving exogenous DNA *via* all three mechanisms (section 7) although it appears to occur principally by transformation (section 7.1). The role of mobile genetic elements like insertion sequences, transposons, and integrons in horizontal dissemination is discussed in section 6.3.

Horizontal transfer of genes encoding antibiotic resistance is a particular concern. Horizontal transfer of antibiotic resistance determinants in a clinical setting between different bacterial species was demonstrated indirectly by sequence homology of the resistance genes amongst geographically- or clinically-related isolates (sections 6.2, 6.3, 7, and 14). Another concern is to evaluate the possibility of horizontal gene transfer of antibiotic resistance determinants in the environment from a transgenic organism to receiving bacteria. Because of its remarkable competency for natural transformation and its ubiquitous distribution, *Acinetobacter* sp. became a model of choice to study horizontal gene transfer in various environments. Natural transformation of *Acinetobacter* sp. was reported in various aquatic and soil microcosms as well as *in situ*, as reviewed in Ray and Nielsen (2005). A few examples are listed here:

1) Transfer of the plasmid pJP4 to *Acinetobacter* sp. from *Pseudomonas fluorescens via* earthworms was demonstrated in a microcosm (Daane *et al.*, 1996).

2) A *Pseudomonas putida* gene for kanamycin resistance was inserted into an *Acinetobacter* chromosome by natural transformation (Kok *et al.*, 1999).

3) Natural transformation of *A. calcoaceticus* was observed both *in vitro* and *in situ*, in a river on a stone surface between 12 and 20°C (Williams *et al.*, 1996). These authors' findings support the idea that recombinant DNA from introduced bacteria could eventually be transferred to the whole population. There is also the possibility that surviving DNA following the death of cells may be taken up, incorporated and expressed in a new host.

4) Transmission of three broad-host range plasmids (RP4, pUPI102 and R57.b) from *Acinetobacter* sp. to various indigenous soil bacteria was demonstrated in three different soil microcosms (Naik *et al*., 1994).

5) Bacterial transformation by *Acinetobacter* sp. in potable water was also demonstrated (Lisle and Rose, 1995), suggesting that natural transformation could occur in water distribution systems and biofilms (see also section 3.6 and 4). The addition of a disinfectant exerted no influence on the capacity of the bacterium to exchange genetic material.

6) Transformation of *A. baylyi* BD413 by DNA harbouring a transgenic construct composed of a kanamycin resistance gene and green fluorescent protein was detected in sterile soil microcosms (using pure plant DNA and ground leaves of transgenic plant) and in non-sterile soil (using pure plant DNA) (Simpson *et al*., 2007b).

7) In another study investigating transformation of rhizosphere bacteria by plasmid DNA or chromosomal DNA from rhizosphere isolates (had been chromosomally tagged with a recombinant selectable marker encoding tetracycline resistance), no naturally transformable strains were detected, although introduction of *A. baylyi* BD413 appeared to stimulate transformation by indigenous *Acinetobacter* strains (Richter and Smalla, 2007).

Horizontal gene transfer to *Acinetobacter* has been studied *in planta*. For example, studies have shown:

1) Conjugative transfer of a broad-host range plasmid and transformation-mediated transfer of chromosomal genes were found to occur at significant frequencies between *Ralstonia solanacearum*, a plant pathogen, and *Acinetobacter* sp. in plants (Kay *et al.*, 2003).

2) A transgenic plant could also transfer its transgene to *Acinetobacter*. However, transformation could only be detected if i) the transgenic plant was co-infected with a bacterial plant pathogen, and ii) if the plant transgene had homologous sequences with *Acinetobacter*, allowing homologous recombination. In absence of these criteria, the probability of natural transformation was very low and fell below the detection limit (Kay *et al.*, 2002).

3) Horizontal transfer of a plasmid from a tobacco plant to *Acinetobacter* (de Vries *et al.*, 2004).

4) Spread of recombinant DNA by roots and pollen of transgenic potato to *Acinetobacter* (de Vries *et al.*, 2003).

5) Natural transformation of an engineered *A. baylyi* BD413 by externally-added DNA was also demonstrated using defrosted and slightly abraded tobacco leaves as a model system for a naturally decaying plant matter (Rizzi *et al*., 2008).

Horizontal genetic transfer to *Acinetobacter via* transformation was studied *in animalia* using *A. baylyi* BD413 and DNA harbouring the kanamycin resistance gene could not be detected in

1) the gut of grass grub larvae (*Costelytra zealandica* (White); Coleoptera: Scarabaeidae), at least not above the detection limit of 1 transformant per 10^3 cells, possibly due to low population density and limited growth of *A. baylyi* cells in grass grub guts (Ray *et al*., 2007),

2) in the gastrointestinal tract (GIT) of mice and rats, even under slightly positive selective pressure, at least not above the detection limit of 1 transformant per 10^3-10^5 bacteria, possibly because exogenous DNA was readily degraded and absorbed in the GIT and the GIT environments was harsh and not conducive to survival of *A. baylyi* BD413 (Nordgård *et al.*, 2007), nor

3) the gut of tobacco hornworm *Manduca sexta* (Lepidoptera) fed transgenic tobacco, even though BD413 survived transfer throught the gut (Deni *et al.* 2005; Brinkmann and Tebbe, 2007). However, fecal matter containing transgenic DNA and DNA extracted from the fecal matter could transform *A. baylyi* BD413 (Brinkmann and Tebbe, 2007).

Efforts were made to detect in the field the possibility of gene transfer from cultivated transgenic plants into soil bacteria. Large amounts of naked DNA can be detected in soils and can persist for periods of time up to several months or years (Paget *et al.*, 1998). However, as reviewed by Gebhard and Smalla (1998), transformation of *Acinetobacter* in the field has not been shown, which Ray and Nielsen (2005) attributed to an insufficient sampling size and insufficient sensitivity of the detection method for rare transformation events.

4.4. Ability to form survival structures (e.g. *spores, sclerotia)*

Acinetobacter does not form spores or sclerotia. The persistence of many strains of *Acinetobacter* in the natural environment and clinical settings, which is probably due to their ability to survive dessication and low temperatures, form biofilms, and the presence of a mucoid cell envelope, has been reviewed under sections 3.5, 3.6, and 13.

4.5. Routes of dissemination in the environment, physical or biological

Dissemination in the clinical and non-clinical environment of medically-relevant *Acinetobacter* spp. has been described in sections 9 and 13. Dissemination in the natural environment has not been extensively studied. However, it is foreseen that various features of *Acinetobacter* spp., in particular their environmental ubiquity, their ability to survive cold stress and dry environments (section 3.5),

their capability to form biofilms on solid surfaces (section 3.6), and their capacity to form associations with foodstuffs, plants, humans, and animals (paragraph 142, section 18, and Table 9), will facilitate their dissemination.

Dissemination of *Acinetobacter* spp. in water distribution systems has been studied. Vess *et al.* (1993) conducted a laboratory investigation to show that *Acinetobacter* (probably *A. baumannii)*, together with other common Gram-negative and acid-fast waterborne micro-organisms, was able to colonize polyvinylchloride pipes and survive extended germicidal exposure. The proposed mechanism envisages that slow-flowing or stagnant contaminated water deposits extracellular material and bacterial cells along the interior of the pipes. Continuous layering with this material results in increased physical thickness and subsequent biofilm production. Once formed, this microbial ecosystem can persist, remain viable and become a continuous reservoir for bacterial contaminants, from which particles can break off and disperse through the water system**.** The potential for biofilm growth by *Acinetobacter* in water distribution systems and for promotion of coagrregation of other bacteria was explored (Chaves-Simões *et al*., 2008; Menaia and Mesquita, 2004; Flemming *et al.*, 2002; Hallam *et al.*, 2001).

4.6. Containment and decontamination in clinical and non-clinical settings

The importance of cleaning, disinfection, and use of appropriate prophylaxis to control outbreaks in hospitals has been emphasized in several reports and in this document (see, for example, section 17), especially considering *Acinetobacter's* capacity to survive extended periods on surfaces and in a dry state (see section 3.5 and 3.6). Effective control of *A. baumannii* can be accomplished with low-technology measures such as irradiation of surfaces with UV C and boiling treatment for hospital clothes (Rastogi *et al*., 2007), pasteurization of material (Wang *et al*., 2006), whole-body washes with 4% chlorhexidine (Borer *et al*., 2007), use of surface disinfectants (Omidbakhsh and Sattar, 2006; Wisplinghoff *et al*., 2007), or copper-based inorganic biocides (Gant *et al*., 2007). However, continuous surveys of susceptibility profiles to chemical disinfectants among clinically-isolated *Acinetobacter* species is necessary from the standpoint of nosocomial infection control as *Acinetobacter* strains isolated from clinical environments can develop decreased susceptibility to surface disinfectants, especially in the presence of organic matter (Kawamura-Sato *et al.,* 2008). Furthermore, care should be taken in the kinds of disinfectants used. For example, ethyl alcohol, which is found in hand rubs, has been observed to enhance growth and heighten virulence of *A. baumannii* (Edwards *et al*., 2007).

No reports were found describing containment or decontamination strategies for *Acinetobacter* in the natural environment.

4.7. Detection and monitoring techniques in clinical and non-clinical environments

4.7.1. General isolation media

Isolation of *Acinetobacter* species can be accomplished by use of standard laboratory media such as trypticase soy agar or brain heart infusion agar. Although most strains of *Acinetobacter* will grow well at 37^{o}C, growing isolates at a range of lower temperatures should be considered in order to take into account the lower growth optima of some species (Towner, 1991a). Temperature requirements for growth of various species of *Acinetobacter* are discussed in section 3.3.

4.7.2. Isolation from non-clinical material

Acinetobacter can be selectively isolated from soil and water using Baumann's enrichment medium (2g/l Sodium acetate (trihydrate), 2g/l KNO_3, 0.2 g/l $MgSO_4.7H_2O$, 0.04M KH_2PO_4 - Na_2HPO_4 buffer (pH 6.0), 20 ml/l Hutner's mineral base) (Baumann, 1968). Liquid enrichment cultures containing 20 ml of Baumann's enrichment medium are inoculated with a 5 ml sample of water or of a filtered 10% soil suspension and vigorously aerated at either 30^{o}C or at room temperature. Cultures are examined

microscopically after 24 or 48 hours and streaked onto suitable isolation media. Strains of *Acinetobacter* prefer a slightly acid medium at a pH of 5.5 to 6.0.

4.7.3. Isolation from clinical material

For clinical specimens, general-purpose, rich media such as blood agar or MacConkey agar are usually preferred because of their broad bacterial coverage (Towner, 1991a). Holton (1983) described a selective medium (Table 10) which may be suitable for the specific isolation and growth of *Acinetobacter* strains from clinical sources:

Table 10 Holton's Selective medium (per litre)

Agar	10.00 g
Casein pancreatic digest	15.00 g
Peptone	5.00 g
NaCl	5.00 g
Desiccated ox bile	1.50 g
Fructose	5.00 g
Sucrose	5.00 g
Mannitol	5.00 g
Phenylalanine	10.00 g
Phenol red	0.02 g

Adjust to pH 7.0. After autoclaving, the medium is cooled to 50°C and the following filter-sterilized ingredients added (final concentration in g/l): 0.010g/l Vancomycin, 0.016 g/l Ampicillin, 0.030 Cefsulodin

Following overnight incubation at 37°C, red colonies on the medium are tested for oxidase reaction and phenylalanine deamination (10% ferric chloride method). Colonies giving a negative reaction with both of these tests are presumptive *Acinetobacter* isolates (see section 2.1).

A similar medium to *Baumann's medium* (see paragraph 204) involving enrichment cultivation has been described in Appendix II of *Acinetobacter* (Bergogne-Bérézin *et al*., 1996).

The Leeds *Acinetobacter* medium was developed in order to screen out *Acinetobacter* (mainly *A. baumannii*) in clinical specimens from other bacteria in the hospital environment (Jawad *et al*., 1994) through the incorporation of several selective antibiotics.

4.7.4. Detection methods that do not require cultivation

The development of detection methods that do not require bacterial plating and cultivation has opened great possibilities in both the fields of routine monitoring in hospital environments and analysis of natural environments. The basis of these methods is to search for one or a few genes specific for a species, using PCR in most cases, among DNA extracted from a complex bacterial population. One example from hospital environments is the routine search for antibiotic resistance genes using PCR primers targeted to integron conserved sequences. Such epidemiological typing approaches are discussed in section 14.4. PCR-based detection of antibiotic resistance genes have also been used in environmental samples to monitor the appearance of resistance genes in bacterial communities from natural sites such as pig manure, a fish farming pond or a pharmaceutical plant outlet, as described in section 14.4.

PCR-based detection methods have also been used to monitor the presence of species-specific or genus-specific genes in complex bacterial communities. These methods are in contrast to traditional culture-based methods such as those described in sections 2.4.1 to 2.4.3. Adoption of these molecular

techniques made scientists realize that microbial populations in the natural environments are much more diverse than previously thought. One community-profiling technique known as PCR-DGGE (Denaturing Gradient Gel Electrophoresis) involves 1) DNA extraction from an environmental sample; 2) PCR amplification of the 16S rDNA; 3) denaturing gradient gel electrophoresis; 4) gel extraction of discrete bands and 5) sequencing. Individual bacteria are then identified by sequence homology searches. Differences in melting behaviors of small DNA fragments (200-700 bp) with as little as one single base substitution can be detected by DGGE (Muyzer *et al.* 1993). DGGE opened new possibilities in the field of molecular microbial ecology, as it allows the analysis of bacterial communities and the effect of various parameters on their diversity. PCR-DGGE was used, for example, to study *Acinetobacter* population dynamics in synthetic brewery wastewater (Tam *et al.*, 2005b), on soybeans in relation to season of isolation from soybean plants, soybean growth phase, and the tissues from which the isolates were obtained (Kuklinsky-Sobral *et al.*, 2004), in the benthic bacterial community of a river (Xu and Leff, 2004), in sediment bacterial assemblages exposed to selenate and acetate amendments (Lucas and Hollibaugh, 2001); to identify unculturable symbionts of a toxic dinoflagellate (Ashton *et al.*, 2003); and to assess *Acinetobacter* diversity in soil (Vanbroekhoven *et al.*, 2004). Application of this technique to food and food-related ecosystems was reviewed (Ercolini, 2004).

Alternatives to PCR-DGGE have been successfully used to detect *Acinetobacter* species in environmental and clinical samples. For example, a DNA microarray consisting of oligonucleotide probes targeting variable regions of the 16S rRNA gene was designed and tested for the investigation of microbial communities in compost, and *A. lwoffi* was detected in several samples (Franke-Whittle *et al.*, 2005). A microarray containing species-specific probes of 15mer oligonucleotides targeting 23S ribosomal DNA sequences was evaluated using reference bacteria and clinical specimens (*e.g.* blood, stool, pus, sputum, urine and cerebrospinal fluid), and *A. baumannii* was successfully detected in 11 out of 13 clinical specimans (Keum *et al.*, 2006). A PCR- and hybridization-based method using microspheres and flow-cytometric-based detection could be used to discriminate amongst 13 different *Acinetobacter* spp. and detect as few as 100 *A. baumannii* cells per mL blood (Lin *et al.*, 2008). A high-throughput method based on PCR-ligase reaction and capillary electrophoresis was described which could detect *A. baumannii* in blood (Pingle *et al.*, 2007). These methods are faster than PCR-DGGE but may be limited to detecting only known or previously described sequences. For example, in the case of DNA microarrays, only DNA completementary to arrayed DNA can be detected.

Methods like PCR-DGGE and DNA microarrays are *ex situ*, non-spatial community analysis methods. They allow an assessment of changes in microbial populations as a function of time or environmental conditions. Sometimes, however, spatial information is needed. In those cases, fluorescent *in situ* hybridization (FISH) is performed. This method consists of probing a bacterial population with a fluorescent taxon-specific probe, and then performing a microscopic examination of the sample. The probe can be Kingdom- or genus-specific (Mudaly *et al.*, 2001). For example, a 16S-rRNA-targeted probe specific for the genus *Acinetobacter* hybridized with any species of the genus, including the seven species newly delineated from activated sludge (Carr *et al.* 2003). An excellent example of *in situ* FISH studies as they pertain to *Acinetobacter* include the use of 16S rRNA *in situ* probing to determine the family-level community structure of micro-organisms implicated in enhanced biological nutrient removal. Several papers reported that according to bacterial counts obtained by FISH, *Acinetobacter* plays an insignificant role in phosphate removal (Wagner *et al.*, 1994; Mudaly *et al.*, 2000, 2001). Moreover, the hybridization signal obtained from an *Acinetobacter*-specific probe did not co-localize with a phosphate-specific dye (Liu *et al.*, 2005). FISH was also used, sometimes in combination with PCR-DGGE, to analyze bacterial communities from natural environments (Liu and Leff, 2002; Olapade and Leff, 2004, 2005; Xu and Leff, 2004; Gao *et al.*, 2005; Olapade *et al.*, 2005). Structure-activity relationships can also be explored using incorporation of ^{13}C-labeled substrates into microbial DNA and RNA to identify metabolically active community members (Wuertz *et al.*, 2004). Sensitivity and specificity of FISH were improved over time (Zwirglmaier, 2005).

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Foreword to the guidance documents relating to micro-organisms

The OECD's Working Group on Harmonisation of Regulatory Oversight in Biotechnology decided at its first session, in June 1995, to focus its work on the development of ***consensus documents*** which are mutually acceptable among Member countries. These consensus documents contain information for use during the regulatory assessment of a particular product.

On reviewing a published consensus document and drafting other consensus documents on micro-organisms, the Working Group felt that these documents did not focus in a straightforward way on questions that are relevant to risk/safety assessment issues.

Responding to the concern, the Working Group decided to take an alternative approach, namely the development of ***guidance documents***, in the micro-organisms area. Guidance documents are intended to provide guidance on specific topics and issues that are relevant to risk/safety assessment in biotechnology. The Working Group also established a sub-working group that consists of experts on micro-organisms and focuses on projects related to the environmental applications involving micro-organisms, especially bacteria. Subsequently, the sub-working group has developed several documents useful for environmental risk/safety of micro-organisms.

The three Guidance documents currently available on Taxonomy (2003); Detection techniques (2004); and Horizontal gene transfer between bacteria (2010), are reproduced below.

Section 2.
Guidance document on the use of taxonomy in risk assessment of micro-organisms: Bacteria

Preface to the guidance document on taxonomy

For risk assessments of micro-organisms used in biotechnology there is, in a general sense, a significant amount of commonality in methods that are used. Regardless of the organism employed or the uses of the organism that are evaluated, certain basic issues always need to be addressed during the course of an assessment. This document addresses one of the basic elements: the use of microbial taxonomy in assigning or confirming the identity of a subject micro-organism. Since the methods of taxonomy and the rules for naming organisms are different for prokaryotes than for eukaryotes or viruses, this document will be limited in scope to the use of taxonomy in the assessment of Eubacteria and Archea (simplified as "bacteria").

In general, the document is primarily intended for risk assessors who must deal with technical information to substantiate the identification of a micro-organism, who must make decisions on the acceptability of such information, and who must ultimately relate the information to the risk assessment of the micro-organism. It is presumed that the reviewer has a limited, but not expert, understanding of taxonomy, or at least has access to resources that can provide a basic background in this subject. The document may also be useful for applicants and other stakeholders in the regulatory process.

More specifically, the document explains why the discipline of bacterial systematics is important to risk assessment. It reviews some of the basic principles of microbial taxonomy and how they might be employed to determine potential risks of a micro-organism used in biotechnology applications. Methods applying these principles are listed and described. However, no single method is best suited for all types of bacteria. Therefore, the document also addresses some of the complexities and limitations that need to be considered in employing these principles. Finally, it addresses the prospect of future developments expected to have a significant effect on how one classifies bacteria and how those classifications may affect risk assessments.

Executive summary

The taxonomic identification of a subject micro-organism is a key element in any risk assessment for a biotechnology product. The uses of taxonomy in risk assessment may be seen as having two components, 1) providing a common frame of reference and 2) use in predictive analysis. In order that predictive analyses can take place, good identification of both the subject and a comparison micro-organism is needed. Inferences derived from a comparison bacterium's characteristics may be used to help formulate questions for risk assessment of the subject micro-organism. Data for subject or comparison bacteria may be acquired directly through testing, or indirectly via *interpretation of published, or otherwise available, information relevant to the issues of the case at hand. Selection of a comparison bacterium may be complex but they can be used in risk assessment, given a good understanding of bacterial systematics and the relationships between the comparison and subject bacteria.*

Identifying an unknown micro-organism is a two-step process requiring methods to characterize the traits of an organism, and approaches to interpret the characterization data. Phenotypic methods include techniques that

directly or indirectly detect, measure or characterize features of an organism resulting from the observable expression of its (total) genetic constitution. Phenotypic characteristics of bacteria include morphological, physiological and biochemical features and require growth of the organism in pure culture under appropriate conditions. Chemotaxonomic methods examine phenotype by using quantitative analysis of the organism's chemical constituents. Genotypic methods directly compare sequences, rather than rely on gene expression.

Approaches to interpret data include determinative approaches, numeric taxonomic approaches and phylogenetic approaches. There are many specific items that risk assessors need to consider to determine the adequacy of data for bacterial identification. Sometimes neither genotypic nor phenotypic methods alone suffice for either classification or identification of some bacteria, but it is possible to combine these methods using polyphasic taxonomy. Experience with all of the above techniques reveals that no single method is perfect for all taxa and all levels of taxonomic hierarchy.

Using bacterial identification in risk assessment is an inexact science and requires significant interpretive work by an assessor. There are constraints on the use of taxonomic identification methods in support of risk due to limits specific to the methods chosen, horizontal gene transfer and its affect on evolution of bacteria, variation in species concepts for different kinds of bacteria, inexact comparisons resulting from use of comparison organisms, and the overall ability to relate specific risk issues to the identification of a bacterium. Thus, interpretation of taxonomic data for use in risk assessment is not trivial, but the complexities should not be construed as preventing its use.

In general one should use methods appropriate for the organism with the objective of ensuring that the subject micro-organism cannot be confused with a member of a different species. Limitations of the techniques make it very difficult to use the simplest of methods and still obtain a reliable identification. In cases where the desired species-level assignment may not be achievable, a designation to the lowest level permissible (usually genus or subgenus) is needed. Sometimes, the best uses of taxonomic methods can narrow down an isolate to a species complex level yet still fail to provide a definitive name assignment. However, providing a single species name for a subject micro-organism, while strongly preferred, is not absolutely essential for risk assessment, provided there exist close taxonomic relatives that have been well characterized.

Knowledge of bacterial taxonomy has improved dramatically, but there is much more to be done. There is an improved ease of classification and identification of bacteria, but new knowledge has highlighted inadequacies in older techniques that may have led to some taxa being misclassified and misidentified in the past. Methods that give precise and unequivocal identifications for some genera of bacteria exist, but for many genera of current interest in biotechnology, only approximations of species assignments can be made with any confidence. To make better use out of taxonomic information, however, several advances will be needed in our knowledge, such as the application of genomics information, a better understanding of the nature of speciation in bacteria and relating taxonomic standing with risk related features.

1. Introduction

1.1. What is taxonomy?

Taxonomy is a means for organizing elements of groups of things in an orderly reproducible manner. Vandamme *et al.* (1996) illustrate that taxonomy for organisms has three components: (i) classification, (ii) nomenclature, and (iii) identification.

Classification involves the organization of relationships among related taxa (*i.e.*, the ordering of organisms in groups) and the creation of schemes for interpreting those relationships.

Nomenclature is a means by which standardized approaches to the formulation of names ensures that people use the same terms in referring to a single taxonomic entity (*i.e.*, the labelling of units in those groups).

Identification is the determination that an unknown isolate belongs to one of the labelled groups (taxa) and is included within the classification schemes.

While classification provides the foundation upon which to base conclusions of relatedness, proper identification is necessary to apply the knowledge of relatedness. Because in systematics (the science of

taxonomy) there are many different ways to organize information, systematists can, and often do, create more than one taxonomy for groups of organisms. Thus, there is no "official" classification of prokaryotes (Staley and Krieg, 1984). Nonetheless, classifications of most taxa become accepted through usage by the microbiology community.

However, there is an official nomenclature. Since 1980 there has been an Approved List of Bacterial Names (Skerman et. al., 1980) that is maintained by the International Committee on Systematics of Prokaryotes and updated with each publication of the *International Journal of Systematic and Evolutionary Microbiology* (IJSEM). Names of bacteria not previously listed in the "Approved List" in 1980, have been added through publication, either published directly in the IJSEM (or its predecessor the *International Journal of Systematic Bacteriology*, IJSB), or through valid publication in other journals, with subsequent listing through validation lists in IJSB/IJSEM.

Great strides are being made in refining the classification process for bacteria. As a result of the application of new methods, especially those that probe the heart of the bacterial genome, significant revisions in traditional taxonomies have been made over the past two decades. These continue to be made, and the new understandings of relationships among bacterial genera and species have allowed a better appreciation for bacterial evolution and the roles bacteria have played in the evolution of life in general. In this light, the modern tools of taxonomy and newly emerging tools of genomics have revealed that many, if not most, prokaryotes exhibit different modes of inheritance as compared with eukaryotic organisms. Whereas the eukaryotic genetic apparatus favors lineal decent with variation provided by mutation and within-species recombination, prokaryotic genomics has shown considerable complex (even chimeric) composition of the bacterial and archeal genomes studied so far (Bult *et al*., 1996; Galibert *et al*., 2001; and Wood *et al*., 2001) that is strongly suggestive of a significant impact of horizontal gene transfer (i. e., between species movement of genetic material) on prokaryotic evolution. The extent of this phenomenon may not be consistent across all bacterial genera but it appears that lineal decent is only one of two major mechanisms of gene inheritance in bacteria. The phenomenon of horizontal gene transfer and its effects on bacterial evolution has an impact on bacterial identification as is discussed in Section III.

In spite of the refinements in methods of taxonomic study, it should be recognized that the use of taxonomic information in risk assessment focuses primarily on proper utilization of identification information, not the creation of new taxonomies through the classification process. Nonetheless, it is helpful to know how organisms are assigned to categories through the classification process in order to understand the basis for the identification methods used for specific taxa. Risk assessors need to know what name to use when referring to a subject organism and how an organism bearing that name relates to other similar bacteria having valid bacterial names.

1.2. Relevance of taxonomy to risk assessment from a regulatory perspective

The risk of any new biotechnology product, particularly those derived through relatively new technologies, must be characterized prior to use. The common components of any risk assessment include a consideration of a number of factors relating to the potential for an organism to cause adverse effects (i. e., hazard) and the persistence and fate (i. e., exposure) of the organism and/or any of its by-products (e. g., toxins). These components are considered in combination to characterize the overall risk of releasing the organism to the environment.

In this context, the relevance of taxonomy as a necessary component of risk assessment is well established. Determining the identification of a micro-organism is usually the first step of a regulatory risk assessment. The uses of taxonomy in risk assessment may be seen as having two components, 1) providing a common frame of reference and 2) use in predictive analysis.

1.2.1. Providing a common frame of reference

One function of proper identification of a subject micro-organism is to establish a common label for the micro-organism. This label, the name assigned to the micro-organism, is to the risk assessment of biological products what the chemical structure and identity are to reviews of chemical substances. Not only is it essential to the basic characterization of the organism during pre-release phases, it also forms the basis for subsequent hazard and exposure assessments, especially those employing information gathered from scientific literature rather than data provided by an applicant. When identifications are reliably done, all who need to refer to a subject micro-organism, whether immediately during pre-release assessment or later after commercialization and widespread use, can feel assured that they are referring to the same entity.

If an organism is improperly or inexpertly identified at the onset of development, misunderstandings of expected or predicted features will be carried throughout the review process and if undiscovered or discovered belatedly, may result in misinterpretations of information and inadequate risk analysis. Ultimately this may lead to undesirable consequences that the risk assessment is designed to protect against.

For example, one *Pseudomonas* having a particularly difficult identification when first reviewed for a release, became a research organism several years after evaluation (Gagliardi *et al*., 2001). In this case extra effort was applied to get as accurate a name as possible with the tools identification available at the time. However, if the results of an initial assessment are used as support for subsequent assessments of the latter variations, and if the initial identification is in error, all the subsequent analyses will carry forward the same error. It may be fortuitous that the error is inconsequential for the initial assessments, but this good fortune may not carry through as more complex genetic modifications are made at latter stages of product development.

1.2.2. Use in predictive analysis

Proper identification can also enable the predictive analysis of a subject micro-organism in the absence of information or direct testing. Characterization information and data from biosafety tests, usually from laboratory studies, is expected upon receipt of an application for review. However, there may be times when information from micro-organisms similar to the subject micro-organism can be used to provide risk-related information when no direct data or information concerning the subject micro-organism exists. This information can be used to identify potential concerns and in this way be used a basis for further inquiries and/or control actions for the subject micro-organism (*e.g.* making sure that monitoring parameters are set appropriately based on expected environmental behaviours).

By having a well supported name for a subject organism one may confidently select appropriate related micro-organisms for comparison and have confidence that the use of data from such related organisms will be meaningful in support of assessing the potential risk of the subject micro-organism. The relevance of comparison organisms in this context is further described in Section III.

2. Methods and approaches for classifying and identifying micro-organisms

Classifying micro-organisms is essentially a two-step process that requires methods to characterize the traits of an organism, and approaches to interpret the characterization data to group organisms with similar traits together. Identifying an unknown micro-organism follows essentially the same two-step process (methods to characterize the traits of an organism, and approaches to interpret the characterization data); the difference being that the data from an unknown organism is compared against an existing classification scheme to arrive at a taxonomic designation.

Methods to generate characterization data range from traditional culture-based phenotypic and biochemical tests to more elaborate molecular techniques. Approaches can reflect the evolutionary inheritance of traits (*e.g.*, lineal decent), the intrinsic properties of the organism regardless of how they were acquired, or a combination of both. The basic approaches to classification and identification have evolved as the science of bacteriology has become more sophisticated, and the methods used to identify bacteria have evolved with these approaches. The intent of this section is to provide a general overview of these approaches and methods, and not to extensively review all of them. For this type of review, the reader is directed to more comprehensive reviews such as those by Rosello-Mora and Amann (2001) and VanDamme *et al*. (1996).

2.1. Methods

2.1.1. Phenotypic methods

Phenotypic methods include techniques that directly or indirectly detect, measure or characterize features of an organism resulting from the observable expression of its (total) genetic constitution. Hence, traits expressed from plasmid-borne genes can be used, along with traits expressed from the organisms's chromosome, to determine taxonomic designations using phenotypic methods. These methods have long been employed to identify unknown organisms, and despite the advent of newer molecular technologies, still have utility in determining taxonomic designations. In part, this is because some phenotypic features are basic and critical to grouping organisms into large classes of similar organisms.

Classical phenotypic characteristics of bacteria have been described as comprising morphological, physiological and biochemical features (Van Damme *et al*., 1996; Rosello-Mora and Amann, 2001), and require growth of the organism in pure culture on appropriate media. Because phenotypic characteristics are culture dependent, it is important that the observed features are attributed to expression of genetic differences and not the conditions in which the organism is cultured. The basis for this limitation is discussed further in Section IV under General Issues.

While not exhaustive, the following provides examples of classical phenotypic features and methods to measure or detect them.

2.1.1.1 Morphological

Morphological features of bacteria are directly observable by the naked eye or under a microscope. While most features do not require specific methodology to observe, some require specific instrumentation (a light or electronic microscope) and/or specific procedures (*e.g.*, spore and flagellar staining). Table 1 outlines some morphological features and associated methods to detect or characterize them.

Table 1 Morphological features and associated methods to detect or characterize bacteria

Feature	Method
Colony shape, colour, surface texture, margin shape	-- Direct observation from culture plate
Cell shape and size	-- Cell stain (*e.g.*, Gram stain, acid-fast stain) and microscopic observation -- Micrometer -- Accurate sizing requires observation under scanning electronic microscope
Inclusion bodies (*e.g.* polyhydroxybutyrate)	-- Microscopic observation
Spore production and morphology	-- Spore staining and microscopic observation
Flagella	-- Flagella staining and (electronic) microscopic observation

2.1.1.2 Physiological

Physiological features characterize how and under what conditions bacteria grow, survive and perpetuate. When a micro-organism is able to grow only under specific (sometimes extreme) conditions, these features are considered robust and can be very useful in arriving at taxonomic designations. At the very least, highly restrictive physiological features can be used to narrow the identity of micro-organisms to just a few options. In general, the number of tests that are applied should be limited to those characters known to have distinguishing value (Steffen, 1998). Table 2 outlines some physiological features and associated methods to measure them.

Table 2 Physiological features and associated methods to detect or characterize bacteria

Feature	Method
Growth temperature (minimum, maximum, optimum)	Establish growth curve over various temperatures
pH range of growth	pH strips, pH meter, colorimetric analysis
Oxygen tolerance (aerobic, anaerobic, facultative)	Incubate at various oxygen tensions; commercial anaerobic jars are available
Carbon dioxide tolerance	Incubate at various CO_2 tensions
Salt tolerance	Grow in various salt concentrations
Requirement for growth supplements	Grow in presence of growth supplements (*e.g.* sheep blood)
Antimicrobial susceptibility or resistance	Grow in a concentration gradient of antimicrobial agents
Susceptibility or resistance to other substrates (*e.g.* heavy metals, sulfur)	Grow in presence of heavy metals or other substrates

2.1.1.3 Metabolic

Metabolic features, for the most part, are indirectly observed because they are based on the cellular metabolism of an organism (*e.g.* biochemical reactions or metabolic activities). The methods used to characterize metabolic features usually involve growth on various substrates, assays for enzymes in biochemical pathways or assays for metabolic by-products resulting from enzyme activity. Direct observation techniques can also be used (*e.g.*, immunological detection of an enzyme or molecular detection of genes encoding enzymes); however, indirect methods are often used because carbohydrate fermentation, metabolic reaction, enzymatic and substrate utilization assays are fairly common, cost-efficient and simple to use. Furthermore, many commercial companies have developed miniaturized identification systems comprised of multiple assays on a single plate which makes characterizing metabolic features much simpler (see Phenetic Approaches/Numeric Taxonomy). While there are numerous enzymes and substrates that can be assayed, Table 3 outlines some enzymes and substrates with discriminating properties, and associated methods to detect them.

Table 3 Enzymes and substrates with discriminating properties and associated methods to detect or characterize bacteria

Feature	Method
Catalase activity	Catalase test ($H_2O_2 \rightarrow H_2O + O_2$)
Oxidase activity	Oxidase test (*e.g.* Swabzyme-oxidase)
Acid production from carbohydrates	Various tests depending on carbohydrate source (*e.g.* API strips, VITEK, BBL, enterotube, etc., or whole tube analysis)
Pigment production	Observation of propagation media, Pseudomonas F and P agar
Carbohydrate utilization	Hugh and Leifson tests depending on various sugars source
Carbon source utilization	Ability to grow on a sole carbon source(s) (*e.g.* BIOLOG, or whole tube analysis)
Enzymatic activities	*e.g.* hydrolase, lipase, proteinase, amylase

2.1.2. Chemotaxonomic methods

Chemotaxonomic methods involve quantitative analysis of the organism's chemical constituents. Because the techniques used in chemotaxonomy are not directed toward DNA or RNA, they are considered phenotypic methods. Consequently, they have the same culture dependent limitations as do phenotypic methods. Growing cultures under carefully standardized conditions is therefore required before any sort of comparative analysis is done. Table 4 outlines some key chemotaxonomic features and methods to characterize them.

Table 4 Chemotaxonomic features and associated methods to detect or characterize bacteria

Feature	Method
Gram behaviour	Gram stain
Teichoic acid	Extraction and purification followed by gas-liquid chromatography
Peptidoglycan type	Acid hydrolysis, HPLC, TLC
Fatty acid	Chromatography (*e.g.* fatty acid methyl ester [FAME] analysis by theMIDI system)
Polar lipids	Chromatography
Lipopolysaccharides	Chromatography, gel electrophoresis
Isoprenoid quinones (*e.g.* ubiquinones)	Chromatography
Polyamines	Gas chromatography, HPLC
Whole cell proteins	Gel electrophoresis, SDS-PAGE

2.1.2.1 Typing

Typing methods often rely on the analysis of chemotaxonomic features of bacteria, permit the identification of features that discriminate micro-organisms below species level (*e.g.* strains) and can be useful to understand intraspecific variability. Table 5 outlines some of the features that can be used to delineate sub-species, and associated methods to detect or characterize them.

Table 5 Features and associated methods to detect or characterize bacteria sub-pecies

Feature	Method
Antigenic cellular components (*e.g.* capsules, flagella, fimbria, etc.)	Serotyping
Total cellular protein profiles	Extraction followed by PAGE, multi-locus enzyme electrophoresis
Toxins	ELISA, cell line testing, molecular probes
Lipopolysaccharide profiles	Extraction followed by PAGE
Total chemical composition	Pyrolysis mass spectrophotometry, Fourier transform infrared spectroscopy, UV resonance Raman spectroscopy

2.1.3. Genotypic methods

Genotypic methods are based on modern technologies and provide alternative techniques that avoid some of the problems associated with methods that depend upon phenotypic expression. Since genotypic methods directly compare sequences, rather than rely on gene expression, they are thought to be more reliable than numeric taxonomic approaches that use phenotypic characters. An important scientific reason for using these methods is that for the first time they provide a potential for development of phylogenetic taxonomies (see Phylogenetic Approaches).

2.1.3.1. DNA base ratios and DNA hybridization

The DNA base ratio is one of the first nucleic acid technology applied to bacterial systematics (Lee *et al.* , 1956), and is calculated by the relative abundance of guanine (G) and cytosine (C) compared to the total genomic content of the micro-organism, *i.e.*, [G+C]/[A+T+C+G]. This technique follows on the premise that the nucleotide ratios [G+C]/[A+T] differ from genome to genome and hence can be used to distinguish phenotypically similar but genomically different organisms at all taxonomic levels (*e.g.* genus, species or sub-species). In general, it is recognized that the greater the differences between the G+C content of two organisms are, the less closely related they are. It has been experimentally shown that organisms differing by greater than 10 mol% do not belong to the same genus and that 5 mol% is the average range for a species (Rosselo-Mora and Amann, 2001).

Other initial efforts focused on DNA-DNA hybridization of whole genomes. For some time, the "standard" comparison for species has been whether genomes were shared at the 70% homology level with a maximum 5° C difference in melting temperature (T_m). However, the scientific basis for this number has never been firmly established and its value seems more indicative than absolute (Vandamme *et al.*, 1996). The basis for this standard is presumably that genomes that hybridize with the greatest affinity are likely to share the greatest sequence similarity and thus be most similar.

2.1.3.2 DNA-based typing methods

Like phenotypic typing methods, DNA-based typing methods allow the delineation of intraspecific boundaries between closely related organisms, that is, below species level. The methods involved in molecular typing evolved as the technology evolved, and two basic techniques are recognized. Early methods concentrated on the digestion of whole genomes using restriction enzymes followed by analysis of restriction patterns in polyacrylamide gels (restriction fragment length polymorphisms, RFLP). Later, the polymerase chain reaction (PCR) was used to amplify specific genome fragments. In this case, primers of 10-20 oligonucleotides in length are used to generate PCR products that vary depending on the organism. When the primers are very short (*e.g.*, 10 base pairs), the amplification is random and the technique is called "randomly amplified polymorphic DNA" analysis (RAPD). When the primers are based on amplification of interspersed, highly repetitive, non-coding elements, and the patterns result from the separation of fragments generated from restriction digestion, the technique is called "repetitive PCR" (rep PCR).

2.1.3.4. RNA-based typing methods

The general conservation of ribosomal RNA (rRNA) genes amongst bacteria, and the presence of certain hypervariable sequences in the genes within different species, can permit discrimination of bacteria to the genera, species and sometimes sub-species level (Woese, 1987; Stackebrandt and Goebel, 1994; Ge and Taylor, 1998). Ribotyping methods take advantage of the variability in rRNA genes. By restriction digest of amplified genomic RNA that contain all or part of the 16S or 23S rRNA genes, a fingerprint of the genetic material unique to the tested organism can be obtained and compared to patterns from other organisms.

2.1.3.5. Sequencing of house keeping genes

The advent of direct sequence analysis has allowed other methods to be developed. Some of these molecular approaches provide a focused insight into those conserved regions of the genome of bacteria that change at a rate fast enough that some variation can be observed in order to discriminate between closely related taxa, yet not so frequently as to provide too wide a separation between otherwise similar organisms. Most seek to sequence all, or portions, of housekeeping genes that fit these criteria. The most commonly used example of this is that part of the genome coding for 16S rRNA, for the reason listed previously. Usually the portion coding for the small subunit (16S) is utilized, but the intergenic spacer

region (ITS) between the 16S and 23S rRNA genes have also been used (Fisher and Triplett, 1999). Sequencing of the ITS region can be used to discriminate bacteria to the sub-species level. Other methods utilize different housekeeping genes, such as DNA gyrase (Yamamoto and Harayama, 1995). Gupta (1998) has suggested using protein sequences rather than nucleic acid sequences for these same purposes.

2.2. Approaches

2.2.1. Determinative approaches

Traditional methods for identifying both bacteria and fungi can be classified as determinative methods. Using these schemes, attempts were made to classify organisms based on a few key characteristics that could easily be observed. Taxonomic designation of an unknown organism is determined following sequential analysis of relatively invariant features characteristic of a taxon based on classification keys derived from known organisms. These approaches have always relied on phenotypic as opposed to genotypic methods.

Keys are often helpful in clinical situations where rapid identification of a pathogen is needed in order to expedite therapy, and where the subset of probable organisms is limited. However, under such circumstances it is possible that, since the paramount need has been to ensure that an identification leads to proper treatment, some bacteria that are otherwise closely related, but require different therapy, may appear separated taxonomically, while others that seem to provide similar host responses, but are distantly related, may be grouped.

As more micro-organisms from diverse environments have been observed, the determinative methods have proved to have limited utility. In part, this has been due to the inability to find enough reliably invariant characteristics that are appropriate for one and only one taxon. Without these, unambiguous identifications in other than clinical situations were found to be increasingly more difficult.

2.2.2. Phenetic approaches/numeric taxonomy

Numeric taxonomic methods have been employed for bacterial classification and identification for many decades. These are essentially statistical methods that use groups of traits that, taken together, point to specific taxa. Application of statistical approaches provides a mechanism for using a wide range of metabolic, biochemical and structural features, each given equal weight (Sneath, 1984) rather than stressing single features over others that may be present and thus have been useful for bacteria where distinguishing morphological features are absent. This analysis is referred to the “unweighted-pair group method with arithmetic mean” (UWPGA) technique. From this perspective, it has been generally accepted that an isolate must have at least 80-85% similarity to belong to a given species based on unweighted-pair group method analysis (Janda and Abbott, 2002).

This approach became the dominant one for classification of bacteria in the latter third of the Twentieth Century. In this method, researchers chose characteristics that strongly differentiated among taxa when strains were directly compared. Thus, as with the determinative approach, phenotypic methods were primarily employed. However, numeric taxonomy is really the application of statistics to any combination of features, phenotypic and genotypic, as long as they are not weighted to favor any given set of features. The intent of avoiding weighting individual elements is to avoid having single features bias the analysis. In practice, it is often difficult to give equal weight to single phenotypic traits with equivalent single genotypic results.

Usually, a battery of many tests are applied to a large number of strains of bacteria known or suspected to be closely related. Most tests are based on binary (yes or no) responses. Features are considered clearly discriminatory and are retained in the test set when strains of the same taxonomic

group respond the same way (either positive or negative) the preponderance of time (*e.g.* >75%). Those features that are equivocal (*e.g.*, gave a response of one test state *versus* its opposite ~50% of the time) are found not useful for the taxa in question. The responses of strains believed to belong to the same taxon are then compared statistically and a matrix of similarities devised. Taxonomic distance using this approach is thus based on an objective numerical value of similarity. Because a large number of tests are used, the consequence of error in interpreting any one test is minimized, and the discriminatory power of each test is reinforced by that of the others.

The successful application of numeric approaches has permitted development of automated methods for identification that have proved helpful in some circumstances. These automated approaches utilize simplified biochemical and physiological tests adapted to machine reading. Standard cultures are extensively tested and the results placed in a reference database. Unknowns receive the same treatment as the reference strains and the results for the tests are compared to those found in the database. Statistical analyses provide a presumptive identification or set of identifications for the isolate in question. Often the identification is accompanied by an estimation of confidence that the isolate matches a species in the database used by the method.

These automated methods have proved useful for certain taxa where a wide range of cultures have been provided for testing and inclusion in the database. Most clinical identifications are done in this fashion. Attempts have been made to extend this to "environmental" isolates, with less success. It may be that the range of variation for features significant for taxonomic placement is greater for environmental isolates than is accommodated by the databases used for clinical ones. It may simply be that too few different isolates of the former type have been collected as reference strains for the comparisons. Or, unlike some clinical isolates which might have pathogenicity factors such as a toxin, it may be that environmental isolates do not have specific, easily measurable defining traits associated with them.

For whatever reason, use of automated methods may be less successful for identification of environmental isolates than clinical ones. This is often reflected in relatively low confidence values for identifications or in multiple "likely" species names, each with "high probability" rankings, for single isolates. Users sometimes fail to understand the implications of the statistical analyses forming the basis of the identifications and will choose the highest ranked name from among those listed, rather than acknowledge that the isolate could be any of the top ranked species coming from the analysis.

2.2.3. Phylogenetic approaches

Unlike higher organisms, the lack of distinguishing morphological features and a sequence of fossils that illustrate genetic variation has frustrated the development of a phylogeny of bacteria. However, with the advent of gene sequencing and the recognition that certain conserved regions of the bacterial genome could serve as slowly varying reference points in lieu of morphological change, efforts to classify bacteria by phylogenetic approaches has move quickly forward and has revolutionized the way classification of all organisms, prokaryotes and higher forms alike, are now viewed.

The phylogenetic approach is based on genotypic methods in which certain genes are sequenced, compared with sequences of other micro-organisms, and then placed in relative standing to other organisms. Housekeeping genes, or predominantly conserved genes with minor areas of hyper-variability, are often used.

In the past it was noted that the structure of ribosomes and their RNA sequences was significantly different between two large groups of prokaryotes, now called Bacteria and Archaea (Woese, 1987). As the study of the differences among the members of these large groups developed, the use of ribosomal RNA/DNA was found to be able to differentiate among finer scale taxonomic units down to the genus and sometimes species level (Palleroni, 1993). Using a statistical approach called cladistics, "trees" have been constructed that show taxonomic relationships found using comparisons of ribosomal nucleic acid

sequences. While there are no consensus guidelines to delineate species based on ribosomal RNA/DNA sequences, it is generally accepted that sequences >97% in similarity belong to the same taxon (Rosello-Mora and Amann, 2001; Janda and Abbott, 2002). However, this figure has been challenged by a number of studies which indicate that unidentified isolates defined a species match with as little as 0.2-1% divergence (Drancourt *et al*., 2000; Woo *et al*., 2001; Janda and Abbott, 2002).

While such molecular approaches can give an insight into the evolutionary relationships existing among bacteria, they really represent the evolution of specific stretches of the genome in the different taxa studied. As suggested by Doolittle in Huynen *et al*. (1999), because there may be different rates of evolution for different parts of a bacterial genome, these methods may fail to provide a complete overview of the genetic basis for differentiation among bacterial species. One reason this may be so is that genes chosen as targets for molecular approaches to bacterial taxonomy are often found on the "chromosome" of bacteria, and are thus stably associated with a particular genome, while many of the important features of bacteria are found on extrachromosomal elements that vary in their ability to transfer among genomes. Genes introduced recently into a species through plasmid exchange and recombination thus have "evolved" differently than genes that originate in a lineal ancestor to the current species and are subsequently modified through a succession of mutational events. Using the evolution of a conserved housekeeping gene as a surrogate for whole genome evolution can thus be misleading.

2.2.4. Polyphasic approaches

Sometimes neither genotypic nor phenotypic methods alone suffice for either classification or identification of some bacteria. Current methods do not rely solely on single molecular features (Steffen, 1998). It is possible to combine these methods to create a more robust method, called polyphasic taxonomy. This approach permits the combination of the information obtained from phenotypic and genotypic methods useful for the taxonomic group of concern.

As Schloter *et al*. (2000) note, most bacteriologists now consider that bacterial species should be defined by a polyphasic taxonomic approach integrating results from numerical pheno- and genotyping and rRNA gene homology studies. Usually this means that a species is delineated from others and can be defined if pheno- and genotyping analyses differentiate the investigated group of strains from related species in consensus, if diagnostic phenotypic characters for the new species are found, and type strains are deposited in culture collections. DNA/DNA hybridization is still preferentially considered over 16S rDNA sequencing.

The polyphasic approach utilizes various methods such as 16S rDNA molecular sequencing methods or gas chromatography/fatty acid methyl esther (GC-FAME) as a form of "range-finding" to provide putative placements of related strains in a classification diagram, usually a dendrogram or "tree". Confirmation of the placements of the members of the grouping is accomplished by independent use of structural, physiological and biochemical phenotypic tests as well as other molecular approaches (such as hybridization). Some may use multilocus enzyme assays in this approach. Others may use more than one set of conserved gene sequences as components in this methodology. Whatever method is used, it is important to be cognizant of what each component is measuring to avoid using complementary methods, which may really be providing the same answers to the same taxonomic questions.

In general, methods useful for placing an unknown into an appropriate genus- or species-level taxon are not as useful for identifying a unique isolate at the strain-level. Often single markers, or groups of unique identifiers are needed for the latter. While this is important, for example, as a means of tracing a released organism in the environment, strain- or isolate-specific methods are not the subject of this document.

Summary

Identifying an unknown micro-organism is a two-step process requiring methods to characterize the traits of an organism, and approaches to interpret the characterization data. Phenotypic methods include techniques that directly or indirectly detect, measure or characterize features of an organism resulting from the observable expression of its (total) genetic constitution. Phenotypic characteristics of bacteria include morphological, physiological and biochemical features and require growth of the organism in pure culture under appropriate conditions. Chemotaxonomic methods examine phenotype by using quantitative analysis of the organism's chemical constituents. Genotypic methods directly compare sequences, rather than rely on gene expression.

Approaches to interpret data include determinative approaches, which involve sequential analysis of relatively invariant features characteristic of a taxon based on classification keys derived from known organisms; numeric taxonomic methods which are essentially statistical methods that use groups of traits that, taken together, point to specific taxa; and phylogenetic approaches, which are based on genotypic methods in which certain genes or proteins are sequenced, compared with sequences of other micro-organisms, and then placed in relative standing to other organisms. The successful application of numeric approaches has permitted development of automated methods for identification that have proved helpful in some circumstances. Sometimes neither genotypic nor phenotypic methods alone suffice for either classification or identification of some bacteria, but it is possible to combine these methods using polyphasic taxonomy. Bacteriologists now consider that bacterial species should be defined by a polyphasic taxonomic approach integrating results from numerical pheno- and genotyping and rRNA gene homology studies. However, experience with all of the above techniques reveals that no single method is perfect for all taxa and all levels of taxonomic hierarchy.

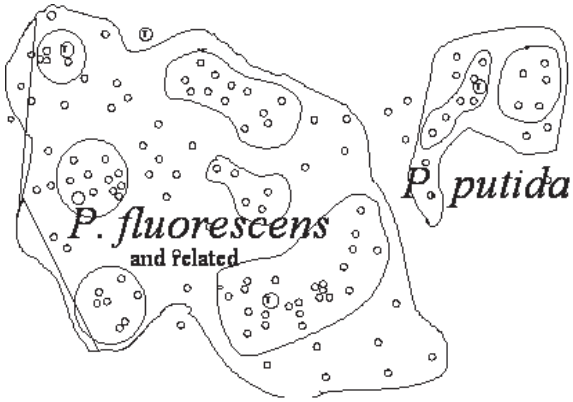
3. Conceptual issues related to identification methods

Section II described the various methods and approaches currently available for bacterial identification. While the methods described there are well advanced and have been shown to be useful in providing identifications for biotechnology risk assessment, there are some scientific issues that arise that can affect the way reviewers interpret the results of such analyses. Some of the more significant ones are described in this section.

3.1. Limits to the ability to assign names to some taxa

Sometimes, the best uses of taxonomic methods fail to provide a definitive name assignment. For example, the genus *Pseudomonas,* is a particularly complex genus. Embedded within this genus is a "supercluster" of species of *Pseudomonas* grouped, in part, based on the production of fluorescent pigments. This subgroup is so complex that one species, *Pseudomonas fluorescens* has been subdivided into at least five biovars, each of which may deserve species rank (Barrett *et al*., 1986). Yet even with this fine subdivision of one species, it is not uncommon that new isolates believed to be part of this grouping cannot be definitively assigned to just one member of this supercluster. This is well illustrated by a diagram showing the relationships of many isolates of "species" of fluorescent *Pseudomonas* (Figure 1).

Figure 1: Scheme showing relationships among species of fluorescent *Pseudomonas*



Modified from Smirnov and Kiprianova, 1990 (Figure 30). The left hand grouping are species and biovars associated with *Pseudomonas fluorescens* and the right hand shows the two primary biovars of *Pseudomonas putida.* Note the large number of isolates that are intermediate between the "species" of the *fluorescens* cluster and the several that are located between the *fluorescens* and the *putida* groups. Individual isolates are shown by circles, type strains by "T".

Even though no existing name can be assigned, the ability to place an isolate close to characterized species is useful information for risk assessment. If there are no pathogens on either "side" of the taxonomic boundary (*e.g.*, at the genus, species or sub-species level), one would not expect the subject micro-organism to be pathogenic. If any of the micro-organisms on either "side" of the boundary is a pathogen, then one might use this as a basis for further inquiry regarding the potential pathogenicity of the subject micro-organism.

Conversely, if there are characteristics found in more distant species, but not in any of the nearby taxa, it implies that there is a far lower probability that those features from the distant species might be found in the isolate. Again to illustrate, lactose utilization is not associated with members of the fluorescent pseudomonads (Palleroni, 1984). One would not expect this feature in any member of that cluster of species. This observation has even been used as a basis for introducing a *lac zy* marker gene into one such micro-organism as a means of tracing movement in the environment (Gagliardi *et al*., 2001) since no *Pseudomonas* found naturally occuring at a release site for this GMM should have that feature and thus be confused with the GMM .

3.2. Effects of the dependence of some methods on stable gene expression

Phenotypic and chemotaxonomic methods measure and depend on the consistent expression of specific sets of genes. An important consideration is that gene regulation can be very complex and thus expression can be variable and environment-dependent. One possible source of apparent inconsistent expression is variability in the conditions under which expression is observed. The use of standard protocols is intended to minimize variation in expression, but this is not always accomplished. Thus, phenotypic methods can be dependent on specific laboratory associated variables. This may lead to some methods being unique to a particular analyzing laboratory and these may be difficult to replicate without experience in that facility, and thus inter-laboratory comparisons can be difficult. While *in vitro* micro-environments can affect the operation of the genomic regulatory components, gene expression can also be impacted when multiple versions of a promoter, or even a whole operon, may be present in a genome. So even if there are scrupulous laboratory controls to limit method variability, some isolates may exhibit variable responses to some tests.

A second source of inconsistent expression is variability in the gene complement of the genome under examination. The phenotype of bacteria, being based on the observation of expressed genes, is dependent on the stability of the genes that produce the features that are measured. Stability, in this case,

refers not to changes in degree of expression of the individual genes of interest, but to the presence or absence, and the frequency of occurrence in the genome of the bacterium in question, of the genes for the feature. Some genes may be mobile *via* the well known mechanisms of conjugation, transduction or transformation. If mobile, such genes may be lost from the original bacterium entirely, if not replicated before transfer, or may be transferred, so as to appear expressed in genomes of bacteria of other taxa. Loss can also occur if the genes are on extrachromosomal elements which themselves are lost (cured) from the genome in question. In either case, whether genes are lost or transferred, unless all features used in creating a phenotype for a taxon are stable, identification based solely on phenotype may be affected by gene mobility. While the mechanism is different than for the effects of variable gene regulation mentioned previously, the result is, similarly, reduced reliability of a measure of identification.

3.3. Horizontal gene transfer

3.3.1. Horizontal gene transfer and its effect on identification of bacteria

Horizontal gene transfer represents a special impediment to interpreting taxonomic data in the context of risk assessment. At the time of preparation of this document a separate Guidance Document addressing this topic in a comprehensive way was being prepared. The current document only considers the importance of horizontal gene transfer to the methods of taxonomy. For other considerations, the reader may want to consult the other document as it becomes available.

Horizontal gene transfer has an important effect on both phenotypic and molecular approaches to taxonomy. In the first case, it reduces the stability of expression of phenotypic features, because they may be gained or lost from the genome by this phenomenon. In conjugation, some plasmids are freely self-transmissible, making those genes located on such plasmids relatively unstable for that genome. Even when genes are located on the main replicon (*i.e.*, the "chromosome") of bacteria, they have the potential to be mobilized if associated with certain genomic features. Transposons and insertion sequences (IS elements) can cause genes to move within a genome, including to and from plasmids. Thus transposition has the potential to expedite transfer *via* a two step process; first within the bacterium, from a portion of the genome on the main replicon to a location on a smaller, often less stable, genetic element such as a plasmid, and next from one bacterium to another if the plasmid itself is transferred. Transduction may also mobilize traits. This is generally a single step process whereby a viral genetic element captures a gene or genes and allows those genes to move out of the bacterium *via* a subsequent "defective" viral infection of an alternate host bacterium. This may sometimes result in modification of an analogous part of the recipient chromosome, but it may be a random insertion in the recipient genome, depending on the type of tranducing phage involved in the transfer. Transduction's importance for taxonomy has not yet been fully evaluated.

To examine the potential for gene transfer within a species, one can consider similarities for specific features among closely related near neighbors of that species. One way to do this is to determine if there is a feature which, if it were to be found in the subject bacterium, would cause concern. If there is substantial experimental evidence to show that the feature of concern is never known to occur in any member of the taxonomic grouping to which closely related species belong, such as the group of fluorescent *Pseudomonas* species in the example above, that would generally be an indication that there may be a barrier to transfer of that feature to, or maintenance in, those taxa. This would suggest that the subject bacterium may also have limited capability to acquire the feature in question *via* gene transfer by natural mechanisms. Conversely, if the feature is present in related taxa, then it follows that one would expect that such barriers are not so effective, and the feature could be mobilized to the subject bacterium in a natural setting.

In the second case, molecular methods as described in Section II often are based on the use of a small portion of the genome that includes relatively conserved, usually lineally inherited, housekeeping genes.

Results from these methods may reflect the similarities of those components only, while not reflecting other relatedness among taxa due to a common source of horizontally, rather than lineally, distributed genes. This may not be important for certain taxa, especially those with single large replicons and small amounts of genomic material found in plasmids. However, some bacteria of importance to biotechnology, such as certain *Burkholderia* and some legume symbionts, have multiple large replicons with substantial genetic material apparently derived *via* horizontal transfer (Lessie *et al*., 1996; Galibert *et al*., 2001). Thus, the potential problem of seemingly different evolutionary pathways for different parts of the same genome makes drawing inferences about the total relationships between bacteria sharing those housekeeping genes used in molecular identification methods much less secure than once thought.

3.3.2. Horizontal gene transfer and its effect on evolution of bacteria

The complexities of classification of bacteria, and thus of their identification, are related to the processes of evolution that affect bacterial speciation. One such process is horizontal gene transfer. Horizontal gene transfer is now at the heart of a controversy over the reality of the species concept in Bacteria and Archea. Genomics research has shown that bacteria may be comprised of associations of functional subunits which have evolved from common ancestral metabolic pathways and gene sequences. Over geological time, natural multiple infusions of foreign DNA may have been responsible for the creation of new genera of bacteria, in addition to those that may have developed through mutational drift away from a common source genome. The evolution of bacteria may include acquisition or rearrangement of these components.

The presence of orthologous genes, those with structural similarity and functional relatedness even when found in different taxa, are now used as ways of illustrating evolutionary relationships among sequenced genomes (Eisen, 2000). Specialized characteristics such as those producing insect-toxic protein crystals or dinitrogen fixation give the appearance of having moved from genome to genome horizontally (Chien and Zinder, 1994; Galibert *et al*., 2001). Other, less obvious, features, no doubt have moved also. For details of this issue see the various reports of annotations of whole bacterial genome sequencing such as Bult *et al*. (1996), Galibert *et al*. (2001) and Wood *et al*. (2001).

Doolittle (1999) has argued that gene transfer precludes establishing a universal tree of life and has further indicated his belief (Doolittle, 1999; Huynen, *et al*. 1999) that speciation in Bacteria and Archea is not meaningful in a systematics sense due to the "chimeric" nature of prokaryote genomes. That is, even if one uses a variety of measures to evaluate the whole of a genome, the different components of the genome may have different "histories" and thus are not comparable in a phylogenetic sense. Others counter with their belief that bacterial genomes contain a core of genes not affected by horizontal transfer and thus subject to lineal descent. These genes would provide an anchor to the concept of species. It appears that this controversy will not be settled until many whole bacterial and archeal genomes are fully sequenced and the ancestry of core genes, if they exist, are worked out.

Most of these issues were addressed in a recent conference on the identification of members of the genus *Pseudomonas* (Workshop on Identification Methods for *Pseudomonas,* 1997, http://www.bif.atcc.org/epa_web/). This is a complex genus, which has undergone extensive reorganization in the past two decades. On the basis of molecular methods, it was split into multiple genera. However, even members of the remaining species assigned to this genus cannot all be readily distinguished using current technologies. What is apparent is that some members of this genus, such as the type species, *P. aeruginosa,* provide coherent species by almost any method, including traditional phenotypic ones. Conversely, as illustrated at the beginning of this Section, other species like *P. fluorescens, P. putida, P. tolassii, P. marginalis,* etc. are better viewed as a species complex, with subunits as broad as species level taxonomic units as described for other genera, yet so diverse that the boundaries among these subunits cannot be defined. The size, proportion included in extrachromosomal elements, and plasticity (including transposons and insertion sequences) of the genomes of these species

makes for a nearly impossible task of clear species separation. A polyphasic approach was considered essential for the identification of members of this genus, but even after proper application of such a method, it was concluded that many of the isolates that fall within the species complexes, like *fluorescens* or *stutzeri,* will not be separable into single named species.

Regardless of how this debate over speciation in bacteria is resolved, gene transfer is seen as having the potential to have some influence over the evolution of bacterial taxa, and thus on bacterial classification and identification. Even if Doolittle's proposal is shown to be correct, there will remain pragmatic approaches, such as polyphasic taxonomy, to revealing relationships among groups of organisms we now consider unique taxa.

3.4. Relevance of comparison organisms

Unless a subject micro-organism has been previously released into the environment, pre-release data specifically on a subject micro-organism's behavior after release cannot be obtained directly. Yet such test data on very similar organisms may exist, and some extrapolations may be made from these similar organisms. Hence, in some cases, a comparison micro-organism can be used for assessing the potential risks of a subject micro-organism prior to deliberate release into the environment.

In order that predictive analyses can take place, good identification of both the subject and comparison bacterium is needed. In addition, for the comparison bacterium, there must be additional useful information beyond that otherwise available for review of the subject organism, *i.e.*, the information on the comparison bacterium must provide added value to the assessment of the subject micro-organism. This capability will always be limited by the degree of functional similarity between the two organisms, but it is often the case that close functional and taxonomic relatedness occur together. One should not expect that analyses of a comparison micro-organism will automatically provide direct answers to risk assessment questions about a subject organism. Nevertheless, inferences derived from a comparison's characteristics may then be used to help formulate questions for risk assessment of the subject micro-organism.

Data for comparison bacteria may be acquired directly through testing, or indirectly *via* interpretation of published, or otherwise available, information relevant to the issues of the case at hand. Such issues as toxin production or pathogenicity potential are obvious ones for evaluation. Other examples of data may include experience with field releases under controlled conditions or microcosm studies (*e.g.*, Gagliardi *et al*., 2001) for strains related to the subject organism.

3.4.1. Use of taxonomy as a basis for further inquiries

The concept of using taxonomic relatedness to choose a comparison bacterium as an aid in risk assessment is predicated on the assumption that most of the features of concern for the subject organism are inherited in a common fashion in both the subject and comparison bacterium. If lineal descent controls the inheritance of the key characteristics of one organism, but not the other, extrapolations based on observations of the comparison bacterium will not be as meaningful as they would otherwise be. However, horizontal gene transfer may affect both organisms similarly, in which case assessments based in part on observations of a comparison bacterium would retain their validity.

No matter how the speciation problems for bacteria are resolved the risk assessor will still be faced with the reality that use of comparison organisms will provide only inexact comparisons in most cases. Unless the comparison bacterium chosen is a direct precursor of the subject micro-organism, there will usually be, at best, an approximate mapping of expressed features from the comparison bacterium to the subject.

One often uses comparisons among members of the same genus since these would be expected to share more functions than distantly related taxa. Consider the example of members of closely related

species within the genus *Pseudomonas*. As mentioned earlier, these species are often difficult to separate taxonomically. For example a new isolate that falls somewhere within the "*fluorescens* supercluster" would be expected to share a limited set of features exhibited by all the members of that group, even though it may not be possible to tell within which species or biovar of that group the new isolate belongs. As an illustration from a risk assessment perspective, a new isolate that is identified as a biovar of *P. fluorescens* might merit further inquiry and/or testing, since the subgroup, *P. fluorescens* biovar II, has embedded within it some members previously known as *P. marginalis*, which is a known plant pathogen (Janse *et al*., 1992). Since not all *P. marginalis*-like isolates fit within the boundaries of *P. fluorescens* biovar II, one cannot automatically assume that only biovar II isolates are likely to be pathogenic, nor can one assume that biovar II isolates must be pathogenic, but the direction of inquiry can be focused by knowing that the isolate falls within the "supercluster" and can be enhanced if own can narrow down that information to one of the categorized biovars of *P. fluorescens*.

In a similar way, many members of the fluorescent *Pseudomonas* group share production of biologically active pigments as a feature of their metabolism (Palleroni, 1984). Therefore a new isolate found to closely resemble members of this cluster of bacteria, but not assignable to just one existing taxon, would nevertheless be expected to possess the capability of producing bioactive pigment molecules related to those made by the other similar bacteria in the supercluster.

Sharing of features is not limited to species in the same genus. As indicated above, one of the revelations of modern bacterial systematics is that some features of bacterial genomes may be derived from distantly related species. This may result in some functional characteristics of bacteria being shared among seemingly remotely related taxa. For example, one current method of classification places three important bacterial species previously seen as distantly related, *Sinorhizobium meliloti*, *Agrobacterium tumefaciens* and *Brucella abortus*, in the same *alpha*-2 subclass of the group of Gram-negative organisms included in the Proteobacteria (Stackebrandt *et al*., 1988). The three species represent a nodule symbiont, a plant pathogen and an animal pathogen respectively; three different ecological niches. Yet all three share a common type of feature, namely, host/bacterium interaction.

Furthermore, the plant pathogenic species *Agrobacterium tumefaciens* and the symbiotic species *Sinorhizobium meliloti* share much genomic sequence material located on the largest replicon, usually called the chromosome, of their respective genomes. Wood *et al*. (2001) found these two genomes so similar that they postulated that they were derived from a recent common ancestor. In addition research has revealed that in both species a gene was independently discovered, and uniquely named, that codes for a 1,2-glucan synthetase which was found in each to have some function in host interactions (Inon De Iannino *et al*., 1998). The respective genes from each species were found to complement defective versions in one of the other species. Apparently a related module of carbohydrate synthesis has been conserved in these related species, even though they are found in distinct genera. Evolution has allowed each to vary the genes which specify the type of host with which they interact, but the production of certain carbohydrates was apparently retained by each as a component of the interactions.

Other examples of features that appear across a wide range of genera considered distantly related by some measures, but which might be closely related by other criteria, include complex metabolic functions important to biotechnology exemplified by the nitrogenase complex genes or aromatic biodegradation genes (Chien and Zinder 1994; Hirsch *et al*., 1995; Harwood and Parales, 1996). How this may happen may be explained by recent developments in genomics, which is the study of an organism's genetic material.

By careful consideration of the relevance of the relatedness between these species, one could conceivably use each as a comparison for the other for those functions known to be shared. To do so, however, requires a sophisticated understanding of the principles of bacterial taxonomy and the limitations of this discipline. Extrapolations from comparisons of distantly related species must only

be undertaken when there is knowledge of specific common functionality that is understood to be derived from a common genetic source.

3.5. Differences in species concepts for different kinds of bacteria

The same criteria for determining what is a species are not applied to all bacteria. Schloter *et al.* (2000) addresses this briefly by illustrating that "inconsistencies in systematics of bacteria arise from the simultaneous application of different species concepts. A serious obstacle to a unified species concept is subjective consideration of practical usefulness for species definition. Particularly in the group of human and animal pathogens, many species are delineated primarily on phenotypic traits such as host range preference and pathogenicity. *Brucella* species, for example, show interspecific DNA relatedness above 98%." It was pointed out that a single species concept (i. e. *Brucella melitensis*) comprising six biovars and respective biotypes for *Brucella* strains, was proposed, but this concept is not accepted in the scientific community working on pathogenic micro-organisms. Several other genera of well known pathogens might also be seen as comprised of a single species, but cannot be reclassified because of the confusion it might cause among public health workers.

Conversely, several genera of well known bacteria have undergone, or are undergoing, a process of ever finer subdivision, rather than consolidation, that is resulting in frequent classification and nomenclature changes. *Burkholderia cepacia* was once considered a single species but recently (Vandamme, *et al*., 1997) underwent the first of many revisions that resulted in designation of many new species level taxa (Coenye, LiPuma *et al*., 2001; Coenye, Mahenthiralingam *et al*., 2001) called genomovars. The frequency of such changes in classification are very difficult to keep up with and could result in assignment of an outmoded species name, based on current nomenclature, to a subject organism during risk assessment. It could mean that different authorities assessing the same bacteria at different times might use different names for the same object, thus defeating the use of taxonomy in providing a common term of reference for subject organisms.

Summary

Using bacterial identification in risk assessment in an inexact science. It requires significant interpretive work by an assessor. There are constraints on the use of taxonomic identification methods in support of risk due to limits specific to the methods chosen, horizontal gene transfer and its affect on evolution of bacteria, variation in species concepts for different kinds of bacteria, inexact comparisons resulting from use of comparable organisms, and the overall ability to relate specific risk issues to identification of a bacterium.

4. Considerations when substantiating a taxonomic designation

4.1. General considerations

Experienced risk assessors have successfully used bacterial taxonomy as an aid in assessment for many years. Nonetheless, the use of taxonomic identification methods in support of risk assessments may be constrained for many reasons. Some of those constraints are imposed by limits specific to the methods chosen. For each method, the strengths and weaknesses must be considered, as some will work well for certain isolates but not others. From this perspective, the purpose of identification must always be kept at the forefront, whether it is to provide a common focus of discourse and information exchange regarding a subject bacterium, or to help choose a comparison for a test micro-organism and, from observations for that comparison bacterium, devise an appropriate question set for developing a risk assessment for the subject organism. Therefore, identifications of micro-organisms are conducted on a case-by-case basis, applying the most appropriate methods for the subject organism.

The previous section provided some illustrations of scientific questions that indicate the need for caution in the use of taxonomic data for risk assessment. While the issues presented suggest that interpretation of taxonomic data for use in risk assessment is not trivial, they nevertheless should not be construed as preventing its use. On the contrary, such information can be very useful provided that care is taken in the generation and use of relevant data. This section is meant to provide guidance on how to identify an unknown micro-organism by indicating the type of information useful in determining an identification, and describing general and specific considerations in generating and applying the data.

4.1.1. Inherent methodological limitations

For many taxa, phenotypic numeric taxonomic (NT) methods have proved especially helpful. However, there are significant problems in its application to bacteria. Numeric taxonomic approaches that include only phenotypic methods sometimes do not provide adequate resolution for species level classification. Since numeric taxonomy often uses a large set of often independently expressed features for developing a taxonomy for bacterial groupings, it is generally not the size of the test set that is the problem with phenotypic NT. Rather, the problems often lie in the requirement of dependable expression of each test under comparable conditions.

There are several reasons why expression of such features may not be dependable. Laboratory variability can lead to unreliable expression. This is illustrated by one study in which split samples from the human periodontal pocket, identified by the same laboratory using immunologic probes and traditional pure culture techniques, yielded levels of agreement between 0% and 81% (M. I.. Krichevsky, personal communication). Hence, because taxonomies are based on test systems devised by individual researchers, other laboratories that choose to replicate the test systems must do so exactly or risk observing different responses than expected. Slight differences in variables such as media composition, temperature, inoculum size and incubation time may result in opposite test results from seemingly identical cultures. Similar, but non-identical test batteries may be used to do comparisons. All of these variables may lead to different results for the same set of strains done at different laboratories.

There may be other reasons that lead to variable test results. Some features may simply not be expressed all the time in certain strains, even when observational variables are kept constant. Thus, variable responses can occur even when the same tests are done by the same technician in the same laboratory. Regulation of the pathways in some strains of bacteria may be unpredictable, possibly because regulatory elements are defective or are controlled by intracellular variables not known to researchers. Usually reliable features may, therefore, vary even if the structural genes for the features are fully functional. It is commonly noted that long term maintenance of cultures can lead to loss of features that are fully functional in new isolates of the same strain. An understanding of why these test responses vary requires an understanding of the biochemical basis for gene function. Unfortunately, such specific genetic research has been performed for a limited number of key pathways in a small set of bacteria. For these reasons, better methods for classifying bacteria that do not rely on potentially unpredictable expression of genes have been sought.

Molecular methods may avoid the problems of phenotypic methods since they have the advantage of directly analyzing sequences, rather than relying on potentially variable gene expression. These methods, such as those that employ housekeeping gene sequencing, including 16S rDNA/DNA or *gyr*B provide a potential for development of phylogenetic taxonomies. However, the molecular methods, by being limited to comparison of a few conserved genes, generally do not provide as broad a comparison as rigorous phenotypic methods. While such molecular approaches can give an insight into the evolutionary relationships existing among micro-organisms, especially bacteria, they really represent the evolution of specific, relatively invariant, stretches of the genome in the different taxa studied.

4.1.2. Interpretation of molecular data

Molecular data, such as gene sequencing and Southern blot analysis using gene probes, can provide information regarding the presence or absence of a particular gene. However, such analysis does not necessarily provide the complete picture with respect to the expression of such genes and hence of the phenotypic characteristic of the organism, *i.e.*, the presence of a gene does not necessarily mean that it is expressed. In recent studies, a number of *B. cereus* hemolytic and non-hemolytic enterotoxin genes were found in non-cereus *Bacillus* species, but these were not expressed (Hansen and Hendriksen, 2000; Rivera *et al*., 2000; P. Gillevet, personal communication 2002). Concluding a taxonomic designation of these non-*cereus* isolates in this situation would be incorrect if based solely on molecular data. Therefore, data such as this should be interpreted with caution by risk assessors. This is particularly important where the expression of structural proteins are encoded by a series of genes (*e.g.* operons, pathogenicity islands). In this instance, the utilization of a probe for one gene in, for example, the operon, may not necessarily mean that the other genes in the operon are present in the genome or that the gene is even transcribed.

The potential limitations of molecular analyses can be mitigated by using follow-up methods, such as reverse transcriptase polymerase chain reaction (RT-PCR) to determine if the gene is transcribed. If RNA transcripts can be detected, the potential for expression of functional formed proteins could be investigated by immunologic probing to determine if the protein is synthesized and cytotoxicity assays to determine if the protein is functional.

4.2. Pragmatic considerations

4.2.1. Dealing with uncertainty

Reasonable efforts to obtain a taxonomic designation for a subject micro-organism should use methods appropriate for the organism. For risk assessment purposes, taxonomic designations should minimally be to the species level, and should follow international codes of nomenclature and standard taxonomic sources where they exist. The objective is to ensure that the subject micro-organism cannot be confused with a member of a different species, especially with relevance to ones having undesirable attributes.

However, providing a species name for a subject micro-organism, while preferred, is not absolutely essential for risk assessment, provided there exist close taxonomic relatives that have been well characterized. For various reasons, even when very sophisticated methods are used, a species-level assignment may not always be achievable. This usually manifests itself when an isolate is incompletely classifiable because it is shown to be very close to the boundaries defining two named species, but not close enough to deserve either name. This knowledge generally suggests that there is a reasonable probability that some characteristics of the two nearby species may also be found in the isolate, but in a combination unique to the isolate.

In these cases, a designation at the lowest level permissible (usually genus or subgenus) is needed. Often, the isolate in such cases may belong to a "species complex", which concept, while generally reflecting the reality of systematic proximity, has no nomenclature standing in bacterial taxonomy. Nevertheless, identification to the level of such groupings, by showing relationships to closely related species, can still be useful in the risk assessment of the micro-organism. As previously discussed, information on functional properties can be implied from knowledge of approximate taxonomic placement, and be useful in predicting potential risks of an unknown micro-organism, if similar properties are found in comparison organisms.

4.2.2. Using appropriate rigor in performing identifications

While it may be economically tempting to use a simple, often automated, approach to identify an unknown micro-organism to the species level, the limitations of the methods (see above and Section III) make it very difficult to use the simplest of methods and still obtain a reliable identification. One must carefully consider and understand the basis for choosing one identification over another, including the limitations of an automated system, in order to ensure that appropriate designations are chosen.

Over the years, reviewer experience has shown that automated systems using phenotypic methods infrequently provide useful identifications for risk assessments of subject organisms derived from environmental isolates. Such methods are dependent upon the strength of the computer database on which the statistics of these methods are based. Even using the most rigorous phenotypic methods, many environmental isolates fall outside of well defined taxa. This problem seems to be amplified when the identification methods are of the automatic variety. These methods are useful, nevertheless, in "range funding", *i.e.* providing an initial indication of the plausible taxonomic neighborhood to which an isolate may belong. Obtaining a single name for a subject bacterium using these methods is not readily accomplished, except for certain specialized sets of taxa (*e.g.* clinically important bacteria) for which the databases behind the methods are most robust.

Simple, automated methods may work for some species, provided the species is one for which the method has an adequately robust database and the strains to be identified are typical of the species. The API 20E strip, for example, is still considered the "gold standard" commercial system for the identification of species in the family *Enterobacteriaceae* (O'Hara *et al*. 1992). Some, however, question even the values of these tests for certain micro-organisms. Given the substantial increase in new described taxa since 1975 (Euzeby, 1997), Janda and Abbott (2002) make the point that many new taxa added to existing commercial databases are based on the results of tests from pre-configured panels, even though the best tests available to identify these new isolates are not on the panels or are not amendable to automated commercial kits.

Frequently, however, organisms used in biotechnology are not in that category and the simple-to-use automated identification systems may not be adequate. Difficulties most often arise when the subject organism is an environmental isolate with no apparent connection to the more common clinically important species, which often dominate the population of the database used in automated systems. Many of the species used in bioremediation, for example, are members of complex genera (*e.g.* fluorescent pseudomonads) in which member species are notoriously difficult to separate and identify. As noted previously in Section IV, for these types of bacteria, some automated methods provide apparently positive species identifications when in fact equivocal results are more appropriate.

Even more commonly, these methods provide equivocal results, but the presentation of the results by commercial suppliers of this information may lead users to misinterpret them as giving unique positive identifications. Often the results of an identification will be a list of several taxa, not all of which need be closely related to each other. Many automated identification systems will often list the most probable identifications in rank order, with the most probable at the top. The difficulty arises when attempting to rely on the "preferred" name that is provided by such systems. Simply choosing the top name is inappropriate since many systems will list only those names that are contained in the system database. It is possible that in some cases, other more similar species have never been tested using the systems method, and thus have no relevant data contained in the database used.

For example, a method report might indicate that an unknown tested micro-organism has a 70% probability of being species "X", and a 50% probability of being species "Y", while the organism is in fact a species "Z". If it turns out that species "Z" is unknown to the database used for the methods of statistical analyses, choosing "X" would be wrong in this case. Simply because there is a strong

separation between the most probable and next most probable does not require that one must choose from the list supplied. Rather, one should be sure that the identification was made in an absolute sense as well as a relative one. Users are generally better served by beginning with the list provided and obtain additional data that can be used to discriminate among the candidate bacterial names listed, rather than to choose the most probable name in the list and assume it is correct.

Therefore, such identifications should be examined carefully, particularly when more than one possible species name is designated for a single culture. In general, if methods used are unable to resolve the identification of an unknown, it is better to provide more than one possible identification than to arbitrarily choose one name from a list of options.

Unless rigorous analyses needed for publishing a new species has been done, however, one should be cautious before declaring an unknown isolate to be a new species. When those who perform identifications do not show strong confidence in a name resulting from an identification of a subject organism, even after extensive follow-up test as suggested by Steffen (1998), it is better to consider all the options revealed by the testing, even those which point to the lack of an existing taxon, and the probabilities associated with those options, than to choose just one.

4.2.3. Who does an identification?

From a practical standpoint, the identification of a genetically modified micro- organism may be affected by the manner in which precursor organisms are obtained and identified. There are three basic ways in which bacteria are obtained and characterized prior to genetic modification: 1) from a service culture collection, 2) from a research culture collection, or 3) from original isolation from a native source. There are considerations which vary among these modes that could affect the confidence in the name assigned to the precursor organism and thus the identification of the modified micro-organism.

Most service collections, whose primary function is generally to store and distribute cultures, usually for a fee, perform extensive characterizations of their cultures. Those who construct modified micro-organisms starting with cultures obtained from such collections usually select cultures with most of the features desired in the final micro-organism and proceed to make stepwise modifications of the precursor. This procedure most often guarantees a significant degree of characterization of the final micro-organism that includes information provided by the service collection and acquired by the developer. The identification of the final organism is usually dependent on the work of the culture collection respective of the supplied culture. Most such identifications are of high quality. Questions may arise for cultures that have been in a collection for many decades and were originally characterized by methods that currently might be construed as less reliable, but many collections are going back to their older materials and re-characterizing the cultures using modern techniques. Many “older” cultures are also characterized by modern techniques when new batches are made for “replenishing” isolates for which there are no more cultures left in stock.

In some ways, research collections can be even more reliable than service collections for some micro-organisms. That is because researchers working with a very few taxa may have done more extensive characterization for each culture than a service collection with thousands of cultures. Sometimes a research collection will use the most sophisticated methods for identifying cultures in which they have an interest, methods that may not be generally available to service collections. However, some of those who maintain a research collection of cultures do so for other than taxonomic purposes. Such research collections may not be as interested in the taxonomic status of their cultures as the performance of the cultures with respect to the investigative interests of the researcher. In these cases, original isolates may be identified by less sophisticated means, to obtain a convenient label, even though other components of characterization are highly sophisticated. Thus the focus of research, and its effects on the methods of taxonomic characterization of cultures in the collection, needs to be taken into account when obtaining micro-organisms to be modified for use in biotechnology.

When micro-organisms are obtained from an original habitat, identification can be obtained in two ways. Some will send a culture to an organization that performs identification of unknowns as a service. Such organizations include service culture collections, research collections or commercial companies using automated identification methods. Others will perform self-characterizations with facilities that they maintain themselves. Such facilities may range from sophisticated methods and equipment equivalent to any in a research or service collection to simple commercial kits for automated analyses.

For the person performing a risk assessment on a micro-organism there are some concerns that must be dealt with in order to evaluate the reliability of a name assigned to a micro-organism used in biotechnology. It is usually appropriate to accept identifications performed by service collections because of their experience and the need for them to provide accurate information to customers about cultures in their collections. However, as mentioned earlier, older holdings in the collection may not have had the benefit of characterization using sophisticated modern methods. This does not necessarily put into question an identification, since many species are as readily identified by older methods rigorously applied as by newer techniques, but some caution is in order for such cases. Good documentation by a service collection should help dispel any such concerns.

Similarly, research collections that specialize in classification of taxa of interest also should provide identifications that need not be questioned. Research collections that do not publish on the systematics of their cultures may use appropriate techniques nevertheless, but it might be helpful to inquire about the methods used to verify this.

Identifications done in-house by an applicant or under contract may need to be scrutinized closely before organism names are accepted. Some commercial services provide fine identifications for some taxa and questionable ones for others. Those using automated systems are dependent on the quality of databases used during the identification procedures. Some of these databases are excellent for a few taxa, but have only a few examples of others. Years of experience by some authorities who have performed risk assessments on GMMs used in biotechnology has revealed that environmental isolates identified by these automated methods are often mis-named or left un-named due to limitations of these databases.

4.2.4. What is the "best" approach?

Experience with all of the above mentioned techniques reveals that no single method is perfect for all taxa and all levels of taxonomic hierarchy (Janda and Abbott, 2002). It is true, however, that great strides in applying molecular methods have been made in the past few decades. When regulatory evaluation of biotechnology products was relatively new in the 1980's, few laboratories were equipped to perform ribosomal nucleic acid analyses. Now rDNA assays are nearly routine. Chemotaxonomic methods such as FAME are also more commonplace. These advances permit the application of more sophisticated analytical methods to the problems of bacterial identification.

Nonetheless, the sophistication of these methods, alone, is insufficient to make bacterial identification trivial in most cases for biotechnology product micro-organisms. The scientific reasons for this have been cited in earlier sections. The best advice that one can use in choosing methods is to have the work performed by those familiar with the presumptive genus of the bacterium and who are prepared, if needed, to perform methods in combination, "polyphasic" methods, to resolve identification problems.

Some years ago Palleroni attempted to illustrate which methods were best for certain purposes (Palleroni, 1993), but an analysis of this work reveals that each method has its limitations and that it usually is best to combine methods in a polyphasic approach. However, there seems to be no single polyphasic taxonomy methodology that is best for all bacteria. Steffen (1998) reported on the approach used by the German Collection of Micro-organisms and Cell Cultures (DSMZ). An extensive set of procedures was outlined. It was reported that for a reliable identification result most bacteria require simultaneously performed identification methods combined with secondary, and in most cases some

tertiary, biochemical tests. By experience of the DSMZ Identification Service, a combination of primary, secondary and tertiary biochemical tests, one or two partly automated commercially available test systems (API, BIOLOG), and the sequencing of the 16S rDNA bacterial gene usually leads to the affiliation of an isolate to a certain species. However, Steffen stated that due to the fact that different organisms or different taxonomic groups have been studied and classified by a wide variety of methods, standardization of identifications cannot be currently achieved. One may have to deviate from a rigid scheme intended for most bacteria. An attempt to devise an alternate scheme only for the genus *Pseudomonas* was described earlier (ATCC, 1997). It was suggested that initial efforts using 16S rDNA and GC-FAME methods often provided a reasonable range-finding for the initial part of an identification scheme. Participants at the workshop that developed this scheme acknowledged that for certain complex species in that genus, even this pragmatic approach might not suffice.

Apparently, as previously mentioned, gene transfer has a role to play in this uncertainty. The significance of the effects of gene transfer on bacterial evolution is that many taxonomies are based on the inheritance of a few stable, rarely mobilized characteristics. Many of these are essential functions, called housekeeping genes. While use of methods that employ these genes, such as 16s rDNA, certainly provide strong evidence for understanding how the main portion of a genome has evolved, this approach might have reduced utility for risk assessment if the features of concern for a subject micro-organism are not part of that main portion and are not primarily inherited in a lineal fashion. A reviewer, therefore, would want to know if, and to what degree, horizontal gene transfer is known to have affected members of the genus of a subject organism before selecting a method for identification of the bacterium to the species level. This is true especially if the methodological options include some that depend on an element of part of the genome affected by this phenomenon.

In short, there is no “best” method. Those who need to identify their cultures must be aware of the advantages and limitations of each type of approach with respect to the presumptive classification of the strain in question. That usually means an initial round of basic “range-finding” tests that rapidly narrow the identification to a few genera, followed by selection of a method that is most likely to give a useful answer. Useful, in this context, may mean a single, species name, arrived at with great confidence, or it could mean an approximation, entailing a choice of several species known to be related in a “complex”, but not always distinguishable from each other. Knowledge that the unknown belongs within the complex may impart enough information to complete a risk assessment, even if the exact species name cannot be determined. The choice of method may be dependent on how precise an identification needs to be for assessment purposes. For example, a method that allows an approximate placement within a complex, where all members of the complex are innocuous or beneficial bacteria, may be sufficient, depending on the intended use of the organism. On the other hand, even one that provides a unique species name may not be enough, if subspecies or individual strains of a named species differ significantly in potential for detrimental effects, such as pathogenicity.

4.2.5. Data needs for the reviewer

The following describes some information that risk assessors find helpful when substantiates the taxonomic designation of a bacterium:

1. Tests and Databases
    - list of tests used to arrive at the taxonomic designation, and a brief description of test conditions, when such conditions are not established as standard for the methods used.
    - data from the tests used to arrive at the taxonomic designation; and second choice
    - any database against which the test data was compared

- other test data to differentiate the notified micro-organism from close relatives and/or pathogens

2. Molecular identification (modified from the Points to Consider for Identifying a Pseudomonad, ATCC 1997 - http://www.bif.atcc.org/epa_web/)

   a) 16S rRNA

      - the method used
      - the sequence used to determine the isolate was in the concluded genus and/or species and the basis for comparison
      - the measure of similarity and the value obtained

   b) DNA homologies

      - description of sequences used (*e.g.* homologous sequences, coding sequences)
      - the method used (*e.g.* PCR, RFLP, fingerprinting, DNA/DNA hybridization)
      - the homology results that led to the conclusion

3. Phenotypic tests

   a) Morphological

      - cellular (*e.g.* shape, Gram stain, size of bacterium, spore production/morphology)
      - colonial (*e.g*, shape, colour, surface texture, margin)

   b) Physiological

      - growth conditions (*e.g.* temperature, type of media, pH, oxygen requirement)
      - metabolic products

   c) Metabolic

      - biochemical reactions (*e.g.* catalase and oxidase activity)
      - substrate utilization (*e.g.* glucose, sucrose, formic acid, lactic acid)

4. Chemotaxonomic tests

      - cellular components (*e.g.* fatty acids, polyamines)
      - cell surface components (*e.g.* antigens, lipopolysaccharides, cell wall components)

4.2.6. Interpretation of "positive" identifications

Not only is risk assessment predictive in nature, it is also an art and often times involves the informed judgement of the risk assessor. The current state of knowledge for some micro-organisms, however, sometimes makes it difficult to make a determination of risk despite the positive identification of the micro-organism.

A case in point here is the *Burkholderia cepacia* complex (Bcc), which is comprised of more than seven distinct genomovars (phenotypically similar but genotypically distinct organisms) [Vandamme, *et al*., 1997; Coenye, *et al*., 2001 (a,b)]. Some members of this complex have beneficial biotechnological applications (environmental isolates) while others cause adverse human health effects, sometimes death, in patients with cystic fibrosis (clinical isolates). These predominant isolates are generally assigned to specific genomovars (*e.g.*, Genomovars I and IV are predominantly environmental strains whereas Genomovars II and III are predominantly clinical strains); however, all genomovars have been found in

clinical settings. Hence, the positive identification of a particular genomovar does not necessarily preclude the micro-organism from being one of clinical importance. Furthermore, a number of virulence factors and markers have been identified with clinical strains but these factors and markers are not present in all clinical strains. Hence, the absence of a virulence factor or marker also does not necessarily preclude the micro-organism from one of being clinical importance.

The risk assessor must therefore take particular caution in the interpretation of these results. Further information in this case, such as testing in appropriate and validated animal models for pathogenicity endpoints, is essential to aid the risk assessor in characterizing risk. It becomes evident, then, that the information requirements required under various legislation, regulations and/or guidelines are critical for the characterization of a micro-organism, and strengthens the fact that identification is a critical element in the risk assessment but not the only element.

4.2.7. Issues for selecting comparison micro-organisms

GMMS present a special case for bacterial identification during pre-release phases because data may not have been yet gathered on the subject micro-organism that best describes that organism, especially when introduced outside the laboratory. This necessitates use of a closely related comparison organism to acquire relevant data. One should choose a comparison bacterium that is expected to most closely mimic the behaviour or characteristics of the subject micro-organism. For GMMs, this comparison bacterium ideally and most likely will be a direct precursor of the subject micro-organism (*i.e.*, the naturally occurring parental organism of the genetically modified micro-organism). Provided proper use of systematics is employed, taxonomic relatedness may then be used as a selection criterion for obtaining a comparison bacterium other than a direct precursor.

It must always be understood that taxonomic similarity is not an exact equivalent to functional similarity (Achenbach and Coates, 2000). Useful information does not necessarily have to come from the closest relative of the subject organism. The farther one gets taxonomically from the subject organism, usually, the lower the confidence that features relevant to risk assessment of the subject organism will be present in the comparison bacterium.

4.2.8. Post-release issues

Use of taxonomic data and its application to selection of comparison bacteria may also be used to help with selection of monitoring and testing methods, should further work on the subject organism be deemed necessary. If the intended use or environmental testing of a micro-organism being readied for release is such that dispersal away from the site of application is a concern, use of test data previously obtained from a comparison bacterium can help in the selection of monitoring methods, test site design and mitigation strategies for dealing with undesirable outcomes. For example, if it is known that a closely related micro-organism has high mobility and potential for dispersal from an application site, then placement of sampling devices can be adjusted to ensure capture of mobilized subject bacteria, which would be expected to behave similarly to its close relative. Knowledge of such things as heat, drying or oxygen tolerances may help establish where, when and, how to sample for the released organism. The presence of resistant forms such as spores, or the observed viable-but-not-culturable (VBNC) state in a comparison bacterium would lead the investigator to prepare for longer and more specialized monitoring regimes than otherwise might be planned. That is, methods that require culturing of micro-organisms would fail to detect a released bacterium that had entered a VNBC state, but certain molecular methods, not needing cultivation of the bacteria, might be able to do so long after the other methods could not. Thus, an extended monitoring period and the use of methods not dependent on culturing, would be called for if it were known that such features were present in the gene pool of the subject organism. Many of these features might not be observed under laboratory testing schemes used to prepare a subject organism for risk review.

Summary

Interpretation of taxonomic data for use in risk assessment is not trivial, but the complexities should not be construed as preventing its use. This section provided both general and specific guidance on generating and applying relevant data. In general one should use methods appropriate for the organism with the objective of ensuring that the subject micro-organism cannot be confused with a member of a different taxon. Limitations of the techniques make it very difficult to use the simplest of methods and still obtain a reliable identification. In cases where the desired species-level assignment may not be achievable, a designation to the lowest level permissible (usually genus or subgenus) is needed. A list of specific considerations for risk assessors, in consideration of the adequacy of data for bacterial identification, was provided in this section. Finally, a practical concern is locating a person or organisation to perform identifications. Factors in making such a decision were described.

5. Issues for the future

Much has changed since regulation of bacteria produced through biotechnology began in the 1980's. Techniques for modifying organisms have improved and the knowledge and experience needed to perform risk assessments of these new bacteria have improved significantly as well. Specifically for this document, knowledge of bacterial taxonomy has improved dramatically, especially within the last decade. However, this improved knowledge has not necessarily made classification and identification of bacteria easier. Some of what the new knowledge has revealed is that it has been easier, in the past, to misclassify and misidentify bacteria through use of techniques that were not as accurate and useful as presumed at the time of their application. The new knowledge has not always brought us closer to understanding speciation in bacteria.

This is not to say that the use of current concepts of bacterial taxonomy is not appropriate when applied to risk assessment. Rather, it means that risk assessors need to be very cautious in taking taxonomic information at face value and to extrapolate with great care. Methods that give precise and unequivocal identifications for some genera of bacteria exist, but for many genera of current interest in biotechnology, only approximations of species assignments can be made with any security.

Because most genetically modified micro-organisms cannot reveal their full potential until release to the environment, and because some testing to observe characteristics relevant to risk assessment also cannot be done until releases take place, any mechanism for anticipating what those characteristics might be and ways of planning to observe them after release requires predictive information that often can only be obtained by observing related bacteria under conditions of interest. Unless some way exists for the use of information on related organisms, this presents a difficult situation for the developer of a new biotechnology bacterial strain - one can't test without release, but one can't get permission to release without testing first.

Selection of an appropriate comparison organism for which data exists requires some way of predicting relatedness. Taxonomic relatedness, while acknowledged as an imperfect predictor, is still a useful indicator. To make better use out of taxonomic information, however, several advances will be needed in our knowledge.

5.1. Understanding the nature of speciation in bacteria

The current debate over what constitutes a bacteria species and even whether a species is definable for prokaryotes, needs to be advanced. Specifically, there needs to be a resolution of the issues regarding the existence of a "core" genome in bacteria. If such cores exist, as revealed by whole genome sequencing and comparative genomics, there is hope for developing new molecular techniques that allow for taxonomies to be based upon the core genomes. It also will help in understanding the role of

horizontal gene transfer in bacterial speciation, possibly reducing the confusing effect of this phenomenon on bacteria systematics.

5.2. Relating taxonomic standing with risk related features

Although taxonomically closely related bacteria are presumed to be related in a more general sense, this is not necessarily the case. Some means needs to be devised that can illustrate when taxonomically related organisms are also similar in their risk potential. When phenotypic information is used to develop taxonomies, it would be helpful to understand if these same features have any bearing on risk. Some such features, such as growth at mammalian body temperature or versatility in use of carbon substrates, may be shown to reflect survival potential in certain environments. There is a need to systematically review these identification characteristics, correlate them with environmental parameters and/or known effects of bacteria on hosts, and develop a scheme to better utilize this information in risk assessment.

5.3. Use of genomics

Some of the issues above may be resolvable through the use of genomics. Genomics is an emerging tool for use during risk assessments of micro-organisms. Whole genome sequencing and the use of sophisticated bioinformatics techniques may enable reviewers to begin to answer questions that have been problematic up until now. For taxonomy, comparisons of whole genomes for multiple examples of related bacteria may overcome the impediments to classification imposed by standard identification methods. Early use of genomics has, for example, shown how specific portions of some genomes must have been derived *via* horizontal, *versus* lineal, inheritance (Garcia-Vallve *et al*., 2000).

The number of microbial genomes is expanding rapidly. About sixty microbial genomes were published by early 2002 and about 175 genomes were undergoing sequencing at that time (TIGR Microbial Database; http://www.tigr.org/tdb/mdb/mdbinprogress.html). Several of these included species from the same genus and strains from the same species, allowing for comparative genomics to take place. From a microbial systematics perspective, once sufficient comparisons are completed it may be possible, for some taxa at least, to determine what may be the "core" genome that is shared by related taxa and thus stabilize some of the rapidly evolving classification schemes. Should this be successful, the next step of developing molecular probes for these "cores" is already within the capabilities of current technology. Thus, identification could, in future, be linked directly to the genome of a micro-organism *via* a specific molecular probe for a unique taxon, or group of taxa, rather than through the use of indirect measurements of indicator features, as is now the case.

There needs to be restraint to the enthusiasm for genomics, however. Given the way taxonomy may be used in risk assessment as a predictive tool to assist in the absence of direct measurement of microbial function during pre-release phases of review, it is important to understand that genomics only can describe the genetic potential of a micro-organism. It is evident that gene expression is highly complex in organisms and that the regulatory networks even of bacteria may be so large and redundant that describing the genetic potential of a bacterium through genomics may not be sufficient to predict post-release functioning of the organism. What genomics may be able to do directly is reveal the absence of a genetic potential for a particular function and provide a measure of assurance if that particular feature is of concern for risk.

This potential is emphasized by a recent report (Stahl and Teidje, 2002) issued by the American Academy of Microbiology, which is comprised of a prestigious set of microbiologists selected for their career accomplishments. A colloquium sponsored by the Academy focused on the effects of advances in genomics on the discipline of microbial ecology. A significant portion of the report dwelled on the importance of microbial systematics to microbial ecology and the influence of advances in genomics on systematics. As the report pointed out:

> "Today, traditional taxonomic concepts *(i.e.*, species, genus, family) do not serve microbial systematics, in which problems of horizontal gene transfer and mechanisms of speciation and evolution are varied and complex. A new framework for taxonomy, one better adapted to genomic information and microbial taxa, needs to be derived. ... (but) taxonomy is far more than an outdated means of classification. It provides a common language for describing microbial forms in the context of a rich literature about their physiology, metabolism, and life history. Molecular phylogenetics has forced us to reevaluate how organisms are related without requiring us to discard traditional taxonomic views....An important feature of sequence-based classification schemes is that they provide a universally applicable and cost-effective method, eliminating much of the ambiguity arising from earlier systems. In addition to providing information about evolution and phylogenetic relationship, sequences will ultimately be mapped to specific phenotypic and ecological characteristics of an organism."

It is especially this last feature of the potential of genomics that provides a tie from taxonomy to risk assessment. The new tools that may elucidate systematic relations among bacteria may simultaneously provide insight into functional relationships. That capability is at the heart of the use of comparison organisms when direct measurement of features is not possible, so that genomics approaches promise to provide significant refinement to methods used to deal with this problem.

In any case the incorporation of genomics into microbial risk assessment is not that far off, with simplistic application of data from the few currently sequenced micro-organisms already being used by some reviewers, though not yet as a taxonomic tool. If the subject micro-organism, or its precursor, has been, fortuitously, the subject of a sequencing project, much speculation about its genetic potential can be eliminated by querying the information published or otherwise available from the genome sequence.

Summary

Knowledge of bacterial taxonomy has improved dramatically, but there is much more to be done. There is an improved ease of classification and identification of bacteria, but new knowledge has highlighted inadequacies in older techniques that may have led to some taxa being misclassified and misidentified in the past. Methods that give precise and unequivocal identifications for some genera of bacteria exist, but for many genera of current interest in biotechnology, only approximations of species assignments can be made with any security. To make better use out of taxonomic information, however, several advances will be needed in our knowledge, such as understanding the nature of speciation in bacteria and relating taxonomic standing with risk related features.

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Section 3.
Guidance document on methods for detection of micro-organisms introduced into the environment: Bacteria

1. Introduction

1.1. Aim and scope

The aim of this Guidance Document is to provide information on the "state-of-the-art" detection methods available for micro-organisms released into the environment. The document is meant to offer guidance to regulators and applicants on how to interpret and evaluate data from scientific studies. It is therefore not an exhaustive list of all detection methods presently available, but a document that discusses the merits and pitfalls of a number of the most current, frequently used methods. However, this can also be helpful for similar discussions on alternative, or newly developed methods.

Although this document focuses on the detection of prokaryotes (bacteria), many of the techniques described are also used or developed for detection of fungi and other eukaryotic micro-organisms. However, detection of micro-organisms belonging to these latter groups may be complicated by specific features (for example, multi-cellular structure, sexual reproduction) and so fall outside the scope of this document.

In fact, the scope of this document is limited to situations where the introduced bacteria have previously been characterised in laboratory studies.

The document is meant to assist in the risk assessment of genetically modified micro-organisms (GMMs or GEMs, genetically engineered micro-organisms) introduced into the environment, or indeed, any strain of bacteria or any other micro-organism that is amenable to the methods and techniques described.

1.2. General background

Due to their metabolic versatility, micro-organisms are present everywhere in the earth's biosphere, playing many vital functions in environmental metabolism, for example, mineralization of organic matter, nitrification, nitrogen fixation, and so on. Because of their versatility, micro-organisms can be used in various geochemical processes, for example, bioremediation and mineral leaching. In addition, many beneficial micro-organisms are being explored for agronomic use in crop protection.

In the future, there may be possibilities to exploit the beneficial properties of micro-organisms in the environment. Genetically engineered strains could be constructed that combine useful traits derived from different sources. However, environmental applications of GMMs have raised concerns about possible hazards to the environment such as that the introduced strains may upset natural balances.

The deliberate introduction of micro-organisms into the environment, and of GMMs, in particular, requires risk assessments based on detailed information about the properties and ecological fate of the GMM in question. These assessments are based on assumptions derived from data from previous studies on the survival of the same or similar organisms in the same or similar environmental conditions.

Following the actual introduction of micro-organisms into the environment, monitoring the fate of these organisms is usually done, both to evaluate the validity of the assumptions made in the risk assessment, and to collect additional data to refine future risk assessments.

An adequate risk assessment requires data on the GMM about survival, dispersal, activity and possible interactions with the indigenous microflora. Recent advances have provided new molecular tools and techniques that can be applied in the risk assessment of GMMs. Both traditional and modern techniques provide researchers with a large variety of methods for marking and detection of micro-organisms in environmental studies, in general, and in risk assessment studies, in particular. Proper review of these studies requires extensive knowledge of the merits and limitations of the various detection methods.

In general, the methods rely on the detection of the heterologous gene(s), or other DNA sequences, or on detection of intrinsic properties of the micro-organism. The specificity of the detection technique depends on the uniqueness of the properties of the particular strain in question. The properties may have been introduced by genetic modification, or they may be the result of traditional mutagenesis and selection; therefore, no specific emphasis will be put on the aspects of genetic modification.

This document does not deal specifically with gene transfer in the environment, but the process is a complicating factor when interpreting the results of detection techniques. The issue will be discussed in this context.

Detection of micro-organisms is different from identification, though both may make use of the same techniques. Identification involves the characterisation of previously unknown strains, whereas detection serves the purpose of monitoring the presence and activity of previously characterised strains.

1.3. Outline of the document

Section 2 presents a discussion of general issues of the detection of micro-organisms. It tries to put the questions around detection methods into perspective, as an aid to the risk assessor.

Section 3 presents detailed discussions of these issues, against the general background of section 2, for individual methods and markers commonly used for detection. The detailed information in section three is cross-referenced to the corresponding paragraphs in section 2 and vice versa.

The reliability of experimental data of environmental studies is dependent on the robustness of the experimental methods. The methods should be properly validated; application of the methods should be subject to quality assurance procedures. Section 4 presents a discussion on these issues.

Section 5 presents information on the importance of quality control and quality assurance of methods used to detect micro-organisms in the environment. Quality control can help to eliminate inter-laboratory variability in test results, allowing a comparison of data from different studies.

In Section 6, examples are presented that show how the interpretation of data on survival of bacteria in the environment is dependent on the detection method that has been used, and how the environmental conditions can affect the results.

1.4. Sources used for the present study

The discussion in section three will focus on the nature and quality of data obtained through different detection methods. Peer-reviewed articles will be used as a source of information, as well as published textbooks. However, it should be noted that, generally, not all of these issues are addressed concomittantly in peer-reviewed literature. Data will be scrutinised as to their quality and relevance.

Special attention will be given to important parameters such as specificity[1], sensitivity[2], reproducibility[3] and repeatability[4]. Literature included in this document does not solely involve field studies, but also studies of microcosm and mesocosm experiments. The latter studies are only included if they contain results that allow extrapolation to environmental situations.

2. Detection of micro-organisms in the environment: Overview of physiology and methods

2.1. Introduction

This section presents an overview of the characteristics of methods for the detection of bacteria introduced into the environment. Section Three presents more detailed information.

The detection of a particular organism in a particular environment requires:

- presence of at least one unique trait or unique nucleotide sequence in the strain that is suitably stable under the physiological conditions set by the environment, and that allows for discrimination of the organism from, in principle, all other organisms present in the sample; and

- a robust detection method that allows a (semi-) quantitative assessment of the trait.

A bacterial strain that is considered for introduction into the environment for some particular use will have been characterised in terms of its general physiology, as well as the specific traits that are needed for the purpose.

This means that a thorough knowledge is available on the growth characteristics and requirements of the strain, as well as its physiological behaviour under laboratory conditions, and possibly concomittantly under micro or mesocosm conditions. At the same time, the strain must have been characterised for traits that can be used for environmental detection.

The traits used for detection may be indigenous to the strain, or they may be acquired from any form of mutation or exchange of genetic information or deliberate genetic modification. As the specificity and sensitivity of the detection depends on the presence of the same trait in the receiving environment before the introduction, a survey must have been made before the introduction of the bacteria, to assess the presence of micro-organisms with the same trait.

Detection can have different goals. It may aim at detection of the number of 'live' viable, bacteria present, or the detection of some specific environmental activity of the bacteria. Here mainly techniques for the enumeration of bacteria will be discussed. But as many of these techniques depend on specific metabolic activity of the bacteria, the problems of assessing such activity will also be covered.

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1 Specificity is defined in general as the capacity to specifically recognize the target organism, distinguishing it from similar non-target organisms. Specificity of a diagnostic test is the probability not to detect a target organism (negative response) in non-infected or non-contaminated test material.

2 Sensitivity is defined in general as the capacity to record small variations in concentration of a target organism in the test material. Sensitivity of a diagnostic test is the probability of detecting a target organism (positive response) in an infected or contaminated test material.

3 Reproducibility is defined as the difference between two single test results with the same method on identical samples under different conditions (*e.g.* different laboratories, different operators, different equipment).

4 Repeatability is defined as the difference between two single test results with the same method on identical samples under the same conditions.

After introduction into the environment, the strain will no longer be under controlled conditions. This may affect its physiology in many ways, changing expression patterns, and possibly even diminishing our ability to isolate it from the environment.

The interpretation of detection results, therefore, requires some understanding of the interplay between the organism, its physiological state, the trait, and the detection method.

This section presents an overview of factors that influence this interplay, as a background for the more detailed description of specific traits and methods in the following sections.

2.2. Physiological and other states of micro-organisms introduced into the environment

The physiological and other states (*e.g.* lysed cells, cell debris) of micro-organisms introduced into the environment determine what techniques can be applied for detection (see Table 1). In general, readily culturable cells introduced into the environment can enter into different states, relevant to detection: viable/culturable; viable but not culturable (due to different mechanisms); ghosts/lysed cells; and cell debris.

2.2.1. Overview of states

Viable/culturable – 'Viability' indicates the ability of an organism to grow; culturability is the ability to grow in a certain growth medium under defined conditions of atmosphere and temperature. For all detection methods that require growth, viability of the organism under the conditions of culture is essential. Optimal culturing conditions in the lab may not be the optimal conditions for retrieval of viable organisms from the environment. Only those organisms will be observed that can adapt from the environmental conditions to culture conditions in the laboratory. Adaptation may occur only after a certain lag time; the number of viable organisms observed may therefore increase with the length of the observation period.

Viable but not culturable (VBNC) - It is now recognised that a large proportion of micro-organisms in the environment are in a physiological state where they may be viable or metabolically active without cell growth. This not only applies to indigenous organisms for which suitable growth conditions have not (yet) been established, but also to micro-organisms that have been cultured in the lab and subsequently intoduced into the environment. The latter may lose their culturability, requiring special media to recover them from environmental conditions. If this is not recognised, the detection method will overlook these bacteria.

Viable, metabolically active organisms will not manifest their complete capabilities all of the time. Conditions in the environment will influence the expression pattern of many genes. Moreover, it has recently been realised that the expression pattern may also be influenced by the complex interactions with other organisms in the environment. Active organisms in the environment may therefore 'look' very different from the same organisms under laboratory conditions.

Ghosts/lysed cells - Immediately after cell death some cells may still possess an intact cell wall. Eventually, the cell wall will lose its integrity (or loss of integrity results in cell death), resulting in empty cell envelopes without cytoplasm. These 'ghosts' will be recognised by methods that rely on markers that are present in or on the cell envelope. The markers that are present on the ghost depends on the physiological activity of the cell before death, and the possible instability of the marker, for example, due to degradation after cell death.

Cell debris and constituents - After lysis of the cell, complex biological molecules may remain present and recognisable by detection techniques for some time. Proteins, RNA and DNA are generally not stable in the environment, but marker molecules bound to soil particles may escape degradation, and

remain biologically intact and available for detection. Some proteins (for example, bacterial Bt pro-toxins and the crystal proteins of baculoviruses) are especially stable in the environment.

In general, the environmental stability of macromolecular compounds is not well understood, and it should be kept in mind that on the one hand, ghosts may lose their distinctive markers, while on the other hand, cell debris may remain intact for some time after cell death.

Enzymes may remain active in ghosts and cell debris. However, enzymes that require cofactors for activity will not be active for long, as the biosynthesis of cofactors like ATP and NAD(H)P require metabolic activity.

Table 1. Methods which can be used to detect micro-organisms which might be present in the environment in various states

	Detection Method				
State	Cultivation based	*gfp* marked	Immuno-based	RNA-based	DNA-based
Viable/culturable	+	+	+	+	+
VBNC	-	+	+	+	+
Ghost/Lysed Cell	-	-	+	-	-
Cell Debris	-	-	-	-	+

+ indicates suitable for detection, - indicates not suitable for detection

2.3. Detection methods

This section deals with the general characteristics of detection methods and the requirements which pose on the physiology and traits of the bacteria that are to be detected.

2.3.1. Methods based on bacterial growth under laboratory conditions

2.3.1.1. Direct plating

Methods typically rely on growth of the organism from a single cell to a visible colony on solid medium. The time required to form a visible colony depends on the growth rate of the micro-organism, which is influenced by intrinsic factors (*e.g.* physiological status) and external parameters (for example, temperature, availability of nutrients). Usually colonies are observed by the naked eye, but by the use of a microscope the visibility of (small) colonies can be enhanced.

Type of information - Numbers of organisms can be assessed by counting the number of colonies derived from a known volume of a known dilution of a sample.

If a selective medium can be employed that only allows for growth of the organism, detection can be straightforward by looking at the number of colonies. If other organisms can also grow, there must be a possibility to recognise the desired organism against this background. In that case, the conditions of growth must allow for 'election' of the organism, based on some specific reaction with the culture medium (for example, substrate conversion due to β-galactosidase activity).

As a spin off, the direct plating methods for single colonies of the organisms can result in pure cultures that can be used for further characterisation.

Physiological requirements - The method only assesses cells that are culturable, and remain so under the conditions of sampling, plating and growth (culture medium, temperature) in the laboratory.

Requirement for marker traits - Depending on the number of other organisms present in the samples, capable of growth under the chosen conditions, it will be necessary to have a selective or, at least, an elective trait in the organism.

Technical requirements - Special techniques may be required to detach organisms from particles in the sample, which is one of the critical steps for reliability of the method.

Speed, sensitivity, reliability - Time required depends on the growth rate of the organism under the conditions of plating.

Sensitivity is typically in the order of 10^2 bacteria per gram soil; growth and recognition of bacteria at low dilutions of the sample may be hindered by a large background of other organisms that also grow under the conditions of plating. Even if plating is carried out under selective conditions (for example, in the presence of antibiotics) unexpected background growth may occur.

The reliability of the method is limited by the impossibility of retrieving all organisms that are viable in the environment as culturable organisms under laboratory conditions. This is not always recognised in environmental studies, which usually assume that plating methods retrieve all viable organisms present in the environment. The first requirement, suitable growth conditions for the bacterial strain in the laboratory are known from the previous characterisation of the strain.

The conditions during sampling and the switch to growth conditions in the laboratory may put extensive physiological stress on the bacteria. Even the sudden dilution of cells from environmental conditions to a situation where they are single and separated from other cells may impose stress on the cells. For some bacterial species this phenomenon has been shown to be related to the loss of signal molecules that play a role in quorum sensing. The term 'quorum sensing' is used to describe the phenomenon whereby the accumulation of signalling molecules enable a single cell to sense the number of bacteria (cell density). In the natural environment, there are many different bacteria living together which use various classes of signalling molecules. As they employ different “languages” they cannot necessarily communicate with all other bacteria. Quorum sensing enables bacteria to co-ordinate their behaviour. As environmental conditions often change rapidly, bacteria need to respond quickly in order to survive. These responses include adaptation to availability of nutrients, defence against other micro-organisms which may compete for the same nutrients and the avoidance of toxic compounds potentially dangerous to the bacteria.

2.3.1.2. Most probable number methods

Most probable number methods are based on detecting the presence of a particular organism in dilutions of a sample. Samples are diluted to concentrations at which there is a high probability that a relatively large number of samples do not contain the organism. The most probable number of bacteria in the original sample can be inferred by statistical methods, from the number of samples in a dilution that contain zero organisms.

In practice, the organisms in the diluted sample are allowed to grow, and the presence of the organism that is to be detected is assayed by some biological effect that is specific to the organism, for example, an enzyme reaction.

Type of information - Similar to direct plating. The method does not, however, yield single, pure colonies for further study and characterisation (still, these could be obtained by sub-culturing from the samples after growth).

Physiological requirements - Similar to direct plating.

Requirements for marker traits - This method requires growth and a unique biological activity of the strain, by which it can be assayed.

Technical requirements - This method requires a homogeneous distribution of the bacteria, and therefore, relies on the detachment of organisms from particles in the sample. Special technical facilities may be

required in particular systems, to assay the biological effect (*e.g.* production of a specific metabolite) by which the presence of the organism is recognised.

Speed, sensitivity and reliability - Dependent on the biological effect, and the way this is measured, the method may require more extensive growth than direct plating methods, and will therefore take longer.

The sensitivity is comparable to direct plating techniques; this is, however, dependent on the sensitivity of the measurement of the biological effect by which the presence of the organism is recognised.

The reliability depends, in the first place, on the soundness of the assessment of the biological effect. The reliability further depends on the experimental approach chosen for the determination of the most probable number, *i.e*. depends on the dilution steps and the number of samples analysed per step. It is generally felt that most probable number determinations are less reliable than results from direct counting.

2.3.2. Detection by assessment of marker gene products

These methods rely on the determination of the product of a marker gene, which is specific to the detected organism.

The gene product may be detected based on its enzyme activity, or on the basis of its physico-chemical properties, for example, a green fluorescent protein that can be detected by its fluorescence when illuminated by light of a suitable wavelength.

Type of information - From the data one can calculate, in principle, the number of protein molecules present in the sample. If it can be assumed the organisms are homogeneous as to their content of the protein, the method is applicable for the assay of relative numbers of bacteria.

In situ detection of the presence of the protein is possible, in principle. It should be kept in mind that data obtained by these methods only show the presence of an intact protein, not necessarily of an active organism.

Physiological requirements - The marker gene must be (or have been) actively transcribed and translated. If the protein needs cofactors for its activity, these must be available for *in situ* measurement. Gene expression can be influenced by environmental conditions. It should be kept in mind that gene expression may be under the control of either constitutive or inducible promoters. Also more global regulation processes may also affect the level of gene expression. It cannot be assumed a priori that gene expression will be the same, for instance, in culturable cells and viable but not culturable cells. The time span during which the gene product can be assayed after its production varies with the stability of the protein under environmental conditions. Gene products that are inserted into the cell envelope may be assayed on ghosts. The presence of proteins that are stable enough to be detectable in cell debris may cause a background noise which interferes with the detection of cells.

Requirements for marker traits - The gene of interest must be present in a stable form in the genome; gene expression must be stable under environmental conditions.

Technical requirements - Extraction of enzymes should be performed under conditions where loss of enzyme activity due to inactivation is minimised. Assay of enzyme activity *in vitro* is usually straightforward.

Assessment of protein activity *in situ* may require specialised equipment. Cofactors or specific substrates may have to be made available during the assay; if the proteins are assayed in whole cells, cell envelopes may have to be made permeable for these substances.

Speed, sensitivity and reliability - The assessments can be done very fast, or even in real time. Preparation of samples may however be time consuming.

The sensitivity strongly depends on the marker gene product and the detection method used.

Reliability is dependent on the reliability of expression and stability of the gene products.

2.3.3. DNA and RNA hybridisation methods

These methods usually rely on the assessment of DNA sequences of a marker gene (although intrinsic markers present in entire genomic DNA may be used as well), that is specific for the detected organism, or of RNA transcribed from the sequence. The provisos mentioned for detection by assessment of marker gene products apply in a similar way to this section.

2.3.3.1. Direct detection by hybridisation

Detection methods are based on hybridisation of probes, marked or tagged for detection, to a specific target sequence. The target sequence is extracted from the sample and immobilised for the hybridisation reaction. Hybridisation may also be performed *in situ*.

Detection of the hybridised probes may be performed on the basis of fluorescence or a chromogenic enzyme reaction, or on radioactive labelling.

Assays of this type usually allow qualitative conclusions: the sequence is either present or absent. Quantitative results may be obtained through the use of extensive controls which are run along with the assay.

Type of information - The presence or absence of the DNA or RNA molecules is determined, but with the proper controls the quantity of target molecules can be calculated. In principle, the number of organisms present in the sample can be calculated if the copy number (for DNA) or the number of molecules per cell (for RNA) is known.

It should be kept in mind that if data obtained by these methods detects the presence of a target DNA sequence, this does not necessarily reflect the presence of an active organism. However, the presence of RNA can serve as an indicator of active cells.

As RNA is usually less stable than DNA, methods based on RNA targets rely on active transcription of the sequence. The regulatory status of the cell will influence the abundance of RNA in a similar way as it influences abundance of proteins.

Physiological requirements - The results indicate the presence in a sample of a sequence, irrespective of whether the sequence is derived from a viable or active organism, or from inactivated organisms or cell debris (lysed cells and ghosts have the status of cell debris in this discussion).

Requirements for marker traits - Marker traits should be unique for the strain in question. The most frequently used intrinsic marker genes, that is, genes coding for ribosomal RNA (rRNA), do not necessarily meet this condition, as they are species specific rather than strain specific. These sequences can only be used if the abundance of strains of the same species in the receiving environment is low. On the other hand, as the concentration of rRNA is very high, at least in active cells, and as rRNA carries species-specific sequences, rRNA is the target of choice in many environmental studies.

Technical requirements - Preparation of probes, if done by oligonucleotide synthesis, requires specialised equipment and sufficient know-how.

DNA and RNA samples for hybridisation do not have to meet very stringent quality criteria. Extensive fragmentation of the material should be prevented, especially when the sequences are separated by gel electrophoresis before hybridisation. Fragments that are heterologous in size will form smears in electrophoresis, which are less easily visualised by hybridisation methods.

Speed, sensitivity and reliability - These methods are relatively time consuming, because of the sample preparation, and because of the time needed for hybridisation and visualisation.

Sensitivity is usually low and is dependent on the number of target sequences present in the cell. If the copy number is high, as is the case for rRNA for instance, the sensitivity for the detection of cells can be quite high. The speed of the procedure and the sensitivity can be enhanced by use of special equipment. In-situ background noise may be a problem with some environmental samples due to autofluorescence. This requires the proper selection fluorophore for different samples

Reliability depends on the specificity of the hybridisation. This is very much influenced by the stringency of the conditions for hybridisation.

Quantitative reliability is low; usually in order of magnitude of the amount of target sequence can be determined.

2.3.3.2. Detection after amplification of DNA or RNA sequences

These methods allow for a much more sensitive assay of specific DNA or RNA sequences by the use of an amplification step by means of a polymerase chain reaction (PCR).

The PCR may be carried out qualitatively, in which case only the presence or absence of a sequence can be tested. Quantitative PCR methods have been developed, and allow for quantitative assays. The method is, however, much more sensitive, as in principle one target molecule can be detected.

Type of information - Similar to direct hybridisation methods (2.3.3.1), but the assay is more sensitive.

Physiological requirements - Similar to direct hybridisation methods (2.3.3.1).

Requirements for marker traits - Similar to direct hybridisation methods (2.3.3.1), but in this case the chosen sequence should additionally contain short sequences on both 3' and 5' side, which can be used as unique primers for the PCR reaction.

Technical requirements - Sample preparation should yield DNA or RNA preparations that do not contain inhibitors of the PCR reaction. This turns out to be difficult and time consuming, especially for preparations from soil samples. A thorough check for the quality of the preparation should always be carried out. Presently various commercial soil DNA extraction kits are available, which claim to extract high quality DNA. Although these are quick and easy to use, they may not yield a satisfying DNA preparation for all soil types.

PCR requires equipment that is no longer very specialised; quantitative PCR, however, still requires special equipment and specific know-how.

Speed, sensitivity and reliability - Speed and reliability are similar to direct hybridisation methods (2.3.3.1), except in the case of real-time PCR, which can be very fast. To obtain reliable results a dedicated laboratory is required, to prevent false positive results. Sensitivity can be very high; as little as one or a few DNA or RNA molecules as the lower detection limit.

Table 2 Overview of considerations on technical requirements, speed, sensitivity and reliability for detection methods addressed in Section 2.3

	Techincal requirements	Speed	Sensitivity	Reliability
Direct plating	Detachment of organisms from particles in sample	Dependent on growth characteristics of detected organisms	Typically 10^2 bacteria per gr soil	Limited by retrieval of organisms in a state viable under laboratory conditions
MPN methods	Detachment of organisms from particles in sample. Recognizable trait.	May require more extensive growth than direct plating	Dependent on sensitivity of measurement of biological effect; comparable to direct plating	Dependent on reliability of assessment of biological effect, and on statistical validity of experimental design
Detection of gene products	Extraction of enzyme without loss of activity	Typically fast (within hours) or real time	High	Dependent on reliabilty of expression and stability of the gene product
DNA/RNA hybridization, direct	Prepration of probes requires equipment and know-how	Time consuming due to sample preparation and time required for hybridization	Dependent on specificity of probes and stringency of hybridization conditions	Dependent on specificity of probes and stringency of hybridization conditions; quantitative reliability is low
DNA/RNA hybridization after amplification	Samples should be free of PCR inhibitors	Time consuming due to sample preparation and time required for hybridization	Very high, up to a few DNA or RNA molecules	Dependent on experimental conditions to avoid false positives

3. Detection of micro-organisms in the environment: Details of methods

The aim of this section is to provide a more detailed description of the most commonly used techniques to enumerate micro-organisms in environmental samples.

It is by no means the aim of the authors to treat and describe all methods that could be used.

3.1. Traits of the organism which can be used for specific detection

Detection and enumeration of specific micro-organisms is of prime importance for the monitoring of the fate of micro-organisms introduced into the environment (Smit *et al*., 1992). One of the major challenges is the specific detection of a certain micro-organism in all of its physiological states within the tremendously diverse natural microbial community. In order to be able to detect only the species of interest, it is essential that the organism possess at least one trait that can be used to distinguish it from all other micro-organisms. Such traits can be intrinsic, that is, a property that the micro-organism possesses naturally, or it can be introduced by selection, for example, spontaneous antibiotic resistance; alternatively the marker genes may be introduced into the micro-organism through genetic modification.

The nature and properties of the marker gene are very important and determine which detection method(s) should preferentially be used, how sensitive the methods will be and in what way the measured data should be interpreted (Akkermans *et al*., 1998). A marker can either be selective such as antibiotic resistance genes or elective. An elective marker allows an organism to be recognized specifically among other, non-target organisms. Some elective markers can also be used for direct detection and enumeration of cells without the need for cultivation, for instance, by directly measuring the signal, by microscopy or by flow cytometry. Some marker systems can be used to detect metabolically active cells. Although such data are highly informative, this approach might be less suited for enumeration in samples where the signal may be low as a result of starvation.

Currently, quite a range of different elective markers is being used such as *lux*, *luc*, *xyl*E, *gus*, *lac*Z, *gfp* and *celB*. There are several prerequisites for the successful use of these markers: 1) they should be present in a stable condition, in the micro-organism; 2) the marker should be expressed at a detectable level; 3) there should not be a high background in the samples which are studied; and 4) there should be a known relationship between the signal produced and the number of cells, in cases where the marker is to be used for enumeration. In order to facilitate the choice of a marker system and the interpretation of the obtained data, it can be of great help to distinguish different classes of markers, which exhibit similar properties. In this document, five classes of marker genes are described:

1) markers which encode antibiotic or heavy metal resistance and which can only be used in a cultivation based detection method;

2) markers which encode compounds which can be detected directly and which can be used without cultivation;

3) markers encoding enzymes which mediate an enzymatic reaction, requiring the addition of a substrate and which can be used for either direct detection or in a cultivation based approach;

4) markers encoding enzymes which mediate energy dependent reactions and which can be used for both direct detection and detection after cultivation; and

5) specific DNA sequences which can be detected without cultivation.

The use of these markers and the choice of the detection method depend on the purpose of the study. The experimental system and the scientific questions will ultimately determine which markers and which detection methods are most suitable.

Markers of class 1 can only be used in culture based detection methods. Markers of class 2, for example, the genes coding for stable *gfp,* are very suitable for direct enumeration of cells in the environment, while markers of class 3 are generally used as confirmation after cultivation, although they are occasionally used for direct detection. Markers of class 4 are more suited for direct detection of cell activity in the environment to distinguish between active and non-active cells. Class 5 markers allow direct detection and do not depend on expression of the DNA sequence. Finally, it should be noted that most markers could also be used in combination with a number of other, less obvious detection methods, *e.g.* direct detection of an antibiotic resistance gene by PCR amplification.

3.1.1. Markers which confer resistance

Traditionally micro-organisms in a certain environment are detected *via* plating or MPN techniques. For these methods, it is essential that the organisms of interest harbor certain selective traits, or at least, traits that enable them to be recognized. For instance, when certain bacteria in soil lacking any known selective trait are plated onto a non-specific medium, they will form colonies along with a large number of colonies from other micro-organisms. When such organisms harbor an elective marker they can be recognized based on color, size or some other characteristic. However, since many other bacteria are able to grow on these plates, the detection limit will still be between 10^5 and 10^6 cells per gram of soil

because colonies of the bacterium of interest will be completely overgrown when lower dilutions are plated. Therefore, it is almost inevitable to use a selective marker as well to counter select other micro-organisms. Markers such as antibiotic resistance genes and heavy metal resistance genes have been used to selectively cultivate the micro-organism of interest from the environment.

3.1.1.1. Antibiotic resistance

The majority of the selective markers used for the specific detection of bacteria introduced into the environment have been antibiotic resistances (Smit *et al*., 1996b). Important prerequisites for the use of antibiotic resistance as selective marker are: 1) the micro-organism has to be culturable; 2) the resistance should be stable; 3) the environment should be investigated for the presence of high numbers of resistant micro-organisms which will produce background growth; and 4) the antibiotic resistance should be expressed. However, there is a world wide trend to limit the environmental introduction of these genes. The use of these genes as markers for environmental use should therefore not be promoted.

There are two methods to mark bacteria with antibiotic resistance. Cells can be cultured and plated onto antibiotic containing medium to select for spontaneous resistant mutants, or an antibiotic resistance gene can be introduced into the bacterium by traditional genetic exchange or by genetic modification. The use of spontaneous mutants resistant against rifampicin has been successful in many environmental studies (Liang *et al*., 1982; van Elsas *et al*., 1986; Turco *et al*., 1986; Compeau *et al*., 1988; Glandorf *et al*., 1992; Nijhuis *et al*., 1993). The number of antibiotic resistant bacteria that can be detected in soil depends on the type of antibiotic and the type of soil. A certain percentage of the micro-organisms in the environment are naturally resistant to certain antibiotics which results in a background level of CFU's on antibiotic containing plates. Generally, a combination of more than one antibiotic resistance will result in a lower background of resistant colonies of the natural microflora.

3.1.1.2. Heavy metal resistance

Another class of selective markers is resistance to heavy metals. Although the natural background of heavy metal resistance or tolerance in soil seems higher than that of antibiotic resistance, heavy metals have the advantage that they do not interfere with therapeutic use (Mergeay, 1995). Researchers from the lab of Mergeay have developed a heavy metal gene cassette consisting of genes encoding for resistance against cobalt, zinc and cadmium, which allows a very specific detection. Factors that can affect the results when using heavy metal resistance are the choice of the medium, since its components should not form complexes with the metals, and the level of expression in the micro-organism.

3.1.2 Markers which code for compounds which can be detected directly

3.1.2.1. Green fluorescent protein

The green fluorescent protein (*gfp*) gene was originally obtained from the jellyfish *Aequoria victoria* and has been expressed in prokaryotic and eukaryotic cells (Chalfie *et al*., 1994). *Gfp* is a protein that emits green light at 508 nm when excited with blue light of 396 nm. *Gfp* fluorescence is independent of the energy charge of the cell. The product of the wild type gene is very stable and there is no need to add a substrate (Chalfie *et al*., 1994). In an experiment with *gfp* marked pseudomonads in phosphate buffer Cassidy *et al*. (2000) showed that fluorescence was almost constant up to 48 days. The use of *gfp* as marker is particularly suited to monitor cell number by detecting individual cells without cultivation. Detection of marked cells can be achieved by epifluorescence microscopy, laser confocal microscopy, flow cytometry and spectrofluorometry (Tombolini and Jansson, 1998). It is possible to study the location of the cells marked by *gfp* on plant roots by studying samples with confocal laser scanning

microscopy (Normander *et al*., 1999; Bloemberg *et al*., 2000). Because of these advantages *gfp* is chosen as a marker instead of *lacZ* and Xyl*E*.

Recent developments can provide even more information on cell physiology by fusing the *gfp* gene to specific promoters which respond to specific environmental conditions in combination with labeled 16S DNA probes (Moller *et al*., 1998). Using different derivatives of the green fluorescent protein, namely enhanced cyan (*ecfp*), enhanced green (*egfp*), enhanced yellow (*eyfp*) and the red fluorescent protein (*rfp*), it is also now possible to simultaneously visualize different populations of micro-organisms. Bloemberg *et al*. (2000) could visualize and distinguish *ecfp*, *egfp* and *rfp* marked *P. fluorescens* cells in the rhizosphere of tomato plants by triple imaging using a confocal laser scanning microscope with negligible cross talk.

The versatility of the *gfp* system is further enhanced by the possibility of using both stable and unstable *gfp* as reporter genes. Especially unstable *gfp* reporter genes are suitable to measure specific physiological reactions to conditions in the environment since they will remain intact only for a short period (Jansson, 2000). Currently, most research is not focussed on simply detecting the introduced micro-organism; the aim is to investigate bacterial activity in relation to the conditions in the environment. For this purpose reporter genes can be combined with the appropriate promoters, such as those which respond to amino acid, carbon, phosphate or nitrogen starvation (Jansson, 2000).

3.1.3. Markers which mediate enzymatic reactions which require a substrate

3.1.3.1. LacZY[5]

LacZY is an elective marker with a selective component, which can be used in microbial ecology studies. The *lacZ* gene codes for ß-galactosidase which can cleave the chromogenic substrate 5-bromo-4-chloro-3-indolyl-ß-D-galactosidase (X-gal) and change the appearance of the colonies when present in the plates. The selective part consists of the *lacY* gene, which codes for lactose permease, which enables the cell to take up lactose. This is particularly useful for pseudomonads since they are unable to utilize lactose as a carbon source. However, lacZY by itself is not selective enough to prevent the naturally occurring microflora from growing on the plates. Therefore, the *lacZY* marker cassette has been used in combination with one or more antibiotic resistances (Hofte *et al*., 1990; Kluepfel, 1993; Drahos *et al*., 1992; Bailey *et al*., 1995; Nairn and Chanway, 1999). The use of *lacZY* as a marker in microbial ecology has proved very useful in studies on dispersion and survival of bacteria. However, it is mainly used in cultivation based detection systems and it is less suited to be used in direct detection procedures. *LacZ* has also been used in experiments in which plant-bacteria interactions were studied, however its use seems limited because of high background in rhizosphere bacteria (Lambrecht *et al*., 2000). To date *lacZY* is gradually being replaced by the more versatile *lux* or *gfp* marker systems.

3.1.3.2. XylE

The marker gene *xylE* codes for catechol-2,3-dioxygenase (C230), which can cleave catechol. Catechol can be sprayed onto plates with colonies of bacteria marked with *xylE*. In this reaction a yellow substance, 2-hydroxymuconic semialdehyde, is formed which can be seen in colonies on plates. As with *lacZY*, *xylE* is an elective marker, which is mainly used in conjunction with antibiotic resistance, and a cultivation based detection method. There are a number of studies in which *xylE* has been used as a marker to facilitate detection of the introduced cells (De Leij *et al*., 1998; Morgan *et al*., 1989; Winstanley *et al*., 1991; Wipat *et al*., 1991). Currently, *xylE* is not often used as a marker to study bacteria introduced into the environment.

[5] Lactose Fermentation is an example of a metabolic trait that is often used for wild type strains in MPN.

3.1.4. Markers which mediate enzymatic reactions which require energy and substrate addition

3.1.4.1. Bioluminescence

There are currently two bioluminescence genes used as markers, *lux* from the bacteria *Vibrio fisheri* and *luc* from the eukaryotic firefly *Photinus pyralis*. The production of light by *lux* or *luc* is mediated *via* the enzyme luciferase and is energy dependent. For luminometry, both *lux*AB and *luc* marked strains require the addition of a substrate. For *lux*AB, it is necessary to add n-tetradecyl aldehyde, dodecanal or decanal and for *luc* one has to add luciferin. The firefly luciferase is very efficient and has a high yield. Application of *luc*, however, is limited by the fact that the substrate luciferin is quite expensive and it can not permeate through bacterial cell walls. Cells must be permeabilized before the measurement can take place. The *luc* system is therefore recommended for use only when high sensitivity is required (Prosser *et al*., 1996). The whole *lux* operon consists of *lux*R, I, C, D, A, B and E. *Lux* A and B are the structural genes for the luciferase enzyme and *lux* C, D and E are involved in the synthesis of the aldehyde. Bioluminescence is regulated by *lux*I and R. In most cases bacteria are marked using the *lux*AB only and the aldehyde, which is freely permeable, is added to the cells. An important advantage is that light can be measured in real time, sometimes even without extraction or cultivation (Prosser, 1994; Yolcubal *et al*., 2000). Light output seems to be correlated with the amount of ATP in the cells and is therefore an indicator of the energy charge (Maechler *et al*., 1998). A very specific property of light is that it can not accumulate in the cells as a result of former activity, so light output gives an instant impression of the energy status of the cell. When the promoter is down regulated or when the amount of ATP is low, production stops, and the signal disappears. Currently, *lux* and *gfp* are the markers, which are employed most frequently in studies on bacterial activity and survival in the environment. In Table 3 a summary is given of advantages and disadvantages of *gfp* and *lux* in combination with various detection methods.

Table 3 Advantages and disadvantages of *lux* and *gfp* in combination with several detection methods

Marker	Detection Method	Advantage (+) or Disadvantage (-)
Lux	MPN cultivation or plating and bioluminescence measurements	+ suited for enumeration + sensitive; strong signal - combination needed with resistance - detects only culturable cells
	Bioluminescence in extract	- not suited for enumeration + suited to measure activity - not sensitive + detects culturable and non-culturables
Gfp	MPN or plating and detection of fluorescence	+ suited for enumeration + sensitive - combination needed with resistance - detects only culturable cells
	Flow cytometry	+ suited for enumeration + sensitive + detects culturable and non-culturables
	Microscopy of cells fixed on filter or slides	+ suited for enumeration - not sensitive + detects culturable and non-culturables
	In situ confocal laser scanning microscopy	- not suited for enumeration + suited to study localization + detects culturable and non-culturables

There are numerous intrinsic traits which can be used for the selective detection of a certain micro-organism (Tas and Lindstom, 2000). Some examples will be given. Bacilli form spores which will

survive heat treatments of 80ºC for a certain period; such a treatment will kill most other bacteria and can be used prior to plating to specifically detect bacilli. Researchers can also make use of outer membrane properties of a micro-organism to develop specific fluorescence labeled poly or monoclonal antibodies. A very elegant example was developed by Raaijmakers *et al.* (1998), who showed that incorporation in the medium of the siderophore pseudobactin 358 allowed the detection of a specific *Pseudomonas* strain. This specific *Pseudomonas* strain is the only bacterium that can take up iron complexed to this siderophore and it is therefore the only strain able to grow on this medium. Since intrinsic traits, which could be used for detection, can be very diverse and used in combination with a great variety of methods they will not be discussed in this document. However, the utilisation of intrinsic markers or of the added functional genes as markers for detection may make the introduction of extra genes into a GMO redundant.

Direct measurement of bioluminescence can be achieved by: 1) visual detection; 2) photographic or X-ray film; 3) charge coupled device (CCD) camera; 4) optical fibre systems; 5) scintillation counter; and 6) luminometry (Prosser *et al*., 1996). In most samples it is necessary to extract the cells first before accurate measurements can be done.3.1.5 Miscellaneous intrinsic markers

Table 4: Most commonly used marker genes in microbial ecology studies

Marker Gene	Detectable Phenotype	Detection Method	Experimental Sensitivity	Back-ground	Pitfalls and Limitations
Antibiotic Resistance (Km^r, Tet^r)	Growth on antibiotic containing media	Plating or MPN	High	Low	Environmental stress can reduce expression and cultivation
Heavy Metal Resistance (Mer^r)	Growth on heavy metal containing media	Plating or MPN	High	Low	Environmental stress can reduce expression and cultivation
Luminescence (*lux*, *luc*)	Light output	Luminometry	Medium	No	Signal determined by activity in extract*1 Stress might reduce signal
		Fibre optic detection	*2		
Chromogenic (LacZ, Xy1E)	Production of a colored product	Plating and Screening	Medium	No	Reduced culturability and expression
		Product Measurement	Medium	Low	Limited sensitivity
Green fluorescent protein (*gfp*)	Fluorescence in blue light	*In situ* microscopy	Medium	Low	Particles producing background Also signal from less active cells
		Facs sort analysis	High	Low	Optimal calibration is required

*1 Reaction (*luc* or *lux*AB only) may require substrate addition; *2 Insufficient data

3.2. Detection methods based on bacterial growth

3.2.1. Direct plating

3.2.1.1. Type of information

Plating techniques are widely used to enumerate micro-organisms introduced into the environment. The main advantage of the plating technique is that the colonies can be counted and that the strain of interest can subsequently be isolated and studied in more detail using any typing method available. For enumerating introduced strains, plating techniques generally have a relatively low detection limit, they are sensitive and easily performed and are, therefore, the usual method of choice for detecting introduced cells.

3.2.1.2. Physiological requirements

Survival of introduced bacteria is determined by the characteristics of the strain and by the environmental conditions to which the cells are exposed, and although an introduced strain is supposed to be well characterized and the re-isolation medium used is optimized, part of the introduced population might shift into a viable but non-culturable state as a result of the stressful conditions in the environment (Wilson and Lindow, 1994; Heijnen *et al*., 1993, Mahaffee and Kloepper, 1997, Warner and Oliver, 1998). Once introduced into the environment bacterial cells can revert to various physiological states. Cells can be: 1) culturable; 2) viable but non-culturable (VBNC); 3) dead, but still intact; or 4) lysed. The choice of markers, used in combination with specific detection methods, determines which of these various physiological states can be detected, and thus has important implications for interpretation of the results for risk assessment purposes (Table 1 and 4). There are several studies which show that introduced cells can revert to a non-culturable state resulting in lower plate count while cells remain detectable by other methods. Wendt-Potthoff *et al*. (1994) compared PCR mediated detection with plating methods for the detection of a recombinant *Pseudomonas amyloliquefaciens* in the phylosphere and found that while the strain was no longer detectable using plate counts, the genetic marker could still be detected by PCR. Troxler *et al.* (1997) observed a progressive decrease in plate counts from 8 to 2 log CFU/g soil of a *Pseudomonas* strain introduced into soil in the field while the number of cells detected by immunofluorescence was several log units higher. This shift to VBNC cells occurred especially in the surface horizon, where the culturable cell numbers declined to less than 2% of the number of viable cells. Mahaffee and Kloepper (1997) determined CFU counts of *Pseudomonas fluorescens* introduced into soil using a rifampicin containing medium, using plates which were screened for bioluminescent colonies and by immunofluorescent colony staining. Both the selective and elective media showed significantly lower CFU counts, which suggested that the selection in combination with environmental stress reduced culturability of the introduced cells.

Binnerup *et al*. (1993) have observed the formation of microcolonies when they tried to detect *Pseudomonas fluorescens* cells that had been introduced in soil. These microcolonies could be seen by epifluorescence imaging and were formed from target cells that ceased multiplying after a limited number of cell divisions. Whether or not these microcolonies are included in the plate count can make a considerable difference.

3.2.1.3. Requirement for marker traits

To enable the specific detection of an introduced micro-organism one can use a general or a specific medium in combination with a selectable trait of the organism (Van Elsas *et al*., 1986; Compeau *et al*., 1988), or with a genetic marker such as antibiotic or heavy metal resistance and *lacZ* or *lux* (Hofte *et al*., 1990; Kluepfel, 1993). It is also possible to use a combination of growth medium with specific fluorescent antibodies (Van Vuurde, 1990). The most important prerequisite is that the micro-organism

can be detected with a minimal background of other micro-organisms. The traits or markers which are selected for should be stable and expressed in the micro-organism (See 3.1). Chabot *et al*. (1996) studied survival and root colonization of rifampicin resistant mutants of *Rhizobium leguminosarum*, *Enterobacter sp*. and *Pseudomonas sp*. marked by inserted kanamycin resistance (*npt*II) and *lux* genes *via* plate counts. Counts were confirmed by measuring light emission from the colonies. The combination of both antibiotic resistances and the additional elective *lux* marker proved to be sufficient to reduce the background of non-target bacteria.

Huertas *et al.* (1998) used both antibiotic resistance and toluene as the sole carbon source to select for the introduced bacteria to assess the survival of several toluene degrading pseudomonads. This resulted in a detection limit of 100 CFU/g soil.

De Leij *et al*. (1998) studied survival of a *Pseudomonas* strain marked *via* insertion of the *aph-I* gene (kanamycin resistance), the *xylE* gene and the *lacZY* genes. Such a triple marked strain appeared to have a reduced environmental fitness since this strain survived less well than the wild type strain. In combination with antibiotic resistances such as nalidixic acid, rifampicin and kanamycin, the *lacZY* marker was shown to be very sensitive with detection limits ranging from 100 CFU to 25 CFU per gram of soil. Winstanley *et al*. (1991) was able to monitor *Pseudomonas* strains in lake water marked with *xylE* without using antibiotics, whereas Morgan *et al.* (1989) studied survival of *xylE* marked pseudomonads by selective plating on streptomycin and ampicillin containing medium and by using an ELISA-based detection method for the *xylE*-enzyme.

3.2.1.4. Technical requirements

In order to enumerate bacteria in soil, the cells have to be dislodged from soil particles and from each other. This can be accomplished by various methods which are usually based on suspending the soil in a buffer followed by shaking and diluting the sample. Buffers which are commonly used are sodium pyrophosphate (0.1% $Na_4P_2O_7$) or $MgCl_2$ (0.1 M). Soil is usually added to, for instance, Erlenmeyer flasks in a ratio of 1:10 (w/w) and sterile gravel is added to improve dispersal of the cells. Samples are subsequently shaken at, for example, 200 rpm for 10 minutes. Smaller amounts of soil can be added to 50 ml tubes with gravel and vortexed for one minute. Alternatively soil samples in buffer can be added to a stomacher. Studies in which these methods are compared for efficiency and reliability are lacking.

Most micro-organisms which are introduced into soil are, in origin, soil micro-organisms. Usually a specific medium is required to culture them. A wide variety of media are available for enumeration of the total number of culturable bacteria or specific groups of bacteria in soil. The optimal medium and culture temperature has to be determined for each individual species. Generally, a low nutrient medium should be used to culture micro-organisms from environmental samples, since cells introduced into the environment generally experience oligotrophic conditions.

The fungal inhibiting antibiotic cycloheximide is always included in the medium to prevent fungal growth.

3.2.1.5. Speed, sensitivity and reliability

If a low detection limit is required then a detection technique based on cultivation using selective markers such as antibiotic resistances can be combined with an elective marker such as *lux* to rule out any background (Cassidy at el., 2000). However, such an approach will fail to detect non-culturable cells or cells which have lost resistance. Most molecular markers can also be used in a hybridization or PCR based detection set-up which will allow detection of non-culturable cells. The major drawback of such an approach is that DNA from inactive and dead cells will also be detected (Table 1).

The sensitivity of the plate count method is determined by the antibiotic(s) used to select for the introduced bacterium and the percentage of the community that is naturally resistant to this antibiotic.

To obtain optimal sensitivity Liang *et al.* (1982) used three antibiotic resistances that lowered the detection limit to 25 CFU per gram of soil. In practice antibiotic resistance is combined with other markers. To enhance sensitivity multiple antibiotic resistance markers can be used simultaneously (Liang *et al*., 1982). Although the use of resistance has been quite successful, there are several reports of problems. Some rifampicin resistant mutants have been shown to have a reduced competitive ability and a diminished nodulation competitiveness (Compeau *et al*., 1986). It is important to investigate the occurrence of possible negative effects of the marker(s) on the environmental fitness of the micro-organisms before they are actually used.

The speed of the method mainly depends on the time of incubation which the micro-organisms require to form a visible colony. While the cell extraction and plating itself is relatively fast, the incubation period can vary between 2 to 14 days or longer.

The reliability of the plate count method is high and can only be negatively affected by differences in marker expression and culturability of the micro-organism.

3.2.2. Most Probable Number culturing

3.2.2.1. Type of information

MPN (Most Probable Number) is an alternative to plating, which can be applied for enumerating viable cells by culturing. The technique uses serial dilutions of the original sample in liquid culture medium and relies on the principle that only a single cell is needed to produce a population of new cells. Growth is detectable by changes in properties of the medium, *e.g.* a color change. After the incubation period each culture is scored either negative or positive, the latter indicating the presence of at least one cell, able to grow in the medium. From the scores of the different dilutions the most probable number of culturable organisms in the original sample can be calculated by statistical methods (Cochran, 1950; Gerhardt *et al*., 1981; Alexander, 1982). Being based on the growth of cells, the MPN culturing technique shares many of the advantages and disadvantages of direct plating methods.

3.2.2.2. Physiological requirements

Being a culture-based technique, MPN only detects cells that will multiply in the liquid medium. Most studies in which both MPN culturing and direct plating were used, have shown a similar result for both methods (*e.g.* Line *et al*., 2001; Massa *et al*., 2001). However, MPN culturing will not detect those target cells that would form microcolonies when plated on a solid medium (Binnerup *et al*., 1993). Cassidy *et al*. (2000) have compared both methods for detection of *gfp*-marked *Pseudomonas fluorescens* cells in soil. They found that the counts from MPN culturing were significantly lower than those from direct plating, due to the presence of microcolonies on the solid medium.

3.2.2.3. Requirement for marker traits

For MPN culturing, both selective and elective markers can be used, but as with direct plating, the best results can be obtained by a combination of both. If the organism of interest only contains an elective marker it may be overgrown by other organisms present in the sample, resulting in a false negative result.

3.2.2.4. Technical requirements

The result of MPN culturing strongly depends on the success of the procedure to extract the target cells from the environmental samples. Inefficient extraction and clumping of cells will lead to an underestimate of the number of culturable target cells present.

3.2.2.5. Speed, sensitivity and reliability

Being based on growth, the speed of an MPN assay is determined by the ability of the organism to grow in the liquid medium and to visibly change the characteristics of this medium. Especially for slow growing cells, such as auxotrophs, this may result in a very lengthy assay. Ekelund *et al*. (1999) have shown that it is possible to automate the reading of an MPN experiment by using a multi-well microtitre plate format for the incubation of the cultures. They could enumerate phagotrophic protist (protozoa) from soil in a medium containing *lux*-labeled bacteria as the growth medium. Wells were scored positive if the light emission had decreased more than 30% compared to a non-inoculated control.

In general, the MPN culturing technique mostly yields results similar to plate counts, but lower numbers than found by methods that also detect non-culturable cells.

3.3. Detection by assessment of marker gene products

There are quite a number of different methods to detect the various marker gene products from introduced bacteria (see Table 4). The methods which are commonly used, such as luminometry, flow cytometry, fluorescent microscopy and confocal laser scanning microscopy, will be discussed.

3.3.1. Enumeration of micro-organisms by luminometry

Bacteria marked with *lux* or *luc* constructs (See 3.1.4.1) can be enumerated in crude extracts by measuring the amount of light which is produced. In this chapter bioluminescence measurements after a cultivation step are excluded, since that method should be regarded as cultivation based rather than direct luminometry. A possible alternative for luminometry might be *in situ* fibre optic detection (Yolcubal, *et al*., 2000), which will not be discussed separately.

3.3.1.2. Type of information

The method will detect the amount of light that is produced. In order to calculate the number of cells, one has to know the relationship between cell number and light output. Since this relationship is dependent on the energy status of the cell, it is difficult to relate a given signal to cell numbers. Actually luminometry of *lux* marked cells gives information on the energy charge of the cells and should be combined with an enumeration method.

Luminometry of bacteria marked with *lux* or *luc* genes is also very suitable to investigate various conditions in the environment when used in combination with certain promoter sequences that respond to environmental stimuli (Prosser *et al*., 1996; Jansson, 2000).

3.3.1.2. Physiological requirements

The measurement is limited both by the level of expression of the *lux* or *luc* genes by the bacteria and by the number of bacteria in the sample. On the other hand the amount of light produced is a direct measurement of the *in situ* activity of the cells and will yield information on the effect of the environmental conditions on gene expression (Meikle *et al*., 1994).

3.3.1.3. Requirement for marker traits

There are quite a number of different *lux* based marker cassettes which have been used in environmental studies. The original *lux* pathway, which consists of *lux*CDABE with its original promoter, is not useful to conduct environmental studies (De Weger *et al*., 1991). Most work is done using the *lux*AB construct with a constitutive promoter. However, it is necessary to add n-decanal and to provide O_2 (Kragelund *et al*., 1997, Meikle *et al*., 1994) and the amount of light production depends on the metabolic activity of the cell. On the other hand *lux* constructs can be combined with specific

promoters which respond to environmental conditions and can be used a biosensor (Kragelund *et al*. 1997, Hestbjerg-Hansen *et al*., 2001).

3.3.1.4. Technical requirements

A disadvantage of the method can be quenching of light by soil particles which will result in a lower value. This can be overcome by separating the cells from the soil particles. Cells can be separated from soil using a Nycodenz density gradient centrifugation procedure (Unge *et al*., 1999; Elväng *et al*., 2001; Unge and Jansson, 2001). However, it is not known what the effect of this procedure is on the physiology of the bacteria and what percentage of the cells is lost.

3.3.1.5. Speed, sensitivity and reliability

Luminometry on crude or purified environmental samples which contain the luminescent bacteria is relatively straightforward and fast. Sensitivity lies between 10^3 and 10^4 cells, which is adequate in most cases. Reliability depends on the environmental conditions and physiology of the introduced cells. Care should be taken when interpreting results from studies in literature which use *lux* since quite a number of studies employ a culture based approach in their detection scheme (Errampalli *et al*., 1998; Tresse *et al*., 1998; Cassidy *et al*., 2000). In such a scheme, the cells are first cultured in an MPN approach and then light emission is measured. These studies do not take the advantage of the possibility to directly detect the marker, and when these studies claim that the luminometry data give similar results to plate counts this is not surprising. A better approach to fully use the advantages of the marker system is direct detection of the product (Unge *et al*., 1999; Yolcubal *et al*., 2000; Elväng *et al*., 2001).

3.3.2. Enumeration of micro-organism by fluorescent microscopy or confocal laser scanning microscopy

Fluorescent microscopy is a sensitive technique to detect marked or labeled cells. Although microcopy is not particularly suited for cell enumeration the method has been successfully applied for this purpose (Putland and Rivkin, 1999; Unge and Jansson, 2001) The strength of the method is in its use to study the *in situ* localization of cells (Bloemberg *et al*., 2000; Unge and Jansson, 2001).

3.3.3. Enumeration of micro-organisms by flow cytometry

Flow cytometry is a relatively new technique for the enumeration of specific bacterial cells in environmental samples. The technique is very promising and seems optimally suited for reliable and fast enumeration of cells. The current disadvantage is the isolation procedure required to separate cells from soil particles of other debris. Currently, work is in progress to optimize such procedures (Ziglio *et al*., 2002).

3.3.3.1. Type of information

Enumeration without cultivation of micro-organisms in environmental samples can be achieved by flow cytometry (Unge *et al*., 1999; Elväng *et al*., 2001). Flow cytometry is commonly used for the analysis of microbial communities in freshwater and marine samples (Rice *et al*., 1997; Chen *et al*., 2001; Lopez-Amoros *et al*., 1995; Marie *et al*., 1996). Soil samples are more complicated because of the presence of fluorescent particles. To eliminate these, the cells have to be separated from the soil which can be achieved by using a Nycodenz density gradient (Unge *et al*., 1999; Elväng *et al*., 2001). The flow cytometer can detect and enumerate cells with a specific fluorescent signal and with a specific shape or size.

3.3.3.2. Physiological requirements

In order to stain cells with a fluorescent dye there are no clear physiological requirements other than that the cell wall and membrane are permeable to the compound. However, *gfp* marked cells should be metabolically active enough to produce amounts of *gfp* that can be detected. On the other hand, once the *gfp* is produced it is generally quite stable. If, however, its metabolic activity in the environment is the subject of the study, *lux* or *luc* in combination with luminometry is a better choice.

Currently, alternatives for such studies have been developed by using marker genes encoding unstable *gfp* mutants. In reporter gene studies the promoter will start expression of the marker once a specific condition in the environment is met. In this situation, it is important that the product formed is not stable, which means that the signal will disappear quickly once the conditions change.

3.3.3.3. Requirements for marker traits

While in some specific cases cells might be detected which produce an autofluorescent signal, in most cases, the micro-organisms can only be detected if they are stained with a fluorescent dye. One can choose a dye which specifically binds to DNA such as DAPI, Hoechst33342, SYBR Green and SYBR Gold, YOYO or YOPRO, PicoGreen. However, these dyes will stain all micro-organisms and can thus not be used to specifically detect introduced ones. The flow cytometer can also detect cells which are specifically stained by *in situ* hybridization probes (FISH) or which have a marker gene inserted into the genome such as *gfp*. Since the excitation peak of natural *gfp* does not match the standard 488nm laser of the flow cytometer, Tombolini *et al.* (1997) used a red shifted mutant gene which displayed a high and stable fluorescence signal. The *gfp* gene was expressed by a constitutive *psb*A promoter from *Amaranthus hybridus* which resulted in a stable and evenly distributed signal in all *Pseudomonas fluorescens* cells. During the growth phase fluorescent intensity varied. The fluorescence intensity decreased to 30% in exponential phase while it increased again in stationary until the intensity per cell was the same as that of the inoculum (Tombolini *et al*., 1997)

The choice of a specific detection method in combination with a certain marker gene and promoter sequence depends on the aim of the experiment. If researchers want to study the survival of a micro-organism, it is best to use a constitutively expressed, stable *gfp* marker gene which is indicative for the presence of a bacterium and suited to determine cell number by FacsSort analysis.

3.3.3.4. Technical requirements

The main disadvantage of flow cytometers is that they are relatively expensive. Less expensive types are limited by the fact that they will have a laser which can only work at one wavelength, which is usually 488 nm. This limits the use to dyes that can only be excited at this wavelength.

The apparatus is designed in such a way that the micro-organisms are forced to go through a capillary with a fluorescence detection device one by one at high speed. While passing the detector the signal is recorded and, after the run, the data can be visualized in a 2-D plot. In this plot the fluorescence is set against the forward light scatter, which is determined by the shape and size of the cell. It is important to distinguish the cells of interest from other things such as particles or cell debris with autofluorenscence. It is recommended, therefore, to analyze positive controls and blank samples. By analyzing blanks (that is, samples without the cells of interest) one can identify the background. By analyzing dilutions of cultures of the organism of interest one can validate if the region in the plot which gates the cells can be separated from the background. Moreover, by analyzing the dilutions researchers can validate the enumeration of the cells.

3.3.3.5. Speed, sensitivity and reliability

The use of flow cytometry for the enumeration of cells introduced into the environment is fast and reliable if the right procedures and controls are used.

3.3.4. Enumeration by enzymatic measurements

A specific *LacZ* construct has been used as reporter to monitor the starvation and stress response of a *Pseudomonas* strain in soil by an extraction procedure. In this experiment cells were lysed and a ß-galactosidase assay was performed on the crude lysate (Van Overbeek *et al*., 1997). In this study ß-galactosidase activity was measured by chemiluminescence as a measure of metabolic activity (Van Overbeek *et al*., 1997). In a later study, the activity of the introduced *Pseudomonas* could even be determined up to 21 days in a wheat rhizosphere. A prerequisite for such an approach is that the introduced micro-organisms survive well and have a sufficiently high level of expression to allow detection.

3.4. Enumeration of micro-organisms by DNA or RNA analysis

DNA or RNA based detection and enumeration techniques for micro-organisms introduced or present in environmental samples are very important in modern microbial ecology studies. These methods are not hampered by cultivation problems, poor expression of markers or changes in physiology of the bacteria and even enable researchers to detect sequences from formerly unknown groups of micro-organisms. Currently, there are quite a number of different methods for the extraction and purification of nucleic acids from the environment (Holben *et al*., 1988; Smalla *et al*., 1993; Van Elsas *et al*., 2000). Briefly, two different approaches can be discerned, one is based on extraction of microbial cells prior to lysis and the other is based on direct lysis of the cells in the sample. The disadvantage of the methods which are based on cell extraction is the impossibility to recover all cells of the microbial community. The advantage is that once the cells are obtained it is relatively easy to extract and purify their RNA or DNA. Methods based on direct cell lysis in the sample are better suited to cover the whole community. Nevertheless, the co-extraction of contaminants from the samples could give problems in the purification. In order to obtain efficient cell lysis, mechanical based methods such as using a bead beater or ribolyser have been shown to give high yields of DNA (Smalla *et al*., 1993; Van Elsas *et al*., 2000; Borneman *et al*., 1996). Currently, there is a kit on the market (Fast DNA Spin Kit for soil, Bio101) which is specifically for the isolation and purification of DNA from soil which should be used in combination with a ribolyser

3.4.1. Enumeration of micro-organisms by hybridization

Direct hybridization of a probe to DNA or RNA extracted from the environment has been used in the past to enumerate bacteria. However, the use of this approach is limited since it has a high detection limit and the data which are generated give only an indication of cell numbers. Therefore simple hybridization assays, dot blots or Southern blots are not particularly suited for the enumeration of micro-organisms. However, current developments in microarray techniques, which are essentially miniaturized hybridization assays, might yield very powerful tools for future microbial ecology studies.

The most important application of currently used techniques based on hybridization is fluorescence *in situ* hybridization (FISH). This technique is based on hybridizing specific oligonucleotides with a fluorescent label to intact whole cells which can be subsequently visualized using a microscope (Moter and Gobel, 2000). Using this technique, cells of a specific taxonomic group for which the oligonucleotide was designed, can be counted. It is very powerful technique, and the selection of the oligonucleotide is of the utmost importance. The much used general prokaryotic oligo EUB 388 has recently been demonstrated not to cover the whole microbial kingdom (Manz *et al*., 1992; Daims *et al*., 1999). This method is very valuable for studying micro-organisms in the environment (Amman *et al*., 1991;

Ramsing *et al*., 1996; Ludwig *et al*., 1997; Felske *et al*., 1998b). However, it is not used to enumerate introduced cells.

3.4.2. Enumeration of micro-organisms by PCR amplification

3.4.2.1. Type of information

DNA or RNA based methods which do not rely on cultivation, might be better suited to enumerate bacteria which are difficult to culture or which can enter a non-culturable state (Van Elsas *et al.*, 2000). PCR techniques for the detection and enumeration of introduced cells seem to offer good possibilities in studying dynamics of micro-organisms. Since the PCR reaction involves an exponential increase of the target molecule, it can not easily be used for quantification. For this purpose three different PCR strategies have been developed: MPN-PCR (Sykes *et al*., 1992), real-time PCR (Blok *et al*., 1997) and competitive PCR (Gilliland *et al*., 1990).

MPN-PCR is based on a normal PCR amplification of serial dilutions of a DNA extract from a sample. The result of each PCR assay is scored as either positive or negative. Using the same statistics as in other MPN procedures the number of target molecules (reflecting the number of cells) in the initial sample can then be calculated.

The advantage of MPN-PCR is that it is relatively easy to perform and that it does not require expensive equipment. A drawback is that the method is very labour intensive and requires a large number of PCR reactions per sample. Moreover, the suitability of MPN-PCR for the quantification of micro-organisms has been questioned (Hermansson and Lindgren, 2001).

In real-time PCR, the accumulation of the PCR product is monitored during amplification, this is in contrast to normal PCR where the amplicons are only detected at the plateau phase of the reaction. This enables monitoring of the product during the exponential phase of the reaction. This exponential phase is usually limited to a few cycles where the amplification curve is log-linear. This part of the curve can be used to accurately determine the original concentration of the target. The advantages are: 1) an accurate quantification; 2) an increased dynamic range and a low detection limit; 3) no post-PCR manipulation and thus a reduced risk of cross contamination; and 4) a quick, reproducible and less labour intensive procedure. A disadvantage is that the efficiency of the PCR reaction in the standard samples can be different from the efficiencies in the environmental samples, which may contain PCR inhibitory substances. Currently, there are various methods to overcome this problem (Hristova *et al*., 2001; Widada *et al*., 2001). Another practical drawback is that real-time PCR requires expensive equipment and reagents. Presently, several different systems for real-time PCR are commercially available such as those which are fast and flexible and use capillaries such as the "LightCycler" (Roche) and the Rotorgene (Corbett research) and the high throughput machines based on 96 or 384 well plate formats such as the ABI Prism Sequence Detection Systems the "iCycler" (Biorad), the MX4000 Multiplex Quantitative PCR system (Stratagene) and the DNA Engine Opticon (MJ Research).

Competitive PCR is based on the simultaneous amplification of the target and a competitor DNA in a single tube. The competitor molecule differs in size, yielding a slightly smaller or larger PCR product than the target. The PCR products are subsequently separated on an agarose gel and by a comparison of the intensities of both bands the number of copies in the original sample can be calculated (Johnsen *et al*., 1999). The advantage of the procedure is that it does not require expensive equipment and that the competitor DNA is amplified in the same reaction mixture as the target. A disadvantage is that the amount of product is compared in the plateau phase of the PCR reaction, which is less accurate than a measurement in the exponential phase.

Several studies describe the use of quantitative PCR for the enumeration of introduced cells in the environment (Rosado *et al*., 1996; Halier-Soulier *et al*., 1996; Moller and Jansson, 1997; Farelly *et al*., 1995; Felske *et al*., 1998a).

Rosado *et al*. (1996) used MPN-PCR for the enumeration of introduced *Paenibacillus azotofixans* in soil. Results between plate counts and MPN-PCR correlated well; however, after prolonged dry conditions plate counts decreased rapidly while MPN-PCR counts decreased little which eventually lead to a difference of 4 log units which could be indicative of either the presence of non-culturable cells or naked DNA. Van Elsas *et al*. (1997) compared immunofluorescence counts with MPN-PCR of *Mycobacterium chlorophenolicum* in soil. Although cell numbers correlated relatively well, there were 10 fold differences in numbers between IF and PCR counts.

Similarly, Hallier-Soulier *et al*. (1996) did not find a clear correlation between the number of colony forming units (CFU) and competitive PCR. Both Lee *et al*. (1996) and Lechner and Conrad (1997) found a good correlation between competitive PCR results and colony counts from soil. However, the presence of dead cells with their DNA still intact could lead to false enumeration results.

3.4.2.2. Physiological requirements

To enumerate cells by quantitative PCR of DNA, cell physiology does not play a role. However, critics have pointed out that these methods will also detect naked DNA from dead and lysed cells. There is only limited and circumstantial evidence that naked DNA is of minor importance. Rosado *et al.* (1996) found that while introduced cells could be easily quantified by MPN-PCR, similar concentrations of naked DNA added to soil were no longer detectable within three hours after introduction. Coolen and Overmann (1998) detected ancient DNA in lake sediment layers up to 9000 years old; however, they also showed that 99% of the DNA was degraded and only small fragments were present. Therefore, amplifying a specific rRNA sequence by competitive or real-time RT-PCR could be more promising (Felske *et al*., 1998a), since RNA has a short half life and will be degraded rapidly when the organism dies. Moreover, the rRNA content of a cell represents the activity of organism and not just its presence, which might be more important from an ecological point of view.

More basic studies are needed to fully investigate the extent to which the presence of naked DNA or dead cells can influence the PCR mediated detection results of an introduced strain.

3.4.2.3. Requirement for marker traits

The markers or genes which are amplified should be specific for the micro-organism which is to be detected and should allow the development of specific primers for the amplification of a DNA sequence of approximately 1.0 kb in length. For real-time PCR the amplification of DNA sequences smaller than 0.5 kb is recommended.

3.4.2.4. Technical requirements

A robust procedure for the extraction of DNA from soil is a key to efficient PCR detection and quantification. Ideally, all micro-organisms are lysed and the isolated DNA is of high quality, that is, pure and not degraded. The extraction procedure should yield high quality DNA/RNA to ensure optimal PCR amplification. Care should be taken to check the occurrence of reduced amplification efficiency due to inhibitory compounds present in the extract.

A common problem with PCR amplification of DNA extracted from environmental samples is the occurrence of inhibitory substances (Van Elsas *et al*., 1997). Van Elsas *et al*. (1997) found that DNA extracted from different soils required different purification steps. A clay soil from the Netherlands did not require any further purification while an organic rich soil from Finland required three additional purification steps to remove inhibitory substances. Chandler and Brockman (1996) observed PCR

inhibition at most 10^{-1} dilutions in a MPN assay aimed at quantifying the presence of a number of biodegradative genes.

For bacteria it is relatively easy to correlate cell number with quantitative PCR amplification; however, for hyphal fungi it is not straightforward.

When detecting a specific micro-organism with PCR, it is of the utmost importance that the primers are specific and will not amplify other DNAs. Therefore, evidence should be given which proves that the primers will amplify only the target. To increase the specificity one can use a hot start technique or touch-down PCR protocol, that is, the gradual decrease in annealing temperature during amplification.

3.4.2.5. Speed, sensitivity and reliability

Although PCR seems to be an ideal detection method there is little evidence that DNA which is amplified from environmental samples is actually representing living micro-organism. Amann *et al.* (1996) provided evidence that PCR amplified cloned 16S rRNA sequences indeed represented the diversity present in their activated sludge sample. The authors used specific oligonucleotide probes for each clone and could detect all micro-organisms by fluorescent microscopy.

The sensitivity of real-time PCR is higher than normal PCR. Cullen *et al*. (2001) showed that the detection of *Helminthosporum solani* by real-time PCR was as sensitive as a nested PCR in a conventional set-up, since both were able to detect 1.5 spore/g of soil. Generally real-time PCR amplification is more sensitive than normal PCR; on the other hand it can be optimized with higher precision. Mygind *et al*. (2001) were able to detect the equivalent of two copies of the genome of *C. pneumoniae* in their samples. The reproducibility was found to be good, by repeating their assays on the standard curve 10 times. The coefficient of variance was found to range from 1.4% to 3.9% (Mygind *et al*., 2001). Only at the lowest concentration (one copy per µl) 3 out of 10 assays were negative. They also found a good correlation between traditional immunohistochemical analysis and the real-time PCR assays.

A lot of parameters can influence the amplification efficiency in real-time PCR. Wilhelm *et al*. (2000) observed with particular samples and primers, differences in amplification efficiencies which were dependent on the place of the capillary in the rotor. These problems could largely be overcome if the samples were completely denatured before starting the amplification. The dynamic range of real-time PCR is high. Böhm *et al*. (1999) were able to detect the arbuscular mycorrhiza fungus *G. mossae* in quantities ranging from 10^{-8} to 10^{-2} µg DNA per ml.

3.5. Determining cell numbers by immunofluorescence techniques

3.5.1. Type of information

Immunofluorescent techniques can also be used to study introduced cells. Using immunological methods, samples are incubated with a specific antibody to which a fluorescent label is attached and cells can be counted using a microscope. Heijnen *et al*. (1988) studied survival of introduced *R. leguminosarum* using a polyclonal antibody for immunofluorescence microscopic counts and found that cell numbers were higher as from day 15 than plate counts. On day 60 cell numbers determined with IF were half a Log unit higher than those obtained by plate counts, suggesting that part of the introduced population had become refractory to cultivation.

Leeman *et al*. (1995) determined the survival of *P. fluorescens* introduced in the radish rhizosphere in a commercial greenhouse using the immunofluorescence colony staining method of Van Vuurde and Roozen (1990). The disadvantage of this method is that it requires a cultivation step; however, the advantage is that the organisms do not need to be marked. Wiehe *et al*. (1996) used strain specific polyclonal antibodies and a chemiluminescence immunoassay to determine colonization of *Pseudomonas*

on roots of *Lupinus albus* and *Pisum sativum.* The use of fluorescently labeled 16S rDNA probes for the microscopic detection of bacteria in environmental samples is not particularly suited for the detection and enumeration of introduced cells. Problems such as high background signals, autofluorescence of soil particles and relative high detection limits have hampered wide scale use. Immunological techniques are gradually being replaced by the use of specific marker genes that can be detected without cultivation by direct measurement of the protein.

4. Sampling introduced micro-organisms, experimental design and sampling practice

4.1. Introduction

Representative sampling of soils is crucial for assessing the survival and distribution of soil micro-organisms. Soils may be very heterogeneous, depending on intrinsic factors, but also on usage, for example, for various agricultural practices. Depending on the purpose of the study, the sampling strategy should take into account the level of precision (defined as the accuracy with which the real mean value of the parameter being assayed is determined) of the data needed in relation to the commonly observed variability. A thorough treatment of sampling methods and strategies is given by Van Elsas (2002). Over the last few years the European Committee for Standardization (CEN) has produced a number of standards which contain useful guidelines for the design of sampling and monitoring strategies of genetically modified micro-organisms, introduced into the environment (for example, EN-12685: "Biotechnology; Modified organisms for application in the environment; Guidance for the monitoring stategies for deliberate releases of genetically modified micro-organisms, including viruses." and EN-12686: "Biotechnology; Modified organisms for application in the environment; Guidance for the sampling strategies for deliberate releases of genetically modified micro-organisms, including viruses.").

4.2. Experimental design

The design of an experiment and the sampling strategies chosen must be clear, in order to yield data that can be analysed by adequate statistical methods (Green, 1979; Totsche 1995). Various experimental designs have been developed to achieve this. In general, the field site to be tested is divided into blocks, according to a pattern (for example, completely randomised, randomised complete block, randomised incomplete block, latin square, or split plot designs) that serves the specific goals of the experiment (Anon, 1992; Totsche, 1995). Sampling of the soil may be done using various strategies, serving different purposes. It must be clear which strategy has been followed, and for what rationale.

Examples of sampling strategies are:

Judgement samples: non-random samples, taken for specific purposes, for example, isolation of organisms, but not suitable for statistical analysis of soil composition;

Simple random samples: samples are collected randomly over the site to be studied, *e.g.* according to a grid pattern, selecting a random sampling site within each section of the grid. Data from simple random samples can be treated statistically, and are appropriate for purposes such as the characterisation of fields by mean parameter values, variation, and spatial distribution;

Stratified random samples: this sample strategy takes into account the different (*e.g.* physical) properties of a plot. The plot is divided according to these properties, and random samples are taken from each subplot;

Systematic sampling: samples are taken in a non-random fashion, of an entire area. They are useful for systematic characterisation of the spatial variability of a parameter across a whole field or area; and

Composite samples: samples are obtained by bulking and mixing individual samples. In this way the variability of individual samples is reduced. This reduces the variance between samples, but composite

samples can only be compared if they are similarly constructed and if there are no statistical interactions between the sampling units.

4.3. Sampling strategies

From the data of detection assays on a limited number of soil samples, conclusions have to be drawn on the population of micro-organisms present in the entire field site. The statistical methods that can be used to derive an adequate description of the total population from the sample populations depend on the population distribution of the tested micro-organism in the field site. The most commonly used mathematical description of a population, the normal distribution, can only be applied to populations that are randomly distributed. However, populations often are non-randomly distributed over fields. Mathematical methods, for example, log transformation, are available for transformation of data from non-random populations to a form that can be analysed as a normal distribution (Isaacs and Srivastava 1989; McIntosh, 1990; Pielou, 1983, McSpadden and Lilley, 1997).

In practice, the distribution of the population under study often will be unknown. In such cases the most likely spatial distribution of the whole population can be approximated from a limited number of samples, but this approach represents just a first rough attempt at characterising the actual distribution. As it is often assumed that the variations in populations of micro-organisms over a field result in log-normal distributions, a log-normal distribution is commonly taken as most likely. However, this assumption can be challenged. Microbial activity and diversity will be influenced by a number of factors of the field site, for example, its history, topography, type of soil, degree of homogeneity, type and variability of vegetation and slope, and presence of water streams. Field history is important, as management or disturbance will certainly impact microbial activity and diversity. For instance, previous use of a fungicide may leave residues that can impact microbial populations in soil. Cropping history is also important for soil microflora and processes such as nitrogen fixation. Knowledge of the site's topography and surroundings is a key to understanding other possible influences such as *via* water movement along slopes.

The deviation of the sample mean from the actual population mean is dependent on the number of samples analysed. This deviation can be determined by statistical methods (McIntosh, 1990; Wollum, 1994; Lamé and Defize, 1993; McSpadden and Lilley, 1997). Using these statistical methods one can calculate the minimal sample number needed to determine the population size in a field site to a given accuracy, with a given confidence interval.

ISO norm 10381-4-1992 (International Organization for Standardization, Geneva, Switzerland[6]) provides general rules for sampling soil, for example, for microbiological analyses. According to this norm, fields with homogeneous utilization of up to 2 ha (most agricultural practice) are well-sampled with one composite sample composed of 15 subsamples per replicate to yield an average whole field estimate. Homogeneous fields of 2 to 5 ha require two such composite samples, fields of 5 to 10 ha require three, fields of 10 to 20 ha five, and so on.

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[6] The International Organization for Standardization (ISO, Geneva Switzerland) has developed standards for adequate soil sampling, which have been described in a series of ISO norms (ISO/CD 10 381-1-1992: Soil quality - Sampling - Part 1: Guidance on the design of sampling programmes; ISO/CD 10 381-2-1992: Soil quality - Sampling - Part 2: Guidance on sampling techniques; ISO/CD 10 381-3-1992: Soil quality - Sampling - Part 3: Guidance of safety; ISO/CD 10 381-4-1992: Soil quality - Sampling - Part 4: Guidance on the procedure for the investigation of natural and cultivated sites; and ISO/DIS 10 381-6-1992: Soil quality - Sampling - Part 6: Guidance on the collection, handling and storage of soil for the assessment of aerobic microbial processes in the laboratory). These norms can be obtained *via* ISO.

4.4. Soil sampling in practice

The results of soil sampling and analysis will be very much dependent on the practical conditions during these processes. Applicants should provide a detailed and well rationalised description of the approaches and techniques chosen, taking into account the following aspects.

Sample size, sampling apparatus: Sample size will depend on statistical considerations, the purpose of the experiment, and the practical requirements of the assays to be performed. Small (up to 100 g), medium (100 g to several kg) or large sample sizes (over several kg) may be required. Most microbiological, biochemical and soil chemical assays will require small (up to 100 g) to medium (100 g to several kg) size samples. Small to medium size samples can be obtained for each soil horizon by using presterilised tools (hand auger, sample corer, spade, shovel or trowel). Rhizosphere soil and rhizoplane (surface of plant roots) samples are obtained by carefully excavating plants from soil with a sterile shovel or trowel. Roots and other plant parts should be left intact as much as possible so as to avoid introducing sampling artefacts. Sampling depth is defined by the type of soil and the experimental requirements. As examples, the plough layer (0-25 cm deep) is commonly sampled in agricultural soils, whereas in grassland, soils from the most densely rooted layer (0-10 cm) are taken.

Conditions during transport and storage: Changes in humidity and temperature as well as exposure to direct sunlight may influence the number of viable organisms that can be retrieved from samples. Long storage periods should be evaded if possible; however, samples may have to be stored for very long periods, *e.g.* for comparison with samples taken later in time.

Sample processing: Information of the spatial distribution of the organisms in the sample will be lost during sample processing unless special precautions are taken, *e.g.* for *in situ* determinations, or for obtaining specific samples of the rhizosphere. Samples should not be exposed to excessive temperatures or conditions that will cause desiccation. Excessively moist samples may be dried, but not to less than 30% of the water holding capacity. If samples are processed according to a 'logical' pattern, this may result in changes during processing that may be interpreted as influences of other, *e.g.* geographical, parameters.

Recovery of the bacterial fraction from a sample: In order to retrieve the bacterial fraction from a sample, the soil aggregates should be dispersed in a suitable liquid medium, which allows for dislodgement of the micro-organisms from the soil particles, and the bacterial fraction should be purified.

Dispersion and dislodgement: Dispersion is brought about by mechanical means such as shaking, blending, ultrasonic treatment (at energy levels that do not disintegrate bacteria) (Ramsey, 1984; Bakken, 1985; Faegri *et al*., 1977; MacDonald, 1986). Dislodgement may be helped by addition of detergents, *e.g.* sodium deoxycholate and the use of ion exchange resins (Hopkins and O'Donnell, 1992; Jacobsen and Rasmussen, 1992; MacDonald, 1986). The applicant may be able to report on their own experience with specific methods; *e.g.* Van Elsas (2002) reports that sodium pyrophosphate is a good soil dispersing agent that allows for recoveries of total and specific bacteria of the same order of magnitude as estimated in soil based on microscopic cell counts.

Separation and purification of the bacterial fraction; This is usually brought about by low speed centrifugation, which removes soil particles and most fungal hyphae, yielding cleared supernatant containing bacteria. The recovery of bacterial cells depends on the efficiency of their previous dislodgement from the soil, which is in general dependent on the type of soil: separation from a sandy soil is easier than from a clay type soil. The bacterial fraction may be further purified by density gradient centrifugation. This is however laborious, and may lead to loss of micro-organisms. It should be kept in mind that recovery of micro-organisms from soil samples is always only partial. It has been estimated that only up to 30% of microscopically detectable bacteria are often recovered from soil by established methods (Steffan *et al*., 1988). This recovery rate may be acceptable if coupled with sensitive analytical methods such as selective plating for viable counts of specific culturable organisms. However, it may be

inadequate for immunofluorescence or DNA-based methods used for monitoring populations because of the enhanced limit of detection. An exception is PCR (Briglia *et al*., 1996) which by its nature can overcome the reduction in sensitivity; however, the presence of PCR inhibitors in humus rich soils is notorious and this should be taken into account.

5. Validation and quality control

Quality control and assurance is an important part of the analysis and procedures in certain microbiological laboratories. For instance, for laboratories which monitor microbiological quality of food, drinking water and recreational water, there are national and international guidelines and standards to work with. Most of these laboratories also have a quality assurance system which involves both technical assessment of the equipment which is used and the documentation of the samples, procedures and data. The extra costs and working hours are insignificant compared to the severe economic and social implications which could be caused by the measures which have to be taken as result of incorrect test results (Lightfoot and Maier, 1998). Most of the research in microbial ecology is generally performed by high-tech experimental R&D laboratories and most of the methods used are highly experimental. These laboratories usually do not work with a quality assurance system and standard procedures. Most laboratories which perform microbial ecology research use "in house" developed protocols, specific microbial strains and study specific environments. There are no standard procedures for sampling and plating of soil bacteria, or for the isolation and purification of DNA from soil. This makes it impossible to compare studies in terms of reproducibility and to distinguish intra- and inter-laboratory variation from actual ecological effects. While there is quite some knowledge on variation in bacterial enumeration using plate counts in food and water analysis, there are hardly any data on the reproducibility of molecular detection methods.

Detection of micro-organisms in complex samples in different laboratories can yield very different results, due to known differences in materials and procedures. However, in collaborative studies it was found that even when uniform samples are examined, using a standardized laboratory protocol, repeatability may show considerable intra-laboratory variability, while reproducibility may show considerable inter-laboratory variability (for example, Mooijman *et al*., 1992). This is an important finding to bear in mind when results from literature are compared which have been obtained in different laboratories, on different samples and with different methods. Collaborative studies to gather information on the variability of results concerning detection of micro-organisms in microbial ecology are rare. In the fields of food and medical microbiology these studies are more generally available and they indicate that the variability of results can be considerable. For example, a multicenter comparison trial was carried out to examine the detection rate of *Chlamidia pneumoniae* in atherosclerotic lesions by PCR (Apfalter *et al*., 2001). It showed that there was no consistent inter-laboratory pattern of positive results and no correlation between the detection rates and the sensitivity of the assay used. There is no reason to assume that the detection results of micro-organisms in ecological studies are not similarly variable.

Another very important aspect of quality control is to check the identity of the strain which is to be introduced (Smalla *et al*., 2002). DNA based methods for identification such as sequencing of the rDNA gene, or rep-, BOX-, or ERIC-PCR are recommended methods (Smalla *et al*., 2002). The strain should not be repeatedly sub-cultured in the laboratory since this might change its genotype and/or its phenotype and affect its environmental fitness (Lenski, 1991). The number of generations that the strain is cultured should be limited and a large number of stocks should be frozen at -80ºC. Especially in the case of large scale introductions, when extensive culturing of bacteria is required to obtain a sufficient quantity of inoculant, contaminants could easily take over.

6. Examples of the use of different detection methods for studies of the fate and survival of bacteria introduced into the environment

6.1. Environmental conditions affecting the detection of introduced bacteria

The interpretation of data on the quantification of bacterial survival in the environment is not straightfoward. Environmental conditions can and will affect the physiological state of the bacteria, which, as has been discussed, may have strong implications for the outcome of a detection technique used. When different detection techniques are applied to identical samples, the results may differ to an extent that they seem incompatible, untill the influence of the physiological state of the bacteria in the sample is sufficiently taken into account. The relevance of data on bacterial survival in the environment, provided in scientific literature or in an application, to answer a specific question should be judged on a case-by-case basis, taking into account these factors. The examples in this section are meant to illustrate how the interpretation of results depends on the method that was used.

Untill recently information on the survival of micro-organisms in ecosystems was obtained mainly by the use of culture based detection and enumeration methods. These methods have indicated that several bacterial genera, such as *Pseudomonas, Rhizobium, Agrobacterium, Azospirillum, Bacillus, Azotobacter, Xanthomona*s and *Erwinia,* have adapted to growth in the rhizosphere. Knowledge of the interaction between micro-organism and evironment is important since introduction of these micro-organisms into bulk soil will not be successful as they will not survive very well. In soil, the presence of plant roots was shown to be the major factor for survival of *Azospirillum* cells (Bashan et. al., 1995). Similarly *Bacillus megaterium* was shown to increase in number in soybean rhizosphere from Log 6.28 one week after inoculation to Log 7.21 four weeks after inoculation (Liu and Sinclair, 1993). This is typical behavior of bacteria adapted to the rhizosphere. While all reports on the survival of pseudomonads in soil demonstrated that their numbers decline fairly rapidly (Table 5), *P. fluorescens* has been shown to increase in number and survive very well when its host plant was continuously grown in microcosms in monthly cycles (Raaijmakers and Weller, 1998). In the rhizosphere, there could be a continouos succession of different species or specific genotypes, types adapted to a certain growth phase of the roots (Duineveld and van Veen, 1999: Semenov *et al*., 1999). This was shown to occur on leaf surfaces by Rainey *et al*. (1994) and Ellis *et al*. (1999).

6.2 Bacterial characteristics and physiology affecting their survival

Generally bacteria introduced into the environment are subject to stress because of the transition from a pure culture in the laboratory to a harsh oligothrophic environment, which may affect the bacterial physiology and thus the number of cells that can be detected with a given method. This should be kept in mind when evaluating the results of survival studies, obtained with culture methods. The humidity (or matric potential) of the soil is an important parameter for the survival of introduced bacteria (Heijnen *et al*., 1993). The survival of *P. azotofixans* in very dry soil as determined by MPN-PCR was 4 Log units higher than plate count values whereas under normal conditions both methods yielded similar data (Rosado *et al*., 1996). This indicates that cells might enter a non-culturable state when experiencing dry conditions. Rattray *et al*. (1992) concluded from their data that both the activity and viable cell counts were negatively influenced by matric potential stress.

To investigate if the physiological conditions of the cells influence their survival, Masher *et al*. (2000) studied the fate of *P. fluorescens* CHAO in soil, which was incubated prior to inoculation under various stress conditions, by IF counts and plating. IF counts and plate counts were similar up to 12 days in soil with cells which had been incubated in minimal medium prior to inoculation. Differences between total cell numbers and viable counts started to occur at day 26 and at day 54 total counts were one Log unit higher than viable counts. This difference was much more pronounced when the cells had been subjected to oxygen and redox stress prior to inoculation. The amount of viable but non-culturable (VNBC) cells,

assessed by Kogure's cells elongation test (Kogure *et al*., 1979), appeared to be intermediate, which suggests that the total cell counts was made up of culturable, viable and dead cells. *P. fluorescens* CHAO appeared to be tolerant to moderate levels of NaCl concentrations at which other strains such as *P. aeruginosa* and *E. coli* were already affected (Masher *et al*., 2000). Tolerance to high NaCl concentrations is suggested to be an important property for rhizosphere bacteria (Miller and Wood, 1996). These results suggest that biotic factors and the culture conditions before the introduction can affect survival. The change from culturable cells to a viable but non-culturable state did not appear to represent a successful adaptive response to adverse environmental conditions (Masher *et al*., 2000). The relation between the non-culturable state and physiological adaptation to the conditions in soil remains unclear. While studying survival of *E. coli* in freshwater systems Dan *et al*. (1997) found a large discrepancy between plate counts and direct counts. The apparent non-culturable cells were shown to be viable since glucose uptake activity was not impaired. Arana *et al*. (1997) could also detect viable but non-culturable transconjugants in river water. Strains of *Xenorhabdus nematophilus* and *Photorhabdus luminescens* genetically marked with kanamycin resistance and *Xyl*E introduced into river water decreased to undetectable levels after 6 days (Morgan *et al*., 1997). However, in sterile water, evidence was found that the strains remained viable but had become non-culturable and had thus escaped detection by plating. England *et al*. (1995) used both plating techniques and PCR mediated detection methods to study the fate of genetically modified *Pseudomonas aureofaciens* introduced into soil. Results suggested the occurrence of non-culturable cells or the persistence of naked chromosomal DNA in the samples. Kluepfel (1993) was able to detect *lacZY* marked pseudomonads released in the field three months after they became undetectable by plate counts. This suggested the presence of non-culturable genetically modified bacteria, since the extraction method used involved isolation of intact cells first. The meaning of the presence of viable but non-culturable cells, dead cells, or naked DNA for DNA based detection techniques remains largely unsolved and will definitely require further study.

The characteristics and physiology of the bacteria also play a key role for their survival. Based on their physiology, bacterial species can roughly be divided into two ecological groups, r-strategists and K strategists. K-strategists have characteristics which make them better adapted to survive in oligotrophic environments and r-strategists thrive better in nutrient rich environments. The fact that the characteristics of the receiving ecosystem and the characteristics of the introduced strain play a key role for survival was shown by Thompson *et al*. (1990) who compared the fate of an *Arthrobacter* and a *Flavobacterium* species. Both strains were introduced at a level of about Log 7 per gram of soil and while the *Flavobactrium* decreased rapidly in number to below the detection limit in less than 20 days *Arthrobacter* decreased to a level Log 5 at day 50 and remained at that level until the end of the experiment at day 100. The *Flavobacterium* survived much better in the rhizosphere of wheat in which it could be detected up to 50 days. Soil is an oligotrophic environment and a major abiotic factor influencing bacterial survival is nutrient limitation, *Arthrobacter* is apparently adapted to such an environment while *Flavobacterium* is not. Respiration measurements on sterile soil microcosms to which both strains had been added showed that the *Arthrobacter* reduced its respiration rate to a lower level than the *Flavobacterium* (Thompson *et al.*, 1990). Differences in physiology of the strains are supposedly responsible for the different survival characteristics. This is strongly supported by the fact that *Flavobacterium* showed much better survival when it was starved prior to inoculation. It should be noted that all experiments were analyzed by plating techniques, so viable but non-culturable cells could have remained undetected. Van Elsas *et al*. (1986) studied the survival of two different bacterial species *P. fluorescens* and *B. subtilus* in two soils of different texture. *P. fluorescens* decreased more slowly in silt loam than in loamy sand, while *B. subtilus* decreased much more rapidly in both soils until it reached a level at which it survived as spores. Kim *et al*. (1997) demonstrated that cell numbers of an introduced *Bacillus* strain remained relatively stable in wheat rhizosphere while the number of *P. fluorescens* cells gradually declined in numbers. *B. megaterium* was shown to survive for two years after introduction into the field for biocontrol purposes (Liu and Sinclair, 1993).

Table 5: Decline rates of introduced cells calculated as a decrease of cell numbers in Log per week and mean decline rates of different bacterial divisions.

Taxon/Species	Decline Rate	Ecosystem	Detection Method[2]	Reference
Proteobacteria				
Alpha subdivision (x=0.11)[1]				
Rhizobium leguminosarum	0.21	Soil	Cult	Heijnen *et al*., 1988
Rhizobium leguminsosarum	0.15	Soil	IF	Heijnen *et al*., 1988
R. leguminosarum RSM2004	<0.01	Soil*	Cult.	Hirsch, 1996
Azospirillum brasilense	-0.1	Rhiz.	Cult	Bashan *et al*., 1995
Azospirillum brasilense	0.46	Rhiz.	Cult.	Bashan *et al*., 1995
Bradyrhizobium japonicum	<0.01	Soil	IF	Brunel *et al*., 1988
Sinorhizobium meliloti	0.07	Soil*	*Luc*	Schwieger *et al*., 2000
Gamma Subdivision (x=0.35)				
Pseudomonas stutzeri	0.26	Soil	Cult. + Cat.	Byzov *et al*., 1996
P. stutzeri	0.22	Soil	Cult. + Cat.	Byzov *et al*., 1996
P. putida	0.42	Soil	Cult.+ Tol.	Huertas *et al*., 1998
P. putida WCS358	0.40	Rhiz.*	Cult.	Glandorf *et al*., 2001
Pseudomonas fluorescens	0.20	Rhiz.	Cult.	Frey-Klett *et al*., 1997
P. fluorescens	1.2	Soil	Cult.	Kozdroj, 1997
P. fluorescens R2f	0.18	Rhiz.*	Cult.	Wernars *et al*., 1996
P. fluorescens Q2-87	-0.06	Rhiz.	Cult.	Raaijmakers and Weller, 1999
P. fluorescens CHAO	0.26	Soil	IF	Masher *et al*., 2000
P. fluorescens CHAO	0.39	Soil	Cult.	Masher *et al*., 2000
CFB Group				
Flavobacterium sp.	2.45	Soil	Cult.	Thompson *et al*., 1990
Firmicutes (x=0.05)				
Paenibacillus azotofixans	-0.2	Rhiz.	MPN-PCR	Rosado *et al*., 1996
P. azotofixans	0.5	Soil	MPN-PCR	Rosado *et al*., 1996
Bacillus megaterium	-0.3	Rhiz.	Cult.	Liu and Sinclair, 1993
Bacillus thuringiensis	0.12	Soil	Cult.	Byzov *et al*., 1996
Arthrobacter globiformis	0.14	Soil	Cult.	Thompson *et al*., 1990

* Data obtained from field experiment; [1] x = mean decline rate for each division, [2] Cult. = cultivation based detection method; *Luc* = *Luc* used as marker for confirmation; Cat. = 2,3 di-oxygenase gene for degradation of catechol was used as marker; Tol. = Toluene degradation was used as marker; IF = immunofluorescent counts; MPN-PCR = quantification by PCR

There are several reports of bacteria which can survive very well for months or even years in certain soils (See Table 5). *Rhizobium Bradyrhizobium* and *Sinorhizobium* have been reported to survive in soil for years sometimes even without the presence of their specific host (Hirsch, 1996; Diatlof, 1977; Brunel *et al*., 1988; Schwieger *et al*., 2000). *Rhizobium* was shown to be able to form nodules when its host plant was planted again after several years (Hirsch, 1996). This shows that not only the ability of the strains to form associations with plant roots affects their survival but also the characteristics of the strain allow them to survive in bulk soil for years. Although *Rhizobium* species have been shown to survive extremely well in soil, both fast and slow growing species with different survival characteristics have been observed (Marshall, 1964). Fast growing *Rhizobium* species were found to be more susceptible to desiccation than the slower growing *Bradyrhizobium* (Marshall, 1964). *Rhizobium* species are sensitive to pH since they are generally not found in soils with pH 5 and lower (Lowendorf et. al., 1981). Competition between introduced and indigenous *Rhizobium* species is also reported to affect inoculant survival. In a study by Vlassak *et al*. (1996) introduced *Rhizobium tropici* which has superior nitrogen fixing capabilities was followed in the field. During successive bean crops an increase in number was

found in the second year after introduction. However, in the third year, only small number of *R. tropici* could be recovered and mainly indigenous *R. etli* and *R. leguminosarum* were found. *Rhizobium* inoculants are difficult to establish when they have to compete with the indigenous strains which are generally much less effective in nitrogen fixation (Thies *et al*., 1991).

6.3. Survival of genetically modified bacteria

Survival of genetically modified bacteria is generally similar to that of non-modified bacteria. Before considering the introduction of an engineered strain, it is important to know in which category the organism fits and to determine which methods will be used to gather data for risk assessment of GMM's. Besides the conditions in the receiving ecosystem, the fate of an introduced GMM is determined by its environmental fitness. The environmental fitness might be impaired as a result of the genetic modification. In a number of experiments in which wild-type and GMM are compared, the GMM's survived less well than their non-modified parent strains (Brockman *et al*, 1991; Bromfield and Jones, 1979; De Leij *et al*., 1998; Van Elsas *et al*., 1991; Wang *et al*., 1991). However, there are also quite a number of studies in which no difference in survival between GMM and parent strain could be detected (Bailey *et al*., 1995; Kline *et al*., 1988; Orvos *et al*., 1990; Wernars *et al*., 1996; Glandorf *et al*., 2001). Only in studies with artificial growth conditions did GMM's survive better then the wild-type strain (Biel and Hartl, 1983, Edlin *et al*., 1984). It is generally assumed that the inserted genes and their expression pose an extra metabolic burden for the strains, which could reduce their environmental fitness (Lenski *et al*., 1991). Results from a study of De Leij *et al*. (1998) showed that the presence of a number of constitutively expressed marker genes in a GMM had a negative effect on its survival in competition with the wild type strain. The site of insertion into the chromosome did not affect survival. The evidence suggested that it was purely the metabolic load which was responsible for the decreased fitness since the study also indicated that this effect did not occur under nutrient rich conditions. An increase in metabolic load resulting from the expression of heterologous genes which do not give the organism a selective advantage resulted in a decrease of fitness. In such experiments, the experimental design is also important. It seems that most studies in which the GMM competed less well with its parent were based on mixed inoculations (Van Elsas *et al*., 1991; De Leij *et al*., 1998). In this way, there was direct competition between the parent and the GMM while studies in which no differences were found, the strains were introduced separately. In a study of van Elsas *et al*. (1991), a reduced survival of a GMM in comparison to the parent strain could only be detected in mixed inoculation experiments.

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Section 4.
Guidance document on horizontal gene transfer between bacteria

1. Introduction

Horizontal gene transfer (HGT) [1] refers to the stable transfer of genetic material from one organism to another without reproduction. The significance of horizontal gene transfer was first recognised when evidence was found for 'infectious heredity' of multiple antibiotic resistance to pathogens (Watanabe, 1963). The assumed importance of HGT has changed several times (Doolittle *et al*., 2003) but there is general agreement now that HGT is a major, if not the dominant, force in bacterial evolution. Massive gene exchanges in completely sequenced genomes were discovered by deviant composition, anomalous phylogenetic distribution, great similarity of genes from distantly related species, and incongruent phylogenetic trees (Ochman *et al*., 2000; Koonin *et al*., 2001; Jain *et al*., 2002; Doolittle *et al*., 2003; Kurland *et al*., 2003; Philippe and Douady, 2003). There is also much evidence now for HGT by mobile genetic elements (MGEs) being an ongoing process that plays a primary role in the ecological adaptation of prokaryotes. Well documented is the example of the dissemination of antibiotic resistance genes by HGT that allowed bacterial populations to rapidly adapt to a strong selective pressure by agronomically and medically used antibiotics (Tschäpe, 1994; Witte, 1998; Mazel and Davies, 1999). MGEs shape bacterial genomes, promote intra-species variability and distribute genes between distantly related bacterial genera.

Horizontal gene transfer (HGT) between bacteria is driven by three major processes: transformation (the uptake of free DNA), transduction (gene transfer mediated by bacteriophages) and conjugation (gene transfer by means of plasmids or conjugative and integrated elements). These will be discussed in more detail below.

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[1] '*Horizontal gene transfer*' is synonymous with '*lateral gene transfer*' and results in unidirectional gene exchange (donor to recipient) between closely related or distantly related organisms; may be accompanied by expression of the introduced genetic material.

'*Transfer*' refers to translocation of genetic material into a cell, followed by stable integration into the recipient genome, including autonomously-replicating components of the genome (*e.g.* a plasmid, accessory chromosome or organelle chromosome). The transferred gene can be perpetuated in the offspring of the recipient organism.

'*Genetic material*' refers to any fraction of the genome (DNA or RNA), usually a gene or part thereof, which can include coding and/or non-coding sequences.

'*Organism*', as used here, includes cellular organism or replication competent virus.

'*Reproduction*' refers to the generation of offspring sexually, parasexually or asexually.

HGT can be differentiated from other types of gene transfer: (1) *Intentional gene transfer* – stable transmission of genes to a recipient organism through directed human intervention; (2) *Transient gene transfer* – transfer of genetic material to a recipient organism, intentionally or unintentionally, that is not perpetuated in the offspring; (3) *Intra-genomic gene transfer* – transfer of genetic material to a different location in the genome of the same organism (transposition); (4) *Vertical gene transfer* – transfer of genetic material from parent to offspring by reproduction, sexual or asexual. HGT contrasts with vertical gene transfer in that it can result in gene transfer between distantly related bacteria.

Mobile genetic elements (MGEs) such as plasmids, bacteriophages, integrative conjugative elements, transposons, IS (insertion sequence) elements, integrons, gene cassettes and genomic islands are the important vehicles in HGT. A brief summary of characteristic properties of MGEs is given in Table 1 (modified from Dobrindt *et al*., 2004). In many species a high proportion of horizontally transferred genes can be attributed to plasmid, phage or transposon-related sequences since remnants of MGEs are often found adjacent to genes identified as horizontally transferred in their complete genome sequences (Ochman *et al*., 2000; Brüssow *et al*., 2004; Gal-Mor and Finlay, 2006). MGEs are essential components that promote rapid adaptation to altered environmental conditions and as a consequence lead to bacterial diversification.

MGEs play a significant role in HGT in three ways:

1) MGEs have evolved mechanisms that enhance the potential for gene transfer between organisms. For example, conjugative elements and viruses have evolved highly efficient mechanisms for the passage of genes into a recipient cell.

2) MGEs can alter the function of genes in the vicinity of the insertion in the host genome. These alterations can include disruption or inactivation of genes at the site of insertion. Conversely, insertional mutagenesis by MGEs can also result in benefits to the host such as provision of regulatory sequences, repair of double stranded DNA breaks, or genome restructuring and speciation.

3) HGT of MGEs can result in the transfer of additional genes through genetic piggy-backing. For example, MGEs are the primary vehicles for the spread of antibiotic genes, pathogenic determinants, and biodegradation pathways amongst bacteria (de la Cruz and Davies, 2000; Top and Springael, 2003; Smets and Barkay, 2005; Gal-Mor and Finlay, 2006; Larraín-Linton *et al*., 2006).

HGT has enabled bacterial populations to occupy entirely new niches (Burrus and Waldor, 2004).

Considering the plasticity of bacterial genomes, the aspect of horizontal gene transfer is of importance for biosafety evaluations of transgenic[2] micro-organisms. For transgenic micro-organisms the transfer of the transgenic DNA to other bacteria as well as the uptake of MGEs, which might change the characteristics of the released strain, are generally to be considered. Bacteria modified by means of genetic engineering as well as natural bacterial populations will exploit the horizontal gene pool to adapt to changing environmental conditions and together with gene loss and genetic alteration, the acquisition of horizontally acquired DNA will play an important role in their adaptive evolution (Dobrindt *et al.,* 2004; Thomas and Nielsen, 2005; Gal-Mor and Finlay, 2006). Thus HGT needs to be understood as a natural process and major driving force of bacterial adaptability and diversity.

The intention of this document is to summarise the present state of knowledge on HGT between bacteria. The mechanisms of bacterial gene transfer and their occurrence under different environmental conditions will be briefly discussed, as well as implications for biotechnological applications.

[2] In this document, the term ‘transgenic’ organisms is used interchangeably for ‘genetically modified’ as well as ‘genetically engineered’ organisms; considered under these terms are organisms that have been obtained by recombinant DNA methods.

Table 1 Characteristics of mobile genetic elements (MGEs)

MGEs	Properties	Review
Plasmids	Circular or linear extrachromosomal replicons; self-transferable or mobilisable plasmids are vehicles for the transmission of genetic information between a broad or narrow range of species	Thomas, 2000; Thomas and Nielsen, 2005
Bacteriophages	Viruses that infect prokaryotes; can integrate into the host genome and then be vehicles for horizontal gene transfer	Canchaya *et al*., 2003
Integrative conjugative elements (ICE)	Self-transferable conjugative elements that integrate into the genome of new hosts like temperate bacteriophages; may promote the mobilisation of genomic islands by utilising conserved integration sites	Burrus and Waldor, 2004
Genomic islands	Large chromosomal regions acquired by horizontal transfer that are flanked by repeat structures and contain genes for chromosomal integration and excision	Dobrindt *et al*., 2004; Gal-Mor and Finlay, 2006
Transposable elements	Genetic elements that can move within or between replicons by action of their transposase; flanked by inverted repeats; transposons typically carry genes for antibiotic resistance or other phenotypes, while IS-elements code only for the transposase; multiple copies of the same IS-element promote genome plasticity by homologous recombination; IS*CR* (common region) s are a newly discovered class of transposable elements which mobilise DNA adjacent to their insertion site by rolling circle replication (those studied so far were closely associated with many antibiotic resistance genes and often located on conjugative plasmids)	Mahillon and Chandler, 1998 Toleman *et al*., 2006
Integrons	Genetic elements that capture promoterless gene cassettes into an attachment site downstream of a promoter by action of the integrase encoded on the integron; non-autonomous, but frequently associated with transposons and conjugative plasmids	Hall and Collis, 1995

2. Natural transformation

Natural transformation is generally understood as the uptake of free DNA by competent bacteria (Lorenz and Wackernagel, 1994; Dubnau, 1999, Chen and Dubnau, 2004). Natural competence is a genetically programmed physiological state permitting the efficient uptake of macromolecular DNA. Natural transformation is a tightly regulated process which requires elaborate machinery with more than a dozen of proteins involved. Transformability seems to be a property which is not shared by all isolates belonging to the same species, and transformation frequencies can vary up to four orders of magnitude among transformable isolates of a species (Sikorski *et al*., 2002; Maamar and Dubnau, 2005). The uptake of DNA can serve as a nutrient source, for DNA repair or as a source of genetic innovation (Dubnau, 1999). The uptake of DNA can be followed by integration into the bacterial genome by homologous recombination, homology-facilitated illegitimate recombination (de Vries and Wackernagel, 2002), or by forming an autonomously replicating element. The absence of homologous sequences or origins of replication were identified as major barriers to HGT by transformation (Thomas and Nielsen, 2005). Furthermore, spontaneous transformation or transformation by lightning (Cérémonie *et al.*, 2004, 2006) was also described for some bacteria which do not possess genes involved in natural competence.

Natural transformation provides a mechanism of gene transfer that enables competent bacteria to generate genetic variability by making use of DNA present in their surroundings (Dubnau, 1999; Nielsen *et al*., 2000). Prerequisites for natural transformation are the availability of free DNA, the development of competence, and the uptake and stable integration or autonomous replication of the captured DNA. However, there is limited knowledge of how important natural transformation is in different environmental settings for the adaptability of bacteria. Transformation may be critical for the establishment, maintenance and gene transfer in bacterial biofilms (Molin and Tolker-Nielsen, 2003; Petersen *et al*., 2005). Two other aspects of natural transformation in the environment have been mainly studied: the persistence of free DNA and the ability of different bacterial species to become competent and take up free DNA under environmental conditions.

Persistence of free DNA in soil - Recent reports have shown that in spite of the ubiquitous occurrence of DNases high molecular weight free DNA could be detected in different environments. It is supposed that free DNA released from micro-organisms or decaying plant material can serve as a nutrient source or as a reservoir of genetic information for indigenous bacteria. Reports on the persistence of nucleic acids in non-sterile soil have been published (Blum *et al*., 1997; Nielsen *et al*., 1997a), and microbial activity was pinpointed as an important biotic factor affecting the persistence of free DNA in soil. Stimulated microbial activity often coincided with an increase in DNase activity in soil (Blum *et al*., 1997). Cell lysates of *Pseudomonas fluorescens*, *Burkholderia cepacia* and *Acinetobacter* spp. were available as a source of transforming DNA for *Acinetobacter* sp. populations in sterile and non-sterile soil for a few days, and Nielsen *et al*. (2000) showed that cell debris protected DNA from degradation in soil. Cell walls may play an important role in protecting DNA after cell death (Paget and Simonet, 1997). Long-term persistence up to two years of transgenic plant DNA was reported by Widmer *et al*. (1996, 1997), Paget and Simonet (1997), and Gebhard and Smalla (1999) in microcosm and field studies. A more rapid break-down of transgenic DNA was observed at higher soil humidity and temperature. Both factors are supposed to contribute to a higher microbial activity in soil (Widmer *et al*., 1996; Blum *et al*., 1997).

Binding of DNA to rather different surfaces such as chemically purified mineral grains of sand, clay, non-purified mineral materials as well as humic substances has been reported (Khanna and Stotzky, 1992; Romanowski *et al*., 1992; Recorbet *et al*., 1993; Gallori *et al*., 1994; Lorenz and Wackernagel, 1994; Crecchio and Stotzky, 1998; Demanèche *et al*., 2001a). In the study of Demanèche *et al*. (2001a) plasmid DNA adsorbed on clay particles was found to be not completely degradable even at high nuclease concentrations. The adsorption of DNA seems to be a charge-dependent process and thus the rate and extent of adsorption of dissolved DNA to minerals depends largely on the type of mineral and the pH of the bulk phase, whereas the conformation and the molecular size of the DNA molecules have a minor effect (Lorenz and Wackernagel, 1994; Paget and Simonet, 1994). Since DNA can persist adsorbed on soil particles or protected in plant or bacterial cells, this DNA could be captured by competent bacteria colonising in close vicinity.

Competence development - Although it is supposed that natural competence is widespread among bacterial species (Lorenz and Wackernagel, 1994; Dubnau, 1999; Chen and Dubnau, 2004), the proportion of bacteria in natural settings which can become competent and the environmental conditions stimulating competence development is largely unknown. Only for a rather limited number of bacterial species have the natural transformation systems been studied in great detail (reviewed by Dubnau, 1999): *Bacillus subtilis, Streptococcus pneumoniae, Haemophilus influenzae, Neisseria gonorrhoeae, Acinetobacter* sp., *Pseudomonas stutzeri*, *Helicobacter pylori.* In the Gram-positive model bacteria studied, the first step in transformation is the binding of the double-stranded DNA to the cell, with no base sequence preference, followed by a fragmentation of the bound DNA. While the single strands are transported across the membrane the non-transported strand is degraded. The efficient uptake of DNA by the two Gram-negative model organisms *Haemophilus influenzae* and *Neisseria gonorrhoeae* require the presence of specific uptake sequences which are often found in the inverted repeats of the

donor sequences. After binding the DNA becomes rapidly DNase resistant and is taken up after fragmentation. In addition to differences in the DNA uptake processes, bacteria do not exhibit the same efficiency to integrate the incoming DNA by heterologous recombination (Sikorski *et al*. 2002). The vast majority of studies on transformation in the context of biosafety research have been performed with strain *Acinetobacter* sp. BD413. Recently, the naturally transformable *Acinetobacter* sp. ADP1 strain and its derivative BD413 were shown to belong to the newly described species *Acinetobacter baylyi* (Vaneechoutte *et al.,* 2006). This strain can be efficiently transformed with DNA of different sources. Although the number of bacterial species for which natural transformation has been observed is growing, the majority of bacterial isolates in culture collections have not yet been tested to determine whether they can be transformed. Even more scarce are the data on natural transformation *in situ*. A few reports on the development of the competence state under environmental condition exist. Nielsen *et al*. (1997a, b) showed that the addition of nutrients can stimulate competence development of *Acinetobacter* sp. in bulk soil. Competence development was reported for the plant pathogen *Ralstonia solanacearum* and the co-inoculated *Acinetobacter* sp. BD413 when colonising tobacco plants. Even more striking is the observation of natural transformation in soil with two soil isolates *Pseudomonas fluorescens* and *Agrobacterium tumefaciens* (Demanèche *et al*., 2001b). However, transformation frequencies were very low and often not reproducible. Marker rescue based on restoration of deleted *nptII* was also used to study transfer of bacterial or transgenic plant DNA in *Streptococcus gordonii*. While *in vitro* transformation could be achieved, no marker rescue was observed in gnotobiotic rats, presumably due to the absence of competence-stimulating factors like serum proteins (Kharazmi *et al*., 2003). A peptide-pheromone system which controls genetic competence in *Streptococcus mutans* functions optimally when cells are living in actively growing biofilms (Li *et al*., 2001). Biofilms seemed also to facilitate natural transformation of *Acinetobacter* sp. BD413 and did not offer a barrier against effective natural transformation (Hendrickx *et al*., 2003). Chitin, an abundant surface particularly in aquatic habitats, induces natural competence in *Vibrio cholerae* (Meibom *et al*., 2005).

3. Transduction

Transduction is a mechanism of DNA acquisition by which non-viral DNA can be transferred from an infected host bacterium to a new host *via* infectious or non-infectious virus particles. Host DNA is mistakenly packaged into the empty phage head when the phage particle is produced. Defective phage particles which are released from lysed host cells can adsorb to new host cells and deliver the DNA carried in the capsid into the new host (Brüssow *et al*., 2004). However, the incomplete phage genome does not allow progeny phage production. The injected bacterial DNA can be integrated into the recipient genome using mechanisms that generally require the presence of homologous DNA sequences or specialised integrases. Based on the phenotype of the bacteriophage mediating the genetic transfer, generalised or specialised transduction are distinguished. While generalised transducing phages can be either temperate or virulent phages which carry any kind of host DNA regardless of location, specialised transducing phages are temperate phages and transfer only specific genes located close to the phage integration site.

An important aspect of prophage acquisition is that the bacterial hosts can gain fitness advantages, *e.g.,* immunity against lytic infections. Furthermore, phages play an important role in the emergence of bacterial pathogens. Many pathogenicity determinants (toxins) have been acquired *via* phages, *e.g.,* by *Corynebacterium diphtheria*, *Clostridium botulinum*, *Streptococcus pyogenes*, *Staphylococcus aureus* and Shiga toxin producing *E. coli* (reviewed by Brüssow *et al*., 2004). Pathogenicity islands (PAI) which are defined as large genomic islands that carry one or more virulence gene and occur in the pathogenic variants of a species but not or less frequently in the non-pathogenic strains often evolved from lysogenic bacteriophages (Hacker *et al*., 2003; Dobrindt *et al*., 2004; Brzuszkiewicz *et al.*, 2006). Forty of 56 sequenced bacterial genomes recently reviewed by Canchaya *et al*. (2003) contain prophage

sequences, which are viral nucleic acids that have been incorporated into the bacterial genome, exceeding 10 kb in length.

Although most bacteriophages infect only a narrow range of hosts, this mechanism of gene transfer has the advantage that transducing phages can be rather persistent under environmental conditions, do not require cell-cell contact, and DNA in transducing phage particles is protected (Wommack and Colwell, 2000). Marine environments are probably a major setting for virus mediated gene transfer between bacteria, where there is an estimated abundance of greater than 10^{29} virus particles (Hendrix *et al*., 1999; Weinbauer and Rassoulzadegan, 2004). For example, virus mediated gene transfer frequencies of around 10^{-8} have been reported from the Tampa Bay estuary, corresponding to around 3.6×10^{11} HGT events each day in the estuary (Jiang and Paul, 1998).

4. Conjugation

The process whereby a DNA molecule is transferred from a donor to a recipient cell *via* a specialised protein complex is termed conjugation. Conjugation requires close physical contact between donor and recipient populations and the DNA (plasmids or conjugative transposons) is transferred *via* the so-called conjugation apparatus (Zechner *et al*., 2000). Although common mechanistic principles are shared by most of the conjugative system, *e.g.*, the synthesis of conjugative pili, there is a remarkable diversity of conjugative systems in Gram-negative and Gram-positive bacteria. Conjugative transfer requires the activity of the mating pair formation complex (Mpf) and of the DNA transfer and replication system (Dtr). While the former is responsible for making the contact between donor and recipient, the latter provides the functions for processing of DNA for transport. The plasmid encoded pili have different properties, *e.g.*, pili encoded by the so-called broad host range plasmids IncP-1, IncN or IncW are short and rigid while other plasmids such as IncF encode for flexible pili. Pilus properties are supposed to determine in which environmental niches the respective plasmid types transfer efficiently. Interestingly, plasmid encoded Mpf complexes are phylogenetically highly related to bacterial transport systems for proteins, the so-called type IV-secretion pathways. However, the question of whether the DNA traverses *via* the Mpf complex remains still to be experimentally proven.

A critical property of plasmids is their host range since this determines how far they can cause spread of antibiotic resistance or other phenotypes without physical recombination with the DNA of the new host. HGT can only affect bacteria that readily exchange genes. Bacterial genome comparisons indicated that members of 'exchange communities' have a tendency to be similar in factors like genome size, genome G/C composition, carbon utilisation, and oxygen tolerance (Jain *et al*., 2003). Host range in general appears to be limited by the interaction of the plasmid and its gene products with host enzymatic machinery. Host range is not an all or nothing property, but in the environment, on the one hand certain species or strains are preferred among the potential hosts (Heuer *et al*., 2007), on the other hand, MGEs are typically not fixed globally but persist in patches of local subpopulations (Berg and Kurland, 2002). This supports the notion that conjugative plasmids and the MGEs carried by them spread among a limited number of host cells and thus contribute to diversification of populations.

IncP-4 (group 4 from *Pseudomonas* species corresponding to IncQ) plasmids appear to be able to replicate in all Gram-negative bacteria and even some Gram-positive bacteria. This promiscuity may be due to their encoding three replication proteins and thus being largely independent of the replication machinery of their host. IncP-1 plasmids synthesise two related replication proteins encoded by overlapping genes and are able to replicate and be stably maintained in all Gram-negative bacteria but not Gram-positive bacteria. However, recently a cultivation-independent examination of the host range of IncP-1 plasmid in the rhizosphere of barley revealed an extremely broad host range and detected the IncP-1 plasmid (pKJK10) in *Arthrobacter* sp., a member of the *Actinobacteria* (Musovic *et al.,* 2006). Plasmids with a broad host range often appear to have lost restriction sites by point mutation and selection enzymes in the strain to which they are transferring. In addition they may carry anti-restriction

systems that minimise the effect of cleavage by special nucleases that protect many bacteria from invasion by foreign DNA. In summary, the host range of a plasmid may be determined by a range of small interactions. A narrow host range plasmid is no guarantee of containment in use of transgenic organisms. Point mutations may well extend the host range (Maestro *et al*., 2003), or environmental hosts may adapt to the plasmid (Heuer *et al*., 2007).

5. Other mechanisms of HGT

In addition to conjugation, transformation and transduction, other less well recognised mechanisms of DNA uptake occur in nature but their significance is uncertain. One such mechanism is *Vesicle-mediated translocation* whereby a range of Gram-negative bacteria such as *Neisseria gonorrheae*, *E. coli* and *Pseudomonas aeruginosa* can bud off vesicle structures that contain genetic material (*e.g.*, antibiotic resistance and virulence genes), which then fuse with another bacterium (Dorward *et al*., 1989; Yaron *et al*., 2000). Another mechanism of HGT involves *Pseudovirus particles*, in which some bacteria have genes that encode proteins capable of forming virus-like particles (gene transfer agent), which can trap random fragments of the genome (about 4,400 – 13,600 base pairs) and transmit them to a second bacterium (Marrs, 1974; Dykhuizen and Baranton, 2001; Lang and Beatty, 2001).

6. Prevalence of mobile genetic elements in bacterial communities from different environmental habitats

The use of genomic approaches to study the prevalence of MGEs resident in environmental bacteria has revealed a large diversity. The presence of MGEs in environmental bacteria is more the rule than the exception. Depending on the isolation procedure, different plasmids, with diverse characteristics with respect to Inc group, host range, avidity to transfer and the type of accessory genes present, can be obtained.

Surveys on the presence of plasmids in bacteria isolated from a wide range of environments have been performed and reveal that a considerable proportion of bacteria from different environments carried plasmids. Approximately 18% of bacterial isolates from the phytosphere of sugar beets were found to contain plasmids (Powell *et al*., 1993) and a large proportion of these plasmids were able to mobilise non-self-transferable but mobilisable IncQ plasmids (Bailey *et al*., 1994). To whatever extent environmental samples have been used, recipients functioning as a genetic sink and introduced under laboratory or *in situ* conditions have acquired MGEs conferring selectable traits such as mercury or antibiotic resistance (Smalla and Sobecky, 2002). In several of the studies increased transfer frequencies were observed when the environmental samples were stressed with pollutants. Mercury resistance was also used as an effective selective marker to exogenously isolate self-transferable Hg^r plasmids from river epilithon (Bale *et al*., 1988), the phylloplane and rhizosphere of different crops or sediments in Gram-negative recipients (Lilley *et al*., 1994, 1996; Smit *et al*., 1998; Schneiker *et al*., 2001; Smalla *et al*., 2006). *In situ* acquisition of mercury resistance plasmids by a transgenic *Pseudomonas fluorescens* SBW25EeZY6KX colonising the phytosphere of sugar beets observed under field conditions in two consecutive years confirmed this result (Lilley and Bailey, 1997a). Transconjugants which acquired Hg^r plasmids were isolated from the rhizosphere and the phyllosphere only at a certain time of plant development. Capturing of degradative genes resident on MGEs has also been demonstrated (Top *et al*., 1995, 1996). Self-transferable plasmids conferring resistance towards a range of antibiotics were captured from activated sludge, sewage or animal manures in green fluorescent protein (*gfp)*-labelled Gram-negative recipients (Dröge *et al*., 2000, Smalla *et al*., 2000; Heuer *et al*., 2002, Van Overbeek *et al*., 2002; Heuer and Smalla, 2007). Many of the exogenously isolated MGEs were shown to belong to the IncP-1 group (Heuer *et al*., 2002, 2004; Schlüter *et al*., 2003; Smalla *et al.*, 2006). Inc-P1 plasmids transfer to and replicate in a wide range of Gram-negative bacterial hosts. Sequencing of a number of IncP-1 plasmids isolated from different sources showed that they all contain blocks of accessory DNA,

e.g., transposons, integrated between backbone modules coding for replication, maintenance and transfer of the plasmid. Transferable antibiotic resistances could be isolated from most of the environments analysed (Heuer *et al*., 2002; Van Overbeek *et al*., 2002). Using this approach not only could conjugative plasmids be isolated from manure bacteria but also mobilisable IncQ-like plasmids indicating the presence of mobilising plasmids in piggery manure (Smalla *et al*., 2000). Mobilising plasmids were isolated by Van Elsas *et al*. (1998) when bacterial communities obtained from the rhizosphere of young wheat plants served as donor in triparental matings. Plasmid pIPO2 was isolated in *R. eutropha* based on its mobilising capacity. Replicon typing and sequencing of the complete plasmid (Tauch *et al*., 2002) revealed that this cryptic plasmid of approx. 45 kbp was not related to any of the known broad host range (BHR) plasmids except to plasmid pSB102 (Schneiker *et al*., 2001). The role of these plasmids for their plant-associated bacterial host remains unknown.

7. Factors affecting transfer efficiency of MGEs

The majority of natural environments - soils and aquatic systems - are restricted by the abundance of resources for microbial growth, which can severely limit population densities and activity. This, in turn, restricts those microbial processes that are dependent on density and activity, such as all HGT mechanisms (Van Elsas *et al*., 2000; Timms-Wilson *et al*., 2001). However, particular sites in these natural habitats, mostly related to soil or plant surfaces or surfaces in aquatic environments, have been shown to provide conditions for bacterial colonisation, mixing and activity, resulting in the occurrence of locally-enhanced densities of active cells. Transformation and conjugation processes were also shown to contribute to biofilm formation (Ghigo, 2001; Reisner *et al.*, 2006). These sites are often conducive to HGT processes, and are regarded as "hot" spots for bacterial gene transfer activity. Key abiotic and biotic factors that affect the extent of HGT in hot spots in natural settings have been reviewed, but quantitative prediction of natural HGT is still not possible presumably due to interactive effects between the different factors (Van Elsas *et al*., 2000; Van Elsas and Bailey, 2002).

Soil and phytosphere - The presence of large surfaces composed of mineral and organic phases in soil plays a key role in determining the physiological status of soil-dwelling bacterial cells. Soil is heterogeneous with regards to the distribution of gaseous, liquid or solid compounds (Stotzky, 1997). Clay-organic matter complexes are important sites for soil micro-organisms, due to their negatively-charged surfaces and enhanced nutrient availability. Availability of water in soil is a second important factor driving microbial activity. In bulk soil, bacterial cells occur mainly adsorbed to surfaces, which often results in micro-colonies that are refractory to movement or to contact with cells at different locations. Hence, most bacterial cells in soil can interact only with other bacteria in their immediate vicinity. Conditions that apply locally, *i.e.,* at the level of the site where bacterial cells are localised, will affect the cells and their involvement in HGT. In spite of the grossly nutrient-poor status of soil (Van Elsas *et al*., 2000; Timms-Wilson *et al*., 2001), nutrients can become concentrated in hot spots, primarily plant phytospheres, decaying organic material of animal or plant origin, and guts of soil animals like earthworms (Daane *et al*., 1996; Thimm *et al*., 2001) and Collembola (Hoffmann *et al*., 1998). The rhizosphere of many plants represents a region in soil with a (transient) high availability of organic carbon and potentially also N, P and S. Moreover, water flow in soil induced by plant roots may enhance bacterial movement. Both mechanisms promote cellular activities and cell-to-cell contacts. Recently, Mølbak *et al*. (2007) showed plant-dependent cell densities and distribution of donors and transconjugants in the rhizosphere of pea and barley. Thus exudation and root growth seem to be key parameters controlling plasmid transfer in the rhizosphere (Mølbak *et al.*, 2007).

Moreover, aboveground plant parts (the phyllosphere) can also provide nutrient-rich surfaces, resulting in similar hot spots (Björklöff *et al*., 1995). Further, as mentioned above, the guts of a range of soil animals represent another class of hot spots, as the mixing of cells and MGEs is enhanced, cells are activated and cell-to-cell contacts are stimulated (Thimm *et al*., 2001; Hoffmann *et al*., 1998). Finally,

the importance for HGT processes of easily-available substrate in soil, such as those provided by manure, has been indicated by Götz and Smalla (1997) and Heuer and Smalla (2007). Thus, soil, on the one hand, poses physical barriers to cell-to-cell contacts and nutritional limitations, whereas, on the other hand, nutrient up-shifts and alleviation of translocation or contact barriers may be found in soil hot spots. HGT rates in soil are certainly affected by the combination of these phenomena.

Selective pressure can be key in exacerbating the impact of gene transfer processes. Effects of selective pressure are most easily seen in cases in which the MGEs transferred confer some type of selective (growth) advantage to their hosts. Top *et al*. (2002) recently reviewed the issue of selection acting on gene transfer in soils. Several studies showed strong effects of selection. Transfer of a catabolic 2,4-dichloropropionate (DCPA) degrading plasmid from an *Alcaligenes xylosoxidans* donor to members of the indigenous community in soil, was only seen in soil treated with DCPA. When *Enterobacter agglomerans* carrying the self-transmissible biphenyl-degradative plasmid RP4::Tn*4371* was introduced as a (non-expressing) donor into soil with or without added biphenyl the introduced donor strain declined to extinction very quickly while indigenous transconjugants belonging to the genera *Pseudomonas* and *Comamonas* appeared. Again, transconjugants were only detected in soil that had received biphenyl. Furthermore, the transfer of the herbicide 2,4-D degradative plasmid pJP4 from *Ralstonia eutropha* JMP134 to *Variovorax paradoxus* was only detectable in soil in the presence of high levels of 2,4-D, and the transfer frequency of pJP4 to indigenous *Pseudomonas* and *Burkholderia* spp. increased as 2,4-D concentrations increased. Proliferation of the new transconjugants formed in soil was noted, which included representatives of *Burkholderia graminis*, *B. caribensis* and *R. eutropha*. The acquisition of novel pathways by HGT resulted in the adaptation of the indigenous bacterial communities to utilise the xenobiotic compounds as sources of nutrients. Evidence has also been provided for a direct role for genetic recombination in the adaptation of bacterial aquifer communities to chlorobenzenes (Van der Meer *et al*., 1998). These and related studies (Herron *et al*., 1998) demonstrated the central role that HGT plays in the adaptation of bacterial communities to changing resources and environmental pressures, such as novel substrate utilisation, antibiotic resistance and toxin production.

Aquatic habitats and biofilm - Aquatic systems contain specific structured (micro)habitats that may represent hot spots for HGT. Conceptually, aquatic systems can be divided in (1) the free (bulk) water phase, (2) the colonisable suspended matter, (3) sediment or sewage, (4) stones and other surfaces that carry biofilms (called epilithon), and (5) aquatic animals. These habitats offer very different conditions to their bacterial inhabitants. The presence of nutrients as well as colonisable surfaces is particularly important as such sites are known to support large densities of metabolically-active micro-organisms (Hill *et al*., 1994). In contrast, bulk water can be a nutrient-poor environment which may induce a (starvation) stress response in bacterial cells. Environments such as sewers, with high inputs of organic matter, form an obvious exception to this generalisation. On the other hand, suspended particles of varying sizes, as well as sediment and stone surfaces, represent nutrient-rich, more hospitable habitats that support microbial communities. Further, sediments, which are often rich in organic material, typically support bacterial populations exceeding those found in bulk water by several orders of magnitude (Van Elsas *et al*., 2000). Biologically diverse and metabolically active communities can also be found in the epilithon of stones in rivers or lakes (Hill *et al*., 1994; Van Elsas *et al*., 2000) and within other biofilms that form at solid/water interfaces. Micro-organisms within the epilithon are components of the extensive polysaccharide matrix which protects cells and adsorbs dissolved and particulate organic matter from the overlying water. Similar biofilms can be found in the percolating filter beds used in sewage treatment processes (Gray, 1992). Finally, a range of aquatic animals provide internal and external surfaces that are colonised by varied micro-organisms.

As bacterial hosts accumulate at surfaces where nutrients are concentrated, their distribution in aquatic systems is not even. The bulk water phase may contain relatively few bacterial cells that are able to participate in HGT processes. On the other hand, aquatic systems tend to provide excellent possibilities for mixing of bacterial cells and MGEs, and thus, for cell-to-cell and cell-to-MGE contacts.

These contacts occur mainly in biofilms at surfaces. The tendency of bacterial cells to stick to suspended particles, sediment or stones (epilithon) in aquatic systems may lead to the development of separate communities. Nevertheless, given the capability of many bacteria to occur in either sessile forms in micro-colonies or biofilms, or in motile forms, and thus to potentially connect spatially-separated biofilms, aquatic habitats provide important sites for cell-to-cell contacts resulting in HGT between bacteria (Van Elsas *et al*., 2000). Using both microcosm and *in situ* experiments, HGT between bacterial hosts has been shown to occur in drinking water, river water and epilithon (Hill *et al*., 1994, 1996), lake water, seawater, marine sediment and wastewater (Van Elsas *et al*., 2000). HGT thus appears to be a common process in aquatic environments, particularly in specific niches where nutrients are more abundant.

Animal ecosystems – Much evidence was found for HGT of antibiotic resistance genes in the intestines of humans, farm animals, and insects, as reviewed by Davison (1999). Transfer of antibiotic resistance genes in the intestines was shown between a variety of Gram-positive and Gram-negative bacteria. Identical nucleotide sequences of resistance genes (*e.g., tetM*, *tetQ*) in bacteria from antibiotic-treated farm animals and humans suggested transfer of these genes between these habitats, raising the possibility of acquisition of resistances by human pathogens through the use of antibiotics as additives in agricultural animal feed. In a recent study, it was shown in the intestine of mice that bacteriostatic compounds like tetracycline could increase spread and establishment of transconjugants which acquired a resistance conferring plasmid (Licht *et al*., 2003). Thus, the use of antibiotics may not only select for resistant populations but also enhance the formation of new resistant strains by HGT. The loss of IncP-1 plasmids from *Escherichia coli* cells colonising the gastrointestinal tract of germ-free rats was shown to be counteracted by the plasmid's ability to conjugate (Bahl *et al*., 2007).

8. Barriers to HGT

Although HGT can increase genetic diversity and promote the spread of novel adaptations, it can also result in excess genetic baggage and the import of deleterious genes. Therefore, organisms possess a number of physical, biochemical and genetic barriers to restrict the frequency of HGT (Kurland, 2005; Matic *et al*., 1996; Nielsen, 1998). Some of the barriers to HGT include the physical integrity of the cell; restriction-modification systems that recognise and hydrolyse foreign gene sequences; requirements for self-recognition sequences (*e.g.*, the genomes of *Haemophilus* and *Neisseria* have multiple copies of short sequences required for recognition and uptake by transformation); sequence specificity for integration into the recipient genome by homologous recombination; presence of inappropriate regulatory signals; nucleotide composition adaptations for optimised gene expression; mismatch repair systems; and natural selection. In general, the stringency of barriers to HGT increases proportionally with genetic distance. Consequently, the frequency of HGT is much greater within species than between unrelated or distantly related species.

9. Survival of MGEs

MGEs are generally agreed to add some, however small, burden to their host, although adaptation can occur to minimise this impact. This burden can be metabolic, *i.e.*, the need to copy and express extra genes, resulting in a change of phenotypic properties, for example, a change in the cell surface that is a disadvantage in specific circumstances, *e.g.*, when bacterial viruses are present. Thus it is generally agreed that the prevalence of plasmids must mean that they can be of benefit to bacteria to compensate for the burden they represent. The way they do this may not be identical for all plasmids. A small, high copy number plasmid and a large, self-transmissible plasmid may benefit its hosts in different ways. However, the broadest view is that MGEs increase the chance of new strains arising with novel or increased selective advantages over their neighbours. Since most naturally occurring plasmids can transfer between strains either by conjugation, transduction or transformation the autonomous replication

ability of plasmids removes the need of a gene to integrate by recombination in to the chromosome of a new host. High copy number plasmids can also modulate phenotype by changing gene dosage as well as increasing mutation frequency. Thus MGEs carry niche traits and survive because they allow their new host to exploit the niche so long as they acquire the MGEs. If no other host presents itself to benefit from the properties that the MGEs can confer then in the long term strains in which the advantageous genes carried by the MGEs have integrated into the chromosome may arise and dominate. Thus MGEs survive because microbial communities, and their environments, are continually changing so that the variability that an MGE allows increases the speed at which adapted strains arise and the adapted strains carry the MGE and propagate it faster. Thus MGEs that increase adaptability evolve and will survive at the expense of those that do not.

10. Mobile genetic elements as tools for biotechnology

The study of degradative pathways is important both from environmental and evolutionary viewpoints. Natural gene exchange in bacterial communities in the environment is an important mechanism that allows the bacteria to acquire new genetic information and thus evolve abilities to degrade persistent chemicals. It has become clear that there is much more diversity among certain catabolic genes and the mobile elements they reside on than we know so far. The source of genetic information for degradative pathways is still largely undiscovered. Plasmids encoding degradation of xenobiotic, often chlorinated compounds, seem to belong mainly to the IncP1 group. These IncP1 plasmids are the most promiscuous (or broad host range, BHR) self-transmissible plasmids characterised to date. If this correlation is true, it could suggest that recent bacterial adaptation to xenobiotics is promoted by plasmid promiscuity. A variety of plasmids involved in chloroaniline degradation have been described (Boon *et al*., 2001; Dejonghe *et al*., 2002). In addition to progress in insights in the role of BHR plasmids in metabolic pathway evolution, MGEs other than plasmids have been shown to carry catabolic genes and to be responsible for their lateral exchange, resulting in the assembly of new pathways. A new transposable element that codes for the degradation of biphenyl and 4-chlorobiphenyl was recently described (Merlin *et al*., 1999) and detected in several PCB degrading bacteria isolated from various environments (Springael *et al*., 2001). These recent findings strongly suggest that such MGEs play a very important role in the dissemination of degradative genes among bacteria, and thus in the natural construction of new degradative pathways. It is of great importance to better understand the underlying rules and mechanisms of these means of gene exchange, since they lead to accelerated removal of unwanted contaminants in natural environments.

Bio-augmentation can be defined as the addition of micro-organisms to a site in order to accelerate biodegradation of pollutants. This has been tested in several laboratory microcosm experiments, but in many cases the introduced bacteria have failed to degrade the pollutants due to their poor survival or low activity in their new environment. This may be due to abiotic and biotic stresses that are not encountered in the usual laboratory environment they have been maintained in for short to very long periods. An alternative approach involves the introduction and subsequent horizontal transfer of appropriate plasmid-borne catabolic genes into well-established and competitive indigenous bacterial populations. In this case, the survival of the introduced donor strain is no longer needed once the catabolic genes are transferred and expressed in the indigenous bacteria.

After inoculation with the 3-chlorobenzoate (3CBA) degrader *Pseudomonas putida* BN10 in a membrane biofilm reactor treating 3CBA containing synthetic wastewater, transfer of its mobile chlorocatechol catabolic genes (*clc*) to various bacterial populations was observed (Springael *et al*., 2002). Since the original inoculum strain always disappeared, the new 3CBA degraders seemed to out-compete the inoculum. Similar observations were made in different wastewater treatment systems inoculated with the same *clc*-element (Springael *et al*., 2002). These results show that treatment of wastewater contaminated with persistent pollutants can be improved by inoculating and spreading

the necessary mobile genetic elements. Furthermore, it was demonstrated that bio-augmentation of soils contaminated with the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) is possible *via* inoculation and subsequent transfer of a degradative self-transmissible plasmid (Dejonghe *et al*., 2000). The transfer of two different 2,4-D degradative plasmids to the indigenous bacteria of the A- and B-horizon of an agricultural soil was shown to positively affect the biodegradation of 2,4-D. This was most striking in the B-horizon without nutrient amendment, where no 2,4-D degradation occurred in the non-inoculated control soil during at least 89 days, while inoculation and subsequent plasmid transfer resulted in complete degradation of 2,4-D within 19 days. Overall, this work clearly demonstrates that bio-augmentation can constitute an effective strategy for clean-up of soils which are poor in nutrients and microbial activity.

As there are very few reports on bio-augmentation mediated by MGEs in soils and wastewater treatment reactors, the results summarised above suggest that natural MGEs that carry degradative genes could indeed be useful as tools in environmental biotechnology.

Since many bacterial species being used in biotechnological applications of various kinds are very distinct from *E. coli*, the cloning tools developed for *E. coli* often do not work for these organisms. Therefore new plasmid vectors, reporter and marker gene constructs, and other MGEs have been developed.

Overall it is clear that MGEs are not just interesting objects to be studied from an evolutionary viewpoint, they can also clearly contribute to solving problems in society. One is environmental pollution, but many other obstacles that can only be overcome realistically by means of biotechnology will continue to need the development of new vectors and other MGEs to optimise micro-organisms for various biotechnological processes.

11. Tools to study the horizontal gene pool

11.1. Endogenous isolation

The traditional approach to analyse the presence of plasmids in bacteria is the so-called endogenous plasmid isolation technique which requires culturability of the plasmid host. Pure cultures of bacteria isolated either on non-selective media or on media supplemented with antibiotics, heavy metals or xenobiotics are subsequently screened for the presence of plasmids by extraction of plasmid DNA. Surveys on the presence of plasmids in bacteria from a wide range of environments have been performed and revealed that a considerable proportion of bacteria from different environments contain plasmids. Determination of plasmid-encoded traits requires curing or subsequent transfer of the plasmid to a well characterised host.

With the use of endogenous plasmid isolation, only plasmids present in culturable bacteria are accessible. The advantage of the endogenous plasmid isolation approach is that the plasmid host is known. Based on the characteristics of the plasmid host, prediction can be made on the occurrence and fate of the plasmid in different environmental niches. A clear limitation of the endogenous plasmid isolation technique is the dependence on culturability of the plasmid host. Another disadvantage of endogenous plasmid isolations is that nothing is known about the relevance of such plasmids to *in situ* gene transfer processes (Smalla *et al*., 2000; Smalla and Sobecky, 2002). Plasmids with a high transfer potential might be overlooked when their hosts are less dominant or belong to the majority of bacteria which are not accessible by the cultivation techniques used.

11.2. Exogenous isolation

The exogenous isolations are performed either in a biparental or triparental mating and MGEs are recovered directly from the bacterial fraction of the environmental sample in recipient cells.

In a biparental mating the donor (bacterial fraction) is mixed for mating purposes with a recipient (Bale *et al*., 1988). The recipient needs an appropriate selectable marker which allows counter-selection against the donor. Due to the commonly low levels of natural background, rifampicin resistant recipients have been used frequently for matings. Recipients which have acquired a plasmid coding for an antibiotic or heavy metal resistance are obtained by plating on media supplemented with rifampicin and antibiotics or heavy metals (Hill *et al*., 1996; Smalla *et al*., 2000; Heuer *et al.*, 2002; Smalla *et al*., 2006; Heuer and Smalla, 2007). Mating conditions and recipients chosen will strongly affect the range of mobile elements obtained. Exogenous plasmid isolations have been applied to retrieve biodegradative genes borne on plasmids (Top *et al*., 1995). *In situ* acquisition of mercury resistance plasmids by a transgenic *Pseudomonas fluorescens* colonising the phytosphere of sugar beets was demonstrated under field conditions (Lilley *et al*., 1994, 1996; Lilley and Bailey, 1997a).

A variety of recipient strains that were rifampicin resistant and/or tagged with a marker gene such as *gfp* or luciferase (*luc*) has been successfully applied to retrieve plasmids from different environments, *e.g.*, *Pseudomonas putida*, *Pseudomonas fluorescens*, *Escherichia coli*, *Enterobacter cloacae*, *Ralstonia eutropha, Sinorhizobium meliloti* and *Agrobacterium tumefaciens.* However, until now exogenous isolation of plasmids into Gram-positive strains (either lab strains or isolates from the rhizosphere) has not yet been reported. The biparental exogenous isolation allows plasmids from environmental samples to be obtained independently from the culturability of the original host. Isolations of MGEs are based on their transfer efficiency under the mating conditions chosen, as well as their ability to replicate and express selectable marker genes in the recipient background. Traits newly acquired by the host are easily identified. A major disadvantage is that the original plasmid host remains unknown. While the biparental approach relies on the expression of selectable markers, the isolation of MGEs in triparental matings is solely based on their ability to mobilise small mobilisable plasmids carrying selectable markers into a new recipient (Hill *et al*., 1992; Van Elsas *et al*., 1998; Smalla *et al*., 2000). Using this methodology gene mobilising capacity was found in most environments analysed.

11.3. Polymerase Chain Reaction (PCR)-based detection of MGEs in directly extracted DNA

Many efficient methods to directly extract nucleic acids from various environmental samples are now available (Van Elsas *et al*., 2000). Two general approaches are used to extract nucleic acids from environmental samples: (I) Cell lysis within the environmental matrix or (II) cell lysis after recovery of the bacterial fraction from the environmental sample. To obtain DNA from environmental samples that are representative of the microbial community, the efficient lysis of diverse cells and spores is critical. Furthermore, co-extracted humic acids, which might inhibit the PCR amplification, need to be removed. The application of MGEs-specific primers to total community DNA can greatly facilitate the screening of and comparison of horizontal pools between different environments for the presence of MGEs such as transposons, plasmid groups or gene cassettes (Götz *et al*., 1996; Smalla *et al.*, 2006; Heuer and Smalla, 2007). The growing sequence database for MGEs facilitates the design of primers specific for their detection. Primer specificity can be predicted based on sequence comparisons and should be confirmed experimentally. The PCR-based approach allows the detection of various MGEs independently from the culturability of their hosts, from the presence and expression of selectable markers, and from their ability to transfer to, and replicate in, a new recipient. MGE-specific sequences can be detected by PCR amplification even though they occur only in a minor fraction of the population. However, the main advantage of this approach is that large sample numbers can be analysed, making extensive screening programmes for a variety of environments more feasible. The obvious limitation is that information regarding the nature of the host(s) containing these various MGEs is not obtained.

11.4. New tools to study **in situ** *transfer processes*

Traditional studies on gene transfer in the environment have relied on cultivation-based techniques, by which donor, recipient and transconjugant, transductant or transformant colonies have been detected

following their dislodgement from the environmental setting. A key factor has been the ability to select for donor, recipient and transconjugant cells using a combination of appropriate markers, including antibiotic resistance (Akkermans *et al*., 1995; Timms-Wilson *et al*., 2001). These approaches are now complemented with molecular methods such as PCR typing, DNA:DNA hybridisation, and sequencing, applied directly to habitat-derived DNA/RNA (Akkermans *et al*., 1995; Götz *et al*., 1996; Smalla *et al*, 2000) and to bacterial isolates. A major objective is the identification of specific sequences and functions relevant to HGT and the persistence of MGEs in response to environmental conditions. Also, reverse transcription (RT)-PCR analysis of environmental mRNA followed by hybridisation (micro-arrays), should facilitate the study of the expression of specific plasmid, phage, transposon or host genes in relation to HGT in the natural environment. Thus, the distribution and activity of genes and transcripts can be determined to identify processes associated with the interaction between MGEs, bacteria and the environment. By contrast, other non-disruptive approaches exploit the potential of fluorescent markers, such as green fluorescent protein (*gfp*), for studying the transfer of plasmids (Christensen *et al*., 1996, 1998; Dahlberg *et al*. 1998a, 1998b; Sørensen *et al*., 2003, 2005). In particular, *in situ* monitoring of plasmid transfer and microbial community physiology in structured microbial communities (through fluorescent reporter systems and confocal laser scanning microscopy) has provided a greater understanding of these complex processes (Christensen *et al*., 1998; Heydorn *et al*., 2000). Using monitoring of expression of unstable fluorescent reporter proteins introduced into chromosomes or on MGEs with assembled biofilm communities, plasmid transfer and cellular activity could be directly recorded (Andersen *et al*., 1998). Furthermore, using fluorescence-labeled reporter plasmids HGT can be detected and quantified independent from cultivation techniques by flow cytometry (Musovic *et al*., 2006). These *in situ* observations illustrate the advances in our knowledge base beyond earlier efforts that were largely system-disruptive (Van Elsas *et al*., 2000). For example, the use of a donor containing a plasmid carrying a *gfp* gene repressed for expression in the donor facilitates the screening for plasmid transfer to nonculturables in which *gfp* is expressed. Recently, *in vivo* expression technology (IVET) was made available to studies on plasmid gene expression in soil and the phytosphere (Bailey *et al*., 2001). Using this method, plasmids can be shown to carry genes that are uniquely expressed in the phytosphere at different periods of colonisation during plant growth. These genes are distinct from those similarly expressed by the host bacteria and share little homology with database sequences. This supports previous data that plasmids can provide periodic fitness advantages to their hosts (Lilley and Bailey, 1997b). The challenge is to identify the phenotypes of ecologically significant genes and the contribution they make to host ecology and evolution.

12. Implications of horizontal gene transfer to risk assessment of transgenic micro-organisms

The occurrence of horizontal gene transfer among bacteria has important implications in the risk assessment of transgenic bacteria. The fact that horizontal gene transfer among bacteria is known to occur in the environment dictates the necessity for evaluation of the potential for subsequent gene transfer of introduced genetic sequences from a transgenic bacterium to indigenous micro-organisms when that bacterium is released into the environment. Transgenic bacteria released into the environment, like their unmodified parent organism, can also capture mobile genetic elements which might improve their adaptation to environmental stresses and thereby enhance their fitness. There are two important concepts in the risk evaluation of the potential for gene transfer from transgenic micro-organism. The first is an exposure component of risk that examines both the likelihood of transfer and the potential extent (*i.e.*, range of recipient organisms) of gene transfer from a transgenic micro-organism to other micro-organisms in the environment resulting from the unique design or construction of the transgenic micro-organism with consideration of the parental micro-organism, the introduced genes, the method(s) of insertion and the environmental habitat into which the GEM is released. The other necessary component in the risk assessment of transgenic micro-organisms in relation to gene transfer is

the evaluation of any adverse effects should gene transfer occur. The evaluation of the potential consequences resulting from gene transfer is the more important consideration in an analysis of risks associated with gene transfer, and therefore, will be discussed first.

12.1. Potential adverse effects of horizontal gene transfer from a transgenic bacterium to indigenous micro-organisms

The risk associated with the horizontal gene transfer of transgenes from a transgenic bacterium is dependent on the likelihood of any adverse effects resulting from the acquisition of the transgenes in other micro-organisms. If any phenotype associated with the inserted gene(s) is potentially unwanted in another host background, such as toxicity, pathogenicity, increased virulence, resistance to antibiotics, competitive advantage, utilisation of novel substrates, or greatly expanded host range, then a close examination of the potential for gene transfer is warranted. On the other hand, if the inserted gene(s) of interest does not impart any adverse effect or novel function, then evaluation of the exposure components of risk are less important. Likewise, if there is already a significant existing gene pool in the environment for genes imparting a particular inserted trait (*e.g.*, degradative pathways such as SAL, TOL, and NAH), then there may be little concern even if gene transfer from the transgenic bacterium to other micro-organisms in the environment readily occurs.

12.2. Likelihood and extent of gene transfer

12.2.1. Construction of the transgenic micro-organism: Implications for horizontal gene transfer

The method of construction of a transgenic bacterium may have an influence upon the likelihood of gene transfer from that bacterium to other micro-organisms in the environment. The potential for transfer by any of the bacterial mechanisms of horizontal gene transfer (*i.e.*, conjugation, transformation, and transduction) warrants consideration in this analysis.

Genes introduced using plasmid vectors - The use of plasmids as vectors that are retained as intact extrachromosomal elements in the transgenic bacterium may allow for likely horizontal gene transfer from that bacterium to other bacteria with the subsequent expression of acquired genes. Both the likelihood and the extent of transfer of a plasmid used as a vector to create a transgenic bacterium, as with plasmids in naturally-occurring bacteria, are dependent upon a number of factors of the plasmid itself, such as host range, the presence of resident plasmids in a potential recipient, and characteristics of potential environmental recipients as discussed previously in this document. Evaluations of the likelihood and extent of transfer of a plasmid vector from the transgenic micro-organism to indigenous micro-organisms through conjugation must take into account whether the plasmid in the final transgenic micro-organism construct still has an intact mobilisation (nick) site and transfer (*tra*) genes on the plasmid. Disruption of these genes may render a conjugative plasmid non-self-transmissible. However, consideration must also be given to the possibility for missing *tra* functions being provided *in trans* by another self-transmissible plasmid.

The use of plasmid vectors for insertion of genes into transgenic micro-organisms may also result in transfer of those genes by the other mechanisms of horizontal gene transfer such as transformation and by transduction. However, the frequency of plasmid transfer through these mechanisms is probably much less than that which would be expected by conjugation of self-transmissible plasmids. The size of the plasmid vector may influence its ability to be transferred by transformation, as there is less likelihood that larger plasmids would remain intact for extended periods in the environment where DNA is subject to degradation by nucleases. Likewise, there are physical limitations in the size of nucleic acid sequences that can be carried by transducing phages. Still, these mechanisms of horizontal gene transfer must be considered in the biosafety evaluation of transgenic micro-organisms.

Genes inserted into the bacterial chromosome - Gene transfer from transgenic micro-organism constructed by the stable incorporation of the inserted genes of interest into the recipient bacterial chromosome may not present as much of a concern as genes introduced on conjugative plasmid vectors. Chromosomal insertion has been successfully accomplished by a variety of different methods such as the use of suicide plasmids or conditional replicons, or through transposon activity. Chromosomal insertion of genes into a recipient may also be accomplished through other mechanisms such as transduction or transformation, or through artificial transformation techniques such as electroporation or use of a gene gun. Regardless of the mechanism, the introduced DNA is integrated at low frequencies into the chromosome by homologous recombination or even through illegitimate/non-homologous recombination.

It should be noted, however, that even though chromosomal integration may decrease the frequency or likelihood of horizontal gene transfer from the transgenic bacterium to other micro-organisms, there is still the potential for gene transfer to occur. Some regions may be inherently more mobile, *e.g.*, genomic islands. Transposition could occur that may cause instability of the inserted gene from one location to another on the chromosome, or even to other replicons within a cell. The evolutionary importance of these processes is illustrated by the presence in microbial populations of genomic islands (including pathogenicity islands) that possess independent mobility through genes encoding integrases and transposons. These islands are examples of known vehicles for transfer of genes from chromosomal regions. They are generally regarded as mobilising components of the flexible gene pool, as opposed to "core genes" (Dobrindt *et al*., 2004) which often code for essential functions and appear to be less subject to mobilisation. In addition, transducing phages also can transfer chromosomal genes. Transformation of chromosomal pieces of DNA from dead cells may still occur. Although the frequency of transfer of chromosomal genes is expected to be lower than that for genes carried on self-transmissible plasmids, consideration of the mobile elements contained within the chromosome, which may lead to instability and thus transfer, must also be considered in risk assessment.

12.2.2. Other factors influencing horizontal gene transfer

There are a number of factors beyond the unique construction methods and final location of introduced genes within the genome of a transgenic bacterium that affect the likelihood and extent of horizontal gene transfer of inserted genes to indigenous micro-organisms. These include (1) the gene itself, including its phenotypic trait, especially if it confers a selective advantage to a recipient, (2) the inherent capability of the parental bacterium to transfer genes in general, (3) the ability of the transgenic bacterium to survive in the environment, (4) the presence of suitable recipient bacteria in the environment, and (5) various environmental factors that may affect microbial activity and transfer such as water content, nutrient status, clay mineralogy, pH, etc. The common experience of using transgenic micro-organisms in laboratory and field tests is a failure to maintain their presence or to transfer the introduced genes unless specifically enhanced for HGT.

As previously mentioned, the trait imparted by a gene(s) is important in assessing whether gene transfer may occur. Genes encoding traits conferring a selective advantage are likely to transfer and establish in bacterial communities regardless of genome location. The existence in microbial populations of genomic islands conferring traits such as pathogenicity, symbiosis, fitness, or resistance is evidence that such traits that are beneficial or useful to the host are commonly transferred (Dobrindt *et al*., 2004). However, novel genes that provide no competitive advantage to a new host may be lost, or not expressed. Some transgenic micro-organisms have been shown to have reduced fitness compared to their unmodified parental strains, which in some cases can be attributed to a metabolic drain on the cell. In other cases, some transgenic micro-organisms have exhibited decreased fitness due to cytotoxic effects of inserted genes. Since gene transfer is affected by the physiological state of the cell, the gene itself may be important in predicting gene transfer.

The inherent capability of the parental bacterium used in the creation of a transgenic micro-organism to exchange genes may influence the potential for horizontal gene transfer. Numerous bacterial species in the environment are known as natural gene exchangers. For example, pseudomonads are notorious for transfer of degradative genes (mostly contained on plasmids) among members in various genera. Closely related genera, such as *Pseudomonas* and *Burkholderia* can readily exchange, most likely due to the existence of the proper host machinery for both transfer and expression of newly acquired genes. Broad host range plasmids such as IncP-1 plasmids (Pukall *et al*., 1996, Top and Springael, 2003) but also the newly discovered groups of BHR such as pIPO2 (Van Elsas *et al*., 1998; Tauch *et al*., 2002) transfer readily between distantly related bacteria. It is important to recognise the plasticity of the bacterial genomes.

An accurate taxonomic identification of the recipient micro-organism used in creation of a transgenic micro-organism may assist in assessing the potential for gene transfer from the transgenic micro-organism to other micro-organisms in the environment. The importance of utilising the proper phenotypic and genotypic tests and methods for accurate identification of bacteria has been previously addressed (OECD, 2003).

For several mechanisms of horizontal gene transfer to occur, the transgenic bacterium must survive, at least transiently. If the transgenic micro-organism is unlikely to survive in the environment, the potential for transfer is greatly diminished, at least for the mechanisms of conjugation and transduction. There is still the possibility for transformation of DNA released from dead cells, although without multiplication of the bacteria, cells would be limited in number. As previously discussed, the persistence of naked DNA in the environment is dependent on a number of factors including adsorption to bacterial membranes, clay minerals, and other surfaces which provide protection from degradation by nucleases.

If the transgenic bacterium survives well in the environment, then the presence and concentration of appropriate recipients affects the extent of potential gene transfer. As previously discussed in this document, there are “hot spots” in soil and water environments and on plant surfaces where microbial concentrations are increased, and thus, there is greater potential for horizontal gene transfer to occur, especially through conjugation. Conjugation could also be stimulated at higher cell densities by quorum sensing systems, *e.g.*, in *Agrobacterium* (Miller and Bassler, 2001). Likewise, horizontal gene transfer by transduction and transformation is more likely to occur in areas of greater concentrations of micro-organisms.

Environmental factors influence the potential for horizontal gene transfer from a transgenic micro-organism to other micro-organisms in the environment. The physiological state of both the donor and recipient micro-organisms may be influenced by a number of abiotic factors, such as water content, temperature, nutrient status, and soil pH. In addition, environmental factors may affect the development of competence for transformation, and abiotic variables such as clay type and content or organic matter content may provide protection of free nucleic acid sequences from degradation. *In situ* acquisition of mobile genetic elements that might confer selective traits by the transgenic micro-organism has recently been shown for the transgenic *Pseudomonas fluorescens* SBW25EeZY6KX under field conditions in two consecutive years (Lilley and Bailey, 1997a). Transconjugants which acquired Hg^r plasmids were isolated from the rhizosphere and the phyllosphere only at a certain time of plant development. The acquisition of new genetic information might change the characteristics of a transgenic micro-organism. The type and frequency of post-release acquisition of additional genetic information will depend on the environmental habitat to which the transgenic micro-organism is released but also on the host organism.

12.2.3. Expression of acquired genes

Horizontal transfer of genes is no guarantee that a particular gene, or all transferred genes, will be efficiently expressed in a new host organism. Transcription is required for expression, and therefore, the new host must possess an RNA polymerase that will recognise the promoter and initiate RNA synthesis. This is not always the case, and the efficiency may vary in different recipient micro-organisms. In addition, a recipient micro-organism may or may not contain the appropriate enzymes for post-translational modification of the protein to produce a biologically active molecule. It should be noted, however, that even if the new host micro-organism does not possess the required enzymes for expression of the foreign gene, the gene sequence still may reside in the genome, and thus may subsequently be transferred to other micro-organisms in the environment capable of efficiently expressing the gene. However, it is often the case that genes that do not confer an advantage to the recipient micro-organism may be lost from the genome, and therefore, subsequent transfer may not be of concern.

12.3. Conclusions

The risk assessment of a transgenic bacterium must consider the potential for transfer of introduced genes to other micro-organisms in the environment. Of greater importance, however, is consideration of whether there would be any adverse consequences posed if gene transfer from a transgenic bacterium occurred. If no adverse effects can be envisioned, then prediction of the likelihood, frequency, and extent of gene transfer are of less concern. If detrimental consequences were to occur if genes were transferred to indigenous micro-organisms, then the exposure components of risk must be carefully evaluated, both from a theoretical perspective depending on potential mechanisms of transfer, and from a real-life perspective given natural barriers to gene transfer in the environment.

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List of OECD consensus documents 1996-2010

	Consensus Document	Lead Country(ies)	Year of Issue	Volume
Biology of Crops	Oilseed Rape (*Brassica napus*)	Canada	1997	Vol. 1
	Potato (*Solanum tuberosum* subsp. *tuberosum*)	Netherlands & United Kingdom	1997	Vol. 1
	Wheat (*Triticum aestivum*)	Germany	1999	Vol. 1
	Rice (*Oryza sativa*)	Japan	1999	Vol. 1
	Soybean (*Glycine max*)	Canada	2000	Vol. 1
	Sugar Beet (*Beta vulgaris*)	Switzerland	2001	Vol. 1
	Maize (*Zea mays* subs. *mays*)	Mexico	2003	Vol. 1
	Sunflower (*Helianthus annus*)	France	2004	Vol. 1
	Papaya (*Carica papaya*)	United States	2005	Vol. 1
	Oyster Mushroom (*Pleurotus* spp.)	Korea	2005	Vol. 1
	Chili, Hot & Sweet Peppers (*Capsicum annuum*)	Korea, Mexico & United States	2006	Vol. 1
	Cotton (*Gossypium* spp.)	Spain	2008	Vol. 4
	Bananas & Plantains (*Musa* spp.)	Spain	2009	Vol. 4
Facilitating Harmonisation	Introduction to the OECD Biosafety Consensus Documents *(Available in English and French)*	Working Group	2005	Vol. 1, 3 & 4
	Points to Consider for Consensus Documents on Biology of Cultivated Plants	Working Group	2006	Vol. 3
	Designation of a Unique Identifier for Transgenic Plants (Revised Version) (Guidance Document)	Working Group	2006	Vol. 3
	Molecular Characterisation of Plants Derived from Modern Biotechnology	Canada	2010	Vol. 3

	Consensus Document	Lead Country(ies)	Year of Issue	Volume
Biology of Trees	*Trees*			
	Norway Spruce (*Picea abies*)	Norway	1999	Vol. 2
	White Spruce (*Picea glauca*)	Canada	1999	Vol. 2
	Poplars (*Populus* spp.)	Canada	2000	Vol. 2
	Sitka Spruce (*Picea sitchensis*)	Canada	2002	Vol. 2
	Eastern White Pine (*Pinus strobus*)	Canada	2002	Vol. 2
	European White Birch (*Betula pendula*)	Finland	2003	Vol. 2
	White Pine (*Pinus monticola*)	Canada	2008	Vol. 3
	Jack Pine (*Pinus banksiana*)	Canada	2006	Vol. 3
	North American Larches (*Larix lyalli, Larix occidentalis, Larix laricina*)	Canada	2007	Vol. 3
	Douglas-Fir (*Pseudotsuga menziesii*)	Canada	2008	Vol. 3
	Lodgepole Pine (*Pinus contorta*)	Canada	2008	Vol. 3
	Black Spruce (*Picea mariana*)	Canada	2010	Vol. 3
	Fruit Trees			
	Stone Fruits (*Prunus* spp.)	Austria	2002	Vol. 2
	Papaya (*Carica papaya*) [listed above in "Crops"]	United States	2005	Vol. 1
	Bananas & Plantains (*Musa* spp.) [listed above in "Crops"]	Spain	2009	Vol. 4

	Consensus Document	Lead Country(ies)	Year of Issue	Volume
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	Genes and their Enzymes that Confer Tolerance to Glyphosate Herbicide	United States, Germany & Netherlands	1999	Vol. 1
	Genes and their Enzymes that Confer Tolerance to Phosphinothricin Herbicide	United States, Germany & Netherlands	1999	Vol. 1
	Herbicide Metabolism and the Residues in Glufosinate-Ammonium (Phosphinothricin)-Tolerant Transgenic Plants	Germany	2002	Vol. 1
	Transgenic Plants Expressing *Bacillus thuringiensis*-Derived Insect Control Protein	United States	2007	Vol. 3

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	Pseudomonas	United Kingdom	1997	Vol. 2
	Acidithiobacillus	Canada	2006	Vol. 2
	Baculovirus	Germany	2002	Vol. 2
	Acinetobacter	Canada	2008	Vol. 4
	Guidance Documents on Biosafety Aspects of Bacteria			
	Use of Taxonomy in Risk Assessment of Micro-organisms: Bacteria	Canada & United States	2003	Vol. 4
	Methods for Detection of Micro-organisms Introduced into the Environment: Bacteria	Netherlands	2004	Vol. 4
	Horizontal Gene Transfer Between Bacteria	Germany	2010	Vol. 4

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- Biology of crops
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    - Sugar Beet (*Beta vulgaris*)
    - Maize (*Zea mays* subs. *mays*)
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    - Genes and their enzymes that confer tolerance to phosphinothricin herbicide
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Safety Assessment of Transgenic Organisms

OECD CONSENSUS DOCUMENTS

Volume 4

The books on "Safety Assessment of Transgenic Organisms" constitute a compilation of the OECD Biosafety Consensus Documents. When published, Volume 1 and 2 contained the documents issued before 2006; Volume 3 and 4 are a continuation of the compilation up to 2010.

The OECD Biosafety Consensus Documents identify elements of scientific information used in the environmental safety and risk assessment of transgenic organisms which are common to OECD member countries and some non members associated with the work. This is intended to encourage information sharing, promote harmonised practices, and prevent duplication of effort among countries.

These books offer ready access to those consensus documents which have been issued on the website thus far. As such, it should be of value to applicants for commercial uses of transgenic organisms (crops, trees, micro-organisms), to regulators and risk assessors in national authorities, as well as the wider scientific community.

More information on the OECD's work related to the biosafety of transgenic organisms is found at BioTrack Online (***http://www.oecd.org/biotrack***).

Further reading

Safety Assessment of Transgenic Organisms – OECD Consensus Documents, Vol 1 (2006)
Safety Assessment of Transgenic Organisms – OECD Consensus Documents, Vol 2 (2006)

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