Antimicrobial resistance and clonality in *Acinetobacter baumannii*

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Nature likes to hide

(Herakleitos)

To Matěj, Štěpán and Šimon

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CHAPTER 1

Introduction

INTRODUCTION

Multidrug resistant pathogens in intensive care units

Developments in intensive care medicine in the last quarter of the 20th century have substantially broadened the options for treatment of critically ill patients. However, this progress was accompanied by the formation of an environment characterized by the presence of patients with impaired defence mechanisms and extensive use of antimicrobial agents and disinfectants. This environment provides a niche for bacteria which are relatively harmless for healthy individuals but have an enhanced ability to survive in the presence of antimicrobial substances and to become resistant to multiple antimicrobial therapeutics. Although many microorganisms isolated from hospital infections remain well susceptible to antimicrobial agents, a group of bacteria has emerged all over the world that escapes the action of nearly all antibiotics. This small but important group includes *Staphylococcus aureus*, *Enterococcus faecium, Klebsiella pneumoniae, Enterobacter* species, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (McGowan 2006, Hawkey 2008, Rice 2008).

Acinetobacter baumannii, a paradigm of a nosocomial pathogen

A. baumannii can serve, perhaps best of the mentioned microorganisms, as a paradigm of a typical nosocomial pathogen (Dijkshoorn et al. 2007). This bacterium causes infections in healthy individuals only rarely and its occurrence in clinical samples from hospitalized patients usually reflects colonization rather than infection. Serious infections are encountered mostly in critically ill patients in intensive care units (ICUs) and these infections are commonly associated with invasive procedures such as mechanical ventilation or catheterization (Peleg et al. 2008). An important property making *A. baumannii* a nosocomial pathogen is its ability to resist environmental pressure. For example, in contrast to other Gram-negative bacteria, it is able to survive on dry surfaces (Jawad et al. 1998). Yet, the most important characteristics that make it the subject of intensive research are the occurrence of strains resistant to most (or rarely all) therapeutic antimicrobial agents and the potential of such strains to cause outbreaks of hospital infections (Dijkshoorn et al. 2007).

Multidrug resistance in *A. baumannii*

A. baumannii is intrinsically resistant to a number of antibiotics used to treat infections caused by Gram-negative bacteria, including aminopenicillins, first- and second-generation cephalosporins and chloramphenicol. As compared to some other Gram-negative species, it also has increased minimal inhibitory concentrations to clinically important antimicrobials, such as third-generation cephalosporins or quinolones. It also has a remarkable capacity to develop clinical resistance to broad-spectrum β-lactams, aminoglycosides, fluoroquinolones and tetracyclines, and in recent years, also to carbapenems, which have been the most

powerful agents against infections caused by multidrug resistant *Acinetobacter* since the late 1980s. Finally, recent reports on *A. baumannii* resistant to polymyxins (Ko et al. 2007) indicate that this organism may become fully refractory to the currently available antimicrobial armoury.

Epidemic clones of *A. baumannii*

Outbreaks of hospital infections caused by *Acinetobacter* were reported already in the 1970s and in the following years it became evident that such outbreaks represent a serious problem in hospital settings, especially in intensive care units. However, at that time studies dealt only with epidemiological investigations of local outbreaks. It was in the late 1980s that strains from different outbreaks in north-western European countries appeared to be very similar. The question was whether the typing methods used did not have sufficient resolution capacity or whether these strains were genetically closely related. This query gave rise to a multicentre study leading to the discovery of two groups of genetically highly similar *A. baumannii* strains (the so-called European (EU) clones I and II) obtained from outbreaks in geographically distant hospitals of north-western Europe (Dijkshoorn et al. 1996). These findings were corroborated by a study showing that strains related to the clones from north-western Europe also prevailed in the Czech Republic (Nemec et al. 1999). As was the case with the first study describing the EU clones I and II (Dijkshoorn et al. 1996), this Czech study also indicated that the strains belonging to the clones were much more resistant to antimicrobials than other strains. This suggests that the multidrug resistance of these clones is an important factor contributing to the progressive increase in resistance in *Acinetobacter* in the 1990s (Nemec et al. 1999). The studies of Dijkshoorn et al. (1996) and Nemec et al. (1999) have set the scope of the present thesis.

Aim of the thesis

The main objective of this thesis was to gain insight into the epidemiology and molecular basis of multidrug resistance of *A. baumannii* at the population level. For this purpose, we used a descriptive and comparative approach to analyse a large number of properties in well-defined collections of strains in order to assess the relationship between multidrug resistance and the genetic population structure of *A. baumannii*. The studied groups of organisms consisted mainly of clinical isolates collected since 1991 from hospitalized patients in the Czech Republic and of isolates from other European countries enrolled in the collection of the Leiden University Medical Centre since the early 1980s.

Overview of the chapters

In the first study (Chapter 2), we investigated the genetic relationship between Czech *A. baumannii* strains assigned previously to the so-called groups A and B and EU clones I and II using AFLP fingerprinting and ribotyping. The study collection included 70 multidrug

resistant and 15 susceptible strains from 1991-2001 from the Czech Republic and reference strains of clones I and II. The aim of the next study (Chapter 3) was to assess the diversity of the genes encoding aminoglycoside-modifying enzymes and their association with class 1 integrons in multidrug resistant strains, mainly belonging to the EU clones. In the next study (Chapter 4), the distribution of the just discovered nonspecific efflux pump AdeABC in *A. baumannii* and its possible impact on the antibiotic susceptibility spectrum of strains were addressed. To this aim a large and genetically diverse collection of 116 clinical isolates from 16 European countries obtained over a period of 23 years were investigated. A panel of methods was used to assess the clonal relatedness of the strains and the presence of the genes associated with the efflux system. The purpose of the last study (Chapter 5) was to analyse the emergence of carbapenem resistance among *Acinetobacter* hospital strains in the Czech Republic, which appeared in the early 2000s. To this aim, *Acinetobacter* clinical isolates were collected prospectively from multiple ICUs in 2005-2006 and were analysed for genomic types and for resistance determinants. Finally, in Chapter 7 the overall results of the studies are discussed within the context of the most recent developments.

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CHAPTER 2

Nemec A, Dijkshoorn L, van der Reijden TJK.

Long-term predominance of two pan-European clones among multiresistant *Acinetobacter baumannii* strains in the Czech Republic.

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Long-term predominance of two pan-European clones among multi-resistant *Acinetobacter baumannii* strains in the Czech Republic

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In a recent study, a large proportion of multi-drug-resistant (MDR) *Acinetobacter baumannii* strains that were isolated from hospitalized patients in the Czech Republic was found to belong to two major groups (A and B). These groups appeared to be similar to epidemic clones I and II, respectively, which were identified previously among outbreak strains from north-western European hospitals. The aim of the present study was to assess in detail the genetic relatedness of Czech *A. baumannii* strains and those of epidemic clones I and II by using ribotyping with *Hin*dIII and *Hin*cII and by AFLP fingerprinting. The study collection included 70 MDR strains that were isolated in 30 Czech hospitals in 1991–2001, 15 susceptible Czech strains from 1991 to 1996 and 13 reference strains of clones I and II from 1982 to 1990. One major *Hin*dIII/*Hin*cIII ribotype (*R1-1*) was observed in 38 MDR Czech strains and eight reference strains of clone I, whereas another major ribotype (*R2-2*) was observed in 11 MDR Czech strains and in three reference strains of clone II. A selection of 59 Czech strains (representative of all ribotypes) and the 13 reference strains were investigated by AFLP fingerprinting. At a clustering level of 83 %, two large clusters could be distinguished: cluster 1 included all reference strains of clone I and 25 MDR Czech strains, whilst cluster 2 contained all reference strains of clone II and 11 MDR Czech strains. There was a clear correlation between the groupings by AFLP analysis and by ribotyping, as all strains with ribotype *R1-1* and four strains with slightly different ribotypes were found in AFLP cluster 1, whereas all strains with ribotype *R2-2* and seven strains with similar ribotypes were in AFLP cluster 2. Thus, 41 and 21 MDR Czech strains could be classified as belonging to clones I and II, respectively. The remaining eight MDR and 15 susceptible strains were highly heterogeneous and were distinct from clones I and II by both AFLP fingerprinting and ribotyping. These results indicate that the two predominant groups observed among MDR Czech *A. baumannii* strains from the 1990s are genetically congruent with the north-western European epidemic clones that were found in the 1980s. Recognition of these clinically relevant, widespread clones is important in infection prevention and control; they are also interesting subjects to study genetic mechanisms that give rise to their antibiotic resistance and epidemic behaviour.

INTRODUCTION

In an extensive review, Henriksen (1973) described acinetobacters as soil and water bacteria of widespread occurrence in the surroundings of man and animals, which have low pathogenic potential, but are opportunistic and capable of causing infection in individuals with reduced resistance. At the time, the genus *Acinetobacter* comprised only one species, *Acinetobacter calcoaceticus* (Lautrop, 1974). Today, acinetobacters are recognized as important nosocomial pathogens (Bergogne-Bérézin & Towner, 1996), but it is not yet well understood to what extent this is caused by increased susceptibility of the host or by expansion of specific strains.

Over the past three decades, considerable progress has been made in resolving the taxonomy of the genus *Acinetobacter* and in the development of methods to identify species and strains. With the inclusion of 10 recently described species, the genus now comprises 32 genomic species, 17 of which have validly published names (Nemec *et al.*, 2001, 2003; Carr

Abbreviation: MDR, multi-drug-resistant.

A table showing data on origin and properties of the strains used in this study is available in JMM Online.

et al., 2003). A few are of undisputed clinical relevance, whereas many others may be true environmental organisms, although the ecology of most species is as yet unrevealed. Among the clinically relevant species, *Acinetobacter baumannii* is the most common in clinical specimens and can give rise to severe infections in critically ill patients. Strains of this species that circulate on intensive-care units are frequently multi-drug-resistant (MDR) and combine this feature with the capacity to spread among patients and to persist in the hospital environment (e.g. Aygun *et al.*, 2002; Wang *et al.*, 2003).

In the mid 1990s, *A. baumannii* strains from 14 outbreaks and 17 sporadic strains from hospitals in different north-western European cities and countries were compared to assess the diversity among outbreak and non-outbreak strains. By using a combination of genotypic and phenotypic methods, the outbreak strains could be allocated to two main groups (designated clones I and II), whereas the sporadic strains were more heterogeneous (Dijkshoorn *et al.*, 1996). In a more recent study, phenotypic and genotypic properties of *A. baumannii* hospital strains from the Czech Republic were studied (Nemec *et al.*, 1999). It was found that MDR strains showed lower variability than susceptible strains. Most MDR strains were classified into two groups (designated A and B), each of which was characterized by a specific ribotype and similarity in other properties. The grouping of two reference strains of clones I and II with strains of groups A and B, respectively (Nemec *et al.*, 1999), and apparent similarities in ribotypes of strains of clones I and II with groups A and B, respectively (Pantophlet *et al.*, 2001), suggested that the respective clones and groups were congruent. Similarity of strains of group A and clone I was corroborated by their common reactivity with O-antigen-specific mAbs (Pantophlet *et al.*, 2001).

The panels of methods that were used to delineate clones I and II and groups A and B were different; therefore, definite conclusions on their genetic relatedness cannot be made until a representative sample of strains is subjected to common methods. The aim of the present study was to analyse in detail genotypic similarities between *A. baumannii* hospital strains from the Czech Republic and those that are representative of north-western European clones, in order to assess whether there is a pan-European presence of particular, genetically highly related, MDR strains (i.e. clones). For this purpose, the collection of Czech strains that was used in the previous study was enlarged with recent Czech MDR isolates. Strains were studied by ribotyping and by high-resolution AFLP fingerprinting, which has been found to be useful for the differentiation of *Acinetobacter* strains at the subspecies level (Dijkshoorn *et al.*, 1996; Janssen & Dijkshoorn, 1996; van Dessel *et al.*, 2003).

METHODS

Bacteria. Two sets of Czech *A. baumannii* strains were used in this study. Set ARC included 52 archive strains that were isolated in the Czech Republic between 1991 and 1999. These strains were selected from more than 700 clinical *Acinetobacter* isolates, in order to comprise hospital strains that were as heterogeneous as possible in terms of their time of isolation and geographical origin (18 cities were included). The ARC strains had been characterized in detail previously and were classified into group A ($n = 23$), group B ($n = 7$), a group of other MDR strains ($n = 7$) and a group of susceptible strains ($n = 15$) (Nemec *et al.*, 1999; Pantophlet *et al.*, 2001).

Set REC comprised 33 recent MDR *A. baumannii* strains from Czech hospitals that were selected, according to biochemical characteristics and susceptibility to antibiotics, from 250 clinical isolates that were referred to the National Institute of Public Health in 2000 and 2001. Strains were selected to be as geographically heterogeneous as possible (from 20 hospitals in 13 cities). Multiple isolates from the same hospital were not considered to be epidemiologically related, as assessed by biotyping (Bouvet & Grimont, 1987) and/or macrorestriction analysis of genomic DNA (Nemec, 1999). REC strains were recovered from sputum ($n = 9$), urine ($n = 7$), blood ($n = 7$), wound swabs ($n = 4$) and other clinical specimens, most of which were taken from intensive-care unit patients.

Reference strains of epidemic clones were RUH 436, RUH 510, RUH 875, RUH 2037, RUH 3238 (= GNU 1084), RUH 3239 (= GNU 1083), RUH 3242 (= GNU 1082), RUH 3247 (= GNU 1078) and RUH 3282 (= GNU 1079) for clone I, and RUH 134, RUH 3240 (= GNU 1086), RUH 3422 (= PGS 189) and RUH 3245 (= GNU 1080) for clone II. These strains were characterized in detail previously (Dijkshoorn *et al.*, 1996; Pantophlet *et al.*, 2001).

Phenotypic characteristics of all strains corresponded to those of the genus *Acinetobacter* (Juni, 1984). Strains were identified as *A. baumannii* according to *Eco*RI ribotypes (Nemec *et al.*, 1999) and were allocated to the biotypes of Bouvet & Grimont (1987) on the basis of utilization of laevulinate, citraconate, L-phenylalanine, phenylacetate, 4-hydroxybenzoate and L-tartrate.

Ribotyping. Ribotyping was carried out as described previously (Nemec *et al.*, 1999), with minor modifications. Total DNA was prepared by using SDS lysis, proteinase K treatment and phenol/chloroform extraction. Digestion was performed with *Hin*dIII and *Hin*cII in two separate steps. These enzymes were selected for the present study because they were found to show an optimal distribution of fragments for pattern analysis, compared to 15 enzymes tested [including *Eco*RI, which was used in previous studies (Nemec *et al.*, 1999; Pantophlet *et al.*, 2001)]. Electrophoretic separation of DNA fragments was done in 0·7 % (*Hin*dIII) or 0·8 % (*Hin*cII) agarose in TBE buffer (45 mM Tris/borate, 1 mM EDTA, pH 8·0) for 16 h. The voltage used was 45 and 35 V for *Hin*dIII and *Hin*cII, respectively. Fragments were blotted onto a nylon membrane, hybridized with a digoxigenin-labelled 16S–23S probe and visualized immunochemically. The resulting patterns were compared visually and distinct ribotypes were numbered arbitrarily. Each strain was characterized by a combined *Hin*dIII/*Hin*cII ribotype, e.g. *R1-1*. For cluster analysis, the presence or absence of a band at each position was scored as plus or minus, respectively. Percentage disagreement was used as a measure of dissimilarity between all pairs of *Hin*dIII/*Hin*cII ribotypes; it was expressed as the percentage of band position differences in a pair of ribotypes out of the total number of band positions (found in all ribotypes). Grouping was obtained by the UPGMA algorithm. All calculations were performed by using Statistica 5.1 software (StatSoft).

AFLP. AFLP fingerprinting was performed according to Nemec *et al.* (2001). Briefly, purified DNA was digested by using *Eco*RI and *Mse*I, while ligation of *Eco*RI and *Mse*I adaptors was performed simultaneously. PCR was done with a Cy5-labelled *Eco*RI + A primer and a *Mse*I + C primer (A and C represent selective nucleotides). The ALFexpress II DNA analysis system (Amersham Biosciences) was used for fragment separation. Fragments of 50–500 bp were subjected to

cluster analysis by using the BioNumerics software package, release 2.5 (Applied Maths), with an overall tolerance setting of 0·11 %. The Pearson product–moment coefficient (r) was used as the measure of similarity and UPGMA was used for grouping.

Antibiotic susceptibility testing. Antimicrobial susceptibility was determined by the disc diffusion method on Mueller–Hinton agar (Oxoid). Antimicrobial agents tested (Oxoid) were (μg per disc): ampicillin + sulbactam (10 + 10), piperacillin (100), ceftazidime (30), imipenem (10), gentamicin (10), tobramycin (10), amikacin (30), netilmicin (30), ofloxacin (5), cotrimoxazole (sulphamethoxazole + trimethoprim: 23·75 + 1·25) and tetracycline (30). Interpretative cut-off values for resistance were adjusted according to the known distribution of inhibition zone diameters among *A. baumannii* strains (Nemec, 1999). These values were identical to those recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 2001) for intermediate categories except for tetracycline and piperacillin, for which the NCCLS values for resistance were used. Multi-resistance was defined as resistance to at least two antibiotics that represent different antibiotic classes.

RESULTS AND DISCUSSION

Ribotyping

Ribotyping of all 98 strains with *Hin*dIII and *Hin*cII separately revealed 33 and 25 different band positions, respectively. In total, 24 different *Hin*dIII ribotypes, 20 *Hin*cII ribotypes and 29 combinations of *Hin*dIII and *Hin*cII ribotypes were identified. Examples of *Hin*dIII and *Hin*cII ribotypes are shown in Fig. 1. The most frequent ribotype was *R1-1*, which was found in 38 MDR Czech strains and in eight reference strains of clone I. Czech strains with ribotype *R1-1* had previously been classified as group A. The second most frequent ribotype was *R2-2*, which was found in 11 MDR Czech strains and three reference strains of clone II. Czech strains with this ribotype had previously been classified as group B. Strain RUH 3242 (clone I) was of ribotype *R3-1*, whereas RUH 3240 (clone II) was of ribotype *R4-2*. These ribotypes were also found in MDR Czech strains. Each of the susceptible strains showed a unique *Hin*dIII/*Hin*cII ribotype.

AFLP

A selection of 72 strains, which included 59 Czech strains that were representative of all different ribotypes and the 13 reference strains of clones I and II, was studied by AFLP. Frequent ribotypes were represented by several strains, which differed mostly in other characteristics (biotype, plasmid profile and antibiotic susceptibility). Clustering of the strains according to their AFLP fingerprints is shown in Fig. 2. At a level of 83 %, two major clusters of MDR strains could be distinguished: cluster 1 included all strains with ribotypes *R1-1* and *R3-1* and one strain with the unique ribotype *R5-3*; whereas cluster 2 included strains with ribotype *R2-2* and four other ribotypes (*R2-4*, *R4-2*, *R2-5* and *R6-4*). AFLP patterns of all susceptible strains and other MDR strains were heterogeneous and clearly distinct from those of strains included in clusters 1 and 2.

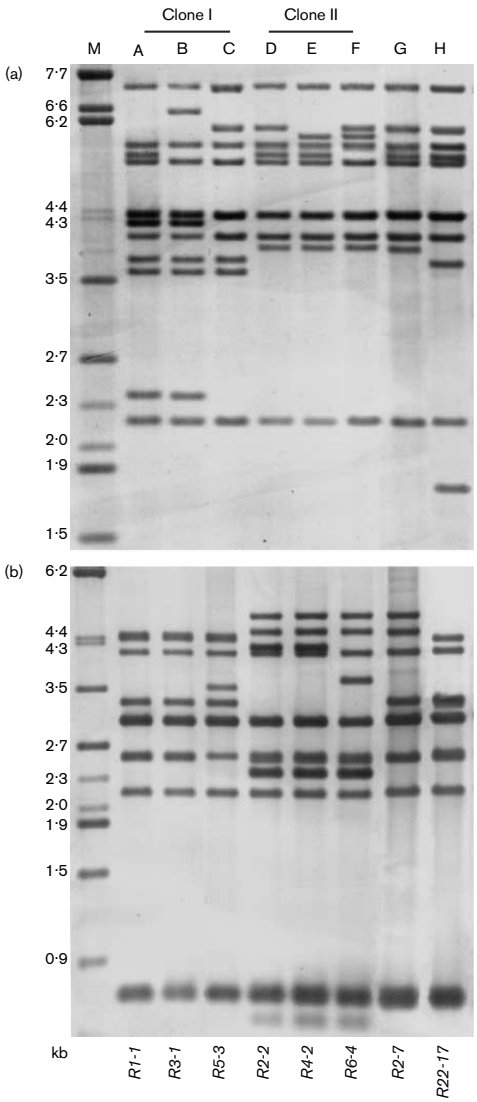

Fig. 1. Examples of (a) *Hin*dIII and (b) *Hin*cII ribotypes observed for *A. baumannii* strains. Strains are indicated by upper-case letters above the lanes: A, NIPH 7; B, NIPH 1605; C, NIPH 10; D, NIPH 24; E, NIPH 1362; F, NIPH 657; G, NIPH 301; H, NIPH 601. M, Molecular size marker (λ-phage DNA digested with *Hin*dIII and *Sty*I). Ribotype designations are given below the lanes.

Correlation between ribotyping and AFLP

There was a good correlation between AFLP and ribotyping results. Both AFLP clusters 1 and 2 contained strains of either identical or similar ribotypes that were specific for each of the

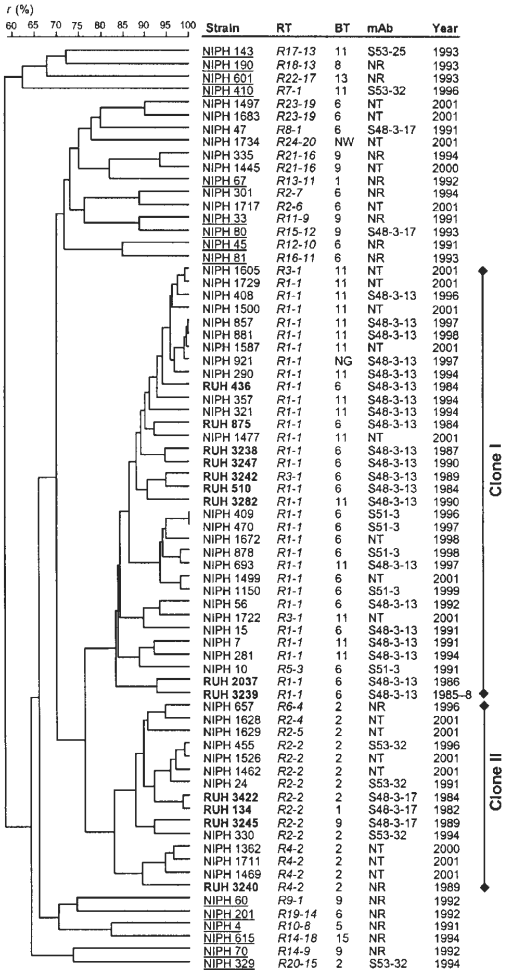

Fig. 2. Cluster analysis of AFLP fingerprints of 59 Czech *A. baumannii* strains (representative of different ribotypes) and 13 reference strains (RUH) for clones I and II. Strains NIPH 4–NIPH 657 belong to set ARC and strains NIPH 1362–NIPH 1729 belong to set REC. Susceptible strains are underlined. RT, *Hin*dIII-*Hin*cII ribotypes; BT, biotypes according to Bouvet & Grimont (1987); mAb, reactivity with O-antigen-specific mAbs (Pantophlet *et al.*, 2001). NG, No growth on mineral medium; NR, no reactivity with any of 20 antibodies tested; NT, not tested; NW, novel biotype.

clusters. This correlation was also found for strains linked in other clusters above 83 %, i.e. NIPH 1497 and NIPH 1683 or NIPH 335 and NIPH 1445 (Fig. 2). Clustering of *Hin*dIII/*Hin*cII ribotypes is shown in Fig. 3. Ribotypes *R1-1* and *R2-2*, which predominated among strains of AFLP clusters 1 and 2, respectively, were clearly distinct from each other (15 band differences in total). Differences between non-identical ribotypes of strains of the same AFLP cluster were small (Fig. 3). However, high similarity of some ribotypes was not confirmed by AFLP, e.g. strain NIPH 410, with a ribotype highly similar to *R1-1* (one band difference), was clearly different from clone I strains according to its AFLP pattern (Fig. 2) and other properties (Nemec *et al.*, 1999). This shows the limitation of ribotyping in estimating genetic relatedness of strains.

Relationship between the Czech groups and clones I and II

So-called epidemic clones I and II were distinguished originally among outbreak *A. baumannii* strains from north-western European hospitals on the basis of similarities in their genotypic and phenotypic properties (Dijkshoorn *et al.*, 1996). Within these clones, there was some intraclonal variability, but AFLP fingerprinting allowed unambiguous allocation of all strains to either clone I or clone II at a clustering level of 90 %. A further study showed that most MDR Czech strains belonged to two main groups, A and B, the delineation of which was based on identity in *Eco*RI ribotypes and supported by similarities in biochemical properties and plasmid profiles. It also appeared that groups A and B were similar to clones I and II, respectively, based on visual comparison of *Eco*RI ribotypes in studies that delineated these groups and clones, on inclusion of two reference strains of clones I and II in the study on Czech strains (Nemec *et al.*, 1999) and on common reactivity of clone I and group A strains with O-antigen-specific mAbs (Pantophlet *et al.*, 2001). However, as intraclonal variability of *Eco*RI ribotypes

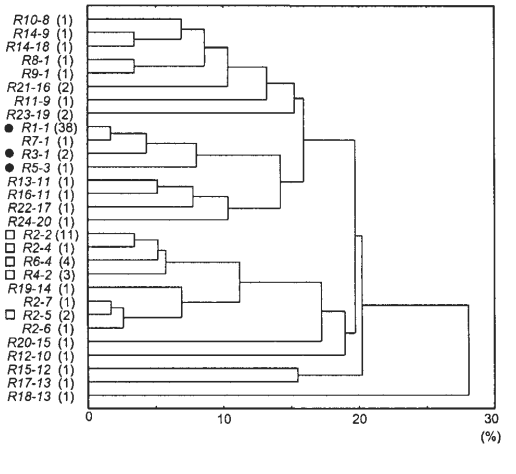

Fig. 3. Cluster analysis of *Hin*dIII/*Hin*cII ribotypes found in 85 Czech *A. baumannii* strains. No. strains with a respective ribotype is indicated in parentheses. Grouping was obtained by the UPGMA algorithm by using percentage disagreement. ●, Ribotypes of strains of AFLP cluster I (clone I); □, ribotypes of strains of AFLP cluster II (clone II).

was found in clone II (Dijkshoorn *et al.*, 1996) and could not be excluded for clone I, the relationship between the clones and some Czech strains remained unclear.

In the present study, a combination of ribotyping and AFLP results allowed the classification of 62 of 70 (89 %) MDR Czech strains into the north-western European clones. The current AFLP protocol was different from that used previously (Dijkshoorn *et al.*, 1996) with respect to the choice of restriction enzymes and selective primers and method of fragment separation. By this modified procedure, reference strains of clones I and II were linked at a level of 83 % in two major clusters. In total, 36 of 44 MDR Czech strains, including the strains allocated previously to groups A and B and strains with ribotypes that were highly similar to those of groups A and B, were found in these respective clusters. According to the positions and interrelatedness of strains in AFLP clusters 1 and 2 and overall similarity of their ribotypes and other characters (biotype, serotype defined by O-antigen-specific mAbs and plasmid content), we conclude that the Czech strains in these clusters belong to the previously described clones I and II (Fig. 2, Table 1). Similarity of AFLP and ribotypes are useful criteria to identify strains that belong to these clones.

Eight MDR and 15 susceptible strains were clearly distinct genotypically from clones I and II. These strains were highly heterogeneous in their AFLP pattern, ribotype (21 *Hin*dIII/*Hin*cII ribotypes), biotype (10 different biotypes), serotype (Pantophlet *et al.*, 2001) and plasmid profile (Nemec *et al.*, 1999). Similarly, remarkable heterogeneity of phenotypic and genotypic features was found among the strains from north-western Europe that were not allocated to clone I or II (Dijkshoorn *et al.*, 1996). These findings are suggestive of high genetic diversity in the general *A. baumannii* population.

Multi-drug resistance in Czech strains

Resistance of the Czech strains to 11 antibiotics is shown in Table 2. It is noteworthy that there was an apparent discontinuity in qualitative resistance between the susceptible and MDR strains, as shown in our previous study (Nemec *et al.*, 1999). Most susceptible strains were not resistant to any of the antibiotics tested, whereas 90 % of MDR strains showed resistance to five or more antibiotics. If susceptible to an antibiotic, MDR strains often had a smaller inhibition zone than susceptible strains (see Supplementary Table in JMM Online), which is indicative of their higher potential for being refractory to antimicrobial therapy.

Intraclonal diversity

Table 1 summarizes the ribotyping and biotyping results of the present study and those of biotyping, serotyping and plasmid analysis that were obtained previously (Nemec *et al.*, 1999; Pantophlet *et al.*, 2001). The data demonstrate some intraclonal variability in ribotype, biotype and serotype. Strains of clones I and II that were analysed in the present study were also heterogeneous in antibiotic resistance profile (see Supplementary Table in JMM Online) and plasmid profile (Nemec *et al.*, 1999). This intraclonal variation may result from ongoing diversification in space and time. One example of this diversification is the clone II strains that

Table 1. Properties of *A. baumannii* strains in clones I and II

Data are from this study, Nemec *et al.* (1999) and Pantophlet *et al.* (2001). Numbers in parentheses indicate no. strains with respective types. NT, Not tested; NR, no reactivity with any of the mAbs.

Clone/set of strains	Year of isolation	No. strains	*Hin*dIII/*Hin*cII ribotype	Biotype*	No. resistances per strain†	Reactivity with mAbs‡	No. of strains with 8·7 kb plasmid pAN1
Clone I:							
ARC	1991–1999	24	*R1-1* (23), *R5-3* (1)	6 (9), 11 (14)	7·1 [2–10]	S48-3-13 (18); S51-3 (6)	24
REC	2000–2001	17	*R1-1* (15), *R3-1* (2)	6 (6), 11 (10), 12 (1)	7·1 [5–10]	NT	NT
Reference strains	1984–1990	9	*R1-1* (8), *R3-1* (1)	6 (8), 11 (1)	6·6 [4–9]	S48-3-13 (9)	9
Clone II:							
ARC	1991–1997	10	*R2-2* (7), *R6-4* (3)	2 (10)	5·7 [3–8]	S53-32 (7); NR (3)	1
REC	2000–2001	11	*R2-2* (4), *R6-4* (1), *R4-2* (3), *R2-5* (2), *R2-4* (1)	2 (11)	5·8 [3–7]	NT	NT
Reference strains	1982–1989	4	*R2-2* (3), *R4-2* (1)	1 (1), 2 (2), 9 (1)	3·8 [1–5]	S48-3-17 (3); NR (1)	1

*Biotype according to Bouvet & Grimont (1987). One clone I strain (set ARC) was auxotrophic.

†Eleven antibiotics were tested (see Methods). Values are means, with range in square brackets.

‡Twenty mAbs against O-antigens were tested (Pantophlet *et al.*, 2001).

Table 2. Antibiotic resistance of Czech *A. baumannii* strains

Figures are percentages of resistant strains.

Antibiotic	Clone I ($n = 41$)	Clone II ($n = 21$)	Other multi-resistant strains ($n = 8$)	Susceptible strains ($n = 15$)
Ampicillin + sulbactam	61	57	25	0
Ceftazidime	41	67	13	0
Imipenem	0	10	25	0
Piperacillin	93	90	75	0
Amikacin	76	38	13	0
Gentamicin	95	76	100	0
Netilmicin	20*	10*	38	0
Tobramycin	39	5	75	0
Ofloxacin	95	71	75	0
Cotrimoxazole	95	57	63	13
Tetracycline	98	100	88	7

*The majority of non-resistant strains showed reduced inhibition zone diameters (15–19 mm) in comparison with the susceptible strains (23–26 mm).

shared ribotype *R4-2* and grouped in a distinct AFLP subcluster at a level of 88 %. Another example, although not reflected in the AFLP clustering pattern, is the Czech clone I strains of biotype 11. This biotype was the most frequent in Czech *A. baumannii* strains (Nemec *et al.*, 1999), but seems relatively rare in western Europe (Bouvet & Grimont, 1987; Seifert *et al.*, 1993). Most Czech strains of biotype 11 showed similarity in other properties (*Apa*I macrorestriction analysis profiles, inability to grow on L-arabinose and the presence of a 6 kb plasmid; data not shown) and are likely to represent a regional subclone. Thus, despite the noted similarity of strains that belong to the same clone, there are still characters that can be used to identify strains for epidemiological purposes.

Geographical spread of the clones

Our results and data from the literature indicate a pan-European spread of strains that are classifiable in clones I or II over a remarkable period of time. These strains were spread widely in Czech hospitals from at least 1991 to 2001 and were found in the Netherlands, the UK, Belgium and Denmark between 1982 and 1990 (Dijkshoorn *et al.*, 1996). They were also recognized by Brisse *et al.* (2000) and van Dessel *et al.* (2003) among quinolone-resistant *A. baumannii* isolates from different parts of Europe, including southern Europe, and one isolate from South Africa. Visual inspection of *Eco*RI ribotypes that were published by Seifert & Gerner-Smidt (1995) also suggests the occurrence of these strains in Danish and German hospitals. Finally, Pantophlet *et al.* (2001, 2002) have shown that serotypes found in strains of clones I and II (Table 1) are spread among *A. baumannii* strains from European countries, including Bulgaria and Hungary.

In conclusion, the results presented here confirm that MDR Czech strains of *A. baumannii* that were isolated from hospitalized patients belong mainly to two genetically distinct groups that were identified originally among strains in north-western Europe. These groups most probably represent old clones in a broad (evolutionary) sense, as can be judged from the noted intraclonal type variation and their wide distribution in space and time, as opposed to recent clonal lineages that are found in local outbreaks, which are usually relatively uniform in type characters. It is not yet known what properties have facilitated the wide spread of these MDR clones. It is possible that the capacity to develop or acquire antibiotic resistance was already an attribute of their ancestors and is a prerequisite for their success. Therefore, these clones, which are of undisputed clinical significance, are challenging targets for research on the evolution and spread of multi-drug resistance and of factors involved in *A. baumannii* epidemicity and pathogenicity.

Deposition of representative Czech strains in the CCM

The following strains were deposited in the Czech Collection of Microorganisms (CCM): CCM 7031 (= NIPH 7; clone I, the reference strain of group A), CCM 7032 (= NIPH 15; clone I/group A), CCM 7034 (= NIPH 281; clone I/group A), CCM 7116 (= NIPH 10; clone I), CCM 7033 (= NIPH 24; clone II, the reference strain of group B), CCM 7117 (= NIPH 657; clone II) and CCM 7118 (= NIPH 1362; clone II). The origin and properties of these strains are available in the Supplementary Table in JMM Online.

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CHAPTER 3

Nemec A, Dolzani L, Brisse S, van den Broek P, Dijkshoorn L.

Diversity of aminoglycoside resistance genes and their association with class 1 integrons among strains of pan-European *Acinetobacter baumannii* clones.

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Diversity of aminoglycoside-resistance genes and their association with class 1 integrons among strains of pan-European *Acinetobacter baumannii* clones

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The purpose of the present study was to investigate the diversity of the genes encoding aminoglycoside-modifying enzymes and their association with class 1 integrons in three pan-European clones of *Acinetobacter baumannii*. The study collection included 106 multidrug-resistant strains previously allocated to clone I ($n = 56$), clone II ($n = 36$) and clone III ($n = 6$) and a heterogeneous group of other strains ($n = 8$), using AFLP fingerprinting and ribotyping. The strains were from hospitals of the Czech Republic ($n = 70$; collected 1991–2001) and 12 other European countries ($n = 36$; 1982–1998). Using PCR, at least one of the following aminoglycoside-resistance genes was detected in 101 (95 %) strains: *aphA1* ($n = 76$), *aacC1* ($n = 68$), *aadA1* ($n = 68$), *aphA6* ($n = 55$), *aadB* ($n = 31$), *aacC2* ($n = 7$) and *aacA4* ($n = 3$). A combination of two to five different resistance genes was observed in 89 strains (84 %), with a total of 12 different combinations. PCR mapping revealed that *aacC1*, *aadA1* and *aacA4* were each associated with a class 1 integron, as was the case with *aadB* for six strains of clone III. Six different class 1 integron variable regions were detected in 78 strains (74 %), with two predominant regions (2·5 and 3·0 kb) in two sets of 34 strains each. The 3·0 kb region contained five gene cassettes (*aacC1*, orfX, orfX, orfX′, *aadA1*) and differed from the 2·5 bp region only by one additional orfX cassette. These two integron regions were confined to clones I and II and were found in strains isolated in seven countries between 1982 and 2001. The clone III strains were homogeneous both in resistance genes and in integron variable regions, whereas clones I and II showed a remarkable intraclonal diversity of these properties, with no clear-cut difference between the two clones. Yet, within the Czech clone I and II strains, the diversity of resistance genes and integron structures was limited as compared to those from other countries. The occurrence of identical resistance genes, gene combinations and class 1 integrons associated with these genes in clonally distinct strains indicates that horizontal gene transfer plays a major role in the dissemination of aminoglycoside resistance in *A. baumannii*.

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Abbreviation: MDR, multidrug-resistant.

The GenBank/EMBL/DDBJ accession number for the sequence of the 3·0 kb integron variable region of NIPH 7 (= CCM 7031 = LMG 22454) is AY577724.

Information on properties and origin of the strains used in this study is available as supplementary data in JMM Online.

INTRODUCTION

Acinetobacter baumannii is an important opportunistic pathogen that has the potential to spread among hospitalized patients and persist in the hospital environment (Bergogne-Bérézin & Towner, 1996). Recent studies have identified

three clones among multidrug resistant (MDR) isolates of *A. baumannii* from hospitals in different European countries. These included clones I and II from north-western Europe in the period 1982–1990 (Dijkshoorn *et al.*, 1996), which were also found to prevail in the Czech Republic between 1991 and 2001 (Nemec *et al.*, 2004), and clone III delineated among western European and Spanish strains from 1997 to 1998 (van Dessel *et al.*, 2004).

Aminoglycosides have long been used for the treatment of infections in hospitalized patients and still are an important alternative for therapy of infections caused by MDR strains. Previous studies have shown a high diversity of mechanisms of resistance to these antibiotics in the genus *Acinetobacter* (Shaw *et al.*, 1993; Miller *et al.*, 1995). Resistance to aminoglycosides has been attributed mainly to enzymic inactivation by acetyltransferases, nucleotidyltransferases and phosphotransferases (Shaw *et al.*, 1993), and *Acinetobacter* strains often contain multiple enzymes of these classes (Miller *et al.*, 1995; Seward *et al.*, 1998). The genes encoding aminoglycoside-modifying enzymes can be located on plasmids and transposons (Devaud *et al.*, 1982), and some of these genes have been found on class 1 integrons in MDR *A. baumannii* strains in Europe (Seward & Towner, 1999; Gallego & Towner, 2001; Gombac *et al.*, 2002; Ribera *et al.*, 2004).

Multidrug resistance is a striking feature of the strains belonging to clones I, II and III, and usually includes resistance to aminoglycosides. Overall, there is a great diversity in resistance phenotypes within the clones (Nemec *et al.*, 2004) but the genetic basis of this diversity has not been studied yet. Since multiple mechanisms may give rise to similar phenotypes, it is not known whether there is an association of particular antibiotic-resistance determinants with specific clones. The aim of the present study was to investigate the genetic basis of aminoglycoside resistance in the pan-European *A. baumannii* clones. To this aim, the occurrence of different genes encoding aminoglycoside-modifying enzymes and their correlation with aminoglycoside-resistance phenotypes was investigated in a set of well-defined strains from the Czech Republic and other European countries belonging to the three described clones. In addition, the structural types of class 1 integron variable regions and their association with aminoglycoside-resistance genes were assessed.

METHODS

Bacteria. A total of 106 MDR clinical *A. baumannii* strains from hospitals in the Czech Republic and other European countries were studied (Table 1). The collection comprised strains classified into clones I ($n = 56$), II ($n = 36$) and III ($n = 6$), and a heterogeneous group of other strains ($n = 8$). The Czech strains ($n = 70$) were isolated in 23 cities between 1991 and 2001 and were described recently (Nemec *et al.*, 2004). The non-Czech strains ($n = 36$) were isolated in 25 cities from 12 European countries between 1982 and 1998 and, except for four strains from Eastern Europe, have also been described previously (Dijkshoorn *et al.*, 1996; van Dessel *et al.*, 2004). All Czech strains and 13 other strains of clones I and II (Dijkshoorn *et al.*, 1996) have been characterized uniformly by AFLP, *Hin*dIII–*Hin*cII ribotyping and biotyping (Nemec *et al.*, 2004). For reasons of harmonization, the remaining strains, i.e. 19 of the study of van Dessel *et al.* (2004) and four Eastern European strains, were analysed by this panel of methods in the present study.

Antibiotic susceptibility testing. Susceptibility was determined by the disk diffusion method according to the National Committee for Clinical Laboratory Standards (NCCLS) recommendations (NCCLS, 2000) using Mueller–Hinton agar (Oxoid) and the following antimicrobial agents (μg per disk): kanamycin (30), gentamicin (10), tobramycin (10), amikacin (30) and netilmicin (30) (Oxoid). MICs of gentamicin, tobramycin, amikacin and netilmicin (MAST Group) were determined by the agar dilution method according to the NCCLS recommendations (NCCLS, 2003).

Detection of aminoglycoside-resistance genes. The presence of genes encoding the following aminoglycoside-modifying enzymes was investigated by PCR: phosphotransferases APH(3′)-Ia (*aphA1*) and APH(3′)-VIa (*aphA6*), acetyltransferases AAC(3)-Ia (*aacC1*), AAC(3)-IIa (*aacC2*) and AAC(6′)-Ib (*aacA4*), and nucleotidyltransferases ANT(2′′)-Ia (*aadB*) and ANT(3′′)-Ia (*aadA1*). The primers were those described by Noppe-Leclercq *et al.* (1999) for *aphA1*, *aacC1*, *aacC2*, *aacA4* and *aadB*, by Vila *et al.* (1999) for *aphA6* and by Clark *et al.* (1999) for *aadA1*. PCR reactions were performed in a final volume of 20 μl containing 10 μl *Taq* PCR Master Mix (Qiagen), 0·2 μM of each primer and 1·5 μl of a DNA suspension obtained by alkaline lysis as described by Nemec *et al.* (2000). The amplification reactions were performed in a FTGENE2D thermal cycler (Techne) with the following parameters: 94 °C for 2 min, followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 60 s at 72 °C. The presence and sizes of amplicons were assessed by electrophoresis in 2 % agarose gels stained with ethidium bromide.

Integron analysis. The presence of class 1 integrons was determined by PCR amplification of an internal fragment of the integrase gene (*intI1*) using the primers described by Koeleman *et al.* (2001). Amplification mixtures and conditions were as specified above. To detect inserted gene cassettes, variable regions of class 1 integrons were amplified with primers 5′CS and 3′CS, which are complementary to 5′ and 3′ conserved segments flanking the inserted DNA (Lévesque *et al.*, 1995). The association of aminoglycoside genes with integrons and the position of the associated genes inside the variable regions were investigated using PCR mapping with primer sets comprising the 5′CS primer and a primer for each individual gene (Lévesque *et al.*, 1995). The amplification protocol used for the 5′CS–3′CS amplification and PCR mapping consisted of 2 min at 94 °C, 35 cycles of 45 s at 94 °C, 45 s at 55 °C and 5 min at 72 °C, and a final extension of 7 min at 72 °C, while the PCR mixtures were prepared as described above. The sequence similarity of amplicons of the same size was investigated by restriction analysis with *Hin*fI and *Rsa*I in separate reactions. The nucleotide sequence of the cloned 3·0 kb variable region from strain NIPH 7 was determined by the dideoxy chain-termination method using an automatic DNA sequencer (ALFexpress II; Amersham Biosciences).

RESULTS AND DISCUSSION

Table 1 shows the distribution of the aminoglycoside-resistance genes, class 1 integron structures and resistance phenotypes of the strains arranged according to their clonal types. A more comprehensive table including quantitative antibiotic susceptibility data is available as supplementary data in JMM Online.

Table 1. Properties of the 106 strains studied

The strains were allocated to clones on the basis of the grouping obtained by AFLP and *Hin*dIII–*Hin*cII ribotyping (Nemec *et al.*, 2004) and are classified successively according to ribotype, resistance gene content, integron type, phenotype and country of isolation. The results of ribotyping and biotyping for NIPH and RUH strains are from Nemec *et al.* (2004). Resistance phenotypes for kanamycin (K), gentamicin (G), netilmicin (N), tobramycin (T) and amikacin (A) were determined using the disk diffusion test and NCCLS breakpoints for resistance (NCCLS, 2000). RT, *Hin*dIII–*Hin*cII ribotypes; BT, biotypes according to Bouvet & Grimont (1987); –, not found; NG, no growth on minimal medium; BE, Belgium; BG, Bulgaria; CZ, Czech Republic; DK, Denmark; ES, Spain; FR, France; GR, Greece; HU, Hungary; IT, Italy; NL, the Netherlands; PL, Poland; PT, Portugal.

Clone/strain designation*	RT	Aminoglycoside-resistance genes†	5′CS–3′CS amplicon (kb)	Resistance phenotype	BT	Country (years of isolation)	Reference‡
Clone I (*n* = 56)							
NIPH 188, NIPH 281, NIPH 307, NIPH 309, NIPH 357, NIPH 1486	1-1	*aacC1*, *aphA1*, *aphA6*, *aadB*	3·0	K, G, T, A	11	CZ (1993–2001)	1
NIPH 290	1-1	*aacC1*, *aphA1*, *aphA6*, *aadB*	3·0	K, G, N, T, A	11	CZ (1994)	1
NIPH 1477	1-1	*aacC1*, *aphA1*, *aphA6*, *aadB*	3·0	K, G, T	11	CZ (2001)	1
LUH 1396	1-1	*aacC1*, *aphA1*, *aphA6*, *aadB*	2·5	K, G, T, A	6	BG (1997)	
LUH 6017	1-1	*aacC1*, *aphA1*, *aphA6*, *aadB*	2·5	K, G, T, A	6	IT (1998)	3
NIPH 1475, NIPH 1693, NIPH 1729	1-1	*aacC1*, *aphA1*, *aphA6*, *aadB*	2·5	K, G, T, A	11	CZ (2001)	1
NIPH 409, NIPH 1150, NIPH 1359, NIPH 1488, NIPH 1499, NIPH 1574	1-1	*aacC1*, *aphA1*, *aphA6*	2·5	K, G, A	6	CZ (1996–2001)	1
NIPH 7, NIPH 321, NIPH 392, NIPH 857, NIPH 881, NIPH 1500	1-1	*aacC1*, *aphA1*, *aphA6*	3·0	K, G, A	11	CZ (1991–2001)	1
NIPH 921	1-1	*aacC1*, *aphA1*, *aphA6*	3·0	K, G, A	NG	CZ (1997)	1
NIPH 207	1-1	*aacC1*, *aphA1*, *aphA6*	3·0	K, G	11	CZ (1992)	1
RUH 3282 (= GNU 1079)	1-1	*aacC1*, *aphA1*, *aphA6*	3·0	K, G, N, A	11	UK (1990)	1, 2
NIPH 15, NIPH 360	1-1	*aacC1*, *aphA1*	3·0	K, G	6	CZ (1991–1994)	1
RUH 436, RUH 510, RUH 2037	1-1	*aacC1*, *aphA1*	3·0	K, G	6	NL (1984–1986)	1, 2
RUH 3238 (= GNU 1084), RUH 3239 (= GNU 1083)	1-1	*aacC1*, *aphA1*	3·0	K, G	6	UK (1985–1988)	1, 2
NIPH 1587, NIPH 1731	1-1	*aacC1*, *aphA1*	3·0	K, G	11	CZ (2001)	1
LUH 6015	1-1	*aacC1*, *aphA1*	2·5	K, G	6	IT (1998)	3
NIPH 1520, NIPH 1672	1-1	*aacC1*, *aphA1*	2·5	K, G	6	CZ (2001)	1
NIPH 408	1-1	*aacC1*, *aphA1*, *aadB*	3·0	K, G, T	11	CZ (1996)	1
NIPH 693	1-1	*aacC1*, *aphA6*, *aadB*	3·0	K, G, T, A	11	CZ (1997)	1
NIPH 878	1-1	*aacC1*, *aphA6*	2·5	K, G, A	6	CZ (1998)	1
RUH 875	1-1	*aphA1*, *aadB*	0·7	K, G, T	6	NL (1984)	1, 2
RUH 3247 (= GNU 1078)	1-1	*aphA1*, *aacA4*	0·8	K, G, N, T	6	BE (1990)	1, 2
LUH 5881	1-1	*aphA1*, *aacA4*	0·8	K, G, N, T, A	6	ES (1998)	3
NIPH 1358	1-1	*aphA6*, *aadB*	–	K, G, T, A	12	CZ (2000)	1
NIPH 470	1-1	*aphA6*	–	K, N, A	6	CZ (1997)	1
NIPH 654	1-1	*aphA6*	–	K, A	6	CZ (1996)	1
LUH 3584	1-1	*aadB*	–	K, G, T	6	HU (1995)	
NIPH 56	1-1	–	–	–	6	CZ (1992)	1
NIPH 1605	3-1	*aacC1*, *aphA1*, *aadB*	2·5	K, G, T	11	CZ (2001)	1
RUH 3242 (= GNU 1082)	3-1	*aacC1*, *aphA1*	3·0	K, G	6	UK (1989)	1, 2
LUH 6125 (= 14C052)	3-1	*aacC1*, *aphA6*	3·0	K, G, A	11	PL (1998)	3
NIPH 1722	3-1	*aphA6*, *aadB*	–	K, G, T, A	11	CZ (2001)	1
NIPH 10	5-3	*aacC1*, *aphA1*, *aphA6*	3·0	K, G, N, A	6	CZ (1991)	1
Clone II (*n* = 36)							
NIPH 455	2-2	*aacC1*, *aphA1*, *aphA6*	2·5	K, G, A	2	CZ (1996)	1
LUH 1398	2-2	*aacC1*, *aphA1*, *aphA6*	2·5	K, G, A	2	BG (1997)	
LUH 6021	2-2	*aacC1*, *aphA1*, *aphA6*	2·5	K, G, A	2	PL (1998)	3

(continued overleaf)

Table 1. cont.

Clone/strain designation*	RT	Aminoglycoside-resistance genes†	5′CS–3′CS amplicon (kb)	Resistance phenotype	BT	Country (years of isolation)	Reference‡
LUH 1245	2-2	*aacC1*, *aphA1*, *aadB*	2·5	K, G, T	2	HU (1993)	
RUH 134	2-2	*aacC1*, *aphA1*	3·0	K, G	1	NL (1982)	1, 2
NIPH 24, NIPH 330, NIPH 471, NIPH 499, NIPH 1526, NIPH 1567, NIPH 1696	2-2	*aacC1*, *aphA1*	2·5	K, G	2	CZ (1991–2001)	1
NIPH 220	2-2	*aacC1*, *aphA1*	2·5	K, G, N, T, A	2	CZ (1993)	1
NIPH 141	2-2	*aacC1*	2·5	G	2	CZ (1993)	1
LUH 6034	2-2	*aphA6*, *aacC2*	–	K, G, N, A	2	ES (1998)	3
RUH 3422 (= PGS 189)	2-2	*aphA1*	–	K	2	DK (1984)	1, 2
NIPH 1462	2-2	*aphA1*	–	K	2	CZ (2001)	1
RUH 3245 (= GNU 1080)	2-2	*aacC2*	–	G, N	9	UK (1989)	1, 2
LUH 6126 (= 15A250)	2-4	–	–	G, N	1	PT (1998)	3
NIPH 1628	2-4	–	–	–	2	CZ (2001)	1
NIPH 1511, NIPH 1629	2-5	*aacC1*, *aphA1*	3·0	K, G	2	CZ (2001)	1
LUH 5868	2-22§	*aphA1*	–	K, G, N, T, A	2	FR (1997)	3
RUH 3240	4-2	*aacC1*, *aacC2*	2·5	G, N	2	UK (1989)	1, 2
NIPH 1469	4-2	*aphA6*	–	K, A	2	CZ (2001)	1
NIPH 1362, NIPH 1711	4-2	–	–	–	2	CZ (2000–2001)	1
LUH 6011	4-21§	*aphA1*, *aacA4*	2·2	K, N, T	1	GR (1997)	3
NIPH 657, NIPH 720, NIPH 732, NIPH 1523	6-4	*aacC1*, *aphA1*, *aphA6*	2·5	K, G, A	2	CZ (1996–2001)	1
LUH 5865	26-2§	*aacC2*	–	G, N	2	ES (1998)	3
LUH 6024	26-2§	*aphA6*, *aacC2*	–	K, G, N, A	2	ES (1998)	3
LUH 6044, LUH 6029	26-2§	*aphA6*, *aacC2*	–	K, G, N, T, A	2	ES (1998)	3
Clone III ($n = 6$)							
LUH 6028, LUH 6037, LUH 6035	25-1	*aphA6*, *aadB*	0·75	K, G, T, A	9	ES (1997–1998)	3
LUH 6009, LUH 5874	25-1	*aphA6*, *aadB*	0·75	K, G, T, A	9	FR (1997)	3
LUH 5875	25-1	*aphA6*, *aadB*	0·75	K, G, T, A	9	NL (1997)	3
Other strains ($n = 8$)							
NIPH 1717	2-6	*aphA1*	–	K	6	CZ (2001)	1
NIPH 301	2-7	*aphA1*	–	K, G, N	6	CZ (1994)	1
NIPH 47	8-1	*aphA1*, *aadB*	–	K, G, T	6	CZ (1991)	1
NIPH 1445	21-16	*aphA1*, *aadB*	–	K, G, T	9	CZ (2000)	1
NIPH 335	21-16	*aadB*	–	K, G, T	9	CZ (1994)	1
NIPH 1497	23-19	*aphA1*	–	K, G, N, T	6	CZ (2001)	1
NIPH 1683	23-19	*aphA1*	–	K, G	6	CZ (2001)	1
NIPH 1734	24-20	*aphA6*, *aadB*	–	K, G, N, T, A	New	CZ (2001)	1

*The strain designations used in the previous studies are in parentheses.

†The *aacC1* gene was always found in association with *aadA1*. Genes integrated in class 1 integrons (as concluded from the results of PCR mapping) are underlined.

‡1, Nemec *et al.* (2004); 2, Dijkshoorn *et al.* (1996); 3, van Dessel *et al.* (2004).

§Novel ribotypes found for clone II strains. These ribotypes differed only in one or two band positions from those typical for clone II.

Aminoglycoside-resistance genes

A total of 102 strains (96 %) were fully resistant to at least one of kanamycin, gentamicin, tobramycin, netilmicin or amikacin, and at least one resistance gene was detected in 101 strains (95 %). The distribution of individual resistance genes among the strains is shown in Table 2. The observed high frequency of *aphA1*, *aadA1*, *aacC1*, *aphA6* and *aadB* is in agreement with the previously published data on clinical isolates of *Acinetobacter* spp. (Shaw *et al.*, 1993) and *A. baumannii* (Seward *et al.*, 1998). There was a good correlation between the content of resistance genes and

Table 2. Distribution of aminoglycoside-resistance genes and class 1 integron variable regions among the *A. baumannii* strains classified into clonal and geographical groups

Resistance gene/ variable region	Clone I		Clone II		Clone III ($n = 6$)	Other Czech strains ($n = 8$)	Total ($n = 106$)
	Czech ($n = 41$)	Non-Czech ($n = 15$)	Czech ($n = 21$)	Non-Czech ($n = 15$)			
Resistance gene							
aacC1	36	11	16	5	–	–	68 (64 %)
aadA1	36	11	16	5	–	–	68 (64 %)
aphA1	34	13	16	7	–	6	76 (72 %)
aphA6	32	4	6	6	6	1	55 (52 %)
aadB	16	4	–	1	6	4	31 (29 %)
aacC2	–	–	–	7	–	–	7 (7 %)
aacA4	–	2	–	1	–	–	3 (3 %)
Variable region (kb)							
3·0	23	8	2	1	–	–	34 (32 %)
2·5	13	3	14	4	–	–	34 (32 %)
2·2	–	–	–	1	–	–	1 (1 %)
0·8	–	2	–	–	–	–	2 (2 %)
0·75	–	–	–	–	6	–	6 (6 %)
0·7	–	1	–	–	–	–	1 (1 %)

resistance phenotypes (Table 1). Strains with *aphA1*, *aacC1* or *aphA6* were found to be resistant to kanamycin, gentamicin or kanamycin+amikacin, respectively, while *aadB* was associated with the resistance to kanamycin, gentamicin and tobramycin. In some strains, the presence of a resistance gene was associated only with intermediate or decreased susceptibility to a given antibiotic, e.g. the MICs for tobramycin in five strains with *aacC2* were in the range 4–8 μg ml^{-1}. High-level resistance to netilmicin was predominantly associated with the genes encoding netilmicin-modifying enzymes (i.e. 10 out of 16 strains with MICs ⩾64 μg ml^{-1} carried either *aacA4* or *aacC2*) while the majority of strains negative for these genes (71 %) showed intermediate or decreased susceptibility to this antibiotic (MICs 4–16 μg ml^{-1}). Low-level resistance to netilmicin, as frequently found in *Acinetobacter* spp., does not seem to be linked to enzymic modification (Miller *et al.*, 1995) but might be associated with the AdeABC efflux pump recently described in *A. baumannii* (Magnet *et al.*, 2001). In several strains with high-level aminoglycoside resistance (e.g. LUH 5868, NIPH 301 or NIPH 1497), none of the genes possibly involved in this resistance was found, which is also suggestive of additional resistance mechanisms.

Eighty-nine strains (84 %) had a combination of two to five different resistance genes, and a total of 12 different combinations were encountered (Table 1). The most frequent combinations, *aadA1* + *aacC1* + *aphA1* ($n = 24$) and *aadA1* + *aacC1* + *aphA1* + *aphA6* ($n = 23$), were detected in both clones I and II, while the combination of *aadA1* + *aacC1* + *aphA1* + *aphA6* + *aadB* ($n = 13$) was confined to clone I. Combinations of two or three genes encoding resistance to the same antibiotic (i.e. gentamicin or kanamycin) were found in 56 strains (53 %). The genes (*aphA1*, *aphA6*, *aadB* and *aacA4*) encoding kanamycin resistance can serve as an example. In contrast to the other genes, *aphA1* encodes resistance to kanamycin but not to other clinically important aminoglycosides such as gentamicin, amikacin or netilmicin. Forty-five out of the 97 strains resistant to kanamycin carried both *aphA1* and at least one of the other kanamycin-resistance genes, 31 strains only contained *aphA1* and 21 strains carried kanamycin-resistance genes other than *aphA1*. Thirteen out of 30 Czech kanamycin-resistant strains from 2000 to 2001 contained both *aphA1* and another kanamycin-resistance gene while other 13 strains carried *aphA1* alone. This, together with a decrease in kanamycin prescribing in the early 1990s, may indicate the stability of the *aphA1* gene in the *A. baumannii* population in the absence of an apparent selective advantage conferred by this gene.

Structural types of the variable regions of class 1 integrons and their association with aminoglycoside-resistance genes

All strains were investigated for the presence of the integrase gene *intI1* and variable regions of class 1 integrons. Seventy-eight strains (74 %) gave a positive reaction for the *intI1* gene and using the 5′CS and 3′CS primers, each of these strains yielded a single PCR product of between 0·7 and 3·0 kb. In total, amplicons of six different sizes were detected (Table 3). Amplicons of the same size gave identical restrictions patterns with *Hin*fI or *Rsa*I, which is indicative of their structural homogeneity. Comparison of the restriction patterns did not suggest a structural similarity between

Table 3. Characteristics of the variable regions of class 1 integrons found in this study

Variable region (kb)	*Hin*fI digestion products (bp)*	Inserted gene cassettes detected by PCR mapping
3·0	660, 490, 460, 400, 330, 250, 210, 110, 40	*aacC1*, *aadA1*
2·5	660, 490, 460, 330, 250, 210, 110, 40	*aacC1*, *aadA1*
2·2	770, 690, 430, 160	*aacA4*
0·8	500, 160	*aacA4*
0·75	350, 220, 190	*aadB*
0·7	470, 260	–

*Fragment sizes as determined by agarose gel analysis; double fragments (as derived from sequence analysis) are underlined.

different amplicons, except for the 2·5 and 3·0 kb amplicons, the *Hin*fI patterns of which differed only by an additional 400 bp fragment present in the 3·0 kb amplicon.

Possible association of integrons with aminoglycoside-resistance genes was investigated by PCR mapping of all integron-positive strains with the 5′CS primer in conjunction with primers from *aacC1*, *aacC2*, *aacA4*, *aadB* or *aadA1*. The results revealed the association of the 3·0 and 2·5 kb variable regions with *aacC1* and *aadA1*, the 2·2 and 0·8 kb regions with *aacA4*, and the 0·75 kb region with *aadB* (Table 3). The *aacC1*, *aadA1* and *aacA4* genes were found exclusively as part of integrons while *aadB* was integron-associated only in the strains of clone III. Taken together with the amplified external non-coding regions, the known sizes of the *aacA4* and *aadB* cassettes (e.g. accession nos AJ313334 and AF221902, respectively) were sufficient to account for the entire regions of the 0·8 and 0·75 kb amplicons, respectively. The results of PCR mapping indicated that the *aacC1* cassette was located at the 5′ end of both the 2·5 and 3·0 kb variable regions while *aadA1* was located at the 3′ end of both of these regions. In the case of the 2·2 kb variable region, the *aacA4* gene was found at the 5′ end of the region.

Gombac *et al.* (2002) characterized a variable region of 2·5 kb of class 1 integrons found in Italian strains of *A. baumannii*. This region consisted of four cassettes, i.e. *aacC1*, two open reading frames of unknown functions (orfX and orfX′) and *aadA1* (accession no. AJ310480). The restriction patterns of the 2·5 kb amplicons of the present study appeared to be identical to those of Gombac *et al.* (2002) (not shown). Sequencing of the 3·0 kb amplicon obtained from isolate NIPH 7 revealed an array of cassettes with the order *aacC1*, orfX, orfX, orfX′, *aadA1*, with both orfXs having the same sequence. Thus the 2·5 and 3·0 kb variable regions differ only by one orfX cassette. To our knowledge, this array of gene cassettes has been found only in a *Serratia marcescens* strain as part of a class 1 integron associated with a Tn*1696*-like transposon (Centrón & Roy, 2002; accession no. AF453999), where the orfX and orfX′ cassettes were termed orfP and orfQ, respectively.

Dissemination and stability of the class 1 integron structures

The structurally related 2·5 and 3·0 kb variable regions were by far the most prevalent integron structures of the present study (Table 2). They were found in both clones I and II but not in the other strains. The 3·0 kb region was detected in 34 strains isolated in four countries between 1982 and 2001 while the 2·5 kb region was found in 34 strains obtained from six countries between 1989 and 2001 (Table 1), indicating the spread of these structures over a relatively large time period. In addition, the comparison of the sizes, restriction patterns and gene cassette contents of the amplicons of 0·75, 0·8 and 2·2 kb with those of other studies (Gallego & Towner, 2001; Gombac *et al.*, 2002; Ribera *et al.*, 2004) suggested the geographical dissemination of other class 1 integrons with structurally related variable regions. It has been proposed that class I integrons comprise conserved and stable variable regions, with resistance genes transferred more often as part of the entire integron structure than as individual cassettes (Martinez-Freijo *et al.*, 1999). Indeed, the complexity of the 2·5 and 3·0 kb regions, their distinctness from other variable regions found in *A. baumannii* and their wide dissemination are indicative of the relative stability of these structures.

Intraclonal and geographical diversity of aminoglycoside-resistance genes and integrons

Table 2 shows the distribution of the resistance genes and integron structures among the strains classified into clonal and Czech or non-Czech groups. The clone III strains were homogeneous in all properties, which suggests a recent expansion from a common ancestor. In contrast, the strains of clones I and II showed a remarkable diversity of both resistance genes and integron variable regions. As many as nine and seven resistance gene combinations (including two to five different genes) were observed among the strains of clones I and II, respectively (Table 1). This is consistent with their intraclonal variability in biotype, serotype and plasmid profile and provides further evidence that these clones are relatively old groups that have been undergoing diversification (Nemec *et al.*, 2004). Thus intraclonal clusters of isolates with identical or highly similar genomic markers and content of resistance genes may represent particular strains (or

subclones) that emerge in a restricted geographical area. For example, all four Czech strains of ribotype R6-4 had the same resistance genes (*aadA1*, *aacC1*, *aphA1*, *aphA6*) while the *aacC2* gene was found in all Spanish isolates of ribotype 26-2 (Table 1).

The strains of clones I and II from the Czech Republic showed a limited spectrum of both resistance genes and integron structures as compared to those from other countries (Table 1). The Czech strains contained only the 2·5 and 3·0 kb integron structures and no *aacA4* or *aacC2* genes. In contrast, the *aacA4* and *aacC2* genes and class I integrons with regions other than those of 2·5 and 3·0 kb were found in clones I and II strains from Western Europe. The variations between geographically separated populations of clonally related strains may reflect local differences in composition of pools of resistance genes and/or differences in antibiotic usage (Miller *et al.*, 1995). Interestingly, Czech populations of both clones I and II shared all resistance genes (except for *aadB* found in clone I only) and integron variable regions. A possible explanation is that the two highly prevalent clonal groups of *A. baumannii* co-occupying a particular ecological niche and geographical region may more readily share genetic pools via horizontal gene transfer.

In conclusion, our study results show a remarkable intraclonal diversity of genes encoding aminoglycoside-modifying enzymes in the pan-European clones I and II. Identical resistance genes, gene combinations and class 1 integrons associated with these genes were found in both clones, indicating that horizontal gene transfer plays an important role in the dissemination of aminoglycoside resistance. In addition, the uniformity of the properties found in clusters of isolates within these clones suggests that clonal expansion further facilitates the spread of resistance. To unravel whether there are properties responsible for the wide occurrence of clones I and II still remains a challenging task. It is conceivable that both the ancient acquisition of some resistance determinants and the high capacity to develop or acquire resistance have contributed to their success. The investigation of mechanisms of resistance to other groups of antibiotics and molecular characterization of mobile structures carrying resistance genes in taxonomically and epidemiologically well-defined strains will contribute to better a understanding of the evolution of multidrug resistance in *A. baumannii*.

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CHAPTER 4

Nemec A, Maixnerová M, van der Reijden TJK, van den Broek P, Dijkshoorn L.

Relationship between the AdeABC efflux system gene content, netilmicin susceptibility and multidrug resistance in a genotypically diverse collection of *Acinetobacter baumannii* isolates.

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Relationship between the AdeABC efflux system gene content, netilmicin susceptibility and multidrug resistance in a genotypically diverse collection of *Acinetobacter baumannii* strains

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***Objectives*: To assess the occurrence of the genes of the AdeABC efflux system and their association with antimicrobial resistance in *Acinetobacter baumannii*.**

***Methods*: A set of 116 strains selected for their diversity both in genotypic properties and geographic origin was investigated for the presence of the structural (*adeA*, *adeB* and *adeC*) and regulatory (*adeR* and *adeS*) genes of the AdeABC system by PCR, for resistance to 11 antimicrobials by disc diffusion, for MIC of netilmicin and for the presence of *aacC2* and *aacA4*, encoding netilmicin-modifying enzymes.**

***Results*: Ninety-five strains were positive for *adeA*, *adeB*, *adeR* and *adeS*, 10 were positive for 1 to 3 of these genes and 11 were negative for all genes. The *adeC* gene was found in 49 strains with one or more of the other genes. Forty-one strains were resistant to a maximum of one agent and 75 strains to two or more agents. Netilmicin MICs showed an almost bimodal distribution with respective peaks of 0.5–1 and 8 mg/L; *aacC2* or *aacA4* was found in six strains with netilmicin MIC of $\geq$64 mg/L. All 61 strains with netilmicin MICs $\geq$ 4 mg/L were both *adeABRS*-positive and resistant to two or more agents, whereas netilmicin MICs $\leq$ 2 mg/L (n = 51) were found for all strains resistant to a maximum of one agent and those negative for one or more of the *adeABRS* genes.**

***Conclusions*: The AdeABC genes are common in *A. baumannii*, but may be absent in some, mostly fully susceptible strains. Decreased susceptibility to netilmicin (MIC 4–32 mg/L) is associated with both the presence of these genes and multidrug resistance and may be indicative of AdeABC overexpression.**

Keywords: European clones, AFLP, PCR detection

Introduction

Acinetobacter baumannii is notorious for its involvement in nosocomial infections and epidemic spread among severely ill patients.[1] The organism is characterized by its ability to evolve resistance to multiple antibiotics and there are recent reports on strains resistant to all clinically relevant drugs.[2] Over the last few decades, many resistance mechanisms have been identified in *A. baumannii*, of which β-lactamases, aminoglycoside-modifying enzymes, tetracycline efflux pumps and alteration in quinolone target sites are considered to be the most important.[2,3]

In 2001, a novel resistance mechanism, the efflux pump system AdeABC, was identified in a multidrug-resistant (MDR) *A. baumannii* strain.[4] This system was shown to be responsible for decreased susceptibility to a broad spectrum of antimicrobials. Although netilmicin and gentamicin appeared to be the best substrates for the pump, the activity of AdeABC has also been associated with other agents including β-lactams, fluoroquinolones, tetracyclines[4] and recently, with tigecycline.[5] Three sequential, clustered genes *adeA*, *adeB* and *adeC* were found to encode proteins homologous to membrane fusion, drug transporter and outer membrane components, respectively, characteristic

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of the RND efflux pump family.[6] It was further revealed that a susceptible *A. baumannii* strain containing the AdeABC genes could produce spontaneous resistant variants with mutations in the *adeS* or *adeR* genes, which encode a two-component system regulating AdeABC expression, and the reduction in susceptibility associated with the efflux system has been attributed to its constitutive overexpression.[7]

Elucidation of the AdeABC complex at the molecular and functional levels has been based mainly on the study of a single *A. baumannii* strain, BM4454.[4,7] Relatively little is known about its distribution and biological significance at the population level. So far, only two studies have reported its occurrence among multiple *A. baumannii* strains. Huys *et al.*[8] found *adeB* in 49 out of 51 strains originating almost exclusively from Europe, whereas in another study, 39 out of 56 strains from Hong Kong tested *adeB* positive.[9] However, the strains of the first study belonged mostly (80%) to three groups of genetically related strains (clones), whereas no epidemiological or microbiological data on strains were shown in the second study.

The aim of the present study was to assess the occurrence of the structural and regulatory genes of the AdeABC system in a genetically and geographically heterogeneous collection of *A. baumannii* strains and to investigate the association of the AdeABC efflux genes with resistance to antimicrobial agents. In addition, the role of reduced susceptibility to netilmicin as a tentative phenotypical marker for the up-regulation of AdeABC was studied.

Materials and methods

Bacteria

One hundred and sixteen *A. baumannii* strains used in the present study were selected from the Leiden University Medical Center (LUMC) collection. This collection comprises more than 8000 *Acinetobacter* isolates of which approximately 2000 have been investigated for their genotype by AFLP whole genome fingerprint analysis (AFLP^{TM}).[10] Cluster analysis of AFLP fingerprints has allowed assessment of similarities among strains and revealed that similarities above 50%, ~80% and 90% are indicative of relatedness at the species, clone and strain level, respectively.[11] Thus, using the dendrogram of all fingerprints (data not shown), unique strains ($n = 45$) or representatives of clusters of strains with similar genotypes ($n = 71$) were selected at a cluster cut-off level of ~80% in order to cover the overall diversity of the *A. baumannii* LUMC collection. The dendrogram of the 116 strains studied is shown in Figure 1. At a cut-off level of 83%, seven AFLP clusters of strains were distinguished; three of them corresponded to European clones I ($n = 24$), II ($n = 19$) and III ($n = 10$) described previously,[11–13] whereas the four others (A–D) may represent new clonal lineages. The strains were obtained from 16 countries between 1982 and 2004 and were from human ($n = 107$) or animal ($n = 8$) specimens or from the hospital environment ($n = 1$), except for three reference strains (ATCC 19606^T, NCTC 10303 and NCTC 7844) which had been isolated before 1963 (Figure 1).

Gene detection

The presence of three structural (*adeA*, *adeB* and *adeC*) and two regulatory (*adeS* and *adeR*) genes of the AdeABC system, and two genes encoding netilmicin-modifying acetyltransferases AAC(3)-IIa and AAC(6′)-Ib (*aacC2* and *aacA4*, respectively), was determined by PCR. The primers were those described for *adeB*,[4] for *adeC*, *adeS* and *adeR*[7] and for *aacC2* and *aacA4*.[14] To detect *adeA*, new primers (A-am: 5′-GCTGAGCCACCACCGGCTAAAG-3′ and A-av: 5′-ACCTTCAACAACGACTCTGTCACC-3′) with an expected amplicon size of 990 bp were used in this study. PCR reactions were performed in a final volume of 20 μL containing 10 μL of Taq PCR Master Mix (Qiagen, Hilden, Germany), 0.2 μM each primer and 1.5 μL of a DNA suspension obtained by alkaline lysis.[15] The PCR reactions were performed in a FTGENE2D thermal cycler (Techne, Duxford, UK) with these parameters: initial denaturation at 94°C for 2 min, 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 2 min and a final elongation at 72°C for 2 min. The presence and sizes of amplicons were assessed by electrophoresis in 2% agarose gels stained with ethidium bromide.

Susceptibility testing

Susceptibility was determined by disc diffusion following the CLSI (formerly NCCLS) guidelines[16] using Mueller–Hinton agar (Oxoid, Basingstoke, UK) and 11 antimicrobial agents, which are primarily effective against susceptible *A. baumannii* strains. The cut-off values for resistance were adjusted according to the known distribution of inhibition zone diameters among *A. baumannii* strains.[11] These values were identical to those recommended by the CLSI guidelines[16] for intermediate susceptibility except for tetracycline and piperacillin, for which the CLSI values for resistance were used.[16] The agents (content in micrograms/disc; resistance breakpoint in millimetres) included gentamicin (10; ≤14), netilmicin (30; ≤14), tobramycin (10; ≤14), amikacin (30; ≤16), ampicillin+sulbactam (10 + 10; ≤14), piperacillin (100; ≤17), ceftazidime (30; ≤17), imipenem (10; ≤15), ofloxacin (5; ≤15), sulfamethoxazole+trimethoprim (23.75 + 1.25; ≤15) and tetracycline (30; ≤14) (Oxoid). MIC of netilmicin (MAST Group, Bootle, UK) was determined by the agar dilution method, according to the CLSI guidelines.[16] The netilmicin susceptibility and resistance breakpoints used were ≤8 and ≥32 mg/L, respectively.[16] All susceptibility tests were carried out in duplicate and were repeated twice if discordant results had been obtained.

Results

Detection of the genes associated with the AdeABC efflux system

PCR results for all five AdeABC-associated genes are shown in Figure 1 and Table 1. Among 116 strains, 47 (40.5%) were PCR positive for all genes, 48 (41.4%) were positive for all genes except for *adeC*, 1 (0.9%) was positive for all genes except for *adeS*, 9 (7.8%) were positive for 1 to 3 genes and 11 (9.5%) were negative for all 5 genes. Strains belonging to the same AFLP cluster yielded the same combination of PCR reactions except for one clone II strain with negative reaction for *adeS* and for the strains of AFLP cluster B, which had variable reactions for *adeB* and/or *adeS* (Table 1). The strains positive for all five genes belonged to clone I ($n = 24$), clone II ($n = 18$) or had unique AFLP genotypes ($n = 5$), whereas strains positive for all genes but *adeC* were associated with 25 unique genotypes and 4 AFLP clusters.

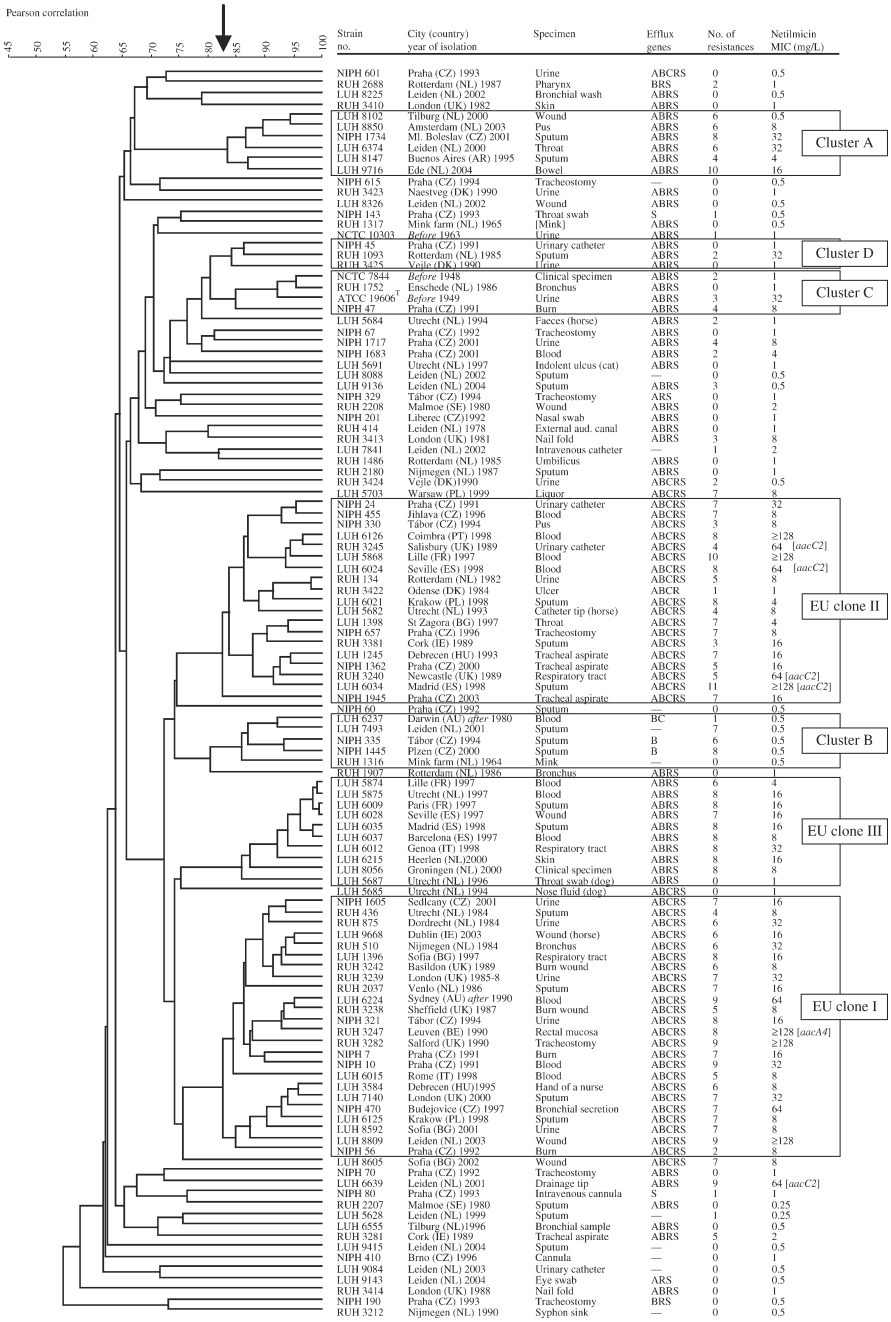

Figure 1. Dendrogram of cluster analysis of AFLP fingerprints of 116 *A. baumannii* strains included in this study. The origin of strains and the results of PCR detection of the AdeABC system-associated genes and of susceptibility testing are indicated. AFLP analysis was performed as described previously;[10] clusters delineated at a cut-off level of 83% are indicated by boxes. The strains were from humans if not stated otherwise. The presence of the netilmicin-modifying genes is indicated in parentheses. A, *adeA*; B, *adeB*; C, *adeC*; R, *adeR*; S, *adeS*; —, no gene detected.

Table 1. PCR detection of the structural and regulatory genes of the AdeABC efflux system in 116 *A. baumannii* strains classified according to their AFLP genotype

AFLP genotype[a]	No. of strains	*adeS*	*adeR*	*adeA*	*adeB*	*adeC*	No. of resistances per strain[b]
Clone I	24	+	+	+	+	+	6.9 (2–9)
Clone II	18	+	+	+	+	+	6.4 (3–11)
	1	−	+	+	+	+	1
Clone III	10	+	+	+	+	−	6.9 (0–8)
Cluster A	6	+	+	+	+	−	6.7 (4–10)
Cluster B	2	−	−	−	+	−	7 (6–8)
	1	−	−	−	+	+	1
	2	−	−	−	−	−	3.5 (0–7)
Cluster C	4	+	+	+	+	−	2.3 (0–4)
Cluster D	3	+	+	+	+	−	0.7 (0–2)
Unique	5	+	+	+	+	+	3.2 (0–7)
	25	+	+	+	+	−	1.2 (0–9)
	2	+	+	−	+	−	1 (0–2)
	2	+	−	−	−	−	1
	2	+	+	+	−	−	0
	9	−	−	−	−	−	0.2 (0–1)

[a]AFLP clusters or clones were delineated as shown in Figure 1.
[b]Eleven antibiotics tested. Values are means, with range in parentheses.

Resistance to antimicrobial agents

The percentages of strains resistant to an antimicrobial agent according to disc diffusion were as follows: gentamicin (56%), netilmicin (18%), tobramycin (25%), amikacin (28%), ampicillin+sulbactam (24%), piperacillin (56%), ceftazidime (32%), imipenem (6%), ofloxacin (42%), sulfamethoxazole+trimethoprim (65%) and tetracycline (62%). Table 2 shows the distribution of the number of agents to which individual strains were resistant and demonstrates that the vast majority of the strains were either susceptible to all antimicrobial agents (29%) or resistant to three or more agents (59%), although only 12% of the strains were resistant to one or two agents.

Susceptibility to netilmicin

The distribution of netilmicin MICs among the 116 strains is shown in Figure 2. The MIC values ranged between 0.25 and $\geq$128 mg/L with MIC_{50} and MIC_{90} being 8 and 16 mg/L, respectively, and showed an almost bimodal distribution with the respective peaks being 0.5–1 and 8 mg/L. The genes *aacC2*

Table 2. Distribution of the strains according to the degree of multiresistance and netilmicin MIC

No. of antibiotics to which a strain was resistant	No. of strains with netilmicin MIC (mg/L)										
	0.25	0.5	1	2	4	8	16	32	64	≥128	Total
0	1	14	18	1							34
1	1	2	3	1							7
2		1	3		1	1		1			7
3		1				2	1	1			5
4					1	4			(1)[a]		6
5				1		3	1		(1)		6
6		2			1	3	1	3			10
7		1			1	6	6	3	1		18
8		1			1	2	6	2	(1)	1+(1)	15
9								1	1+(1)	2	5
10							1			1	2
11										(1)	1

[a]Strains PCR positive for the genes encoding netilmicin-modifying enzymes (*aacC2* or *aacA*) are indicated in parentheses.

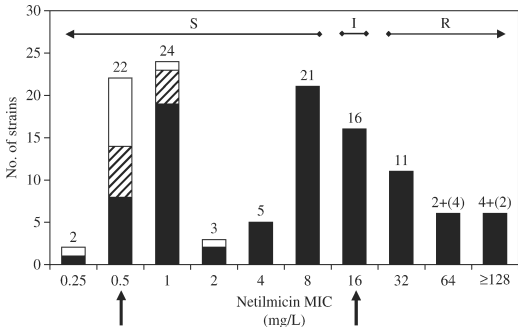

Figure 2. Distribution of netilmicin MICs among the 116 *A. baumannii* strains classified according to the presence of the genes associated with the AdeABC system. Black bars, positive for *adeA*, *adeB*, *adeR* and *adeS*, and variable reactions for *adeC*; white bars, negative reactions for all five genes; shaded bars, the remaining combinations of positive and negative reactions for all genes (Table 1). The vertical arrows indicate the published data for strain BM4454 with the up-regulated system (16 mg/L) and its derivative with inactivated *adeB* (0.5 mg/L).[4] Numbers of strains with the genes encoding netilmicin-modifying enzymes are indicated in parentheses. Interpretation according to the CLSI breakpoints[16] is depicted above the columns. S, susceptible; I, intermediate; R, resistant.

and *aacA4*, which encode the netilmicin-modifying enzymes most commonly found in *A. baumannii*, were found in five strains and one strain (Figure 1), respectively, all of which had netilmicin MIC $\geq$ 64 mg/L.

Relationship between the AdeABC system gene content, netilmicin susceptibility and multidrug resistance

Figure 2 and Table 2 show the relationships between the content of the efflux genes, MICs to netilmicin and multidrug resistance. As *adeC* was shown not to be essential for the efflux function,[7] this gene was not included in the following comparisons. Thus, the strains could be classified into three groups: (i) positive for all four genes ($n = 95$); (ii) negative for all genes ($n = 11$) and (iii) positive for one to three genes ($n = 10$) (Figure 2). Except for four, all strains ($n = 75$) resistant to two or more agents were positive for all essential genes, whereas among 41 strains resistant to less than two agents, as many as 17 (41%) strains yielded negative results for one or more genes (Figure 1). Figure 2 shows that all strains with netilmicin MICs $\geq$ 4 mg/L were positive for all four genes, whereas all strains negative for one or more of these genes had netilmicin MICs $\leq$ 2 mg/L. As indicated in Table 2, all strains ($n = 59$) with netilmicin MICs $\geq$ 4 mg/L were resistant to two or more agents, whereas strains ($n = 51$) with netilmicin MICs $\leq$ 2 mg/L included all fully susceptible strains.

Discussion

The results of the present study suggest that the AdeABC system occurs in the vast majority of the *A. baumannii* population. At least one of the five genes associated with AdeABC was found in 91% of all strains. Even if only one representative per AFLP cluster is considered, as many as 82% of strains were positive for at least one gene (Figure 1). Most of the 116 strains (82%) yielded positive PCR results for all efflux genes, except for *adeC*, which was found in only 35% of them. The absence of *adeC*, which is thought to encode an outer membrane porin, may be explained by the fact that this porin is not essential for the efflux function.[7] However, negative PCR results obtained with the specific primers inferred from one sequence have to be interpreted with caution, as they may result from the polymorphism of DNA regions targeted by primers. Similarly, 10 strains yielded different combinations of positive and negative results for *adeA*, *adeB*, *adeS* or *adeR* (Table 1) and further studies are required to determine whether the negative results truly indicate the absence of parts of the efflux system gene cluster considered essential for the system to function.

To assess the involvement of the AdeABC system in antimicrobial susceptibility, a simple phenotypic marker of the efflux up-regulation is needed. Ideally, such a marker should clearly differentiate between the strictly regulated and up-regulated efflux forms and should not be affected by other resistance mechanisms. Even though such a marker is unlikely to be found among clinically used antimicrobials, the published data show that decreased susceptibility to netilmicin might serve as an indication of the up-regulated AdeABC. First, among different antimicrobial agents, netilmicin and gentamicin showed the highest differences between the MICs for the MDR strain BM4454 and its derivative with the disrupted *adeB* gene.[4] Secondly, although clinically relevant aminoglycoside resistance in *A. baumannii* has been attributed mainly to enzymatic modification of antibiotics,[17,18] modification of netilmicin—in contrast to gentamicin—may be infrequent, especially in countries where the prescription of netilmicin is low.[15] For the identification of modifying enzymes which can decrease susceptibility to netilmicin, the detection of genes known to encode these enzymes in *A. baumannii* can be used.[15]

In the present study, the netilmicin MIC values showed an almost bimodal distribution (Figure 2) with the respective peaks near to the published values for strain BM4454 with up-regulated AdeABC efflux system (16 mg/L) and its derivative with inactivated *adeB* (0.5 mg/L).[4] Another study[7] showed that the challenge of a susceptible strain (gentamicin MIC, 1 mg/L) with gentamicin resulted in the selection of mutants with an up-regulated AdeABC system (gentamicin MIC, 12 mg/L). These results are also consistent with our data, assuming that the up-regulation of the AdeABC system leads to similar increased MIC values of both gentamicin and netilmicin.[4] Such congruency between our data and the quoted data may suggest that the *A. baumannii* population forms two major groups according to efflux activity. It can be hypothesized that MICs of $\sim$0.5–1 mg/L may reflect the inactivity of the system because of its stringent regulation, alteration or absence, whereas values $\sim$8–16 mg/L could result from the constitutive expression of the system. This hypothesis is indirectly supported by our other data. First, all strains with netilmicin MICs $\geq$ 4 mg/L harboured all four genes essential for the efflux activity,[4,7] whereas all strains negative for at least one of these genes had MICs $\leq$ 2 mg/L. Secondly, the genes encoding netilmicin-modifying enzymes were not detected in any of the strains with netilmicin MICs $\leq$ 32 mg/L. Finally, comparison of inhibition zone diameters produced by different aminoglycosides in individual strains did not indicate the involvement of permeability resistance

(data not shown), which is indicated by a proportional decrease in susceptibility to all aminoglycosides.[19] Thus, all strains with netilmicin MICs of 4–32 mg/L were associated with all essential efflux genes, although in none of them were non-efflux mechanisms detected. In 6 of 12 strains with MICs $\geq$ 64 mg/L, the genes encoding netilmicin-modifying enzymes were found, which could explain the high level of netilmicin resistance, whereas the absence of these genes in the other 6 strains may indicate the involvement of other factors.

It is noteworthy that studies conducted by the Schering-Plough Research Institute in the 1980s indicated a high prevalence of the aminoglycoside resistance phenotype similar to that of BM4454[4] among worldwide *Acinetobacter* spp. strains.[18] This phenotype was characterized by low-level resistance to netilmicin and gentamicin, resistance to two non-clinical derivatives of netilmicin (2′-*N*-ethylnetilmicin and 6′-*N*-ethylnetilmicin) and by susceptibility to the other aminoglycosides. The hypothetical mechanism underlying this phenotype was provisionally designated AAC(3)-?, yet the corresponding acetyltransferase has never been identified.[18] It is conceivable that the AdeABC system was responsible for AAC(3)-?, suggesting that the up-regulated form of this system was widespread among *A. baumannii* strains more than two decades ago.

In conclusion, the results of the present study are suggestive of the association between resistance to multiple agents and the up-regulated AdeABC system in *A. baumannii*, as indicated by the observed link between the presence of the genes essential for the activity of the AdeABC, decreased netilmicin susceptibility and multidrug resistance. However, although the up-regulated AdeABC system was shown to transport a wide range of structurally dissimilar compounds, the level of resistance to individual agents conferred by the system usually does not reach clinical resistance breakpoints.[4,7,20] Values exceeding these breakpoints are therefore likely to result from the presence of other, more effective, mechanisms or from a combination of different mechanisms.[6,21] It has been suggested that increased expression of chromosomally encoded efflux systems may be the first step in the bacterium becoming fully resistant.[6] Thus, originally susceptible, AdeABC-positive strains may produce mutants with constitutive expression of the AdeABC. It confers low-level protection that may facilitate the initial survival of the organisms in an antimicrobial-rich environment such as the hospital and enable them to acquire subsequently specific, high-level resistance mechanisms. This could explain why genotypically distinct MDR strains harbouring different combinations of specific resistance mechanisms share the up-regulated AdeABC system.

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Transparency declarations

None to declare.

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CHAPTER 5

Nemec A, Krizova L, Diancourt L, Maixnerova M, J. K. van der Reijden T, Brisse S, van den Broek P, Dijkshoorn L.

Emergence of carbapenem resistance in *Acinetobacter baumannii* in the Czech Republic is associated with the spread of multidrug resistant strains of European clone II.

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Emergence of carbapenem resistance in *Acinetobacter baumannii* in the Czech Republic is associated with the spread of multidrug-resistant strains of European clone II

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***Objectives*: The aim of this study was to analyse the emergence of carbapenem resistance among hospital strains of *Acinetobacter* in the Czech Republic.**

***Methods*: *Acinetobacter* isolates were collected prospectively in 2005–06 from 19 diagnostic laboratories. They were identified to species level by AFLP, typed using AFLP, pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing, and tested for susceptibility to 14 antimicrobials and for the presence of 20 genes associated with antimicrobial resistance.**

***Results*: A total of 150 *Acinetobacter* isolates were obtained from 56 intensive care units of 20 hospitals in 15 cities. They were identified as *Acinetobacter baumannii* ($n = 108$) or other species. *A. baumannii* isolates were allocated to EU clone I ($n = 5$), EU clone II ($n = 66$) or other, mostly unique genotypes. Two-thirds of the clone II isolates had nearly identical AFLP and PFGE fingerprints. As many as 85% and 88% isolates were susceptible to meropenem and imipenem ($\leq$4 mg/L), respectively. Carbapenem MICs of $\geq$8 mg/L were found in 23 *A. baumannii* isolates, of which 20 belonged to clone II. Isolates with $bla_{\text{OXA-58-like}}$ ($n = 3$), $bla_{\text{OXA-24-like}}$ ($n = 1$) or IS*Aba1* adjacent to $bla_{\text{OXA-51-like}}$ ($n = 34$) had carbapenem MICs of 2 to >16 mg/L, while those without these elements showed MICs of $\leq$0.5–4 mg/L. Clone II isolates varied in susceptibility to some antibiotics including carbapenems and carried 6–12 resistance genes in 17 combinations.**

***Conclusions*: The emergence of *Acinetobacter* carbapenem resistance in the Czech Republic is associated with the spread of *A. baumannii* strains of EU clone II. The variation in susceptibility in these strains is likely to result from both the horizontal spread of resistance genes and differential expression of intrinsic genes.**

Keywords: European clonal lineages, AFLP, PCR gene detection, OXA-type carbapenemases

Introduction

Bacteria of the genus *Acinetobacter*, with *Acinetobacter baumannii* in particular, are notorious for their involvement in nosocomial infections and spread among severely ill patients.[1] These organisms are frequently resistant to multiple antimicrobial agents and there are recent reports on strains resistant to virtually all clinically relevant drugs. Extensive genotypic characterization has shown that, within *A. baumannii*, clusters of highly similar strains occur, which are assumed to represent distinct clonal lineages. Of these, the so-called European (EU) clones I, II and III are widely spread across Europe and include strains that are usually multidrug-resistant (MDR) and associated with outbreaks of hospital infections.[1–3]

Carbapenem resistance in *Acinetobacter* spp. has emerged as a significant health problem over the last decade, leaving limited

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options for antimicrobial therapy.[1] This resistance has been attributed to the production of carbapenem-hydrolysing β-lactamases (carbapenemases), although other mechanisms can also be involved, including those that reduce membrane permeability, alter penicillin-binding proteins or expel drugs from the cell.[1] Carbapenemases found in *Acinetobacter* belong to molecular class D (OXA enzymes) or class B (metalloenzymes of IMP- and VIM-type or SIM-1). The OXA carbapenemases of *Acinetobacter* are divided into four phylogenetic subgroups: acquired enzymes OXA-23-like, OXA-24-like and OXA-58-like, and OXA-51-like enzymes that are intrinsic to *A. baumannii*. OXA-51-like enzymes are normally expressed at low levels but can be overexpressed as a consequence of the insertion of an IS*Aba1* sequence upstream of their genes.[4,5]

Our previous studies showed that resistance of *Acinetobacter* isolates to carbapenems was rare in the Czech Republic till the early 2000s.[3,6] However, in 2003 and 2004, *A. baumannii* isolates resistant to these antibiotics were received by the National Institute of Public Health (NIPH) in Prague from several hospitals. This observation gave rise to the current study to analyse the emergence of carbapenem resistance among clinical *Acinetobacter* isolates at the country level. We investigated prospectively collected *Acinetobacter* isolates for their species and strain diversity, and for susceptibility to carbapenems and the presence of genes linked to carbapenem resistance. Furthermore, to obtain a comprehensive view of the situation, the susceptibility and presence of genes conferring resistance to other clinically important agents were determined.

Materials and methods

Collection of Acinetobacter isolates

Acinetobacter strains were collected prospectively from 19 diagnostic laboratories in the Czech Republic between January 2005 and April 2006. The laboratories were asked to send clinically relevant isolates of *Acinetobacter* spp. obtained from patients hospitalized at intensive care units (ICUs) with no more than one isolate per patient and 10 isolates per ICU. Isolates sent to the NIPH were confirmed for the genus identity and presumptively identified to species using a set of biochemical tests[7] and assessed for susceptibility to 12 antimicrobial agents using disc diffusion (see below). Isolates from the same ICU that were indistinguishable from each other according to phenotype were further typed using *Apa*I macrorestriction analysis by pulsed-field gel electrophoresis (PFGE). From each group of isolates with a common PFGE profile, sharing phenotypic properties and originating from the same ICU, one isolate was selected for further investigation. Thus, a final set of 150 *Acinetobacter* isolates remained from a total of 265 isolates received by the NIPH. The 150 isolates were from 56 ICUs of 20 hospitals in 15 cities and were recovered from sputum ($n = 69$), wounds or pus ($n=19$), urine ($n = 17$), blood or intravenous catheters ($n = 15$) or from other clinical specimens ($n = 30$).

Genomic fingerprinting and multilocus sequence typing (MLST)

AFLP genomic fingerprinting performed as described[8] was used both to identify strains to species and to classify them at the subspecies (clone, strain) level. DNA macrorestriction analysis by PFGE included digestion of agarose plugs containing genomic DNA with *Apa*I (New England Biolabs; 30 U per plug) for 2 h at 25°C, followed by separation of restriction fragments with a CHEF-DRII device (Bio-Rad) through a 1.2% SeaKem LE agarose gel (Cambrex) in TBE buffer at 14°C for 19 h (pulse times 5–20 s at 6 V/cm). The resulting PFGE fingerprints were compared visually: patterns that differed in the position of more than six bands were designated by different capitals, while those differing in the positions of one to six bands were marked with the same letter followed by different numerals (Figure 1). MLST was based on a sequence analysis of the internal portions of seven housekeeping genes (*cpn60*, *fusA*, *gltA*, *pyrG*, *recA*, *rplB* and *rpoB*). Details of the MLST scheme including amplification and sequencing primers, allele sequences and STs are available at Institut Pasteur's MLST Web site (www.pasteur.fr/mlst).

Susceptibility testing

Resistance to 12 antimicrobial agents that are primarily effective against susceptible *A. baumannii* strains was determined by disc diffusion following the CLSI guidelines.[9] The cut-off values for resistance were adjusted according to the distribution of inhibition zone diameters among *A. baumannii* strains.[3] The agents (content in μg/disc; resistance breakpoint in mm) included gentamicin (10; ≤14), netilmicin (30; ≤14), tobramycin (10; ≤14), amikacin (30; ≤16), ampicillin + sulbactam (10 + 10; ≤14), piperacillin (100; ≤17), ceftazidime (30; ≤17), meropenem (10; ≤15), imipenem (10; ≤15), ofloxacin (5; ≤15), sulfamethoxazole + trimethoprim (23.75 + 1.25; ≤15) and doxycycline (30; ≤15) (Oxoid). MICs were determined by the agar dilution method according to the CLSI guidelines using the CLSI susceptibility and resistance breakpoints.[9] Etest MBL strips (AB Biodisk, Solna, Sweden) as well as a synergy test using imipenem- and EDTA-containing discs[10] were used to screen for metallo-β-lactamase production.

Gene detection

The presence of the following genes was determined by PCR amplification of: the genes encoding the class D carbapenemases OXA-23-like, OXA-24-like, OXA-51-like and OXA-58-like;[11] the genes encoding the metallo-β-lactamases IMP, VIM and SIM-1;[12] the genes encoding aminoglycoside-modifying phosphotransferases APH(3′)-Ia (*aphA1*) and APH(3′)-VIa (*aphA6*), acetyltransferases AAC(3)-Ia (*aacC1*), AAC(3)-IIa (*aacC2*) and AAC(6′)-Ib (*aacA4*), and nucleotidyltransferases ANT(2″)-Ia (*aadB*) and ANT(3″)-Ia (*aadA1*);[13] the $bla_{\text{TEM-1-like}}$ gene encoding TEM-1-like β-lactamases;[14] the *ampC*-like gene encoding class C β-lactamases intrinsic to *A. baumannii*;[15] the *tet*(A) and *tet*(B) genes encoding the respective tetracycline-specific efflux pumps;[16] the class 1 integrase gene *intI1*;[13] the *adeB* and *adeR* genes encoding the structural and regulatory proteins of the AdeABC efflux system, respectively,[8] and the IS*Aba1* insertion sequence gene.[4] To determine the structure of class 1 integron variable regions, PCR mapping and restriction analysis of amplicons obtained by PCR with primers targeting 5′ and 3′ conserved integron segments were carried out as previously.[13] The location of IS*Aba1* in the upstream region of the chromosomal genes encoding OXA-51-like and AmpC-like β-lactamases was determined according to Turton *et al.*[4] and Ruiz *et al.*,[17] respectively.

Results

Species diversity and antimicrobial susceptibility of non-A. baumannii isolates

Using AFLP analysis, the 150 *Acinetobacter* isolates were identified as *A. baumannii* ($n = 108$), genomic sp. 3 ($n = 30$),

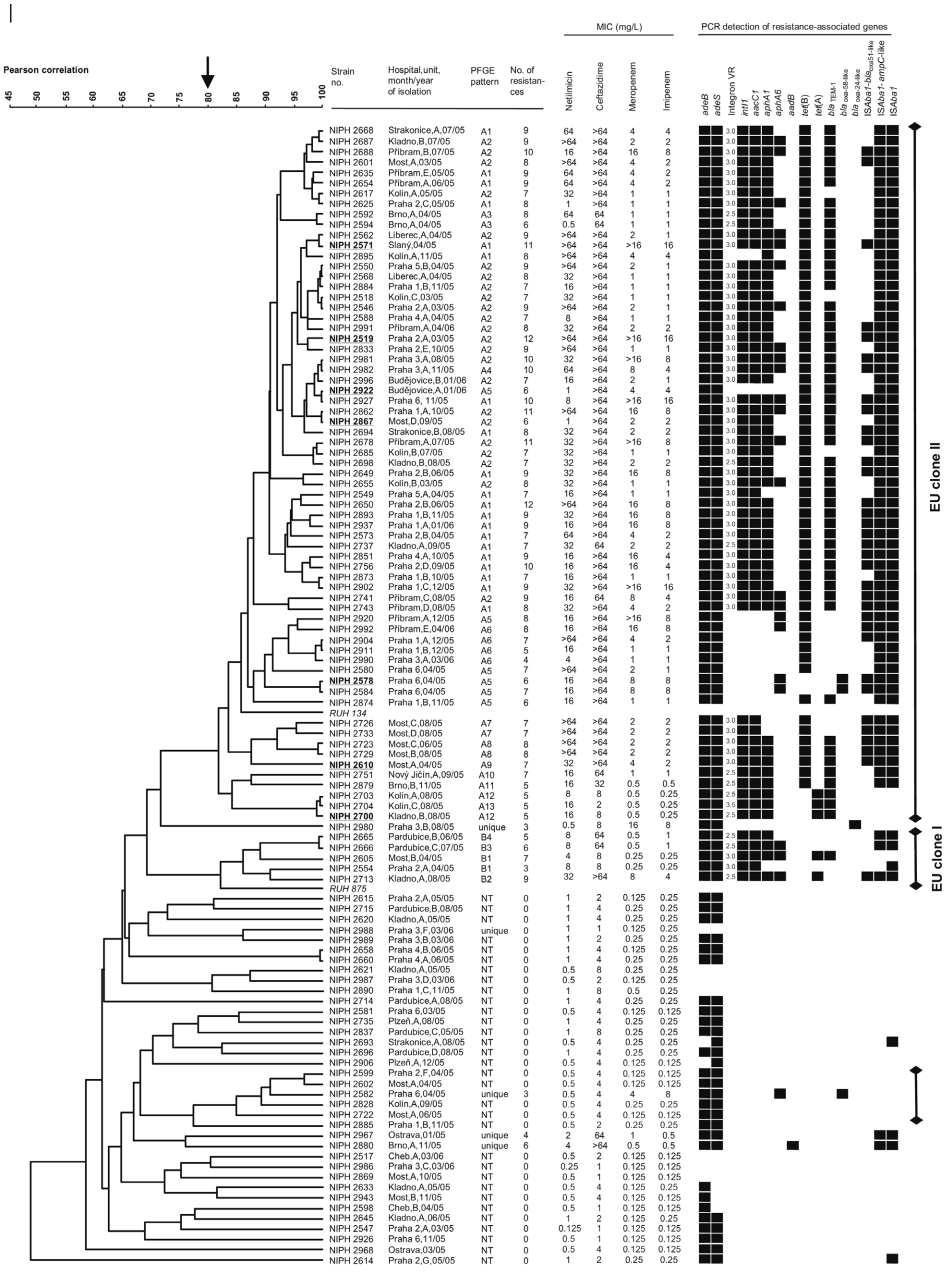

Figure 1. Dendrogram of cluster analysis of AFLP fingerprints of 108 *A. baumannii* isolates in the present study. Clusters of more than two isolates defined at 80% are marked by vertical lines. Numbers following the city name indicate different hospitals in the city; capitals denote different ICUs in the same hospital. Indicated are the numbers of antimicrobial agents to which an isolate was resistant using disc diffusion with 12 antimicrobial agents. Positive PCR results are presented by black boxes. All strains were positive both for *ampC*-like and $bla_{\text{OXA-51-like}}$, and negative for $bla_{\text{IMP-like}}$, $bla_{\text{VIM-like}}$, $bla_{\text{SIM-1}}$, *aacC2* and *aacA4*. All strains positive for *aacC1* were also positive for *aadA1*. VR 2.5, 3.0 and 3.5 denote three respective variable class 1 integron regions. Positive PCR results for primer combinations IS*Aba1*-$bla_{\text{OXA-51-like}}$ and IS*Aba1*-*ampC*-like indicate the location of IS*Aba1* in the promotor regions of $bla_{\text{OXA-51-like}}$ and *ampC*-like, respectively. The designations of isolates studied by MLST are underlined. RUH 134 and RUH 875 are the reference strains of EU clones II and I, respectively.[2] NT, not tested; blank, negative (except for RUH 134 and RUH 875 for which no data are shown).

genomic sp. 13 TU ($n = 8$), *Acinetobacter calcoaceticus* ($n = 1$), *Acinetobacter schindleri* ($n = 1$) or *Acinetobacter junii* ($n = 1$). One isolate could not be allocated to any of the known *Acinetobacter* species. The MICs of imipenem and meropenem for the non-*A. baumannii* isolates ranged from $\leq$0.125 to 0.5 mg/L. All non-*A. baumannii* isolates were fully susceptible to the 12 antimicrobials tested by disc diffusion, except for four isolates of gen. sp. 3, which were resistant to gentamicin and tobramycin and/or ofloxacin, and for one gen. sp. 13 TU isolate that was resistant to gentamicin and netilmicin. The non-*A. baumannii* isolates were negative for all resistance genes except for three aminoglycoside resistant isolates of gen. sp. 3, which were PCR-positive for *aadB*, and for all gen. sp. 13 TU isolates, which were positive for at least one of the genes associated with the AdeABC efflux system.

Population structure of A. baumannii isolates

The results of cluster analysis of the AFLP fingerprinting of 108 *A. baumannii* isolates are shown in Figure 1. Using a cut-off of 80% (which corresponds to the approximate grouping level of strains of the same clone[3,18]), the isolates were classified into one major cluster with 66 isolates, two clusters with 5 isolates each, 5 pairs and 22 single isolates. The major cluster and one of the small clusters corresponded to EU clones II and I, respectively, while none of the strains was found to group with strains of EU clone III (data not shown). Most clone II isolates yielded identical or highly similar PFGE patterns (Figure 2) and 45 (68%) of the clone II isolates clustered together according to their AFLP patterns at $\geq$90% (Figure 1), indicating that they were genetically related at the subclonal level.[18] MLST was performed for seven clone II isolates that differed from each other in PFGE/AFLP patterns or/and in resistance phenotype (Figure 1). Six of them had ST2 (2-2-2-2-2-2-2), which seems to be the typical ST of EU clone II (L. Diancourt, V. Passet, A. Nemec, L. Dijkshoorn and S. Brisse, unpublished results), while NIPH 2578 yielded ST47 (2-13-2-2-2-2-2), a single locus variant of ST2.

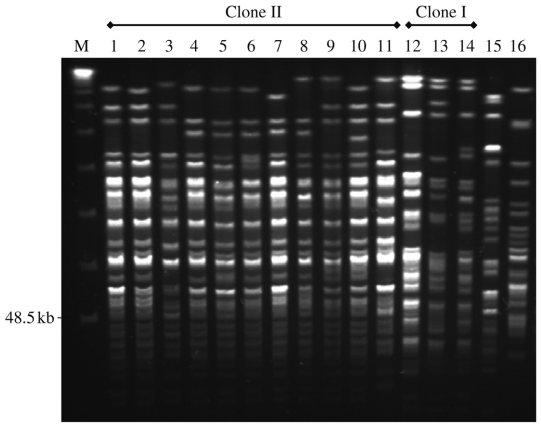

Figure 2. Examples of the *Apa*I macrorestriction patterns of *A. baumannii* isolates. Strains are indicated by the numbers above the lanes: 1, NIPH 2571; 2, NIPH 2895; 3, NIPH 2700; 4, NIPH 2519; 5, NIPH 2981; 6, NIPH 2867; 7, NIPH 2982; 8, NIPH 2990; 9, NIPH 2874; 10, NIPH 2610; 11, NIPH 2578; 12, NIPH 2713; 13, NIPH 2605; 14, NIPH 2666; 15, NIPH 2988; 16, NIPH 2706. Lane M, molecular size markers (48.5 kb ladder).

Resistance of A. baumannii isolates to carbapenems

According to MICs, 85 (79%) *A. baumannii* isolates were susceptible (MIC $\leq$ 4 mg/L) to both imipenem and meropenem, while 23 (21%) isolates were either intermediate (8 mg/L) or resistant ($\geq$16 mg/L) to at least one carbapenem (Figure 1). Out of the 85 susceptible isolates, 40 had MICs $\leq$ 0.5 mg/L for both carbapenems, but 45 showed reduced susceptibility (MICs 1.0–4.0 mg/L) to at least one carbapenem. All 68 isolates with carbapenem MICs $\geq$ 1 mg/L were also resistant to at least one other antimicrobial agent and belonged to clone II, clone I or three unique AFLP genotypes. None of the isolates was PCR-positive for the genes encoding metallo-β-lactamase IMP-like, VIM-like or SIM-1. In addition, no metallo-β-lactamase activity was detected in any of 16 isolates with imipenem MICs $\geq$ 16 mg/L, using Etest MBL and a double-disc synergy test.

The results of PCR detection of OXA-type carbapenemases are shown in Table 1. All isolates were positive for $bla_{\text{OXA-51-like}}$, three were positive for $bla_{\text{OXA-58-like}}$ and one for $bla_{\text{OXA-24-like}}$. Using the IS*Aba1* forward primer and the OXA-51-like gene reverse primer, 34 isolates yielded a PCR amplicon of ~1.2 kb, which indicates the location of IS*Aba1* in the upstream region of the OXA-51-like gene of these isolates.[4] The remaining 74 isolates showed no PCR product, although 37 of them were positive for the IS*Aba1* sequence (Figure 1). The isolates with $bla_{\text{OXA-58-like}}$, $bla_{\text{OXA-24-like}}$ or/and IS*Aba1* adjacent to $bla_{\text{OXA-51-like}}$ had carbapenem MICs of 2 to >16 mg/L (MIC_{50} 8 mg/L and $MIC_{90} \geq 16$ mg/L), while those without evidence of these genetic structures showed MICs of $\leq$0.5–4 mg/L (MIC_{50} 0.5 mg/L and MIC_{90} 2 mg/L) (Table 1).

Resistance of A. baumannii isolates to non-carbapenem agents

According to disc diffusion, 33 isolates were fully susceptible to all 12 antimicrobials, while 75 isolates showed resistance to $\geq$3 agents (Figure 1). The MICs of imipenem and meropenem for the 33 fully susceptible isolates ranged from $\leq$0.125 to 0.5 mg/L, while those of the 75 isolates resistant to $\geq$3 agents were between 0.25 and >16 mg/L. Out of these 75 isolates, 5 and 66 belonged to clones I and II, respectively.

There was a good correlation between the presence of the genes associated with resistance to non-carbapenem agents and MIC of these agents. All isolates positive for *aphA6* ($n = 24$), *aacC1* ($n = 60$) and *aadB* ($n = 1$) were resistant to or, in a few cases, intermediate to amikacin, gentamicin and tobramycin + gentamicin, respectively. All 61 *tet*(B)-positive isolates showed doxycycline MICs $\geq$ 32 mg/L, while the *tet*(B)-negative isolates had MICs of $\leq$8 mg/L. The MICs of ampicillin/sulbactam of $\geq$16/8 mg/L were found only in isolates harbouring the $bla_{\text{TEM-1-like}}$ gene ($n = 53$), while all but one isolate with MICs of $\leq$8/4 mg/L were $bla_{\text{TEM-1-like}}$-negative. Using the IS*Aba1* forward primer and the *ampC*-like gene reverse primer, 68 isolates yielded a PCR product of ~750 bp, which indicates the presence of IS*Aba1* in the promoter region of the *ampC*-like gene.[17] Ceftazidime MICs against these 68 isolates were

Table 1. Relationship between carbapenem MICs and the presence of genes associated with the decreased susceptibility or resistance to carbapenems in 108 *A. baumannii* isolates

	No. of isolates								
	MIC of imipenem/meropenem (mg/L)								
Genes[a]	≤0.25	0.5	1	2	4	8	16	>16	Total no. of isolates
No gene[b] (non-EU clone II)	35/34	2/4	2/1	0/0	0/0	0/0	0/0	0/0	39
No gene (EU clone II)	3/0	1/4	22/17	4/7	3/5	0/0	0/0	0/0	33
IS*Aba1*-$bla_{\text{OXA-51-like}}$[c]	0/0	0/0	0/0	13/8	5/5	10/3	4/9	0/7	32[d]
IS*Aba1*-$bla_{\text{OXA-51-like}}$ + $bla_{\text{OXA-58-like}}$	0/0	0/0	0/0	0/0	0/0	2/2	0/0	0/0	2[e]
$bla_{\text{OXA-58-like}}$	0/0	0/0	0/0	0/0	0/1	1/0	0/0	0/0	1[f]
$bla_{\text{OXA-24-like}}$	0/0	0/0	0/0	0/0	0/0	1/0	0/1	0/0	1[f]

[a]All isolates were positive for the $bla_{\text{OXA-51-like}}$ gene and negative for the genes encoding OXA-23-like, IMP, VIM and SIM-1 carbapenemases.
[b]Neither $bla_{\text{OXA-24-like}}$, $bla_{\text{OXA-58-like}}$ nor IS*Aba1* located in the $bla_{\text{OXA-51-like}}$ promoter region was detected.
[c]IS*Aba1* located in the promoter region of the $bla_{\text{OXA-51-like}}$ gene.
[d]All except one were EU clone II isolates.
[e]EU clone II isolates.
[f]Non-EU clone II isolates.

≥32 mg/L, whereas those against 40 isolates without IS*Aba1* in the promoter region were ≤8 mg/L (Figure 1).

Both the *adeB* and *adeS* genes that are associated with the AdeABC efflux system were detected in 96 isolates, while seven isolates were negative for both genes and five isolates were positive for only one of the genes. Only the isolates positive for both genes showed increased netilmicin MICs (≥4 mg/L) (Figure 1), which may indicate up-regulation of the efflux.[8] The class 1 integrase gene was found in 60 isolates (belonging to either clone I or II) and was unequivocally associated with the *aacC1* and *aadA1* genes and with PCR products obtained with the primers aimed to amplify variable integron regions. Three different variable regions with the respective sizes of 2.5, 3.0 and 3.5 kb were detected (Figure 1), and restriction analysis and PCR mapping of these structures revealed that they contained the same genes in the same order [*aacC1*-(orfX)$_{1\text{-}3}$-orfX′-*aadA1*], differing only in the number of orfX copies.[13]

Heterogeneity of resistance phenotypes and genotypes within EU clone II isolates

The susceptibility rates of clone II isolates ($n = 66$) according to the MIC and the CLSI breakpoints[9] were as follows (% susceptible isolates): imipenem (76), meropenem (70), ceftazidime (5), piperacillin (0), ampicillin + sulbactam (23), gentamicin (14), tobramycin (80), amikacin (68), netilmicin (12), sulfamethoxazole + trimethoprim (12), doxycycline (6), ciprofloxacin (0) and colistin (98). As many as 21 different resistance profiles were identified among these isolates and a similar heterogeneity was revealed at the gene level. The isolates were positive for the tested genes as follows (% PCR-positive isolates): $bla_{\text{TEM-1-like}}$ (80), *tet*(B) (92), *tet*(A) (5), *aacC1* (83), *aphA1* (80), *aphA6* (30), $bla_{\text{OXA-58-like}}$ (3), *intI1* (83) and IS*Aba1* (95). The integron variable regions of 2.5, 3 and 3.5 kb were found in 8, 46 and 1 isolate, respectively. Individual strains carried from 6 to 12 resistance-associated genes in 17 different combinations. Some isolates with the same PFGE patterns and obtained from the same ICU (e.g. NIPH 2893 and NIPH 2873) differed in the combination of resistance genes, whereas other isolates indistinguishable from each other by genotype and phenotype originated from different cities (e.g. NIPH 2601 and NIPH 2991) (Figure 1).

Discussion

Of the 150 *Acinetobacter* isolates in the present study, 146 (97%) were identified as *A. baumannii* (72%) or other genomic species of the *Acinetobacter calcoaceticus–A. baumannii* complex (25%). Nearly all strains resistant to multiple antimicrobial agents belonged to *A. baumannii*, and the vast majority of these MDR isolates were allocated to EU clone I or II. These results are consistent with those of our retrospective study on the *A. calcoaceticus–A. baumannii* complex isolates collected in Czech hospitals in 1991–97.[3,6] However, whereas in the present study, 5 and 66 isolates were allocated to clones I and II, respectively, 39 and 9 isolates from the 1990s were classified into the respective clones. Even though the results of the two studies are not directly comparable as the strain inclusion criteria differed, the data suggest a shift in the recent *A. baumannii* population towards clone II.

In the present study, 90% of the isolates with decreased susceptibility or resistance to carbapenems (≥1 mg/L) and 83% of those resistant to one or more non-carbapenem agents belonged to EU clone II. The wide spread of clone II may have resulted from its selective advantage in the antibiotic-rich hospital environment and could further be facilitated by the absence of effective measures to prevent the transmission of MDR microorganisms, a problem commonly encountered in Czech hospitals. Other European studies have also recently reported on the spread of strains of clone II and on the association of carbapenem resistance with these strains.[18,19] EU clone II thus seems to be particularly successful in its spread in European countries and it is conceivable that the ability of clone II strains to develop carbapenem resistance has substantially contributed to this spread.

Neither metallo-β-lactamase activity nor the genes encoding these enzymes were detected in any of the studied isolates.

The genes encoding OXA-23-type carbapenemases were not found either, and those for OXA-24-type or OXA-58-like enzymes were identified only in four carbapenem-resistant isolates. These data indicate that carbapenem resistance in Czech *Acinetobacter* strains does not result from the presence of acquired carbapenemases. It has recently been shown that the insertion of an IS*Aba1* sequence upstream of the chromosomal genes encoding OXA-51-type β-lactamases can increase the expression of these genes, which are normally expressed at a low level, and result in carbapenem resistance.[4,5] In the present study, IS*Aba1* was located in the promoter region of the $bla_{\text{OXA-51-like}}$ gene in half of the clone II isolates and most of these isolates had higher carbapenem MICs when compared with those devoid of IS*Aba1* in the promoter region. However, similar MICs (2–4 mg/L) were obtained for some isolates regardless of the presence or absence of IS*Aba1* adjacent to the $bla_{\text{OXA-51-like}}$ gene. Carbapenem resistance thus seems to result from the overexpression of the $bla_{\text{OXA-51-like}}$ gene, but other mechanisms are probably also involved.

In conclusion, the present study shows that the emergence of *Acinetobacter* resistance to carbapenems in the Czech Republic was associated with the spread of MDR *A. baumannii* strains belonging to EU clone II. Carbapenem resistance of these strains is likely to result from up-regulation of the chromosomal OXA-51-like β-lactamase rather than from acquisition of other OXA- or metallo-β-lactamases, but the precise molecular basis of this resistance remains to be resolved. Although the high genomic similarity of most clone II isolates suggests that they represent a recent lineage within the clone, these isolates show a striking variation in the phenotype and genotype of resistance to several clinically important antibiotics. This variation is likely to result from a relatively frequent horizontal acquisition and/or loss of resistance genes as well as from differential expression of intrinsic genes. This striking genetic versatility may endow EU clone II with the ability to develop resistance to nearly all clinically relevant agents.

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Transparency declarations

None to declare.

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CHAPTER 6

Disccussion

DISCUSSION

There has been a great interest in antibiotic resistance of *Acinetobacter baumannii* over the last years. Given the striking increase in the number of reports published since 2004, the findings of the present thesis are discussed in the context of the current knowledge on the subject.

Resistance mechanisms in *A. baumannii*

The resistance of *A. baumannii* to antimicrobial agents is mediated by all major mechanisms that are known to occur in Gram-negative bacteria (Figure 1). These mechanisms include enzymatic hydrolysis or modification of antimicrobials, modification or bypassing of target sites, active efflux and decreased influx of drugs (Table 1). To date, at least 80 different resistance mechanisms have been identified in clinical isolates of *A. baumannii* (Dijkshoorn et al. 2007; Peleg et al. 2008). The vast majority of them are represented by drug-inactivating enzymes which account for most of the clinically relevant resistance to β-lactams and aminoglycosides: more than 50 different β-lactamases including their allelic forms and at least nine distinct aminoglycoside-modifying enzymes have so far been identified in *A. baumannii*. In addition, clinically relevant resistance to fluoroquinolones has been correlated with mutations in genes that encode DNA topoisomerases, while resistance to tetracyclines

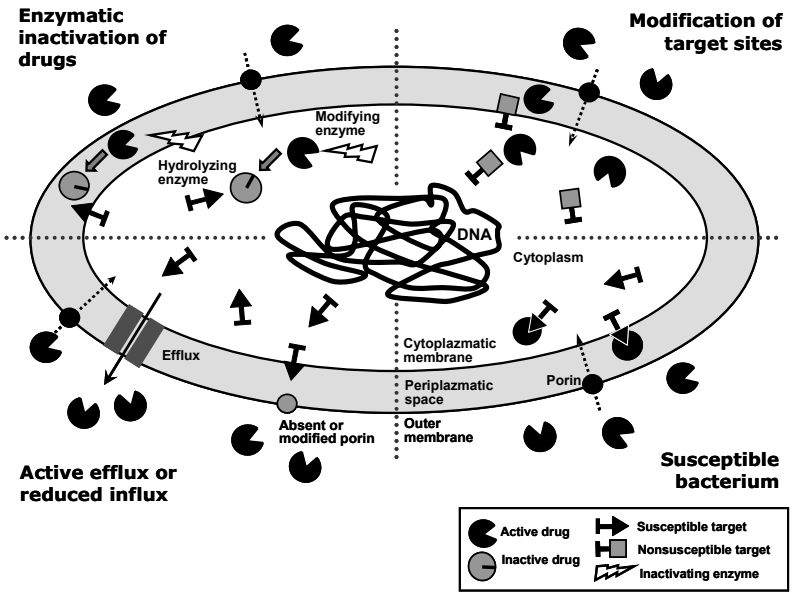

Figure 1. General mechanisms of resistance to antimicrobial agents in Gram-negative bacteria.

has been predominantly associated with genes encoding tetracycline-specific efflux pumps. It is of note that single *A. baumannii* strains may harbour multiple mechanisms that confer resistance to multiple classes of antimicrobials (Hujer et al. 2006, Fournier et al. 2006, Mak et al. 2009) while a combination of several mechanisms may contribute to a high-level resistance to a particular group of antibiotics (Bou et al. 2000). These features make some strains high-level resistant to nearly all clinically relevant antimicrobials.

Horizontally acquired mechanisms

Most genes that encode inactivating enzymes and specific efflux pumps in *A. baumannii* are associated with genetic elements capable of incorporating and/or transferring genetic information such as transposons, integrons or plasmids (Dijkshoorn et al. 2007). These genes can also be found in other species, which suggests that they were acquired by horizontal transfer, i.e. they are part of the accessory genome of *A. baumannii*. Even though most of these genes occur in multiple bacterial genera, some of them are predominantly associated with the genus *Acinetobacter* (e.g. the APH(3′)-VI gene conferring amikacin resistance, Lambert et al. 1990) or even with *A. baumannii* (the genes encoding OXA-24 carbapenemases; Poirel & Nordmann 2006), which is likely to reflect different abilities of particular resistance genes to spread between evolutionary distant species.

Table 1. Mechanisms of resistance of *A. baumannii* to clinically important antibiotics

Mechanism responsible component	Typical resistance to	Desription of the mechanism
β-lactam hydrolysis		
ADC*	Cephalosporins†	AmpC-type β-lactamase intrinsic to *A. baumannii*
TEM-1, CARB-5, SCO-1	Carboxypenicillins	Narrow spectrum class A β-lactamases
VEB-1, PER*, TEM-92, CTX-M-2	Cephalosporins	Broad spectrum class A β-lactamases
OXA-51*	Carbapenems†	Class D β-lactamase intrinsic to *A. baumannii*
OXA-23*, OXA-24*, OXA-58*	Carbapenems	Class D β-lactamases
IMP*, VIM*, SIM-1	Carbapenems	Metalo-β-lactamases
Aminoglycoside modification		
AAC(3)-Ia	Gentamicin	Ubiquitous acetyltransferase
AAC(3)-IIa	Gentamicin, tobramycin	Acetyltransferase
AAC(6')-Ib, AAC(6')-Ih	Tobramycin, amikacin	Acetyltransferases
APH(3')-I	Kanamycin	Ubiquitous phosphotransferase
APH(3'')-VI	Amikacin	Phosphotransferase typical for *Acinetobacter* spp.
ANT(2'')-Ia	Gentamicin, tobramycin	Ubiquitous nucleotidyltransferase
Target alteration		
GyrA, ParC	Quinolones	Subunits DNA topoisomerases
ArmA	Aminoglycosides	16 rRNA methylase
Active efflux		
AdeABC	Aminoglycosides†	Chromosomally encoded nonspecific efflux pump
Tet(A), Tet(B)	Tetracyclines	Tetracycline-specific efflux pumps
Changes in outer-membrane proteins		
CarO	Carbapenems	Protein responsible for drug infflux

In addition to the above list, a number of resistance mechanisms and/or genes have been reported to occur in *A. baumannii* that have known or putative role in natural or acquired resistance of this species (Dijkshoorn et al. 2007, Peleg et al. 2008). Mechanisms so far identified in strains from the Czech Republic are underlined. *, Several allelic variants known; †, upregulation of the system necessary for resistant phenotype.

Intrinsic β-lactamases

A few chromosomal genes encoding β-lactamases are present in most, if not all, *A. baumannii* strains. They are normally expressed at a very low level but can be overexpressed as a result of genetic events. The genes encoding chromosomal ADC-type β-lactamases can be upregulated as a consequence of the upstream insertion of an IS*Aba1* sequence, which provides an efficient promoter (Héritier et al. 2006). This insertion sequence is thought to serve as a 'moving switch' to turn on those genes with which it is juxtaposed (Livermore & Woodford 2006). IS*Aba1* also seems to have this role in some carbapenem-resistant strains by enhancing the expression of the intrinsic OXA-51-like carbapenemases (Turton et al. 2006). IS*Aba1* is thought to be customized for *A. baumannii* (Segal et al. 2005) and can be present in multiple copies in the genome of a single strain (Vallenet et al. 2008). In Chapter 5, we investigated the distribution of IS*Aba1* in 108 *A. baumannii* isolates which were classified into 26 different genotypes using AFLP fingerprinting. IS*Aba1* was found in 70% of the isolates; yet these isolates belonged only to six out of 31 different AFLP genotypes. This suggests that that there is a non-random distribution of IS*Aba1* in the population of *A. baumannii*. As nearly all IS*Aba1*-harbouring isolates were MDR whereas those without IS*Aba1* were fully susceptible, it is possible that the presence of IS*Aba1* in the genome provides an important selective advantage for the development of resistance based on activation of silent intrinsic genes.

AdeABC efflux system

Another chromosomal system typical for *A. baumannii* is the AdeABC efflux system which was identified in a multidrug resistant strain from France (Magnet et al. 2001). Although hydrophobic aminoglycosides such as netilmicin and gentamicin appeared to be the best substrates for this pump, its activity has also been associated with decreased susceptibility to other aminoglycosides, some β-lactams, fluoroquinolones, tetracyclines and, recently, tigecycline (Magnet et al. 2001, Peleg et al. 2007, Ruzin et al. 2007). The system includes three structural components homologous to membrane fusion, drug transporter and outer membrane components, characteristic of the Resistance-Nodulation-Division (RND) efflux pump family. It was shown that a susceptible *A. baumannii* strain containing the AdeABC genes could produce spontaneous resistant variants with mutations in the *adeS* or *adeR* genes, which encode a two-component system regulating AdeABC gene expression (Marchand et al. 2004). The reduction in susceptibility associated with the efflux system has therefore been attributed to its constitutive overexpression. As little was known about the distribution and clinical significance of the AdeABC system at the population level, we set up a study (Chapter 4) to assess the occurrence of the structural and regulatory genes of the AdeABC system in a genotypically and geographically heterogeneous collection of *A. baumannii* strains and the association of the AdeABC efflux genes with resistance to antimicrobial agents. We found that the AdeABC genes are common in *A. baumannii*, but may be absent

in some, mostly fully susceptible strains. In addition, our results suggested an association between resistance to multiple agents and the up-regulated AdeABC system, as inferred from the observed link between the presence of the genes essential for the activity of the AdeABC, multidrug resistance and decreased netilmicin susceptibility, which was used as a putative phenotypic marker for the up-regulation of AdeABC. It is of note that, although the up-regulated AdeABC system transports a wide range of structurally dissimilar compounds, the level of resistance to individual agents conferred by the system usually does not reach clinical resistance breakpoints (Magnet et al. 2001). Susceptibility values exceeding these breakpoints are likely to result from the presence of other, more effective mechanisms. Considering this, we hypothesized that increased expression of the system may be the first step in the evolution of the bacterium to become multidrug resistant (Chapter 4). Thus, originally susceptible, AdeABC-positive strains may produce mutants with constitutive expression of the AdeABC. This would confer low-level resistance against multiple antimicrobials and/or biocides which might facilitate the initial survival of the organisms in patients and in the hospital environment and enable them to acquire subsequently specific, high-level resistance mechanisms.

Genetics of multidrug resistance

Despite the progress in the elucidation of the function and genetic basis of particular resistance mechanisms, knowledge of the genetic factors that contribute to the complexity of resistance mechanisms in particular *A. baumannii* strains is limited. In our study focused on aminoglycoside resistance (Chapter 3), we investigated the presence of seven genes encoding aminoglycoside-modifying enzymes in 106 multidrug resistant (MDR) *A. baumannii* strains. Eighty-nine of these strains showed the presence of two to five different resistance genes in as many as 12 different combinations. This emphasizes the remarkable diversity in acquired genes among *A. baumannii* strains that apparently developed during successive events. Some other research groups have reported similar findings (Seward et al. 1998, Hujer et al. 2006, Mak et al. 2009) but only very recent studies provided an insight into the organization of the acquired resistance genes within a particular genome (Fournier et al. 2006, Smith et al. 2007, Iacono et al. 2008, Vallenet et al. 2008, Adams et al. 2008). The first important observation was made by Fournier et al. (2006) who compared the complete genomes of a MDR (AYE) strain and a susceptible (SDF) strain of *A. baumannii*. Whereas AYE contained an 86-kb genomic region, termed a resistance island AbR1, in which 45 putative genes associated with resistance were clustered, SDF exhibited a 20-kb genomic island at the homologous location that comprised genes that encode transposases but no resistance genes. The authors hypothesized that such a genetic structure could serve as a 'hot spot' that facilitates chromosomal integration of horizontally acquired resistance genes. It is of particular interest that a structure (designated AbaR2) homologous to AbR1 was recently identified in an Italian MDR strain (ACICU) of *A. baumannii* (Iacono et al. 2008).

Although AbaR2 comprises only 17 coding DNA sequences, its composition and structure is highly similar to the 3' region of AbaR1. As AYE and ACICU belong to two distinct main clonal lineages (EU clone I and II, respectively), this finding indicates that highly similar genetic structures associated with antimicrobial resistance may be integrated at the same chromosomal site of unrelated strains.

Taxonomic considerations on *A. baumannii*

With the recent description of the species *A. berezinae* and *A. guillouiae*, the genus *Acinetobacter* currently consists of 22 validly named species and 12 genomic species with provisional designations (Dijkshoorn et al. 2007, Nemec et al. 2009a, Nemec et al. 2009b, Vaneechoutte et al. 2009). The genus also encompasses a number of unique strains and groups of strains that may represent new species. *A. baumannii* is, clinically and epidemiologically, the most important species, although some other species (e.g. *A. ursingii*, *A. junii*, *A. haemolyticis*, *A. schindleri*, *A. lwoffii*) can also cause infections in humans. In a recent study (Chapter 5), we collected prospectively 150 *Acinetobacter* spp. clinical isolates from multiple intensive care units in the Czech Republic. It was found that 108 (72%) of these isolates belonged to *A. baumannii*, which corroborates the relative importance of this species in intensive care units. However, the prevalence of *A. baumannii* may depend on many factors including the source of isolates (ICU or non-ICU patients) or the endemic situation and may be lower in the absence of MDR and/or outbreak strains of *A. baumannii* (van den Broek et al., unpublished).

Despite the considerable progress made in resolving the taxonomy of the genus *Acinetobacter*, our knowledge of the spread and clinical significance of species other than *A. baumannii* is limited. This is mainly owing to the absence of a reliable and practical identification method that can be used in diagnostic laboratories. Diagnostic systems currently available to clinical microbiology laboratories (e.g. API20NE, Vitek 2) suffer from a number of drawbacks such as a small number of relevant characters tested or the insufficient quality of reference data in identification matrices (Bernards et al. 1996). In particular, *Acinetobacter calcoaceticus*, *A. baumannii*, genomic species 3 and 13TU (together referred to as the *A. calcoaceticus–A. baumannii* complex) cannot be unambiguously separated by phenotypic methods (Gerner-Smidt et al. 1991). Genomic species 3 and 13TU can occur relatively frequently in clinical specimens of hospitalized patients (Tjernberg & Ursing 1989, van den Broek et al. unpublished). Genomic species 3 and 13TU encompassed together 25% of 150 clinical isolates of *Acinetobacter* spp. from ICU patients in our prospective study (Chapter 5) and 23% of 103 retrospective clinical isolates of the *A. calcoaceticus–A. baumannii* complex isolated in Czech hospitals in the 1990s (Nemec et al. 1999). These observations imply that acinetobacters identified by routine procedures as *A. baumannii* may also represent other species of the *A. calcoaceticus–A. baumannii* complex. It is of note that many studies describing new resistance

mechanisms in *A. baumannii* used unreliable methods for species identification. Consequently, such mechanisms may be incorrectly associated with this species.

Population structure of *A. baumannii*

Recognition of geographically widespread clones

The term clone in the epidemiological sense was coined by Ørskov & Ørskov (1983) to denote bacterial cultures isolated independently from different sources, in different locations, and possibly at different times, but sharing so many phenotypic and genotypic traits that the most likely explanation for this identity is a common origin. The first information suggesting the existence of such clones in *A. baumannii* was published in 1996 (Dijkshoorn et al. 1996). In that study, *A. baumannii* strains from 14 outbreaks and 17 sporadic strains from hospitals in different northwestern European cities and countries were compared to assess the diversity among outbreak and non-outbreak strains. By using a combination of genotypic and phenotypic methods, a so-called polyphasic approach (Vandamme et al. 1996), the outbreak strains could be allocated to two main groups (designated European (EU) clones I and II), whereas the sporadic strains were more heterogeneous. This observation was followed by a study, in which phenotypic and genotypic properties of *A. baumannii* hospital strains from the Czech Republic were investigated (Nemec et al. 1999). Most MDR Czech strains were classified into two groups (designated A and B), each of which was characterized by a specific ribotype and similarity in plasmid DNA profiles and in the ability to grow on a particular set of carbon sources. This study also showed that the two reference strains (RUH 875 and RUH 134) of clones I and II were allocated to groups A and B, respectively. In addition, similarity of strains of group A and clone I was further corroborated by their common reactivity with O-antigen-specific monoclonal antibodies (Pantophlet et al. 2001). Altogether, these findings suggested that the respective clones and groups were congruent. Another report supporting the spread of EU clones I and II in European hospitals was published by Brisse et al. (2000). In this study, fluoroquinolone-resistant isolates of *Acinetobacter* spp. collected in hospitals from all over Europe were analysed by automated ribotyping. Several groups of *A. baumannii* isolates, each with a specific ribotype, were identified. Comparison of these groups with strains of EU clones I and II showed that ribotypes of two groups corresponded to those of the respective clones. Later analysis of a subset of these isolates by additional methods corroborated the assignment of these strains to the two clones, and also resulted in the delineation of a new epidemic lineage, EU clone III (van Dessel et al. 2004). It is of note, that additional geographically widespread MDR clones can be expected to exist as indicated by our results of AFLP cluster analysis on a geographically diverse collection of *A. baumannii* isolates (Chapter 4). Overall, there is ample evidence that, apart from fully susceptible sporadic strains, the species *A. baumannii* comprises groups of MDR strains that are genetically closely related. Strains of these groups seem to represent clonal lineages of

strains that are characterized by multidrug resistance and the ability to spread epidemically.

Methods to identify EU clones

Original delineation of EU clones I and II was based on the congruence of the results obtained by several typing methods which were known to differentiate epidemiologically unrelated *Acinetobacter* isolates (Dijkshoorn et al. 1996). These methods included antibiotic susceptibility determination, biotyping, cell envelope protein profiling, ribotyping, and AFLP fingerprinting. Despite some variability in the characteristics of isolates belonging to the same clone, clonal groups could be unambiguously differentiated from each other and from all unique isolates by AFLP (Dijkshoorn et al. 1996, Nemec et al. 2001). By this method, organisms are compared for similarity in their whole genomic fingerprints as obtained by selective amplification of restriction fragments followed by fragment separation on a sequencing system. The obtained profiles are compared for similarity by cluster analysis. It was shown that strains allocated to each of the EU clones have a similarity of ~ 80%. New strains can be identified to clones by comparison of their AFLP profile to a library of > 2000 fingerprints of the Leiden University Medical Center comprising strains of different clones and unique strains. In Leiden, this high-resolution DNA fingerprinting method has become a main tool to delineate and identify clonal groups among *A. baumannii* strains (van Dessel et al. 2004, van den Broek et al. 2006, Da Silva et al. 2007). Also in the present thesis (Chapters 2-5), AFLP analysis appeared to be a powerful tool to assess the diversity within the genus *Acinetobacter* and to identify clones within *A. baumannii*. However, despite the local robustness of this method and the possibility to archive digitalized AFLP fingerprints, it suffers from general drawbacks of fingerprinting methods such as low interlaboratory reproducibility and the difficulty of definitive classification of types. To overcome these drawbacks, several sequence-based typing methods have been proposed for population analysis of *A. baumannii* over the last four years. The first of them was the multilocus sequence typing (MLST) scheme proposed by Bartual et al. (2005), which is based on sequence analysis of conserved regions of seven housekeeping genes (*gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi*, *rpoD*). Although the classification based on this scheme was in a good agreement with the typing results generated by PFGE and AFLP analysis, only a limited number of geographically related strains were tested. Ecker et al. (2006) described a high-throughput modification of MLST that can be used both for species identification of *A. baumannii* and for determination of clonality. This method is based on amplification of the conserved regions of six housekeeping genes (*trpE*, *adk*, *efp*, *mutY*, *fumC*, *ppa*), purification of amplicons and determination of their mass spectra. Even though this method is very quick, its results cannot directly be compared to standard sequencing data and therefore, the method lacks the data portability, the major advantage of MLST. Another scheme, proposed by Turton et al. (2007), is based on sequence analysis of three genes intrinsic to *A. baumannii*, i.e. *ompA* (encoding outer-membrane protein A), *csuE* (pilus assembly

component) and $bla_{\text{OXA-51-like}}$ (β-lactamase). This method and a multiplex PCR based on the clone-specific allelic combinations of the three genes could identify EU clones I-III (Turton et al. 2007), although the usefulness of this approach for population genetic analysis still needs to be evaluated using a comprehensive set of strains from different geographic areas.

The last and best evaluated MLST scheme was proposed by Diancourt et al. (2007) (www.pasteur.fr/mlst). It is based on the comparative analysis of internal portions of seven housekeeping genes (*cpn60, gltA, recA, fusA, pyrG, rplB, rpoB*). Three of these regions are included also in the scheme of Bartual et al. (2005), whereas the other genes are different. The scheme was evaluated using 155 *A. baumannii* isolates, i.e. 80 isolates belonging to EU clone I, II or III and 75 other strains representing the breadth of the known AFLP diversity of a library of > 2000 fingerprints. Phylogenetic analysis based on allelic profiles revealed that isolates from clones I, II and III fell into three respective clonal complexes, each formed by a central, predominant genotype and a few single locus variants. Notably, these clonal complexes differed from each other and from the other 75 strains in at least three distinct loci. In addition, analysis of the recombination/mutation rate indicated that recombination is unlikely to disrupt the clonal frame of the clones. Thus the clonal complexes can be expected to be stable over long periods of time, which is consistent with the occurrence of the EU clone I and II genotypes in Europe at least since the late 1970s (see below).

Spread of EU clones in space and time

In the foregoing, evidence was presented of the existence of the so-called EU clones, genotypically and evolutionary coherent groups of strains which are distinct from other strains or clonal lineages within *A. baumannii*. These groups have been defined on the basis of overall similarity rather than in terms of definite molecular markers. Although taxonomically correct, this stochastic definition makes it basically difficult to compare the results of studies in which different typing methods were used. However, as several recent studies have employed the above mentioned high-resolution DNA sequence-based typing methods and included sets of reference strains of the EU clones, their results can be used to obtain information about the geographic spread of these clones. Table 2 summarizes data on the origin of strains belonging or related to these clones as indicated by published reports. These data illustrate a pan-European spread of strains classifiable in EU clones I and II over three decades. The first known strain (HK302) of clone I was isolated from a hospital outbreak in Switzerland in 1977 (Devaud et al. 1982; Krizova & Nemec, unpublished). Notably, our recent unpublished results show that HK302 harbours many of the acquired resistance genes and genetic structures that are present in EU clone I or II strains isolated up to 30 years later. These structures include a class 1 integron with a 2.5 bp variable region comprising the *aacC1*, orfX, orfX‘ and *aadA1* gene cassettes as well as a resistance island related to AbaR1/AbaR2 (Krizova & Nemec, unpublished). Dijkshoorn et al. (1996) identified EU

Table 2. Published data on the origin of *A. baumannii* isolates related to EU clones I-III

Isolates related to	Reference	Country (year) of isolation	Main method of the clonal assigment	Study
EU clone I	Dijkshoorn et al. 1996	Belgium (1990), the Netherlands (1984-6), UK (1987–1990)	AFLP	Comparison of European outbreak strains
	Devaud et al. 1982	Switzerland (1977)	AFLP*	Genetic analysis of an outbreak strain
	Nemec et al. 2004 (= Chapter 2)	Czech Republic (1991–2001)	AFLP	A country-wide retrospective study
	van Dessel et al. 2004	Italy, Poland, Spain, South Africa (all 1997–1999)	AFLP	Population analysis of European fluoroquinolone-resistant isolates
	Abbott et al. 2005	Ireland (≤2004)	AFLP*	Analysis of a horse isolate
	Fournier et al. 2006	France (≤2002)	AFLP*, Turton et al. 2007	Completelly sequenced strain AYE
	Turton et al. 2007	UK (2002–2006)	Turton et al. 2007	Evaluation of a sequence based method for identification of EU clones
	Nemec et al. 2007 (= Chapter 4)	Australia (> 1990), Bulgaria (2001), Hungary (1995)	AFLP	Analysis of the AdeABC efflux in a diverse set of retrospective isolates
	Nemec et al. 2008 (= Chapter 5)	Czech Republic (2005–2006)	AFLP	A country-wide prospective study
	Towner et al. 2008	Bulgaria, Croatia, Estonia, Greece, Norway, Poland, Slovenia (all 2001–2004)	Turton et al. 2007	Population analysis of European carbapanem-resistant isolates
	Post & Hall 2009	Austraila (1997)	Turton et al. 2007	Genetic analysis of a MDR strain
	D'Arrezo et al. 2009	Italy (2004–2005)	Turton et al. 2007	Analysis of isolates from ICUs in Rome
EU clone II	Dijkshoorn et al. 1996	Denmark (1984), the Netherlands (1982), UK (1989)	AFLP	Comparison of European outbreak strains
	Nemec et al. 2004 (= Chapter 2)	Czech Republic (1991–2001)	AFLP	A country-wide retrospective study
	van Dessel et al. 2004	France, Greece, Poland, Portugal, South Africa, Spain, Turkey (all 1997–1999)	AFLP	Population analysis of European fluoroquinolone-resistant isolates
	Bartual et al. 2004	Spain (1997, 2000-2002)	MLST (Bartual et al. 2004)	Evaluation of a MLST scheme
	Turton et al. 2007	UK (2002–2006), Spain, Israel	Turton et al. 2007	Three subclones of EU clone II from UK
	Nemec et al. 2008 (= Chapter 5)	Czech Republic (2005–2006)	AFLP	A country-wide prospective study
	Nemec et al. 2007 (= Chapter 4)	Bulgaria (1997), Hungary (1993)	AFLP	Analysis of the AdeABC efflux in a diverse set of retrospective isolates
	Towner et al. 2008	Bulgaria, Czech Republic, Germany, Greece, Spain, Poland, Slovakia, UK (all 2001–2004)	Turton et al. 2007	Population analysis of European carbapanem-resistant isolates
	Iacomo et al. 2008	Italy (2005)	Turton et al. 2007	Completelly sequenced strain ACICU
	Whitman et al. 2008	USA (2006)	PCR/ESI-MS (Ecker et al. 2006)	Cause of pneumonia in a health care worker
	Shelburne et al. 2008	USA (2005–2006)	MLST (Bartual et al. 2004)	Endemic analysis in a Houston hospital
	D'Arrezo et al. 2009	Italy (2004–2005)	Turton et al. 2007	Analysis of isolates from ICUs in Rome
EU clone III	van Dessel et al. 2004	Spain, the Netherlands, France, Italy (all 1997–1999)	AFLP	Population analysis of European fluoroquinolone-resistant isolates
	Huys et al. 2005a	Belgium (1991–1993)	Rep-PCR fingeprinting (Huys et al. 2005b)	Study on EU clone III
	Turton et al. 2007	UK (2002–2006), Spain	Turton et al. 2007	Evaluation of a sequence based method for identification of EU clones
	Nemec et al. 2007 (= Chapter 4)	The Netherlands (2000)	AFLP	Analysis of the AdeABC efflux in a diverse set of retrospective isolates

* Unpublished. Clonal assignment based on additional AFLP analysis performed in the Leiden University Medical Center.

clones I and II among epidemic strains isolated in the Netherlands, the UK, Belgium and Denmark between 1982 and 1990. Investigation of large sets of strains from the Czech Republic indicated that these clones had also spread widely in Czech hospitals from at least 1991 to 2006 (Chapters 2 and 5, see below). Furthermore, they were identified by Brisse et al. (2000) and van Dessel et al. (2004) among fluoroquinolone-resistant *A. baumannii* isolates from different parts of Europe, Turkey and South Africa. Finally, recent papers have reported on the occurrence of MDR strains belonging or related to the EU clones, especially

clone II, in many additional European countries as well as in the USA (Shelburne et al. 2008, Whitman et al. 2008). Overall, although precise genomic relationships between many of these isolates still have to be assessed, these data suggest pan-European and perhaps global spread of two highly successful MDR lineages of *A. baumannii*.

A. baumannii in the Czech Republic

Comparison of the studies from 1991-1997 and from 2005-2006

Two systematic studies were performed since 1991 to assess the population structure and resistance determinants in hospital strains of *Acinetobacter* spp. in the Czech Republic (Nemec et al. 1999, Chapters 2 and 5). The first study included 103 clinical isolates of the *A. calcoaceticus–A. baumannii* complex obtained in 17 hospitals between 1991 and 1997 (Chapter 2), while the second study comprised 150 *Acinetobacter* spp. isolates collected prospectively from 56 intensive care units of 20 hospitals in 2005-2006 (Chapter 5). As many as 75% and 72% of the isolates were identified as *A. baumannii* in the first and second study, respectively, and all isolates which were MDR and/or associated with outbreaks of hospitals infections belonged to this species. In addition, nearly all MDR isolates were allocated to either EU clone I or II in both studies, which is indicative of the long-term predominance of these clones among hospital *Acinetobacter* strains in Czech hospitals.

The comparison of these two studies yet revealed some important differences between the population of strains from the 1990s and that from 2005-2006 (Figure 2). In the latter study, 5 and 66 isolates were allocated to clone I and II, respectively, in contrast to 39 and 9 isolates from 1991-1997 classified into the respective clones (Nemec et al. 1999, Chapters 2 and 5). Even though the results of the two studies are not directly comparable as the strain inclusion criteria differed, the data are suggestive of a shift in the recent *A. baumannii* population towards clone II. It is furthermore noteworthy that 21% of *A. baumannii* strains from 2005-2006 were resistant or intermediately susceptible to carbapenems whereas no carbapenem resistant strain was found among the isolates from the 1990s. Importantly, nearly 90% of carbapenem non-susceptible isolates from the 2000s belonged to EU clone II. It is likely that the emergence of carbapenem resistance was a response to the escalating use of these antibiotics. Indeed, although the data on the consumption of particular drugs at the country level are not available, several hospitals included in this study have reported an increase in carbapenem prescription since 2000 (Ježek, unpublished). The question is whether the relative success of EU clone II as compared to EU clone I results from its greater propensity to develop carbapenem resistance. Notably, studies in several other European countries and regions have also reported on the association of carbapenem resistance with epidemic strains of clone II (Turton et al. 2007, Da Silva et al. 2007), although the carbapenem resistance mechanisms in these strains varied with geographic region and carbapenem resistance could also be found in a number of strains not belonging to clone II (Towner et al. 2008). Altogether,

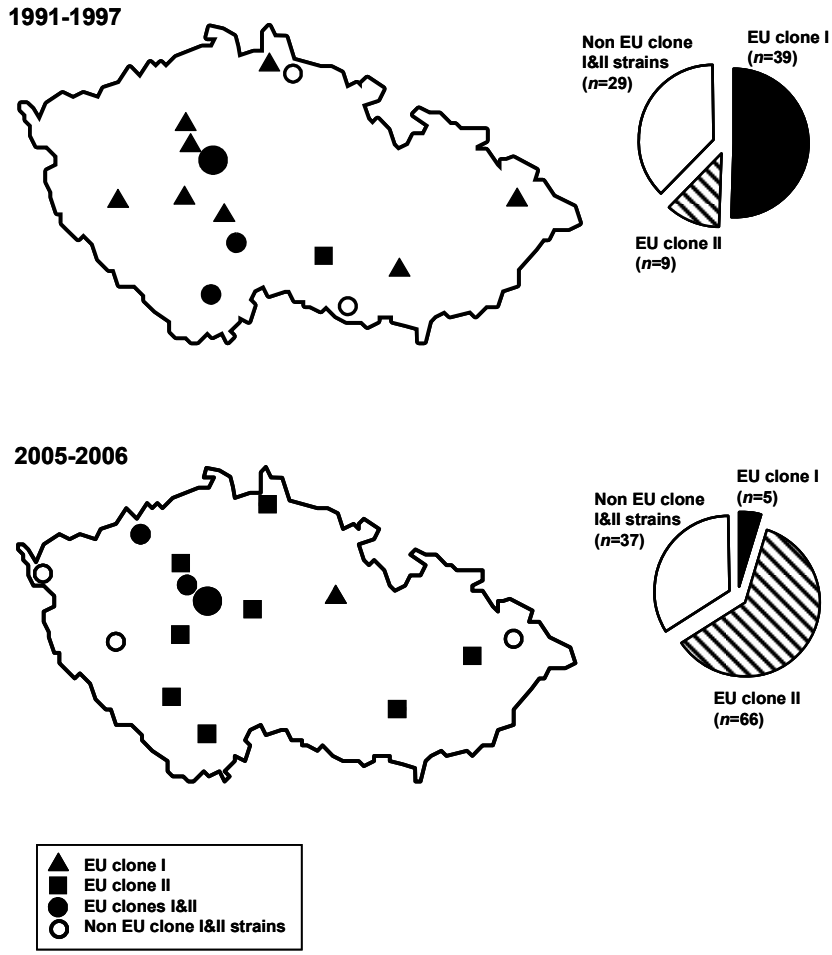

Figure 2. Geographic origin and the population structure of the in-detail studied isolates of *A. baumannii* from the Czech Republic. A. Results of 77 retrospective isolates from the period between 1991 and 1997 (Nemec et al. 1999, Chapter 2). B. Results of 108 prospective isolates of 2005-2006 (Chapter 5).

apart from intrinsic and acquired factors of clone II to emerge it is of note that its spread in Czech hospitals was likely to be facilitated by frequent inter-hospital spread of patients and the lack of effective measures to prevent the transmission of MDR microorganisms.

Mechanisms of antimicrobial resistance

Apart from the elucidation of the population structure of acinetobacters in the Czech Republic, a major aim of the thesis was to investigate the mechanisms resulting in antimicrobial resistance of the organisms (Chapters 3-5). A summary of the resistance mechanisms identified in Czech *A. baumannii* strains on the basis of the association between the

presence of resistance genes and corresponding phenotype is given in Table 1. It was found that high-level resistance to aminoglycosides was mainly caused by modifying enzymes, such as phosphotransferases APH(3')-I and APH(3')-VI, acetyltransferase AAC(3)-I and nucleotidyltransferases ANT(2'')-I and ANT(3'')-I (Chapters 3 and 5). However, the genes encoding acetyltransferases AAC(3)-II or AAC(6')-I and the resistance profiles associated with these enzymes that have been observed in other countries were not found in the Czech strains. This suggests that the genes encoding these mechanisms were absent or rare in the gene pool available for *A. baumannii* in the country. The up-regulated chromosomal AdeABC efflux system is likely to contribute to the increased MICs of aminoglycosides and other antimicrobial drugs which are substrates for this pump, such as carbapenems or fluoroquinolones (Chapter 4). The AdeABC activity is probably best reflected by reduced susceptibility to netilmicin because this antibiotic is effectively expelled by AdeABC (Magnet et al. 2001) and Czech strains seem to be devoid of other mechanisms of netilmicin resistance (Chapters 3, 4 and 5). The results of Chapter 5 revealed putative resistance mechanisms for clinically important β-lactams. High-level resistance to ceftazidime is likely to result from up-regulation of the chromosomal ADC β-lactamase as indicated by the presence of IS*Aba1* in the promoter region of the bla_{ADC} gene in ceftazidime-resistant strains. Similarly, carbapenem resistance seems to result from the overexpression of OXA-51-like as suggested by the fact that carbapenem-resistant strains harboured IS*Aba1* located upstream of $bla_{OXA\text{-}51\text{-}like}$ gene whereas only few of them were positive for the genes encoding acquired OXA-type carbapenemases (OXA-58 and OXA-24-like). So far, no strain produced metallo-ß-lactamases, enzymes that have already been identified especially in Asia but also in some European countries (Poirel & Nordman 2006, Wroblewska et al. 2007, Towner et al. 2008). However, apart from the mentioned carbapenem resistance mechanisms, other mechanisms may also be involved in carbapenem resistance as suggested by a relatively wide range of carbapenem MICs found in clonally related strains with IS*Aba1* associated with $bla_{OXA\text{-}51\text{-}like}$ (Chapter 5). Sulbactam, a ß-lactamase inhibitor with unusual antibiotic activity against *Acinetobacter*, has been proposed for the treatment of infections caused by some carbapenem resistant strains (Peleg et al. 2008). Although resistance to sulbactam in MDR *A. baumannii* has been reported (Peleg et al. 2008), no information on the responsible mechanism could be found in the literature. Notably, all sulbactam-resistant Czech isolates harboured the genes encoding TEM-like ß-lactamases whereas all but one susceptible isolates were negative for these genes (Chapter 5). It remains to be elucidated whether or not TEM-like enzymes play a role in resistance of *A. baumannii* to sulbactam.

The diversity in resistance genotypes

In Chapters 3 and 5, the diversity of acquired resistance genes in association with the clonal type and origin of *A. baumannii* strains was investigated. In Chapter 3, a remarkable intraclonal heterogeneity of genes encoding aminoglycoside-modifying enzymes and their

combinations was found in strains belonging to EU clone I or II, with no clear-cut difference between the two clones. In contrast, strains of EU clone III were homogeneous both in resistance genes and other resistance-associated genetic structures such as integrons. This observation can be explained by the fact that clones I and II are relatively old lineages, which each encompass a geographically heterogeneous group of strains isolated over a large period of time whereas clone III seems to be a relatively young lineage with limited geographic spread. Nevertheless, considerable differences in resistance genotypes between EU clone II isolates were also shown in Chapter 5 dealing with strains collected recently in the Czech Republic over a one-year period. In this study, as many as 17 combinations of different genes associated with horizontally acquired resistance were found among EU clone II isolates, with 10 such combinations identified in two thirds of these isolates, which showed nearly identical genomic fingerprints and are therefore considered a young subclone within EU clone II. Notably, the genomically highly related isolates that shared resistance gene content were collected in different cities while those differing in the presence of one or more genes could originate from the same ICU. This is likely to result from a relatively frequent acquisition and/or loss of resistance genes rather than from the concomitant spread of strains with distinct resistance genotypes. Some of these genes (e.g. *aacC1*, *aadA1*, *aphA1*, *tet(B)*, $bla_{\text{TEM-1}}$) are known to be transposon-associated, which may explain their instability even if they are chromosomally integrated. Importantly, most of these genes have been identified as part of resistance islands located in a specific chromosomal locus in several *A. baumannii* strains (Fournier et al. 2006, Iacono et al. 2008, Vallenet et al. 2008). Our preliminary, unpublished results indicate that nearly all Czech MDR strains belonging to EU clone I or II carry structures related to resistance islands AbaR1 (Krizova & Nemec, unpublished) and it remains to be established whether these genetic structures are important sources of the diversification of resistance genotypes in *A. baumannii*.

Options for treatment of infections caused by MDR strains

Rational treatment of nosocomial infections caused by *A. baumannii* ultimately relies on the determination of in-vitro susceptibility to antimicrobial agents. This requirement results from the frequent occurrence of MDR strains and high variability of the susceptibility patterns of these strains (Chapters 3 and 5). In Chapter 5, susceptibilities to antimicrobial agents in 108 isolates prospectively collected in 2005-2006 were assessed. As many as 75 *A. baumannii* isolates were classified as MDR (i.e. resistant to three or more of 13 antimicrobials tested; Figure 3). The antimicrobial agents most effective in-vitro against MDR strains were colistin (99% susceptible), tobramycin (81%), imipenem (76%), meropenem (71%) and amikacin (67%) whereas less than 30% of isolates were susceptible to other drugs. Despite the unfavourable pharmacological properties of colistin, this antibiotic is increasingly used for therapy of infections caused by strains resistant to the other antimicrobials, especially carbapenems (Peleg et al. 2008). It is of note, however, that one of the clone II isolates showed

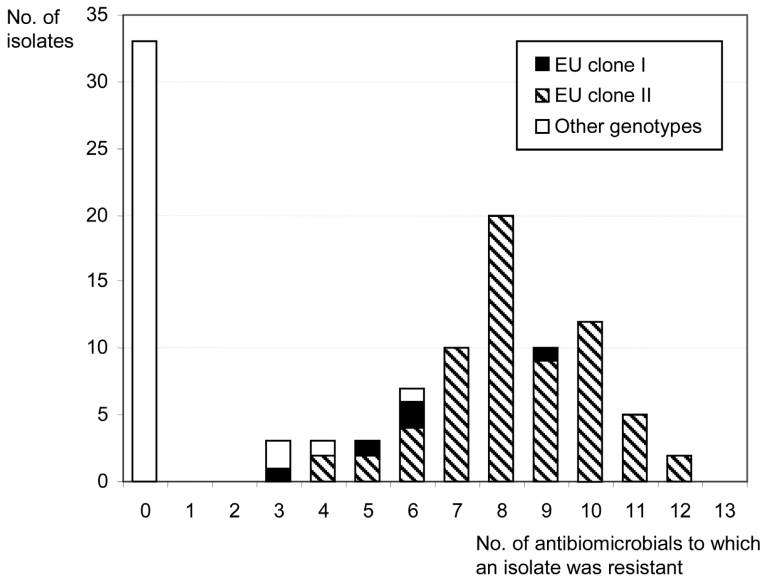

Figure 3. Distribution of 108 *A. baumannii* clinical isolates from the Czech Republic according to the level of multidrug resistance and allocation to EU clones. The isolates were prospectively collected from as many as 56 ICUs in 20 hospitals in the period of 2005-2006 (for the geographical distribution of hospitals see Figure 2) (Chapter 5). The antimicrobials used were imipenem, meropenem, ceftazidime, piperacillin, ampicillin-sulbactam, ciprofloxacin, gentamicin, netilmicin, amikacin, tobramycin, co-trimoxazole, doxycycline and colistin. The 33 fully susceptible isolates belonged to 25 different AFLP genotypes.

Table 3. Distribution of minimal inhibitory concentration for carbapenems and aminoglycosides in 108 *A. baumannii* isolates from the Czech Republic from 2005-2006.

Isolates	No. of isolates								Total
	MIC for imipenem/meropenem (mg/l)*								
	0.125	0.25	0.5	1	2	4	8	≥16	
Multidrug resistant†	0/0	5/2	3/7	24/18	17/15	8/11	14/5	4/17	75
Susceptible‡	13/19	20/13	0/1	0/0	0/0	0/0	0/0	0/0	33

Isolates	No. of isolates								Total
	MIC for tobramycin (mg/l)								
	0.25	0.5	1	2	4	8	16	>16	
Multidrug resistant	0	10	22	19	10	12	1	1	75
Susceptible	7	26	0	0	0	0	0	0	33

Isolates	No. of isolates								Total
	MIC for amikacin (mg/l)								
	≤1	2	4	8	16	32	64	≥128	
Multidrug resistant	8	21	8	6	7	2	4	19	75
Susceptible	23	11	0	0	0	0	0	0	33

* Intermediate and resistance values are highlighted

† Resistant to more than two antibiotics which are primarily effective against *A. baumanni* (see below).

‡ Susceptible to aminoglycosides, carbapenems, ofloxacin, doxycycline, ceftazidime and piperacillin.

colistin MIC ≥8 mg/L, which indicates the capacity of this clonal lineage for developing resistance to this drug. Good susceptibility to tobramycin can be explained by the absence of tobramycin-modifying enzymes in Czech strains of clone II and by the fact that this antibiotic has been withdrawn from the market in the Czech Republic more than 10 years ago. Although carbapenems also seemed to be relatively effective according to the CLSI (2005) susceptibility breakpoints (4 mg/l), most MDR strains show increased MIC values for these antibiotics (1-4 mg/l) as compared to fully susceptible strains (0.125-0.5 mg/l) (Table 3). These increased MICs might facilitate the selection of carbapenem-resistant variants of these strains and result in treatment failure. Several recent publications have reported on the in-vitro efficacy of sulbactam and tigecycline against carbapenem-resistant strains of *A. baumannii* (Dijkshoorn et al. 2007, Peleg et al. 2008). However, less than one third of the Czech MDR isolates were susceptible to sulbactam and although the in-vitro activity of tigecycline against these isolates still has to be assessed, the activity of this antibiotic can be compromised in strains with the up-regulated AdeABC efflux system (Peleg et al. 2007, Ruzin et al. 2007). Given the mentioned limitations of the current therapeutic options as well as the lack of prospect that a new agent effective against MDR *A. baumannii* strains will be introduced in the coming years, optimization of the use of existing antimicrobials is crucial. This includes a thorough understanding of the pharmacokinetic and pharmacodynamic parameters that predict maximal drug efficacy yet minimize the evolution of drug resistance, as well as an evidence-based approach to therapeutic strategies for highly drug resistant strains (Peleg et al. 2008).

Concluding remarks

The aim of the studies of the present thesis was to obtain insight into the epidemiology and molecular basis of multidrug resistance of *A. baumannii* at the population level. To this aim we performed a number of studies on strains mainly from the Czech Republic. Results of these studies have provided a detailed insight into the diversity of the organisms and genes conferring resistance to antibiotics.

In particular, the studies have shown that (i) the vast majority of MDR clinical isolates of *A. baumannii* from the Czech Republic belong to two geographically widespread clonal lineages termed EU clone I and II; (ii) these two clones have predominated among MDR hospital isolates in the Czech Republic since at least 1991; (iii) emergence of *A. baumannii* resistance to carbapenems in the Czech Republic in the early 2000s was associated with the country-wide spread of a subclone of EU clone II; (iv) multidrug resistance is a general feature of strains of EU clones I and II, yet strains belonging to each of these clones may differ from each other in the content of resistance determinants; (v) there is no clear-cut difference between the two clones in terms of the presence of particular resistance genes and the genetic structures associated with these genes; (vi) up-regulation of the resistance genes

intrinsic to *A. baumannii*, such as chromosomal β-lactamases or a non-specific efflux system as well as horizontal acquisition of resistance genes are probable sources of development of multidrug resistance and intraclonal diversification; (vii) the genes encoding the non-specific efflux system AdeABC are present in nearly all *A. baumannii* strains, yet overexpression of this system is seen mostly in MDR strains harbouring additional resistance genes.

In conclusion, the studies of the thesis have contributed to a growing body of evidence that the problem of resistance to multiple antimicrobial agents in *A. baumannii* is significantly associated with some geographically widespread clonal lineages. Among them, the so-called EU clones I and II seem to have prevailed among MDR *Acinetobacter* strains in some European countries since the early 1980s. Whereas clone I dominated over clone II among the strains isolated in the 1980s, more recent studies indicate an increasing importance of clone II. Our results as well as other studies show that resistance to carbapenems, the most important group of antibiotics against MDR acinetobacters, can be associated with some geographic subclones of EU clone II, which suggests that this clone plays an important role in the development and spread of carbapenem resistance. Recent studies including ours have identified a number of resistance mechanisms in *A. baumannii* and have shown that multiple mechanisms can be present in particular strains. It has become clear that both activation of intrinsic mechanisms and horizontal acquisition of resistance genes play a role in the evolution of antibiotic resistance of this organism. In addition, the most recent studies (Fournier et al. 2006, Iacono et al. 2008, Vallenet et al. 2008) have reported unique genetic structures termed resistance islands which integrate acquired resistance genes and can be responsible for a striking variation in resistance genotypes in clonally and epidemiologically related isolates. Thus, recent findings including the observations of the present thesis are bringing up new questions and opening a window to experimental studies that can lead to a better understanding of genetic factors which contribute to the ability of *A. baumannii* to develop resistance to all clinically relevant agents.

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CHAPTER 7

Summary

SUMMARY

Acinetobacter baumannii is an important nosocomial pathogen which gives rise to colonization and infection of patients, in particular in intensive care units. The strains involved are frequently resistant to multiple antimicrobial agents, leaving limited therapeutic options, and these strains can spread among patients. In 1996, it was shown that two groups (termed European (EU) clones I and II) of genotypically highly similar *A. baumannii* strains prevailed among isolates from outbreaks in geographically distant hospitals of north-western Europe. This observation was corroborated by the study published in 1999 that showed that strains related to EU clones I and II predominated among clinical isolates of *A. baumannii* in the Czech Republic. Both studies also showed that the strains belonging or related to EU clones I and II were more resistant to antimicrobial agents than other strains and it has been suggested that the association of multi-drug resistance and clonality might have played a role in the progressive increase in *Acinetobacter* resistance. These findings and considerations gave rise to the attempt to gain insight into the epidemiology and molecular basis of multidrug resistance of *A. baumannii* at the population level. To this aim, a descriptive and comparative approach was used to analyse a large number of properties in well-defined collections of strains in order to assess the relationship between multidrug resistance and the population structure of *A. baumannii*. The studied organisms consisted mainly of clinical isolates collected since 1991 from hospitalized patients in the Czech Republic and of isolates from various other European countries enrolled in the collection of the Leiden University Medical Centre over the recent two decades.

In the first study, we investigated the genotypic relationship between Czech *A. baumannii* strains previously assigned to the so-called groups A and B and the north-western EU clones I and II using AFLP fingerprinting and ribotyping. The study collection included 70 multidrug resistant (MDR) and 15 susceptible strains from 1991-2001 from the Czech Republic and reference strains of EU clones I and II. The results confirmed that the two predominant groups A and B observed among the MDR Czech *A. baumannii* strains from the 1990s were genomically congruent with clones I and II, respectively. In the next study, we investigated the diversity of the genes encoding aminoglycoside-modifying enzymes and their association with class 1 integrons in the EU clones including also the newly described clone III. We found that, whereas the clone III strains were relatively homogeneous both in resistance genes and integrons, clone I and II showed a remarkable intraclonal diversity of these properties, with no clear-cut difference between the two clones. Yet, within the Czech clone I and II strains, the diversity of resistance genes and integron structures was limited as compared to those from other countries. These findings suggest the occurrence of local pools of resistance genes and the possibility of horizontal transfer of resistance genes between the two clones.

In the third study, the question of the clinical-epidemiological relevance of the recently discovered nonspecific efflux pump AdeABC in *A. baumannii* was addressed. A total of

116 strains from 16 European countries isolated over a period of 23 years were investigated for the presence of genes associated with the efflux system. Furthermore, the diversity of the strains (clonal relatedness or unrelatedness) was assessed by AFLP fingerprinting. Results showed that the AdeABC genes were present in most *A. baumannii* strains including fully susceptible strains, and that overexpression of this system is likely to be a common property of MDR strains. The purpose of the last study was to analyse the emergence of carbapenem resistance among *Acinetobacter* hospital strains in the Czech Republic, which has been noted from the early 2000s onwards. To this aim, *Acinetobacter* clinical isolates were collected prospectively from multiple ICUs in 2005-2006 and were analysed for their genomic types and for resistance determinants. The results showed that the emergence of carbapenem resistance was associated with the spread of *A. baumannii* strains of a subclone of EU clone II. An impressive variation in resistance determinants in this group of highly related strains was observed. Finally, in chapter 6 of the thesis the overall results of the studies are discussed within the context of the most recent developments.

The present thesis provides an important contribution to the concept that the increasing resistance to multiple antibiotics in *A. baumannii* is significantly associated with the occurrence of a limited number of widespread groups of genetically closely related strains (clonal lineages). Among them, the so-called EU clone I and II seem to have prevailed among MDR *Acinetobacter* strains in some European countries since the early 1980s. Whereas clone I dominated over clone II among the strains isolated in the 1980s, more recent studies indicate a growing importance of clone II. Our results as well as other studies have shown that resistance to carbapenems, the most important group of antibiotics against MDR acinetobacters, can be associated with some geographic subclones of EU clone II, which suggests that this clone plays an important role in the development and spread of carbapenem resistance. Recent studies including ours have identified a number of resistance mechanisms in *A. baumannii* and have shown that multiple mechanisms can be present in particular strains. It has become clear that both activation of intrinsic resistance mechanisms and horizontal acquisition of resistance genes play a role in the evolution of antibiotic resistance of this organism. In addition, very recent studies have reported unique genetic structures termed resistance islands which harbour acquired resistance genes and can be responsible for a striking variation in resistance genotypes and phenotypes in clonally and epidemiologically related isolates. Thus, recent findings including the observations of the present thesis are bringing up new, more basic, questions and opening a window to experimental studies that can lead to a comprehensive understanding of factors which contribute to the ability of *A. baumannii* to develop resistance to all clinically relevant agents.

CHAPTER 8

Samenvatting

SAMENVATTING

Acinetobacter baumannii is een belangrijke ziekenhuisbacterie die vooral bij patiënten op intensivecare-afdelingen aanleiding kan geven tot kolonisatie en infecties. De betrokken stammen zijn dikwijls resistent voor meerdere antibiotica, waardoor behandeling van infecties moeilijk of zelfs onmogelijk is. Hoewel ook goed gevoelige *A. baumannii* stammen voorkomen, zijn het vooral de multiresistente stammen die zich epidemisch onder patiënten kunnen verspreiden. In een publicatie die verscheen in 1996 werd vastgesteld dat twee groepen (de zogenaamde Europese (EU) klonen I en II) van genotypisch verwante *A. baumannii* stammen overheersten onder isolaten uit epidemieën in ziekenhuizen verspreid over Noordwest-Europa. Er was geen bekende epidemiologische relatie tussen deze ziekenhuizen. Deze waarneming werd ondersteund door een publicatie uit 1999 waarin werd beschreven dat stammen die verwant waren aan de EU-klonen I en II ook overheersten onder klinische isolaten van *A. baumannii* uit ziekenhuizen verspreid over Tsjechië. In beide studies bleken de stammen die behoorden tot de EU-klonen I en II veel resistenter te zijn voor antibiotica dan andere stammen. Dit was aanleiding te veronderstellen dat de koppeling van multiresistentie en klonaliteit een rol heeft gespeeld bij de immer voortschrijdende toename van resistentie bij *Acinetobacter*. Deze bevindingen waren aanleiding tot dit proefschrift dat zich richt op de relatie tussen de epidemiologie en de moleculaire basis van meervoudige resistentie voor antibiotica in *A. baumannii* op populatieniveau. Hierbij is een beschrijvende en vergelijkende benadering gevolgd waarbij een groot aantal eigenschappen van goed gedefinieerde stammencollecties werd onderzocht om de relatie tussen multiresistentie en populatie-structuur van *A. baumannii* vast te stellen. De bestudeerde organismen bestonden in hoofdzaak uit klinische isolaten die sinds 1991 waren verkregen uit ziekenhuizen in Tsjechië en uit isolaten uit verscheidene andere Europese landen die in de afgelopen decennia in de de cultuurcollectie van het Leids Universitair Medisch Centrum waren opgenomen.

In de eerste studie onderzochten wij met behulp van AFLP fingerprinting en ribotypering de genotypische relatie tussen enerzijds Tsjechische *A. baumannii* stammen die eerder waren ingedeeld in de zogenaamde groepen A and B en, anderzijds, stammen van de Noordwest-Europese klonen. De studiecollectie omvatte 70 multiresistente en 15 gevoelige stammen uit Tsjechië over de periode 1991-2001 en referentiestammen van de EU-klonen I and II. De resultaten bevestigden dat de twee overheersende groepen A en B uit Tsjechië genomisch congruent waren met de klonen I en II. In de volgende studie onderzochten wij de diversiteit van de genen die coderen voor aminoglycoside-modificerende enzymen alsmede de associatie van deze enzymen met klasse I integronen in de bekende EU-klonen inclusief de nieuw beschreven kloon III. Wij vonden dat kloon III zowel in resistentiegenen en integronen relatief homogeen was, terwijl kloon I en II een opvallende intraklonale diversiteit hadden, maar zonder scherp onderscheid tussen de klonen. Echter, binnen de Tsjechische klonen I en II was de diversiteit van resistentiegenen en integronstructuren beperkt in vergelijking tot

elders. Dit zou kunnen worden verklaard door aan te nemen dat er locale reservoirs zijn van resistentiegenen en dat er mogelijk horizontale overdracht is van resistentie determinanten tussen de twee klonen. In the derde studie stond de vraag naar de klinisch-epidemiologische betekenis van de recent ontdekte niet-specifieke effluxpomp AdeABC in *A. baumannii* centraal. Een collectie van 116 stammen, afkomstig uit 16 Europese landen en ingezameld over een periode van 23 jaar, werd onderzocht op de aanwezigheid van genen die waren geassocieerd met het effluxsysteem. Bovendien werd de diversiteit van de stammen (klonaal verwant of niet) onderzocht met verschillende methoden. De resultaten toonden aan dat de AdeABC-genen aanwezig waren in de meeste *A. baumannii* stammen inclusief sporadische stammen, en dat overexpressie van dit systeem een algemene eigenschap is van multiresistente stammen. Het doel van de laatste studie was om inzicht te krijgen in de opkomst van carbapenemresistentie vanaf het begin van de jaren 2000 in Tsjechië. Voor dit doel werden klinische isolaten van *Acinetobacter* in de periode 2005-2006 prospectief ingezameld van een groot aantal intensivecare-afdelingen verspreid over het land. Van deze stammen werd het genotype en de aanwezigheid van resistentiedeterminanten bepaald. De resultaten toonden aan dat de opkomst van carbapenemresistentie verband hield met de verspreiding van *A. baumannii* stammen die behoorden tot een subkloon van EU-kloon II. Er werd een indrukwekkende diversiteit in resistentie determinanten waargenomen in deze groep van nauw verwante stammen. Tot slot worden in hoofdstuk zes de verschillende studies in samenhang met elkaar en in het licht van de meest recente ontwikkelingen besproken.

De resultaten van dit proefschrift dragen bij tot het beeld dat de toenemende resistentie voor antibiotica van *A. baumannii* in belangrijke mate samenhangt met de aanwezigheid van een beperkt aantal wijdverspreide groepen van nauw verwante stammen (klonen). Het waren vooral de EU-klonen I en II die in verscheidene Europese landen vanaf de tachtiger jaren van de vorige eeuw domineerden onder epidemische stammen. Terwijl kloon I aanvankelijk vaker werd gevonden dan kloon II, tonen recente studies een toenemende aanwezigheid van kloon II aan. Onze bevindingen en ook die van andere studies laten zien dat resistentie voor carbapenems, de belangrijkste antibiotica voor behandeling van multiresistente acinetobacters, gekoppeld kan zijn aan bepaalde geografische subgroepen van kloon II. Dit suggereert dat juist deze kloon een belangrijke rol speelt in de ontwikkeling en verspreiding van carbapenemresistentie. Onderzoek uit de afgelopen tijd heeft een aantal resistentiemechanismen in *A. baumannii* ged'entificeerd en ook aangetoond dat verschillende mechanismen tegelijkertijd in een stam aanwezig kunnen zijn. Inmiddels is bekend dat zowel activatie van intrinsieke resistentiemechanismen als horizontale overdracht van resistentiegenen een rol kunnen spelen bij de evolutie van resistentie voor antibiotica van dit organisme. Daarnaast hebben zeer recente studies een aantal unieke genetische structuren ged'entificeerd, de zogenaamde resistentie-eilanden, die een groot aantal resistentiegenen kunnen omvatten en verantwoordelijk zijn voor een enorme variatie aan resistentiegenotypen en -fenotypen binnen klonaal en epidemiologisch verwante stammen. Alles bij elkaar

geven de resultaten van dit proefschrift en die uit de literatuur aanleiding tot nieuwe, meer fundamentele, vragen. Deze vragen vormen de basis voor verder onderzoek met als doel een beter begrip te krijgen van de factoren die bijdragen tot het vermogen van *A. baumannii* om resistentie te ontwikkelen tegen alle mogelijke klinisch relevante therapeutica.

CURRICULUM VITAE

Alexandr Václav Nemec was born on February 7, 1963 in Hradec Králové, Czech Republic. He has three sons. After graduation from the secondary school in Klatovy (southern Bohemia) in 1981, he studied general biology and microbiology at the Faculty of Science, Charles University in Prague. In 1987, he received the degree RNDr. (*Rerum Naturalium Doctoris*) and in 2003 obtained his Ph.D. in medical microbiology at the 3rd Faculty of Medicine, Charles University, with a thesis on the taxonomy and molecular typing of strains of the genus *Acinetobacter*. In 2006, he defended a habilitation thesis entitled "Study of medically relevant strains of *Acinetobacter*" to become an associate professor in medical microbiology at the Charles University.

After graduation in 1987, he worked as a bacteriologist in the Bacteriological Laboratory of the Agricultural Cooperative at Jílové u Prahy. In 1990, he joined the Working Group of Clinical Microbiology headed by Professor Jiri Schindler at the Institute of Hygiene and Epidemiology (currently the National Institute of Public Health). At present, he is a senior researcher and head of the Department of Bacterial Genetics and Mycobacteria at the National Institute of Public Health. In 1993-1994 and 2003-2004, he was a part-time lecturer at the Department of Medical Microbiology of the 3rd Medical Faculty, Charles University. From 2005 to 2009, he was a part-time senior researcher at the Department of Immunology of the 2nd Medical Faculty in Prague. Since 2007, he has been a lecturer in medical bacteriology at the Faculty of Science, Charles University. From 1997 onwards he has collaborated with Dr. Lenie Dijkshoorn at the Department of Infectious Diseases of the Leiden University Medical Center on the taxonomy and epidemiology of *Acinetobacter* and has been a frequent visitor of the Department. He was the principal investigator of seven successfully completed projects supported by Czech grant agencies. His current research is focused on the taxonomy of the genus *Acinetobacter*, population structure of *Acinetobacter baumannii* and *Pseudomonas aeruginosa* and the genetic basis and evolution of resistance mechanisms in clinical strains of these species.

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