The Diversity of the Genus *Acinetobacter*

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Lenie Dijkshoorn and Alexandr Nemec

Abstract

The genus *Acinetobacter* comprises 17 validly named and 14 unnamed (genomic) species. Some unrelated (genomic) species have common designations, while some other species seem to be congruent but have different names. The knowledge of the biology or ecology of acinetobacters at species level is limited. This is due to the fact that identification of acinetobacters at species level is difficult. A phenotypic species identification system comprising c. 20 tests has been described (Bouvet and Grimont, 1986) but is not widely used and some closely related species cannot be separated well with this system. A variety of genotypic methods has been explored and applied to investigate the diversity or phylogeny in the genus. These methods include high resolution fingerprinting with AFLP, PCR-RFLP with digestion of PCR amplified sequences, and analysis of various DNA sequences. Of these, AFLP analysis and amplified 16SrDNA ribosomal DNA restriction analysis have been validated with large numbers of strains of all described species. Nucleotide sequence based methods are expected to be the standard for identification in the near future, but a prerequisite for their successful application is the availability of libraries of sequences of strains of all described genomic species. For each species, the sequences should cover the intra-species diversity. Sequence comparisons will also provide a valuable tool to study the phylogenetic relatedness of species.

Introduction

Bacteria that belong to the genus *Acinetobacter* constitute a heterogeneous group of organisms. Yet, it was not before the mid 1980s that different species could be distinguished within the genus. Since, up to 33 genomic groups have been identified by DNA–DNA hybridization, 17 of which have been given species names (Table 1.1). Unfortunately, in applied microbiology the knowledge on the existence of the many named and unnamed species and the availability of methods for species identification are lagging behind. Hence, relatively little is still known about the biology of the different (genomic) species except for the clinically relevant species *Acinetobacter baumannii*.

Progress in the field of the diversity of the genus *Acinetobacter* has largely been made by the use of molecular methods including DNA-based methods. It is of note that different methods may provide different groupings. The challenge of present-day taxonomy or

epidemiology is to find the grouping which reflects the natural diversity and evolutionary history of the bacteria of study.

It is the aim of the present chapter to discuss the diversity of the genus *Acinetobacter* as currently known. We will start with an overview of the taxonomy of the genus from a historic perspective. This section is followed by a paragraph on the problems of the current taxonomy of *Acinetobacter*. For those not familiar with bacterial systematics, an introduction to the basic concepts of taxonomy is included. This may help to understand the particular problems of the classification and nomenclature of acinetobacters. The body of the chapter provides an overview of the diversity of the genus as assessed by different methods and the use of these methods in classification and identification.

The taxonomy of the genus *Acinetobacter* from a historic perspective

It was at the beginning of the 20th century that Martinus Willem Beijerinck, a Dutch microbiologist and father of the Delft School of Microbiology (la Riviere, 1997), described an organism that was isolated from soil by enrichment cultivation on a calciumacetate-mineral medium (Beijerinck, M.W., 1911). This organism was named *Micrococcus calco-aceticus*. In the following decades similar organisms were described independently and assigned different genus and species names like e.g. *Herellea vaginicola*, *Mima polymorpha*, *Bacterium anitratum* and *Moraxella lwoffii* (Henriksen, 1973). By the late 1960s a comprehensive study was published in which strains from these different taxa were compared for a large number of physiological characters (Baumann *et al.*, 1968). The conclusion was that the organisms represented a single genus, but no clear-cut criteria for subdivision of the genus into species could be given. Thus, it was proposed to name the genus *Acinetobacter* and to consider only one species, named *Acinetobacter calco-aceticus*. The Beijerinck strain described in 1911, being the oldest living and documented *Acinetobacter* strain, was presented as the type strain for both the genus and the species. Interestingly, the report of that study (Baumann *et al.*, 1968) included unpublished evidence from M. Mandel on DNA composition and from J. Johnson on DNA homologies indicating that multiple species could be recognized. A first published DNA hybridization study assigned strains to five DNA groups with four strains remaining ungrouped (Johnson *et al.*, 1970). Twelve DNA–DNA hybridization groups—then named "genospecies"—were described in 1986, seven of which were given species names while the others were provisionally given numbers (Bouvet and Grimont, 1986) (Table 1.1). In a next report, five additional DNA–DNA hybridization groups—we will use the term genomic species or gen. sp. for these groups—with the designations 13–17 were described (Bouvet and Jeanjean, 1989). Independently, a concurrent report also described three new genomic species numbered 13–15, where gen. sp. 13 of this paper (Tjernberg and Ursing, 1989) appeared to correspond to genomic species 14 of the other paper (Bouvet and Jeanjean, 1989). It was also shown by DNA hybridization that the previously described species *Acinetobacter radioresistens* (Nishimura *et al.*, 1988) corresponds to gen. sp. 12 (Bouvet and Grimont, 1986), and that *A. lwoffii* (gen. sp. 8) and gen. sp. 9 belong to one hybridization group (Tjernberg and Ursing, 1989). Likewise, recent results indicate that *A. grimontii* and *A. junii* represent one species making *A. grimontii* a junior synonym (Vaneechoutte, unpublished data). Furthermore, *A. calcoaceticus, A. baumannii,*

and gen. sp. 3 and 13TU were found so closely related (~65%) that lumping them into one species—to be named *A. calcoaceticus*—was considered but not formally proposed.

Ten additional species have been validly described over the past five years. These include three species of possible clinical relevance, *A. ursingii*, *A. schindleri* (Nemec *et al.*, 2001) and the small colony forming *A. parvus* (Nemec *et al.*, 2003), and seven species from activated sludge, *A. baylyi*, *A. bouvetii*, *A. grimontii*, *A. tjernbergiae*, *A. towneri*, *A. tandoii*, and *A. gerneri* (Carr *et al.*, 2003). It is of note that the well studied mutant strain ADP1—originally described as BD413 and derived from the soil isolate BD4 (Juni and Janik, 1969)—has recently been found to belong to one of these species, *A. baylyi* (Young *et al.*, 2005; Vaneechoutte *et al.*, 2006). DNA–DNA hybridization studies have also revealed the existence of two additional genomic species which have not yet been validly described. These were designated "between 1 and 3" and "close to 13TU" indicating their position based on DNA homology as relatively close to *A. calcoaceticus* and gen. sp 3, and to gen. sp. 13, respectively (Gerner-Smidt and Tjernberg, 1993). Another species, "*Acinetobacter venetianus*" which includes the biochemically important, oil-degrading strain RAG-1 has not yet been validly described either, although it fulfils the criteria for validation (Vaneechoutte *et al.*, 1999).

In conclusion, considering that the respective pairs of designations *A. lwoffii* and gen. sp. 9, 13BJ and 14TU, and *A. junii* and *A. grimontii* are synonyms, the actual number of published (genomic) species is 30 (Table 1.1).

Current problems related to the taxonomy of *Acinetobacter*

In the preceding paragraph we have seen that considerable progress has been made to elucidate the diversity of the genus *Acinetobacter* at species level. Nevertheless, there are still several problems and inconsistencies (Box 1.1). For example, only 17 described DNA–DNA hybridization groups (genomic species) have valid names, while the remaining groups have numbers or other designations that do not contribute to their familiarity. The reasons for not giving species names to these DNA hybridization groups were that the groups were considered too small (< 10 strains) to describe their characteristics as a group properly, and that criteria for phenotypic differentiation were insufficient (Bouvet

Table 1.1 Hitherto described genomic species of the genus *Acinetobacter*

Designation				
Named species	Genomic species	Type or reference strain	Main origin in reference studies	Reference*
A. calcoaceticus	1	ATCC 23055T	Soil, human specimens	1, 2
A. baumannii	2	ATCC 19606T	Human clinical specimens	1, 2
A. haemolyticus	4	ATCC 17906T	Human clinical specimens	1, 2
A. junii	5	ATCC 17908T	Human clinical specimens	1, 2
A. johnsonii	7	ATCC 17909T	Human and animal specimens	1, 2

Table 1.1 continued

Designation				
Named species	Genomic species	Type or reference strain	Main origin in reference studies	Reference*
A. lwoffii	8, 9	ACTC 15309T	Human specimens	1, 2
A. radioresistens	12	IAM 13186T	Human clinical specimens, soil, cotton	1, 2, 3
A. ursingii		LMG 19575T	Human clinical specimens	4
A. schindleri		LMG 19576T	Human clinical specimens	4
A. parvus		LMG 21765T	Human clinical specimens	5
A. bouvetii		DSM 14964T	Activated sludge	6
A. baylyi		DSM 14961T	Activated sludge	6
A. towneri		DSM 14962T	Activated sludge	6
A. tandoi		DSM 14970T	Activated sludge	6
A. grimontii		DSM 14968T	Activated sludge	6
A. tjernbergiae		DSM 14971T	Activated sludge	6
A. gerneri		DSM 14967T	Activated sludge	6
"A. venetianus"		ATCC 31012	Sea water	7
	3	ATCC 19004	Human clinical specimens	1, 2
	6	ATCC 17979	Human specimens	1, 2
	10	ATCC 17924	Human clinical specimens	1, 2
	11	ATCC 11171	Human specimens	1, 2
	13BJ, 14TU	ATCC 17905	Human clinical specimens	2, 8
	14BJ	CCUG 14816	Human clinical specimens	8
	15BJ	SEIP 23.78	Human specimens	8
	16	ATCC 17988	Human specimens	8
	17	SEIP Ac87.314	Human clinical specimens	8
	13TU	ATCC 17903	Human clinical specimens	2
	15TU	151a	Human specimens	2
	"Between 1 and 3"	10095	Human clinical specimens	9
	"Close to 13TU"	10090	Human clinical specimens	9

*1, Bouvet and Grimont 1986; 2, Tjernberg and Ursing 1989; 3, Nishimura *et al*. 1988; 4, Nemec *et al*. 2001; 5, Nemec *et al*. 2003; 6, Carr *et al*. 2003; 7, Vaneechoutte *et al*. 1999; 8, Bouvet and Jeanjean 1989; 9, Gerner-Smidt and Tjernberg 1993.

Box 1.1 Problems of the taxonomy of the genus *Acinetobacter*

16 Genomic species (DNA-DNA hybridization groups) have no valid names yet

Several named/unnamed species are congruent, i.e. the names are synonyms:

- Gen. sp. *sensu* Bouvet and Jeanjean (BJ) and gen. sp. 14 *sensu* Tjernberg and Ursing (TU)
- *A. lwoffii* and gen. sp. 9
- *A. junii* and *A. grimontii*

Different unnamed species have identical designations:

- Gen. sp. 14 BJ and gen. sp. 14 TU
- Gen. sp. 15 BJ and gen. sp. 15 TU

Six named species and most unnamed species comprise only one or a few strains per species

Identification of most species is problematic

and Grimont, 1986; Bouvet and Jeanjean, 1989; Tjernberg and Ursing, 1989; Gerner-Smidt and Tjernberg, 1993). Another problem is the already mentioned coincidence of two studies using the common designations 13–15 for new genomic species (Bouvet and Grimont, 1986; Tjernberg and Ursing, 1989). (To avoid confusions for these species, it is recommended to add the extensions of the respective authors Tjernberg and Ursing or Bouvet and Jeanjean to designate the genomic species from either studies, for example "gen. sp. 14BJ" to distinguish it from "gen. sp. 14TU").

It is of note that only four unnamed genomic species have been described in the International Journal of Systematic and Evolutionary Microbiology (formerly the International Journal of Systematic Bacteriology), the official journal of record for novel prokaryotic taxa. The remaining unnamed species were described in other journals and may have escaped the attention of some microbiologists.

Not only unnamed genomic species comprise a few strains only. Recently also seven named, environmental species were described which included at the time of publication 11 strains only (Carr *et al.*, 2003). It has been argued that species can be considered as a cluster of strains in a character space (Sneath, 1977) and, preferably, 25 but no less than 10 strains are needed to approximate the variation in a species. Thus, although phenotypic characteristics were given for the named and unnamed *Acinetobacter* species that include a few strains only, it will be difficult to identify new strains to these species since the variation within these species is not known. The current practice in taxonomy to describe novel named species based on one or a few strains is a matter of debate (Christensen *et al.*, 2001). A recent comparison of six genomes of a single species, *Streptococcus agalactiae*, has shown a considerable variation in gene content among these strains, giving rise to the term "pan-genome" to denote the overall gene content of a species (Tettelin *et al.*, 2005). This finding further emphasizes that the description of a single strain is not sufficient for inductive generalization about the characters of the species as a whole.

Many of the problems related to the taxonomy of *Acinetobacter* are temporary and can be solved in the near future by performance of a comparative study with strains of all

described species. Such a study would require that the number of strains included in the yet-low-strain-number-species is extended. As a result, an improved description of the species would be obtained and a proposal for names for species with readily identifiable characters could be made. However, having noted this, we are confronted with a major problem in *Acinetobacter* taxonomy, the lack of a practical identification scheme.

The difficulty of *Acinetobacter* species identification

The fact that the genus *Acinetobacters* has been split up into a large number of species but that no practical identification scheme for these species is available must be difficult to understand to applied microbiologists. Indeed, the lack of practical species identification methods is a serious obstacle in the development of knowledge on the biology, pathogenicity or ecology of acinetobacters at the species level. In the clinical setting, the problems of correct species identification may have consequences in diagnosis and infection control.

Only few methods have been validated for *Acinetobacter* species identification with large numbers of strains that had also been identified by DNA–DNA hybridization, the gold standard. These methods include a phenotypic system developed to identify the first 12 described genomic species (Bouvet and Grimont, 1986). The frequency matrix of this system has later been improved by inclusion of a collection of strains, all identified by DNA–DNA hybridization (Gerner-Smidt et al., 1991). Unfortunately, this system is not able to separate the genetically closely related species *A. calcoaceticus*, *A. baumannii*, and gen. sp. 3 and 13TU which gave rise to the suggestion to refer to these species as a group, the *A. calcoaceticus–A. baumannii* (ACB) complex (Gerner-Smidt et al., 1991).

Well validated genotypic identification methods are amplified 16S ribosomal DNA restriction analysis (ARDRA) (Vaneechoutte et al., 1995; Dijkshoorn et al., 1998) and high resolution fingerprint analysis by AFLP analysis (Janssen et al., 1997; Janssen et al., 1996; Nemec et al., 2001). By these methods, which are discussed in detail in the following paragraphs, the profiles of novel strains are compared to a library of profiles of reference strains for species identification. Despite their usefulness for identification, these methods are not widely used and can hardly be considered options for applied microbiology. A challenge for the immediate future is to search for more practical identification methods.

A brief introduction to taxonomy

Taxonomy is a specialized field and practiced by a minority of microbiologists, while the consequences of classification and the development of identification methods are important to all microbiologists. To help the reader understand the difficulties of classification and identification of the genus *Acinetobacter*, an outline of the basic concepts of taxonomy is provided in Box 1.2.

The diversity within the genus *Acinetobacter*

The inter- and intraspecific diversity in the genus *Acinetobacter* has been assessed with a variety of methods (Table 1.2). Some have been used for classification and/or species identification, while others have been used to assess relatedness below species level (typing). In the following paragraphs the diversity of the genus *Acinetobacter* as assessed by these methods is discussed.

Box 1.2 A summary of taxonomic practices

Taxonomy comprises three interrelated, sequential processes including classification, the giving of names (nomenclature) and identification. It is the task of the taxonomist to classify organisms into genera and species, to give names to these taxa and to provide criteria for their identification. Originally, classifications were based on phenotypic relationships of organism. However, these classifications do not always correlate with the genetic relatedness of organisms. The aim of current taxonomy is to provide natural classifications that would reflect the phylogenetic origin of organisms. It is assumed that relatedness derived from the genetic material or other conserved macromolecules is useful to assess phylogenetic relationships. Ideally, the complete DNA sequence of organisms would be the reference standard to determine phylogeny, but—although great progress in this field is being made—up to now either global comparisons of chromosomal DNA have to be made by DNA–DNA hybridization, or particular sequences are used.

In biology, the species is the basic unit of classifications but the prokaryotic species concept is a continuous source of debate (Cowan, 1965; Moreno, 1997; Dijkshoorn *et al*., 2000; Rossello-Mora and Amann, 2001; Cohan, 2002; Gevers *et al*., 2005). The following definition "a species is a category that circumscribes a (preferably) genomically coherent group of individual isolates/strains sharing a high degree of similarity in (many) independent features, comparatively tested under highly standardized conditions" (Rossello-Mora and Amann, 2001) has the approval of an ad-hoc committee for the re-evaluation of the species definition in bacteriology (Stackebrandt *et al*., 2002). Furthermore, it has been agreed that the parameters DNA–DNA similarity and, whenever determinable, ΔTm—the difference in melting temperature between homologous and heterologous duplexes—remain the standard for species delineation (Wayne *et al*., 1987; Grimont *et al*., 1980). In practice, apart from DNA homology studies, the description of novel species is further substantiated by 16S rDNA sequence analysis, and extensive phenotypic characterization, in a so-called polyphasic approach (Colwell, 1968; Vandamme *et al*., 1996). Although DNA–DNA hybridization remains the standard, it is generally agreed that there is a need for alternative genomic methods for species delineation, provided that they are validated with collections of strains for which DNA–DNA similarity data are available (Stackebrandt *et al*., 2002).

Where only recommendations can be given to delineate novel species, there are strict rules for the giving of names. The process of giving names, nomenclature, is laid down in the International Code of Nomenclature of Bacteria (Lapage S.P. and Sneath, 1992). This code has to be followed for a name to be valid. One living example strain of a novel species has to be designated to be the type strain and a precise description of this strain is required when the species is proposed. A subculture of this type has to be deposited at two Public Culture Collections like, e.g., the American Type Culture Collection (ATCC) and the National Collection of Type Cultures (NCTC).

Valid descriptions of novel species and genera are published in the International Journal of Systematic and Evolutionary Microbiology (IJSEM), formerly the International Journal

of Systematic Bacteriology (IJSB), which is the official journal of record for novel prokaryotic taxa. The names of newly described taxa in other scientific journals will not be valid until reported in IJSEM. The list of bacterial names with standing in nomenclature is available on the Internet (http://www.bacterio.cict.fr/) (Euzeby, 1997).

The development of the classification of a genus is a continuous, long lasting process during which novel species may be added or during which a genus may be split up. Methods to delimit the new taxa differ over time which may provide an unclear picture since—in the end—there may not be a comprehensive scheme for identification of all species in a genus.

It is further of note that the activity of identification and development of identification schemes is not the exclusive field of the taxonomist. Once a classification and description of a genus is available, additional identification methods may be developed in applied microbiology. A prerequisite for a reliable identification method is that it has been validated by using a sufficient number of reference strains of the described species.

DNA–DNA hybridization

Despite rapid advances in DNA-sequence based approaches, DNA–DNA hybridization is still the gold standard to assess relatedness of bacteria. By this methodology the whole genomes of strains are compared. The similarity can be estimated as a percentage of relative binding or as differences in thermal stability of the hybrids. Although DNA hybridization has great value in bacterial taxonomy—for example, the current taxonomy of *Acinetobacter* is primarily based on classification by DNA–DNA similarity—it has also several disadvantages. First, different hybridization results can be obtained with different methods (Grimont *et al.*, 1980). It is of note in this context, that no less than five different methods or protocols have been used in studies describing novel *Acinetobacter* species. These included (i) the S1 endonuclease method in which the S1 nuclease hydrolyses single stranded DNA leaving double stranded DNA intact for quantification (Bouvet and Grimont, 1986), (ii, iii) a modified hydroxyapatite (HA) method in which single stranded DNA is separated from double stranded DNA by HA, with either radioactive labeling (Tjernberg and Ursing, 1989) or with non-radioactive labeling of DNA (Carr *et al.*, 2003), (iv) a quantitative dot method with a two-step elution which quantifies the DNA released from a filter by denaturation of the duplex at two temperature steps (Nemec *et al.*, 2001), and (v) a microplate methods with immobilized unlabelled DNA and hybridization with photo-activable biotin labeled DNA (Nemec *et al.*, 2003).

Another disadvantage of DNA hybridization is that it is laborious and technically demanding. Consequently, it is performed on a limited scale only, which seriously impedes the development of bacterial taxonomies. For example, ideally, to resolve relatedness within a genus, DNAs of all species, and of representatives of novel species have to be hybridized reciprocally pairwise. In practice, only selected strains are investigated by hybridization and it has become common practice to first compare strains for similarity of the 16S rDNA sequence and use only strains for DNA–DNA hybridization if they have interspecies 16 rDNA nucleotide sequence similarities > 97% (Stackebrandt and Goebel, 1994). This "rule of the thumb" cut-off value above which strains should be investigated for their

Table 1.2 Methods used to assess the diversity of the genus *Acinetobacter* and their application

Method	Target structure	Application*	Reference**
DNA–DNA hybridization	Whole genome	Classification	1, 2, 3, 4, 5
DNA sequence analysis	16S rDNA	Phylogenetic analysis/ identification	6
	gyrB	Phylogenetic analysis/ identification	7
	recA	Phylogenetic analysis/ identification	8
	rpoB	Phylogenetic analysis/ identification	9
	16S-23S spacer rDNA	Phylogenetic analysis/ identification	10
MLST	Seven housekeeping genes	Assessing population diversity, typing	11
PCR-RFLP	16S rDNA	Identification	12, 13
	recA	Identification	8
	16S-23S spacer rDNA	Identification	14
tRNA spacer PCR fingerprinting	tRNA spacer	Identification	15
Oligonucleotide probes	16S-23S spacer rDNA	Identification of *A. baumannii* and gen. sp. 3	16
Ribotyping	rDNA and adjacent regions	Identification of ACB complex spp, typing	17
AFLP analysis	Whole genome	Classification, identification, typing	3, 18
RAPD analysis	Whole genome	Typing	19
PFGE	Whole genome	Typing	20
Cell envelope protein SDS-PAGE	Cell envelope proteins	Identification, typing	21, 22
LPS analysis	LPS	Identification, typing	23

* "Identification" corresponds to the identification to species while "typing" means the identification at the strain level.

** The cited references are either pioneer papers or comprise well validated protocols. 1, Bouvet and Grimont 1986; 2, Tjernberg and Ursing 1989; 3, Nemec *et al*., 2001; 4, Nemec *et al*., 2003; 5, Carr *et al*., 2003; 6, Vaneechoutte and De Baere, this volume; 7, Yamamoto *et al*., 1999; 8, Krawczyk *et al*., 2002; 9, La Scola *et al*., 2006; 10, Chang *et al*., 2005; 11, Bartual *et al*., 2005; 12, Vaneechoutte *et al*., 1995; 13, Dijkshoorn *et al*., 1998; 14, Dolzani *et al*., 1995; 15, Ehrenstein *et al*., 1996; 16, Lagatolla *et al*., 1998; 17, Gerner-Smidt 1992; 18, Janssen *et al*., 1997; 19, Grundmann *et al*., 1997; 20, Seifert and Gerner-Smidt 1995; 21, Dijkshoorn *et al*., 1987; 22, Dijkshoorn *et al*., 1990; 23, Pantophlet *et al*., 2002.

genomic uniqueness by DNA–DNA hybridization has recently been overturned, and a similarity range of 98.7–99% forms the new threshold (Stackebrandt and Evers, 2006).

DNA–DNA hybridization was—apart from being used for delineation of novel *Acinetobacter* species—also used to assess relatedness between several *Acinetobacter* species (Tjernberg and Ursing, 1989). Thus, as already mentioned, *A. calcoaceticus*, *A. baumannii* and the unnamed species 3 and 13TU (together the ACB complex) were so closely related that "lumpers" in taxonomy would like to consider them as one species. Other species that were relatively closely linked together by DNA–DNA hybridization, albeit at lower levels than within the ACB complex, were gen. sp. 10 and 11, and *A. haemolyticus* and gen. sp. 6 (Tjernberg and Ursing, 1989). Furthermore, hemolytic unnamed genomic species 13–15BJ and 16–17 have been reported to be relatively highly related by DNA–DNA hybridization (Bouvet and Jeanjean, 1989).

It is concluded that DNA–DNA hybridization is a crucial method for the current classification of species within the genus *Acinetobacter*. However, due to its complexity and the large number of hybridization groups within the genus, DNA-hybridization can hardly be considered an option for the delineation of additional novel species, let alone as a method for species identification. This is a problem of general importance in taxonomy and emphasizes the recommendations of the *ad hoc* committee for the re-evaluation of the species definition in bacteriology that other genomic methods for species delineation are required (Stackebrandt *et al.*, 2002).

DNA sequence based analysis

Bacterial taxonomy, originally an intuitive process, is currently dominated by the view that phylogeny should determine taxonomy (Wayne *et al.*, 1987). The use of particular DNA sequences to assess the (phylogenetic) relatedness between organisms has undergone an explosive development over the past decades. The general assumption is that the phylogeny of these sequences—as inferred from their similarity—is congruent with the phylogeny of the organisms of study. Sequences can be generated at any location in the world and be added to international databases and used at any location to deduce the phylogeny/taxonomic position of organisms. Thus, in applied microbiology, identification of local isolates is increasingly achieved by comparing a sequence to those in a database, e.g., http://www.ridom.de (Harmsen *et al.*, 2002). The 16S rRNA gene is the most widely used sequence in microbial classification and phylogenetics, but other highly conserved genes including protein-coding genes may provide a higher resolving capacity to classify closely related species. With the increased availability of complete genomes it will be possible to develop systems based on multiple protein-coding genes (multilocus sequence analysis, MLSA) for species delineation (Gevers *et al.*, 2005).

For *Acinetobacter*, several DNA sequences have been used to study interspecies relationships and/or the usefulness of these sequences for species identification. The sequences include the 16S rDNA sequence (Ibrahim *et al.*, 1997; Vaneechoutte *et al.*, 2006); (Vaneechoutte and De Baere, this volume), the 16S-23S spacer region (Chang *et al.*, 2005), *recA* which encodes the recA protein (Krawczyk *et al.*, 2002), *gyrB*, the structural gene for the DNA gyrase B subunit (Yamamoto *et al.*, 1999), and *rpoB*, RNA polymerase β-subunit gene (La Scola *et al.*, 2006). In addition, a multilocus sequence typing scheme

based on seven housekeeping genes (Bartual *et al.*, 2005) has been described, but this is primarily aimed to assess the diversity within *A. baumannii*.

A detailed discussion of 16S rDNA sequence analysis for *Acinetobacter* is given by Vaneechoutte and De Baere in this volume. Figure 1.1 shows the frequency distribution of interspecies 16S rDNA sequence similarity values of strains representative of 31 genomic species. The frequencies were calculated from pairwise comparisons of sequences of type and reference strains of these 31 species (kindly provided by Vaneechoutte and DeBaere). The similarity values among this set of strains ranged from 94.1–99.6%. Seventeen out of 453 similarities (3.7%) were at or above the 98.7% threshold recently proposed (Stackebrandt and Evers, 2006), the cut-off for recognition of strains that may represent novel species. Thus, these 3.7% of pairs of strains with high 16S rDNA sequence similarities were found to belong to distinct species by DNA–DNA hybridization. The lack of congruence between DNA homology and 16S rDNA sequence similarity may also occur the other-way-around as is illustrated by the 16SrDNA sequence analysis of species of the ACB complex (Vaneechoutte and De Baere, this volume). In the tree based on 16SrDNA similarities, *A. baumannii* and unnamed sp. 13TU, and *A. calcoaceticus* and unnamed sp.
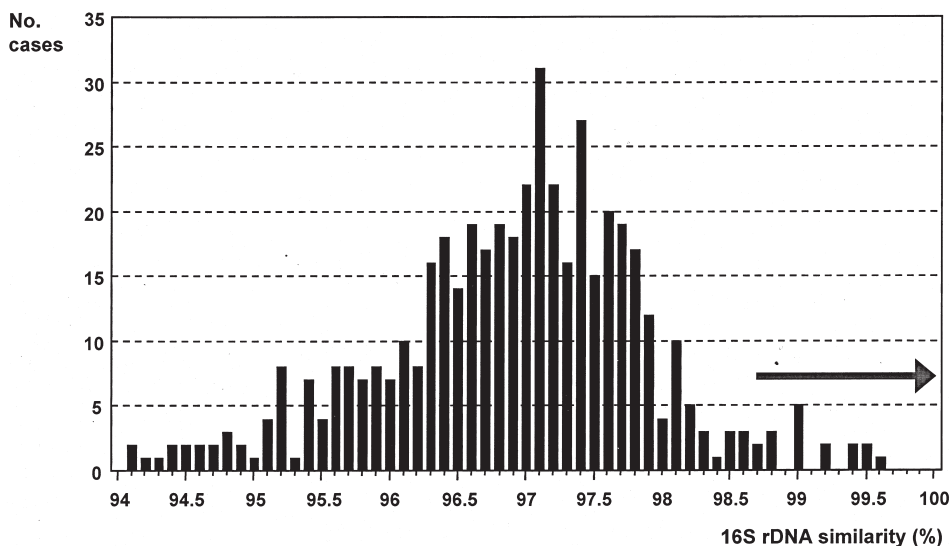

Figure 1.1 Distribution of interspecies 16S rDNA sequence similarity values obtained for pairs of the type or reference strains of 31 known (genomic) species of the genus *Acinetobacter*. The arrow indicates the range of similarity values, which may reflect species identity of compared organisms (Stackebrandt and Ebers, 2006). Out of all possible (465) sequence pairs, the following 17 pairs revealed a similarity value of equal or higher than 98.7%: *A. radioresistens* and "*A. venetianus*" (98.7%); *A. johnsonii* and gen. sp. 16 (98.7%); *A. junii* and gen. sp. 13TU (98.8%); *A. calcoaceticus* and gen. sp. CT13TU (98.8%); *A. johnsonii* and gen. sp. 15BJ (98.8%); *A. junii* and *A. grimontii* (99.0%); gen. sp. 3 and CT13TU (99.0%); gen. sp. 1–3 and CT13TU (99.0%); *A. haemolyticus* and gen. sp. 16 (99.0%); *A. haemolyticus* and *A. johnsonii* (99.0%); *A. calcoaceticus* and gen. sp. 1–3 (99.2%); *A. haemolyticus* and gen. sp. 15BJ (99.2%); gen. sp. 10 and 11 (99.4%); *A. calcoaceticus* and gen. sp. 3 (99.4%); gen. sp. 15TU and 16 (99.5%); gen. sp. 3 and 1–3 (99.5%); *A. baumannii* and gen. sp. 13TU (99.6%)

3, are in different branches while they have 65% DNA–DNA similarity. This 16S rDNA clustering is not consistent with the phylogenetic trees derived from other sequences either (Yamamoto *et al.*, 1999; La Scola *et al.*, 2006; Krawczyk *et al.*, 2002). These findings suggest that 16S rDNA sequence analysis may neither reflect the phylogenetic relationship of some *Acinetobacter* species correctly nor be a reliable tool for their differentiation.

Sequence analysis of the 16S-23S rRNA gene intergenic spacer region (ITS) has led to the discovery of short sequences specific for *Acinetobacter* spp., *A. baumannii* and gen. sp. 3. Probes corresponding to these sequences appeared useful for identification of these species in a hybridization assay (Lagatolla *et al.*, 1998). Comparative analysis of the complete 16S-23S rRNA gene spacer sequence and small fragments of the flanking regions of strains of 10 named and 11 unnamed genomic species revealed intraspecies similarities ranging from 0.52 to 0.92 (Chang *et al.*, 2005). Relatively high interspecies similarities were observed for genomic species 10 and 11 (0.88), *A. calcoaceticus*, *A. baumannii*, and gen. sp. 3 and 13TU of the ACB complex (0.86–0.92), and between gen. sp. 14TU, 14BJ, 15BJ, 16 and 17 and "*A. venetianus*" (0.81–0.92). Intraspecies similarities for strains of the ACB complex was 0.99–1.00, except for 13TU. It was concluded that the ITS sequence was a useful marker for strains of the ACB complex with an identification rate of 96.2

The phylogenies from the cited studies of the sequences of 16S rDNA, 16S-23S rRNA gene spacer, *recA* and *gyrB* are not fully comparable since different strain sets were used. A common finding is, however, the clustering tendency of hemolytic gen. sp. species 13–15BJ, 16 and 17, gen. sp. 14TU, *A. haemolyticus* and gen. sp. 6, of *A. lwoffii* and gen. sp. 9, and of gen. sp. 10 and 11. The study of the *rpoB* gene (La Scola *et al.*, 2006) revealed that partial sequences of this gene and its flanking spacers are promising for *Acinetobacter* species identification.

Multilocus sequence typing (MLST) is a relatively novel approach of comparative analysis of sequences of housekeeping genes of strains within a species to study their epidemiological relatedness or population structure (Enright and Spratt, 1999). The advantage of the system is that the data are transportable and that international databases of clinically important species have been set up (http://www.mlst.net/). Two MLST systems have been developed for *A. baumannii*. One system used 305- to 513-bp internal fragments of seven housekeeping genes—*gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi* and *rpoD* (Bartual *et al.*, 2005). The collection of tested strains was relatively small and from a few cities only, while some genes were at relatively short distances from each other. Nevertheless, results correlated well with typing by AFLP and pulsed field gel electrophoresis and the method may have useful applications in population studies of *A. baumannii*. An alternative, very rapid multilocus system with great potential is based on PCR amplification of information-rich regions of the *trpE*, *adk*, *mutY*, *fumC*, and *ppa* genes followed by electrospray ionization mass spectrometry (PCR/ESI-MS) (Ecker *et al.*, 2006). This method detects amplicon base compositions to type the organisms. A good correlation with PFGE typing was found and several species including *A. baumannii*, unnamed genomic species 3 and 13TU could be identified correctly.

In summary, several nucleotide sequences have been used for classification of organisms and for identification of organisms to species by comparing their sequence with those of described species. Two MLST systems which are primarily for intraspecies diversity studies have been developed for *A. baumannii*, while one of the two was also applicable to

the closely related genomic species 3 and 13TU. Inclusion of other species with the aim of species identification will largely depend on the finding of consensus anchor sites for primers in information-rich regions of suitable genes.

Restriction analysis of PCR amplified DNA sequences (PCR-RFLP)

PCR-RFLP of DNA sequences assumed to be phylogenetic or taxonomic markers is an easy method to generate profiles for characterization of organisms. By this approach, conserved sequences are amplified by PCR and the amplification products are digested with one or several restriction enzymes. The restriction fragments obtained are separated by agarose electrophoresis; the resulting restriction patterns can be used to create a library for identification. Several sequences have been the target for such an approach including the 16S rDNA sequence (Vaneechoutte *et al.*, 1995; Dijkshoorn *et al.*, 1998), the nearly complete 16–23s rDNA sequence (Garcia-Arata *et al.*, 1997), the 16S-23S intergenic-spacer sequence (Dolzani *et al.*, 1995), and the *recA* gene (Nowak and Kur, 1996; Jawad *et al.*, 1998; Krawczyk *et al.*, 2002).

Amplified ribosomal DNA restriction analysis (ARDRA)

ARDRA has appeared a powerful method for species identification of acinetobacters. Five enzymes, CfoI, AluI, MboI, RsaI and MspI, eventually supplemented with BfaI and BsmaI, are used for restriction; electrophoretic fragment separation is done in 2.5% or 3% agarose gels. Restriction patterns are examined visually and compared to a library of profiles of reference strains of described species to assign the profiles a code conform the reference list of the library.

In an exploratory study, ARDRA profiles of 53 strains of all 18 species at the time known were determined (Vaneechoutte *et al.*, 1995). In a next study, the database of profiles was extended to 202 strains of the same number of species (Dijkshoorn *et al.*, 1998) (http://users.ugent.be/~mvaneech/ARDRA/Acinetobacter.html). All strains had been identified to species by DNA–DNA hybridization, the gold standard. This study showed that multiple profiles may occur in one species indicating sequence polymorphism within species. On the other hand, common profiles were found in a number of different species. In this case supplementation with a few phenotypic tests (Vaneechoutte *et al.*, 1995) or all tests of the scheme of Bouvet and Grimont (1986)—an approach designated "consensus identification" (Nemec *et al.*, 2000)—can lead to definitive identification. More recently, ARDRA profiles of the novel species *A. ursingii* and *A. schindleri* and *A. parvus* have been reported as characters for identification for these species (Nemec *et al.*, 2001; Nemec *et al.*, 2003). Further ongoing research has revealed numerous additional, new profiles or new combinations of restriction patterns (L. Dijkshoorn, unpublished results). Most of these profiles are indicative for novel species, but others were found in already described species, which emphasizes that the polymorphism of the 16S rDNA sequence in *Acinetobacter* is considerable.

16S-23S rRNA intergenic spacer RFLP

Dolzani *et al.* (1995) showed that restriction analysis of the 16S-23S rRNA intergenic spacer region was a promising method for identification of *Acinetobacter* isolates of the ACB complex. It is of note that with Southern blotting five to six target sequences in

the genomic DNA of tested strains were identified while only one fragment per strain was detected by 16S-23S spacer amplification. It was concluded that the length of the sequences is well conserved both between copies in the same chromosome and between isolates of the same species.

RecA-RFLP

The *recA* gene, has also been investigated as a marker for PCR-RFLP identification of *Acinetobacter* species. An initial study with type and reference strains of 17 genomic species and MboI and HinfI were promising (Nowak and Kur, 1996), but another study with a greater number of strains per species was disappointing (Jawad *et al.*, 1998). In a further study, a larger number of strains and three enzymes, HinfI, MboI and Tsp509I were used (Krawczyk *et al.*, 2002). *In-silico* restriction analysis of the sequences digested with the respective enzymes were followed by "wet" laboratory experiments. Tsp509I was found to be the most discriminatory enzyme, generating unique profiles for each of 23 genomic species with 43 reference strains tested.

Conclusions regarding PCR-RFLP for species identification

Altogether, PCR RFLP of conserved genes is a useful and relatively easy-to-perform method for species identification. ARDRA is the method most extensively tested on a large numbers of reference strains, but restriction with five enzymes makes it also a laborious method. As is the case with any definitive identification system, a new strain can only be identified by any of the PCR-RFLP methods if a database of reference strains of the different species is available and if the profile of this strain is included in this database.

Ribotyping

Ribotyping is a special application of Southern blotting. By this method, bacterial genomic DNA is digested, followed by electrophoretic fragment separation and transfer of fragments to a membrane. Next, hybridization with a labeled probe specific for ribosomal DNA is done. The resulting profiles can be species or strain specific. Gerner-Smidt pioneered the method for *Acinetobacter* with EcoRI, ClaI and SalI as restriction enzymes and a digoxenin-11-UTP labeled cDNA probe derived from 16S and 23S rRNA of *Escherichia coli* (Gerner-Smidt, 1992). Since, it has been used in numerous studies to type strains. The method is robust and profiles can be compared between laboratories. Species of the ACB complex could be separated by ribotyping, emphasizing its usefulness for identification of these species, but—as a typing method—ribotyping was found less discriminatory than pulsed field gel electrophoresis (PFGE) typing (Seifert and Gerner-Smidt, 1995). Ribotyping with HindIII and/or HincIII has been found in concordance with AFLP and protein typing to identify epidemic clones of *A. baumannii* strains circulating in Europe (Dijkshoorn *et al.*, 1996; Nemec *et al.*, 2004) (Figure 1.2). An automated ribotyping system, RiboPrinter®, has been used in several studies to type acinetobacters with EcoRI as restriction enzyme (Brisse *et al.*, 2000; van Dessel *et al.*, 2004).

In summary, ribotyping is a robust method for identification of species, clones and strains of the ACB complex. At the strain level its discriminatory capacity is relatively limited, probably due to the fact that the ribosomal DNA and the flanking regions it detects are relatively stable over time. It is also quite a laborious method and has gradually been replaced by other methods like, e.g., pulsed field gel electrophoresis.
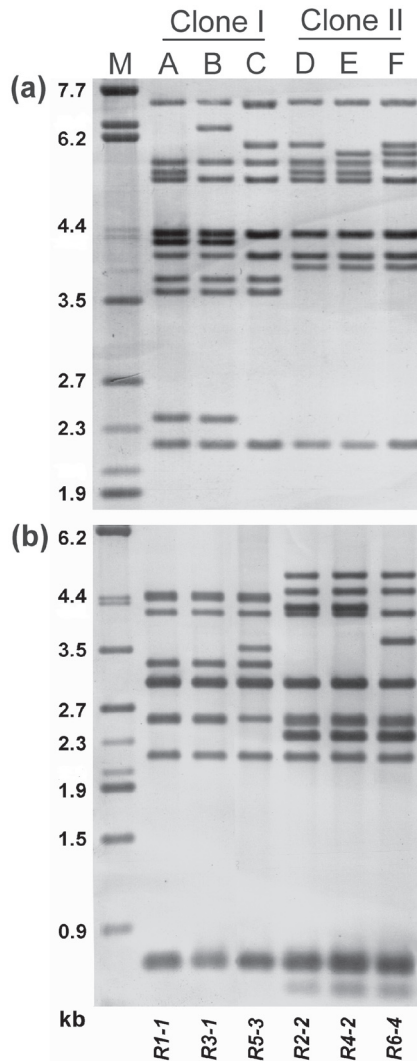
Figure 1.2 Ribotypes of *Acinetobacter baumannii*. HindIII (a) and HincII (b) ribotypes typical of *A. baumannii* European clone I and II. Strains are indicated by the upper-case letters above the lanes: A, NIPH 7; B, NIPH 1605; C, NIPH 10; D, NIPH 24; E, NIPH 1362; F, NIPH 657. M, molecular size markers (phage λ DNA digested with HindIII and StyI). Combined ribotype designations are given below the lanes. Ribotypes *R1–1* and *R2–2* have been most frequently found among isolates of clone I and II, respectively.

Genome analysis by selective amplification of restriction fragment, AFLP™

General aspects
AFLP is the name for a high resolution genomic fingerprinting method that can be used on any DNA disregarding its origin (Vos *et al.*, 1995). Janssen pioneered the method for bacteria including acinetobacters (Janssen *et al.*, 1996). The method comprises the following steps: digestion of the cellular DNA with one or two enzymes and ligation of adaptors to the restriction fragments, selective amplification of fragments, electrophoretic

separation of amplified fragments, and visualization of profiles. Primers consist of the adaptor-derived core sequence, the 3′-part of the restriction half-site, and an extension sequence of one or more selective nucleotides at the 3′-end of the primer. Amplification starts at a high annealing temperature (63°-65°C) and elongation will only take place if the selective nucleotides are complementary to those of the target molecule.

The method can be designed for fragment separation by conventional agarose electrophoresis (Valsangiacomo *et al.*, 1995), or—with radioactive or fluorescent primer labeling—for separation by sequencing systems with autoradiography or fluorescence detection systems. Currently, AFLP is most frequently performed as a semi-automated procedure with fluorescent primer labeling and laser detection of fragments on sequencing machines. The resulting, complex profiles comprising several tens to > 100 bands are analyzed by computer assisted cluster analysis resulting in dendrograms with strains grouped together according to similarity in banding patterns.

Application of AFLP analysis
AFLP analysis has appeared a most important technique in resolving the diversity within the genus *Acinetobacter*. Initially, a protocol was used with HindIII and TaqI as restriction enzymes, and with primers T05 and 32P-labelled H01 with two and one adenosines as 3′ extensions, respectively (Janssen *et al.*, 1996). With this protocol, 151 reference strains of 18 named and unnamed species were allocated to the correct (genomic) species (Janssen *et al.*, 1997). All groups were properly separated and intraspecies grouping for most species was around 45%. The procedure was also useful to identify outbreak strains (Janssen and Dijkshoorn, 1996) and groups of highly similar, multidrug resistant strains from different locations in Europe that were postulated to represent clonal lineages (European clone I and II) (Dijkshoorn *et al.*, 1996).

Simplification of the AFLP procedure was achieved with EcoRI and MseI as restriction enzymes which offered the possibility to perform digestion and ligation in a single step (Koeleman *et al.*, 1998). From 2000 onward, the Leiden University Medical Center (LUMC) used a protocol with these restriction enzymes. A Cy-5 labeled EcoRI+A and Mse+C (A and C = selective nucleotides) primer were used for amplification. Fragments are separated on the ALF express sequencing machine (Pharmacia, Roosendaal, Netherlands) with automated laser detection. Images in Tiff format are used for BioNumerics pattern analysis (Applied Maths, Sint-Martens-Latem, Belgium) with Pearson product moment correlation coefficient as similarity measure and the unweighted pair group average linkage method (UPGMA) as clustering algorithm. With this protocol, more than 2000 *Acinetobacter* strains have been processed and the resulting AFLP database is a powerful resource for studying relatedness of strains at different levels.

Using this system, the overall grouping level for identification of species was found to be around 50% derived from analysis of type and reference strains of all described (genomic) species (Figure 1.3). At this "species delineation" clustering level, three novel species could be identified with AFLP analysis, which was confirmed by other methods including DNA–DNA hybridization (Nemec *et al.*, 2001; Nemec *et al.*, 2003). AFLP analysis of the c. 2000 profiles of the LUMC database by November 2006 has revealed the presence of at least 31 additional putative species and 26 unique strains at the 50% clustering level (Dijkshoorn, unpublished data). Some described species had a relatively high linkage level
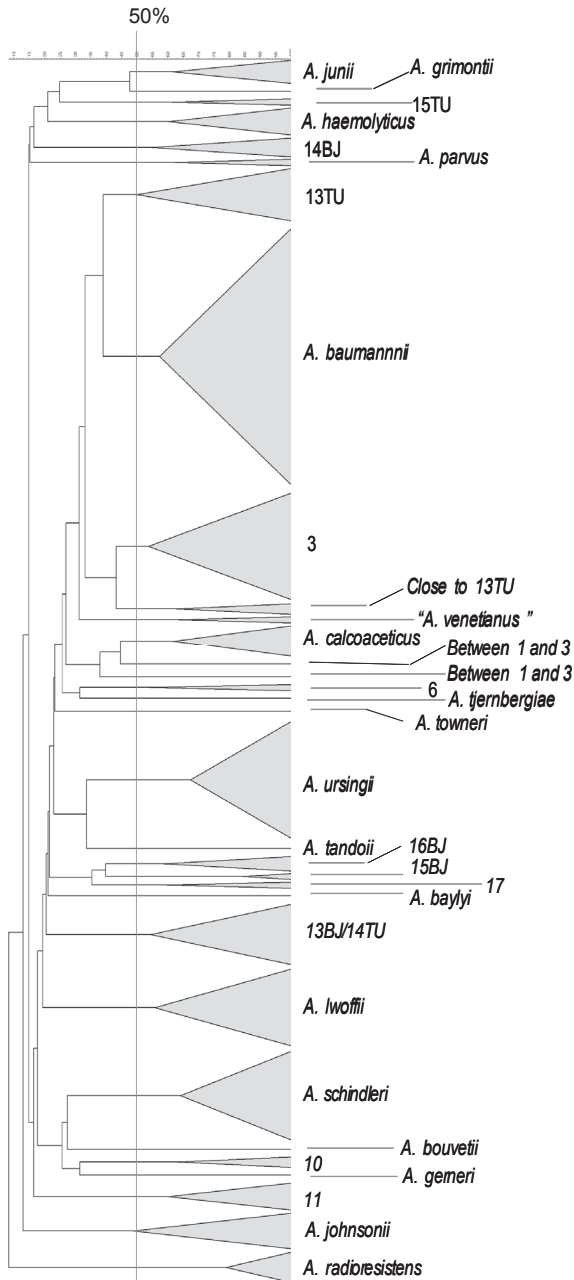
Figure 1.3 Dendrogram of cluster analysis of AFLP profiles of 267 type and reference strains of the 31 described named and unnamed (genomic) species of *Acinetobacter*. All strains have been allocated to species by DNA–DNA hybridization. Similarities between all possible pairs of patterns were expressed by the Pearson product moment correlation coefficient. Strains were clustered by using the unweighted pair group average (UPGMA) linkage method. The vertical line indicates the 50% cluster cut-off level above which strains of the same species are linked together.

including e.g. *A. radioresistens* (79%) (Figure 1.3), and *A. baylyi* (78%) (Vaneechoutte *et al.*, 2006) indicating a high degree of homogeneity. AFLP analysis was also useful for identification of *A. baumannii* outbreaks with a clustering level of strains from the same outbreak being well above 90% (Wroblewska *et al.*, 2004; Bernards *et al.*, 2004; Dobrewski *et al.*, 2006) (Figure 1.4a). Within *A. baumannii*, clusters of similar strains were found that grouped together at a level of ≥ 80%. These strains were also similar in other characters as noted by methods like PFGE or protein electrophoresis and were considered to represent clonally related strains. Thus, AFLP analysis appeared an important tool in the identification and spread of multidrug resistant clones of *A. baumannii* in European countries, including European clone I–III [(Nemec *et al.*, 2004; van Dessel *et al.*, 2004), the South East England clone (Turton *et al.*, 2004), and recently, a Portuguese clone (Da Silva *et al.*, 2007). An overview of the grouping patterns of strains of EU clones I–III and some unrelated strains is given in Figure 1.4b. It is of note that there are differences in clonal grouping levels—e.g. strains in clone III are linked > 90%—which may be related to the time–space frame of origin of strains and the associated degree of diversification within the lineage.

It can be concluded that AFLP analysis is a robust method which makes it suitable for setting up a database of fingerprints. Such a system is a powerful tool for longitudinal comparison of large numbers of strains and for species, clone and strain identification. The time of processing of c. 30 strains is about four days including computer analysis, although the actual hands-on time is less. A disadvantage is that the data are difficult to transport between laboratories, mainly due to different sequencing platforms.

Macrorestriction analysis with pulsed field gel electrophoresis

Digestion of genomic DNA with rare cutting enzymes generates large DNA fragments which can be separated by pulsed field gel electrophoresis (PFGE). The most widely used application of PFGE is contour-clamped homogeneous electric field (CHEF) electrophoresis. With this system, fragments migrate zigzag-wise according to size through an agarose matrix in response to electric fields that alternate at an angle of, usually, 120°.

Although DNA sequence-based methods are now emerging rapidly, PFGE is still the method of choice for epidemiological typing of many microorganisms. It has been used for typing of *Acinetobacter* strains in numerous studies, usually with ApaI or SmaI as restriction enzyme. When applied on a set of strains of the ACB complex, PFGE with ApaI was found to be more discriminating than ribotyping (Seifert and Gerner-Smidt, 1995). In contrast to ribotyping it was not useful for taxonomic identification of the species in the complex. A comparative study of a selection of *Acinetobacter baumannii* strains, performed by three laboratories, showed that PFGE profiles can be compared between laboratories if the procedure is rigorously standardized (Seifert *et al.*, 2005). Thus, this standard procedure offers the opportunity to set up an international database for monitoring strains spreading regionally or globally. An analysis of *A. baumannii* strains of European clones I–III showed that the grouping of strains according to their PFGE profiles generated with this protocol largely agreed with their allocation to the respective clones, although there were exceptions (Figure 1.5) (Dijkshoorn, unpublished results).

In hospital epidemiology, it is common practice to consider strains with few—e.g. up to three—PFGE band differences as closely related and between four and six band
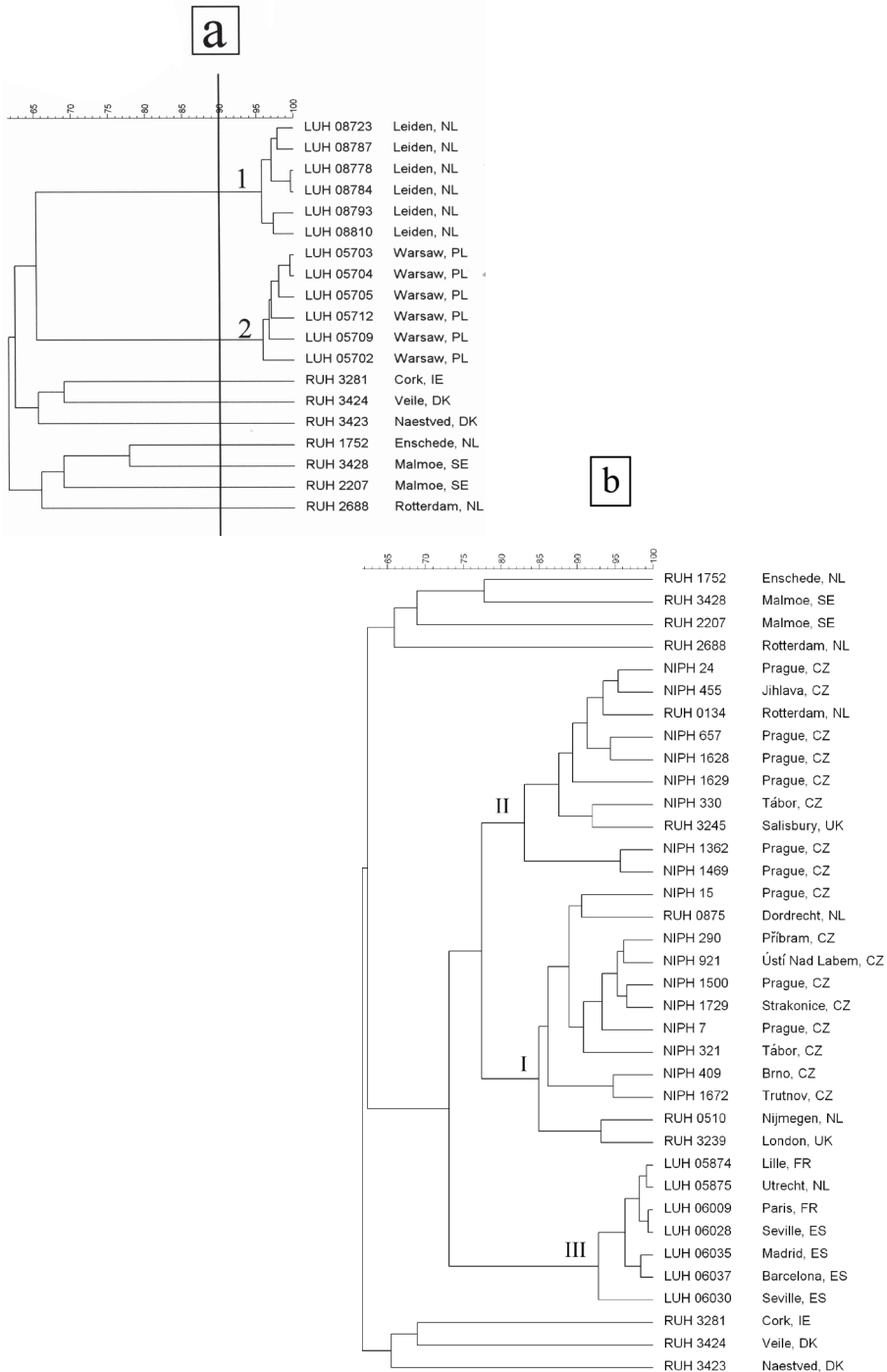
Figure 1.4 Identification of epidemic strains and clones of *Acinetobacter baumannii* by AFLP analysis. (a) Isolates from each of two outbreaks (1) and (2) linked > 90%, while they were distinct from seven remaining, unrelated strains; (b) strains in groups I–III represent the corresponding EU clones I–III, respectively, and linked at > 80%.
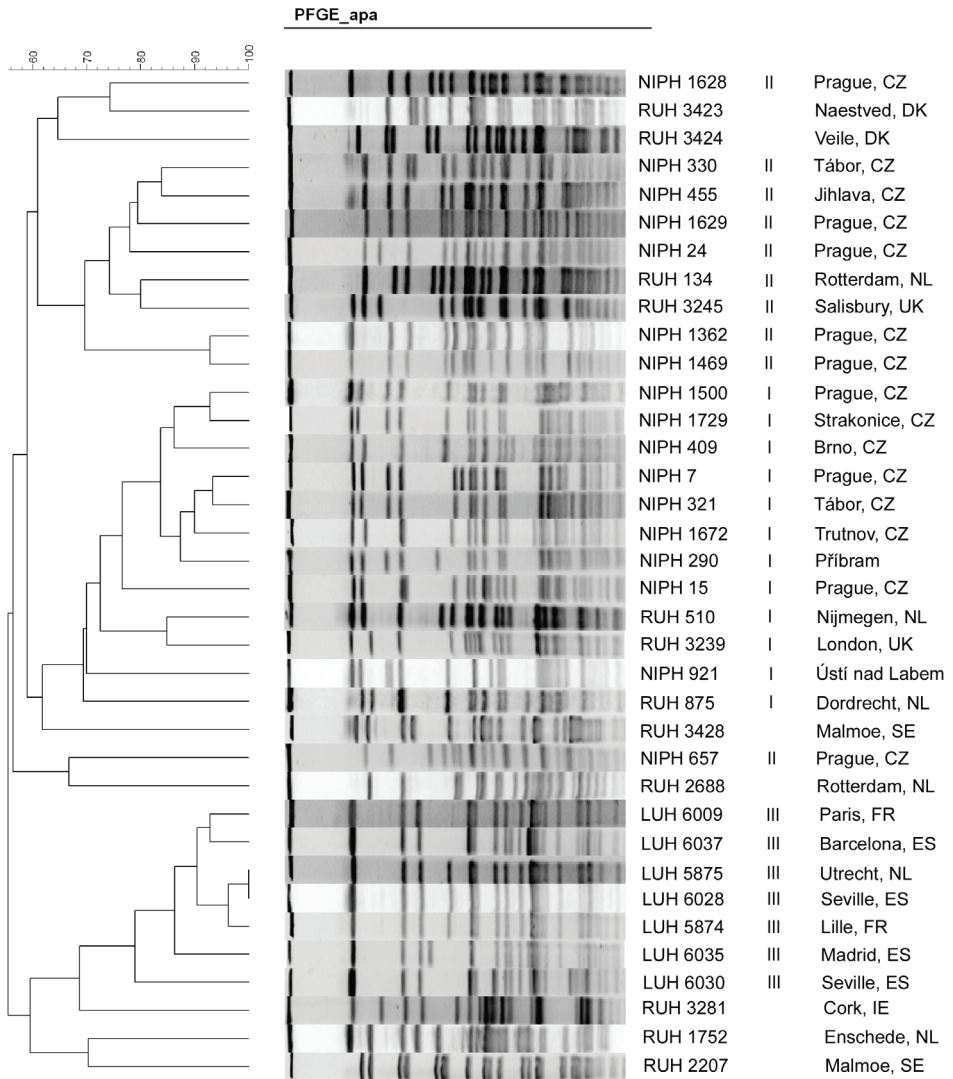
Figure 1.5 Pulsed field gel electrophoresis profiles of *A. baumannii* strains grouped according to similarity. The strains are the same as those in Figure 4. Strains of respective clones I–III showed a clustering tendency according to their lineage, but there were exceptions. The Dice coefficient was used to calculate similarity; grouping was obtained with the UPGMA method.

differences as possibly related during outbreaks (Tenover *et al.*, 1995). To our knowledge no systematic study has yet been performed to assess the band variation of acinetobacters during outbreaks or endemic episodes. In the light of the occurrence of clonally but not directly epidemiologically related strains, typing results to assess sources and mode of spread have to be interpreted with caution.

RAPD analysis and tDNA fingerprinting

RAPD analysis
One of the most easy-to-perform genotypic methods to assess strain relatedness is PCR fingerprinting by random amplification of polymorphic DNA (RAPD) analysis. The primer anchor sites for PCR may vary in number and location over the genome and, consequently, the number and sizes of amplified fragments may vary among strains. Thus, the electrophoretic RAPD profiles can be used to type strains. A variety of primers and protocols have been used for epidemiological typing of acinetobacters (Graser *et al.*, 1993; Reboli *et al.*, 1994; Webster *et al.*, 1996; Snelling *et al.*, 1996). With the introduction of commercial, standardized reagents it was a challenge to standardize RAPD fingerprint analysis to such a degree that profiles could be compared and exchanged between laboratories. Thus, in a multicenter study comprising seven laboratories four PCR protocols with different primers were compared for a set of 40 isolates of the ACB complex (Grundmann *et al.*, 1997). Epidemiologically related isolates grouped between laboratories at a similarity coefficient (SAB) of ≥ 0.7. In a follow-up, attempts by three centers to improve the level of interlaboratory reproducibility were disappointing (Seifert, Dolzani and Dijkshoorn, unpublished results). Nevertheless, PCR with standardized reagents and the respective primers DAF4 and core sequence of phage M13 with conditions as described (Grundmann *et al.*, 1997) provides clear banding patterns (Figure 1.6). These patterns are strain specific and useful for epidemiological typing (Wroblewska *et al.*, 2004). Thus, RAPD analysis is a useful approach to resolve urgent questions regarding the possible epidemiological relatedness of small sets of strains at the hospital level. However, it is not useful for longitudinal or interlaboratory studies with large numbers of strains.

Repetitive-DNA-element PCR fingerprinting with (GTG)$_5$-primer
Study results from a laboratory with, to our knowledge, outstanding experience in intralaboratory standardizaton have shown that PCR fingerprints can be sufficiently robust to set up a (local) database. Thus, PCR fingerprinting with the repetitive (GTG)5-primer allowed for differentiation of *A. baumannii* strains and identification of strains to European clone I–III (Huys *et al.*, 2005b; Huys *et al.*, 2005a).

tDNA fingerprinting
tRNA genes are dispersed in multiple copies over the genome and contain common sequence motifs. PCR products generated with primers containing consensus tRNA sequences are species specific and can be used for species identification. The potential for tDNA fingerprinting for *Acinetobacter* species identification has been documented (Wiedmann-Al-Ahmad *et al.*, 1994; Ehrenstein *et al.*, 1996). A library of tDNA profiles of *Acinetobacter* species can be found on the Internet at http://users.ugent.be/~mvaneech/All_C.txt.

Summary PCR fingerprinting applications
The studies have shown that, generally, PCR fingerprinting with RAPD primers are useful for local typing but not for interlaboratory or longitudinal comparisons of *Acinetobacter* strains. In contrast, strict standardization and use of conserved primers like (GTG)5 may
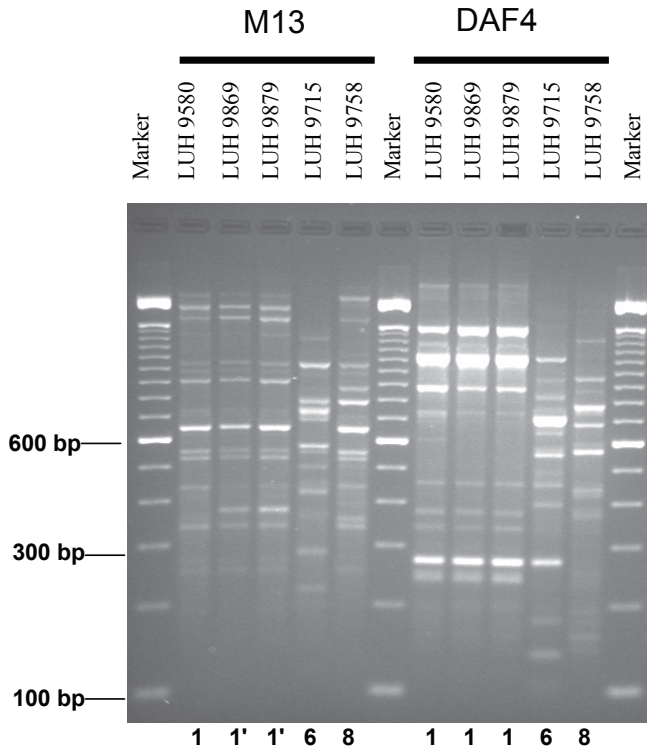
Figure 1.6 RAPD-PCR fingerprints of *Acinetobacter* isolates generated with primers M13 and DAF4 in a standard procedure (Grundmann *et al.*, 1997). Isolates with profiles 1 (or 1′) represent isolates that were likely to be epidemiologically related.

make PCR fingerprinting more robust and useful for identification of *A. baumannii* strains and clones. tDNA fingerprinting for species identification seems to be underestimated for its usefulness in species identification.

Plasmid analysis

Characterization of plasmids by electrophoresis was an early application of electrophoresis to analyze microbial DNA. In a comprehensive review on plasmids in acinetobacters (Towner, 1991) it was noted that indigenous plasmids have been found in the majority of *Acinetobacter* isolates examined. Many plasmids had unidentified functions, while others had been associated with antibiotic and heavy metal resistance, aromatic hydrocarbon degradation, conjugation and restriction/modification functions. A study performed in the late 1980s showed that glucose-acidifying clinical isolates contained less plasmids than glucose-non-acidifying strains (Gerner-Smidt, 1989). Since glucose-non-acidifying strains do not belong to *A. baumannii* and are generally well susceptible to antibiotics, the genes located on their plasmids are likely to have functions not related to antibiotic resistance. Most of these plasmids (70%) were less than 23 kb in size, indicating that the genes on these plasmids are not accompanied by genes for the mobilization of the plasmids. Over the past fifteen years, numerous reports have documented the occurrence of antibiotic

resistance genes on plasmids and the possible importance of plasmids in the spread of antibiotic resistance (Seward *et al.*, 1998). Other studies have recorded the role of plasmid associated genes in metabolic activities or metal resistance (Fujii *et al.*, 1997; Schembri *et al.*, 1994; Winstanley *et al.*, 1987; Rusansky *et al.*, 1987; Furukawa and Chakrabarty, 1982; Deshpande and Chopade, 1994).

Plasmid electrophoretic profiles can be used for epidemiological typing of strains (Seifert *et al.*, 1994; Hartstein *et al.*, 1990). However, due to the fact that plasmids can easily be lost or transferred horizontally among strains, plasmid typing has to be interpreted with caution. Today, plasmid typing for epidemiological purposes has largely been superseded by other genotypic typing methods. In the medical field, an important application of plasmid analysis of *Acinetobacter* strains is to elucidate particular antibiotic resistance mechanisms (Poirel and Nordmann, 2006). A particular cryptic plasmid of ca 8.7 kb, designated pAN1, was found almost exclusively in strains of European clone I and, therefore, can be considered a marker for this clone (Figure 1.7) (Nemec *et al.*, 1999; Nemec *et al.*, 2004).

Altogether, plasmids seem to be highly prevalent in acinetobacters but the knowledge on the genes associated with plasmids is largely limited to genes encoding antibiotic resistance and transfer mechanisms. The prevalence of plasmids in environmental acinetobacters and the genes associated with these plasmids is still largely unknown.
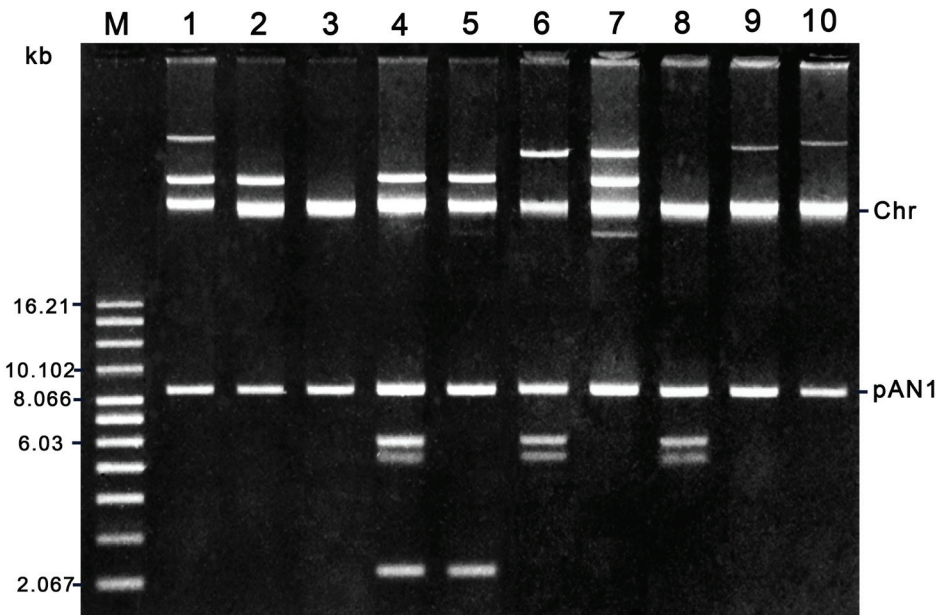

Figure 1.7 Plasmid profiles of epidemiologically unrelated isolates of *A. baumannii* European clone I. Isolates are indicated by the numbers above the lanes: 1, NIPH 110; 2, NIPH 321; 3, NIPH 921; 4, NIPH 472; 5, NIPH 409; 6, NIPH 309; 7, NIPH 303; 8, NIPH 281; 9, NIPH 15; 10, NIPH 7. M, supercoiled DNA ladder; pAN1, a cryptic plasmid (8.7 kb) found so far in all tested clone I isolates but only in two non-clone I isolates of *A. baumannii* (Nemec *et al.* 2004); Chr, chromosomal DNA.

Protein analysis

Cell protein patterns obtained by sodium dodecylsulphate-polyacrylamide electrophoresis (SDS-PAGE) have been shown to vary among microorganisms. In the 1980s and 1990s cell protein SDS-PAGE was a frequently used method for classification and typing of bacteria including acinetobacters. Generally, three types of samples are used, whole cell (WC) preparations and, for Gram-negative bacteria, cell envelope (CE) preparations comprising the complex of layers surrounding the cytoplasm, and outer membrane (OM) fractions. WC preparations can easily be obtained by boiling cells in denaturing lysis buffer and SDS-PAGE of these preparations is still often used in taxonomy. For *Acinetobacter*, CE SDS-PAGE has been found a powerful method for the discrimination of strains and species. CE fractions were obtained by sonication of cultured cells followed by fractionated centrifugation; staining of proteins was done with Fast Green FCF (Dijkshoorn *et al.*, 1987a). Thus, complex profiles with a great diversity of weak and heavy bands in the range of 14.0–100.0 K can be obtained (Figure 1.8).

CE protein analysis of *Acinetobacter* strains has been applied to study the temporal and topical carriership of these organisms by patients (Dijkshoorn *et al.*, 1987b), and to
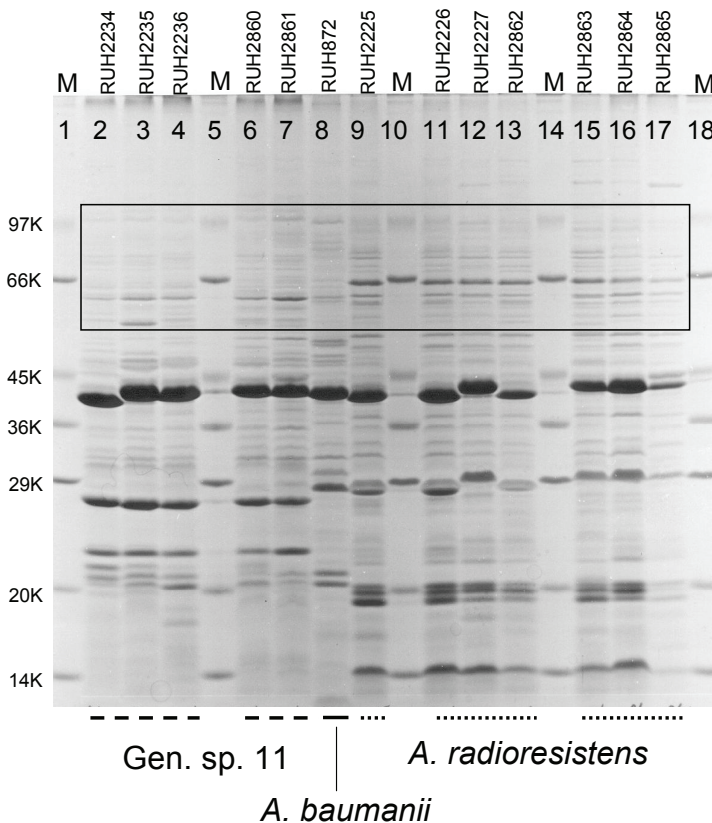

Figure 1.8 Cell envelope protein profiles of *Acinetobacter* gen.sp. 11 (lane 2–4, 6,7) *A. baumannii* (lane 8), and *A. radioresistens* (lane 9, 11–13,15–17). M, size marker. The fragments of the marked size range > 55–97K were found useful for differentiation of species.

establish sources and mode of spread of strains within hospitals (Crombach *et al.*, 1989; Weernink *et al.*, 1995; Bernards *et al.*, 1997). CE profiles also allowed for identification of strains belonging to European clones I and II (Dijkshoorn *et al.*, 1996). In another study, relatively weakly stained bands of > 55 K of CE profiles were found species specific, and it was concluded that protein profiling could be used for species identification (Dijkshoorn *et al.*, 1990) (Figure 1.8). In the above cited studies the profiles were used without any knowledge of the function of the proteins involved. It is likely, as inferred from studies on the cell envelope of *Escherichia coli* that the heavily stained bands of the CE profiles represent outer membrane proteins, many of which are porins. Various studies of the past decade have clarified the role of particular outer membrane proteins of acinetobacters, e.g. in siderophore-mediated iron acquisition (Dorsey *et al.*, 2004), as bioemulsifier (Toren *et al.*, 2002), in activation of gastrin expression and IL-8 expression (Ofori-Darko *et al.*, 2000) or in the influx of antibiotics (Mussi *et al.*, 2005). Novel approaches like the combination of SDS-PAGE, 2-dimensional electrophoresis and mass spectrometry have recently been used to characterize numerous membrane proteins (the membrane subproteome) of several *A. baumannii* strains (Siroy *et al.*, 2006; Marti *et al.*, 2006).

Altogether, cell envelope protein profiles have shown a great variation among *Acinetobacter* strains and species and can, therefore, be used as epidemiological and taxonomic markers. However, DNA based methods have now replaced one-dimensional SDS-PAGE for differentiation within and between *Acinetobacter* strains. The challenge of the near future is to elucidate the function of the cell envelope proteomes and to relate the protein diversity among strains and species to their function in their natural environment.

Lipopolysaccharide analysis

Acinetobacters, being Gram-negative bacteria, have been shown to possess lipopolysaccharide (LPS) of the so-called smooth type, which implies that it contains—besides the lipid A moiety and the core—an O-polysaccharide chain which is at the outer side of the outer membrane. Initially, this O-polysaccharide has been overlooked since proteinase K treatment of whole cell lysates did not show a ladder type electrophoretic profile upon SDS-PAGE and silver staining (Brade and Galanos, 1982; Brade and Galanos, 1982). It was, however, possible to visualize the ladder type O-polysaccharide if SDS-PAGE was combined with western blotting using antisera against LPS (Pantophlet *et al.*, 1998). A series of studies aimed at the characterization and occurrence of certain LPS O-antigens have shown that a variety of these molecules occur both within and between *Acinetobacter* species. Hence, they are useful markers for species and strain identification (Pantophlet *et al.*, 2002). A detailed discussion of these studies on LPS in acinetobacters is presented by Pantophlet elsewhere in this volume.

Interestingly, prior to the studies of Pantophlet *et al.*, O-antigen has been observed in strains of several *Acinetobacter* species when proteinase K treated cell envelopes were processed by SDS-PAGE followed by silver staining but without immunoblotting (Draaisma and Dijkshoorn, unpublished results). It was also noted that the electrophoretic migration of the lipid A moiety differed among species which indicates structural differences between species (Draaisma and Dijkshoorn, unpublished results). In an animal model, the LPS of an *Acinetobacter baumannii* strain, like the LPS of other Gram-negative bacteria, was an

important molecule in the interaction with the host (Knapp *et al.*, 2006). It is a challenge to assess whether differences in the lipid A moiety of LPS will explain the differences in pathogenicity between *Acinetobacter* strains and species.

Fatty acid analysis

Fatty acid composition of microorganisms is frequently used as a taxonomic marker. A study of cellular fatty acid composition of, at the time recognized, 19 named and un-named *Acinetobacter* species showed that patterns were characterized by major amounts of hexadecanoic acid, cis-9 hexadecanoic acid and octadecenoic acid and minor amounts of 3-hydroxy dodecanoid acid (Kampfer, 1993). Only two groups could be differentiated on the basis of the presence or absence of 3-hydroxy dodecanoic acid. One group, comprising *A. lwoffii*, gen. sp. 9, which are actually one species, and 15TU did not contain this fatty acid, while strains of the remaining groups did with few exceptions. These findings indicate that fatty acid analysis is not a useful method for *Acinetobacter* species identification.

Raman spectrometry and other spectrometric methods

Spectrometric methods with dedicated instruments, originally developed for use in analytical chemistry, are increasingly used in microbiology to assess the biochemical composition of cells. With these instruments fingerprints are generated which can be can be used for intra- and interspecies identification without any knowledge of the precise chemical components that determine the profiles. Examples are pyrolysis mass spectrometry, Fourier transform infra-red spectrometry and dispersive Raman spectrometry, high throughput methods which have been promising for *Acinetobacter* species and/or strain identification (Freeman *et al.*, 1997; Carr *et al.*, 2001; Winder *et al.*, 2004; Maquelin *et al.*, 2006). However, the usefulness and level of resolution—genus, species or strain level—will have to be assessed with sets of well characterized strains. Once this turns out to be successful, these methods will likely get a place in applied microbiology.

Phenotypic methods

Acinetobacter strains and species show a great diversity in nutritional and physiological characters. Bouvet and Grimont have described a phenotypic scheme of 19 tests for identification of the 12 firstly distinguished genomic species including seven named species (Bouvet and Grimont, 1986). This system was also used for phenotypic characterization of novel species (Nemec *et al.*, 2001; Nemec *et al.*, 2003) and showed, in combination with ARDRA, a good performance to identify non-*baumannii* species (Nemec *et al.*, 2000). However, the system also has limitations as already mentioned in previous paragraphs. For example, identification of the species included in the ACB complex (Gerner-Smidt *et al.*, 1991) or differentiation of hemolytic species is not well possible with this system (Bouvet and Jeanjean, 1989). Unfortunately, commercial phenotypic identification systems have also appeared insufficient for identification of acinetobacters according to the recent taxonomy (Bernards *et al.*, 1996; van Dessel *et al.*, 2004; Rodriguez-Bano *et al.*, 2006). The difficulties of phenotypic species identification in clinical diagnostics—and the apparent inability to implement methods like ARDRA—has led to the recommendation to allocate acinetobacters to two major groups only, the glucose acidifying and the glucose-non-acidifying acinetobacters (Schreckenberger *et al.*, 2003). This recommendation turns

the clock back to more than 20 years ago when only glucose-acidifying strains—sometimes designated by the obsolete species name *Acinetobacter anitratus*—and the glucose-non-acidifying, *A. lwoffii*, were recognized. The challenge for the near future is to find a limited number of novel phenotypic identification criteria, e.g., by investigation sets of reference strains of all species on arrays with large numbers of phenotypic tests, and to include these tests in commercially available identification systems.

Discussion

Diversity at species level

In the foregoing we have discussed a variety of methods to assess the diversity of acineto-bacters at the species and strain level. Several methods or combinations of methods have been found useful to delineate species and this has led to the description of 31 genomic species, while there are, as indicated by AFLP analysis, several tens of potential additional species. DNA–DNA hybridization and/or analysis of particular sequences have revealed that, within the genus, some species are relatively highly related. Examples are the species in the ACB complex, unnamed species 10 and 11, and the hemolytic unnamed species 6, 13BJ/14TU, 14BJ, 15BJ, 16 and 17. Future studies, including those based on comparative analysis of multiple genes, will provide a more comprehensive insight into the (phylogenetic) relatedness of species within the genus. However, DNA sequence similarity groups and groups of living organism may not be congruent. Therefore, a comprehensive polyphasic characterization is required to develop a robust classification. Such an approach should take into account not only genomic features but also the following, sequential DNA-expression levels: (i) proteins, (ii) other cell structure components, and (iii) morphology and behavior (Norris, 1980). In addition, the ecology of certain groups is a parameter to approximate the natural coherence of related organisms (Cohan, 2002).

Diversity below species level

The diversity within *Acinetobacter* species is not yet well elucidated since many strains have not been speciated due to the lack of practical identification methods. Some insight into the homogeneity of species has been obtained with AFLP analysis. With two different protocols AFLP grouping levels of *Acinetobacter* species were at 45 or 50%, respectively (Janssen *et al.*, 1996; Nemec *et al.*, 2001) (Figure 1.3). It is of note that the strains enrolled into the Leiden University Medical Center AFLP database, from which the dendrogram of Figure 1.3 is derived, may not reflect the variation in nature since the strains are mainly from clinical origin and, therefore, subject to sampling bias. For many *Acinetobacter* species only a limited number of strains is available. Nevertheless, some findings are noteworthy. For example, some species were relatively homogeneous, including *A. radioresistens* and *A. baylyi*, despite the fact that the strains of each species were from different origin. In particular, the degree of homogeneity of *A. baylyi* (~78% similarity level) is striking if one considers that the organisms are from environmental origin at different hemispheres and are easily transformable by DNA (Vaneechoutte *et al.*, 2006; Young *et al.*, 2005). This indicates that this species must have a remarkable capacity to maintain the integrity of its DNA.

A. baumannii, a clinically important species, is the only species which has been intensively studied for its diversity with a variety of methods. Within this species groups of strains were distinguished that were highly similar despite the fact that they were from different locations. It has been postulated that these organisms represent clonal lineages but the routes along they have spread and the selective forces that contributed to their emergence in clinical settings are yet to be elucidated.

Remaining questions and challenges for the near future

The great diversity observed within the genus *Acinetobacter* has, so far, mainly been used to classify and identify strains. Only a limited number of strains have been studied in depth to elucidate either certain metabolic pathways or the potential to produce important biochemical agents.

Most of the research on *Acinetobacter* so far has been undertaken to serve certain human interests, e.g., in clinical diagnosis, in the study of genetics, metabolism and antibiotic resistance, or in biotechnology. It is known that acinetobacters occupy a great variety of ecological niches, but very little is known about the relevance of the diversity of acinetobacters in relation to their environment. Another challenging question regarding clinically important species is which mechanisms have led to their adaptation from their presumed environmental origin to the human host.

In these days, a wide array of methodologies is becoming available to microbiologists including genome analysis, proteomics, use of phenotype arrays, chromatography and spectrometry to characterize important cell constituents. The way ahead is to apply these methods in an integrated approach and in association with the ecology of the organisms to get a better understanding of the significance of the diversity of acinetobacters in their natural environment.

Finally, the existence of clusters of species within the genus and the availability of large numbers of well characterized strains of all described species makes *Acinetobacter* an interesting model for taxonomists and evolution biologists to study the discontinuities within a genus.

Web resources

Site	Subject
http://www.bacterio.cict.fr/	List of prokaryotic names with standing nomenclature (LPSN)
http://www.ridom.de	Ribosomal differentiation of medical microorganisms
http://www.mlst.net/	Multi locus sequence typing
http://users.ugent.be/~mvaneech/All_C.txt	tDNA profiles of *Acinetobacter* species
http://users.ugent.be/~mvaneech/ARDRA/Acinetobacter.html	ARDRA protocol and profiles of

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