# *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<br>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.<br>*A. haemolyticus* | Francisco *et al.*, 2002<br>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<br>Ice-stored or frozen for food<br>Diseased (Syndrome 93) | Lee and Pfeifer, 1977<br>Guardabassi *et al.*, 1999; Lakshmanan *et al.*, 2002<br>Costa *et al.*, 1998 |
| Crab (various spp.) | Arthropoda | As food<br>In haemolymph of normal crab | Lee and Preifer, 1975<br>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.<br>As potential candidates for genetic manipul. to control the disease transmission capabilities of the host.<br>Increase susceptibility to viral disease. | Zayed and Bream, 2004<br>Pidiyar *et al.*, 2004<br>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<br>In diseased fish<br>In fish meat<br>Antibiotic resistance | Horsley, 1973; Esteve and Garay, 1991; Huber *et al.*, 2004<br>D'Souza *et al.*, 2000<br>Acuff *et al.*, 1984; Gennari and Tomaselli, 1988; Middlebrooks *et al.*,1988; Gonzalez *et al.*, 2000<br>Petersen *et al.*, 2002; Miranda *et al.*, 2003 |
| Chicken | Aves | Meat, carcass<br>From septicaemic hens<br>With antibiotic resistance | Grimont and Bouvet, 1991; Olivier *et al*., 1996<br>Erganis *et al*., 1988; Kaya *et al*., 1989<br>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<br>Rat thigh abscess model<br>Mice with gastritis and hypergastrinemia<br>Large intestines (tissue and luminal contents) of restricted-flora and specific-pathogen-free mice | Montero *et al*., 2002, 2004<br>Fetiye *et al.*, 2004<br>Zavros *et al.,* 2002<br>Scupham *et al*., 2006 |
| Cats | Mammalia | Skin, vagina, uterus of healthy animal<br>Nosocomial disease<br>Cat suffering from necrotizing fasciitis with septic shock | Krogh and Kristensen, 1976 ; Clemetson and Ward, 1990<br>Francey et al., 2000; Boerlin *et al.*, 2001<br>Brachelente *et al*., 2007 |
| Dog | Mammalia | Mouth, large airways of diseased animal<br>Normal or infected skin<br>Nosocomial disease | Johnson and Fales, 2001; Kasempimolporn *et al.,* 2003<br>Krogh and Kristensen, 1976, 1981; Kristensen & Krogh, 1978\<br>Francey *et al*. 2000; Boerlin *et al*. 2001 |
| Pigs | Mammalia | Bacteriosperma in porcine semen | Althouse and Lu, 2005 |
| Horses | Mammalia | Normal nose, conjunctiva<br>Animal with chronic haematuria.<br>Animal with respiratory symptoms.<br>Nosocomial strain. | Cabassi *et al*., 1975; Cattabiani *et al*., 1976; Moore *et al*., 1988; Gemensky-Metzler *et a*l., 2005<br>Rajasekhar *et al.,* 1978<br>Boguta *et al*., 2002; Newton *et al*., 2003<br>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°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°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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