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Genetic basis of multidrug resistance in *Acinetobacter baumannii*

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ABSTRACT

Acinetobacter baumannii has emerged as a significant bacterial pathogen pre-eminently associated with hospital-acquired infections. Strains of this species may currently exhibit resistance to nearly all or even all clinically relevant drugs. The vast majority of epidemic and multidrug-resistant *A. baumannii* strains belong to a few globally spread lineages, in particular to the so-called European (EU) clones I, II, and III. Complex resistance patterns displayed by these strains result from their marked capacity to develop, acquire, and combine secondary resistance mechanisms against originally effective agents. The aim of this thesis was to broaden our knowledge on the genetic basis and epidemiology of multidrug resistance in *A. baumannii*. The obtained results have been published in the form of six studies which are part of this thesis.

In the first study, we analysed the epidemiology of carbapenem resistance among hospital strains of *Acinetobacter* in the Czech Republic. We have shown that the emergence of this resistance was associated with the spread of *A. baumannii* strains of EU clone II and it was predominantly caused by the overexpression of the intrinsic $bla_{\text{OXA-51}}$-like gene. Furthermore, the striking variation in the susceptibility to other clinically relevant drugs in these strains appeared to result from both the horizontal spread of resistance genes (e.g. *aacC1*, *aphA6*, or $bla_{\text{TEM-1}}$) and differential expression of the AdeABC efflux pump.

The second and third studies dealt with the genotypic characterization of a high-level carbapenem resistant strain of *A. baumannii* imported to the Czech Republic from Egypt in 2011. The strain co-harboured genes encoding five β-lactamases (NDM-1, OXA-23, OXA-51-like, TEM-1, and ADC) and at least five other resistance mechanisms, which made it resistant to all clinically relevant drugs except for colistin and tobramycin.

In the fourth study, we have identified and described a clinically relevant mechanism of sulbactam resistance in *A. baumannii* based on the production of the TEM-1 β-lactamase. Its role was supported especially by the correlation between the level of sulbactam resistance and the expression of the $bla_{\text{TEM-1}}$ gene, by the transferability of sulbactam resistance via a $bla_{\text{TEM-1}}$-carrying plasmid, and by the susceptibility of a clinical strain expressing TEM-19, a low activity variant of TEM-1.

In the fifth and sixth studies, we investigated the structural diversity of AbaR genomic resistance islands in the population of *A. baumannii* EU clone I in order to find additional clues for a better understanding of the evolution of antibiotic resistance in this multidrug-resistant lineage. We have described nine novel AbaR islands which were truncated variants of AbaR3. These variants resulted either from IS*26*-mediated deletions or homologous recombination. We suggested that AbaR3 is the original form of AbaR in EU clone I, which may have provided strains of the lineage with a selective advantage facilitating their spread in European hospitals in the 1980s or before.

SOUHRN

Acinetobacter baumannii je významný původce infekcí u pacientů v nemocniční péči. Rostoucí podíl multirezistentních a panrezistentních kmenů tohoto druhu se v posledním desetiletí stal celosvětovým problémem. Z populačně-genetického hlediska je významné, že tyto kmeny náležejí pouze do několika klonálních linií, tzv. Evropských klonů I, II a III. Odolnost těchto kmenů vůči původně účinným antimikrobním látkám je dána jejich schopností účinně vyvíjet, získávat a kombinovat nejrůznější mechanismy rezistence. Tato dizertační práce obsahuje šest studií, které přispěly k rozšíření znalostí o epidemiologii a genetice multirezistence u *A. baumannii*.

První studie se zabývá epidemiologií rezistence ke karbapenemům u nemocničních kmenů acinetobakterů. Nárůst této rezistence v letech 2005-2006 byl spojen s rozšířením kmenů *A. baumannii* náležejícím k Evropskému klonu II a hlavním mechanismem této rezistence byla zvýšená exprese druhově inherentního genu pro OXA-51. U studovaných kmenů byla dále zjištěna značná variabilita v citlivostech k dalším klinicky významným antibiotikům plynoucí z horizontální akvizice různých genů rezistence (např. *aacC1*, *aphA6* nebo $bla_{\text{TEM-1}}$) a/nebo rozdílné exprese efluxního systému AdeABC.

Druhá a třetí studie se věnuje molekulárně-genetické charakterizaci kmene *A. baumannii*, který byl vysoce rezistentní ke karbapenemům a který byl v roce 2011 importován do České republiky z Egypta. Kmen nese geny pro pět různých β-laktamáz (NDM-1, OXA-23, OXA-51-like, TEM-1 a ADC) a nejméně pět dalších mechanismů rezistence, což vysvětluje jeho citlivost pouze na kolistin a tobramycin.

Ve čtvrté studii popisujeme klinicky významný mechanismus rezistence *A. baumannii* k sulbaktamu založený na produkci získané ß-laktamázy TEM-1. Úlohu TEM-1 jsme experimentálně potvrdili na základě (i) pozitivní korelace mezi mírou exprese genu $bla_{\text{TEM-1}}$ a rezistence k sulbaktamu, (ii) přenosu sulbaktamové rezistence na původně citlivý kmen *A. baumannii* pomocí transformace genem $bla_{\text{TEM-1}}$ a (iii) citlivosti kmene produkujícího β-laktamázu TEM-19 (enzymaticky méně aktivní variantu TEM-1) k sulbaktamu.

Pátá a šestá studie popisují strukturní rozmanitost genomových ostrovů rezistence (AbaR) u kmenů *A. baumannii* náležejících k Evropskému klonu I ve snaze porozumět evoluci multirezistence u této klonální linie. Výsledky ukázaly, že ostrov rezistence AbaR3 byl u klonu I původní strukturou, která mohla tomuto klonu v nemocničním prostředí poskytnout výraznou selektivní výhodu. Dále jsme popsali devět nových variant ostrovů, jež pravděpodobně vznikly delecemi v původní struktuře AbaR3, a to skrze homologní rekombinaci nebo transpoziční aktivitu inzerční sekvence IS*26*.

CONTENTS

1. INTRODUCTION

This thesis deals with the issue of multidrug resistance in *Acinetobacter baumannii*. The main emphasis is on the molecular genetic basis, epidemiology, and evolution of resistance mechanisms and their significance both in the biological and clinical contexts. It also contributes to our understanding of the organisation of resistance determinants in the *A. baumannii* genome. In addition, the association of antimicrobial resistance with the major clonal lineages of *A. baumannii* is investigated, owing to its clinical and epidemiological importance. The findings of the thesis are discussed in the light of the current knowledge and with the accent on the epidemiological situation in the Czech Republic.

2. OVERVIEW OF LITERATURE

2.1. The genus *Acinetobacter*

The genus *Acinetobacter* encompasses Gram-negative, strictly aerobic, generally non-motile, catalase-positive, and oxidase-negative coccoid bacteria (Peleg *et al*., 2008). Currently, the genus comprises 30 distinct species with valid names and several provisionally designated genomic species that were delineated by DNA-DNA hybridization (Dijkshoorn *et al*., 2007; www.bacterio.net/a/acinetobacter.html). *Acinetobacter* species are generally isolated from environmental samples such as soil and sludge, but also from human clinical specimens. Since some *Acinetobacter* species cannot be reliably differentiated based on their phenotypic characteristics, molecular methods were designed for species identification. The identification methods relevant for this thesis are listed in Table 1.

Table 1. Molecular methods for *Acinetobacter* species indentification

Method	Reference
Whole-genome DNA-DNA hybridisation	Bouvet & Grimont, 1986
AFLP (whole-genome fingerprinting)	Janssen *et al.,* 1997
ARDRA* (restriction enzyme analysis of amplified 16S *r* DNA)	Vaneechoutte *et al.,* 1995
Direct sequencing of a single gene	
16S *r* DNA	Vaneechoutte & De Baere, 2007
gyrB	Yamamoto & Harayama, 1996
rpoB	La Scola *et al.,* 2006
Whole-cell protein fingerprinting by MALDI-TOF†	Álvarez-Buylla *et al.,* 2012

*Amplified Ribosomal DNA Restriction Analysis

†Matrix-Assisted Laser Desorption Ionization Time-Of-Flight mass spectrometry

2.2. *Acinetobacter baumannii*: a paradigm of an emerging hospital pathogen

Over the last decades, *A. baumannii* has emerged as a significant bacterial pathogen pre-eminently associated with hospital-acquired infections (Dijkshoorn *et al*., 2007; Peleg *et al*., 2008). This bacterium has generated alarm among the medical fraternity worldwide, largely owing to its extensive armamentarium of potent antibiotic resistance mechanisms and capability to epidemically spread in the hospital environment (Dijkshoorn *et al*., 2007). *A. baumannii* together with *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella*

pneumoniae, *Pseudomonas aeruginosa*, and *Enterobacter* spp., collectively known as the ESKAPE pathogens, have become able to escape the action of nearly all antimicrobial agents (Howard *et al*., 2012).

A. baumannii has been increasingly reported as the cause of hospital infections including ventilator-associated pneumonia, septicemia, urinary tract infections, secondary meningitis, wound infections, and skin and soft tissue infections (Bergogne-Bérézin & Towner, 1996). The most predisposed hosts to *Acinetobacter* infections are critically ill patients in intensive care units (ICUs), burn and trauma patients, and other debilitated patients with indwelling blood or urinary catheters or with invasive mechanical ventilation (Peleg *et al*., 2008).
This organism has been shown to be responsible for up to 8 % of all healthcare-associated infections (Vincent *et al*., 1995; Gaynes & Edwards, 2005) with crude case fatality rates of about 40%, reaching up to 75% in systemic infections (Chastre *et al*., 1996; Seifert, 2009). However, it is not clear to what extent these deaths are attributable to underlying diseases in infected patients and/or to the contribution of other co-infecting organisms.

Even though *Acinetobacter* clinical strains were commonly susceptible to a number of traditional antibiotics in the 1960s and 1970s, the hospital population of *A. baumannii* currently exhibits resistance to nearly all or even all clinically relevant drugs. In the light of such a striking evolution from a clinically unimportant bacterium towards a multidrug-resistant (MDR) pathogen, *A. baumannii* represents a typical example of an emerging pathogen which demands our undivided attention (Falagas *et al*., 2006). Together with *K. pneumoniae* and *P. aeruginosa*, *A. baumannii* belongs to the microorganisms with the greatest gap between the current antimicrobial research and development pipeline and the unmet medical need for new effective antimicrobials (Boucher *et al*., 2009).

An important property making *A. baumannii* a nosocomial pathogen is its ability to resist physical and chemical factors of the hospital environment. For example, unlike other Gram-negative bacteria, *A. baumannii* can effectively survive desiccation. Thus, in addition to its ability to develop antimicrobial resistance, *A. baumannii* excels in persistence for prolonged periods in the various hospital settings as well as on the hands of healthcare workers (Jawad *et al*., 1998). All these properties contribute to the marked ability of *A. baumannii* to spread among patients and cause outbreaks in hospitals. Some MDR strains or epidemic lineages of *A. baumannii* have been shown to have the potential to spread internationally. This has a

serious impact on the management of hospital infections worldwide. Although much less frequent as compared to the hospital situation, *A. baumannii* may cause community-acquired infections, especially in countries with tropical climates, which are often associated with underlying conditions such as alcoholism, smoking, diabetes mellitus, or chronic obstructive pulmonary disease (Anstey *et al*., 2002).

2.3. Population diversity in *A. baumannii*

A number of phenotypic and genotypic typing methods have been employed for the differentiation of epidemiologically unrelated *Acinetobacter* isolates especially during investigations of hospital outbreaks. Traditional methods included antibiotic susceptibility determination, cell envelope protein profiling, and biotyping, but these were later replaced by high-resolution DNA fingerprinting approaches such as ribotyping, AFLP analysis, PCR-fingerprinting, and macrorestriction analysis by pulsed-field gel electrophoresis (PFGE), with the latest having become a standard for analysis of local epidemics (Dijkshoorn *et al*., 1993; 1996). Even though these comparative fingerprinting methods were found to be useful for epidemiological typing of isolates at the local scale, they generally suffer from low inter-laboratory reproducibility and the incapability to provide information on the relatedness of isolates in a broader phylogenetic sense or to delineate an epidemic strain in a precise way (Brisse, 2009). To overcome these drawbacks, sequence-based typing methods have been proposed for population analysis, with multilocus sequence typing (MLST) being the most widely accepted approach. MLST consists of the sequencing of internal portions of seven (house-keeping) protein-coding genes (*cpn60*, *fusA*, *gltA*, *pyrG*, *recA*, *rplB*, and *rpoB*) providing unambiguous and portable data which can be easily compared. For a given house-keeping gene, each unique sequence (allele) is assigned with a number and a combination of these numbers, the so-called allelic profile (e.g. 1-1-1-1-5-1-1), defines a sequence type (e.g. ST1) (www.pasteur.fr/recherche/genopole/PF8/mlst/Abaumannii.html).

In their 1996 study, Dijkshoorn *et al.* (Dijkshoorn *et al*., 1996) were the first to report the high genetic similarity of *A. baumannii* strains isolated in geographically distant hospitals. They proposed that these strains were clonally related, i.e. representing a clone in the epidemiological sense. The term clone in this sense was first used by Ørskov & Ørskov (Ørskov & Ørskov, 1983) to denote bacterial isolates originating independently from different

sources, in different locations, and possibly at different times, but being genotypically and phenotypically homogeneous and distinct from other isolates. Isolates of a clone most likely descend from a common ancestral strain (Brisse, 2009; Nemec, 2009). Dijkshoorn *et al.* (Dijkshoorn *et al*., 1996) compared outbreak and sporadic hospital strains from different northwestern European countries using a combination of genotypic and phenotypic methods, and found that the outbreak strains could be allocated to two main groups, which are currently known under the designations European (EU) clone I and II, respectively. This observation was confirmed by the following studies (Nemec *et al*., 1999; Diancourt *et al*., 2010) which further revealed that strains of these epidemic clones were commonly multidrug resistant (MDR). It is currently well accepted that a limited number of successful MDR clonal lineages prevail within the global population of *A. baumannii* (Nemec *et al*., 2004a; Turton *et al*., 2004; van Dessel *et al*., 2004; Diancourt *et al*., 2010; Higgins *et al*., 2010; Zarrilli *et al*., 2013). Among these lineages, EU clones I, II, and III (Figure 1) are most commonly encountered (van Dessel *et al*., 2004; Diancourt *et al*., 2010) although additional clones have been reported (Higgins *et al*., 2010).

The first known strains of EU clones I and II were isolated in the 1980s (Dijkshoorn *et al*., 1996) while EU clone III strains seem to represent a more recent epidemic lineage (Huys *et al*., 2005). Most (80%) MDR *A. baumannii* strains isolated from inpatient clinical specimens in the Czech Republic between 1991 and 2001 were assigned to EU clones I and II (Nemec *et al*., 1999; Nemec *et al*., 2004a). Although data from the other European countries indicate a more complex diversity of the MDR *A. baumannii* population (Spence *et al*., 2002), its structure appeared to be relatively stable in the Czech Republic between 1991 and 2001 (Nemec *et al*., 1999, Nemec *et al*., 2004a). However, a more recent study (included in this thesis) has shown a striking expansion of EU clone II in this country in 2005-2006, with the vast majority (>90%) of MDR isolates classified into this clone. Furthermore, the study has suggested that the expansion of EU clone II had been associated with the emergence of carbapenem resistance (Nemec *et al*., 2008). Several other studies focussing on the epidemiology of resistance to carbapenems have also reported that EU clone II has spread worldwide and plays a major role in the dissemination of resistance to these antibiotics (Turton *et al*., 2004; Higgins *et al*., 2010; Zarrilli *et al*., 2013).

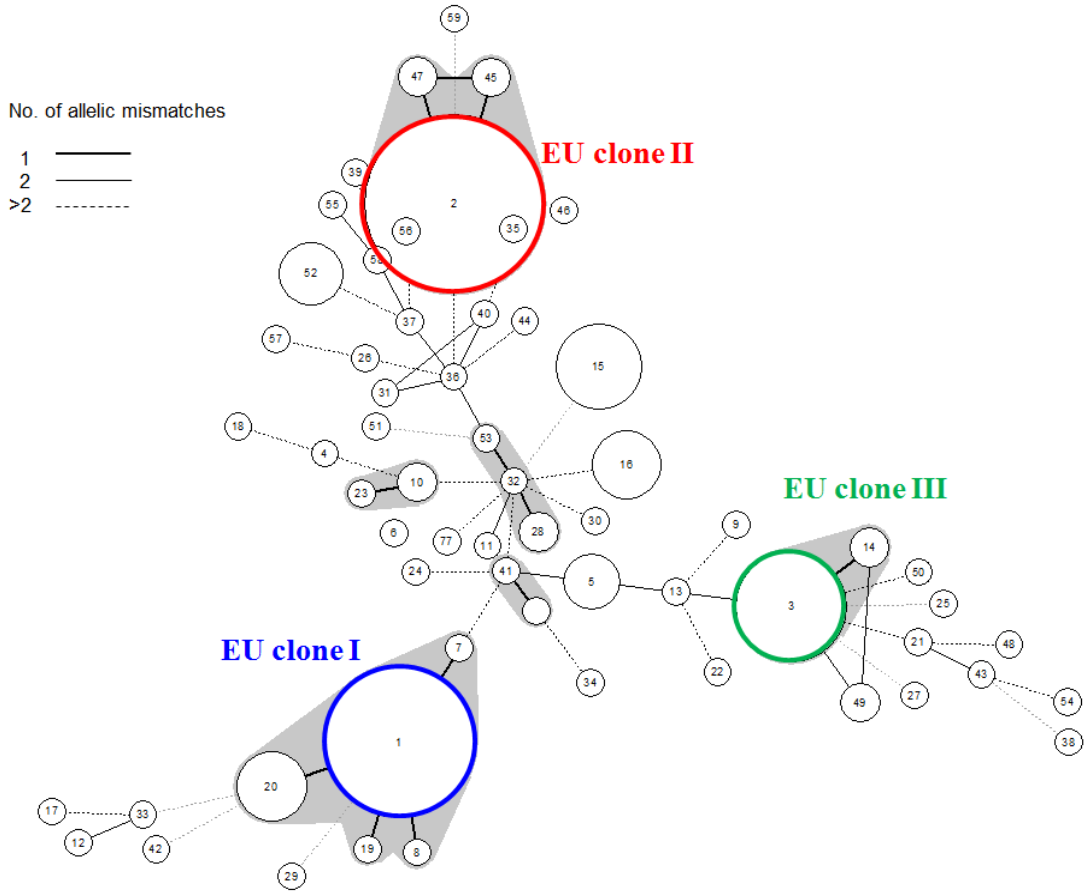


Figure 1. Minimum spanning tree analysis of the *A. baumannii* sequence types (STs) (Diancourt *et al*., 2010). The number of allelic mismatches among ST profiles was used as the distance measure. Each circle corresponds to one sequence type (ST), with its designation indicated inside. Circle size increases logarithmically with the number of isolates that had this ST, from one (smallest circles) to many of them (large circles). Grey zones that surround some groups of circles indicate that these profiles belong to the same clone, meaning that they have a single allelic mismatch with at least one other member of the group. EU clones I, II, and III are coloured in blue, red, and green, respectively.

2.4. Antimicrobial resistance of *A. baumannii*

In the early 1970s, the genus *Acinetobacter* first began to be recognized as a significant nosocomial pathogen being defeated by available antimicrobial agents relatively easily.

However, over time, *A. baumannii* became resistant to most originally effective antibiotics including new generations of broad spectrum *β*-lactams, aminoglycosides and fluoroquinolones (Joly-Guillou *et al*., 1988; Bergogne-Bérézin & Towner, 1996). The emergence of MDR strains prompted the introduction of carbapenems (imipenem and later meropenem) that became the mainstay of treatment for *Acinetobacter* infections. Until recently, carbapenems were the only agents to remain broadly effective in the treatment of serious infections caused by MDR strains (Towner, 2009). However, since the 1985, hospital outbreaks caused by carbapenem-resistant strains have been reported (Patron *et al*., 1993; Tankovic *et al*., 1994; Bogaerts *et al*., 2006; Lolans *et al*., 2006) and since the 2000s, the proportion of these strains among MDR *A. baumannii* has dramatically increased worldwide (Jones *et al*., 1999; Yucesoy *et al*., 2000; Van Looveren *et al*., 2004). Carbapenem-resistant strains were often resistant also to other therapeutically relevant antibiotics except polymyxins. Nevertheless, strains non-susceptible to polymyxins (Li *et al*., 2006) have been described, which indicates that this species has the potential to become pandrug resistant.

Table 2 lists the classes of antimicrobial agents that are currently considered to have potential activity against *A. baumannii*. As different strains may show diverse combinations of resistance to these antibiotics, therapy should always be based on the results of properly performed antimicrobial susceptibility tests.

2.5. Resistance mechanisms in *A. baumannii*

A. baumannii is amongst a few bacterial pathogens displaying the most complex resistance patterns. This results from both the presence of intrinsic resistance and low susceptibility to some antimicrobial agents such as aminopenicillins, narrow spectrum cephalosporins, or chloramphenicol and enormous capacity of *A. baumannii* to develop, acquire, and combine secondary resistance mechanisms against primarily effective agents (Dijkshoorn *et al*., 2007). The secondary resistance mechanisms are encoded by genes acquired by horizontal transfer or associated with an enhanced expression of intrinsic resistance genes. The enhanced expression mostly results from the insertion of genetic elements into their promotor regions or mutations of their regulatory genes (Roca *et al*., 2012).

Table 2. Antimicrobial agents with potential activity against *Acinetobacter baumannii*

Antipseudomonal penicillins (ticarcillin, piperacillin)
Antipseudomonal penicillins with inhibitors (ticarcillin/clavulanic acid, piperacillin/tazobactam)
Extended-spectrum cephalosporins (ceftazidime, cefepime)
Carbapenems (imipenem, meropenem)
Sulbactam
Aminoglycosides (gentamicin, amikacin, netilmicin, tobramycin)
Fluoroquinolones (ciprofloxacin, levofloxacin)
Folate pathway inhibitors (trimetoprim-sulphametoxazole)
Tetracyclines (doxycyclin, minocyclin)
Glycylcyclines (tigecycline*)
Polymyxins (colistin)

* Clinical breakpoint for tigecyclin susceptibility or resistance in *A. baumannii* is currently not available in CLSI (CLSI, 2011) or EUCAST (EUCAST, 2013) guidelines.

The mechanisms of resistance to antimicrobial agents in *A. baumannii* are summarized in Table 3. They include (i) enzymatic inactivation of antibiotics (*β*-lactamases and aminoglycoside modifying enzymes), (ii) modification of a target site (DNA gyrase/topoisomerase IV alterations), (iii) decreased outer membrane permeability (loss of porins), or (iv) active drug efflux (Tet(A), Tet(B), and AdeABC) (Limansky *et al*., 2002; Bonomo & Szabo, 2006; Poirel & Nordmann, 2006).

2.5.1. Diversity of acquired resistance mechanisms

As shown in Table 3, many acquired resistance determinants responsible for resistance to *β*-lactams have been reported in *A. baumannii.* These include TEM-1-like (TEM-1, TEM-19), SHV (SHV-5, SHV-12, TEM-92, TEM-116), and SCO-1 *β*-lactamases, which hydrolyse carboxypenicillins/ureidopenicillins, and extended spectrum *β*-lactamases (ESBLs) such as CTX-M-2, GES (GES-11, GES-14), CARB (RTG-4, CARB-5), PER (PER-1, PER-2, PER-7), and VEB-1 (Bonomo & Szabo, 2006; Potron *et al*., 2009; Poirel *et al*., 2012b; Bonnin *et al*., 2013; Krizova *et al*., 2013).

Table 3. Antimicrobial resistance mechanisms in *Acinetobacter baumannii*

Mechanism Responsible component	Note	Origin	Typical target drug
ß-lactam hydrolysis			
ADC*	AmpC-type ß-lactamase intrinsic to *A. baumannii*	Intrinsic	Cephalosporins
TEM-1-like	Narrow spectrum class A ß-lactamases	Acquired	Penicillins
SHV	Narrow spectrum class A ß-lactamases	Acquired	Penicillins
SCO-1	Narrow spectrum class A ß-lactamase	Acquired	Penicillins
CARB	Broad spectrum class A ß-lactamases	Acquired	Penicillins
VEB-1	Broad spectrum class A ß-lactamase	Acquired	Cephalosporins
PER	Broad spectrum class A ß-lactamases	Acquired	Cephalosporins
GES	Broad spectrum class A ß-lactamases	Acquired	Cephalosporins
CTX-M-2	Broad spectrum class A ß-lactamase	Acquired	Cephalosporins
OXA-51-like*	Class D ß-lactamase intrinsic to *A. baumannii*	Intrinsic	Carbapenems
OXA-23-like	Class D ß-lactamases	Acquired	Carbapenems
OXA-40-like	Class D ß-lactamases	Acquired	Carbapenems
OXA-58-like	Class D ß-lactamases	Acquired	Carbapenems
OXA-143-like	Class D ß-lactamase	Acquired	Carbapenems
OXA-235-like	Class D ß-lactamase	Acquired	Carbapenems
IMP	Metallo-ß-lactamases	Acquired	Carbapenems
SIM	Metallo-ß-lactamase	Acquired	Carbapenems
VIM	Metallo-ß-lactamases	Acquired	Carbapenems
NDM†	Metallo-ß-lactamases	Acquired	Carbapenems
Aminoglycoside modification			
AAC(3)-Ia	Acetyltransferase	Acquired	Gentamicin
AAC(3)-IIa	Acetyltransferase	Acquired	Gentamicin, tobramycin
AAC(6´)-Ib, AAC(6´)-Ih	Acetyltransferases	Acquired	Tobramycin, amikacin
APH(3´)-I	Phosphotransferase	Acquired	Kanamycin
APH(3´)-VI	Phosphotransferase	Acquired	Amikacin
ANT(2´´)-Ia	Nucleotidyltransferase	Acquired	Gentamicin, tobramycin
ANT(3´´)-Ia	Nucleotidyltransferase	Acquired	Streptomycin
Mutation			
GyrA	DNA gyrase subunit	Intrinsic	Fluoroquinolones
ParC	DNA topoisomerase IV subunit	Intrinsic	Fluoroquinolones
Methylation			
ArmA	16S rRNA methylase	Acquired	Aminoglycosides
Active efflux			
AdeABC*	Chromosomally encoded nonspecific efflux pump	Intrinsic	Aminoglycosides
Tet(A), Tet(B)	Tetracycline-specific efflux pumps	Acquired	Tetracyclines
Changes or loss of OMPs			
CarO	Protein responsible for drug infflux	Intrinsic	Carbapenems

Adopted form Dijkshoorn *et al.,* 2007 and Nemec, 2009, modified and updated. For detailed description see the text 2.5.1. and 2.5.2.

Mechanisms identified in *A. baumannii* strains isolated in the Czech Republic since 1991 are underlined.

*Mechanisms overexpression of which results in *in vitro* resistance.

†NDM-1 was detected in an *A. baumannii* strain imported from Egypt to the Czech Republic in 2011 (Krizova *et al*., 2012).

ESBLs hydrolyse penicillins and most cephalosporins at a high level, but most carbapenems are hydrolysed weakly by these enzymes. The genes encoding the above *β*-lactamases are commonly part of integrons or transposons (Poirel, 2009).

The diversity of acquired resistance determinants can be illustrated by the wide range of enzymes associated with carbapenem resistance. These are VIM (VIM-1, VIM-2, VIM-4), IMP (IMP-1, IMP-2, IMP-4, IMP-5, IMP-6), SIM-1, and NDM (NDM-1, NDM-2) enzymes belonging to Ambler class B metallo-*β*-lactamases (MBLs) (Nordmann & Poirel, 2002; Poirel & Nordmann, 2006). MBLs confer high level of resistance to carbapenems and to all other *β*-lactams except for aztreonam. They are zinc dependent metalloproteins inhibited by EDTA but not by the inhibitors of serine *β*-lactamases such as clavulanic acid, tazobactam, and sulbactam. A technique using an Etest strip containing imipenem or imipenem and EDTA has been developed to screen for the production of MBLs (Bonnin *et al*., 2012a). However, specificity of this test can be affected by the production of other *β*-lactamases occurring in *A. baumannii*, particularly by OXA-23 (Segal *et al*., 2005; Krizova *et al*., 2012). MBL genes are mostly located on transposons.

The most widespread *β*-lactamases with carbapenemase activity in *A. baumannii* are carbapenem-hydrolysing Ambler class D *β*-lactamases (CHDLs) (Poirel & Nordmann, 2006; Higgins *et al*., 2009). So far, six phylogroups of CHDLs have been identified in this bacterium, i.e. OXA-51-like, OXA-23-like, OXA-40-like, OXA-58-like, OXA-143-like, and OXA-235-like (Higgins *et al*., 2009; Higgins *et al*., 2013). The $bla_{\text{OXA-51-like}}$ gene encoding the OXA-51-like enzyme is intrinsically located on the chromosome of *A. baumannii* (Turton *et al*., 2006b) whereas the genes encoding the other (acquired) OXAs can be located on plasmids or chromosomes and are usually associated with insertion elements, providing them with mobility and/or increased expression (see 2.5.2.).

Acquired resistance to aminoglycosides has been attributed mainly to drug inactivation by acquired aminoglycoside modifying enzymes (AMEs) such as acetyltransferases (AAC), nucleotidyltransferases (ANT), and phosphotransferases (APH) (Shaw *et al*., 1993). In *A. baumannii*, at least eight distinct groups of AMEs (Table 3) have been occurring either separately or in different combinations (Nemec *et al*., 2004b). Genes encoding AMEs are often a part (a gene cassette) of class 1 intergons (Nemec *et al*., 2004b). Figure 2 illustrates the combination of different mechanisms of aminoglycoside resistance expressed in a clinical strain: namely the overexpression of the nonspecific chromosomal efflux pump AdeABC and

compared between a fully susceptible (NIPH 33) and a MDR (NIPH 281) clinical *A. baumannii* strains isolated in the Czech Republic in 1991 and 1994, respectively.

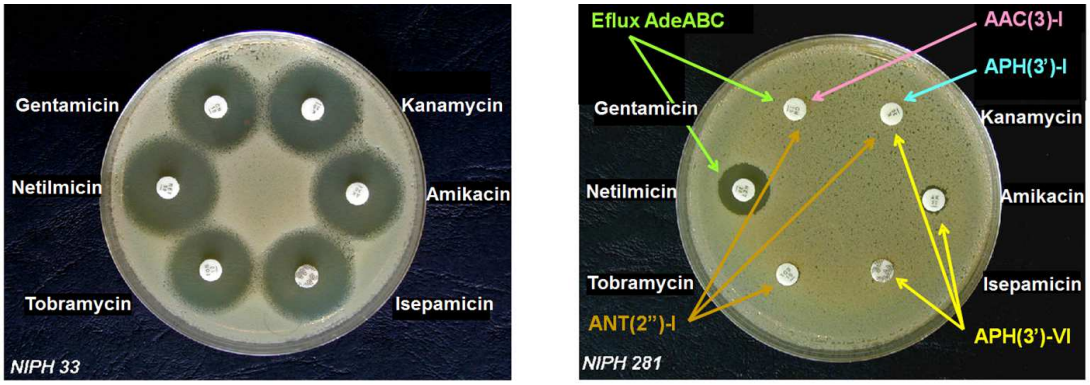


Figure 2. The effect of the combination of different resistance mechanisms on aminoglycoside resistance in *A. baumannii*. Shown are the results of disk diffusion for a fully susceptible (left) and MDR (right) clinical isolates collected in the Czech Republic in the early 1990s. The mechanisms include the overexpression of the nonspecific chromosomal efflux pump AdeABC and the activities of four different AMEs (AAC(3)-I, APH(3')-I, APH(3')-VI, and ANT(2'')-I).

In addition to AMEs, the plasmid-mediated RNA methylase ArmA that confers high-level resistance to most aminoglycosides by modifying 16S rRNA has been increasingly reported in India and China (Peleg *et al*., 2008). Recently, imported *armA*-positive *A. baumannii* strains have also been detected in European countries (Karah *et al*., 2011; Strateva *et al*., 2012).

The acquired resistance determinants found in *A. baumannii* are generally harboured on mobile genetic elements such as transposons (e. g., $bla_{\text{NDM-1}}$) or plasmids (e. g., $bla_{\text{OXA-58}}$) (Poirel *et al*., 2011). These well-known structures are per se mobile and thus, they mediate transfer of the harboured resistance genes. Other important vehicles for the spread and accumulation of resistance genes in *A. baumannii* are integrons (Nemec *et al*., 2004b). These genetic elements, although not mobile by themselves as a whole unit, are located on transposons and/or plasmids or are IS-bordered which allow them to be disseminated effectively in a horizontal manner (Roca *et al*., 2012). Integrons cluster (or exchange or remove) different gene cassettes and direct their coordinated expression. Based on the sequence similarity of integrases (enzymes mobilizing gene cassettes), several classes of

integrons have been described (Hall, 2012). In *A. baumannii*, the most important are class 1 integrons which integrate gene cassettes encoding AMEs (AAC(3)-I, AAC(6')-I, ANT(3'')-I, ANT(2'')-I; Nemec *et al*., 2004b), MBLs (VIM, IMP, SIM; Poirel *et al*., 2011), ESBLs (VEB-1; Fournier *et al*., 2006), and OXAs (OXA-10; Fournier *et al*., 2006). A positive correlation between the carriage of integrons and the degree of MDR in *A. baumannii* has been found (Huang *et al*., 2008).

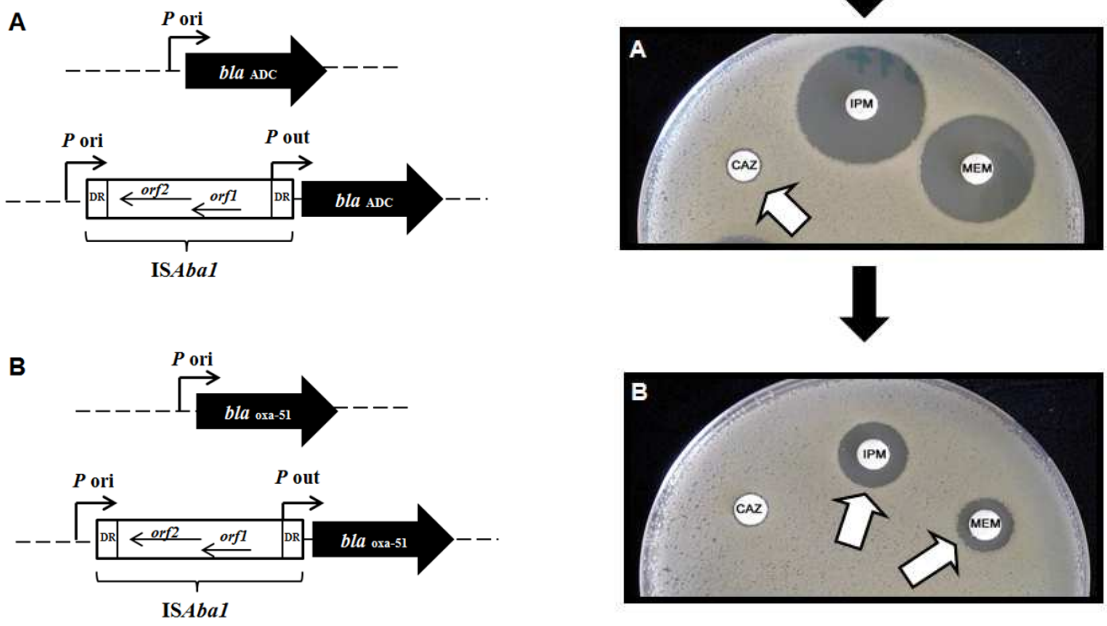
2.5.2. Activation of intrinsic resistance mechanisms

Recent studies have shown that the upregulation of some chromosomal genes which are species intrinsic to *A. baumannii* may also play an important role in the development of resistance. Chromosomal AmpC-type *β*-lactamase termed ADC (*Acinetobacter*-Derived Cephalosporinase) is not inducible and normally expressed only at a low level (Bou *et al*., 2000b); however it can be overexpressed as a result of the upstream insertion of the IS*Aba1* element, which provides the ADC gene with a more efficient promoter (P_{out}) than is the original one (P_{ori}) (Figure 3) (Héritier *et al*., 2006). IS*Aba1* is widespread in *A. baumannii*, with up to 21 copies per cell, and it is believed to serve as a "moving switch" to turn on the genes which are juxtaposed (Livermore & Woodford, 2006). Similarly to ADC, the gene encoding OXA-51-like enzyme, an intrinsic CHDL, is expressed poorly in most strains but its expression can be enhanced by the insertion of IS*Aba1* upstream of $bla_{\text{OXA- 51-like}}$ (Figure 3) (Turton *et al*., 2006a).

Another intrinsic chromosomal system is the AdeABC efflux pump of as yet unknown physiological function (Magnet *et al*., 2001). The expression of AdeABC is regulated by a two-component system composed of AdeS (a sensor) and AdeR (a response regulator). Fully susceptible *A. baumannii* strains containing the AdeABC genes can spontaneously produce resistant variants with mutations in the *adeS* or *adeR* genes. These mutations affect susceptibility to a broad spectrum of antimicrobials, yet the level of resistance to individual agents usually does do not reach clinical breakpoints (Marchand *et al*., 2004). Notably, recent studies have shown that the high–level overexpression of the AdeABC system was associated with an insertion of the IS*Aba1* element upstream of *adeS* in a tigecycline-resistant isolate (Ruzin *et al*., 2007; Sun *et al*., 2012). It has also been suggested that the overexpression of

this efflux pump can underlie the reduction of susceptibility to carbapenems in *A. baumannii* (Higgins *et al.*, 2004; Heritier *et al.*, 2005).

Figure 3. The effect of the IS*Aba1*-mediated upregulation of the chromosomal genes bla_{ADC} (A) and bla_{OXA-51} (B) on the susceptibility to *β*-lactams in *A. baumannii*. Presented are the possible successive insertions of IS*Aba1* harbouring a strong promoter (P_{out}) into the promotor sequences (P_{ori}) of these genes which resulted in the resistance to (A) ceftazidime (CAZ) followed by resistance to (B) imipenem (IPM) and meropenem (MEM).



Several other chromosomal mechanisms are known to confer resistance to clinically important drugs in *A. baumannii*. The mutations in the genes encoding the GyrA and ParC subunits of DNA gyrase and DNA topoisomerase IV, respectively, result in high resistance to fluoroquinolones (Vila *et al.*, 1997; Wisplinghoff *et al.*, 2003). Furthermore, susceptibility to

carbapenems can be decreased by the loss or modifications of outer membrane proteins responsible for the influx of these antibiotics (Roca *et al*., 2012). The best studied example of these porins is the 29-kDa CarO protein (Mussi *et al*., 2005; Siroy *et al*., 2005).

Overall, a single *A. baumannii* strain may harbour multiple mechanisms that confer resistance to multiple classes of antimicrobial agents (Nemec *et al*., 2004b; Hujer *et al*., 2006) and, in some combination, may contribute to high-level resistance to a particular group of antibiotics (Bou *et al*., 2000a). These traits make some strains high-level resistant to nearly all or even all clinically relevant drugs.

2.6. Genetic basis of multidrug resistance in *A. baumannii*

Over the last decades, significant progress has been made in the clarification of the function and genetic basis of particular resistance mechanisms (Roca *et al*., 2012; Zhao & Hu, 2012). However, until recently, very little was known about the genetic architecture of different resistance mechanisms in particular *A. baumannii* strains. This has been changed by the findings of several studies based on comparisons of the whole genome sequences obtained from several MDR strains (Fournier *et al*., 2006; Adams *et al*., 2008; Iacono *et al*., 2008; Vallenet *et al*., 2008). These studies have revealed that acquired genes encoding resistance to antibiotics and other chemical agents can form large clusters integrated into specific loci of the genome. These structures are termed resistance genomic islands.

2.7. Resistance islands (AbaRs)

Resistance islands are mostly chromosomal genetic structures of alien origin which may differ from the core genome of a host in the content of GC nucleotide pairs and codon preferences (Hacker & Carniel, 2001; Guy, 2006). These structures are diverse in their length (~10^3 – 10^5 bp) and are usually inserted into a gene, disruption of which results in loss of its function. Resistance islands consist mainly of mobile genetic elements such as transposons or integrons. They commonly harbour genes or operons conferring resistance to multiple antibiotics, heavy metals

or biocides, which results in selective advantage for host bacteria to survive and spread in hostile environments such as hospitals (Evans *et al*., 2013).

The term AbaRs is generally used to denote resistance islands found in *A. baumannii* which share a homologous core represented by a large composite transposon and are mostly integrated into a unique locus on the chromosome (Figure 4). In 2006, Fournier *et al*. (Fournier *et al*., 2006) reported an 86.2–kb resistance island, termed AbaR1, integrated into the ATPase gene (*comM*) of MDR *A. baumannii* strain AYE and harbouring 45 genes putatively associated with antimicrobial resistance. At that time, AbaR1 was the largest resistance island known in bacteria. In the same study, a 20-kb island flanked by transposases but devoid of resistance genes, was identified in the *comM* gene of another *A. baumannii* strain susceptible to antibiotics (Fournier *et al*., 2006). Further studies have found various sequences related to AbaRs integrated into the same locus in different *A. baumannii* strains (Adams *et al*., 2008; Iacono *et al*., 2008; Post & Hall, 2009; Shaikh *et al*., 2009). It was therefore suggested that AbaR1-like sequences in *A. baumannii* arose from an ancestral (Tn*7*-related) transposon which successively accumulated multiple transposable elements associated with resistance genes (Rose, 2010).

Based on in-depth comparative genomic analyses, Adams et al. (Adams *et al*., 2008) and Post & Hall (Post & Hall, 2009) suggested that out of the known AbaRs, only AbaR3 and AbaR5 were highly related to AbaR1 in terms of the composition and order of resistance genes. Notably, these three homologous AbaRs were found to be associated with EU clone I. The hypothesis that clonally related strains, e.g. those belonging to EU clone I, harbour homologous AbaRs was further supported by the results of Krizova *et al*. (Krizova *et al*., 2011) and Post *et al*. (Post *et al*., 2010b). In these studies, the AbaR core transposon (known as either Tn*6019* or Tn*AbaR3-like*) and the presence of some AbaR components were shown to be EU clone I-specific.

Overall, the findings of the above and other studies have revealed that AbaRs found in EU clone I and II differ structurally from each other, suggesting that these structures have evolved independently in these lineages, even though integrated in the same chromosomal locus (*comM*). AbaRs associated with *comM* in EU clone I and II are termed AbaR3-like (Krizova *et al*., 2011) and AbaR4-like (Seputine *et al*., 2012), respectively (Figure 5). The few exceptions found thus

far are AbaR2 in an EU clone II strain (Iacono *et al*., 2008) and some variants of AbaR4-like (Adams *et al*., 2008; Hamidian & Hall, 2011).

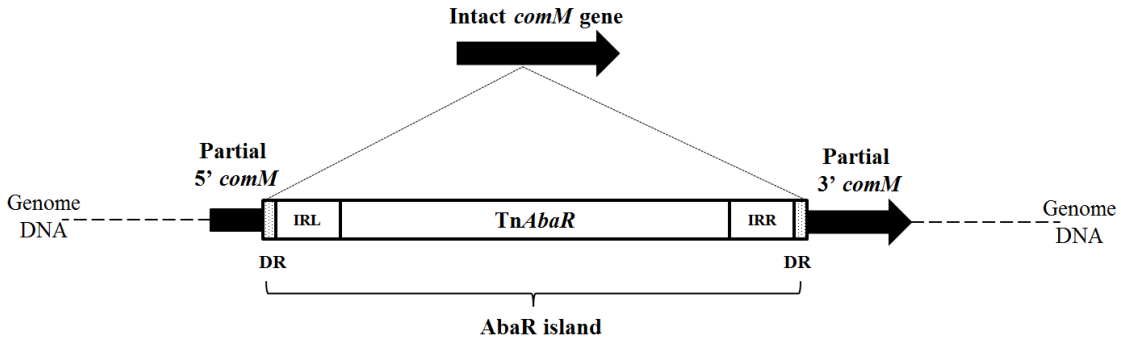


Figure 4. The layout of an AbaR resistance island inserted in the *comM* gene. AbaRs are formed by a core composite transposon (Tn*AbaR*) bounded by right (IRR) and left (IRL) inverted repeats and two copies of direct repeats (DR).

2.7.1. AbaRs in EU clone I

The results of our recent studies (included in this thesis) strongly support the hypothesis that the variants of AbaRs found in EU clone I strains derive from AbaR3 and, accordingly, these structures are termed AbaR3-like (Krizova & Nemec, 2010; Krizova *et al*., 2011). What AbaR3-like islands have in common is that they share the backbone Tn*6019* and the adjacent Tn*6018* transposons (Adams *et al*., 2008; Post & Hall, 2009; Post *et al*., 2010b; Krizova *et al*., 2011; Nigro *et al*., 2011).

It has been suggested that AbaR1, the so far the largest known AbaR3-like island, underwent the insertion of a 29-kb class 1 integron carrying the genes encoding ß-lactamases VEB-1 and OXA-10, which could thereby advantage the outbreak host strain (Post & Hall, 2009). Among AbaR3-like islands, a number of variants have been identified which most likely resulted from deletions

triggered by homologous recombination or were mediated by insertion sequence IS*26* (Post & Hall, 2009; Adams *et al*., 2010; Post *et al*., 2010b; Krizova *et al*., 2011).

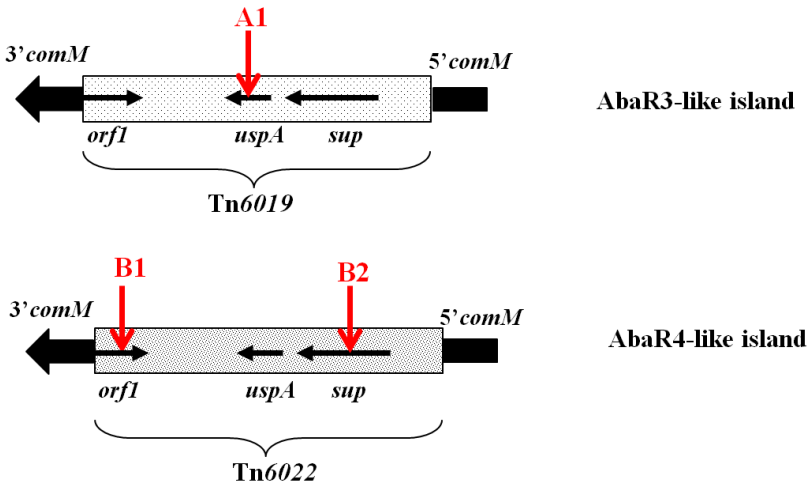


Figure 5. Outline of the main differences between the structures of AbaR3-like and AbaR4-like. Different dot densities in the AbaR cores represent different but related transposons. Red arrows indicate the positions at which the sequences specific for AbaR3-like or AbaR4-like are inserted. The *uspA* gene is usually disrupted by the so-called MARR (multiple-antibiotic resistance region, A1) in most AbaR3-like islands. B1 denotes the site of the integration of insertion sequences and/or deletion events, B2 stands for Tn*2006* harbouring the $bla_{\text{OXA-23}}$ gene or insertion sequence IS*CR2* in AbaR4-like islands.

An exception against the suggested paradigm that AbaR3-like islands are located within *comM* in EU clone I strains was reported by Iacono *et al.* (Iacono *et al*., 2008). In that study, a largely truncated variant of an AbaR3-like island (termed AbaR2) inserted within *comM* was found in a MDR strain of EU clone II while no AbaR4-like was detected in this strain. It is also of note that a strain (AB0057) belonging to EU clone I was found to co-harbour both AbaR3-like and AbaR4-like islands (Adams *et al*., 2008). While the AbaR3-like island was typically inserted in the *comM* gene, the AbaR4-like one was located at a different chromosomal locus.

2.7.2. AbaRs in EU clone II

Compared to EU clone I, the knowledge of AbaR islands in EU clone II is more limited although a number of AbaR variants have been described in this clone (Zhou *et al*., 2011; Kim *et al*., 2012; Nigro & Hall, 2012a; Seputine *et al*., 2012). This may result from the fact that the so far published studies on AbaRs from EU clone II strains dealt mostly with single structures while a compilation study attempting to draw a general conclusion from these scattered reports is still missing.

The common feature of AbaRs typically integrated in the *comM* gene of EU clone II strains is the presence of the backbone transposon Tn*6022* or its variants (Tn*6021*, Tn*6166*, Tn*6167*). Tn*6022* and its variants differ from each other in the presence of an insertion sequence in *orf1* and/or internal deletions (Figure 5) (Zhou *et al*., 2011; Nigro & Hall, 2012b). The first such structure was identified in EU clone I strain AB0057 out of the *comM* gene and termed AbaR4 (Adams *et al*., 2008). However, it was later revealed that structures related to AbaR4 (therefore termed AbaR4-like) are typically present in the *comM* gene of EU clone II strains (Zhou *et al*., 2011; Kim *et al*., 2012; Nigro & Hall, 2012a; Seputine *et al*., 2012).

In contrast to most AbaR3-like islands, AbaR4-like carry only a few resistance determinants. However, AbaR4-like islands typically harbour genes encoding a carbapenemase or ESBL (i.e. $bla_{\text{OXA-23}}$ or $bla_{\text{PER-1}}$, respectively) (Zhou *et al*., 2011; Kim *et al*., 2012; Nigro & Hall, 2012a; Seputine *et al*., 2012) which limit the current therapeutic options. Thus, AbaR4-like structures may have contributed to the recent global success of EU clone II. In addition, the IS*CR2* insertion sequence located in some AbaR4-like islands represents a powerful system, which can mobilize any section of DNA, and, consequently, be responsible for the mobilization of antibiotic resistance genes (Poirel *et al*., 2009; Seputine *et al*., 2012).

In general, as Tn*6019* of the AbaR3-like islands and Tn*6022* of the AbaR4-like islands are both associated with *comM* and found to be interrelated, a common progenitor of these structures has been suggested (Hamidian & Hall, 2011; Zhou *et al*., 2011; Seputine *et al*., 2012).

Table 4 shows an overview of the hitherto described and termed AbaR islands. Since the numbers of new AbaR variants have grown rapidly and because there is no accepted rule for their nomenclature, recently described islands (mostly those in EU clone II strains) have been left undesignated.

Table 4. AbaR-entitled islands in *A. baumannii*

AbaR	AbaR-type	Size (kb)	Strain	EU clone	*comM* insertion	Reference
AbaR1	AbaR3-like	86	AYE	I	+	Fournier *et al.,* 2006
AbaR2	AbaR3-like	17	ACICU	II	+	Iacono *et al.,* 2008
AbaR3	AbaR3-like	63	AB0057	I	+	Adams *et al.,* 2008
AbaR4	AbaR4-like	18	AB0057	I	-	Adams *et al.,* 2008
AbaR5	AbaR3-like	56	3208	I	+	Post & Hall, 2009
AbaR6	AbaR3-like	27	D2	I	+	Post *et al.,* 2010
AbaR7	AbaR3-like	20	A92	I	+	Post *et al.,* 2010
AbaR8	AbaR3-like	29	D13	I	+	Post *et al.,* 2010a
AbaR9	AbaR3-like	39	AB056	I	+	Adams *et al.,* 2010
AbaR10	AbaR3-like	31	AB058	I	+	Adams *et al.,* 2010
AbaR11	AbaR3-like	20	NIPH 470	I	+	Krizova *et al.,* 2011
AbaR12	AbaR3-like	38	LUH 6013	I	+	Krizova *et al.,* 2011
AbaR13	AbaR3-like	45	LUH 6015	I	+	Krizova *et al.,* 2011
AbaR14	AbaR3-like	21	LUH 5881	I	+	Krizova *et al.,* 2011
AbaR15	AbaR3-like	55	LUH 6125	I	+	Krizova *et al.,* 2011
AbaR16	AbaR3-like	39	LUH 7140	I	+	Krizova *et al.,* 2011
AbaR17	AbaR3-like	58	LUH 8592	I	+	Krizova *et al.,* 2011
AbaR18	AbaR3-like	52	NIPH 2713	I	+	Krizova *et al.,* 2011
AbaR19	AbaR3-like	30	NIPH 2554	I	+	Krizova *et al.,* 2011
AbaR20	AbaR3-like	42	NIPH 2665	I	+	Krizova *et al.,* unpublished
AbaR21	AbaR3-like	61	RUH 875	I	+	Nigro *et al.,* 2011
AbaR22	AbaR4-like	39	MDR-ZJ06	II	+	Zhou *et al.,* 2011
AbaR23	AbaR3-like	48	A424	NI	+	Kochar *et al.* , 2012
AbaR24	NI	NI	A1	I	NI	Hamidian & Hall, unpublished
AbaR25	AbaR4-like	47	K51-65	II	+	Saule *et al.,* 2013

NI - not identified

3. AIMS OF THE THESIS

This thesis addresses the following three questions related to the genetic basis and epidemiology of multidrug resistance in *A. baumannii*.

- The first aim was to assess the genetic basis and molecular epidemiology of carbapenem resistance in *Acinetobacter* spp. in the Czech Republic. This issue was motivated by the increase of carbapenem resistance among hospital *Acinetobacter* strains, which appeared in the early 2000s. In **Study I**, we investigated the emergence of carbapenem resistance using a collection of clinical isolates prospectively collected in 2005-2006. **Studies II** and **III** were aimed at the molecular characterization of a high-level carbapenem resistant *A. baumannii* strain isolated in a Czech hospital in 2011.

- The second aim (addressed in **Study IV**) was to test the hypothesis that β-lactamase TEM-1 is responsible for sulbactam resistance in *A. baumannii*. The starting point of this study was the fact that although the resistance of acinetobacters to this agent had been known for more than 30 years, virtually nothing was known about the molecular basis of sulbactam resistance in this organism.

- The last aim (addressed in **Studies V** and **VI**) was to obtain insight into the structural diversity of AbaR islands in the *A. baumannii* population of EU clone I in order to determine whether these structures were responsible for the heterogeneity of resistance determinants in this multidrug resistant lineage. This issue was initiated by the discovery of an 86-kb resistance island (AbaR1) in a MDR strain of EU clone I, which was published in 2006 and opened a new perspective on the understanding of the evolution of antibiotic resistance in *A. baumannii*.

4. RESULTS

Study I

Nemec A, Krizova L, Diancourt L, Maixnerová 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. *J Antimicrob Chemother* 2008; 62: 484-9.

Study II

Krizova L, Bonnin RA, Nordmann P, Nemec A, Poirel L. Characterization of a multidrug-resistant *Acinetobacter baumannii* strain carrying the $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-23}}$ carbapenemase genes from the Czech Republic. *J Antimicrob Chemother* 2012; 67:1550-2.

Study III

Nemec A, Krizova L. Carbapenem-resistant *Acinetobacter baumannii* carrying the NDM-1 gene, Czech Republic, 2011. *Euro Surveill* 2012; 15: 17(11).

Study IV

Krizova L, Poirel L, Nordmann P, Nemec A. TEM-1 β-lactamase as a source of resistance to sulbactam in clinical strains of *Acinetobacter baumannii*. *J Antimicrob Chemother* 2013; 68: 2786-91.

Study V

Krizova L, Nemec A. A 63 kb genomic resistance island found in a multidrug-resistant *Acinetobacter baumannii* isolate of European clone I from 1977. *J Antimicrob Chemother* 2010; 65:1915-18.

Study VI

Krizova L, Dijkshoorn L, Nemec A. Diversity and evolution of AbaR genomic resistance islands in *Acinetobacter baumannii* strains of European clone I. *Antimicrob Agents Chemother* 2011; 55: 3201-06.

Study I

Nemec A, Krizova L, Diancourt L, Maixnerová 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. *J Antimicrob Chemother* 2008; 62: 484-9.

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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 (≤ 4 mg/L), respectively. Carbapenem MICs of ≥ 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 ≤ 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),

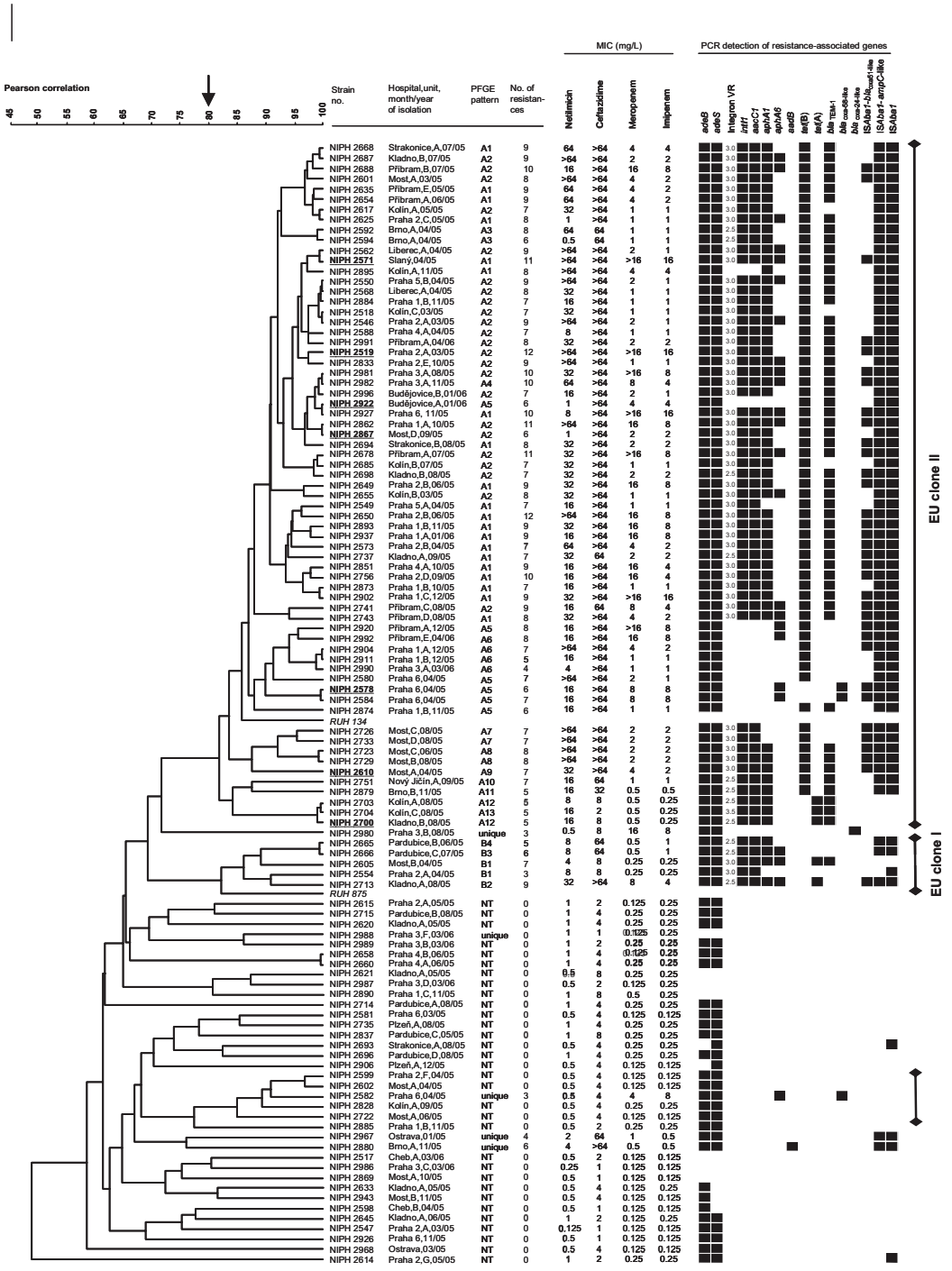

Figure 1. Dendrogram of cluster analysis of AFLP fingerprints of 108 *A. baumannii* isolates in the present study. Clusters of more than two isolates defined at 80% are marked by vertical lines. Numbers following the city name indicate different hospitals in the city; capitals denote different ICUs in 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.

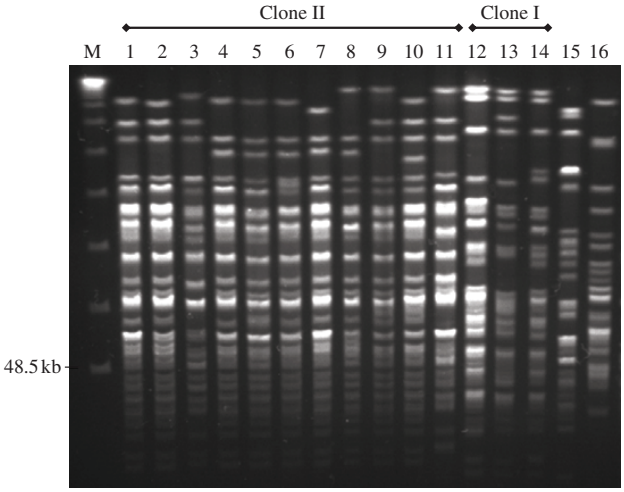

Figure 2. Examples of the *Apa*I macrorestriction patterns of *A. baumannii* isolates. Strains are indicated by the numbers above the lanes: 1, NIPH 2571; 2, NIPH 2895; 3, NIPH 2700; 4, NIPH 2519; 5, NIPH 2981; 6, NIPH 2867; 7, NIPH 2982; 8, NIPH 2990; 9, NIPH 2874; 10, NIPH 2610; 11, NIPH 2578; 12, 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 $\sim$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 $\sim$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)$_{1-3}$-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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Study II

Krizova L, Bonnin RA, Nordmann P, Nemec A, Poirel L. Characterization of a multidrug-resistant *Acinetobacter baumannii* strain carrying the $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-23}}$ carbapenemase genes from the Czech Republic. *J Antimicrob Chemother* 2012; 67:1550-2.

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Characterization of a multidrug-resistant *Acinetobacter baumannii* strain carrying the bla_{NDM-1} and bla_{OXA-23} carbapenemase genes from the Czech Republic

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Keywords: metallo-β-lactamases, Tn*125*, Tn*2008*, European clone I

Sir,

Since its discovery in 2008, the metallo-β-lactamase (MBL) NDM-1 has been identified in different Enterobacteriaceae species and recently also in a number of other bacterial species isolated from water supplies in India, such as *Vibrio cholerae* and *Pseudomonas* spp.[1] Genes encoding NDM β-lactamases have been additionally identified in multidrug-resistant *Acinetobacter* spp.[2] Here, we report on the identification and characterization of an *Acinetobacter baumannii* strain carrying the bla_{NDM-1} gene that was isolated in the Czech Republic in 2011.

A. baumannii strain ANC 4097 was isolated in mid-2011 during a prospective, nationwide study in the Czech Republic focused on the *Acinetobacter* population structure and resistance mechanisms. It was recovered from the sputum of an elderly patient hospitalized in an intensive care unit (ICU) of a hospital located in the north-western part of the Czech Republic. The patient, who had a malignant tumour, died of complications of the underlying disease several days after the isolation of the strain.

The MICs of β-lactams for ANC 4097 determined using Etest (bioMérieux, Solna, Sweden)[3] showed high-level resistance to all β-lactams, including carbapenems (MICs of both imipenem and meropenem >32 mg/L) and broad-spectrum cephalosporins (MICs of both ceftazidime and cefepime >256 mg/L), as well as penicillins in combination with inhibitors (MICs of ampicillin/sulbactam and piperacillin/tazobactam >256 mg/L). The strain was also resistant to fluoroquinolones (MIC of ciprofloxacin >32 mg/L), aminoglycosides (MIC of amikacin >64 mg/L and MIC of gentamicin 32 mg/L) and tetracycline (MIC 128 mg/L). An MBL Etest (bioMérieux) revealed a 24-fold reduction of imipenem MIC when combined with EDTA, which suggested production of an MBL. Isolate ANC 4097 remained susceptible only to tigecycline (MIC 2 mg/L), doxycycline (MIC 2 mg/L), netilmicin (MIC 4 mg/L), tobramycin (MIC 0.5 mg/L) and colistin (MIC 0.25 mg/L).

Strain ANC 4097 was genotyped by multilocus sequence typing (MLST) using the Pasteur Institute scheme (http://www.pasteur.fr/recherche/genopole/PF8/mlst/). It belongs to sequence type (ST) 1 (allelic profile 1-1-1-1-5-1-1), which is typical of the European clone I epidemic lineage.[4] PCR screening followed by sequencing of resistance determinants[2–4] revealed that the strain harboured genes encoding five β-lactamases: NDM-1, class D carbapenemases OXA-23 (acquired) and OXA-69 (intrinsic), acquired narrow-spectrum class A β-lactamase TEM-1 and intrinsic AmpC-type β-lactamase ADC. This strain co-harboured the *tetA*(A) tetracycline resistance gene and the *aacC1*, *aphA1*, *aphA6* and *aadA1* aminoglycoside resistance genes, but no *armA* 16S RNA methylase gene was identified. Insertion sequence IS*Aba1* was detected upstream of each of bla_{OXA-23}, bla_{OXA-69} and bla_{ADC}, thus providing strong promoter sequences likely to enhance the expression of these genes in ANC 4097.[3] In addition, the *comM* gene (encoding a polypeptide containing an ATPase domain) was found to be disrupted, which, together with the presence of the bla_{TEM-1}, *aacC1*, *aphA1*, *aadA1* and *tetA*(A) genes, suggested the presence of an AbaR3-like genomic resistance island typical of the European clone I lineage.[5]

Genetic structures associated with the bla_{OXA-23} and bla_{NDM-1} genes were analysed using PCR mapping and sequencing as described elsewhere.[2,5] The bla_{NDM-1} gene was located in the previously characterized composite transposon Tn*125* (two copies of IS*Aba125* bracketing a 7925 bp region),[2] while the bla_{OXA-23} gene was found to be part of transposon Tn*2008*.[4]

In order to determine the genetic location of the bla_{OXA-23} and bla_{NDM-1} genes, plasmid DNA of ANC 4097 was isolated as described previously.[6] Transfer of the ticarcillin resistance marker was attempted by both electroporation of the ANC 4097 plasmid suspension into *A. baumannii* BM4547 and liquid mating-out assays of the ANC 4097 and BM4547 strains at 37°C.[6] Selection was performed on agar plates supplemented with ticarcillin (100 mg/L). Transformants or transconjugants harbouring the bla_{NDM-1} and bla_{OXA-23} genes were not obtained, suggesting a chromosomal location of both genes, as described previously.[2,4]

This study reports on the first bla_{NDM-1}-positive *A. baumannii* strain in the Czech Republic and adds to the body of evidence of the current spread of multidrug-resistant *Acinetobacter* harbouring this MBL in Europe.

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Transparency declarations

None to declare.

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Study III

Nemec A, Krizova L. Carbapenem-resistant *Acinetobacter baumannii* carrying the NDM-1 gene, Czech Republic, 2011. *Euro Surveill* 2012; 15: 17(11).

Carbapenem-resistant *Acinetobacter baumannii* carrying the NDM-1 gene, Czech Republic, 2011

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To the editor: We read with interest the paper of Hrabák et al. [1] on the isolation of a bla_{NDM-1}-carrying *Acinetobacter baumannii* strain. However, although the authors believed to be the first in the Czech Republic to report on a New Delhi metallo-beta-lactamase-1 (NDM-1)-producing bacterium, there have been other reports on the same *A. baumannii* strain in the country [2,3]. Here, we would like to comment on some points of these studies.

In our report published in a Czech national bulletin in September 2011 [2], we presented epidemiological and microbiological data on a high-level carbapenem-resistant strain (designated ANC 4097) isolated from a patient hospitalised in an intensive care unit in the Czech Republic. Based on the available information, we concluded that the strain had been imported to the Czech Republic from Egypt in mid-2011 [2]. The strain was shown to be resistant in vitro to all beta-lactams and to most other clinically relevant antimicrobial agents, and to carry the genes encoding the NDM-1 and OXA-23 carbapenemases together with a number of other resistance determinants [2]. In a later paper, we provided additional genetic characterisation of ANC 4097 focused on the genetic structures associated with the bla_{NDM-1} and bla_{OXA-23} genes [3]. We have only recently learned that the laboratory where the original clinical specimens were processed had provided the same bacterial strain to two research groups. Thus, two independent investigations on the same strain were conducted leading to our papers [2,3] and that of Hrabák et al. [1].

Even though the data on the NDM-1 carrying strain presented in the independent studies are mostly congruent, some findings and conclusions by Hrabák et al. [1] deserve a commentary. Firstly, Hrabák et al. claimed that the strain was a producer of the NDM-1 carbapenemase based on the presence of the bla_{NDM-1} gene, carbapenemase activity, and the inhibitory effect of ethylenediaminetetraacetic acid (EDTA) on the carbapenem resistance phenotype. However, ANC 4097 was also shown by us to harbour the bla_{OXA-23} and bla_{OXA51}-like genes, both carrying IS*Aba1* in their promoter regions [2,3]. Therefore, the strain may produce at least three different carbapenemases, each of which can be responsible for the carbapenem resistance. As EDTA inhibition of carbapenemase activity is not a specific marker to detect the metallo-beta-lactamase production in *A. baumannii* [4,5], unambiguous evidence that ANC 4097 is a genuine producer of NDM-1 (and not only a carrier of a silent bla_{NDM-1} gene) is still missing.

Secondly, the minimum inhibitory concentration (MIC) for chloramphenicol (8 mg/L) reported by Hrabák et al. was surprisingly low seeing as *A. baumannii* is typically resistant to this antibiotic. In contrast, we found a chloramphenicol MIC of ≥256 µg/mL in ANC 4097 using Etest (bioMérieux), and the strain yielded a positive PCR signal for the *catA1* gene encoding chloramphenicol acetyltransferase (unpublished data). It is of note that the catA1 gene is part of the AbaR3 resistance island which, or variants of which, are commonly present in *A. baumannii* European clone I to which ANC 4097 belongs [3].

Finally, although Hrabák et al. [1] have reported their isolates to be susceptible only to colistin, we found that ANC 4097 was also susceptible to at least tobramycin and doxycycline [2,3]. Even though these antimicrobials may have limited value in the treatment of systemic *Acinetobacter* infections they have been recommended for consideration when defining the level of multidrug resistance in *A. baumannii* for epidemiological purposes [6].

Despite these points, the epidemiological part of the report of Hrabák et al. [1] has valuably contributed to the comprehensiveness of the information on the first bacterial strain with the bla_{NDM-1} gene isolated in the Czech Republic.

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Study IV

Krizova L, Poirel L, Nordmann P, Nemec A. TEM-1 β-lactamase as a source of resistance to sulbactam in clinical strains of *Acinetobacter baumannii*. *J Antimicrob Chemother* 2013; 68: 2786-91.

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TEM-1 β-lactamase as a source of resistance to sulbactam in clinical strains of *Acinetobacter baumannii*

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Objectives: Sulbactam is well known to have clinically relevant intrinsic activity against *Acinetobacter baumannii*. Although secondary resistance to this drug has long been reported in acinetobacters, virtually nothing is known about its molecular basis. The aim of this study was to test the hypothesis that β-lactamase TEM-1 is responsible for sulbactam resistance in *A. baumannii*.

Methods: Seventeen clinical strains of *A. baumannii* were selected to represent different combinations of quantitative susceptibilities to sulbactam and molecular typing characteristics. The strains were screened by PCR for the presence of the bla_{TEM-1} gene and its variants. Amplicons encompassing the bla_{TEM} genes, including their promoters, were sequenced. The expression and copy number of the bla_{TEM} genes were assessed using semi-quantitative real-time PCR. Transfer of the bla_{TEM-1} gene into a susceptible *A. baumannii* strain was achieved by electroporation.

Results: Six strains were negative for the bla_{TEM} gene and had sulbactam MICs of 0.5–1.0 mg/L, 10 strains harboured bla_{TEM-1} and showed MICs ≥8.0 mg/L, except for one strain with an MIC of 2 mg/L, while the remaining strain carried bla_{TEM-19} and had an MIC of 1 mg/L. The level of bla_{TEM-1} expression positively correlated with the MICs of sulbactam ($r=0.92$). Promoter P4 was linked to the bla_{TEM} gene in all strains except for a P3-carrying strain (an MIC of 2 mg/L). Transformation of the susceptible *A. baumannii* strain with bla_{TEM-1} resulted in a 64-fold increase in sulbactam MIC and in resistance to ticarcillin and piperacillin, but no change in susceptibility to broad-spectrum generation cephalosporins, aztreonam or carbapenems.

Conclusions: The results presented suggest that TEM-1 represents a clinically relevant mechanism of sulbactam resistance in *A. baumannii*.

Keywords: multidrug resistance, transformation, gene expression, mechanisms of resistance

Introduction

Acinetobacter baumannii is an important nosocomial pathogen that mostly affects patients with serious underlying diseases.[1] This bacterium has a remarkable capability to acquire and develop multiple cellular mechanisms that confer resistance to virtually all antimicrobial agents applicable to *Acinetobacter* infections. Until recently, carbapenems were considered to be the gold standard in the treatment of infections caused by multidrug-resistant *A. baumannii* (MDRAB) strains. However, the rate of carbapenem resistance among MDRAB strains has dramatically increased worldwide since the early 2000s, resulting in a need to search for other therapeutic strategies.[2] Sulbactam is one of the few antimicrobial agents that are currently considered a plausible option to combat MDRAB infections.[1,3] Although this β-lactam has been primarily known as an inhibitor of Ambler class A penicillinases, it also has intrinsic and clinically relevant antimicrobial activity against *Acinetobacter* spp.[4,5] Previous studies have shown that sulbactam (given alone or in a commercially available combination with either ampicillin or cefoperazone) retains *in vitro* activity against a substantial number of MDRAB strains including carbapenem-resistant and colistin-resistant isolates.[6–8]

In contrast to the other antimicrobial agents relevant for the therapy of *A. baumannii* infections, virtually nothing is known about the molecular basis of resistance to sulbactam. This is rather surprising considering the ongoing discussion on the potential of sulbactam to combat MDRAB[3] and the fact that the resistance of acinetobacters to this drug has been known for more

than 30 years.[9] To our knowledge, no published studies have addressed the issue of the mechanism of sulbactam resistance, although Joly-Guillou *et al.*[10] reported as long ago as 1995 that TEM-1-producing *Acinetobacter* strains showed increased MICs of sulbactam compared with penicillinase-negative strains. Notably, several recent studies have found high proportions of bla_{TEM-1}-carrying isolates among MDRAB strains.[11,12] The inspection of *in vitro* antibiotic susceptibilities and genotypic data in our collection of *A. baumannii* strains collected over the last two decades revealed a strong association between the presence of the gene encoding TEM-1 and sulbactam resistance. Based on these findings, we designed the present study to test the hypothesis that TEM-1 is responsible for resistance to sulbactam in *A. baumannii*.

Methods

Strains and their genotypic characteristics

The 17 clinical *A. baumannii* strains investigated in the present study are listed in Table 1. These were selected to represent different combinations of quantitative susceptibilities to sulbactam and molecular typing characteristics as revealed by multilocus sequence typing (MLST)[13] and by ApaI macrorestriction profiling of genomic DNA.[12] Based on MLST, the strains were classified into the following sequence types (STs): ST1, ST7 (both of these belonging to clonal complex CC1/EU clone I), ST2 (EU clone II), ST3 (EU clone III), ST39, ST46 and ST52 (Table 1). The strains belonging to one of two main lineages (EU clone I and II) differed from each other in their ApaI patterns except for two pairs (NIPH 2862/NIPH 2884 and NIPH 2873/ANC 3943), each pair including EU clone II strains with identical ApaI patterns. Strains NIPH 2862 and NIPH 2884 were collected in one hospital over the same period of time but clearly differed in their sulbactam MICs (Table 1). Strain ANC 3943 (representative of a carbapenem-resistant *A. baumannii* population currently prevailing in the Czech Republic) differed from NIPH 2873 only in carbapenem MIC (Table 1) and the presence of IS*Aba1* upstream of the bla_{OXA-51} gene. All 17 strains were PCR-negative for the presence of the genes encoding the following β-lactamases known to occur in *A. baumannii*: NDM, VIM, IMP, VEB-1, PER-1, OXA-10, OXA-23-like, OXA-40-like, OXA-58-like, OXA-235-like and OXA-143-like (for primers and references, see Table S1, available as Supplementary data at *JAC* Online). Only strains NIPH 2862, ANC 3943 and ANC 4030 harboured IS*Aba1* upstream of the bla_{OXA-51}-like gene, which is in accordance with their increased carbapenem MICs (Table 1).

Susceptibility testing

Susceptibilities to nine β-lactam agents or their combinations (Table 1), which are primarily effective against *A. baumannii*, were assayed by Etest (bioMérieux, Marcy l'Étoile, France) using Mueller–Hinton II agar (BBL, BD, USA) and following the manufacturer's guidelines and the CLSI criteria for interpretation[17] (Table 1). As clinical breakpoints for sulbactam are not available, we used a provisional susceptibility breakpoint of ≤4 mg/L derived from the CLSI breakpoint for ampicillin/sulbactam (≤8/4 mg/L).[17]

PCR and sequencing

Genes encoding TEM-1 or its variants (hereafter termed bla_{TEM}) were amplified by PCR using primers C1 and C2 as previously described.[12] For the bla_{TEM}-positive strains, a second PCR to amplify the entire gene including the promoter region was carried out using primers TEM prom F and R.[18] Amplicons were sequenced using primers TEM prom F and R and a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). DNA sequences were obtained on an ABI 3130xl genetic analyser (Applied Biosystems) and analysed using KODON software (Applied Maths, St-Martens-Latem, Belgium).

Gene copy number assay

To assess differences between the number of the bla_{TEM} genes in bla_{TEM}-positive strains, a copy number assay based on quantitative PCR (qPCR) was performed using primer pairs targeting the bla_{TEM-1} gene (RT TEM-F, 5′-TTTTTGCGGCATTTTGCCTT-3′; RT TEM-R, 5′-GGGCGAAAACTCTCAAGG ATC-3′) and the *rpoB* (housekeeping) gene as previously described.[19] To prepare a DNA standard for qPCR, the *rpoB* amplicon was cloned into the pCR plasmid using a TOPO TA Cloning Kit (Invitrogen). The resulting recombinant plasmid pCR (*rpoB*) harboured a single copy of bla_{TEM-1} and a 902 bp fragment of *rpoB*. Dilutions of pCR (*rpoB*) over the range 8.6×10^{-1}–2.6×10^{4} copies/μL were used as DNA templates to generate standard curves. qPCR was performed using a C1000 Real-Time system (Bio-Rad) and QuantiTect SYBR Green (Qiagen, Hilden, Germany) according to the manufacturer's guidelines. Real-time cycling parameters for both genes were: an initial hot start of 95°C for 5 min and then 40 cycles of 95°C for 15 s, followed by both annealing and extension at 55°C for 1 min. The relative copy number difference was determined using the $2^{-\Delta\Delta CT}$ calculation as previously described.[20]

Semi-quantitative reverse transcription PCR (qRT–PCR)

For qRT–PCR, cells of the bla_{TEM}-positive strains were cultured in Luria–Bertani (LB) broth until mid-log phase [optical density measured at 600 nm ($OD_{600}=0.5$)], harvested by brief centrifugation and resuspended in the RNAprotect medium (Qiagen). Extraction of total RNA and reverse transcription were performed as previously described.[21] To detect transcripts of the bla_{TEM} and *rpoB* genes, the same primers as those in the gene copy number assay were used. Expression was monitored using real-time PCR as described above. Triplicate samples and non-template and non-reverse transcriptase controls were included in each run. All qRT–PCRs were performed three times. Dissociation curves were obtained to identify the PCR products. Expression of the bla_{TEM} gene was quantified relative to that of the *rpoB* gene, and the relative expression was calculated as previously described.[22]

Transformation with the bla_{TEM-1} gene

The recombinant plasmid pAT801 harbouring the entire bla_{TEM-1} gene with the P3 type promoter[23] was extracted from a donor *Escherichia coli* strain using a Plasmid Mini Kit (Qiagen) and transformed into both *E. coli* Top10 and the sulbactam-susceptible, bla_{TEM-1}-negative *A. baumannii* strain NIPH 56 (Table 1 and Table S2, available as Supplementary data at *JAC* Online) by electroporation. Transformants were selected onto LB agar plates containing 100 mg/L ticarcillin after overnight incubation at 37°C and tested for the bla_{TEM-1} gene by PCR and for susceptibilities to β-lactams. The presence of pAT801 in the transformants was assayed by plasmid analysis as previously described.[24]

GenBank accession number

The sequence of the bla_{TEM-19} gene was deposited in the GenBank database under accession number JX042489.

Results and discussion

To test the hypothesis that the TEM-1 β-lactamase is responsible for sulbactam resistance in *A. baumannii*, we first explored the relationship between sulbactam susceptibility and the presence of the bla_{TEM-1} gene in the 17 clinical strains. The MICs of sulbactam

Table 1. Characteristics of the *A. baumannii* strains studied

Strain	City, country, year of isolation	Specimen	ST[a] (EU clone)	ApaI profile[b]	bla_{TEM}	bla_{TEM} promoter	Relative expression of bla_{TEM}[c]	Copy number of bla_{TEM}[d]	MICs (mg/L)[e]									reference
									SUL	SAM	CAZ	CTX	FEP	IPM	MEM	PIP	TIM	
ANC 4030	Prague, Czech Republic, 2011	urine	2 (EU II)	1	bla_{TEM-1}	P4	110.83±0.17	6.02±0.23	>128	>128	128	>256	256	>32	>32	>256	>256	NP
NIPH 1605	Sedlčany, Czech Republic, 2001	urine	1 (EU I)	2	bla_{TEM-1}	P4	16.52±0.32	0.88±0.01	16	16	4	16	16	1	0.25	256	>256	14,16
HK302	Zurich, Switzerland, 1977	respiratory tract	1 (EU I)	3	bla_{TEM-1}	P4	18.8±0.38	1.07±0.07	16	16	2	8	64	0.25	0.5	>256	>256	15
NIPH 7	Prague, Czech Republic, 1991	burn	7 (EU I)	4	bla_{TEM-1}	P4	16.5±0.24	0.96±0.05	16	16	8	16	32	1	0.5	>256	>256	13
ANC 3943	Prague, Czech Republic, 2011	sputum	2 (EU II)	5	bla_{TEM-1}	P4	12.2±0.15	0.64±0.02	8	16	128	>256	256	≥32	≥32	>256	>256	NP
NIPH 2873	Prague, Czech Republic, 2005	wound	2 (EU II)	5	bla_{TEM-1}	P4	13.94±0.13	0.5±0.08	8	16	128	>256	128	1	1	>256	>256	12
NIPH 2862	Prague, Czech Republic, 2005	sputum	2 (EU II)	6	bla_{TEM-1}	P4	21.73±0.20	0.5±0.11	8	16	128	>256	256	8	16	>256	>256	12
NIPH 10	Prague, Czech Republic, 1991	blood	1 (EU I)	7	bla_{TEM-1}	P4	10.77±0.57	1.19±0.12	8	16	16	32	>256	2	2	256	256	13
NIPH 24	Prague, Czech Republic, 1991	urinary catheter	2 (EU II)	8	bla_{TEM-1}	P4	14.77±0.25	0.65±0.14	8	16	8	16	32	1	1	256	256	13
NIPH 1717	Prague, Czech Republic, 2001	urine	46	9	bla_{TEM-1}	P3	8.27±0.21	1.5±0.01	2	4	8	32	64	1	1	128	≥256	14
NIPH 2884	Prague, Czech Republic, 2005	urine	2 (EU II)	6	bla_{TEM-19}	P4	6.09±0.17	0.76±0.04	1	2	256	>256	64	2	1	>256	64	12
NIPH 1815	Genoa, Italy, 1998	respiratory tract	3 (EU III)	10	none	none	NT	NT	1	1	16	128	256	2	2	64	32	13
NIPH 657	Prague, Czech Republic, 1996	tracheostomy	2 (EU II)	11	none	none	NT	NT	1	2	16	256	32	2	1	>256	>256	13
NIPH 2554	Prague, Czech Republic, 2005	sputum	1 (EU I)	12	none	none	NT	NT	0.5	1	8	32	32	0.25	0.25	8	8	12,16
NIPH 410	Brno, Czech Republic, 1996	cannula	39	13	none	none	NT	NT	0.5	1	2	4	2	0.5	0.25	4	2	13
ATCC 19606^T	Not known, before 1949	urine	52	14	none	none	NT	NT	0.5	1	8	32	64	1	1	16	8	13
NIPH 56	Prague, Czech Republic, 1992	burn	1 (EU I)	15	none	none	NT	NT	0.5	1	4	8	8	1	0.25	8	8	13,16
NIPH 56 (pAT801)[f]					bla_{TEM-1}	P3	73.17±0.25	99±0.18	32	64	4	8	8	1	0.5	>256	>256	NP

NT, not tested; NP, not published.
[a]STs based on the Institute Pasteur MLST scheme (www.pasteur.fr/mlst).
[b]ApaI macrorestiction profiles of genomic DNA.
[c]Expression of bla_{TEM} genes normalized to *rpoB* expression.
[d]Copy number of bla_{TEM} transcripts normalized to *rpoB* transcripts.
[e]Antibiotic abbreviations and breakpoints (mg/L) according to the CLSI:[17] SUL, sulbactam; SAM, ampicillin/sulbactam (2:1), S (≤8/4), R (≥32/16); CAZ, ceftazidime, S (≤8), R (≥32); CTX, cefotaxime, S (≤8), R (≥32); FEP, cefepime, S (≤8), R (≥32); IPM, imipenem, S (≤4), R (≥16); MEM, meropenem, S (≤4), R (≥16); PIP, piperacillin, S (≤16), R (≥128); TIM, ticarcillin/clavulanic acid, S (≤16/2), R (≥128/2).
[f]NIPH 56 transformed by plasmid pAT801 harbouring the bla_{TEM-1} gene.

and other β-lactams for these strains are shown in Table 1. Eight strains were susceptible (≤4 mg/L) to sulbactam, while five and four strains showed sulbactam MICs of 8 mg/L and ≥16 mg/L, respectively. The susceptibility value to ampicillin/sulbactam (2:1) corresponded to that observed for sulbactam alone in each strain (Table 1), which confirms that sulbactam is responsible for the effect of this drug combination against *A. baumannii*. PCR detection of the genes encoding TEM-1 or its variants revealed an amplicon in all strains with a sulbactam MIC ≥ 8 mg/L. In addition, amplicons were produced by NIPH 1717 and NIPH 2884, with a sulbactam MIC of 2 mg/L and 1 mg/L, respectively. Sequencing of the amplicons showed the presence of the $bla_{\text{TEM-1}}$ gene in all strains, except NIPH 2884, in which the $bla_{\text{TEM-19}}$ gene was identified (GenBank accession no. JX042489). Thus, with the exception of strain NIPH 1717, the presence of the $bla_{\text{TEM-1}}$ gene was associated with either resistance or intermediate susceptibility to sulbactam, while the absence of this gene correlated with sulbactam susceptibility.

To assess the relationship between the level of sulbactam resistance and bla_{TEM} expression at the transcriptional level, qRT–PCR was carried out for the 11 $bla_{\text{TEM-1/TEM-19}}$-positive strains. The results shown in Table 1 indicate that the bla_{TEM} genes were expressed in all strains. Moreover, there was a positive correlation between $bla_{\text{TEM-1}}$ gene expression and MICs of sulbactam, as indicated by a Pearson's correlation coefficient value of 0.92 between the values ($\log_2$) of the relative expression of $bla_{\text{TEM-1}}$ and the corresponding sulbactam MICs ($\log_2$) calculated for the $bla_{\text{TEM-1}}$-positive strains.

The copy number of the bla_{TEM} genes was further assessed as a parameter potentially influencing the expression of the genes (Table 1). In all but one strain, the copy number of the $bla_{\text{TEM-1/TEM-19}}$ genes relative to the *rpoB* housekeeping gene ranged from 0.5 to 1.5, which estimates that one copy of bla_{TEM} was present in the genome of each of these strains. This finding is in accordance with recent data indicating that a single chromosomal copy of the $bla_{\text{TEM-1}}$ gene is commonly present in the *A. baumannii* genome. For example, EU clone I strains were shown to carry typically one copy of this gene located in the AbaR3 resistance island.[15,16,25] In line with this, all $bla_{\text{TEM-1}}$-positive EU clone I strains included in the present study (NIPH 7, NIPH 10, NIPH 1605 and HK302) were calculated to have $bla_{\text{TEM-1}}$ copy numbers ranging from 0.88 to 1.19 (Table 1). Strain ANC 4030 (EU clone II) was the only one with a significantly higher $bla_{\text{TEM-1}}$ copy number (6.02). Compared with the other strains, ANC 4030 also showed a markedly higher relative expression level of the $bla_{\text{TEM-1}}$ gene (110.83) and a high sulbactam MIC (>128 mg/L). Moreover, the normalized relative expression (per copy of the $bla_{\text{TEM-1}}$ gene) was 18.4 for ANC 4030, which falls into the range of values found for the other $bla_{\text{TEM-1}}$-positive strains (8.27–21.73). Thus, multiple copies of $bla_{\text{TEM-1}}$ are likely to contribute to the high level of sulbactam resistance in ANC 4030.

Different levels of TEM-1 activity have been associated with distinct promoter sequences in *E. coli*.[26] We therefore examined the possible influence of promoter type on the level of sulbactam resistance and on the expression of the bla_{TEM} genes in the $bla_{\text{TEM-1/TEM-19}}$-positive strains. Two promoter types were found to be linked to the $bla_{\text{TEM-1/TEM-19}}$ genes: promoter P3 in one strain (NIPH 1717) and promoter P4 in the other 10 strains (Table 1). Compared with P3, P4 has been previously reported to be associated with up to 32-fold increases in MICs of β-lactams for *E. coli*.[26] Notably, our P3-carrying strain had the lowest sulbactam MIC (2 mg/L) of the $bla_{\text{TEM-1}}$-positive strains, although we did not find significant differences in the relative expression of $bla_{\text{TEM-1}}$ between NIPH 1717 and the other $bla_{\text{TEM-1}}$-positive strains.

To evaluate experimentally the involvement of TEM-1 production in sulbactam resistance, the sulbactam-susceptible strains *A. baumannii* NIPH 56 and *E. coli* Top10 were transformed by electroporation with the pAT801 plasmid harbouring the $bla_{\text{TEM-1}}$ gene. Transformants were successfully obtained, as evidenced by the detection of the $bla_{\text{TEM-1}}$ gene and plasmid pAT801 by PCR and plasmid analysis, respectively, in the ticarcillin-resistant derivatives of the parental strains. In the transformed strains *A. baumannii* NIPH 56 (pAT801) and *E. coli* Top10 (pAT801), sulbactam MICs increased from 0.5 mg/L to 32 mg/L and from 16 mg/L to ≥256 mg/L, respectively. *A. baumannii* NIPH 56 (pAT801) also acquired resistance to ticarcillin, ticarcillin/clavulanate and piperacillin, but no change in susceptibility values was recorded for piperacillin/tazobactam, ceftazidime, cefotaxime, cefepime, imipenem, meropenem and aztreonam as evidenced by Etest and/or disc diffusion (Tables 1 and Table S2, available as Supplementary data at *JAC* Online). Notably, a more than 32-fold increase in the MIC of ticarcillin/clavulanate was observed for *A. baumannii* NIPH 56 (pAT801) compared with NIPH 56, indicating that clavulanate did not significantly inhibit the activity of TEM-1. Previous studies have shown that a high-level expression of the $bla_{\text{TEM-1}}$ gene at the transcriptional level resulted in the overproduction of TEM-1 and consequently in clavulanate resistance in *E. coli*.[27,28] In line with this, the overexpression of the $bla_{\text{TEM-1}}$ gene in *A. baumannii* NIPH 56 (pAT801) was supported by the high copy number and relative expression values of the gene (Table 1).

The finding of the $bla_{\text{TEM-19}}$ gene in one of the strains studied deserves comment. To our knowledge, this gene has not yet been reported to occur in *A. baumannii*. The $bla_{\text{TEM-19}}$ gene differs from $bla_{\text{TEM-1}}$ by a single nucleotide (706G>A), leading to an amino acid change.[29] This change takes place in the enzyme catalytic domain[30] and may lead to a more than 64-fold decrease in hydrolytic activity against sulbactam in *E. coli*.[31] As we found the $bla_{\text{TEM-19}}$ gene in *A. baumannii* NIPH 2884, which was genotypically closely related and epidemiologically linked to *A. baumannii* NIPH 2862 harbouring the $bla_{\text{TEM-1}}$ gene, the conversion of $bla_{\text{TEM-1}}$ into the $bla_{\text{TEM-19}}$ gene may have occurred during the in-hospital spread of the bacterium. The sulbactam MIC for NIPH 2884 (1 mg/L) was 8-fold lower than that for NIPH 2862, suggesting that the shift from $bla_{\text{TEM-1}}$ to $bla_{\text{TEM-19}}$ may have restored clinical susceptibility to this antibiotic.

Besides its intrinsic antibiotic activity against acinetobacters, sulbactam acts as a time-dependent irreversible inhibitor protecting β-lactams from hydrolysis by various serine β-lactamases, including TEM-1. It has been suggested that TEM-1 first hydrolyses ∼7000 sulbactam molecules and then sulbactam functions as an irreversible inhibitor of the enzyme.[32,33] Furthermore, the concentration inhibiting 50% of the TEM-1 activity for sulbactam was shown to be much higher (900 nM) than those for clavulanate and tazobactam (60 nM and 97 nM, respectively). More specifically, ∼10 000 molecules of sulbactam are needed to inactivate a single TEM-1 molecule.[33] Therefore, it is likely that TEM-1 effectively protects *Acinetobacter* spp. cells against sulbactam unless the drug reaches concentrations that inhibit the enzyme activity.

In conclusion, our findings are congruent with the assumption that the production of TEM-1 results in sulbactam resistance or non-susceptibility in *A. baumannii*. This is especially supported by

the correlation between the level of sulbactam resistance and the expression of the bla_{TEM-1} gene, by the transferability of sulbactam resistance via a bla_{TEM-1}-carrying plasmid, and by the susceptibility of a clinical strain expressing TEM-19, a low-activity variant of TEM-1. Although other mechanisms of resistance to sulbactam are expected to occur in acinetobacters, the high prevalence of TEM-1 producers among MDRAB strains makes this particular mechanism of major clinical importance.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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Table S1. PCR primers used for the detection of β-lactamase genes

Gene/locus	Primer name	Primer sequence 5'→3'	Reference
IS*Aba1*	ISAba1F	CACGAATGCAGAAGTTG	Segal H *et al. FEMS Microbiol Lett* 2005; **243**: 425-9.
	ISAba1R	CGACGAATACTATGACAC	
bla$_{\text{OXA-51-like}}$	OXA51-likeF	ATGAACATTAAAGCACTC	Turton JF *et al. FEMS Microbiol Lett* 2006; **258**: 72-7.
	OXA51-likeR	CTATAAAATACCTAATTGTTC	
bla$_{\text{OXA-23-like}}$	OXA23-likeF	GATCGGATTGGAGAACCAGA	Woodford N *et al*. *J Antimicrob Agents* 2006; **27**: 351-3.
	OXA23-likeR	ATTTCTGACCGCATTTCCAT	
bla$_{\text{OXA-40-like}}$	OXA24-likeF	GGTTAGTTGGCCCCCTTAAA	Woodford N *et al*. *J Antimicrob Agents* 2006; **27**: 351-3.
	OXA24-likeR	AGTTGAGCGAAAAGGGGATT	
bla$_{\text{OXA-58-like}}$	OXA58-likeF	AAGTATTGGGGCTTGTGCTG	Woodford N *et al*. *J Antimicrob Agents* 2006; **27**: 351-3.
	OXA58-likeR	CCCCTCTGCGCTCTACATAC	
bla$_{\text{OXA-143-like}}$	pre-OXA-143A	AGTTAACTTTCAATAATTG	Higgins PG *et al. J Antimicrob Agents* 2010; **35**: 305.
	pre-OXA-143B	TTGGAAAATTATATAATCCC	
bla$_{\text{OXA-235-like}}$	OXA-235F	TTGTTGCCTTTACTTAGTTGC	Higgins PG *et al. Int Antimicrob Agents Chemother* 2013; **57**: 2121-6.
	OXA-235R	CAAAATTTTAAGACGGATCG	
bla$_{\text{OXA-10}}$	OXA-10F	ATCCCCAACGCAATTATCGGC	this study
	OXA-10R	ATATTCAGGTGCCGCCTCCG	
bla$_{\text{NDM}}$	NDM-R	AGCGCAGCTTGTCGGCCAT	this study
	NDM-F	TTGCCCAATATTATGCACCCG	
bla$_{\text{VEB-1}}$	VEB-1F	ATGAAAATCGTAAAAAGGATATT	Hujer KM *et al. Antimicrob Agents Chemother* 2006; **50**: 4114-23.
	VEB-1R	TTATTTATTCAAATAGTAATTCC	
bla$_{\text{PER-1}}$	PER-1F	ATGAATGTCATTATAAAAG	Hujer KM *et al. Antimicrob Agents Chemother* 2006; **50**: 4114-23.
	PER-1R	TTGGGCTTAGGGCAG	
bla$_{\text{IMP}}$	IMP-F	GTTTATGTTCATACWTCG	Hujer KM *et al. Antimicrob Agents Chemother* 2006; **50**: 4114-23.
	IMP-R	GGTTTAAYAAAACAACCAC	
bla$_{\text{VIM}}$	VIM-F	TTTGGTCGCATATCGCAACG	Hujer KM *et al. Antimicrob Agents Chemother* 2006; **50**: 4114-23.
	VIM-R	CCATTCAGCCAGATCGGCAT	

Table S2. Antibiotic susceptibilities of *A. baumannii* NIPH 56 and *E. coli* Top 10 and their transformants based on Etest and disc diffusion[a]

ß-Lactam	MIC (mg/L)				Inhibition zone diameters (mm)			
	A. baumannii		*E. coli*		*A. baumannii*		*E. coli*	
	NIPH 56	NIPH 56 (pAT801)[b]	Top 10	Top 10 (pAT801)[c]	NIPH 56	NIPH 56 (pAT801)[b]	Top 10	Top 10 (pAT801)[c]
Sulbactam	0.5	32	16	≥256	NT	NT	NT	NT
Ampicillin	32	>256	2	>256	8	6	24	6
Ampicillin/sulbactam (2:1)	1	64	2	128	NT	NT	NT	NT
Piperacillin	8	>256	1	>256	24	6	36	8
Piperacillin/tazobactam (2 mg/L)	≤0.125	≤0.125	2	2	26[e]	24[e]	35[e]	18[e]
Ticarcillin	NT	NT	NT	NT	26	6	31	6
Ticarcillin/clavulanic acid (2 mg/L)	8	>256	1	>256	NT	NT	NT	NT
Ceftazidime	4	4	0.125	0.25	21	21	34	34
Cefotaxime	8	8	0.125	0.125	20	20	40	39
Cefepime	8	8	0.125	0.5	20	18	42	38
Imipenem	1	1	1	1	31	31	35	33
Meropenem	0.25	0.5	0.125	0.125	29	28	42	40
Aztreonam	NT	NT	NT	NT	19	19	38	37

[a]Determined by Etest (bioMérieux) using Mueller-Hinton II agar (BBL, BD, USA) following the manufacturer guidelines and the CLSI interpretative criteria.[17] Disc diffusion was performed according to CLSI guidelines[17] with the following antibiotic discs (Oxoid) (content of antibiotic in µg/disc): ampicillin (10), piperacillin (100), piperacillin/tazobactam (100/10), ticarcillin (75), ceftazidime (30), cefotaxime (30), cefepime (30), imipenem (10), meropenem (10) and aztreonam (30).

[b]*A. baumannii* NIPH 56 transformed by plasmid pAT801 harbouring the $bla_{\text{TEM-1}}$ gene.

[c]*E. coli* Top 10 transformed by plasmid pAT801 harbouring the $bla_{\text{TEM-1}}$ gene.

NT, not tested.

Study V

Krizova L, Nemec A. A 63 kb genomic resistance island found in a multidrug-resistant *Acinetobacter baumannii* isolate of European clone I from 1977. *J Antimicrob Chemother* 2010; 65:1915-18.

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A 63 kb genomic resistance island found in a multidrug-resistant *Acinetobacter baumannii* isolate of European clone I from 1977

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Objectives: Multidrug-resistant *Acinetobacter* strain HK302 was isolated from an outbreak of nosocomial infections in Switzerland in 1977. The aim of the present study was to assess whether this archive strain belongs to one of the known international clonal lineages of *Acinetobacter baumannii* and whether it harbours a genomic structure related to the AbaR1-like resistance islands.

Methods: Multilocus sequence typing (MLST) and HindIII ribotyping were used to determine the taxonomic position of HK302 at the species and subspecies (clonal) levels. The position and structure of the putative resistance island were investigated by AbaR1-based PCR mapping followed by restriction analysis and partial sequencing of amplicons. *A. baumannii* AYE harbouring AbaR1 was used as a positive control for PCR mapping.

Results: The MLST allelic profile (1-1-1-1-5-1-1) and HindIII ribotype of HK302 were typical of *A. baumannii* European (EU) clone I. In addition, an AbaR1-related region inserted into the ATPase gene at the same position as AbaR1 was found in HK302. PCR mapping and partial sequencing revealed that this region is structurally congruent with AbaR3, a 63.4 kb island described in an *A. baumannii* isolate from 2004.

Conclusions: *A. baumannii* HK302 belongs to EU clone I and harbours an AbaR3-like island related to resistance islands described in EU clone I strains. Our findings suggest that variants of these sophisticated genomic structures already existed in *A. baumannii* in the late 1970s.

Keywords: international clonal lineages, multilocus sequence typing, PCR mapping, AbaR3

Introduction

Acinetobacter baumannii strains resistant to most or even all clinically relevant antimicrobial agents are of growing concern in hospitals worldwide.[1] These strains have been associated, although not exclusively, with a small number of clonal lineages.[1–3] Two of these lineages, termed European (EU) clones I and II, have been isolated worldwide, with their oldest known isolates dating back to 1987 and 1984, respectively.[1,4] Even though it is assumed that these clones had been circulating among hospitalized patients even earlier, no such isolates have been reported yet.

Fournier *et al.*[5] identified an 86.2 kb resistance genomic island, termed AbaR1, integrated into the ATPase gene of an *A. baumannii* strain and harbouring 45 genes putatively associated with resistance to antimicrobials or biocides. Further studies have found that various related sequences were integrated into the same position of the ATPase gene in a number of genotypically unrelated *A. baumannii* strains[6–8] and it was suggested that these sequences had arisen from an ancestral transposon related to Tn*7*.[9] So far, only two of these sequences (AbaR3 and AbaR5) have been shown to be highly similar to AbaR1 in terms of both the composition and the order of resistance genes.[6,7] The three strains harbouring AbaR1, AbaR3 and AbaR5 were isolated in three continents between 1997 and 2004, and were found to belong to EU clone I.[5–7]

In 1977, a multidrug-resistant *Acinetobacter* strain was isolated from an outbreak of nosocomial infections in a Swiss hospital.[10] This strain (HK302) carried multiple resistance determinants, mostly associated with mobile genetic elements.[10] The genes encoding some of these determinants have later been shown to be associated with the AbaR1-like islands.[5–7] In addition, the original data[10] suggested that HK302 harboured an 8.7 kb cryptic plasmid, which is typically found in EU clone I strains.[11] The aim of the present study was to test the hypothesis that strain HK302 belongs to EU clone I and harbours a structure related to the AbaR1-like resistance islands.

Materials and methods

Bacterial strains

Strain HK302, originally recovered from the respiratory tract of a patient in the intensive care unit of Zürich University Hospital, Switzerland, in 1977[10] and then stored in a lyophilized form, was revitalized for the purpose of the present study in 2005. Strain AYE was used as a positive control for the PCR detection and mapping of genes associated with AbaR1.[5]

HK302 characterization

Susceptibility to antimicrobial agents was assayed by Etest (BioMérieux, Marcy l'Étoile, France) using Mueller–Hinton II agar (BBL™, BD, USA), as suggested by the manufacturer. Multilocus sequence typing (MLST) was based on sequence analyses of the internal portions of seven housekeeping genes (*cpn60*, *fusA*, *gltA*, *pyrG*, *recA*, *rplB* and *rpoB*), as described by Diancourt *et al.*[2] Analysis of rRNA gene restriction fragment length polymorphisms (RFLPs) (ribotyping) with the HindIII restriction endonuclease (Fermentas, Vilnius, Lithuania) and biochemical profiling based on the assimilation of six different compounds as the sole source of carbon were carried out as described previously.[11]

Gene detection and mapping

The presence of genes associated with AbaR1 was tested by PCR[12] using primers and annealing temperatures indicated in Table S1 [available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)]. The primers were derived from the known sequence of AbaR1 (GenBank accession no. CT025832). PCR mapping was carried out using a Long Range PCR Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol, with 1 μL of genomic DNA obtained by the method of Boom *et al.*[13] The cycling parameters for mapping reactions were as follows: initial denaturation at 93°C for 3 min; first 10 cycles of 93°C for 15 s, 56–62°C for 30 s and 68°C for 1 min/kb; and then 28 cycles of 93°C for 15 s, 56–62°C for 30 s and 68°C for 1 min/kb plus 20 s in each additional cycle. Amplicons were analysed by RFLP analysis using the AccI, ApaI, BfaI, BglI, BsmAI, ClaI, EcoRI, HincII, HindIII or StyI enzymes (Fermentas).

Sequence analysis

PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) or, after visualization in agarose gel, using a High Pure PCR Product Purification Kit (Roche). Sequencing reactions were carried out using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the primers listed in Table S1. Sequences were determined using an ABI 3130 XL genetic analyser (Applied Biosystems) and were analysed using BLASTN (www.ncbi.nlm.nih.gov/blast) and KODON software (Applied Maths).

GenBank accession numbers

The sequences of parts X, Ya and Yb of the HK302 island (Figure 1) have been deposited in GenBank with the accession number HM357806.

Results and discussion

Strain HK302, isolated in 1977, was originally identified as *Acinetobacter calcoaceticus* subsp. *anitratus*, according to the taxonomy of that time.[10] In the present study, MLST was used to determine the precise taxonomic position of HK302, at the species and subspecies (clonal) levels. HK302 showed the allelic profile 1-1-1-1-5-1-1 (sequence type ST1), which is typical of *A. baumannii* EU clone I (clonal complex CC1) and is considered the founder genotype of this lineage.[2] In addition, HK302 yielded a HindIII ribotype identical to that of the reference strain of EU clone I and belonged to biotype 6, which is characteristic of EU clone I strains.[11] Thus, HK302 seems to be a typical member of EU clone I, currently being the oldest known strain belonging to this clonal lineage. The results of antimicrobial susceptibility testing of HK302 based on Etest were as follows (MIC in mg/L): gentamicin (384); netilmicin (16); kanamycin (>256); tobramycin (3); amikacin (12); piperacillin (>256); ceftazidime (2); cefotaxime (8); sulbactam (12); imipenem (0.19); chloramphenicol (96); tetracycline (48); doxycycline (2); tigecycline (0.75); ciprofloxacin (0.125); trimethoprim (4); sulfamethoxazole (>1024); and colistin (0.19). These data are in general agreement with the original report,[10] which is indicative of no substantial changes in the susceptibility of HK302 over the storage period.

Previous studies showed that AbaR1 and most of the related islands were integrated into the same position of the ATPase gene.[5–7] In HK302, the presence of the ATPase gene was confirmed using two separate PCRs targeting, respectively, the 3′ and 5′ ends of the gene (Table S1). In addition, the disruption of the ATPase gene by an integrated sequence was revealed by the negative result of PCR with two primers derived from the 3′ and 5′ ends of the gene (3ATP-F and 5ATP-R, respectively; Table S1). The precise location of the integrated sequence was assessed by sequence analysis with primers targeting the junctions between the sequence and the ATPase gene (Table S1). The results corroborated that the integrated sequence was located at the same position of the ATPase gene as known for AbaR1, AbaR3 and AbaR5. In addition, both ends of this sequence (~300 bp each) were identical to those of AbaR1, AbaR3 and AbaR5, including the flanking 5 bp perfect direct repeats (ACCGC) and short imperfect terminal inverted repeats (19/26 bp matches).[5–7]

To investigate the structure of the putative genomic island of HK302, we first screened for the presence of genes harboured by AbaR1. Of 25 genes tested, 18 were identified in HK302 (indicated by striped arrows in Figure 1), which suggests that some AbaR1 genes are missing in this strain (i.e. bla_{VEB-1}, *aadB*, *arr-2*, *strA*, *strB*, *cmlA1* and *dfrA1*). Notably, all these missing genes are part of a 29 kb region located in AbaR1, but are absent from AbaR3 and AbaR5.[5–7] As the next step, the structure of the HK302 island was investigated using PCR mapping with primers derived from the AbaR1 genes found in HK302 (Table S1), followed by RFLP analysis of amplicons. The results are summarized in Figure 1. The HK302 island was completely mapped by 13 overlapping long-range PCRs. The comparison of amplicons obtained with the same sets of primers from both HK302 and AYE, and their RFLP patterns suggested that 11 amplicons (marked by unbroken lines in Figure 1) were of the same size and structure in both strains. Two remaining amplicons of HK302, termed X (7.7 kb) and Y (5.6 kb), differed from those obtained from AYE and were sequenced using primer walking (Figure 1). Region X encompassed a part of transposon Tn*6018*, the *topA* gene and four other genes. Sequences nearly identical to region X are present in both AbaR3 (4417/4422 matches; GeneBank accession no. CP001182) and AbaR5 (4419/4422 matches; FJ172370) at the same position as in the

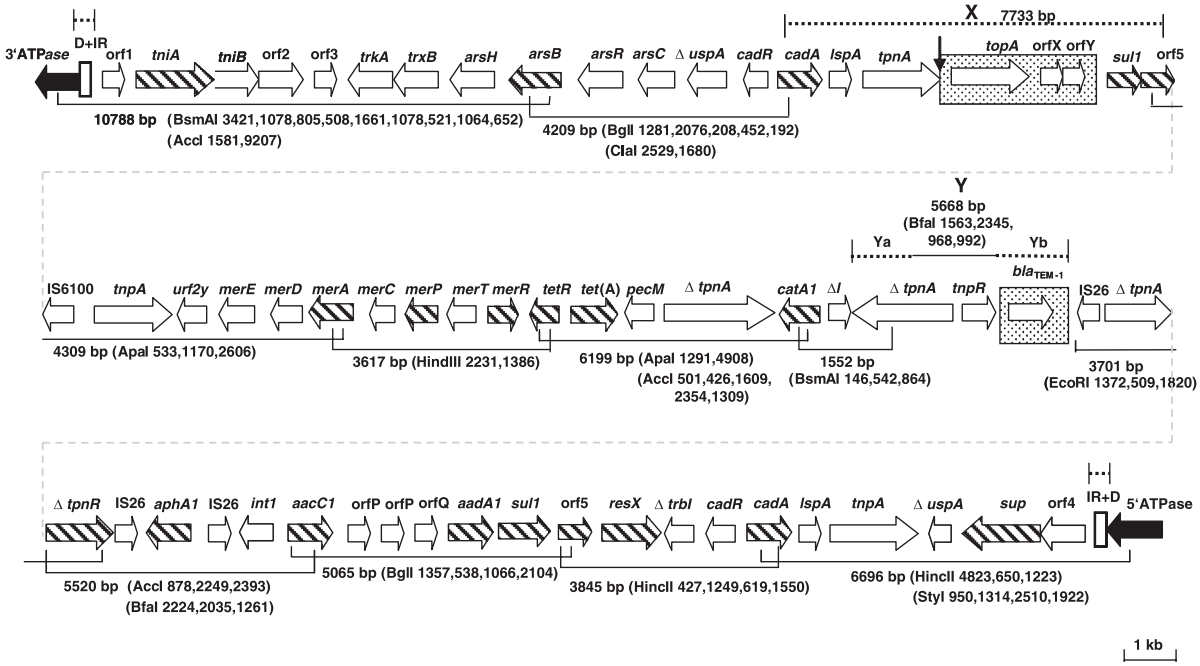

Figure 1. Putative structure of the HK302 resistance island based on PCR–RFLP mapping and partial sequencing. Horizontal arrows indicate genes associated with the island flanked by the segments of a disrupted ATPase gene (black arrows); the striped arrows denote the genes identified in HK302 by PCR screening of individual genes. Rectangles represent 5 bp direct repeats (D) and 26 bp imperfect terminal inverted repeats (IR). Small vertical lines indicate the positions of primers used for PCR mapping. Unbroken horizontal lines denote individual PCR amplicons corresponding in both size and RFLP patterns to those of AbaR1; indicated below or above each line are the size of the amplicon and, in parentheses, the lengths of restriction fragments obtained by digestion of the amplicon with the given restriction enzyme. Dotted horizontal lines show the sequenced regions; Ya (1567 bp) and Yb (1484 bp) indicate the sequenced segments of region Y. Dotted backgrounds represent the regions missing in AbaR1 but present in AbaR3. The vertical arrow indicates the position of the 29 kb specific sequence of AbaR1.

HK302 island (Figure 1). Partial sequencing of region Y revealed that its ends corresponded to the AbaR3 region harbouring transposon Tn*3* associated with the bla_{TEM-1} gene.[6] RFLP analysis of this region with the BfaI enzyme yielded fragments of the sizes predicted from the AbaR3 sequence, indicating that this region is identical to that in AbaR3. Based on these results, it could be concluded that strain HK302 harbours a 63.4 kb resistance island that is structurally congruent with AbaR3. Even though AbaR3 was originally reported to be sized only 49 kb,[6] its corrected size based on the AbaR3 GenBank sequence (CP001182) is 63.4 kb.

Devaud *et al.*[10] reported HK302 to possess multiple acquired mechanisms that confer resistance to aminoglycosides, β-lactams, chloramphenicol, tetracycline and sulphonamides. Although these mechanisms were shown to be chromosomally encoded, some laboratory variants of HK302 had less extensive resistance patterns. One of these variants lost both phosphotransferase APH(3′)-I (resistance to kanamycin) and TEM β-lactamase (ampicillin) activities, and its resistance to other antimicrobials was transferable to another strain using a helper plasmid. Additional experiments indicated that this transfer was due to the transposition activity of an ∼24 kb DNA sequence encoding acetyltransferase AAC(3)-I (gentamicin), adenyltransferase AAD(3″) (streptomycin) and chloramphenicol-inactivating enzyme, and conferring resistance to sulfamethoxazole and tetracycline. As shown in Figure 1, all genes corresponding to the mentioned resistance mechanisms, i.e. *tet*(A), *catA1*, bla_{TEM-1}, *aphA1*, *aacC1*, *aadA1* and *sul1*, are clustered in a large fragment of AbaR3 that was probably derived from a Tn*21*-like transposon by the successive integration of additional transposable elements, such as Tn*3* with bla_{TEM-1} or Tn*6020* with *aphA1*.[7] It is, therefore, likely that the variant strain lost the region encompassing the last two adjacent elements while retaining the transposable 24 kb sequence.

It has been recently suggested that AbaR1-like sequences in *A. baumannii* arose from a Tn*7*-related transposon, termed Tn*AbaR1*, which successively accumulated multiple transposable elements associated with resistance genes.[9] Sixteen Tn*AbaR1*-related sequences have so far been described in *A. baumannii*, seven of which have been fully sequenced.[5–9,14] Three large islands, i.e. AbaR1, AbaR3 and AbaR5, were found to be highly structurally related to each other and to be carried by strains of EU clone I. Our finding that an isolate of EU clone I from 1977 harbours an AbaR3-like island indicates that these sophisticated genomic structures were associated with this clonal lineage already in the late 1970s. As resistance mechanisms encoded by AbaR3 are effective against many antimicrobial drugs used at that time, it can be speculated that the selective advantage provided by AbaR3 or its variants may have played a role in the spread of EU clone I in European hospitals in the 1980s.[4]

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Transparency declarations

None to declare.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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Supplementary data

Table S1. Primers

Gene/Location	Name	Sequence 5' - 3'	Product (bp)	Annealing temperature	Reference[a]
Beta-lactamase genes					
bla$_{TEM-1}$	TEM-1-F	gggaattctcggggaaatgtgcgcggaac	998	55	1
	TEM-1-R	gggatccgagtaaacttggtctgacag			
bla$_{VEB-1}$	VEB-1-F	atgaaaatcgtaaaaaggatatt	900	46	2
	VEB-1-R	ttatttattcaaatagtaattcc			
Aminoglycoside resistance genes					
aac(3)-Ia	AAC3-IA1	gacataagcctgttcggtt	372	55	3
	AAC3-IA2	ctccgaactcacgaccga			
aph(3')-Ia	APH3-IA1	cgagcatcaaatgaaactgc	623	55	3
	APH3-IA2	gcgttgccaatgatgttacag			
ant(3")-Ia	ANT3-IA1	tgatttgctggttacggtgac	248	55	4
	ANT3-IA2	cgctatgttctcttgcttttg			
ant(2")-Ia	ANT2-IA1	atctgccgctctggat	404	55	3
	ANT2-IA2	cgagcctgtaggact			
strA	STRA-F	aacaggagggcgcatgcct	400	50	this study
	STRA-R	cgcccaaggtcgatcagacc			
strB	STRB-F	acgggactcctgcaatcgtca	400	50	this study
	STRB-R	cgcagttccgaggcattgc			
Tetracycline resistance genes					
tet(A)	TETA2-F	gtaattctgagcactgtcgc	950	50	5
	TETA2-R	ctgcctggacaacattgctt			
tetR	TETR-F	cctgctcgaacgctgcgtc	400	50	this study
	TETR-R	gaagccatgctggcggagaat			
Chloramphenicol resistance genes					
cmlA1	CLMA-F	aggcgcaacggctttcgtt	595	50	this study
	CMLA-R	cgctacgcatcccgcgata			
catA1	CAT-F	gatgaacctgaatcgccagcg	398	48	this study
	CAT-R	tcacattcttgcccgcctgat			
Sulphonamide resistance gene					
sul1	SUL1-F	tgtccgatcagatgcaccgtg	300	55	this study
	SUL1-R	gatgagccggtcggcagcg			
Trimethoprim resistance genes					
dfrA1	DFRA1-F	tggctgttggttggacgca	352	52	this study
	DFRA1-R	ccttttgccagatttggtaa			
Mercuric ion resistance genes					
merA	MERA-F	cgtgcgtggtggtcagcac	780	50	this study
	MERA-R	taagcccagtggcaacgaacg			
merP	MERP-F	gatagcccgcgtcctcggtc	252	50	this study
	MERP-R	aaaaactgtttgccgccctcg			
merR	MERR-F	gccggggtcaatgtggagac	400	50	this study
	MERR-R	tagtcaccccgtgactccccc			

Table S1. Primers *continued*

Gene/Location	Name	Sequence 5' - 3'	Product (bp)	Annealing temperature	Reference[a]
Arsenic resistance gene					
arsB	ARSB-F	gcaatcgctacagccagtgcc	850	50	this study
	ARSB-R	ggcattggggattgcgatagg			
Rifampicin resistance gene					
arr-2	ARR-2F	caagcaggtgcaaggaccgtt	344	50	this study
	ARR-2R	caacaggatgcccctcccagt			
Class 1 integron					
int1	INT1-F	cagtggacataagcctgttc	160	55	6
	INT1-R	cccgaggcatagactgta			
5'CS	5'CS	ggcatccaagcagcaag	variable	57	7
3'CS	3'CS	aagcagacttgacctga			
Other AbaR1- associated genes					
3'ATPase	3ATP-F	tccggcgaactttcagctca	400	52	this study
	3ATP-R	gcaacccgtaaaacgcgatga			
5'ATPase	5ATP-F	aggcaggcgtgaggccaat	490	52	this study
	5ATP-R	tgctcctgcagatttgccca			
uspA	USPA-F	tggaatgaccataagccccaa	401	50	this study
	USPA-R	ggggaaacacggcactcagac			
cadA	CADA-F	ctaggcgcctcgcttcagga	400	46	this study
	CADA-R	caatcaatgcgacgaatgcga			
tpnA	TPNA-F	acgtcggggctaaatcgcg	358	50	this study
	TPNA-R	ttccactgagcgtcagacccc			
tpnR	TPNR-F	gcttgcagatcgacgcgct	400	50	this study
	TPNR-R	catcagggcctttgcccgt			
resX	RESX-F	cgcaggtgtcgaacgggac	425	50	this study
	RESX-R	aagtatcgcgctgggccatg			
orf5	ORF5-F	ggagcctccgaacgttcgg	461	50	this study
	ORF5-R	tgaaggttggatcccagccg			
tniA	TNIA-R	ttggaaatgaacccgcagcag	variable	55	this study
IS26	IS26-F	tccatttcaggcgcataacgc	128	50	this study
	IS26-R	ggccgtacgctggtactgcaa			
sup	SUP-F	gcccgactttgggatcgaca	variable	55	this study

Table S1. Primers *continued*

Gene/Location	Name	Sequence 5' - 3'	Product (bp)	Annealing temperature	Reference[a]
Sequencing primers					
J3[b]	J3-F	atttgccgagctgcacgtgaa	300	55	this study
	J3-R	accattcaggcactcgtgcct			
J5[c]	J5-F	tttgggaagcaatcaatagtc	310	55	this study
	J5-R	attggcctcacgcctgcct			
cadA	1822-F	ggcgctcaaggattaagcaa			this study
lspA	LSPA-SEQF	aaacaccaatgctgagtcgg			this study
	2266-F	ggctgcatgagtacgtggag			this study
	LSPA-NEXT-F	atatcgggcctgctggcagc			this study
tpnA	TNPASEQ-R	gcctcatcgctaactttgc			this study
	TNPASEQ-F	tgctgttacgacgggagga			this study
	TPNA2-R	tccggtttcgtggacacgtt			this study
topA	TOPSEQ-F	attgcagcctatgcccgcag			this study
	TOPSEQ-R	acggcccgccatgccgtgc			this study
	TOPA2-F	cgatgcgcttgtaggccgg			this study
	TOPASTART-R	tagttccttcggcggcaagtc			this study
	4974-F	cggtgcgccttgtggtggac			this study
	5347-F	gacgacgccggtatggagga			this study
orfX[d]	HP3208-R	cgtggatcgaggcgatgga			this study
	8099NEW-F	gaaaggcgtgacgtggaagt			this study
sul1	SUL3208-R	cgtttaacggacattcgtaa			this study
	SULNEXT-R	cctcgccgggctggcaatcg			this study
bla_{TEM-1}	TEMSEQ-R	ggccggtgcccgtctgacgt			this study
Δ*tpnA*	IS15-F	tggtatcccagcagccagaa			this study
	TPNANEXT-F	agaggcgtgcgatatcttca			this study

[a] Reference for primers
[b] J3, junction between 5'end of the island and 3'end of the ATPase gene
[c] J5, junction between 3'end of the island and 5'end of the ATPase gene
[d] Gene encoding a hypotetical protein

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Study VI

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Diversity and Evolution of AbaR Genomic Resistance Islands in *Acinetobacter baumannii* Strains of European Clone I▽†

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To assess the diversity of AbaR genomic resistance islands in *Acinetobacter baumannii* European clone I (MLST clonal complex 1), we investigated 26 multidrug-resistant strains of this major clone isolated from hospitals in 21 cities of 10 European countries between 1984 and 2005. Each strain harbored an AbaR structure integrated at the same position in the chromosomal ATPase gene. AbaR3, including four subtypes based on variations in class 1 integron cassettes, and AbaR10 were found in 15 and 2 strains, respectively, whereas a new, unique AbaR variant was discovered in each of the other 9 strains. These new variants, designated AbaR11 to AbaR19 (19.8 kb to 57.5 kb), seem to be truncated derivatives of AbaR3, likely resulting from the deletions of its internal parts mediated by either IS*26* elements (AbaR12 to AbaR19) or homologous recombination (AbaR11). AbaR3 was detected in all 10 strains isolated in 1984 to 1991, while AbaR11 to AbaR19 were carried only by strains isolated since 1997. Our results and those from previous publications suggest that AbaR3 is the original form of AbaR in European clone I, which may have provided strains of the lineage with a selective advantage facilitating their spread in European hospitals in the 1980s or before.

Nosocomial infections caused by multidrug-resistant (MDR) strains of *Acinetobacter baumannii* have become a serious therapeutic and epidemiological problem worldwide (9). Resistance to multiple antimicrobials in the species has been associated with several international lineages, particularly with the so-called European (EU) clones I and II (7, 9). The earliest known MDR strain of EU clone I was isolated in a Swiss hospital in 1977 (16), and the lineage prevailed among outbreak and MDR *Acinetobacter* strains in some European countries in the 1980s and 1990s (8, 18). Further studies in these countries have demonstrated a shift toward the predominance of EU clone II among hospital *A. baumannii* strains in the early 2000s (19, 25), considered to be a result of the spread of carbapenem-resistant strains (subclones) of this clone (19). Although strains belonging to or related to EU clone II seem to dominate in the current global population of MDR *A. baumannii* strains (5, 13, 14), EU clone I and other MDR lineages can be common or even prevail in some regions (10, 26).

Recent studies have revealed that the chromosomes of some *A. baumannii* strains harbor large clusters of horizontally transferred genes conferring resistance to multiple antibiotics and heavy metals, which are integrated at a specific site in a particular ATPase gene (12, 22). Nine such genomic resistance islands (*A. baumannii* resistance islands [AbaRs]) have been fully characterized, eight of which were found in strains of EU clone I, i.e., AbaR1 (12), AbaR3 (2), AbaR5 (20), AbaR6, AbaR7 (22), AbaR8 (21), AbaR9, and AbaR10 (1). These AbaRs share a structure represented by a 16.3-kb backbone transposon (Tn*6019*) interrupted by a large compound transposon that contains a variable-resistance region bounded by directly oriented copies of Tn*6018* (Fig. 1) (22). Exceptions are AbaR6 and AbaR7, each with a large deleted region that includes the left-hand copy of Tn*6018* and part of the Tn*6019* backbone (22). Some strains of EU clone I were also found to harbor a $bla_{\text{OXA-23}}$ gene-carrying island termed AbaR4, which was integrated at a chromosomal site different from that of the ATPase gene (2, 24) and the backbone of which is formed by a Tn*6021* transposon instead of Tn*6019* (22). Much less is known about AbaRs in EU clone II. The resistance islands harbored by this clone are integrated at the same site of the ATPase gene as is known for AbaRs in EU clone I. Apart from AbaR2, a largely truncated AbaR island found in a fully sequenced strain (15), AbaRs from two EU clone II strains were partially characterized and shown to contain a transposon related to Tn*6021* (22). In addition, an epidemic strain of EU clone II from the United Kingdom was shown to carry an AbaR4-type island integrated in the ATPase gene (24). These findings suggest that the genomic islands associated with the ATPase gene evolved independently in two main *A. baumannii* clones.

The EU clone I strains harboring the completely characterized AbaRs were isolated in Europe (AbaR1), the United States (AbaR3, AbaR9, and AbaR10), or Australia (AbaR5 to AbaR8) between 1997 and 2009 (1, 2, 12, 20, 21, 22). Comparative analysis of these AbaRs has suggested that AbaR1 and AbaR5 to AbaR10 are truncated variants of AbaR3, while AbaR1 is the only known AbaR structure that contains additional DNA compared to AbaR3 (1, 22). Recently, we identified an intact AbaR3 island in an outbreak MDR strain of EU clone I from 1977 (16), indicating that AbaR variants already existed in *A. baumannii* in the late 1970s. The aim of the

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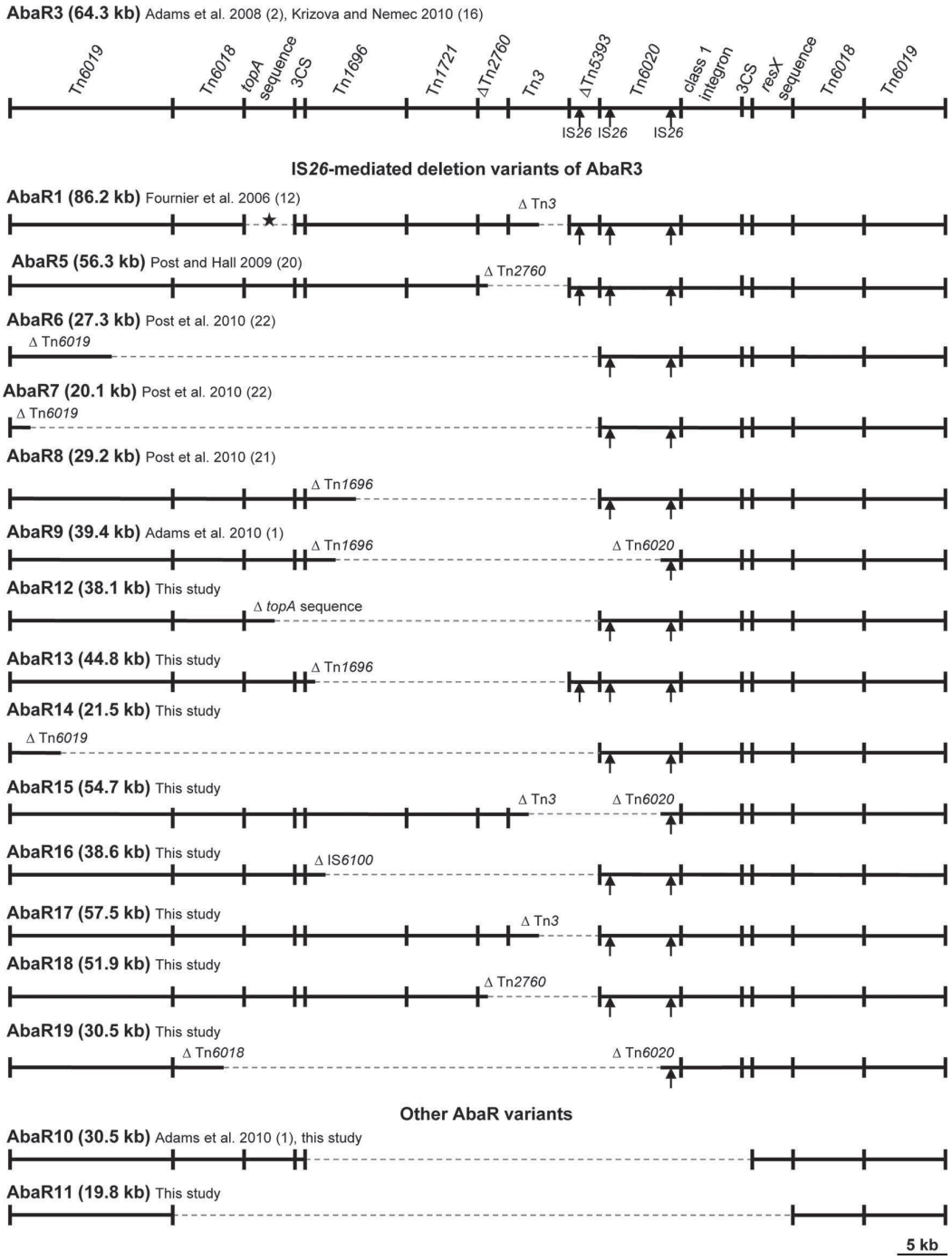

FIG. 1. Schematic overview of AbaR variants in European clone I. A diagram of AbaR3 is shown at the top. Only AbaR3 components discussed in the text are shown (demarcated by vertical lines); for a more detailed structure of AbaR3, see reference 16. The other AbaRs are lined up below AbaR3 according to the presence of the regions homologous to those of AbaR3. Unbroken and dashed horizontal lines indicate, respectively, theAbaR3 regions present or absent in each of the AbaRs. Partial deletion of an AbaR3 component is indicated by the absence of a vertical line. The arrows indicate the positions of three copies of IS*26* sequences present in AbaR3. The location of the 29-kb specific sequence in AbaR1 is denoted by an asterisk. The sizes of the individual AbaRs are proportional to the scale, except for the regions with class 1 integrons (Table 1). The sequences of AbaR1, AbaR3, AbaR5, AbaR6, AbaR7, AbaR8, AbaR9, and AbaR10 are available from the GenBank database under accession numbers CT025832, CP001182, FJ172370, GQ406245, GQ406246, HM590877, ADGZ00000000, ADHA00000000, respectively.

TABLE 1. Characteristics of the *A. baumannii* strains of European clone I used in the present study

Strain	City, country,[a] yr of isolation	ST[b] (allelic profile)	AFLP type[c]	AbaR island[d]	Gene cassettes of the AbaR-associated class 1 integron	Reference(s)
RUH 875	Dordrecht, NL, 1984	1 (1111511)	1	AbaR3	*dfrA1*	7, 8, 17
RUH 510	Nijmegen, NL, 1984	8 (1111111)	2	AbaR3	*aacC1*-(*orfP*)$_2$-*orfQ*-*aadA1*	7, 8, 17
RUH 3239	London, UK, 1985-8	1 (1111511)	3	AbaR3	*aacC1*-(*orfP*)$_2$-*orfQ*-*aadA1*	7, 8, 17
RUH 2037	Venlö, NL, 1986	1 (1111511)	3	AbaR3	*aacC1*-(*orfP*)$_2$-*orfQ*-*aadA1*	7, 8, 17
RUH 3238	Sheffield, UK, 1987	1 (1111511)	4	AbaR3	*aacC1*-(*orfP*)$_2$-*orfQ*-*aadA1*	7, 8, 17
RUH 3242	Basildon, UK, 1989	1 (1111511)	2	AbaR3	*aacC1*-(*orfP*)$_2$-*orfQ*-*aadA1*	7, 8, 17
RUH 3247	Leuven, BE, 1990	1 (1111511)	4	AbaR3	*aacA4*	7, 8, 17
RUH 3282	Salford, UK, 1990	1 (1111511)	5	AbaR3	*aacC1*-(orfP)$_2$-orfQ-*aadA1*	7, 8, 17
NIPH 7	Praha, CZ, 1991	7 (1112511)	6	AbaR3	*aacC1*-(*orfP*)$_2$-*orfQ*-*aadA1*	7, 17, 18
NIPH 10	Praha, CZ, 1991	1 (1111511)	6	AbaR3	*aacC1*-(*orfP*)$_2$-*orfQ*-*aadA1*	7, 17, 18
NIPH 56	Praha, CZ, 1992	1 (1111511)	7	AbaR10	None	7, 17, 18
NIPH 321	Tábor, CZ, 1994	1 (1111511)	4	AbaR3	*aacC1*-(*orfP*)$_2$-*orfQ*-*aadA1*	7, 17, 18
NIPH 783	Debrecen, HU, 1995	1 (1111511)	8	AbaR10	None	
NIPH 827	Sofia, BG, 1997	1 (1111511)	9	AbaR3	*aacC1-orfP-orfQ-aadA1*	
NIPH 470	Č. Budějovice, CZ, 1997	1 (1111511)	10	AbaR11	None	17, 18
LUH 6013	Rome, IT, 1997	1 (1111511)	11	AbaR12	*aacC1*-(*orfP*)$_2$-*orfQ*-*aadA1*	7
LUH 6015	Rome, IT, 1998	1 (1111511)	12	AbaR13	*aacC1-orfP-orfQ-aadA1*	7
LUH 5881	Madrid, ES, 1998	1 (1111511)	13	AbaR14	*aacA4*	7
LUH 6125	Krakow, PL, 1998	1 (1111511)	10	AbaR15	*aacC1*-(*orfP*)$_2$-*orfQ*-*aadA1*	7
LUH 7140	London, UK, 2000	1 (1111511)	10	AbaR16	*aacC1-orfP-orfQ-aadA1*	7
NIPH 1605	Sedlčany, CZ, 2001	1 (1111511)	14	AbaR3	*aacC1-orfP-orfQ-aadA1*	17
LUH 8592	Sofia, BG, 2001	1 (1111511)	10	AbaR17	*aacC1-orfP-orfQ-aadA1*	7
LUH 9668	Dublin, IE, 2003	1 (1111511)	2	AbaR3	*aacC1-orfP-orfQ-aadA1*	7
NIPH 2605	Most, CZ, 2005	1 (1111511)	15	AbaR3	*aacC1*-(*orfP*)$_2$-*orfQ*-*aadA1*	19
NIPH 2713	Kladno, CZ, 2005	1 (1111511)	16	AbaR18	*aacC1-orfP-orfQ-aadA1*	19
NIPH 2554	Praha, CZ, 2005	1 (1111511)	16	AbaR19	*aacC1*-(*orfP*)$_2$-*orfQ*-*aadA1*	19

[a] Country abbreviations: BE, Belgium; BG, Bulgaria; CZ, Czech Republic; ES, Spain; HU, Hungary; IE, Ireland; IT, Italy; NL, Netherlands; PL, Poland; UK, United Kingdom.
[b] ST of the Institut Pasteur MLST scheme available at http://www.pasteur.fr/mlst.
[c] AFLP types distinguished by comparative cluster analysis of the current set of strains at 90% similarity level and numbered for the purpose of this study.
[d] The AbaR3 designation is used for all four subtypes of AbaR3 based on variations in class 1 integron cassettes.

present study was to obtain more insight into the structural diversity of AbaR islands in the European population of EU clone I. To this end, we investigated 26 strains isolated in 10 countries over a period of 21 years.

MATERIALS AND METHODS

Selection and properties of bacterial strains. Twenty-six *A. baumannii* strains isolated from hospitals in 21 cities of 10 European countries between 1984 and 2005 were investigated (Table 1). The strains known to belong to EU clone I were selected from published (7, 8, 17, 18, 19) or unpublished (strains NIPH 783 and NIPH 827) studies to be as diverse as possible in time and place of isolation without any known epidemiological link, with the emphasis on isolates from the 1980s and 1990s. Strains from the same city were included only if they differed in molecular-typing characteristics and/or in the content of genes associated with the known AbaRs. The strains had been previously assigned to EU clone I by multilocus sequence typing (MLST) as developed at the Institut Pasteur (7) and by amplified fragment length polymorphism (AFLP) analysis (9). By MLST, all strains had either sequence type (ST) 1 or one of its single-locus variants (Table 1), which together form clonal complex 1, corresponding to EU clone I (7). By AFLP analysis, the isolates were further classified into 16 types at a similarity level of 90% (Table 1). The minimal inhibitory concentrations of 10 antimicrobial agents known to be primarily effective against *A. baumannii* were determined by the agar dilution method using CLSI guidelines (4) and are shown in Table 2.

Gene detection and AbaR mapping. The genes associated with AbaR islands were detected by PCR using primers derived from the sequences of AbaR3 (GenBank accession no. CP001182) and AbaR1 (GenBank accession no. CT025832). The 25 genes studied, primers, and annealing temperatures are listed in Table S1 in the supplemental material. To investigate the structures of AbaRs, PCR mapping experiments were performed as described previously (16). DNA fragments (up to 12 kb in size) corresponding to overlapping internal segments of AbaR3-like islands were amplified using a Long Range PCR Kit (Qiagen, Hilden, Germany) and primers listed in Table S2 in the supplemental material. The resulting amplicons were analyzed for restriction fragment length polymorphism (RFLP) using AccI, ApaI, BfaI, BclI, BglI, BsmAI, ClaI, EcoRI, EcoRV, HincII, HindIII, or StyI restriction enzymes (Fermentas, Vilnius, Lithuania). EU clone I strains AYE (12) and HK302 (16), carrying AbaR1 and AbaR3, respectively, were used as positive controls for PCR detection of the AbaR-associated genes, while strain HK302 was a positive control for PCR-RFLP mapping experiments.

DNA sequence analysis. Sequence analysis was performed on the regions different from those of AbaR3 using a primer-walking strategy. PCR amplicons were purified with a QIAquick PCR Purification Kit (Qiagen) or, after visualization in agarose gel, with a High Pure PCR Product Purification Kit (Roche Diagnostic GmbH, Mannheim, Germany). DNA sequencing was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI 3130 XL genetic analyzer (Applied Biosystems). DNA sequences were assembled using the software KODON (Applied Maths, St-Martens-Latem, Belgium) and annotated using BLASTN (http://www.ncbi.nlm.nih.gov/blast) and the sequence annotation tools integrated into the Sequin program (available at http://www.ncbi.nlm.nih.gov/Sequin).

Nucleotide sequence accession numbers. The partial nucleotide sequences of the AbaR structures have been deposited in the GenBank database under accession numbers JF262165 (AbaR10 from NIPH 56), JF262166 (AbaR10 from NIPH 783), JF262167 (AbaR11), JF262168 (AbaR12), JF262169 (AbaR13), JF262170 (AbaR14), JF262171 (AbaR15), JF262172 (AbaR16), JF262173 (AbaR17), JF262174 (AbaR18), and JF262175 (AbaR19).

RESULTS AND DISCUSSION

Structural analysis of AbaRs. To analyze the structure of AbaR resistance islands, we used a strategy based on the structural homology of the AbaR-type islands in strains of EU clone I (16). This strategy included (i) PCR determination of the presence and interruption of the ATPase gene, (ii) PCR analysis of the junctions between the inserted DNA and the

TABLE 2. MICs of the *A. baumannii* EU clone I strains included in the study

Strain	MIC (μg/ml)[a]									
	CAZ	CTX	FEP	MEM	AMK	GEN	NET	TOB	CIP	CST
RUH 875	8	32	16	1	8	>32	32	>16	1	0.5
RUH 510	8	16	8	0.5	8	>32	32	4	0.5	0.5
RUH 3239	8	32	16	1	8	>32	32	4	1	0.5
RUH 2037	128	>64	128	2	8	>32	16	4	>32	0.5
RUH 3238	8	16	8	0.5	4	>32	8	2	0.25	0.5
RUH 3242	8	32	16	0.5	4	>32	8	2	0.5	0.5
RUH 3247	8	16	16	0.25	16	32	>64	>16	8	0.5
RUH 3282	128	>64	32	1	>128	>32	>64	4	8	0.25
NIPH 7	8	16	8	0.5	>128	>32	16	4	2	0.5
NIPH 10	16	32	32	1	>128	>32	64	8	8	0.5
NIPH 56	4	8	4	0.25	4	4	8	2	0.25	0.5
NIPH 321	8	16	8	0.5	128	>32	16	4	8	0.5
NIPH 783	8	32	4	0.25	4	>32	8	>16	32	0.5
NIPH 827	64	>64	16	0.5	>128	>32	16	>16	0.5	0.5
NIPH 470	64	>64	16	0.5	128	8	64	2	4	0.5
LUH 6013	128	>64	16	1	>128	>32	8	2	>32	0.5
LUH 6015	>128	>64	16	0.5	4	>32	8	2	>32	0.5
LUH 5881	16	64	16	0.25	32	>32	>64	>16	>32	0.25
LUH 6125	16	>64	16	0.125	>128	>32	8	2	8	0.5
LUH 7140	64	>64	32	1	8	>32	32	4	>32	0.5
NIPH 1605	4	16	8	0.25	8	>32	16	>16	32	0.5
LUH 8592	128	>64	16	0.5	128	>32	8	2	32	0.5
LUH 9668	4	16	8	0.25	8	>32	16	4	4	0.5
NIPH 2605	8	32	4	0.25	128	>32	8	2	16	0.5
NIPH 2713	>128	>64	16	8	128	>32	32	2	4	0.5
NIPH 2554	4	16	8	0.25	4	>32	8	2	32	0.25

[a] AMK, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; CST, colistin; CTX, cefotaxime; FEP, cefepime; GEN, gentamicin; MEM, meropenem; NET, netilmicin; TOB, tobramycin.

ATPase gene, (iii) PCR detection of the genes corresponding to those found in the known AbaR in EU clone I, (iv) PCR-RFLP mapping based on AbaR3 and on the content of the AbaR-associated genes in a given strain, and (v) sequence analysis of the amplicons which differ in size and/or RFLP pattern from those of AbaR3.

All 26 strains carried the ATPase gene interrupted by a DNA insert at the same position as in the known AbaRs (16, 22). PCR analysis of the junctions using primers designed to distinguish between Tn*6019*- and Tn*6021*-like transposons revealed that the ends of the inserted sequence corresponded to Tn*6019*, known to be associated with AbaRs in EU clone I (22). PCR screening for the presence of 25 genes harbored by AbaR1 or AbaR3 showed that individual strains carried from 5 to 20 of these genes in 10 different combinations (data not shown). Thus, at least nine variants of AbaRs different from AbaR1 and AbaR3 occurred in the strains of the study.

AbaR3 and its integron-based subtypes. The structures of AbaRs were further investigated using an array of 14 overlapping long-range PCRs designed to cover the whole sequence of AbaR3 (16) (see Table S2 in the supplemental material), followed by RFLP analysis of the resulting amplicons. Between 3 and 14 PCRs were performed for each strain, depending on the presence of AbaR1- or AbaR3-associated genes. In 10 strains, all 14 amplicons corresponded in size and RFLP pattern to those of AbaR3, which indicated that these strains carried AbaRs structurally congruent with AbaR3 (Table 1). Each of five other strains yielded 12 amplicons identical to those of AbaR3, but the two amplicons spanning the variable region of a class 1 integron were different. Sequence analysis revealed that these five strains carried gene cassette arrays different from that originally identified in AbaR3 (*aacC1-orfP-orfP-orfQ-aadA1*). While one copy of *orfP* was absent from this cassette array in three strains (NIPH 827, NIPH 1605, and LUH 9668), the variable region in two other strains (RUH 875 and RUH 3247) included only a single gene cassette, either *dfrA1* or *aacA4* (Table 1). These results indicate that the already established AbaR3 islands may have undergone diversification through changes in the structure of class 1 integrons, possibly via loss, acquisition, or replacement of integron gene cassettes. For reasons of simplicity, we do not propose new designations for these integron-based subtypes of the complete AbaR3 island.

Truncated AbaR3 variants. Structural analysis using PCR-RFLP and sequencing revealed that all 11 remaining strains carried AbaR structures that can be considered truncated versions of the AbaR3-type islands. Except for AbaR10, found in two strains, each of these structures was detected in a single strain and represented a unique, yet-undescribed variant of AbaR. The nine new AbaRs have been termed AbaR11 to AbaR19, and their nearly complete or partial sequences are available from GenBank under accession numbers JF262167 to JF262175.

Compared to AbaR3, all of these new AbaRs lacked internal regions of different lengths, most likely as a result of deletions mediated by an IS*26* element, except for AbaR11 (Fig. 1). IS*26* is known to create deletions of adjacent regions, especially those containing antibiotic resistance genes (6, 11, 22). Three copies of IS*26* are located in the right half of AbaR3, and as

depicted in Fig. 1, each of them could be associated with 5′-ward deletions in AbaRs. In AbaR13, the IS*26* located next to the right end of Tn*3* is likely to have caused deletions reaching to the left part of Tn*1696*, and the same IS*26* copy seems to have been responsible for deletions in AbaR1 and AbaR5 (20). The IS*26* located at the left end of Tn*6020* has most likely created deletions of different lengths in AbaR12 (reaching to the *topA* sequence and partially removing *orfY* in this sequence), AbaR14 (reaching to the left part of Tn*6019* and partially deleting the *tniB* gene), AbaR16 (leaving only part of IS*6100* in Tn*1696*), AbaR17 (completely removing $bla_{\text{TEM-1}}$ carried by Tn*3*), and AbaR18 (partially deleting the *catA1* gene located in ΔTn*2760*). It may have also been involved in deletions in AbaR6, AbaR7 (22), and AbaR8 (21). Finally, the IS*26* located at the right end of Tn*6020* has probably removed internal regions in AbaR15 (partially deleting the *tpnR* gene of Tn*3*), AbaR19 (reaching to the left-hand copy of Tn*6018* and partially deleting the *tpnA* gene), and AbaR9 (1). Although multiple genetic events might have been responsible for the formation of the AbaRs discussed above, it is also possible that, except for AbaR1 (Fig. 1), all these structures arose from the complete AbaR3-type islands through a single deletion event mediated by IS*26*. Given the nonspecificity of IS*26*-mediated deletions, a number of additional AbaR variants can be expected in the current EU clone I population. Therefore, it should be considered whether to continue numbering IS*26*-based variants, especially when confronted with tiny and genetically irrelevant differences.

AbaR10 and AbaR11 represent AbaRs with no apparent deletions mediated by IS*26*. In the present study, two strains carried AbaRs, the partial sequences of which corresponded to that of the recently described AbaR10 (1). Compared to AbaR3, AbaR10 lacks a 31.5-kb region, which is located between the left-hand copy of the class 1 integron 3′ conserved segment (3′CS) and the *resX* sequence, while AbaR11 is missing several additional segments, including one copy of Tn*6018*, the *topA* and *resX* sequences, and the second copy of the 3′CS (Fig. 1). Even though AbaR10 and AbaR11 might also be less developed progenitors of the AbaR3-type islands, it is likely that each of these AbaRs has arisen from a larger AbaR structure through a deletion resulting from recombination between homologous sequences present in AbaR3. In AbaR10 and AbaR11, such deletions could be caused by recombination between two copies of 3′CS (1) and two copies of Tn*6018*, respectively. Thus, similar to the vast majority of the AbaRs with IS*26*-mediated deletions, both AbaR10 and AbaR11 may have arisen from an AbaR3-type structure via a single genetic event.

AbaRs in space and time. The available data indicate that AbaR3 is the most widespread of the AbaRs in space and time among EU clone I strains. So far, AbaR3 has been found in two strains isolated at the Walter Reed Army Medical Center in 2004 (1, 2) and in a Swiss isolate from 1977 (16). In the present study, strains with AbaR3 (including all its integron-based subtypes) were isolated in six European countries between 1984 and 2005 (Table 1). In addition, we identified AbaR3 (carrying an integron with *aacC1-orfP-orfP-orfQ-aadA1*) in an EU clone I strain isolated in Australia in 1995 (L. Krizova and A. Nemec, unpublished data). In contrast, each of the non-AbaR3 variants was found in a single strain isolated not earlier than 1997. The only exception was AbaR10, found in two European strains from 1992 and 1995 (NIPH 56 and NIPH 783, respectively), and in a U.S. isolate from 2003 (1).

Concluding remarks. The results of the present study and previously published data suggest that AbaR3 is the original genomic structure from which the hitherto known AbaRs in EU clone I have been derived. This assumption is supported by the distribution of different AbaRs in time and space and by the observation that nearly all non-AbaR3 islands are most likely truncated derivatives of AbaR3 that might have arisen from AbaR3 through a single genetic event. Notably, AbaR3 and its integron-based subtypes harbor a number of genes conferring resistance to antimicrobials used in the 1970s and 1980s, including early generations of β-lactams (the $bla_{\text{TEM-1}}$ gene), aminoglycosides (*aphA1*, *aacC1*, *aadA1*, and *aacA4*), tetracyclines (*tetA*), chloramphenicol (*catA1*), sulfonamides (*sul1*), and trimethoprim (*dfrA1*). Therefore, it is conceivable that these structures provided strains of EU clone I with a selective advantage, which facilitated their dissemination in European hospitals in the 1980s or before (8, 16). Reportedly, genomic islands integrated into the ATPase gene of *A. baumannii* represent a hot spot that could explain the rapid acquisition of resistance markers under antimicrobial pressure (12). However, AbaR1, found in an epidemic strain in France, is the only known AbaR in EU clone I that has incorporated additional genes encoding antibiotic resistance compared to AbaR3. Although recent isolates of EU clone I can harbor horizontally acquired genes encoding resistance to modern antibiotics, such as carbapenems, these genes (e.g., $bla_{\text{OXA-23}}$) have not been located on any of the AbaRs integrated in the ATPase gene in this clone (1, 24). In addition, many mechanisms conferring resistance to antimicrobials currently used against MDR strains of *A. baumannii* result from the upregulation or mutational modification of intrinsic chromosomal genes rather than from horizontal gene transfer (3, 9, 23). Consequently, the accumulation of truncated derivatives of AbaR3 in the recent population of EU clone I may reflect the diminishing role of these structures in resistance to antimicrobial therapy. In light of these considerations, AbaRs may be seen as footprints left behind by an important phase in the evolution of EU clone I, but currently they do not seem to play a substantial role in the ongoing development of antimicrobial resistance in *A. baumannii*.

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TABLE S1. Primers for the detection of the AbaR1 and AbaR3 associated genes

Gene or location	Primer pair	Sequence 5' - 3'	Amplicon (bp)	Annealing temperature	Reference
Beta-lactamase genes					
bla $_{TEM-1}$	TEM-1-F	gggaattctcggggaaatgtgcgcggaac	998	55	1
	TEM-1-R	gggatccgagtaaacttggtctgacag			
Aminoglycoside resistance genes					
aac(3)-Ia	AAC3-IA1	gacataagcctgttcggtt	372	55	2
	AAC3-IA2	ctccgaactcacgaccga			
aph(3')-Ia	APH3-IA1	cgagcatcaaatgaaactgc	623	55	2
	APH3-IA2	gcgttgccaatgatgttacag			
ant(3'')-Ia	ANT3-IA1	tgatttgctggttacggtgac	248	55	3
	ANT3-IA2	cgctatgttctcttgcttttg			
ant(2'')-Ia	ANT2-IA1	atctgccgctctggat	404	55	2
	ANT2-IA2	cgagcctgtaggact			
strA	STRA-F	aacaggagggcgcatgcct	400	50	This study
	STRA-R	cgcccaaggtcgatcagacc			
aac(6')-Ib	AAC6-IB1	tatgagtggctaaatcgat	395	55	4
	AAC6-IB2	cccgctttctcgtagca			
Tetracycline resistance genes					
tet (A)	TETA2-F	gtaattctgagcactgtcgc	950	50	5
	TETA2-R	ctgcctggacaacattgctt			
tetR	TETR-F	cctgctcgaacgctgcgtc	400	50	This study
	TETR-R	gaagccatgctggcggagaat			
Chloramphenicol resistance genes					
cmlA1	CMLA-F	aggcgcaacggctttcgtt	595	50	This study
	CMLA-R	cgctacgcatcccgcgata			
catA1	CAT-F	gatgaacctgaatcgccagcg	398	48	This study
	CAT-R	tcacattcttgcccgcctgat			
Sulphonamide resistance gene					
sul1	SUL1-F	tgtccgatcagatgcaccgtg	300	55	This study
	SUL1-R	gatgagccggtcggcagcg			
Trimethoprim resistance genes					
dfrA1	DFRA1-F	tggctgttggttggacgca	352	52	This study
	DFRA1-R	ccttttgccagatttggtaa			
Mercuric ion resistance genes					
merA	MERA-F	cgtgcgtggtggtcagcac	780	50	This study
	MERA-R	taagcccagtggcaacgaacg			
merP	MERP-F	gatagcccgcgtcctcggtc	252	50	This study
	MERP-R	aaaaactgtttgccgccctcg			
merR	MERR-F	gccggggtcaatgtggagac	400	50	This study
	MERR-R	tagtcaccccgtgactcccc			
Arsenic resistance gene					
arsB	ARSB-F	gcaatcgctacagccagtgcc	850	50	This study
	ARSB-R	ggcattggggattgcgatagg			
Class 1 integron					
int1	INT1-F	cagtggacataagcctgttc	160	55	6
	INT1-R	cccgaggcatagactgta			
5'CS	5CS	ggcatccaagcagcaag	variable	57	7
3'CS	3CS	aagcagacttgacctga			
Other AbaR-associated genes					
3' end of the ATPase gene	3ATP-F	gcaacccgtaaaacgcgatga	400	52	This study
	3ATP-R	tgagctgaaagttcgccgga			
5' end of the ATPase gene	5ATP-F	aggcaggcgtgaggccaat	490	52	This study
	5ATP-R	tgctcctgcagatttgccca			
uspA	USPA-F	tggaatgaccataagccccaa	401	50	This study
	USPA-R	ggggaaacacggcactcagac			
cadA	CADA-F	ctaggcgcctcgcttcagga	400	46	This study
	CADA-R	caatcaatgcgacgaatgcga			
tpnA (Tn*3*)	TPNA-F	acgtcggggctaaatcgcg	358	50	This study
	TPNA-R	ttccactgagcgtcagacccc			
resX	RESX-F	gcttgcagatcgacgcgct	425	50	This study
	RESX-R	catcagggcctttgcccgt			

TABLE S2. *Continued*

Gene or location	Primer pair	Sequence 5' - 3'	Amplicon (bp)	Annealing temperature	Reference
orf5[a]	ORF5-F	ggagcctccgaacgttcgg	461	50	This study
	ORF5-R	tgaaggttggatcccagccg			
IS26	IS26-F	tccatttcaggcgcataacgc	128	50	This study
	IS26-R	ggccgtacgctggtactgcaa			
J3[b]	J3-F	atttgccgagctgcacgtgaa	300	55	This study
	J3-R	accattcaggcactcgtgcct			
J5[c]	J5-F	tttgggaagcaatcaatagtc	310	55	This study
	J5-R	attggcctcacgcctgcct			

[a] The gene encoding acetyltransferase related to puromycin acetyltransferases.

[b] J3, junction between the 5 end of the island and the 3 end of the ATPase gene.

[c] J5, junction between the 3 end of the island and the 5 end of the ATPase gene.

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TABLE S2. Primers used for PCR mapping of AbaR islands

Region	Primers	Sequence 5'-3'	Amplicon length (bp)	Amplicon analysis (RE[a])	AbaR variant
3'ATPase-Tn*6019*	3ATP-F	gcaacccgtaaaacgcgatga	10788	RFLP (AccI, BsmAI)	AbaR3,10-19
	ARSB-R	ggcattggggattgcgatagg			
Tn*6019*-Tn*6018*	ARSB-F	gcaatcgctacagccagtgcc	4209	RFLP (BglI, ClaI)	AbaR3,10-13, 15-19
	CADA-R	caatcaatgcgacgaatgcga			
Tn*6018*-*topA* sequence	CADA-F	ctaggcgcctcgcttcagga	3132	RFLP (AccI, BclI)	AbaR3,10,13,15-18
	TOPA-R	tccggtttcgtggacacgtt			
topA sequence-3CS	TOPA-F	cgatgcgcttgtaggccgg	3669	RFLP (AccI, EcoRV)	AbaR3,10,13,15-18
	ORF5-R	tgaaggttggatcccagccg			
3CS-Tn*1696*	ORF5-F	ggagcctccgaacgttcgg	4309	RFLP (ApaI)	AbaR3,15,17,18
	MERA-R	taagcccagtggcaacgaacg			
Tn*1696*-Tn*1721*	MERA-F	cgtgcgtggtggtcagcac	3617	RFLP (HindIII)	AbaR3,15,17,18
	TETR-R	gaagccatgctggcggagaat			
Tn*1721*-ΔTn*2760*	TETR-F	cctgctcgaacgctgcgtc	6199	RFLP (ApaI, AccI)	AbaR3,15,17
	CAT-R	tcacattcttgcccgcctgat			
ΔTn*2760*-Tn*3*	CAT-F	gatgaacctgaatcgccagcg	1552	RFLP (BsmAI)	AbaR3,15,17
	TPNA-R	ttccactgagcgtcagacccc			
Tn*3*-ΔTn*5393*	TPNA-F	acgtcggggctaaatcgcg	5864	RFLP (HindII, EcoRV)	AbaR3
	IS26-R	ggccgtacgctggtactgcaa			
ΔTn*5393*	IS26-F	tccatttcaggcgcataacgc	3701	RFLP (EcoRI)	AbaR3,13
	TPNR-R	aagtatcgcgctgggccatg			
ΔTn*5393*-class 1 integron	TPNR-F	cgcaggtgtcgaacgggac	5520	RFLP (AccI, BfaI)	AbaR3,13
	AAC3-IA2	ctccgaactcacgaccga			
class 1 integron	AAC3-IA2	ctccgaactcacgaccga	5065	RFLP (BglI)	AbaR3[b],12,13,15-19
	ORF5-R	tgaaggttggatcccagccg			
3CS-Tn*6018*	ORF5-F	ggagcctccgaacgttcgg	3845	RFLP (HincII)	AbaR3,12-19
	CADA-R	caatcaatgcgacgaatgcga			
Tn*6018*-*5*'ATPase	CADA-F	ctaggcgcctcgcttcagga	6696	RFLP (HincII, StyI)	AbaR3,10-19
	5ATP-R	tgctcctgcagatttgccca			
3CS-*resX* sequence	ORF5-F	ggagcctccgaacgttcgg	1661	Sequencing	AbaR10
	RESX-R	catcagggcctttgcccgt			
topA sequence-Tn*6020*	TOPA-F	cgatgcgcttgtaggccgg	1925	Sequencing	AbaR12
	IS26-F	tccatttcaggcgcataacgc			
3CS-ΔTn*5393*	ORF5-F	ggagcctccgaacgttcgg	1465	Sequencing	AbaR13
	IS26-R	ggccgtacgctggtactgcaa			
Tn*6019*-Tn*6020*	3ATP-F	gcaacccgtaaaacgcgatga	5957	Sequencing	AbaR14
	APH3-IA2	gcgttgccaatgatgttacag			
Tn*3*-Tn*6020*	TPNA-F	acgtcggggctaaatcgcg	3865	Sequencing	AbaR15
	IS26-F	tccatttcaggcgcataacgc			
3CS-Tn*6020*	ORF5-F	ggagcctccgaacgttcgg	12831	Sequencing	AbaR16
	IS26-F	tccatttcaggcgcataacgc			
Tn*3*-Tn*6020*	TPNA-F	acgtcggggctaaatcgcg	4344	Sequencing	AbaR17
	IS26-F	tccatttcaggcgcataacgc			
Tn*1721*-Tn*6020*	TETR-F	cctgctcgaacgctgcgtc	6084	Sequencing	AbaR18
	IS26-F	tccatttcaggcgcataacgc			
Tn*6018*-Tn*6020*	CADA-F	ctaggcgcctcgcttcagga	2192	Sequencing	AbaR19
	IS26-F	tccatttcaggcgcataacgc			

[a] Restriction endonuclease (RE).

[b] AbaR3 islands carrying a class 1 integron with *aacC1*-orfP-ofrP-orfQ-*aadA1*.

5. DISCUSSION

This thesis has contributed to a better understanding of the mechanisms that make *A. baumannii* multidrug resistant in order to assist the development of strategies for combating this emerging pathogen.

In **Study I**, we investigated the epidemiology of resistance to carbapenems and other drugs in clinical strains of *Acinetobacter* spp. collected in the Czech Republic during 2005 and 2006. We have shown that *A. baumannii* was a predominant species accounting for as many as 72% of all isolates and that all multidrug-resistant (MDR) strains including those non-susceptible to carbapenems belonged to this species. The *A. baumannii* strains showed a bimodal distribution in terms of resistance phenotype and genotype: while fully susceptible strains were genotypically highly heterogeneous, MDR strains mostly belonged to one of the two main clonal lineages, i.e. EU clones I and II. Interestingly, the proportion of strains belonging to EU clone I was significantly lower in 2005-2006 (5%) as compared to 1991-1997 (51%). At the same time, the proportion of EU clone II strains within the *A. baumannii* population increased from 12% to 61%, respectively. Although the two studies were not directly comparable as the strain inclusion criteria differed, the data strongly suggested a significant shift towards EU clone II over one decade. Others have also reported that while EU clone I and other MDR lineages can be common or even prevail in some regions (Di Popolo *et al*., 2011; Villalón *et al*., 2011), strains belonging to EU clone II seem to dominate in the current global population of MDR *A. baumannii* (Fu *et al*., 2010; Higgins *et al*., 2010; D'Arezzo *et al*., 2011).

Our analysis of the genetic basis of carbapenem resistance has shown that the predominant mechanism in the Czech hospital population of *A. baumannii* was the IS*Aba1*-mediated up-regulation of the chromosomal OXA-51 β-lactamase rather than the acquisition of other enzymatic mechanisms except for three strains with the $bla_{\text{OXA-58}}$-like or $bla_{\text{OXA-40}}$-like genes. This and our other studies have revealed, despite the relative homogeneity of the typing characteristics of the EU clone II isolates, a remarkable variability in resistance phenotypes and genotypes, even when isolates with identical genomic typing patterns originated from the same locality (Krizova *et al*., 2008; Nemec *et al*., 2008; Krizova *et al*., 2011; Krizova *et al*., 2013). This variability is likely to result from the high genetic versatility of EU II which

might contribute to the ability of this lineage to develop resistance to nearly all clinically relevant antibiotics. The relatively frequent horizontal acquisition or loss of resistance genes, differences in the expression of intrinsic genes, or mutational changes influencing enzymatic activity or the binding of an antibiotic to its primary target are likely to play a role in this variability (Nemec *et al*., 2008; Krizova *et al*., 2011; Krizova *et al*., 2013).

In summary, **Study I** strongly suggests that the increase of *Acinetobacter* resistance to carbapenems in the Czech Republic has resulted from the spread of MDR *A. baumannii* strains belonging to EU clone II. In line with this, the spread of carbapenem resistant EU clone II strains have been observed worldwide (Higgins *et al*., 2012; Zarrilli *et al*., 2013). Thus, it could be hypothesised that carbapenem resistance has advantaged EU clone II over EU clone I strains in the hospital population of *A. baumannii* challenged with the growing consumption of these antibiotics.

Massive international travel has undoubtedly led to the dissemination of highly successful epidemic clones or strains (Higgins *et al*., 2010; Karah, 2011; Zarrilli *et al*., 2012). The emergence and spread of strains producing New Delhi metallo-β-lactamase (NDM) (Yong *et al*., 2009) are illustrative in this regard (Bonnin *et al*., 2012b; Johnson & Woodford, 2013; Patel *et al*., 2013;). A therapeutically important property of NDM is its potent carbapenemase activity and ability to degrade all *β*-lactams except for monobactams (Cornaglia *et al*., 2011).
It has been suggested that NDM-encoding genes can spread effectively between strains of the same or even different species owing to IS*Aba125* which borders on these bla_{NDM} genes (Poirel *et al*., 2012a). In **Study II**, we investigated a high-level carbapenem-resistant *A. baumannii* strain imported to a Czech hospital from Egypt. This strain was shown to belong to EU clone I and to carry the genes encoding carbapenemases NDM-1 and OXA-23 associated with IS*Aba125* and IS*Aba1*, respectively, as well as the intrinsic $bla_{\text{OXA-51-like}}$ gene preceded by IS*Aba1*. Thus, the unusually high resistance to carbapenems in this strain could be attributed to as many as three different *β*-lactamases. As discussed in **Study III**, it is not trivial to define the precise role of individual resistance mechanisms under such complex circumstances unless experiments targeting their expression are employed. Besides the carbapenemase genes, the strain carried a number of additional mechanisms which explains its high-level resistance to all clinically relevant drugs except for colistin and tobramycin.

Overall, the results of **Studies II** and **III** as well as **Study I** illustrate the importance of a detailed genetic analysis for the assessment of the epidemiology of resistance and, on the practical basis, emphasize the need to screen for MDR strains in patients admitted from other healthcare settings (especially from abroad) together with parallel improvements in hygiene and epidemiological measures against the spread of MDR bacteria.

In **Study IV**, we described a clinically relevant mechanism of sulbactam resistance in *A. baumannii* based on the production of the TEM-1 β-lactamase. This outcome was supported by (i) the correlation between the level of sulbactam resistance and the quantitative expression of the $bla_{\text{TEM-1}}$ gene, (ii) the transferability of sulbactam resistance via a $bla_{\text{TEM-1}}$-carrying plasmid, and (iii) susceptibility to sulbactam in a clinical strain expressing TEM-19, a rare low-activity variant of TEM-1. The $bla_{\text{TEM-19}}$ gene was found in a sulbactam susceptible *A. baumannii* strain which was epidemiologically linked to another strain harbouring $bla_{\text{TEM-1}}$ and being sulbactam resistant. Therefore, the conversion of $bla_{\text{TEM-1}}$ into $bla_{\text{TEM-19}}$ may have occurred during the in-hospital spread of this bacterium, which led to the restoration of sulbactam susceptibility.

Despite the common presence of the $bla_{\text{TEM-1}}$ gene in *A. baumannii* (Ben *et al*., 2011; Manageiro *et al*., 2012), a number of MDR strains including carbapenem-resistant ones do not carry this gene and retain susceptibility to sulbactam. The results of **Study I** (2005-2006) and our unpublished data from 2011 and 2012 show that approximately 65% of MDR isolates (mainly carbapenem non-susceptible) are susceptible to sulbactam and this proportion has not significantly changed over this period. Thus, sulbactam can be considered as a valuable therapy in some cases where therapeutic options are very limited (Dijkshoorn *et al*., 2007; Tripodi *et al*., 2007; Peck *et al*., 2012; Santimaleeworagun *et al*., 2011; **Study I**; **Study IV**; Vila & Pachón, 2012)

Studies V and **VI** have engaged in the issue of genomic resistance islands (AbaRs) which are believed to accrete multiple mobile elements associated with drug resistance in *A. baumannii* (Roca *et al*., 2012; Ramirez *et al*., 2013). Our pilot experiments aimed to estimate the occurrence of AbaRs in *A. baumannii* suggested that these structures were commonly present in both EU clone I and clone II but absent from EU clone III. Similarly, other authors have detected AbaRs in MDR strains of EU clones I and II (Arduino *et al*., 2012; Huang *et al*.,

2012; Kim *et al*., 2012; Nigro & Hall, 2012a, 2012b; Seputiene *et al*., 2012; Farrugia *et al*., 2013; Ramirez *et al*., 2013; Saule *et al*., 2013; Sung *et al*., 2012;). However, our preliminary data also indicated the presence of AbaR-like structures lacking resistance genes in some strains which were fully susceptible to antibiotics and belonged neither to EU clone I nor to clone II (Krizova *et al*., 2009). Congruently, Fournier *et al.* (Fournier *et al*., 2006) have found an AbaR island (AbaG1) in a susceptible strain isolated from a louse. These observations suggested that AbaRs were widespread in the general population of *A. baumannii* and it has been hypothesised that these structures may have functioned as chromosomal "hotspots" for the acquisition of resistance genes (Fournier *et al*., 2006).

In **Study VI**, we showed that genetic structures related to AbaR3 were common and spread globally in strains of EU clone I. Our finding of a 63-kb island structurally congruent with AbaR3 in the oldest known MDR isolate of EU clone I from 1977 (**Study V**) indicates that highly sophisticated AbaR3-like islands were associated with this clonal lineage already in the late 1970s. Similarly, Post & Hall (Post & Hall, 2009) have concluded that AbaR1, the largest known AbaR3-like island, possibly evolved from an ancestral AbaR3 by an additional acquisition of a 29-kb integron carrying AbaR1-specific resistance genes such as that encoding extended spectrum *β*-lactamase VEB-1. This genetic event could therefore advantage the AbaR1-carrying strain in the hospital environment.

To analyse the structures of AbaR-like islands, we developed PCR mapping strategies (**Study V**) based on the publicly available sequences of four AbaRs (Fournier *et al*., 2006; Adams *et al*., 2008; Iacono *et al*., 2008; Post & Hall, 2009). Even though this approach consists of time-consuming long-range PCR reactions requiring stringent standardization and optimization, it was successfully applied in a number of the following studies (**Study VI**, Post *et al*., 2010b; Nigro & Hall, 2012a, 2012b; Ramirez *et al*., 2013). However, recent advances in whole genome sequencing have brought about more effective methodologies to gain precise information about primary genomic structures. Thus, it is expected that rapidly developing bioinformatics approaches will provide more precise insights into the structure and genomic context of AbaRs as well as other genetic structures playing a role in antibiotic resistance.

In **Study VI**, two main types of the modification of AbaR3 were revealed in MDR EU clone I strains: complete AbaR3 islands differing only in the internal composition of class 1 integrons

and truncated AbaR3 variants. We suggested that the already established AbaR3 islands could have undergone changes in the structure of integrons via the exchange, loss, or acquisition of internal gene cassettes. Furthermore, we assumed that truncated AbaR3 variants had resulted from IS*26*-mediated deletions or homologous recombination. Overall, it is likely that the origin of AbaR3 laid in the antibiotic regimes administered in Europe during the 1970s and 1980s. This structure provided *A. baumannii* with resistance to antimicrobial agents commonly used at that period and thus might have facilitated the spread of the bacterium. Subsequent changes in the use of antibiotics in hospitals might have further weakened selective pressure on the original AbaR3 structure and gave rise to truncated derivatives of AbaR3. In contrast to AbaR3-like structures, EU clone II-associated AbaR4-like islands commonly harbour genes ($bla_{\text{OXA-23}}$, $bla_{\text{PER-1}}$) conferring resistance to modern antibiotics such as carbapenems or broad-spectrum cephalosporins (Hamidian &Hall, 2011; Kim *et al*., 2012; Seputine *et al*., 2012) which may have contributed to the current global predominance of EU clone II.

Although AbaR3-like and AbaR4-like islands typically present in EU clone I and II, respectively, share the same integration locus (*comM*), the concept of a common ancestor for these two types of AbaRs is not conclusive. The possible reason for the hotspot homology was proposed by Rose (Rose, 2010) and Nigro & Hall (Nigro & Hall, 2011) who have shown that both core transposons (Tn*AbaR3*-like and Tn*AbaR4*-like) are related to the Tn*7* transposon and therefore may independently recognise the same locus. Furthermore, the structural differences between AbaR3-like and AbaR4-like islands (Roca *et al*., 2012) and the distinctive evolutionary history of the clones (Diancourt *et al*., 2010) support the assumption that AbaRs have evolved independently in each of these clones (Post *et al*., 2010b; Krizova *et al*., 2011; Zhou *et al*., 2011; Roca *et al*., 2012; Seputine *et al*., 2012).

It is conceivable that AbaRs may be horizontally transferred *en bloc.* Given the high spectrum of resistance genes carried by these structures, such an ability might have a serious impact on the spread of multidrug resistance in susceptible bacterial populations. However, the only known experiments attempting to transpose or delete AbaRs *en bloc* gave ambiguous results or failed (Kochar *et al*., 2012; Bonnin, unpublished). Therefore this important question still remains unanswered.

6. CONCLUSIONS

- The emergence of *Acinetobacter* resistance to carbapenems in the Czech Republic was shown to be associated with the spread of MDR *A. baumannii* strains belonging to EU clone II. The predominant mechanism of carbapenem resistance in these strains appeared to be the IS*Aba1*-mediated up-regulation of the chromosomal gene for ß-lactamase OXA-51. A striking variation in antibiotic resistance of strains of EU clone II was noted, which likely resulted from the differences in the presence of acquired resistance genes and possibly from the effect of IS*Aba1* on the expression of intrinsic genes. This high genetic versatility of EU clone II might contribute to its ability to develop resistance to nearly all clinically relevant antibiotics.

- A high-level carbapenem resistant *A. baumannii* strain imported to the Czech Republic from Egypt in 2011 was assigned to epidemic EU clone I. We found that the strain harboured the $bla_{\text{NDM-1}}$ gene associated with IS*Aba125* together with the $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-51}}$-like genes, both carrying IS*Aba1* in their promoter regions. The combination of these mechanisms was responsible for the high-level resistance of the strain to all *β*-lactams.

- The production of TEM-1 *β*-lactamase was shown to result in sulbactam resistance in *A. baumannii*. This conclusion was supported by the correlation between the level of sulbactam resistance and the expression of the $bla_{\text{TEM-1}}$ gene, by the transferability of sulbactam resistance via a $bla_{\text{TEM-1}}$-carrying plasmid, and by the susceptibility of a clinical strain expressing TEM-19, a low activity variant of TEM-1. The expression of the $bla_{\text{TEM-1}}$ gene in MDR *A. baumannii* strains might play an important role in clinical resistance to sulbactam.

- Highly sophisticated AbaR3-like genomic islands were shown to be common in strains belonging to EU clone I from as early as the late 1970s. The truncated AbaR3-like variants are likely to arise from IS*26*-mediated deletions or by homologous recombination. We suggested that these islands may have provided strains of EU clone I with a selective advantage facilitating their spread in European hospitals in the 1980s or

before but currently, they do not seem to play a substantial role in the ongoing development of antimicrobial resistance in *A. baumannii*.

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8. CURRICULUM VITAE

Lenka Křížová was born on 26 December, 1981 in Nymburk, Czech Republic. After graduation from the secondary school (Gymnázium Jiřího z Poděbrad) in Poděbrady (central Bohemia), she studied at the Faculty of Education, University of Hradec Králové (eastern Bohemia). In 2007, she graduated with a Master's degree in Biology and Chemistry Education.

After graduation, she joined the Laboratory of Bacterial Genetics headed by Associate Professor Alexandr Nemec at the National Institute of Public Health in Prague where she has been working as a bacteriologist. She completed two research fellowships abroad: (i) at the Department of Infectious Diseases of the Leiden University Medical Center (November 2008, one month) and (ii) at the Laboratoire de Bacteriologie of the Hospital de Bicetre in Paris (February-July 2011, five months) within the ERASMUS programme. She has been involved in four research grant projects focused on the taxonomy of the genus *Acinetobacter*, population structure of *Acinetobacter baumannii*, and the genetic basis and evolution of resistance mechanisms in clinical strains of this species. Her current interests include the molecular genetic basis and evolution of multidrug resistance in *Acinetobacter baumannii* and molecular genetic methods in classification, identification, and typing of Gram-negative bacteria.

9. LIST OF PUBLICATIONS

Original articles in peer reviewed international journals

Périchon B, Goussard S, Walewski V, **Krizova L**, Cerqueira G, Murphy C, Feldgarden M, Wortman J, Clermont D, Nemec A, and Courvalin P. Identification of fifty class D β-lactamases and of sixty-five Acinetobacter-Derived Cephalosporinases in *Acinetobacter* spp. *Antimicrob Agents Chemother* 2014; 58(2): 936-49.

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Original articles in non-peer reviewed journals

Alexandr Nemec, **Lenka Křížová**, Martina Maixnerová, Andrea Šmejcová, PSA. Multirezistentní *Acinetobacter baumannii* nesoucí geny pro karbapenemázy NDM-1 a OXA-23 importovaný do České republiky. Zprávy CEM 2011; (20)8: 295-8. In Czech.

Other presentations

A total of 19 abstracts in journals or abstract books.

A total of 4 oral presentations at international conferences.