Diversity of strains of the genus *Acinetobacter* isolated from humans

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Summary of PhD thesis

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INTRODUCTION

The genus *Acinetobacter* includes non-motile, coccobacillary, strictly aerobic, Gram-negative bacteria that can be cultured on simple growth media (Juni, 1986). These organisms are widespread in nature, and can be recovered from water, soil and living organisms. In view of their low pathogenicity, acinetobacters remained out of focus of clinical microbiologists for a long time. However, over the last three decades they have increasingly been recognised as important causative agents of hospital infections. Acinetobacters cause severe infections including bronchopneumonia and septicemia that can have a fatal course in debilitated patients and new-borns. Particularly problematic is the fact that multiple resistant strains can spread epidemically among patients, while due to their resistance to antibiotics, infections by these organisms are difficult to treat (Bergogne-Bérézin, 1995; Bergogne-Bérézin & Towner, 1996).

Taxonomy. The basis for the present classification of *Acinetobacter* was laid in 1986 by Bouvet & Grimont with the description of 12 DNA-DNA hybridisation groups (genomic species) within the genus. In the period 1986 up to 1999, this scheme was extended with 11 additional genomic species (Tjernberg & Ursing, 1989; Bouvet & Jeanjean, 1989; Gerner-Smidt & Tjernberg, 1993; Vaneechoutte et al., 1999). Eight genomic species were given names (*A. calcoaceticus*, *A. baumannii*, *A. haemolyticus*, *A. junii*, *A. johnsonii*, *A. lwoffii*, *A. radioresistens*, and ‘*A. venetianus*’) while the others are designated by numbers. Strains of *A. calcoaceticus*, *A. baumannii*, the unnamed groups 3 and 13TU are genetically closely related and difficult to separate phenotypically, and are therefore sometimes unified in the so-called *A. calcoaceticus-A. baumannii* (ACB) complex (Gerner-Smidt et al., 1991). Apart from the known genomic species, additional strains of unknown taxonomic status were found in a number of studies (Tjernberg & Ursing, 1989; Bouvet & Jeanjean, 1989). The percentage of these strains ranged between 2 and 15%, depending on the origin of the organisms. These unidentifiable strains may indicate either the existence of additional genomic species or yet unrecognised intraspecies diversity.

Ecology. Strains of *A. baumannii*, and the unnamed groups 3 and 13 TU are recovered predominantly from clinical specimens, with *A. baumannii* being the most prominent as an agent able to colonise and infect severely ill, hospitalised patients (Horrevorts et al., 1995; Bergogne-Bérézin and Towner, 1996; McDonald et al., 1999). Strains of the latter species can persist in hospitals and give rise
to outbreaks; they are usually highly resistant to antibiotics (Seifert et al., 1993b). Most other (genomic) species of Acinetobacter have been found in different environments, e.g. A. calcoaceticus was isolated predominantly from soil, and A. johnsonii was found in activated sludge and frozen food, although representatives of these and other species have also been recovered occasionally from human specimens (Seifert et al., 1993a; Bergogne-Bérézin & Towner, 1996). Overall, the natural habitats of most Acinetobacter (genomic) species have not been well-studied.

Species identification. DNA-DNA hybridisation is the gold standard for identification of Acinetobacter strains, but this method is not applicable in most laboratories. A phenotypic identification scheme, including nutritional tests and growth at different temperatures, was devised by Bouvet & Grimont (1987). However, it has been shown that some species are difficult to identify by these tests (Gerner-Smidt et al., 1991). Similarly, commercial phenotypic identification systems show only moderate performance (Bernards et al., 1995; Bernards et al., 1996). Several genotypic methods have been proposed for identifying acinetobacters to genomic species level, including ribotyping (Gerner-Smidt, 1992), tDNA fingerprinting (Ehrenstein et al., 1996), amplified ribosomal DNA restriction analysis (ARDRA) (Vaneeschoutte et al., 1995; Dijkshoorn et al., 1998), and AFLP™ fingerprinting (Janssen et al., 1997). Nevertheless, identification to genomic species using one method is still problematic (Dijkshoorn et al., 1998; Jawad et al., 1998) and the development of practical reliable methods for identification within the genus Acinetobacter remains a challenging task.

Population structure at the subspecies level. Virtually all currently available typing methods have been used for epidemiological typing of hospital strains of A. baumannii. These methods include phenotypic (biotyping, cell envelope protein electrophoresis, antibiogram typing etc.) or genotypic techniques (plasmid typing, ribotyping, pulsed-field gel electrophoresis (PFGE), PCR fingerprinting and AFLP analysis), often used in combination (Dijkshoorn et al., 1993; Seifert and Gerner-Smidt, 1995; Janssen & Dijkshoorn, 1996; Grundmann et al., 1997). The application of the methods has revealed high diversity at the strain level. However, most of these studies dealt with geographically limited collections of isolates and results obtained in different laboratories were difficult to compare because of variations in the methods used. A recent study has shown that epidemic strains from different cities and countries in Europe are highly similar (Dijkshoorn et al., 1996), which may indicate that the emergence of epidemic A. baumannii at different locations is associated with the occurrence of specific clones (i.e., isolates showing so many identical phenotypic and genotypic traits
that the most likely explanation for this identity is a common origin [Ørskov & Ørskov, 1983]).

**OBJECTIVES**

The present study was conceived as a taxonomic analysis of a large group of clinical isolates of the genus *Acinetobacter* isolated at different locations in the Czech Republic with the aim to establish the prevalence of different (genomic) species, and to analyse the strain (clonal) diversity among hospital isolates of the clinically most relevant species, *A. baumannii*. In the course of the study, two novel phenotypically and genotypically distinct groups were discovered, including most strains that could not be identified to any of the hitherto known species. At the same time, it was found that Czech multiresistant *A. baumannii* strains belong almost exclusively to two phenotypically and genotypically homogeneous groups (clones). That is why collaboration with several European laboratories was sought to analyse the groups discovered in more detail by additional methods and to compare them with other strains from various European locations.

In summary, the steps undertaken are as follows:

- Study of the (genomic) species diversity of clinical isolates of the genus *Acinetobacter* originating from the Czech Republic.
- Classification of hitherto unidentifiable strains and definition of their taxonomic status.
- Study of the diversity of *A. baumannii* hospital isolates at the subspecies (strain, clonal) level.
- Study of characteristics of *A. baumannii* epidemic clones.
- Development of a practical method for epidemiological typing of *A. baumannii* hospital isolates.
**THESIS CHAPTERS**

The results of the PhD thesis have been published as follows:

1. **Nemec A.** [Taxonomy of the genus *Acinetobacter.*]. *Epidemiol Mikrobiol Imunol* 1996;45:23-29. [Czech]


RESULTS

Species diversity

- Genomic species diversity was studied among 700 *Acinetobacter* clinical isolates obtained between 1991 and 1999 from diagnostic laboratories in the Czech Republic (Chapter 5). The isolates were recovered from a variety of specimens from patients in hospitals and general practice and represented different levels of clinical significance, ranging from colonisation to life-threatening infections. All isolates were analysed by biochemical tests of Bouvet and Grimont (1987) and identified by numerical probabilistic identification using two reference probability matrices. If not identified concordantly by both matrices as belonging to the *ACB* complex, the strains were further analysed by ARDRA. The final identification was then derived from the combined biochemical and ARDRA results ("consensus identification"). Identification of 700 isolates yielded the following results: the *ACB* complex (*n*=553), *A. lwoffii* (*n*=63), genomic species 13BJ (*n*=9), *A. johnsonii* (*n*=7), *A. haemolyticus* (*n*=6), *A. junii* (*n*=5), and other genomic species (<5 isolates each). Forty-five isolates could not be identified as belonging to any known (genomic) species. Among the unidentified isolates two large groups of non-glucose-acidifying, non-hemolytic and non-gelatinase-producing isolates were distinguished. These groups, designated phenon 1 (*n*=17) and phenon 2 (*n*=15), had distinctive phenotypic features and novel ARDRA profiles, which suggested that they represented hitherto undescribed *Acinetobacter* species.

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**Fig. 1.** Rooted 16S rDNA sequence based tree showing the relationship of *A. ursingii* sp. nov. (phenon 1), *A. schindleri* sp. nov. (phenon 2), the other members of the genus *Acinetobacter*, *Moraxella lacunata* and *Psychrobacter immobilis* (an outgroup). The tree was constructed using the neighbour-joining method. EMBL accession numbers are given in parentheses. Bar, 1% estimated sequence divergence.
Taxonomic positions of phenon 1 and phenon 2 were studied in collaboration with three European laboratories (Chapter 7). For this purpose, the collection of Czech strains was enlarged with strains from other European countries that showed characters similar to those of the two phenons. These additional strains (n=21) were selected from a set of c. 100 archive Acinetobacter strains that remained unidentifiable. Polyphasic taxonomic analysis (analysis of biochemical characteristics, ARDRA, AFLP fingerprinting, DNA-DNA hybridisation, 16S rRNA gene sequence analysis) showed that phenons 1 and 2 represented two novel species of the genus Acinetobacter, for which the names Acinetobacter ursingii sp. nov. and Acinetobacter schindleri sp. nov. were proposed (Fig. 1). A. ursingii and A. schindleri differed in their distribution in patients. While the majority of the A. schindleri strains were isolated from non-sterile body sites of outpatients, A. ursingii comprised mainly clinically significant isolates from seriously ill, hospitalised patients. Almost half of the A. ursingii strains were isolated from blood cultures and at least some of them were recovered from patients with diagnosed bacteraemia or septicaemia. Practical identification of both species based on specific biochemical characteristics and ARDRA profiles (Fig. 2) was proposed. An analogous procedure was used for defining the taxonomic position of Acinetobacter strains (n=7) showing unusually small colonies on agar plates (Fig. 3) and low biochemical activity (Chapter 9). Polyphasic analysis confirmed,
that these strains represent a new species, for which the name *Acinetobacter parvus* sp. nov. was proposed.

**Genomic species diversity within the ACB complex** was studied among 103 clinical isolates obtained between 1991 and 1997 from 17 Czech hospitals (*Chapter 3*). These isolates were selected from c. 400 ACB complex isolates to include strains as heterogeneous as possible in time and place of isolation. According to the *Eco*RI ribotypes, all but one of these isolates were identified to species: 77 (75 %) isolates were allocated to *A. baumannii*, 14 to genomic species 3, 10 to genomic species 13TU and one to *A. calcoaceticus*. The remaining isolate could not be unambiguously identified as any of these (genomic) species; its *Eco*RI ribotype was closest to that of one of two additionally described groups of the ACB complex (Gerner-Smidt & Tjernberg, 1993). All multiresistant and epidemic strains were found to belong to *A. baumannii*.

**Subspecific classification of Acinetobacter baumannii strains**

**Diversity at the subspecific level** (strain, clonal) was studied in the 103 Czech isolates of the ACB complex (*Chapter 3*) by ribotyping, plasmid analysis, biotyping and antibiotic susceptibility testing. *Eco*RI ribotyping showed high genotypic diversity (50 different ribotypes); most of the isolates had a unique ribotype (Fig. 4). However, more than 50% of the *A. baumannii* isolates belonged to two ribotypes only. The isolates with identical ribotypes were also similar in other characteristics such as plasmid profile, biotype and susceptibility to antimicrobial agents. These two genotypically and phenotypically relatively homogeneous groups were designated as groups A and B and it was hypothesised that they represented two discrete clonal lineages of *A. baumannii* spread in Czech hospitals. Group A comprised 37 isolates of *Eco*RI ribotype I belonging to biotypes 11 or 6 and containing an 8.7 kb cryptic plasmid (designated pAN1; Fig. 5).
Group B consisted of eight isolates of EcoRI ribotype II, belonging to biotype 2, in most of which plasmid DNA was not detected. Both groups contained epidemic and sporadic isolates resistant to more antibiotics than the remaining isolates (groups A and B included 85% of all multiresistant isolates).

- A taxonomically and epidemiologically well-defined set comprising representative Czech hospital strains of 1991-1999 (n=52) and reference strains for epidemic clones I (n=9) and II (n=4) taken from the original study of Dijkshoorn et al. (1996) was selected for further analysis of *A. baumannii* clonal groups (Chapter 6). The Czech collection comprised strains of groups A (n=23) and B (n=7) and other multiresistant (n=7) and susceptible (n=15) strains. In collaboration with Dr. R. Pantophlet (Borstel, Germany), the strains were characterised using newly developed monoclonal antibodies (MAbs) against acinetobacter O-antigens. The results confirmed homogeneity of both Czech clonal groups A and B and their difference from the other Czech strains. All group A strains reacted with *MAb* S48-3-13 or S51-3 and all group B strains reacted with *MAb* S53-32, while most of the other resistant and susceptible strains did not show reactivity with any of 20 *MAbs* tested. The group A characteristics were congruent with

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**Fig. 4.** Examples of EcoRI ribotypes showing those for 15 Czech clinical isolates and four reference strains of the *A. calcoaceticus - A. baumannii* complex. Isolates (NIPH) 7 and 24 represent clone I (group A) and clone II (group B), respectively; isolate 417, ungrouped. HG, DNA hybridisation group (= genomic species); HG 2, *A. baumannii*; HG 1, *A. calcoaceticus*; MW, mol. wt markers (phage λ DNA digested with HindIII and Styl).
clone I (identical EcoRI ribotype, biotypes 6 or 11, reactivity with MAb S48-3-13, presence of plasmid pAN1) and most group B characteristics were congruent with clone II (identical EcoRI ribotype, biotype 2), although reactivity with MAbS was different since clone II strains reacted with S48-3-17, and not with S53-32. The results of the following study (Chapter 8) showed that reactivity with the above MAbS is frequently found in hospital A. baumannii isolates from other European countries as well, which is indicative of pan-European spread of the epidemic clones.

- Ribotyping with enzymes HincII and HindIII and AFLP fingerprinting were used for quantitative analysis of genotypic similarity among A. baumannii strains of the previous collection completed with recent multiresistant isolates (n=33) from the Czech Republic (Chapter 10). Numerical analysis of combined HincII/HindIII ribotypes and AFLP profiles confirmed genotypic congruency between group A and clone I and group B and clone II. Furthermore, the results showed that both clones included some strains with slightly different ribotypes, that initially had not been classified into groups A or B. Comparison of recent isolates (2001-2002) with strains of 1991-1999 revealed that the structure of the Czech A. baumannii population had not undergone any significant change in the last decade.

- A practical method for epidemiological typing of multiresistant A. baumannii strains was introduced, based on numerical analysis of inhibition zone diameters in the disk diffusion test (Chapter 4). Eleven antibiotics representative of the major groups of effective antimicrobial drugs were selected for typing: ampicillin+sulbactam, piperacillin, ceftazidime, imipenem, co-trimoxazole, ofloxacin, gentamicin, tobramycin, amikacin, netilmicin and tetracycline. Each isolate was characterised by a set of inhibition zone diameters for different antibi-
otics and these sets were compared by cluster analysis with the Euclidean distance as a similarity coefficient. Compared to ribotyping and biotyping, the discriminatory power of quantitative antibiogram typing was highest for the multiresistant strains. As chromosomal DNA macrorestriction analysis (pulsed-field gel electrophoresis) did, this method effectively differentiated genotypically similar strains belonging to epidemic clone I. Theoretical limits to the method are given by the instability of the resistance markers and phenotypic convergence, and therefore, definitive conclusions on strain identity should be confirmed by genotypic methods.

- **Strain and clonal diversity of the ACB complex in an endemic situation** was studied in 95 isolates obtained between 1991 and 1993 in the Prague Burn Centre (*Chapter 2*). Classification based on restriction fragment length polymorphism of total DNA, plasmid profiles, ribotypes, antibiograms and biotypes allowed distinguishing of 12 relatedness groups (each including isolates of the same strain) and 19 single strains. *EcoRI* ribotyping of 28 strains yielded the following results: 18 strains of *A. baumannii*, 5 strains of genomic species 13TU and 5 strains of genomic species 3. Two multiresistant *A. baumannii* strains belonging to epidemic clone I persisted in the burn centre throughout the whole study period and one of them caused an outbreak in summer 1993. Isolates of these two strains (*n* = 39) presented 41% of all isolates studied. Short-term occurrence and patient-to-patient transmission were also made conceivable on the basis of epidemiological typing for a strain belonging to epidemic clone II. Illustrative of the significance of the polyphasic description is that three almost simultaneously isolated multiresistant strains belonged to epidemic clone I and were distinguishable only by a combination of at least two of the methods used (e.g. ribotyping and biotyping).

**CONCLUSIONS**

In summary, the present study has significantly contributed to the current knowledge of species and subspecies diversity within the genus *Acinetobacter*. A method termed "consensus identification" was developed, using a combination of practical, complementary methods, that together allowed for reliable species identification. Furthermore, groups of hitherto unidentifiable strains were defined and *A. baumannii* multiresistant/epidemic clones were described. Cooperation with other European laboratories enabled to complete the study set with additional strains from other geographically distinct localities and to analyse the most relevant groups by supplementary taxonomic methods (polyphasic taxonomy). This collaboration resulted in description of three novel *Acinetobacter* species, i.e. *A.*
A. ursingii sp. nov., A. schindleri sp. nov., and A. parvus sp. nov. A. ursingii is a clinically important taxon isolated from severely ill, hospitalised patients. The results of the polyphasic subspecific analysis of strains from the Czech Republic and northwestern Europe showed the existence of pan-European clonal lineages of A. baumannii. Strains belonging to these clones are usually multiresistant and can spread epidemically within hospitals, but they also occur at different locations in Europe. Multiresistant strains isolated in the Czech Republic were found to belong almost exclusively to these two clones. The distinguished multiresistant/epidemic clones will be the subject of future research addressing the factors involved in pathogenicity, epidemicity and multiresistance of A. baumannii.

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PROFESSIONAL BIOGRAPHY OF ALEXANDR NEMEC

Born in 1963, Hradec Králové.

Education. Graduated from the Faculty of Sciences, Charles University, Prague in 1987. A degree of RNDr. (Rerum Naturalium Doctor) received in 1987.

Professional career. Since 1990, working at the Unit of Bacterial Resistance to Antibiotics and Collection of Type Cultures of the National Institute of Public Health, Prague (NIPH). In 1992-1994, worked as a part-time assistant professor of medical microbiology at the 3rd Faculty of Medicine, Charles University, Prague.

General interests. Molecular-biology and phenotypic methods in classification and identification of bacteria; numerical taxonomy; typing of multiresistant nosocomial strains; taxonomy and epidemiology and of the genus *Acinetobacter*; genetic basis of resistance to antibiotics in *A. baumannii*.

Principal investigator of the following research grants


Fellowships abroad

- Study of the secondary structure of ribosomal protein S20 by comparative analysis of the primary structure of the homologous genes in selected eubacterial species, Department of molecular biology of the University of Western Ontario, London, Canada (1992, three months).
- Complex genomic typing of unclassified strains of the genus *Acinetobacter*, University Medical Centre, Leiden, the Netherlands (1997, one month).

Organisation of international meetings

- The international conference "The Taxonomy and automated identification of bacteria". (Prague, 20.-24.7. 1992)", a member of the organising committee.
- The symposium: "Classification methods and classification in epidemiology", the 7th IUMS international congress of bacteriology and applied microbiology (Prague, 3.-8.7. 1994), a chairman.
LIST OF PUBLICATIONS

Original articles in peer reviewed journals and book chapters


**Posters**


*Other 13 articles and notes in non-peer reviewed journals or abstract books*
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