Distribution and genetic composition of carbapenem-resistance determinants in clinical *Acinetobacter* isolates – focus on carbapenem-hydrolysing β-lactamases

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1. Introduction

Robert Koch (1843-1910) first confirmed the role of bacteria as causative organisms of infectious diseases by his studies on *Bacillus anthracis*, the causative organism of anthrax.[1] The targeted treatment of a bacterial infection using an antimicrobial agent was initially performed in 1910 by Paul Ehrlich (1845-1915) and Sahachiro Hata (1873-1938) by the successful treatment of syphilis by the use of the chemically synthesized arsenic compound arsphenamine (*Salvarsan*®) (Figure 1).[2] This was the foundation of modern antimicrobial chemotherapy, the medical treatment of infectious microbial diseases. Since then, multiple antimicrobial compounds were developed, illustrated by the timeline in Figure 1. The development of novel antimicrobial agents was driven forward by the identification of naturally occurring antibiotics. The first antibiotic identified was the β-lactam penicillin, which was discovered by Alexander Fleming (1881-1955) in 1928 by the observation that growth of a *Staphylococcus aureus* culture on a solid medium was inhibited in the presence of *Penicillium notatum*.[3] Penicillin was introduced into clinical therapy in 1941.

In parallel to the introduction of novel antimicrobial agents, resistant bacteria, which were able to overcome the antimicrobial effect of certain drugs, emerged. β-lactam resistance was initially discovered in 1940 with the observation of a penicillin-resistant *S. aureus*.[4] An emerging threat in health-care facilities nowadays is the spread of multi-drug resistant (MDR) and pan-drug resistant (PDR) pathogens. Multi-drug resistance implies non-susceptibility to one or more agents of at least three antimicrobial classes, whereby available treatment options can be dramatically reduced. Pan-drug resistance implies resistance to all currently available antimicrobials which are usually active against the respective organism, which leads to untreatable infections. A global challenge in health-care facilities is *Acinetobacter baumannii*, which possesses a high propensity to multi-drug resistance and epidemic spread.[5, 6] Until 1970 *Acinetobacter* spp. isolates retained susceptibility to almost all available antimicrobials, but since the early 1980s the rate of antimicrobial resistance in *Acinetobacter* spp. isolates is raising.[7]

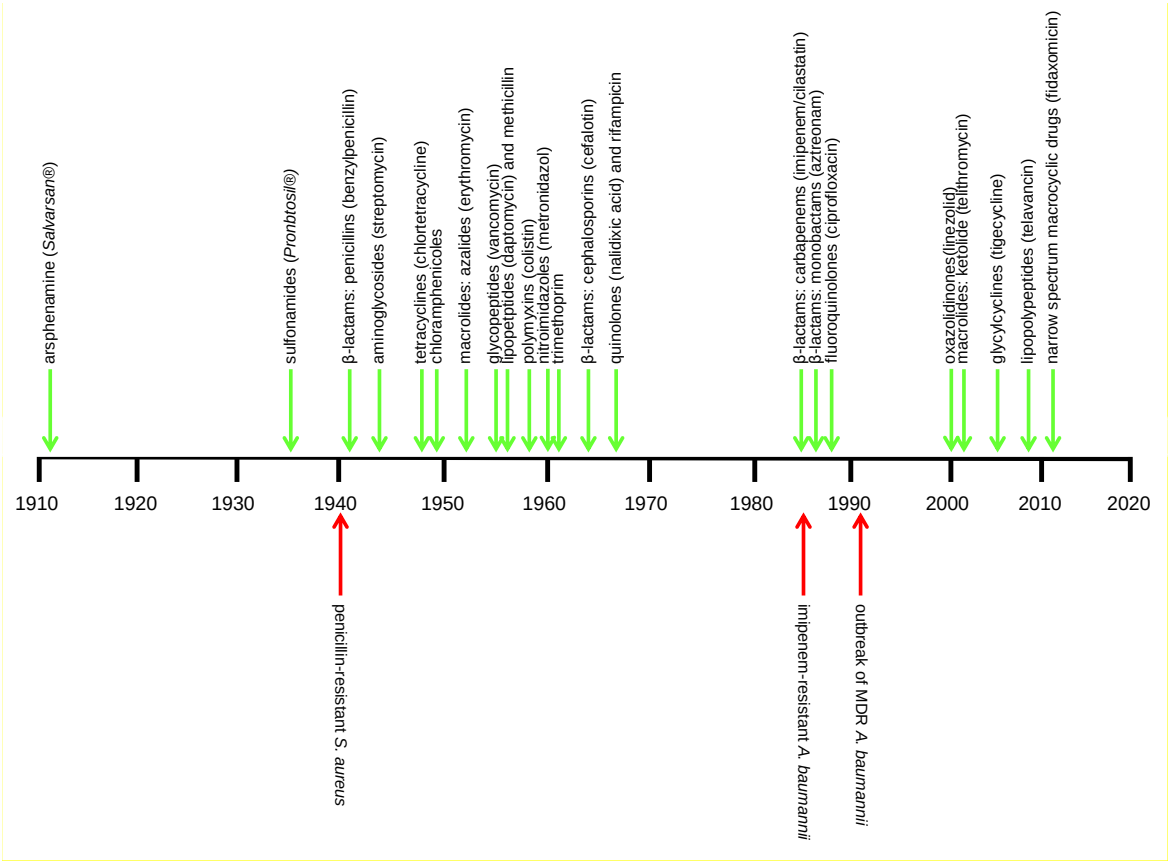

Figure 1: Introduction of antimicrobial agents and classes from 1911 to 2011. Green arrows highlight the date of introduction of antimicrobial agents and classes. Red arrows highlight the primarily detection of β-lactam resistance in 1940, the first detection of a carbapenem-resistant *Acinetobacter* isolate in 1985 and the first report of an outbreak of MDR *A. baumannii* in a hospital in New York City, USA, in 1991.[4, 8, 9]

Since the early 1990s, the majority of *A. baumannii* isolates retained susceptibility to the β-lactam class of carbapenems. Interestingly, the first carbapenem-resistant *A. baumannii* isolate was reported in 1993, but was isolated in 1985 in parallel to the introduction of carbapenems in the clinical treatment of infections.[10] Interestingly, at the time of isolation, carbapenems have not been in use in the respective hospital.[10] To date carbapenems are the drugs of choice to treat infections with MDR *A. baumannii*. However, carbapenem resistance in *A. baumannii* has increased over the years and emerged as a global threat in health-care facilities. To illustrate the consequence of *Acinetobacter* spp. in the clinical setting the *Acinetobacter* genus will be discussed below with a special focus on *A. baumannii* and antimicrobial resistance.

1.1 The genus *Acinetobacter*

The genus *Acinetobacter* belongs to the family of *Moraxellaceae* in the class of γ-proteobacteria. *Acinetobacter* spp. are Gram-negative, strictly aerobic, non-fermentative bacteria. Untypical for *Moraxellaceae*, the cytochrome c oxidase test of *Acinetobacter* spp. is usually negative, indicating the presence of an alternative cytochrome in the respiratory chain. The genus *Acinetobacter* was first proposed in 1954 by Brisou and Prévot, while initial detection of an *Acinetobacter* strain goes back to 1911 when the Dutch microbiologist Beijerinck isolated `*Micrococcus calcoaceticus*´ which was finally re-named as *Acinetobacter calcoaceticus*.[11, 12] The term *Acinetobacter* has originated from the greek term `akinetos´ which means "non-motile". However, the dogma of *Acinetobacter* being non-motile has been disproved. Recent studies have shown that some *Acinetobacter* species, in particular *A. baumannii*, encode several genes necessary for motility and in fact can be motile when growing on semi-solid media in the absence of light.[13, 14]

1.1.1 Taxonomy of the genus *Acinetobacter*

The genus *Acinetobacter* currently comprises 33 distinct species with valid names (http://www.bacterio.cict.fr/a/Acinetobacter.html) and 15 provisional taxa which include either genomic species (*Acinetobacter* genomic species) delineated by DNA-DNA hybridization[6] or species with effectively (but not validated) published names (Table 1). Once a proposed novel species name is published on the validation list of the International Journal of Systematic and Evolutionary Microbiology (IJSEM), it is accepted as a valid species name (http://ijs.sgmjournals.org/site/misc/about.xhtml#VL). Effectively published species names are not yet validated by the IJSEM.

Table 1: The species composition of the genus *Acinetobacter* (adapted from http://apps.szu.cz/anemec/Classification.pdf). Species names in parentheses are effectively published, but have not been validated yet.

Valid species names	Main sources of isolation	Reference
A. baumannii	Human, animals	Bouvet & Grimont[15]
A. baylyi	Soil	Carr *et al.*[16], Vaneechoutte *et al.*[17]
A. beijerinkii	Human, animals, soil, water	Nemec *et al.*[18]
A. bereziniae (formerly AGS 10)	Human	Nemec *et al.*[19], Bouvet & Grimont[15]
A. boissieri	Floral nectar	Álvarez-Pérez *et al.*[20]
A. bouvetii	Activated sludge	Carr *et al.*[16]
A. brisouii	Peat	Anandham *et al.*[21]
A. calcoaceticus	Soil	Bouvet & Grimont[15]
A. gerneri	Activated sludge	Carr *et al.*[16]
A. grimontii (= *A. junii*)	Activated sludge	Carr *et al.*[16], Vaneechoutte *et al.*[22]
A. guillouiae (formerly AGS 11)	Human, soil	Nemec *et al.*[19], Bouvet & Grimont[15]
A. gyllenbergii	Human	Nemec *et al.*[18]
A. haemolyticus	Human	Bouvet & Grimont[15]
A. harbinensis	River water	Li *et al.*[23]
A. indicus	Dumpsite	Malhotra *et al.*[24]
A. johnsonii	Human, animals	Bouvet & Grimont[15]
A. junii	Human	Bouvet & Grimont[15]
A. kookii	Soil	Choi. *et al.*[25]

AGS, *Acinetobacter* genomic species

Table 1 continued: The species composition of the genus *Acinetobacter* (adapted from http://apps.szu.cz/anemec/Classification.pdf). Species names in parentheses are effectively published, but have not been validated yet.

Valid species names	Main sources of isolation	Reference
A. lwoffii (formerly AGS 8 and 9)	Human, animals	Bouvet & Grimont[15], Tjernberg & Ursing[26]
A. nectaris	Floral nectar	Álvarez-Pérez *et al.*[20]
A. nosocomialis (formerly AGS 13TU)	Human	Nemec *et al.*[27], Tjernberg & Ursing[26]
A. parvus	Human, animals	Nemec *et al.*[28]
A. pittii (formerly AGS 3)	Human, soil	Nemec *et al.*[27], Bouvet & Grimont[15]
A. puyangensis	Canker bark	Li *et al.*[29]
A. qingfengensis	Canker bark	Li *et al.*[30]
A. radioresistens (formerly AGS 12)	Human, soil, cotton	Nishimura *et al.*[31], Bouvet & Grimont[15]
A. rudis	Raw milk, wastewater	Vaz-Moreira *et al.*[32]
A. schindleri	Human	Nemec *et al.*[33]
A. soli	Human, soil	Kim *et al.*[34]
A. tandoii	Activated sludge	Carr *et al.*[16]
A. tjernbergiae	Activated sludge	Carr *et al.*[16]
A. towneri	Activated sludge	Carr *et al.*[16]
A. ursingii	Human	Nemec *et al.*[33]
A. venetianus	Sea water	Vaneechoutte *et al.*[35], Di Cello *et al.*[36]

AGS, *Acinetobacter* genomic species

Table 1 continued: The species composition of the genus *Acinetobacter* (adapted from http://apps.szu.cz/anemec/Classification.pdf). Species names in parentheses are effectively published, but have not been validated yet.

Provisional designations and published species names	Main sources of isolation	Reference
AGS 6	Human	Bouvet & Grimont[15]
AGS 13BJ/14TU	Human	Bouvet & Jeanjean[37], Tjernberg & Ursing[26]
AGS 14BJ	Human	Bouvet & Jeanjean[37]
AGS 15BJ	Human	Bouvet & Jeanjean[37]
AGS 15TU	Human	Tjernberg & Ursing[26]
AGS 16	Human, vegetables	Bouvet & Jeanjean[37]
AGS 17	Human, soil	Bouvet & Jeanjean[37]
AGS `between 1 and 3´	Human	Gerner-Smidt & Tjernberg[38]
AGS `close to 13TU´	Human	Gerner-Smidt & Tjernberg[38]
`*A. antiviralis*´	Tobacco plant roots	Lee *et al.*[39]
`*A. kyonggiensis*´	Sewage	Lee & Lee[40]
`*A. marinus*´	Sea water	Yoon *et al.*[41]
`*A. oleivorans*´	Rice paddy	Kang *et al.*[42]
`*A. oryzae*´	Rice	Chaudhary *et al.*[43]
`*A. seohaensis*´	Sea water	Yoon *et al.*[41]
`*A. septicus*´ (= *A. ursingii*)	Human	Kilic *et al.*[44], Nemec *et al.*[45]

AGS, *Acinetobacter* genomic species

As shown by Vaneechoutte *et al. A. grimontii* is identical to *A. junii*, which means that both species names belong to the same taxon.[22] In addition, Nemec *et al.* have shown that `*A. septicus´* is *A. ursingii*.[45] Several *Acinetobacter* genomic species harbor specific abbreviations which correspond to the initials of the authors who first described them, with BJ referring to Bouvet and Jeanjean and TU referring to Tjernberg and Ursing. During the last years, several provisional designations received valid species names. For example, Bouvet & Grimont proposed the species name *A. lwoffii* for *Acinetobacter* genomic species 8.[15] The taxon includes *Acinetobacter* genomic species 9, as suggested by Tjernberg & Ursing, which also showed that *Acinetobacter* genomic species 12 corresponds to *A. radioresistens*.[26] Furthermore, Nemec *et al.* recently proposed species names for *Acinetobacter* genomic species 3, 10, 11 and 13TU, which are now validated as *A. pittii*, *A. bereziniae*, *A. guillouiae* and *A. nosocomialis*, respectively.[19, 27]

1.1.2 Natural habitats and clinical relevance of *Acinetobacter* spp.

Species of the genus *Acinetobacter* can be recovered from environmental samples, like soil, vegetables and surface water. For example, *A. calcoaceticus*, *A. pittii*, *A. johnsonii* and *A. guillouiae* have been isolated from soil and water.[5] Furthermore, *Acinetobacter* spp. can also be commensals of the human skin, mucous membranes and intestinal tract and are mainly isolated as colonizing organisms. Previous studies have reported *Acinetobacter* carriage rates in healthy humans between 25% (fecal colonization) and 44% (skin colonization).[46-48] The predominant *Acinetobacter* spp. detected in fecal samples were *A. johnsonii* and *A. guillouiae*. Frequently skin colonizing *Acinetobacter* spp. include *A. lwoffii*, *A. johnsonii*, *A. junii*, *A. pittii*, *A. radioresistens* and *Acinetobacter* genomic species 15BJ. Although *A. baumannii* and *A. nosocomialis* have been recovered from vegetables, human skin, body lice or animals, both species are mainly recovered from clinical specimens and infected patients.[5, 49] In summary, certain *Acinetobacter* spp., like *A. calcoaceticus*, *A. johnsonii* and *A. guillouiae*, seem to be widely distributed in the environment, while the natural habitat for *A. baumannii* and *A. nosocomialis* remains unclear.

To date the most prevalent clinically relevant species of the *Acinetobacter* genus is *A. baumannii*, which possesses a high propensity to multi-drug resistance and is frequently associated with clinical outbreaks, which means infection of multiple patients in a hospital by the same strain. However, *A. pittii* and *A. nosocomialis* are also increasingly reported in association with outbreaks and antimicrobial resistance.[13]

1.1.3 *Acinetobacter* species identification

A wide range of molecular methods and semi-automated systems have been described to identify *Acinetobacter* isolates to the species level. These include for example, DNA-DNA hybridization, amplified fragment length polymorphism (AFLP), amplified ribosomal DNA restriction analysis (ARDRA), matrix-assisted laser desorption ionization-time-of-flight mass spectrometer (MALDI-TOF MS), semi-automated systems such as the Vitek2 system, amplification of the gyrase β-subunit gene (*gyrB*) by multiplex PCR and partial sequencing of the RNA-polymerase β-subunit gene (*rpoB*).

The reference standard for *Acinetobacter* species identification is **DNA-DNA hybridization**.[15] The technique is based on the determination of the melting temperature of hybridized complementary DNA strands. The DNA of a strain of interest and a reference strain are denatured. The reference DNA is radioactively labeled and mixed with an excess of the DNA of interest. Single-stranded DNA of both strains will bind complementary. These hybrid DNA-duplexes will be subsequently denatured (DNA-melting). The radioactive labeling allows tracing of the melting of heteroduplex DNA (double-stranded DNA which comprises single strands of the reference DNA and the DNA of interest). The more differences the DNA strands possess, the weaker the covalent bonds are, which results in a lower melting temperature. Therefore less related strains reveal a lower melting temperature than closely related strains. Although DNA-DNA hybridization is a suitable method for phylogenetic analysis and species identification, it is very laborious and thereby impractical for routine identification of *Acinetobacter* species.[50]

AFLP and **ARDRA** are based on restriction digest and amplification of DNA fragments.[51, 52] AFLP operates with a frequent cutting restriction enzyme (4 bp recognition site) and a rare cutting enzyme (usually 6 bp recognition site). After digestion, fragments which possess restriction sites of a frequent and a rare cutting enzyme are specifically amplified and radioactively or fluorescently labeled in two PCR reactions. In contrast, ARDRA is based on the amplification of species-specific ribosomal genes which are subsequently digested by multiple restriction enzymes. Species identification is performed by the visual analysis of PCR products (AFLP) or restriction patterns (ARDRA) of a strain of interest in comparison to reference strains.

In routine laboratories the semi-automated **MALDI-TOF MS** (Bruker, Bremen, Germany) and **Vitek2** (bioMérieux, Nürtingen, Germany) system are frequently used for *Acinetobacter* species identification.[53, 54] MALDI-TOF MS is based on the crystallization and laser-mediated ionization of matrix-embedded samples and generates a species-specific mass spectrum, which can be compared to an online database.[53] Vitek2 uses specifically designed cards which contain cavities either with dehydrated substrates for species identification or dehydrated antimicrobial agents for susceptibility testing. A standard inoculum of an isolate is prepared and added to the cavities of the card. The incubation, reading and interpretation of the card is automatically performed by a computer-based system which enables species identification within 5-8 hours. Phenotypically it is very hard to distinguish between the environmental species *A. calcoaceticus* and the clinically important organisms *A. baumannii*, *A. pittii* and *A. nosocomialis*.[5] Thereby the latter species were combined in the "*A. baumannii* group", whereas for all the four species the designation "*A. calcoaceticus-A. baumannii* complex" had been proposed.[55] Based on their phenotypic similarity, the Vitek2 system is not suitable to differentiate among species of the "*A. calcoaceticus-A. baumannii* complex".[54]
A simple method to distinguish between these species is the ***gyrB* multiplex PCR**, recently established by Higgins *et al.*[56, 57] The PCR is based on species-specific primer pairs that amplify partial regions of the *gyrB* gene, whereby specific amplicon sizes allow the differentiation between *A. baumannii*, *A. calcoaceticus*, *A. nosocomialis* and *A. pittii*.

Partial *rpoB* sequencing has been found to be a reliable tool for species identification in the genus *Acinetobacter*. La Scola *et al.* identified two variable regions within the gene which are flanked by conserved sequences.[58] Sequencing of these 350-450 bp variable regions can be used to distinguish between *Acinetobacter* species. Based on its polymorphism, *rpoB* sequencing seems to be a more reliable method for species identification in *Acinetobacter* then sequencing of the 16S rRNA gene.[58]

1.1.4 *Acinetobacter baumannii*: characteristics of a successful pathogen

A. baumannii, also denoted as the "Gram-negative methicillin-resistant *S. aureus* (MRSA)", is a hospital-acquired pathogen, which mainly infects patients with impaired host defenses. Therapy of *A. baumannii* infections can be difficult due to wide distribution of **multi-drug resistance** in combination with increasing rates of carbapenem resistance.[5, 6] Accumulation of multiple resistance mechanisms in *A. baumannii* is based on great genome plasticity, the upregulation of innate resistance mechanisms and acquisition of foreign resistance genes.[13] **Infections** caused by *A. baumannii* include bloodstream infections, urinary tract infections, wound infections and meningitis, but particularly ventilator-associated pneumonia (VAP). Although community-acquired pneumonia has been reported in tropical climates, *A. baumannii* is rarely isolated outside of the hospital environment.[5] **Risk factors** for acquisition of *A. baumannii* infections are advanced age, immune suppression and severe underlying diseases, indwelling catheters, mechanical ventilation, invasive procedures, for example major surgeries and cardiac catheter examination, contact with patients that are infected or colonized with *A. baumannii*, extended hospital stay, particularly on intensive care units (ICU), and previous antimicrobial therapy.[5, 6] The clinical impact of nosocomial *A. baumannii* infections is controversial. Although high mortality rates have been reported, this could be influenced by underlying diseases.[59] *A. baumannii* is regarded as a low virulence pathogen, as it mainly affects immunocompromised patients. However, the organism possesses several attributes which support its ability to cause an infection. Recently identified **virulence factors** include lipopolysaccharides, capsular polysaccharides, phospholipases, the penicillin-binding protein 7/8, outer membrane vesicles (OMV) and an acinetobactin-mediated iron acquisition system.[60]

An additional virulence factor is the outer membrane protein OmpA, which triggers adherence and invasion of epithelial cells and can induce apoptosis in host cells. The dissemination of OmpA into host cells is provided by OMVs.[60]

Main **reservoirs** for *A. baumannii* in the hospital seem to be contaminated medical equipment, as well as colonized and infected patients.[6] For example, *A. baumannii* can be easily spread through the air from the respiratory tracts of infected or colonized patients and contaminate the environment. Subsequent **transmission** of *A. baumannii* is supported by its prolonged viability outside of the human body. For example, the species can persist up to 4 weeks on dry surfaces from where it can easily spread via the hands of the hospital staff, patients and visitors.[61] Furthermore, *A. baumannii* possesses the ability to form biofilms on plastic and glass surfaces which might support colonization of medical devices like catheters and intubation equipment.[62, 63] In addition, *A. baumannii* isolates can be resistant to disinfectants which results in dramatically reduced infection control opportunities.[64, 65]

Long-term viability and transmission of *A. baumannii* in the hospital environment increases the likelihood of *A. baumannii* strains to colonize multiple patients. Consistently, *A. baumannii* outbreaks have been reported in hospitals around the world which highlights the potential for *A. baumannii* to spread epidemically.[59] This is also mirrored by the presence of several distinct *A. baumannii* clonal lineages. Several studies have demonstrated worldwide distribution of at least eight **international clonal lineages** (IC1 to IC8) of closely related *A. baumannii* isolates. Initially IC1, IC2 and IC3 were found to be distributed in European countries whereby these lineages were termed European clones I to III (EUI to EUIII).[66] Recent worldwide epidemiological studies identified global distribution of EUI to EUIII and revealed presence of additional *A. baumannii* clonal lineages, which were assigned as worldwide clonal lineages 1 to 8 (WW1 to WW8) with WW1 to WW3 corresponding to EUI to EUIII.[67, 68] To avoid confusion the terms “European clonal lineages” and “worldwide clonal lineages” were subsequently converted into “international clonal lineages”. To date IC1 and IC2 are the predominant lineages worldwide, while IC3 seems to be more prevalent in Europe.[69]

1.1.5 Molecular typing of *A. baumannii*

Various molecular typing methods have been established to investigate the clonal relatedness of *A. baumannii* isolates, whereby the global epidemiology can be monitored and outbreaks can be analyzed. Frequently used typing methods include restriction-based pulsed-field gel electrophoresis (PFGE), amplification of repetitive extragenic palindromic regions (rep-PCR), multi-locus sequence typing (MLST) and sequence group typing.

PFGE is considered to be the "gold-standard" of *A. baumannii* typing.[70] The genomic DNA is restricted and fragments are separated on an agarose gel. Based on periodical changes of the electrical field during the electrophoresis, PFGE is suitable to separate high molecular nucleic acids of >10 kb, but this increases the gel running time compared to normal vertical gel electrophoresis. According to Tenover *et al.* isolates are regarded as epidemiologically related if the number of band differences in their restriction patterns is ≤ 6.[71]

With regards to *A. baumannii*, **MLST** is based on the partial sequencing of seven chromosomal housekeeping genes. The method can be used for epidemiological studies, but is not suitable for fine-typing, for example to confirm clonal identity between different isolates. Two schemes exist, which can be used to distinguish between *A. baumannii* sequence types (ST); the Bartual (http://pubmlst.org/abaumannii/) and the Pasteur scheme (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Abaumannii.html).[72-74] Both schemes share three loci for sequence analysis; *cpn60* (60 kDa chaperonin), *gltA* (citrate synthase) and *recA* (homologues recombination factor). In addition, the Bartual scheme includes sequences of *gyrB* (DNA gyrase β-subunit), *gdhB* (glucose dehydrogenase B), *gpi* (glucose-6-phosphate isomerase) and *rpoD* (RNA polymerase σ^{70} factor), while the Pasteur scheme includes sequences of *fusA* (elongation factor EF-G), *pyrG* (CTP synthase), *rplB* (50S ribosomal protein L2) and *rpoB* (RNA polymerase β-subunit). According to the websites, 770 and 493 *A. baumannii* ST have been so far identified by the Bartual and Pasteur scheme, respectively.

rep-PCR based DiversiLab (bioMérieux, Nürtingen, Germany) is a semi-automated method, which has been recently used to identify IC1 to IC8.[67] Primers amplify short repetitive extragenic palindromic regions which are spread in the *Acinetobacter* genome. rep-PCR products of the isolates of interest are loaded on a DNA chip and the software generates a virtual gel, a dendrogram and a similarity matrix. The results can be compared to the personal strain library. A similarity of > 95% indicates clonal relatedness, while a similarity of ≥ 98% indicates clonal identity.[75]

Furthermore, clonal relatedness of *A. baumannii* isolates can be investigated by **sequence group typing** which is based on two allele-specific multiplex PCRs, which amplify the chromosomal genes *bla*$_{\text{OXA-51-like}}$ (β-lactamase), *csuE* (part of a pilus assembly system for biofilm formation) and *ompA* (outer membrane protein A).[76] This method is suitable for the identification of IC1, IC2 and IC3.

1.2 Mode of action and target sites of antimicrobial classes

Target sites of antimicrobial compounds in the bacterial cell include the cell wall, cell membrane, DNA synthesis, RNA synthesis, protein synthesis and metabolism. The mode of action of an antimicrobial agent can be bacteriostatic or bactericidal. A bacteriostatic action inhibits bacterial replication, while a bactericidal action causes cell death. With regards to different target sites, the mode of action of antimicrobial classes will be explained below, with a special focus on β-lactams. As glycopeptides (e.g. vancomycin and teicoplanin), lincosamides (e.g. clindamycin) and oxazolidinones (e.g. linezolid) are inactive against Gram-negative bacteria, these classes are not included.

1.2.1 Overview of antimicrobial classes active against Gram-negative bacteria

An overview of the target sites of antimicrobial agents in Gram-negative bacteria is given in Figure 2. **Cell wall synthesis** is affected by β-lactams, which inhibit peptidoglycan cross-linking which results in cell death (this is further explained in 1.2.2).

As cell wall synthesis is only active in growing cells, the bactericidal effect of β-lactams does not impair cells in stationary phase. In contrast to β-lactams, polymyxins (e.g. polymyxin E (colistin) and polymyxin B) possess a high affinity for negatively charged surfaces, for example the lipopolysaccharide (LPS) layer and the outer membrane (see Figure 5). Interaction of polymyxins with these structures leads to their perforation, which results in cell death. It should be noted that polymyxins can also affect cell membranes of human cells and can cause nephrotoxicity.[77]

The **RNA- and DNA synthesis** are affected by rifampicin (RNA synthesis), quinolones (RNA- and DNA synthesis) and folate metabolism antagonists, like sulfonamides and trimethoprim (folate metabolism and consequently DNA synthesis). Rifampicin inhibits the transcription initiation by binding to the active site of the DNA-dependent RNA polymerase.

In contrast, quinolones (e.g. nalidixic acid) target the bacterial DNA gyrase (topoisomerase II) and topoisomerase IV which are needed during DNA replication, transcription and cell division to relax negative supercoilings and decatenation. By stabilizing topoisomerase-DNA complexes quinolones are able to inhibit these cellular processes. Fluoroquinolones (e.g. ciprofloxacin) possess a fluorine atom at the C6 carbon which increases their affinity to topoisomerases.

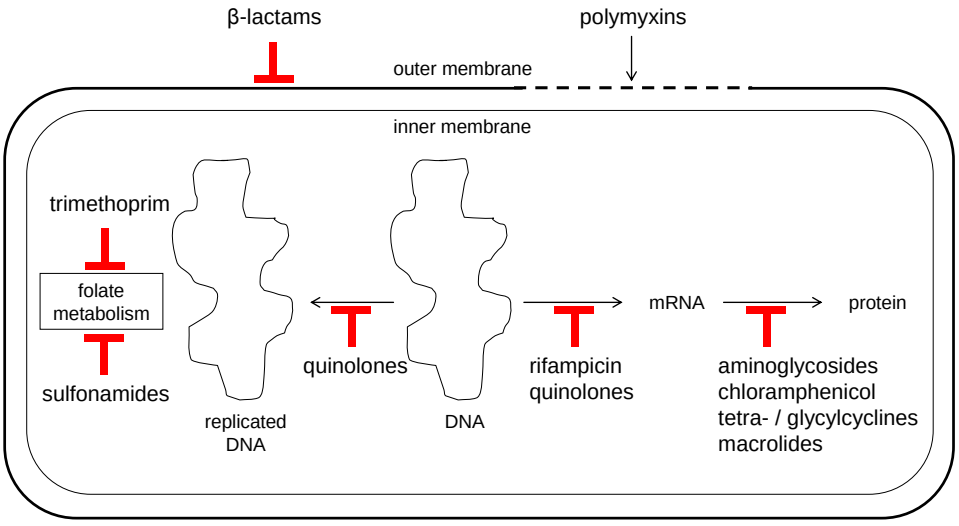

Figure 2: Target sites of antimicrobial agents in Gram-negative bacteria. Inhibition of cellular processes or products (cell wall) is indicated with red bars. Perforation of cell wall and outer membrane by polymyxins is indicated by dashed lines.

Sulfonamides (e.g. sulfamethoxazole) and trimethoprim act on the bacterial folate metabolism at two different positions. While sulfonamides are active against the dihydropteroic acid-synthetase at an earlier stage of the pathway, trimethoprim inhibits the diahydrofolic acid-reductase at the end of the pathway. To block the whole metabolism pathway sulfonamides are often combined with trimethoprim, which results in a bactericidal effect. The natural product of the pathway is tetrahydrofolate (THF) which, in form of formyl-THF, is a cofactor during purine synthesis. Therefore inhibition of THF synthesis subsequently inhibits DNA synthesis.

Protein synthesis is targeted by aminoglycosides (e.g. amikacin and gentamicin), chloramphenicol, tetracyclines (e.g. tetracycline and doxycycline) and their structural derivatives gycylcylines (e.g. tigecycline), as well as macrolides (e.g. erythromycin). The bacterial ribosome comprises of the 30S and 50S subunit, which are both targets for antimicrobial agents. For example, aminoglycosides act on the translation and elongation in protein biosynthesis via irreversible binding to the 30S subunit of the ribosome which causes misreading of the mRNA and generates non-functional proteins. These nonsense proteins can be integrated into the cell membrane, leading to reduced stability and cell lysis. Tetracyclines and glycylcylines are also active against the 30S ribosomal subunit, but in contrast to aminoglycosides they block the tRNA binding site whereby attachment of the aminoacyl-tRNA to the mRNA is prevented and translational elongation is inhibited. The 50S ribosomal subunit is targeted by chloramphenicol and macrolides, which prevent translocation of the aminoacyl-tRNA within the ribosome, whereby the translation is terminated.

1.2.2 Detailed focus on β-lactams

Structural basis of the class of β-lactams is the four-membered β-lactam ring (Figure 3). With regards to additional structural components β-lactams are divided into four subclasses; penicillins, cephalosporins, monobactams and carbapenems. Furthermore, penicillins and cephalosporins can be subdivided into four groups, respectively. The subclass of penicillins includes penicillin G and derivatives, isoxazolylpenicillins, aminobenzylpenicillins and acylaminopenicillins, which are distinguished on the basis of structural differences.

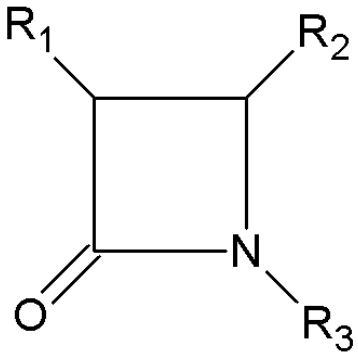

Figure 3: Structure of the β-lactam ring.

In contrast, cephalosporins are divided into group 1 to 4, based on their mode of action. The activity against Gram-negative bacteria is increased from group 1 to 4, while the activity against Gram-positive bacteria can be decreased. Groups 3 and 4 possess a broad spectrum of activity against Gram-negative species and group 4 additionally exhibits a broad spectrum against Gram-positive species. Therefore agents of these groups are also termed extended-spectrum cephalosporins. In contrast to penicillins and cephalosporins, the monobactam aztreonam is inactive against Gram-positive bacteria.

As this dissertation focuses on carbapenem resistance in *Acinetobacter* spp., the carbapenems will be explained in more detail. The first naturally occuring carbapenem, thienamycin produced by *Streptomyces cattleya*, was identified in 1976 (Figure 4).[78] To date four semi-synthetic derivatives of thienamycin are used in the treatment of bacterial infections; imipenem, meropenem, doripenem and ertapenem (Figure 4).

The bactericidal action of carbapenems is independent of the concentration, but depends on the time the minimal inhibitory concentration is exceeded. Resorption of carbapenems in the intestinal tract is inadequate. Therefore the application of carbapenems occurs parenterally (intervenously), which is the reason why these agents are mainly used in the hospital. In contrast to other carbapenems, imipenem is hydrolyzed by the dehydropeptidase I which is produced in the kidneys. To ensure a stable concentration of the substance in the body, imipenem has to be applied in combination with cilastatin which reversibly inhibits the dehydropeptidase I.

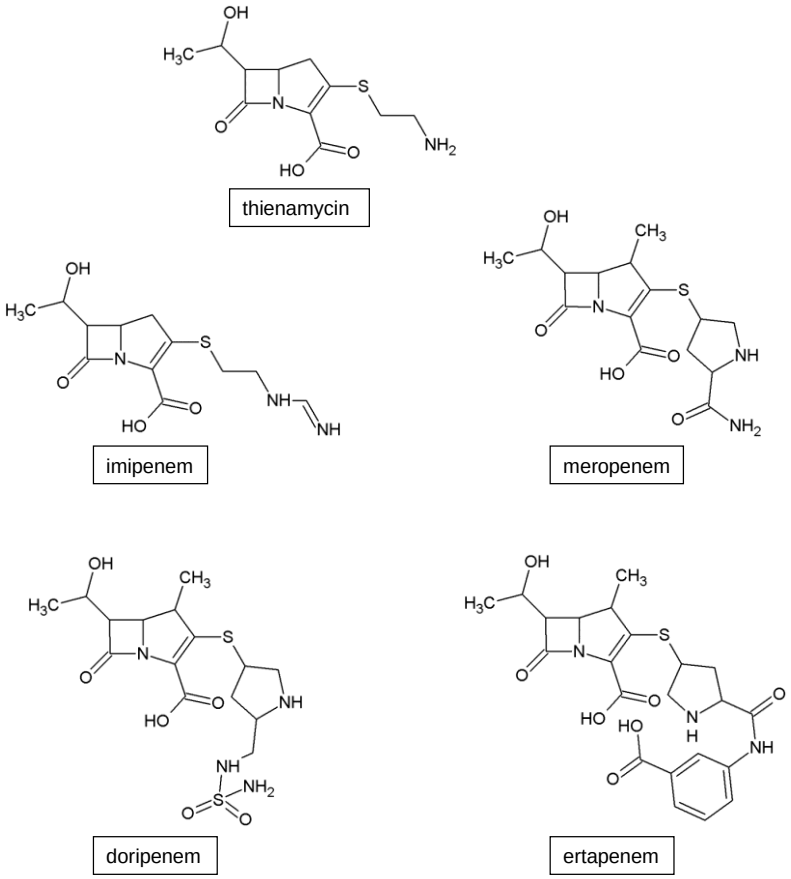

Figure 4: Structure of the β-lactam subclass of carbapenems.

The addition of a methylgroup at C1 provides stability of the other carbapenem compounds against the renal dehydropeptidase I, wherefore the combination with cilastatin is unnecessary. MDR Gram-negative bacteria, including *A. baumannii*, often retain susceptibility against carbapenems, which is the reason why this subclass of β-lactams is used as the drugs of choice to treat infections with these pathogens.

Target sites and mode of action of β-lactams

β-lactams affect the cell wall synthesis in Gram-negative and Gram-positive bacteria. In Gram-negative bacteria the cell wall consists of 2-5 cross-linked peptidoglycan layers (murein) and an outer membrane, which contains phospholipids, proteins, channel-forming porins and lipopolysaccharides (LPS) (Figure 5). The outer membrane is linked to the peptidoglycan via lipoproteins.

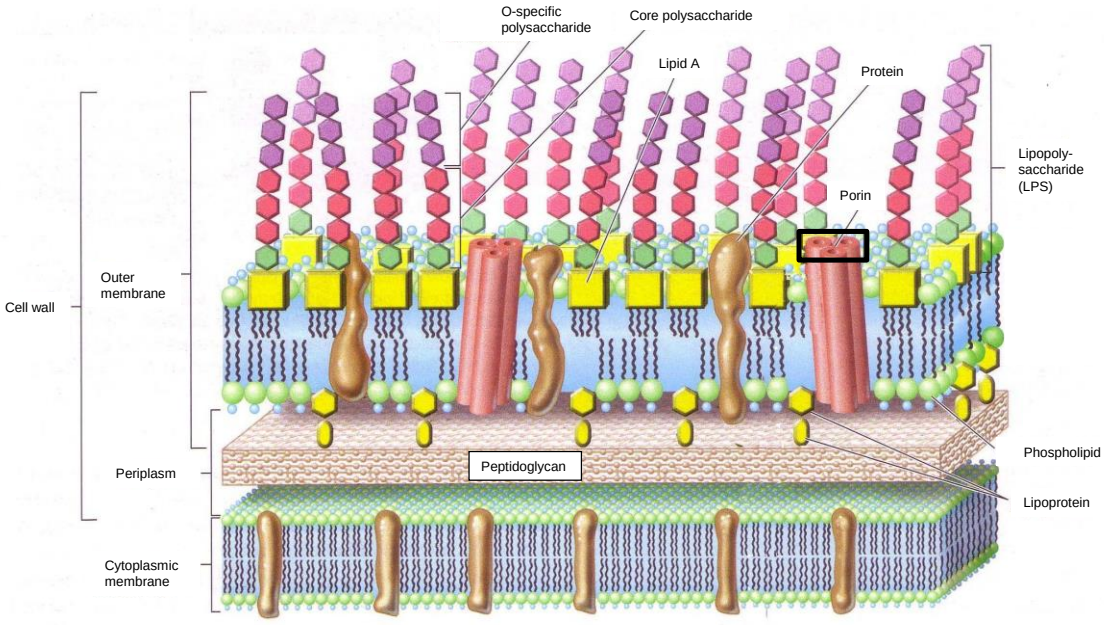

Figure 5: Composition of the Gram-negative cell wall; adapted[79].

The target sites of β-lactams are transpeptidases and carboxypeptidases, enzymes which are involved in murein synthesis. The peptidoglycan layers are based on long chains of the sugar derivatives N-acetyl glucosamine and N-acetyl muramic acid (Figure 6). The N-acetyl muramic acids are further linked to pentapeptides which are essential for the cross-linking of two peptidoglycan strands.[80] These pentapeptides terminate in two D-alanine residues.

In Gram-negative bacteria transpeptidases catalyse a bond between the peptide chains of two peptidoglycan strands by connecting the penultimate D-alanine with meso-diamino pimelic acid. The terminal D-alanine residues are cleaved by carboxypeptidases (Figure 6). Although β-lactams target both enzymes, the inhibition of carboxypeptidases is negligible. The bactericidal effect of β-lactams is mainly based on the inhibition of transpeptidases. The ability of transpeptidases to bind β-lactam agents has led to the alternative term "penicillin binding proteins" (PBPs).[80] Reaction of the transpeptidase with the β-lactam results in a covalent bond of a serine residue in the active centre of the transpeptidase with the β-lactam ring (acyl-enzyme complex), which obstructs the cross-linking reaction. Based on poorly cross-linked murein the cell wall is unable to resist the turgor of the cell, which leads to cell lysis.

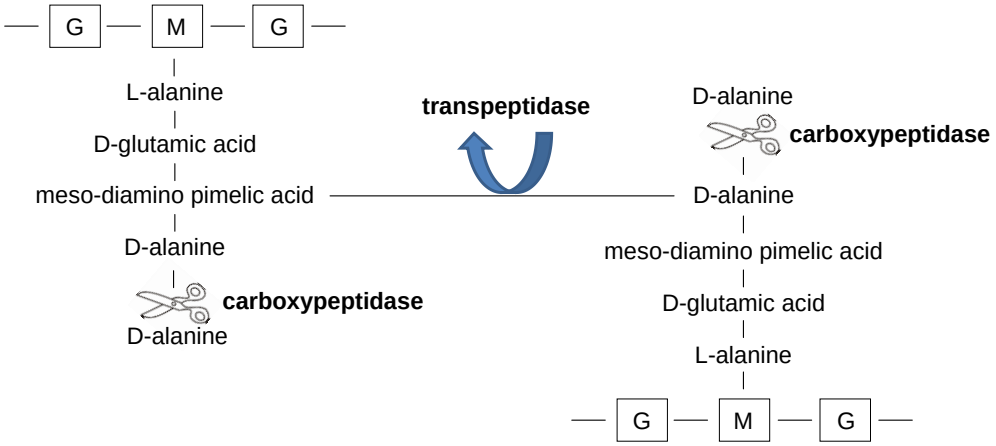

Figure 6: Peptidoglycan crosslinking in Gram-negative bacteria; adapted[79]. Transpeptidase- and carboxypeptidase-mediated reactions are indicated with an arrowhead and scissors, respectively. G, N-acetyl glucosamine; M, N-acetyl muramic acid

1.3 Antimicrobial susceptibility testing

Antimicrobial susceptibility of bacterial isolates is determined on the basis of the minimal inhibitory concentration (MIC), which defines the lowest concentration of a drug (mg/L) at which bacterial growth is inhibited. The susceptibility data are compared to given clinical breakpoints, which allow interpretation of an isolate as susceptible (S), intermediate (I) or resistant (R) to a certain drug. The breakpoints are established by official committees of various institutions, for example the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST).[81] The susceptibility of a bacterial isolate to antimicrobial agents can give suggestions for appropriate treatment options. For example, if an isolate is susceptible to a certain drug, this agent possesses a high activity against the isolate and might be considered for therapy. In contrast, resistance to a certain agent implies low activity of the drug and indicates potential treatment failure. The intermediate level implies uncertainty of a treatment success. For example, the drug might retain a therapeutic effect if a high dosage can be used or if the drug can be concentrated at the site of infection. However, depending on the institution which set the breakpoints, the intermediate level might be missing.

Standardization of susceptibility testing methods has been established by various institutions, including CLSI and EUCAST.[82, 83] Frequently used methods are for example the epsilometer test (Etest), disc diffusion, agar dilution and microbroth dilution. Etest, disc diffusion and agar dilution are performed on agar plates, while microbroth dilution is performed in microtiter plates which contain 96 cavities. The type of solid and liquid media can vary depending on the institution that established the guidelines, but commonly Mueller-Hinton media are recommended. Single colonies of a freshly subcultured strain are used to prepare the standard inoculum for the susceptibility test. Using a cotton swab colonies are taken from the plate and suspended in 0.9% NaCl solution to achieve a cell density of McFarland 0.5 which equals 1-1.5 x 10^8 cells. To perform Etest or disc diffusion, the swab is used to inoculate the testing agar plate. Afterwards an Etest strip or an antibiotic disc is placed on top of the inoculated agar and incubated for 16-18 (-24) hours.

Etest strips contain a gradient of an antimicrobial agent, which diffuses into the agar where it can inhibit bacterial growth. After overnight incubation an elliptic zone of inhibition might be visible which allows MIC determination. If the zone of inhibition is missing, the MIC is interpreted as higher than the maximum concentration of the agent on the strip. For example, the maximum concentration of imipenem and meropenem Etest strips is 32 mg/L, whereby the lack of an inhibition zone would be interpreted as an MIC of >32 mg/L. In contrast to an antimicrobial gradient, antibiotic discs which are used for **disc diffusion** contain a fixed concentration. The antimicrobial agent diffuses into the agar and can inhibit growth of the surrounding cells during overnight incubation. The susceptibility is interpreted on the basis of the diameter of this zone of inhibition which corresponds to the MIC.

In contrast to Etest and disc diffusion, the **agar dilution** plates contain serial dilutions of a certain drug. A 1:100 dilution of the standard cell suspension is used to inoculate the agar plate. The inoculation can be performed semi-automatically, which allows investigation of multiple isolates simultaneously. After overnight incubation the MIC values can be determined.

Microbroth dilution allows susceptibility testing of multiple antimicrobial agents on one microtiter plate. The cavities of the plate contain different concentrations of antimicrobial agents, which are present as either liquid or freeze-dried. The standard inoculum is diluted in liquid medium, according to the manufacturer´s instruction, and a defined volume of the dilution is supplied into the cavities. After overnight incubation the MIC values can be determined.

1.4 Antimicrobial resistance in *A. baumannii*

Bacterial defense against naturally occurring antibiotics, like penicillin produced by some fungi or *Actinobacteria*, is an ancient mechanism. However, excessive usage of antimicrobial agents during the last decades has supported the evolution and the spread of resistance mechanisms. Resistance can be intrinsic, based on innate mechanisms which are present in every organism of a species, or acquired. Resistance mechanisms in Gram-negative bacteria include enzymatic hydrolysis or modification of the drug, mutations in the drug target, reduced permeability, active efflux of the drug, ribosomal mutations or modifications, lipopolysaccharide (LPS) mutations and rarely metabolic bypass pathways.[84]

1.4.1 Distribution of antimicrobial resistance determinants

The spread of antimicrobial resistance determinants in *A. baumannii* is supported by the transfer of plasmids, or mobile genetic elements, like insertion sequences (IS elements) and transposons. Furthermore, genetic structures like integrons and resistance islands contribute to the accumulation of multiple resistance genes. Plasmids can harbor IS elements, transposons and integrons which further highlights the potential for rapid spread of antimicrobial resistance genes.

Insertion sequences

Insertion sequences are small genetic elements of approximately 0.8 to 2.5 kb. They encode one or more transposase genes (*tnp*) which are flanked by two inverted repeats and mediate transposition of the IS element (Figure 7).[85] Transposition can either generate a copy or excise the full-length IS element which will be inserted in the same parental DNA or a different one, for example a plasmid. Insertion of the IS element at a new position leads to the duplication of the insertion- or target site presented by two direct repeats.

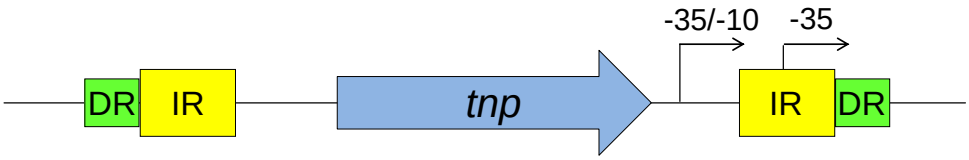

Figure 7: Schematic composition of an insertion sequence. Possible promoter regions provided for adjacent genes are indicated with thin arrows (-35/-10: full-length promoter; -35: partial promoter). *tnp*, transposase gene; IR, inverted repeat; DR, direct repeat

Insertion sequences often contain strong promoter sequences which can confer overexpression of adjacent antimicrobial resistance genes (Figure 7). Furthermore, IS elements often provide -35 regions which can generate a hybrid promoter in the presence of a downstream located -10 region. A common IS element in *A. baumannii* is IS*Aba1* (insertion sequence *Acinetobacter baumannii* 1) which can be found in multiple copies on *A. baumannii* genomes and is often associated with β-lactam resistance genes.[86]

Transposons

Transposons are large mobile genetic structures. Similar to IS elements they harbor transposase genes and are flanked by inverted repeats (Figure 8). In addition, transposons can contain other mobile genetic structures (e.g. integrons (Figure 9)) and often encode additional genes (e.g. resistance genes and chaperone genes). The flanking inverted repeats are commonly part of IS elements. If these are identical or closely related to each other the genetic structure is called composite transposon.

Comparable to IS elements, transposition results in target site duplication, indicated by two direct repeat regions (DR) at both ends of the transposon (Figure 8).

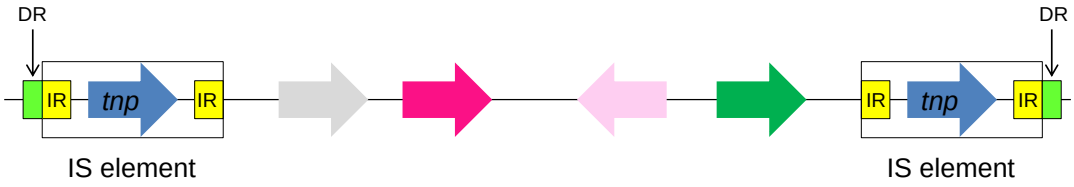

Figure 8: Schematic composition of a transposon. Flanking IS elements are indicated with boxes. The middle part contains a variable number of structural and resistance genes, which are indicated by coloured arrowheads. *tnp*, transposase gene; IR, inverted repeat; DR, direct repeat

In *A. baumannii* β-lactamases of the acquired OXA-23 subclass are frequently encoded on transposons, for example Tn*2006*, a composite transposon which is flanked by two copies of IS*Aba1*.[68]

Integrons

Integrons vary in size depending on their genetic composition (Figure 9) and are often part of large transposons. These elements encode a site-specific integrase which conducts accumulation of resistance genes at the *attI1* recombination site.[85] The integrase gene (*intI*) provides the promoter (Pc) for downstream located cassettes which can contain multiple resistance genes. These gene cassettes are separated by small terminator sites with possible stem-loop structures (59-base-pair elements (59 be) or *attC*).

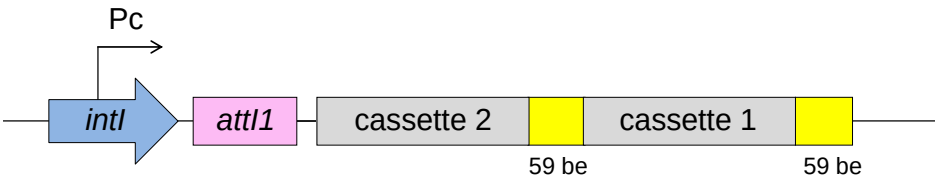

Figure 9: Schematic composition of an integron. The promoter Pc is indicated with a thin arrow. Gene cassettes are highlighted in grey, while resistance genes are not separately illustrated. *intI*, integrase gene; *attI1*, recombination site; 59 be, 59-base-pair element

Integrons are widespread genetic structures in MDR *A. baumannii* where they are frequently associated with aminoglycoside, chloramphenicol and sulfonamide resistance.[87]

Resistance islands

Probably the largest mobile elements in *A. baumannii* are the recently identified resistance islands. These genetic structures show diverse compositions based on multiple transposons, integrons and IS elements, as well as stress response proteins, proteins involved in translocation processes, chromosomal DNA and putative open reading frames (ORF). To date the largest resistance island identified in *A. baumannii*, AbaR25, is 121.7 kb in size and harbors 141 ORFs of which seven encode antibiotic resistance genes.[88] In contrast, AbaR1 is 86 kb large and carries 88 ORFs, including 45 resistance genes.[89] Resistance islands in *A. baumannii* seem to have similar integration sites in chromosomal genes, like *comM* (encodes an ATPase) or *pho* (encodes a phosphatase).[90]

Plasmids

Plasmids are self-replicating, circular DNA molecules of 1 to >100 kb which bacteria can take up via conjugation from different species of their own genus or other related genera. Conjugation is based on the assembly and disassembly of a filamentous pilus, providing close contact of two bacterial cells, which is needed for the generation of a direct cell to cell contact. The respective channel spans the cytoplasmic membrane and the outer membrane and provides transfer of the replicated plasmid from the donor to the recipient cell. Self-transmissible or conjugative plasmids harbor all genes which are necessary for channel formation, plasmid replication and transfer. Plasmids which lack the genes for the channel formation are unable to trigger their transfer, but can be mobilized in the presence of a conjugative plasmid. Plasmids often encode several resistance genes, which can be part of transposons and integrons. If transposons and IS elements are part of the plasmid they might be transposed from the plasmid to the chromosome, providing a high potential for bacterial genome plasticity and further distribution of resistance determinants. In *A. baumannii* the characterization of plasmids is performed by replicon typing, recently described by Bertini *et al.*[91] The six established multiplex PCRs distinguish between 19 different replicase genes of widespread plasmids in *A. baumannii*.

1.4.2 Resistance of *A. baumannii* to multiple antimicrobial classes

A. baumannii exhibits a high propensity to multi-drug resistance. To illustrate the reduction of treatment options in *A. baumannii*, the variety of resistance mechanisms against aminoglycosides, chloramphenicol, macrolides, tetracyclines and glycylcyclines, fluoroquinolones, polymyxins (colistin), rifampicin and folate antagonists will be summarized.

Drug-modifying enzymes, target site mutations and drug efflux play important roles in the development of antimicrobial resistance in *A. baumannii*. For example, resistance to **aminoglycosides** is commonly mediated by drug-modifying enzymes including phosphotransferases (e.g. APH(3´)), acetyltransferases (e.g. AAC(6´)I-ad) and adenyltransferases (e.g. AAD(3´´)-Ia).[62, 92, 93] Another aminoglycoside-resistance determinant in *Acinetobacter* spp. is ArmA, an RNA-methylase that modifies 16S rRNA and reduces affinity for aminoglycosides to bind to the 30S ribosomal subunit.[62] Resistance to **chloramphenicol** is frequently conferred by plasmid-encoded proteins, including a drug-modifying acetyltransferase (*cat* gene) or an efflux pump (*cmlA* gene). Additional efflux pumps associated with chloramphenicol resistance are CraA (chloramphenicol specific pump), the small multidrug resistant (SMR) pump AbeS and the multi antimicrobial extrusion (MATE) pump AbeM.[62] **Macrolide** resistance has been found to be associated with resistance genes *mph* (encodes a drug-modifying enzyme) and *mel2* (encodes an efflux pump).[94] In contrast, **tetracycline** resistance can be mediated by efflux pumps of the multi facilitator superfamily (MFS) (TetA and TetB) and ribosomal protection proteins (TetM and TetO). Ribosomal protection proteins are cytoplasmatic proteins which are assumed to be related to elongation factors and are able to release tetracycline from the ribosome.[95] Although MDR *A. baumannii* often retain susceptibility to the **glycylcycline** tigecycline, efflux-mediated resistance via RND pumps is increasingly reported.[62] Common resistance mechanism in *A. baumannii* also include mutations in the chromosomal DNA gyrase genes *gyrA* and *gyrB* or the topoisomerase IV genes *parC* and *parE*, which can lower the affinity of **fluoroquinolones** to the topoisomerase-DNA complex. Ciprofloxacin-resistant isolates often harbor mutations in *gyrA* (Ser-86-Leu substitution) and *parC* (Ser-80-Leu substitution), but efflux has also been reported.[62]

Plasmid-mediated fluoroquinolone-resistance determinants, as found in *Enterobacteriaceae* (e.g. *qnrA*) have so far not been detected in *A. baumannii*. Furthermore, reduced **colistin** susceptibility has been proposed to be associated with LPS modification and increased expression of the two-component system PmrAB which might have to be coupled with amino acid changes in PmrB.[96] Furthermore, total loss of the LPS via mutations in the lipid A synthesis genes *lpxA*, *lpxC* or *lpxD* was associated with decreased colistin susceptibility in a laboratory mutant.[96] Resistance to **rifampicin** in *A. baumannii* can be mediated by mutations in the chromosomally encoded RNA polymerase β-subunit gene (*rpoB*) that alter the amino acid composition of the active centre of the polymerase or an acquired rifampicin ADP-ribosylating transferase (encoded by *arr-2*) which inactivates rifampicin by ribosylation.[62] Widespread resistance to **sulfonamides** and **trimethoprim** in *A. baumannii* is conferred by the generation of isoenzymes which exhibit reduced affinity to these compounds and are usually encoded on plasmids (*sul* or *dfr* genes).[62]

In addition to the above mentioned mechanisms, a MDR phenotype in *A. baumannii* can be based on overexpression of the common resistance-nodulation-cell division (RND) efflux pump family (see 1.4.3), which typically exhibits a broad substrate spectrum including many antimicrobial classes.[97] For example, the *A. baumannii* efflux pump systems (Ade) ABC and IJK have been found to be associated with resistance to aminoglycosides (AdeABC), β-lactams, chloramphenicol, fluoroquinolones, lincosamines (AdeIJK), macrolides (AdeABC), tetracyclines, tigecycline, and trimethoprim (AdeABC).[98] Furthermore, overexpression of AdeFGH has been reported to confer resistance to chloramphenicol, clindamycin (lincosamines), fluoroquinolones and trimethoprim, as well as reduced susceptibility to tetracycline, tigecycline and sulfamethoxazol.[98]

The spread of resistance determinants and development of novel resistance mechanisms, has led to global emergence of MDR *A. baumannii*. Although carbapenems are used as the drugs of choice to treat infections with these pathogens, carbapenem resistance in *A. baumannii* is increasingly reported.[99] Mechanisms which contribute to β-lactam resistance, particularly carbapenem resistance, will be explained in the following section.

1.4.3 Resistance of *A. baumannii* to β-lactams

The main mechanism for β-lactam resistance in *Acinetobacter* spp., particularly in *A. baumannii*, is the production of drug-hydrolyzing enzymes (β-lactamases).[100] Via channel-forming proteins (porins), which span the outer membrane, β-lactam agents from the surrounding medium enter the Gram-negative cell and reach the periplasm, the acting site of the β-lactamases.

Enzyme-mediated β-lactam resistance

β-lactamases inactivate β-lactam agents by hydrolysis of the amide bond in the β-lactam ring. According to their mode of action, β-lactamases are divided into serine- and metallo-β-lactamases which either need a serine residue or one or two zinc ions in their active centre.[80]

- **Enzymatic hydrolysis of β-lactam agents**

The reaction of **serine-β-lactamases** is based on acylation and deacylation.[101] The hydroxyl-group of the serine residue in the active centre of the β-lactamase reacts with the carboxyl-carbon of the β-lactam ring, whereby the carbon-nitrogen bond is opened and an acyl-enzyme intermediate is generated. This intermediate reacts with a water molecule which results in regeneration of the hydroxyl-group in the active serine residue of the β-lactamase and release of the inactivated β-lactam agent. To overcome the problem of widespread β-lactamase mediated resistance, β-lactam derivatives like tazobactam, sulbactam and clavulanic acid have been generated. These β-lactamase inhibitors irreversibly bind to the enzyme, preventing its interaction with the β-lactam and therefore leading to inactivation of the β-lactamase. However not all serine-β-lactamases are significantly affected by these inhibitors. Moreover, extensive use of β-lactams during the last decades has supported the evolution and selection of more effective enzymes which are active against an extended spectrum of substrates (**extended-spectrum β-lactamases** (ESBLs)), including extended-spectrum cephalosporins and monobactams.[80]

In contrast to serine-β-lactamases, metalloenzymes harbor one (mononuclear) or two (binuclear) zinc ions in their active centre. As a consequence these enzymes are inactivated by chelators, like EDTA, but are not affected by β-lactamase inhibitors. **Metallo-β-lactamases** are divided into three subclasses; B1 (e.g. VIM-4 from *Pseudomonas aeruginosa*), B2 (e.g. CphA from *Aeromonas* spp.) and B3 (e.g. L1 from *Stenotrophomonas maltophilia*).[101] Enzymes of subclasses B1 and B3 are active with two zinc ions in their active centre and exhibit a broad substrate spectrum which includes penicillins, cephalosporins and carbapenems. In contrast, B2 enzymes possess a narrow substrate spectrum, including carbapenems, and need one zinc ion for proper activity. The catalytic mechanism of metallo-β-lactamases is not fully understood yet. However, it is proposed that the hydrolysis of the amide bond in the β-lactam ring is generated via the attack of either a hydroxide ion (subclasses B1 and B3) or a water molecule (subclass B2) in the active site of the enzyme on the carboxyl-carbon of the β-lactam ring.[101] Zinc ions are proposed to interact with functional groups of the β-lactam agent and stabilize the hydroxide ion as well as anionic intermediates which are generated during hydrolysis.

- **Classification of β-lactamases**

β-lactamases are generally classified by two different schemes which have been proposed by Ambler *et al.* and Bush *et al.*[102, 103] Bush *et al.* analysed functional characteristics and the molecular structure of β-lactamases and identified 4 classes (1 to 4), with the second class being further divided into eight subclasses. This classification gives additional information about the substrate spectrum and inhibitor susceptibilities. In contrast Ambler *et al.* based their classification scheme on the amino acid sequences to analyse clonal relatedness of β-lactamases. The Ambler classification contains four classes (A to D), with classes A, C and D containing serine-β-lactamases, while metallo-β-lactamases are represented by class B. An overview of the different subclasses of β-lactamases in *Acinetobacter* spp. according to the Ambler classification is given in Table 2 and Table 3.

β-lactamase (*bla*) genes can be encoded on the chromosome or on plasmids and are widespread in the genus *Acinetobacter*.[67, 104, 105] *A. baumannii* harbors two innate, chromosomally encoded β-lactamases; AmpC and OXA-51-like. In addition numerous acquired β-lactamases have been recently identified in *A. baumannii* and other *Acinetobacter* spp.[62]

- **β-lactamases with no activity against carbapenems**

A wide range of β-lactamases without carbapenem-hydrolysing activity have been reported in *A. baumannii*, including class A, class C and class D enzymes (Table 2). Class A β-lactamases without carbapenem-hydrolysing activity in *A. baumannii* include several narrow-spectrum and extended-spectrum cephalosporinases. The class C cephalosporinase AmpC is intrinsic to *A. baumannii* but is usually expressed at low level.[106] IS-mediated overexpression of the chromosomal AmpC or acquisition of the extended-spectrum AmpC-type β-lactamase (ESAC) can affect susceptibility against penicillins and cephalosporins. In addition to class A and class C, class D β-lactamases without carbapenem-hydrolysing activity from *P. aeruginosa* have been described in *A. baumannii* (Table 2).[107]

Table 2: β-lactamases without carbapenem-hydrolysing activity in *A. baumannii*.

Ambler class	Substrate	Enzyme	Reference
A	cephalosporins	narrow spectrum: TEM-1, TEM-2, CARB-5, SCO-1	Poirel *et al.*[62]
	cephalosporins with extended spectrum and aztreonam	extended spectrum: PER-1, PER-2, PER-7, VEB-1, CTX-M-2, CTX-M-15, CTX-M-43, TEM-92, TEM-116, SHV-5, SHV-12, RTG-4	Poirel *et al.*[62], Potron *et al.*[108]
C	penicillins and cephalosporins	AmpC-type (intrinsic)	Jacoby *et al.*[106]
	penicillins and cephalosporins with extended spectrum	extended spectrum: ESAC	Poirel *et al.*[62]
D	penicillins and cephalosporins	narrow spectrum: OXA-20, OXA-21, OXA-37	Roca *et al.*[107]

AmpC, ampicillinase; CARB, carbenicillinase; CTX-M, cefotaximase from Munich, Germany; ESAC, extended-spectrum AmpC-type β-lactamase; OXA, oxacillinase; PER, *Pseudomonas* extended resistant; RTG, enzyme with conserved RTG (arginine, threonine, glycine) triad; SCO, carbenicillinase from *E. coli*; SHV, sulfhydryl variable (variable susceptibility to sulfhydryl inhibitors); TEM, Temoniera (name of the patient the enzyme was first isolated from); VEB, Vietnam extended-spectrum β-lactamase

- **Carbapenem-hydrolysing β-lactamases**

Carbapenem-hydrolysing enzymes identified in *Acinetobacter* spp., mainly *A. baumannii*, include class A β-lactamases, class B metallo-β-lactamases and class D β-lactamases (Table 3). Carbapenem-hydrolysing class A and B β-lactamases previously identified in *A. baumannii* include carbapenemases from *Klebsiella pneumoniae* (KPC), extended-spectrum GES-type cephalosporinases, as well as several B1 metalloenzymes of the IMP-, NDM-, SIM- and VIM-type (Table 3).[107]

Table 3: Carbapenem-hydrolysing β-lactamases in *A. baumannii*.

Ambler class	Additional substrates	Representative enzyme	Reference
A	-	KPC-2 to KPC-4, KPC-10	Poirel *et al.*[62]
	cephalosporins and aztreonam	extended spectrum: GES-11, GES-14, GES-22	Karah *et al.*[68], Cicek *et al.*[109]
B	most β-lactams, except aztreonam	IMP-1, IMP-2, IMP-5, IMP-6, IMP-11, SIM-1, NDM-1, NDM-2 VIM-1, VIM-2	Poirel *et al.*[62]
D	penicillins	OXA subclasses: OXA-51-like (intrinsic), OXA-23-like, OXA-40-like, OXA-58-like, OXA-143-like, OXA-235-like	Poirel *et al.*[104], Higgins *et al.* [97]

GES, Guinea extended-spectrum β-lactamase; IMP, imipenemase; KPC, *Klebsiella pneumoniae* carbapenemase; NDM, New Delhi metallo-β-lactamase; OXA, oxacillinase (carbapenem-hydrolysing class D β-lactamase (CHDL)); SIM, Seoul imipenemase; VIM, Verona integron-encoded metallo-β-lactamase

However, class D β-lactamases with carbapenem-hydrolysing activity are the most frequently detected carbapenem-resistance determinants in *A. baumannii*. The abbreviation OXA might be confusing, as it is also used for class D β-lactamases which lack carbapenem-hydrolysing activity (see Table 2). The class D β-lactamases are a large group of enzymes which were designated oxacillinases (OXA) according to their preferential hydrolysis of oxacillin. Depending upon the substrate spectrum, the group can be subdivided into three subclasses. The first two subclasses include narrow spectrum oxacillinases (see Table 2) and extended-spectrum oxacillinases (ES-OXA) which are inactive against carbapenems and are prevalent in *P. aeruginosa*. The third subclass is represented by carbapenem-hydrolysing class D β-lactamases (CHDL), which are widespread in the genus *Acinetobacter*, particularly *A. baumannii*. To date, six different subclasses of CHDL have been identified in *A. baumannii*; the intrinsic OXA-51-like and the acquired OXA-23-like, OXA-40-like, OXA-58-like, OXA-143-like and OXA-235 (see 2.4.2).[110, 111] In contrast to OXAs which lack carbapenem-hydrolysing activity (see Table 2), these enzymes are not inhibited by clavulanic acid, sulbactam and tazobactam.

It has been proposed that the acquired *bla*$_{\text{OXA-23-like}}$ in *Acinetobacter* spp. derived from *A. radioresistens* to which the gene is intrinsic.[112] The origin of the other acquired OXA subclasses is unknown. The subclass-specific numbers (e.g. OXA-**51**-like) represent the first identified enzyme of the respective group. Generally, OXA-specific numbers are assigned by the Lahey β-lactamase database (http://www.lahey.org/Studies/). To show that each subclass possesses multiple amino acid variants each subclass notation includes the term "-like". Representative enzymes of different OXA subclasses show amino acid identities between 49% (OXA-23 and OXA-58) and 88% (OXA-40 and OXA-143). Although OXAs are weak carbapenem hydrolysers they can confer resistance to carbapenems in the presence of additional resistance determinants and when expression of the *bla* gene is increased. This can be mediated by strong promoters which are mainly provided by IS elements (see 1.4.1).[104] However, OXA-40-like and OXA-143-like are usually not associated with these genetic structures. Table 4 summarizes IS elements which have already been detected in association with *bla*$_{\text{OXA}}$ in *A. baumannii*.

Table 4: *bla*$_{\text{OXA}}$ associated IS elements in *A. baumannii*.

Subclass	Associated IS elements	Reference
OXA-51-like	IS*Aba1*, IS*Aba9*, IS*Aba825*	Roca *et al.*[107], Lopes *et al.*[113] and Poirel *et al.*[114]
OXA-23-like	IS*Aba1*, IS*Aba4*, IS*Aba10*	
OXA-58-like	IS*Aba1*, IS*Aba2*, IS*Aba3*, IS*Aba8*, IS*Aba125*, IS*Aba825*, IS*18*, IS*26*	
OXA-235-like	IS*Aba1*	Higgins *et al.*[111]

The diversity of OXAs and their association with mobile genetic structures in *A. baumannii* might indicate further spread in non-*baumannii Acinetobacter* species which could also play an important role in clinical settings in the future.

Non-enzymatic β-lactam resistance

β-lactam resistance in *A. baumannii* might be enhanced by additional resistance mechanisms. Characteristic for Gram-negative bacteria are channel-forming proteins (porins) which span the outer-membrane and provide material exchange between the cell and the surrounding medium but also allow β-lactams to enter the periplasm. Compared to other Gram-negative bacteria *A. baumannii* possesses a **naturally low permeability**, which is based on low porin expression and diffusion rates.[115] Therefore reduced expression or total loss of one of these porins can be associated with β-lactam resistance. For example, loss of the porins CarO, the 43 kDa OMP (a homologue of the *P. aeruginosa* porin OprD) and the 33-36 kD OMP has been suggested to be associated with imipenem resistance in clinical *A. baumannii* isolates.[5, 116, 117]

Several **efflux systems** have been identified in *Acinetobacter* spp. which actively extrude antimicrobial agents and toxic compounds out of the cell. As mentioned before (see 1.4.2) RND pumps are common efflux systems in the genus *Acinetobacter*. These pumps are compromised of three parts (an outer membrane pore, an inner membrane pump and a membrane fusion protein) and translocate their substrates out of the cell coupled with proton transport (antiporter). While AdeABC and AdeIJK can be involved in β-lactam resistance, including extended-spectrum cephalosporins (AdeABC) and aztreonam (AdeIJK), AdeFGH seems to have no effect on β-lactam susceptibility.[98] In the presence of carbapenem-hydrolysing OXA, overexpression of AdeABC has been proposed to enhance meropenem resistance in *A. baumannii*.[118] However, the effect on other carbapenems remains unclear.

2. Results

2.1 Molecular characterization of *bla*$_{NDM-1}$ in an *Acinetobacter baumannii* strain isolated in Germany in 2007

The New Delhi metallo-β-lactamase 1 (NDM-1) was identified in 2009 by Yong *et al.* and constituted a novel subclass of B1 metalloenzymes.[119] The *bla*$_{NDM-1}$ gene was encoded on transmissible plasmids in two MDR *K. pneumoniae* and *E. coli* strains, isolated from urine and feces of the same patient, respectively. After hospitalization in New Delhi, India, the patient acquired a urinary tract infection, caused by the NDM-1 producing *K. pneumonia*, and was repatriated to Sweden.[119] The NDM-1 enzyme shares little amino acid identity with other metallo-β-lactamases; VIM-1 and VIM-2 show the closest similarity, sharing 32.4% amino acid identity with NDM-1.[119] Like other metallo-β-lactamases NDM-1 is active against almost all β-lactam agents, except aztreonam. However, transfer of NDM-1 into a susceptible *E. coli* strain has led to significant reduction in aztreonam susceptibility.[119] NDM-1 is usually encoded on plasmids which often harbor other resistance genes. Commonly, NDM-1 expressing isolates retain susceptibility only against tigecycline and colistin.[120] Recent studies analyzed the epidemiology of *bla*$_{NDM-1}$ in *A. baumannii* and *Enterobacteriaceae*, while little information was given on the genetic environment of the gene.[120, 121]

In the following publication, we characterized the genetic composition of *bla*$_{NDM-1}$ of a clinical *A. baumannii* isolate from Germany. The gene was located on a large composite transposon that inserted into a chromosomal major facilitator superfamily (MFS) symporter gene and was flanked by two copies of IS*Aba125*, a common IS element in *A. baumannii*. In addition, parts of the transposon revealed genetic similarity to other Gram-negative species. For example, *bla*$_{NDM-1}$*-trpF* was similar to *K. pneumoniae* plasmid pKpANDM-1 and the downstream located Δ*groS-groL-insE* showed similarity to *E. coli* plasmids pEH4H and pAR060302.

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Molecular characterization of bla_{NDM-1} in an *Acinetobacter baumannii* strain isolated in Germany in 2007

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Objectives: To investigate the genetic environment of the metallo-β-lactamase gene bla_{NDM-1} in an *Acinetobacter baumannii* isolated in 2007 in a German hospital.

Methods: Antimicrobial susceptibility testing was performed and resistance genes were characterized by PCR amplification and sequencing. Transferability of β-lactam resistance was tested by broth mating assays and transformation of plasmids. The genetic background of bla_{NDM-1} was analysed by primer walking. Typing of the *A. baumannii* strain was performed by repetitive extragenic palindromic sequence-based PCR (rep-PCR) using the DiversiLab system.

Results: The multidrug-resistant *A. baumannii* isolate harboured β-lactamase genes bla_{NDM-1} and intrinsic bla_{OXA-64}, but without the insertion sequence IS*Aba1* often located upstream. Transfer of carbapenem resistance by conjugation and transformation failed. Hybridization of isolated plasmid DNA with bla_{NDM} probes was not successful. Shotgun cloning of whole genomic DNA and sequence analyses revealed that bla_{NDM-1} was located between two insertion elements of IS*Aba125*. Furthermore, this bla_{NDM-1}-containing transposon structure was integrated into a chromosomal gene encoding a putative *A. baumannii* major facilitator superfamily (MFS) metabolite/H^+ symporter.

Conclusions: The metallo-β-lactamase gene bla_{NDM-1} in this *A. baumannii* strain was integrated in the chromosome on a new transposon structure composed of two copies of insertion sequence IS*Aba125*. The variability of the genetic environment of bla_{NDM-1} likely facilitates the rapid dissemination of this gene within many Gram-negative bacterial species.

Keywords: multidrug resistance, metallo-β-lactamases, carbapenemases, transposons, DiversiLab

Introduction

In recent years an increasing number of reports on the emergence of multidrug-resistant Gram-negative pathogens have been published and in particular it is the emergence of bla_{NDM-1} that has created the most concern.[1,2] Indeed, since its first description in 2009, this metallo-β-lactamase (MBL) has now been found worldwide.[3,4] NDM-1 has been identified in various Enterobacteriaceae due to localization of bla_{NDM-1} on conjugative plasmids, enabling transfer and rapid dissemination of multidrug resistance.[4] In 2010 bla_{NDM-1} and the related bla_{NDM-2} gene were also found in *Acinetobacter baumannii*, whereby either plasmid transfer of these genes was confirmed or the genetic location was not further investigated in detail.[5–8] Infections with carbapenemase-producing *A. baumannii* are a serious threat because colistin is often the only treatment option. Carbapenem resistance in *A. baumannii* is mainly due to expression of various OXA β-lactamases, such as OXA-23, OXA-58, OXA-40 and OXA-143-related enzymes, as well as overexpression of the intrinsic OXA-51-like enzyme.[9] Non-OXA-mediated carbapenem resistance is still rare. Here we report the molecular characterization of the genetic environment of bla_{NDM-1} detected in a clinical *A. baumannii* strain from a German hospital.

Materials and methods

Bacterial strains

A. baumannii strain 161/07 was isolated from a patient who had been repatriated to Germany from Serbia in 2007. The case history surrounding

this bla_{NDM-1}-positive *A. baumannii* strain 161/07 has been described previously.[6] Standard *A. baumannii* ATCC 19606 and ATCC 17978 were used as recipients for plasmid transformation. Sodium azide-resistant *Escherichia coli* J53 was used as the recipient for transformation and broth mating assays.

Antimicrobial susceptibility

Antimicrobial drug susceptibilities were determined according to the guidelines of the CLSI by broth microdilution (Table S1, available as Supplementary data at *JAC* Online) and Etest (bioMérieux, Nürtingen, Germany).[10] The MBL phenotype was confirmed with MBL-Etest and a combined disc diffusion test CDDT (KPC+MBL Confirm ID Kit; Alere GmbH, Cologne, Germany).

Presence of carbapenem resistance genes

Detection of OXA-type carbapenemases and associated insertion elements was performed by PCR and sequence analyses were performed as previously described.[11,12] Detection of the bla_{NDM-1} gene was performed by PCR using primers ndm1_F (5′-CTGAGCACCGCATTAGCC-3′) and ndm-1_R (5′-GGGCCGTATGAGTGATTGC-3′). For detection of other common bla_{OXA} genes ($bla_{OXA-1,2,9,10}$), the following primers were used: oxa1_ F (5′-TATCTACAGCAGCGCCAGTG-3′); oxa1_ R (5′-TAAATTCGACCCCAAGTTTC C-3′); oxa2_ F (5′-GCAAGAAGGCACGCTAGAAC-3′); oxa2_ R (5′-CAGCGTCC GAGTTGACTG-3′); oxa9_ F (5′-TTTGCTGCTGCATATGTTGG-3′); oxa9_ R (5′-CCCATCAACACGGGTAATTC-3′); oxa10_ F (5′-TTCGAGTACGGCATTAGCTG-3′); and oxa10_ R (5′-CAATGATGCCCTCACTTTCC-3′).

Molecular typing

Strain typing was performed by rep-PCR using the DiversiLab system (bioMérieux) and results were compared with our database of worldwide clonal lineages.[9] In addition, multiplex PCR-based typing in combination with ApaI PFGE was performed.[13]

Characterization of bla_{NDM-1}

Transfer of β-lactam resistance was tested by broth mating assays with *E. coli* J53 as the recipient. Selection of transconjugants was performed on Mueller–Hinton agar plates that contained sodium azide (200 mg/L) and ampicillin (100 mg/L). Plasmid DNA was isolated using the QIAGEN Plasmid Mini Kit (QIAGEN, Hilden, Germany).

Transformation of plasmids into an electrocompetent *E. coli* J53 and *A. baumannii* ATCC 19606 and ATCC 17978 recipients was performed using standard procedures.[14] Plasmid size was determined by performing S1 nuclease restriction of whole genomic DNA combined with PFGE.[15] Southern hybridization using digoxigenin-dUTP-labelled probes and signal detection using CDP-*Star* were performed following the manufacturer's guidelines (Roche Diagnostics Ltd, West Sussex, UK).

Whole genomic DNA was isolated using the QIAGEN DNeasy kit and digested with EcoRV, ligated into EcoRV-cut plasmid pBBR1MCS, transformed into *E. coli* NEB 5-alpha and selected on ticarcillin (25 mg/L).[16] Inserts of isolates that grew on ticarcillin were amplified by PCR using M13 primers and sequenced. Insert DNA was sequenced on both strands by primer walking.

Additionally, transfer of naked whole genomic DNA of *A. baumannii* 161/07 into an ampicillin-susceptible *A. baumannii* 102/07 recipient by natural transformation was performed. We have recently identified conditions promoting the uptake of naked DNA by a significant number of *A. baumannii* isolates. These results will be published elsewhere (E. Skiebe and G. Wilharm, unpublished results).

Nucleotide sequence accession number

The nucleotide and protein sequences of the bla_{NDM-1}-containing transposon and gene *aphA-6* have been registered in GenBank under accession numbers HQ857107 and JF949760, respectively.

Results and discussion

Strain typing by rep-PCR (DiversiLab) revealed that the NDM-1-producing multidrug-resistant *A. baumannii* strain 161/07 clustered with isolates of the clonal lineage WW7. Previous work has shown that *A. baumannii* strains belonging to the WW7 cluster harboured the carbapenemase genes bla_{OXA-58} or bla_{OXA-23} and were from different countries in South America, Europe and Asia.[9] Furthermore, all *A. baumannii* of the WW7 cluster harboured bla_{OXA-64}, a variant of the intrinsic bla_{OXA-51}.[17] It was confirmed by PCR that *A. baumannii* 161/07 was positive for the chromosomally located bla_{OXA-64}, but this gene was not associated with insertion element IS*AbaI*. Other bla_{OXA} genes were not detected. PCR-based *A. baumannii* typing in combination with ApaI PFGE analysis confirmed that *A. baumannii* 161/07 was not related to European clonal lineages 1–3.[13]

Transfer of resistance genes by *in vitro* conjugation and transformation of plasmids into *E. coli* or *A. baumannii* recipients was not successful. Analysis of S1-digested DNA fragments revealed the presence of two plasmids (125 kb, 75 kb) in the NDM-1-producing *A. baumannii* 161/07 strain. However, hybridization signals with a bla_{NDM-1} probe were not detected for these plasmids, and PCR with bla_{NDM-1}-specific primers failed to amplify a PCR product, suggesting a chromosomal location of bla_{NDM-1}.

Using naked whole genomic DNA from *A. baumannii* 161/07 to transform the naturally competent ampicillin-susceptible *A. baumannii* recipient 102/07, the gene bla_{NDM-1} was successfully transferred, as confirmed by PCR. All β-lactams tested against the transformant 161/07-102 showed an increase in MIC, with imipenem and meropenem MICs increasing from 0.25 mg/L to >32 mg/L, and 0.5 mg/L to 32 mg/L, respectively (Table S1). No other class of antibiotic was affected.

Sequence analysis of shotgun cloned *A. baumannii* 161/07 genomic DNA revealed a 3.9 kb insert containing the bla_{NDM-1} gene and parts of the plasmid sequence pKpANDM-1 described previously (Figure 1C).[3] However, the IS*26* transposase upstream of bla_{NDM-1} in previously characterized plasmids pKpANDM-1 and pNDM-HK was not present in *A. baumannii* 161/07. Instead, the insertion sequence IS*Aba125* was identified (Figure 1B). Interestingly, pKpANDM-1 includes a partial sequence of IS*Aba125* adjacent to bla_{NDM-1}. BLAST analysis of IS*Aba125* revealed seven copies of IS*Aba125* on the chromosome of *A. baumannii* strain ACICU in the GenBank database, and IS*Aba125* was found disrupting the *carO* gene, leading to carbapenem resistance.[18] Furthermore, IS*Aba125* can also be plasmid located, and has been described recently upstream of the bla_{OXA-58} gene.[19]

To determine whether bla_{NDM-1} was located in a transposon, PCR was performed with inverse primers to IS*Aba125* [TRANSIS_R (5′-AAACAACGGATCGCTTCAAC-3′) and TRANSIS_F (5′-CGAGCAT TACCAAAGGGTGA-3′)] using genomic DNA of *A. baumannii* 161/07 as template. Two products of 2 kb and 9 kb were amplified. Sequencing of the 2 kb amplicon revealed *aphA-6*, an

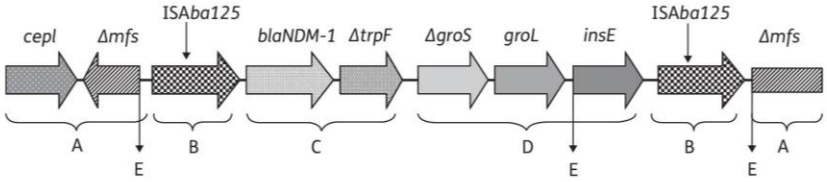

Figure 1. Schematic diagram showing the genetic environment of bla_{NDM-1} in *A. baumannii* 161/07. (A) Chromosomal genes *cepI* and the truncated *mfs*; (B) IS*Aba125*; (C) bla_{NDM-1} and truncated *trpF*; (D) truncated chaperonin subunit *groS*, chaperonin *groL* and the transposase *insE*; (E) 8 bp target duplication.

aminoglycoside resistance gene bracketed between two copies of IS*Aba125*.

Primer walking from both ends of the 9 kb amplicon revealed a composite transposon structure containing bla_{NDM-1} bracketed between two copies of IS*Aba125* (Figure 1B). Both copies of IS*Aba125* were flanked by 17 bp inverted repeats. Both 5′ inverted repeats and both 3′ inverted repeats were identical, respectively, but there were two nucleotide differences between 5′ and 3′ inverted repeats. The two IS*Aba125* transposase genes differed by four nucleotides whereby one resulted in amino acid substitution, Arg-41→Gln. Including both IS*Aba125* insertion elements, the composite transposon was 10093 bp in length. Furthermore, it was integrated into a chromosomal gene encoding a putative *A. baumannii* major facilitator superfamily (MFS) metabolite/H^+ symporter (Figure 1A) that has previously been disrupted by IS*Aba1*-bla_{OXA-23}.[20] Further PCR and sequencing with primers for IS*Aba125* and the disrupted MFS transporter confirmed the chromosomal location of the bla_{NDM-1}-containing transposon. Evidence of this being a transposition event was an 8 bp target site duplication at the point of insertion in the gene encoding MFS (Figure 1E). Adjacent to the MFS gene we identified a chromosomal homoserine lactone synthase gene (*cepI*). In addition, inside the bla_{NDM-1}-containing transposon there was a 4 kb element 93% similar to that described in *E. coli* plasmids pEH4H and pAR060302, encoding the chaperonin subunits *groS* and *groL*, and the transposase *insE*, which was not associated with inverted repeats (Figure 1D). The 8 bp target site duplication was also present at the 5′-end of the *insE* gene (Figure 1E). Further BLAST analysis revealed a 297 bp sequence, including 41 bp of the 3′-end of *insE* and extending towards IS*Aba125*, that showed 99% identity to insertion sequence IS*CR19*-like and *ori*IS previously described in a *Pseudomonas aeruginosa* isolate.[21] Deletions were found in gene *trpF* (79 bp, 3′-end) and gene *groS* (154 bp, 5′-end). No significant DNA homology or open reading frames were detected in the 1.6 kb region between the truncated *trpF* and *groS* genes.

In conclusion, analysis of the genetic environment of bla_{NDM-1} in *A. baumannii* 161/07 revealed a transposon structure composed of two copies of insertion sequence IS*Aba125* that is integrated into the bacterial chromosome. However, since bla_{NDM-1} is flanked by these insertion elements, we cannot discount the possibility of integration into a plasmid and subsequent horizontal spread. The variability of the genetic environment of bla_{NDM-1}, as evidenced by the transposon structure described here, with DNA seemingly originating from Enterobacteriaceae, *P. aeruginosa* and *A. baumannii*, may explain the observed rapid dissemination of this gene within many Gram-negative bacterial species and across genera. Thus there is an urgent need for further investigations to find the origin of this gene and its mechanisms of spread.

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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. MICs of a range of antibiotics for the NDM-1-producing clinical strain *A. baumannii* 161/07, the NDM-1-producing *A. baumannii* transformant 161/07-102 and the recipient *A. baumannii* strain 102/07

	MIC (mg/L)		
		transformant	
Antibiotic	strain 161/07	161/07-102	strain 102/07
Ampicillin	>16	>16	8
Mezlocillin	>32	>32	16
Mezlocillin/sulbactam	>32	>32	≤2
Cefotiam	>8	>8	>8
Cefotaxime	>16	>16	≤1
Ceftazidime	>32	32	≤2
Cefoxitin	>32	>32	32
Aztreonam	32	16	16
Gentamicin	>8	≤0.5	≤0.5
Kanamycin	>32	≤2	≤2
Amikacin	>32	≤2	≤2
Streptomycin	>64	≤4	≤4
Rifampicin	2	4	4
Chloramphenicol	>32	>32	>32
Ciprofloxacin	>64	4	2
Sulfameracin	>512	≤32	≤32
Trimethoprim/sulfamethoxazole	>128	≤4	≤4
Colistin[a]	0.38	0.38	0.38
Tigecycline[a]	1	0.75	0.75
Meropenem[a]	>32	32	0.5
Imipenem[a]	>32	>32	0.25
Ertapenem[a]	>32	>32	0.19
MBL-Etest [imipenem/imipenem+EDTA (MBL inhibitor).]	positive	positive	negative
KPC+MBL Confirm ID Kit[b]	MBL positive	MBL positive	MBL negative

[a]Determined by Etest.

[b]Alere GmbH, Cologne, Germany.

2.2 Characteristics of the intrinsic OXA-51 subclass in *A. baumannii*

2.2.1 Detection of intrinsic *bla*$_{\text{OXA-51-like}}$ by multiplex PCR on its own is not reliable for the identification of *Acinetobacter baumannii*

The intrinsic OXA-51-like has so far been detected in every investigated *A. baumannii* isolate. Therefore, detection of a *bla*$_{\text{OXA-51-like}}$-specific amplicon in the OXA-multiplex PCR described by Woodford *et al.*, is often used for species identification.[122] In the present publication we report unusual *bla*$_{\text{OXA-51-like}}$ amplicons detected by multiplex PCR in three clinical *A. baumannii* isolates, originating from different countries. Recently, Lopes *et al.* identified disruption of the intrinsic OXA gene by IS*Aba16* in a single *A. baumannii* isolate.[123] Comparably, sequencing in our isolates revealed disruption of *bla*$_{\text{OXA-51-like}}$ by IS elements. In contrast to Lopes *et al.* we detected insertion of IS*Aba19* into *bla*$_{\text{OXA-78}}$ in two epidemiologically related carbapenem-susceptible *A. baumannii* isolates, as well as insertion of IS*Aba15* into *bla*$_{\text{OXA-66}}$ in a carbapenem-resistant isolate which also harbored an acquired OXA-23. Our results show that disruption of the intrinsic OXA-51-like gene in *A. baumannii* has occurred on more than one occasion and that the intrinsic gene is not necessary as an additional determinant for carbapenem resistance in the presence of acquired OXA. Furthermore our results indicate that detection of *bla*$_{\text{OXA-51-like}}$ by multiplex PCR may not be a fully reliable tool for *A. baumannii* species identification and should be confirmed by additional methods.

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Short communication

Detection of intrinsic $bla_{\text{OXA-51-like}}$ by multiplex PCR on its own is not reliable for the identification of *Acinetobacter baumannii*

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ABSTRACT

Three clinical *A. baumannii* isolates Ab-508, Ab-511, and Ab-653 were recovered from South Africa, South Korea, and Turkey, respectively. Multiplex PCR to detect OXA-type carbapenemases showed atypical $bla_{\text{OXA-51-like}}$ amplification products. The aim of this study was to investigate the background of changes in $bla_{\text{OXA-51-like}}$ PCR products. Isolates were confirmed as *A. baumannii* using *gyrB* multiplex and *rpoB* sequencing and were epidemiologically unrelated by rep-PCR-based DiversiLab. Sequencing of $bla_{\text{OXA-51-like}}$ revealed an insertion of IS*Aba15* in $bla_{\text{OXA-66}}$ (isolate Ab-511) and an insertion of the novel IS*Aba19* in $bla_{\text{OXA-78}}$ (isolates Ab-508 and Ab-653). Detection of the intrinsic $bla_{\text{OXA-51-like}}$ by OXA-multiplex PCR should not be considered a fully reliable method for identification of *A. baumannii* when used without an additional independent method. Other species identification methods such as *gyrB* multiplex PCR and *rpoB* sequencing should be used to reliably identify *A. baumannii*.

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2.2.2 Association between β-lactamase-encoding *bla*$_{\text{OXA-51}}$ variants and DiversiLab rep-PCR-based typing of *Acinetobacter baumannii* isolates

Some *A. baumannii* clonal lineages are more prevalent than others, for example IC2. Various typing methods can be used to identify clonal lineages of *A. baumannii*, including sequence group typing, MLST and rep-PCR (see 1.1.5).[73, 75, 76] The OXA-51-like gene is intrinsic to *A. baumannii* and shows great diversity, whereby *bla*$_{\text{OXA-51-like}}$ sequencing might be considered as a single locus typing method. Previous studies have investigated the correlation between *bla*$_{\text{OXA-51-like}}$ sequenced-based typing and MLST or sequence group typing.[76, 124] However, these studies were limited to IC1 to IC3 and did not include other international clonal lineages.

On the basis of a large collection of clinical *A. baumannii* isolates with worldwide origin we identified correlation between rep-PCR and eight previously described international (worldwide) clonal lineages,[67] indicating that certain OXA-51-like genes belong to certain clonal lineages. Therefore *bla*$_{\text{OXA-51-like}}$ typing by sequencing might be a suitable indicator of IC1 to IC8. In contrast to a previous study we discovered that the nucleotide sequence is more convenient to analyze OXA-51-like relatedness, compared to the amino acid sequence.[125] In addition, we identified five novel OXA-51 amino acid variants, OXA-200, OXA-201, OXA-202, OXA-219 and OXA-223, as well as a novel nucleotide variant, OXA-113a.
Interestingly, the association of IS*Aba1* was restricted to certain OXA-51-like, for example OXA-82 and OXA-201, and was only detected in carbapenem-resistant isolates in the absence of acquired OXA. In contrast, more ancestral OXA variants like OXA-66 were not associated with IS*Aba1* and were detected either in carbapenem-susceptible isolates in the absence of other OXAs or in carbapenem-resistant isolates only when an acquired OXA was present. This might indicate influence of carbapenem selection on OXA-51-like evolution.

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Association between β-Lactamase-Encoding bla_{OXA-51} Variants and DiversiLab Rep-PCR-Based Typing of *Acinetobacter baumannii* Isolates

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This study investigated the correlation between bla_{OXA-51} variants and *Acinetobacter baumannii* worldwide clonal lineages 1 to 8 (WW1 to -8). The $bla_{OXA-51-like}$ genes of 102 *A. baumannii* isolates were sequenced. Using DiversiLab repetitive-sequence-based PCR (rep-PCR) typing, 92 of these isolates had previously been assigned to WW1 to -8 and 10 were unclustered. Clustering of DNA sequences was performed using the neighbor-joining method and the Jukes-Cantor phylogenetic correction. bla_{OXA-51} variants were in good correlation with DiversiLab-defined clonal lineages. Sequence-based typing of bla_{OXA-51} variants has the potential to be applied for epidemiologic characterization of *A. baumannii* and to identify worldwide clonal lineages 1 to 8.

Acinetobacter baumannii is a Gram-negative hospital-acquired pathogen which commonly causes pneumonia, bloodstream infections, meningitis, wound infections, and urinary tract infections, especially in patients with impaired host defenses (3, 14). Until recently, the majority of *A. baumannii* isolates, while being resistant to many antimicrobial classes (fluoroquinolones, tetracyclines, cephalosporins, and aminoglycosides), remained susceptible to carbapenems (3). However, today carbapenem resistance is more frequently encountered, with rates of up to 70% of isolates reported in some countries (3, 11, 13, 14, 19). Predominantly in *A. baumannii*, carbapenem resistance is conferred by carbapenem-hydrolyzing class D oxacillinases (CHDLs) (8, 15). These include the acquired OXA-23-like, OXA-40-like, OXA-58-like, and OXA-143 oxacillinases, as well as the intrinsic OXA-51-like oxacillinase, of which there are currently 68 variants identified. Although CHDLs exhibit weak carbapenem hydrolysis, they can confer resistance when overexpressed. This is mediated through a combination of naturally low permeability to β-lactams and IS*Aba* elements located upstream of the gene, providing a strong promoter (the OXA-40-like and OXA-143 oxacillinases appear to be exceptions to this) (16, 21).

Molecular typing of isolates obtained from various locations in Europe has shown the existence of three distinct lineages that have been termed European clone I (EUI), EUII, and EUIII (2, 3). More recently, repetitive-sequence-based PCR (rep-PCR) typing using the DiversiLab system has identified eight carbapenem-resistant *A. baumannii* clonal lineages (WW1 to -8) that are distributed worldwide (8). WW1 to -3 have been shown to correspond to EUI to -III (8). A correlation of OXA-69, OXA-66, and OXA-71 to EUI to -III, respectively, has been utilized in a multiplex PCR-based method to identify the three lineages (20). The aim of this study was to investigate the correlation between $bla_{OXA-51-like}$ sequences and worldwide clonal lineages 1 to 8.

(This work was presented in part at the 21st ECCMID/27th ICC, Milan, Italy, 7 to 10 May 2011.)

MATERIALS AND METHODS

Bacterial isolates. One hundred two *A. baumannii* isolates were selected from a worldwide collection of imipenem-nonsusceptible *A. baumannii* clinical isolates collected between 2004 and 2010. These had previously been molecularly typed using DiversiLab and assigned to worldwide clonal lineages 1 to 8 or to other (sporadic) genotypes (8). The isolates selected for this study comprised at least eight isolates representing each clonal lineage (Table 1). In addition, 10 isolates with unique DiversiLab genotypes were included. Isolates within a lineage were chosen to represent as many countries of origin as possible.

Sequence group multiplex PCR. Isolates which had been identified as WW1, -2, or -3 using DiversiLab or which were found to be in possession of a bla_{OXA-51} variant that was associated with these lineages were also investigated by multiplex PCR based on amplification of the *ompA*, *csuE*, and $bla_{OXA-51-like}$ genes as previously described (20).

PCR amplification and $bla_{OXA-51-like}$ sequencing. Template DNA was extracted from an overnight culture on blood agar plates. A 1-μl loopful was resuspended in 100 μl sterile water and boiled for 10 min before snap cooling on ice. Amplification of $bla_{OXA-51-like}$ genes was performed as described previously, using primer pair OXA-69A/OXA-69B or preABprom^{+}/OXA-69B when IS*Aba1* was found upstream of the gene (4, 21). PCR products were sequenced in both directions. bla_{OXA-51} variants were identified by BLAST query. To confirm sequences of novel bla_{OXA-51} variants, PCR and sequencing reactions were repeated using Phusion hot-start high-fidelity DNA polymerase (Thermo Fisher Scientific, Schwerte, Germany). Products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced. Novel sequences were assigned by the Lahey β-lactamase database (http://www.lahey.org/Studies/) and submitted to GenBank.

Phylogenetic analysis. Based on the nucleotide sequences covering the whole coding regions of $bla_{OXA-51-like}$ genes, phylogenetic trees were constructed by using the neighbor-joining clustering algorithm and the Jukes-Cantor distance model using Bionumerics 5.1 software (Applied-Maths, St-Martens-Latem, Belgium). An initial analysis looked at clustering of only the bla_{OXA-51} variants sequenced in this study. A second analysis compared clustering of all published bla_{OXA-51} variants.

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TABLE 1 bla_{OXA-51} variants and origins of 92 isolates belonging to WW1 to -8

Lineage	bla_{OXA-51} variant product (*n*)	Amino acid substitution(s)[a]	Country(ies) of origin
WW1 (EUI)	OXA-69 (7 + 1[b])		Germany, Spain, Pakistan,[b] India, Greece, Italy
	OXA-92 (1)	W234→S	Greece
	OXA-107 (3)[c]	L167→V	Poland
WW2 (EUII)	OXA-66 (13)		UK, Portugal, Australia, Austria, Greece, Ireland, Italy, South Africa, Poland
	OXA-82 (5)[c]	L167→V	USA, Poland, Taiwan
	OXA-172 (1)[c]	I129→V, W222→L	Taiwan
	OXA-201 (1)[c]	L167→V, P130→Q	Spain
	OXA-202 (1)[c]	I129→M	USA
WW3 (EUIII)	OXA-71 (2)		Spain, South Africa
	OXA-113 (7)[c]	L167→V	USA
WW4	OXA-51 (8)		Turkey, Argentina, India, Germany, Brazil, Chile
	OXA-219 (1)[c]	L167→V	Chile
WW5	OXA-65 (12 + 2[b])		Spain,[b] Argentina, USA, Colombia, Venezuela, Germany, Mexico
WW6	OXA-90 (3)		Italy
	OXA-200 (5)[c]	P130→L, W222→L	Honduras
WW7	OXA-64 (10)		Latvia, Switzerland, Venezuela, Mexico, Colombia, Singapore, Germany
WW8	OXA-68 (7)		Spain, Turkey, South Korea, China
	OXA-128 (2)	D68→V	France, Bulgaria

[a] Amino acid changes compared to OXA-69 for WW1, OXA-66 for WW2, OXA-71 for WW3, OXA-51 for WW4, OXA-90 for WW6, and OXA-68 for WW8.
[b] IS*Aba1* upstream of bla_{OXA-51} variant in an isolate with an acquired OXA.
[c] IS*Aba1* upstream of bla_{OXA-51} variant in an isolate without an acquired OXA.

Nucleotide sequence accession numbers. The nucleotide sequences of the novel bla_{OXA-51} variants reported in this paper have been submitted to the EMBL/GenBank database under accession numbers JN790646 (OXA-113b), HQ734811 (OXA-200), HQ734812 (OXA-201), HQ734813 (OXA-202), JN215211 (OXA-219), and JN248564 (OXA-223).

RESULTS

Sequencing of $bla_{OXA-51-like}$ genes. To investigate the correlation between bla_{OXA-51} variants and DiversiLab clonal lineages, $bla_{OXA-51-like}$ genes of 102 clinical isolates were sequenced. Ninety-three isolates were in possession of known bla_{OXA-51} variants, and we identified five novel OXA-51-like oxacillinases: OXA-200, OXA-201, OXA-202, OXA-219, and OXA-223. Tables 1 and 2 summarize these results and include the strains' countries of origin. With the exception of $bla_{OXA-113}$, which we found to have a nucleotide sequence different from that published (two nucleotide mismatches; henceforth denoted as $bla_{OXA-113b}$), DNA polymorphisms were not found in the bla_{OXA-51} variants.

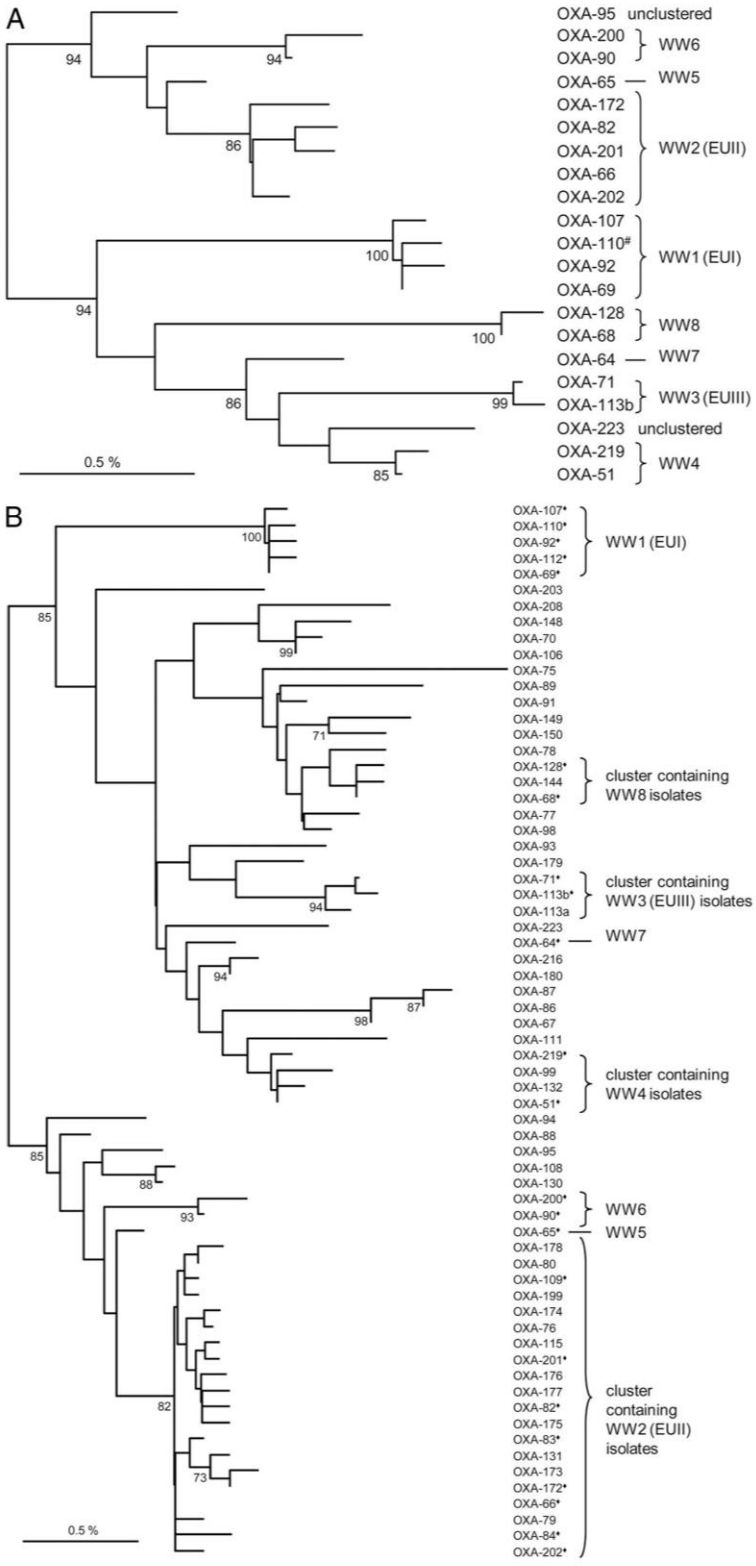
***bla*$_{OXA-51-like}$ typing and DiversiLab clustering.** The bla_{OXA-51} variant groups were compared to DiversiLab clusters. Isolates belonging to the same clonal lineage were in possession of similar bla_{OXA-51} variants. These had either the same bla_{OXA-51} variant or an amino acid variant (Table 1). For example, WW1 isolates were in possession of OXA-69 or variants of this, i.e., OXA-92 (W234→S) or OXA-107 (L167→V) (Table 1).

Interestingly we found that variants of OXA-66, OXA-69, and OXA-71 were identified in carbapenem-resistant isolates without an acquired OXA, and in each case the $bla_{OXA-51-like}$ gene was associated with IS*Aba1* (with the exception of bla_{OXA-92}) (Table 1).

TABLE 2 Isolates that did not cluster in DiversiLab and their expected worldwide lineage (where applicable) based on their $bla_{OXA-51-like}$ sequence

bla_{OXA-51} variant (*n*)	Expected worldwide lineage based on bla_{OXA-51} variant[a]	Highest similarity (%) to worldwide lineage using DiversiLab	Sequence group	Country(ies) of origin
OXA-69 (2)	WW1	WW1, 87.3	SG2 (EUI, WW1)	Italy, Argentina
OXA-110 (1)	WW1 (see Fig. 1A)	WW1, 87.6	SG2 (EUI, WW1)	Poland
OXA-66 (1)	WW2	WW2, 88.6	SG1 (EUII, WW2)	Australia
OXA-82 (1)	WW2	WW6. 81.3	$bla_{OXA51-like}$ band of SG1	Taiwan
OXA-51 (2)	WW4	WW5, 77.9	ND[b]	Chile, Brazil
OXA-68 (1)	WW8	WW8, 84.9	ND	India
OXA-223 (1)	UNC[c]	WW4, 88.8	ND	USA
OXA-95 (1)	UNC	WW6, 79	ND	Singapore

[a] See Table 1.
[b] ND, not determined.
[c] UNC, unclustered.


A
OXA-95 unclustered
OXA-200
OXA-90
WW6
OXA-65
WW5
OXA-172
OXA-82
OXA-201
OXA-66
OXA-202
WW2 (EUII)
OXA-107
OXA-110#
OXA-92
OXA-69
WW1 (EUI)
OXA-128
OXA-68
WW8
OXA-64
WW7
OXA-71
OXA-113b
WW3 (EUIII)
OXA-223 unclustered
OXA-219
OXA-51
WW4
0.5 %
B
OXA-107*
OXA-110*
OXA-92*
OXA-112*
OXA-69*
WW1 (EUI)
OXA-203
OXA-208
OXA-148
OXA-70
OXA-106
OXA-75
OXA-89
OXA-91
OXA-149
OXA-150
OXA-78
OXA-128*
OXA-144
OXA-68*
cluster containing WW8 isolates
OXA-77
OXA-98
OXA-93
OXA-179
OXA-71*
OXA-113b*
OXA-113a
cluster containing WW3 (EUIII) isolates
OXA-223
OXA-64*
WW7
OXA-216
OXA-180
OXA-87
OXA-86
OXA-67
OXA-111
OXA-219*
OXA-99
OXA-132
OXA-51*
cluster containing WW4 isolates
OXA-94
OXA-88
OXA-95
OXA-108
OXA-130
OXA-200*
OXA-90*
WW6
OXA-65*
WW5
OXA-178
OXA-80
OXA-109*
OXA-199
OXA-174
OXA-76
OXA-115
OXA-201*
OXA-176
OXA-177
OXA-82*
OXA-175
OXA-83*
OXA-131
OXA-173
OXA-172*
OXA-66*
OXA-79
OXA-84*
OXA-202*
cluster containing WW2 (EUII) isolates
0.5 %

Similarly $bla_{OXA-200}$, a variant of bla_{OXA-90}, and $bla_{OXA-219}$, a variant of bla_{OXA-51}, were also associated with IS*Aba1* and carbapenem resistance. IS*Aba1* was also upstream of one bla_{OXA-69} and two bla_{OXA-65} genes, but the isolates possessing these genes also had an acquired bla_{OXA}.

Ten isolates had unique DiversiLab genotypes (Table 2). Two of these were in possession of bla_{OXA-95} or $bla_{OXA-223}$, which did not cluster with the other bla_{OXA-51} variants (Fig. 1A; Table 2). Five isolates possessed a bla_{OXA-51} variant that suggests that they should cluster with WW1 or WW2. However, DiversiLab did not cluster them with either of these lineages. Therefore, all isolates with OXA-66 or OXA-69 variants were investigated using sequence-based multiplex PCR (Table 2). This confirmed the WW1/WW2 clustering as previously described (8). The four unclustered isolates possessing bla_{OXA-69}, $bla_{OXA-110}$, or bla_{OXA-66} amplified either the sequence group 1 (SG1) or SG2 pattern (Table 2). In addition, the unclustered bla_{OXA-82} isolate was positive for an SG1 $bla_{OXA-51-like}$ gene, while *ompA* and *csuE* were negative.

Phylogenetic analysis. bla_{OXA-51} variant gene sequences could be classified into six distinct clusters and four unique sequences (Fig. 1A). Correlation of OXA-69, OXA-66, and OXA-71 to EUI, -II, and -III, respectively, was shown by the linkage map published by Evans et al., which was based on amino acid sequences (4). OXA-65 was placed in the center of the map, and all variants radiated out from there. However, this linkage map can be misleading. For example, OXA-91 and OXA-95 differ by five amino acids and are distant on the map. OXA-104 and OXA-95 differ by three amino acids and are situated close together on the map. However, at the DNA sequence level, bla_{OXA-91} and bla_{OXA-95} are 98.4% similar, while $bla_{OXA-104}$ is <76% similar to either of these genes. Therefore, we chose to analyze OXA-51-like variants at the DNA level because this may allow for a more sensitive approximation of relatedness.

Figure 1B represents the hypothetical phylogenetic relationship of all bla_{OXA-51} variants published to date, with the exceptions of $bla_{OXA-116}$ and $bla_{OXA-117}$, whose sequences were incomplete, and $bla_{OXA-104}$, which occupied a separate position in the tree, based on very low similarity (<76%) to all other variants (data not shown). Although there was a lack of a clear phylogenetic structure, some putative monophyletic sequence groups were present, as indicated by bootstrapping values of >70% (Fig. 1B). Two large, well-defined clusters encompass bla_{OXA-51} variants associated with WW1 and WW2, respectively.

DISCUSSION

Commonly used methods to identify the clonal relatedness of *A. baumannii* isolates are macrorestriction analysis by pulsed-field gel electrophoresis (PFGE), sequence group typing based on the amplification of three chromosomal genes (20), and multilocus sequence typing (MLST) based on the amplification of seven housekeeping genes (1, 2). Unfortunately these methods are often time-consuming, expensive, or labor-intensive. The major advantage of $bla_{OXA-51-like}$ gene typing is that sequencing is based on a single gene; therefore, this method appears to be an easier, faster, and cheaper way of *A. baumannii* typing. Single-locus sequencing has proved useful with typing of other species, for example, Shiga toxin-producing *Escherichia coli* (STEC) (5) and *Staphylococcus aureus* (*spa* typing) (12, 22). However, some *S. aureus* single-locus methods, such as SCC*mec* and *agr* typing, while useful, are not as discriminatory as *spa* typing (22).

Several studies have also investigated sequence-based $bla_{OXA-51-like}$ gene typing in comparison to other typing methods, where it was shown that $bla_{OXA-51-like}$ gene sequencing corresponded to MLST and sequence group typing (6, 20) but not to PFGE (6). However, the PFGE-derived dendrogram did not fully differentiate between the different EU clonal clusters, highlighting that PFGE is not suited for population studies. In our study, we found a correlation between $bla_{OXA-51-like}$ sequences and worldwide clonal lineages 1 to 8. It was shown that some clonal lineages possess more OXA-51 variants than others, which may result from either the stability of some variants or their association with relatively young lineages. For example, WW5 isolates were geographically widespread but possessed only OXA-65, while WW1 and WW2 were equally widespread but possessed several OXA-51 variants. It was speculated that based on known intraclonal heterogeneity, EUI and -II are relatively old compared to EUIII, and this may explain why they have a greater number of OXA-51 variants (2). Based on this, WW5 is likely to be a more recently established clonal lineage. However, the relative age of a clonal cluster may not be the only factor behind the variability of bla_{OXA-51} variants.

It has been shown that carbapenem resistance is commonly associated with acquired OXAs or overexpression of OXA-51-like oxacillinases (7, 9, 21). We found that the majority of carbapenem-resistant isolates either possessed an acquired OXA or had IS*Aba1* associated with a $bla_{OXA-51-like}$ gene. Interestingly, IS*Aba1* was associated predominantly with $bla_{OXA-51-like}$ genes which encoded amino acid variants of OXA-66, OXA-69, OXA-51, OXA-71, and OXA-90 but mainly where it was the only carbapenem resistance mechanism detected. Not only do insertion sequence (IS) elements lead to overexpression, but published data suggest a role of IS elements in the evolution of β-lactamases. For example, an *in vivo* mutation was described in *A. baumannii* that converted the acquired $bla_{OXA-164}$ into bla_{OXA-58} and was associated with carbapenem therapy and the presence of IS*Aba3* (10). Similarly, it was shown in *E. coli* that the IS*Ecp1*-associated extended-spectrum β-lactamase CTX-M-3 exhibited an amino acid substitution after selection on ceftazidime (18). It can therefore be hypothesized that carbapenem therapy may play a role in the selection of bla_{OXA-51} variants when it is the sole carbapenem resistance determinant and associated with IS*Aba1*-mediated overexpression.

As the association between DiversiLab types and bla_{OXA-51} variants suggests the coevolution of $bla_{OXA-51-like}$ sequences with other parts of the *A. baumannii* genome (seen as different rep-PCR patterns), it would be interesting to further investigate isolates carrying each of the known OXA-51 variants to gain further insights into the correlation between sequence-based $bla_{OXA-51-like}$

FIG 1 (A) Unrooted neighbor-joining tree based on $bla_{OXA-51-like}$ nucleotide sequences of 102 clinical isolates. Bootstrap percentages of >70% after 1,000 replicates are shown. Horizontal bar, 0.5% sequence divergence. #, the isolate possessing OXA-110 did not cluster with WW1 to -8 by rep-PCR but was positive for SG2 (WW1). (B) Unrooted neighbor-joining tree based on nucleotide sequences of all published bla_{OXA-51} variants except $bla_{OXA-116}$, $bla_{OXA-117}$, and $bla_{OXA-104}$. Horizontal bar, 0.5% sequence divergence. Bootstrap percentages of >70% after 1,000 replicates are shown. ◆, bla_{OXA-51} variants ($bla_{OXA-109}$, $bla_{OXA-112}$, bla_{OXA-83}, and bla_{OXA-84}) that have previously been shown to correlate with EUI to -III (6, 20).

typing and other typing methods. The results for our rep-PCR-unclustered isolates indicate that amplification of repetitive regions of the genome may not always be in agreement with sequence-based multiplex PCR. Repetitive sequences may be subject to rapid changes brought about by recombination events, which may explain the lack of correlation between the two methods with these strains. In a recent study, recombination hot spots were found to include genomic regions that encode proteins associated with cell surface molecules but to our knowledge were not associated with $bla_{\text{OXA-51-like}}$ genes (17). Therefore, the use of alternative methods such as the Bartual and/or Pasteur MLST schemes may help to resolve this issue (1, 2).

In summary, despite the variation in DNA sequences, we observed a striking correlation between $bla_{\text{OXA-51}}$ monophyletic groups and *A. baumannii* worldwide clonal lineages 1 to 8. Therefore, sequencing of $bla_{\text{OXA-51-like}}$ genes has the potential to contribute to the population analysis of *A. baumannii* and be used to identify not only European clones I to III but also *A. baumannii* isolates belonging to worldwide clonal lineages 1 to 8.

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We have no transparency declarations.

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2.2.3 Conversion of OXA-66 into OXA-82 in clinical *Acinetobacter baumannii* isolates and association with altered carbapenem susceptibility

In the present study three outbreak related *A. baumannii* isolates were investigated which were clonally related to each other but showed differences in their carbapenem susceptibility. The isolates were part of an outbreak in a hospital in Krakow as has been reported elsewhere.[126] As no acquired OXAs were detected, the intrinsic OXA-51-like genes were further investigated. In *A. baumannii* the chromosomal $bla_{\text{OXA-51-like}}$ gene is located between the *fxsA* and *yncA* genes, which encode a suppressor for F exclusion of phage T7 and a phosphinothricin acetyltransferase, respectively. Based on these flanking genes primers were designed to confirm the chromosomal location of the OXA genes. Furthermore $bla_{\text{OXA-51-like}}$ and flanking regions were sequenced. This revealed presence of OXA-66 in the carbapenem-susceptible isolate, while the carbapenem-resistant isolates harbored IS*Aba1* and a point mutation in OXA-66, converting it into OXA-82. In correlation to our previous study (see 2.2.2), OXA-82 was associated with an upstream located IS*Aba1*. As expected, OXA-82 was overexpressed in both carbapenem-resistant isolates compared to OXA-66 in the carbapenem-susceptible isolate. Figueiredo *et al.* have recently detected a comparable IS*Aba1*-mediated level of OXA-66 overexpression in a carbapenem-susceptible *A. baumannii* isolate.[127] Therefore our results suggest that the single amino acid substitution contributes to carbapenem resistance in *A. baumannii* when the OXA gene is overexpressed. This was further investigated on the basis of an isogenic background and preliminary data are presented below (see: Unpublished results I). Transfer of $bla_{\text{OXA-82}}$ with the IS*Aba1*-mediated promoter into the *A. baumannii* reference strain ATCC 17978 conferred carbapenem resistance. In addition, no change in the outer membrane profile of the clinical isolates was observed. Therefore we considered the OXA conversion, coupled with IS*Aba1*-mediated overexpression, as the sole carbapenem-resistance mechanism in both OXA-82-expressing *A. baumannii* isolates.

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Conversion of OXA-66 into OXA-82 in clinical *Acinetobacter baumannii* isolates and association with altered carbapenem susceptibility

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Objectives: Three clinical *Acinetobacter baumannii* isolates (A–C) were isolated from three separate patients during an outbreak in a hospital in Krakow, Poland. Isolate A was recovered first and was susceptible to carbapenems, whereas isolates B and C were resistant. The aim of this study was to investigate the differences in carbapenem susceptibility in these outbreak-related isolates.

Methods: Clonal relatedness was determined using rep-PCR-based DiversiLab. The $bla_{\text{OXA-51-like}}$ genes and their upstream regions were sequenced. Expression of the genes encoding OXA-51-like and the three major porins CarO, OprD-like and 33–36 kDa Omp were investigated by semi-quantitative RT–PCR. Comparison of outer membrane protein (OMP) profiles was performed using SDS–PAGE. IS*Aba1*-$bla_{\text{OXA-82}}$ was cloned into the shuttle vector pWH1266 and transferred into *A. baumannii* ATCC 17978.

Results: The isolates were identical by rep-PCR and clustered with international clonal lineage 2. Sequencing of $bla_{\text{OXA-51-like}}$ revealed a conversion of OXA-66 (isolate A) into OXA-82 (isolates B and C). $bla_{\text{OXA-82}}$ was also associated with IS*Aba1*. Expression analysis revealed overexpression of $bla_{\text{OXA-82}}$. There was no difference in OMP expression between the isolates. IS*Aba1*-$bla_{\text{OXA-82}}$ conferred carbapenem resistance in ATCC 17978.

Conclusions: Carbapenem resistance in outbreak-related isolates was mediated by conversion of OXA-66 into OXA-82 and its subsequent overexpression. This further highlights the genome plasticity of *A. baumannii*, leading to carbapenem resistance.

Keywords: qRT–PCR, $bla_{\text{OXA-51-like}}$, SDS–PAGE, porin expression

Introduction

Acinetobacter baumannii is a Gram-negative, non-fermentative pathogen that causes particular concern in the hospital. *A. baumannii* infections, including pneumonia, wound infections, bloodstream infections, urinary tract infections and meningitis, mainly affect hospitalized patients, especially the immunocompromised and those in intensive care units.[1] *A. baumannii* has the ability to survive on dry surfaces for prolonged periods, whereby it can be easily spread.[1] It is also characterized by its innate and acquired resistance to multiple antimicrobial classes (e.g. β-lactams, tetracyclines, aminoglycosides, cephalosporins and fluoroquinolones), including the development of carbapenem resistance in recent years.[1]

Carbapenem resistance in *A. baumannii* can be mediated by various mechanisms, including impermeability due to loss of one of its major porins (e.g. CarO, OprD-like and the 33–36 kDa Omp) and, possibly, efflux, as shown recently for meropenem.[1–4] Most frequently, though, it is mediated through enzymatic hydrolysis of the drug, particularly by carbapenem-hydrolysing class D β-lactamases (CHDLs). The CHDLs, also called oxacillinases, can be intrinsic (OXA-51-like) or acquired (OXA-23-like, -40-like, -58-like and -143-like).[5] Although they are weak carbapenem hydrolysers, they confer resistance when overexpressed. This overexpression of OXA is often associated with insertion elements (IS) that are located upstream of the gene, providing a strong promoter.[6,7] For example, carbapenem resistance mediated through overexpression of $bla_{\text{OXA-51-like}}$ is associated with IS*Aba1*.[6,8] Based on a previous study, IS*Aba1*-$bla_{\text{OXA-51-like}}$ was found to be the most prevalent carbapenem resistance mechanism in *A. baumannii* isolates around the world.[9]

In this study, we investigated the mechanism of carbapenem resistance in three clinical outbreak-related *A. baumannii* isolates recovered from different patients in a hospital in Krakow, Poland.

The isolate recovered first was susceptible to carbapenems, whereas the other two isolates were carbapenem resistant.

Materials and methods

Bacterial isolates, clonal relatedness and carbapenem susceptibility

Three outbreak-related *A. baumannii* isolates were recovered from three patients in a hospital in Krakow during 2009 and 2010 (Table 1). Their clonal relatedness was investigated using the rep-PCR-based DiversiLab system (bioMérieux, Nürtingen, Germany) and $bla_{OXA-51-like}$ typing.[10] Carbapenem susceptibility was determined by imipenem and meropenem Etest. Carbapenem-susceptible *A. baumannii* ATCC 17978 was used for cloning experiments.

PCR and sequencing

All primers used in this study are listed in Table 2. The presence of CHDLs and IS*Aba1* located upstream of $bla_{OXA-51-like}$ was investigated as previously described.[6,11] The $bla_{OXA-51-like}$ genes and their upstream regions were sequenced using previously published primers OXA69A/OXA69B (isolate A) or preABprom+/OXA69B (isolates B and C).[8] To confirm its chromosomal location, PCR was performed using primers yncA-F and fxsA-R, which anneal to the genes flanking $bla_{OXA-51-like}$.

Cloning experiments

IS*Aba1*-bla_{OXA-82} from isolate B was amplified by PCR using the preABprom+/OXA69B primer pair and blunt ligated into the ampicillin resistance gene of the shuttle vector pWH1266 before transforming into electrocompetent *A. baumannii* ATCC 17978 as previously described.[12,13] The transformants were selected on Luria–Bertani agar supplemented with 30 mg/L tetracycline.

Semi-quantitative reverse transcription PCR (qRT–PCR)

RNA extraction, cDNA synthesis and real-time PCR to measure the expression of $bla_{OXA-51-like}$, *carO*, *oprD*-like and the 33–36 kDa *omp* were performed as previously described using *rpoB* as a reference gene.[14] The primer sequences are shown in Table 2. All qRT–PCR was done in triplicate using freshly prepared RNA and cDNA. qRT–PCR was repeated at least three times. The relative expression of $bla_{OXA-51-like}$, *carO*, *oprD*-like and the 33–36 kDa *omp* from isolates B and C was compared with that of isolate A.

Outer membrane protein profiles

SDS–PAGE of the outer membrane from isolates A, B and C was performed as previously described with minor modifications.[15] Briefly, cells were grown until mid-log phase and harvested by centrifugation. Disruption of the cells was performed in a Constant Cell Disrupter System (Constant Systems Limited, Daventry, UK). The cell debris was removed by centrifugation and the supernatant centrifuged for 1 h at 100000 **g** (4°C) to pellet the membranes. Separation of the inner and outer membranes was performed by 30 min incubation in 2% *N*-lauroyl sarcosinate (Sigma–Aldrich, Steinheim, Germany) at room temperature, followed by a second ultracentrifugation step. The outer membrane pellets were resuspended in phosphate-buffered saline (Biochrom AG, Berlin, Germany). The protein concentration was measured by the BCA Assay (Thermo Scientific, Rockford, IL, USA). The OMP samples (10 ng) were prepared with XT reducing agent (Bio-Rad, Munich, Germany) and separated on Criterion XT 12% Bis-Tris pre-cast gels (Bio-Rad) using MOPS running buffer and stained with Coomassie brilliant blue. SDS–PAGE was performed three times using freshly prepared OMP preparations.

Results and discussion

The carbapenem susceptibility results of clinical *A. baumannii* isolates A, B and C are summarized in Table 1 and show that isolate A was susceptible to imipenem (4 mg/L) and meropenem (2 mg/L), whereas isolates B and C were resistant to both (>32 mg/L). No acquired OXAs were detected by multiplex PCR. By rep-PCR, the isolates showed 98.3% similarity, which indicates clonality. DiversiLab also identified the isolates as clustering with international clone 2 and this was confirmed by $bla_{OXA-51-like}$ typing.[10] PCR with primers yncA-F and fxsA-R amplified PCR products of 1630 bp in isolate A and 2809 bp in isolates B and C, confirming the chromosomal location of $bla_{OXA-51-like}$.

IS*Aba1* was detected in the three isolates; however, it was only associated upstream of $bla_{OXA-51-like}$ in isolates B and C (Table 1). A recent study comparing the genome sequences of six *A. baumannii* strains found multiple copies of IS*Aba1* spread throughout the genomes.[16] Sequencing of $bla_{OXA-51-like}$ revealed that isolate A possessed bla_{OXA-66}; however, in isolates B and C, bla_{OXA-66} was converted into bla_{OXA-82} through a point mutation, leading to a Leu167Val amino acid substitution. Sequences upstream of bla_{OXA-82} in carbapenem-resistant isolates B and C were identical. Similar mutations and the development of carbapenem resistance associated with the appearance of IS*Aba1* upstream of $bla_{OXA-51-like}$ in clonally related isolates has previously been observed.[6] qRT–PCR experiments revealed that isolates B and C expressed similar amounts of bla_{OXA-82} and this was a >40-fold overexpression compared with bla_{OXA-66} from isolate

Table 1. Phenotypic and genotypic characterization of clinical isolates

Isolate	Date of isolation	OXA-51 variant and presence of IS*Aba1*	MIC (mg/L)		Relative expression of $bla_{OXA-51-like}$
			IPM	MEM	
A	30/01/2009	OXA-66	4	2	1
B	09/09/2010	IS*Aba1*-OXA-82	>32	>32	46
C	15/09/2010	IS*Aba1*-OXA-82	>32	>32	46

IPM, imipenem; MEM, meropenem.

Table 2. List of primers used in this study

Primer	Target/purpose	Sequence 5′–3′	reference
yncA-F	chromosomal location of $bla_{OXA-51-like}$	CATTTAGAGCGTTTCCAG	this study
fxsA-R		CCCAGAACCACAATAAGAAG	this study
rpoB_for	*rpoB* qRT–PCR	GAGTCTAATGGCGGTGGTTC	Hornsey *et al.*[20]
rpoB_rev		ATTGCTTCATCTGCTGGTTG	Hornsey *et al.*[20]
OXA-51F	$bla_{OXA-51-like}$ standard curve for qRT–PCR	TAATGCTTTGATCGGCCTTG	Woodford *et al.*[11]
OXA-51R		TGGATTGCACTTCATCTTGG	Woodford *et al.*[11]
RT–PCR_OXA-51F	$bla_{OXA-51-like}$ qRT–PCR	GGAAGTGAAGCGTGTTGGTT	this study
RT–PCR_OXA-51R		CAAACTGTGCCTCTTGCTGA	this study
carO_F	*carO* standard curve for qRT–PCR	GCGGTGACATTTCTTGGTCT	this study
carO_R		CTGCAGAACCTGACGAAACA	this study
carO_rt_F	qRT–PCR *carO*	TGCCGATGGTGTCAAAATTA	this study
carO_rt_R		CGAATACGCCCCAGTTTTTA	this study
oprD_F	*oprD* standard curve for qRT–PCR	AGCAAAGTGAGGCAAAAGGA	this study
oprD_R		AATTGCACGGTCAAGACCTC	this study
oprD_rt_F	qRT–PCR *oprD*	GTTTCGGTGTAGGCGTTGTT	this study
oprD_rt_R		GGTCGTAAGCTGAACCATCG	this study
33–36 kDa_F	33–36 kDa *omp* standard curve for qRT–PCR	AAGGTGAGGCATACGTTCCA	this study
33–36 kDa_R		TTTACGTTACCACCCCAAGC	this study
33–36 kDa_rt_F	qRT–PCR 33–36 kDa *omp*	AATCGGTTTTGAAGCTGCTG	this study
33–36 kDa_rt_R		CCTACGAAAGTAGCGCCAAC	this study

A (Table 1). Previously, the *in vivo* selection of a mutation in bla_{OXA} was associated with meropenem therapy and transposition of IS*Aba1* was shown in the presence of imipenem.[7,14] Therefore, it is likely that a carbapenem was involved in the selection of the carbapenem-resistant isolates B and C.

The porins have been found to be associated with reduced carbapenem susceptibility in Gram-negative bacteria, e.g. decreased expression of OmpC in *Escherichia coli*.[17] In *A. baumannii*, loss of CarO[3] or the 33–36 kDa Omp[2] can confer carbapenem resistance and this is also proposed for the OprD homologue.[1] To determine the potential role of OMPs in conferring carbapenem resistance to isolates B and C, we investigated the expression of the porins *carO*, *oprD* and the 33–36 kDa *omp*. qRT–PCR revealed no significant changes in porin expression among the three isolates (data not shown). This was confirmed by SDS–PAGE, where we saw no difference in the OMP profiles (Figure 1).

Our results suggested that IS*Aba1*-mediated overexpression of bla_{OXA-82} was the sole carbapenem resistance mechanism in clinical isolates A, B and C. To confirm this hypothesis, we cloned IS*Aba1*-bla_{OXA-82} into the shuttle vector pWH1266 and transformed it into carbapenem-susceptible *A. baumannii* ATCC 17978. The imipenem and meropenem MICs increased from 0.25 to >32 mg/L in the transformant.

We do not have an intermediary between isolates A and B; therefore, we do not know what came first, IS*Aba1* or the OXA conversion. Previous work suggests that overexpression of bla_{OXA-66} has a minor effect on carbapenem susceptibility.[18] It was shown that the appearance of IS*Aba1* upstream of bla_{OXA-66} led to a 50-fold overexpression of bla_{OXA-66}, but with only a 2-fold increase in imipenem and meropenem MICs (from 2 to 4 mg/L and from 3 to 6 mg/L, respectively).[18] In the present study, we observed a similar overexpression of the intrinsic bla_{OXA}, but, critically, it was the conversion into bla_{OXA-82} that was necessary for carbapenem resistance. Interestingly, we have previously described bla_{OXA-82} associated with

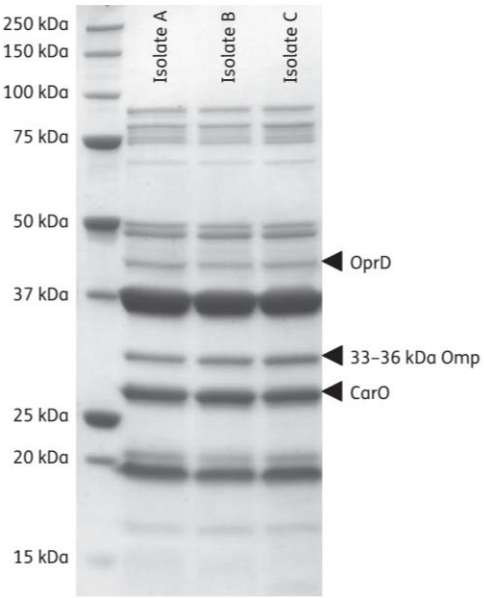

Figure 1. SDS–PAGE of outer membrane protein extractions of isolates A, B and C. No significant changes in porin expression were detected. CarO, OprD and 33–36 kDa Omp bands are marked with arrowheads; identification was based on the predicted protein sizes.

IS*Aba1* as the sole carbapenem resistance determinant in unrelated strains, but we did not find IS*Aba1* with bla_{OXA-66}.[10]

IS*Aba1*-$bla_{OXA-51-like}$ was recently detected in *Acinetobacter* species other than *A. baumannii*, e.g. *Acinetobacter calcoaceticus* and *Acinetobacter nosocomialis* (formerly *Acinetobacter* genomic species 13TU).[19] In contrast to in *A. baumannii*, the $bla_{OXA-51-like}$ genes were encoded on plasmids, highlighting their potential to spread between different *Acinetobacter* species. This also indicates the propensity to increasing carbapenem resistance within the genus *Acinetobacter*.

In conclusion, this study shows that a conversion of bla_{OXA-66} into bla_{OXA-82} in association with IS*Aba1* conferred carbapenem resistance in clonally related *A. baumannii* isolates. The overexpression of bla_{OXA-82} was identified as the sole carbapenem resistance mechanism.

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Transparency declarations

None to declare.

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Unpublished results I

To investigate the question whether it might be beneficial for *A. baumannii* to harbor one OXA-51 variant over another, the effect on carbapenem susceptibility of related $bla_{\text{OXA-51-like}}$, overexpressed in the same strain, was analyzed. The initial idea was to perform a gene replacement of the chromosomal $bla_{\text{OXA-51-like}}$ in the carbapenem-susceptible *A. baumannii* reference strain ATCC 17978 using a double crossover reaction. As OXA-51-like genes usually harbor only a few point mutations, the replacement would be performed in two steps (Figure 10).

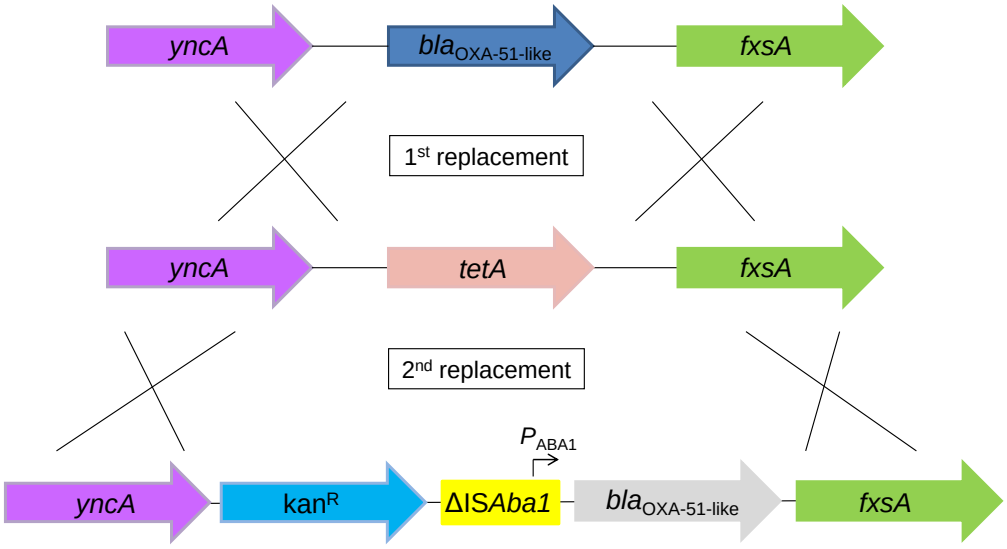

Figure 10: Two-step $bla_{\text{OXA-51-like}}$ replacement. Different colors of the $bla_{\text{OXA-51-like}}$ gene highlight that the parental gene (dark blue) will be replaced by other variants (grey). *yncA*, phosphinothricin acetyltransferase gene; *fxsA*, suppressor gene F exclusion of phage T7; IS*Aba1*, partial insertion sequence *A. baumannii* 1, including the $bla_{\text{OXA-51-like}}$ promoter (P_{ABA1}); *tetA*; tetracycline resistance gene; kan^{R}, kanamycin resistance gene; $bla_{\text{OXA-51-like}}$, β-lactamase gene

Together with parts of the $bla_{\text{OXA-51-like}}$ surrounding areas, including partial *fxsA* and *yncA* sequences, a tetracycline resistance gene (*tetA*) was cloned into the suicide vector pUC18. This vector does not contain an origin of replication for *Acinetobacter* and should therefore be unstable in the cell. Selective pressure should favor homologous recombination via double crossover and replace the chromosomal $bla_{\text{OXA-51-like}}$ with *tetA*.

This deletion mutant should have been used for a second round of gene replacement, where the *tetA* is replaced by different OXA-51-like variants together with the same promoter region provided by a partial IS*Aba1* to confer *bla*$_{OXA}$ overexpression. It is important not to clone the full IS element, i.e. a functioning transposase, to avoid its transposition.

Although the first gene replacement was successful, we could not use the deletion mutant for further experiments, because unexpectedly the pUC18 construct persisted in the cell. All efforts to cure the plasmid from the ATCC 17978 *bla*$_{OXA\text{-}51\text{-}like}$ deletion mutant failed, including serial passages for two weeks under plasmid curing conditions, e.g. different concentrations of sodium-dodecyl sulfate (SDS) and incubation at 42°C. Therefore we decided to use the *E. coli* and *A. baumannii* shuttle vector pWH1266 as the backbone for cloning the constructs in cis. The use of a replicable plasmid was not the first choice for our investigation, as transformants might end up having multiple plasmid copies which would influence *bla*$_{OXA\text{-}51\text{-}like}$ expression levels. However, pWH1266 is considered to be a low copy plasmid, whereby the possibility of multiple plasmids in the cell should be negligible. Closely related *bla*$_{OXA\text{-}51}$ variants, including *bla*$_{OXA\text{-}66}$, *bla*$_{OXA\text{-}82}$ and *bla*$_{OXA\text{-}201}$ (see 2.2.2) were amplified from clinical *A. baumannii* isolates. All of these OXAs belong to the IC2 lineage and differ by only few amino acid substitutions. With regards to these substitutions, either OXA-82 or OXA-109 can be considered as an intermediate between OXA-66 and OXA-201 (Figure 11). To include OXA-109 in our analyses, we generated the appropriate gene by site-directed mutagenesis.

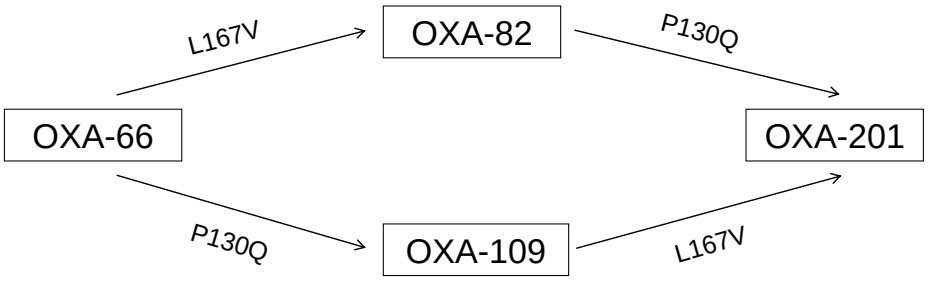

Figure 11: Representative OXA-51 variants of *A. baumannii* IC2 and amino acid substitutions responsible for OXA conversion. As indicated, OXA-201 might have evolved from either OXA-82 or OXA-109. L, leucine; P, proline; Q, glutamine; V, valine

The *bla*$_{OXA}$ genes were cloned together with partial *bla*$_{OXA-51-like}$ flanking regions from ATCC 17978, approximately 300 bp of the IS*Aba1* element and a kanamycin resistance gene into a pWH1266 derivative, which lacked the ampicillin resistance gene. The cloned constructs were transferred into ATCC 17978 and *bla*$_{OXA-51-like}$ gene expression, as well as carbapenem susceptibility by Etest, was investigated. Although we observed overexpression of all cloned *bla*$_{OXA-51}$ variants in the transformants, the effect on carbapenem susceptibility was different. Overexpression of OXA-66 slightly increased carbapenem MICs in ATCC 17978 (Table 5). In contrast, OXA-82, OXA-109 and OXA-201 conferred carbapenem resistance in the reference strain, suggesting an advantage of these enzymes over OXA-66 with regards to carbapenem susceptibility. However, the advantage of OXA-201 over OXA-82 or OXA-109 remained unclear.

Table 5: Carbapenem susceptibility of ATCC 17978 wildtype and *bla*$_{OXA-51-like}$ harboring transformants.

Strain	Carbapenem MIC [mg/L]	
	Imipenem	Meropenem
ATCC 17978	0.25	0.25
ATCC 17978-OXA-66	1	4
ATCC 17978-OXA-82	>32	>32
ATCC 17978-OXA-109	>32	>32
ATCC 17978-OXA-201	8 (h^R >32)	16 (h^R >32)

MIC, minimal inhibitory concentration; h^R, MIC including heteroresistant colonies

Interestingly, appearance of a heteroresistant phenotype was discovered in the OXA-201 transformant, but so far it remains unclear if this phenotype provides any advantage for the transformant. Heteroresistance describes a phenomenon of diverse antimicrobial susceptibility to an agent within one isolate (Figure 12). This means the presence of a drug-resistant subpopulation among a more susceptible background population.[128] For example, the inhibition zone next to an Etest stripe can harbor multiple colonies which grow up to higher drug concentrations (Figure 12). To avoid treatment failure, the MIC should be interpreted on the basis of the resistant population. The mechanism behind the heteroresistant phenotype has not been determined.

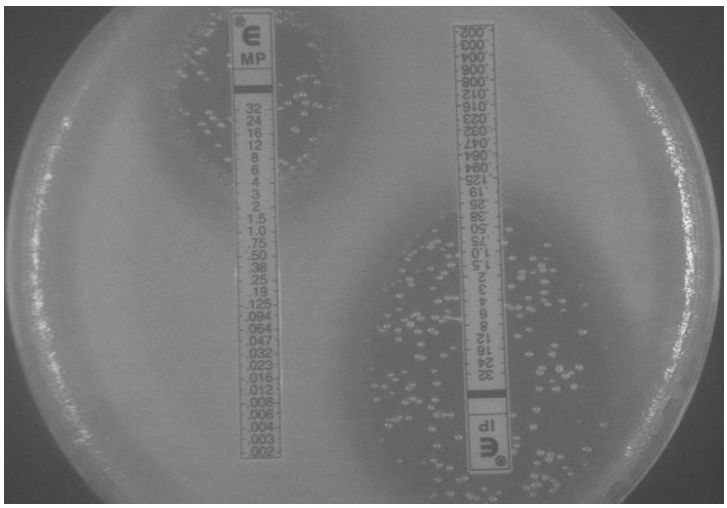

Figure 12: Carbapenem-heteroresistant *A. baumannii*. IP, imipenem; MP, meropenem

I presented preliminary data of this work as part of an oral presentation at the 24th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) in Barcelona, Spain, in May 2014.[129]

2.3 Insertion sequence mediated overexpression of intrinsic carbapenem-hydrolysing OXA in non-*baumannii Acinetobacter* species

2.3.1 Identification of a novel insertion sequence element associated with carbapenem resistance and the development of fluoroquinolone resistance in *Acinetobacter radioresistens*

OXA-23 (formerly ARI-1) was first identified in 1995 and was detected in an imipenem-resistant *A. baumannii* isolate from Scotland.[8] To date, OXA-23-like is the most commonly detected acquired OXA subclass in *A. baumannii*, but it has also been detected in non-*baumannii Acinetobacter* species.[54, 130] The origin of this subclass remained unknown until in 2008 OXA-23 was identified in carbapenem-susceptible *A. radioresistens* isolates.[112] To date, six amino acid variants have been identified in this species; OXA-23, OXA-102, OXA-103, OXA-105, OXA-133 and OXA-134 (see 3.2).[112, 131] Acquired *bla*$_{\text{OXA-23-like}}$ are usually associated with carbapenem resistance due to IS-mediated overexpression. In contrast, the intrinsic OXA-23 variants in *A. radioresistens* do not confer carbapenem-resistance and have so far not been detected in association with IS elements.

The following publication reports the first detection of an IS element upstream of *bla*$_{\text{OXA-23}}$ in two carbapenem-resistant *A. radioresistens* isolates, recovered before and after institution of ciprofloxacin therapy from the same patient. In addition, a shift in ciprofloxacin susceptibility was discovered between the two isolates which was associated with a *gyrA* mutation. A novel insertion sequence IS*Acra1* was identified, which conferred overexpression of the intrinsic OXA in the clinical isolates compared to the carbapenem-susceptible *A. radioresistens* reference strain SH164. Cloning and transfer of *bla*$_{\text{OXA-23}}$ together with a partial IS*Acra1* conferred carbapenem resistance in *A. baumannii* and *A. radioresistens* reference strains. Association of the intrinsic OXA-23 in *A. radioresistens* with an IS element indicates potential spread of carbapenem resistance in this species and dissemination of IS*Acra1*-*bla*$_{\text{OXA-23}}$ in the genus *Acinetobacter*.

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Identification of a novel insertion sequence element associated with carbapenem resistance and the development of fluoroquinolone resistance in *Acinetobacter radioresistens*

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Keywords: GyrA, imipenem, ciprofloxacin, QRDR, oxacillinase

Sir,

Acinetobacter radioresistens, although sometimes isolated on the skin of healthy humans, rarely causes serious illness.[1,2] In a recent study, *A. radioresistens* was isolated from blood and urine cultures, but made up <1% of the total *Acinetobacter* spp. isolated, while

other studies found a higher incidence of *A. radioresistens* when only looking at *Acinetobacter* bloodstream isolates, where bacteraemia is most often associated with indwelling devices.[3–5] Compelling evidence suggests that the origin of OXA-23, the most commonly acquired oxacillinase in *Acinetobacter baumannii*, is *A. radioresistens*, to which this enzyme is intrinsic.[6] In *A. baumannii*, bla_{OXA-23} mediates carbapenem resistance when overexpressed and this is caused by the insertion element IS*Aba1*, which is located upstream where it provides a strong promoter; however, this has not been found in *A. radioresistens*, so the mechanism of bla_{OXA-23} mobilization is still not fully understood. Despite possessing OXA-23, carbapenem resistance is rarely described in *A. radioresistens* and has only been reported when the additional acquired carbapenemases IMP-1 and OXA-58 were present.[7,8]

In this study, we investigated carbapenem resistance and the development of fluoroquinolone resistance in two *A. radioresistens* isolates that were recovered from a patient 17 days apart. The primer sequences used in this investigation are shown in Table S1 (available as Supplementary data at *JAC* Online). The IS*Acra1*-bla_{OXA-23} nucleotide sequence reported in this paper has been submitted to the EMBL/GenBank nucleotide sequence database under accession no. JQ326202. Isolate F1244 was isolated from a blood culture of a patient with a catheter-related bloodstream infection. Ciprofloxacin was used for treatment. Isolate A1474 was recovered 17 days later from a sputum sample. Species identification was confirmed by *rpoB* sequencing and the isolates were typed by rep-PCR (DiversiLab) as previously described.[9] The isolates showed 98% similarity in their rep-PCR patterns and confirms their clonality. The only difference we found between the isolates was in their fluoroquinolone susceptibility: isolate F1244 was susceptible to ciprofloxacin, levofloxacin and moxifloxacin, whereas isolate A1474 was resistant (Table 1). Carbapenem MICs were investigated by Etest and showed that both isolates were carbapenem resistant (Table 1). The isolates were negative for other bla_{OXA} genes often associated with *Acinetobacter* and we investigated the expression of the intrinsic bla_{OXA-23} by qRT–PCR. qRT–PCR was performed three times in triplicate using freshly prepared RNA and cDNA as previously described,[9] with *rpoB* as a reference gene. This revealed that F1244 and A1474 expressed similar levels of bla_{OXA-23} and that it was overexpressed >100-fold when compared with the carbapenem-susceptible control strain *A. radioresistens* SH164 (data not shown). PCR to detect IS*Aba1* adjacent to bla_{OXA-23} proved negative. Analysis of the *A. radioresistens* genome sequence revealed putative *O*-sialoglycoprotein- and ATPase-encoding genes flanking bla_{OXA-23}. The primer pair OXA-23-up/OXA-23-down was designed to amplify and sequence the region between these flanking genes and revealed an ~700 bp insertion in the 5′ region of bla_{OXA-23}. Sequencing revealed a novel insertion element in a non-coding region 62 bp upstream of the bla_{OXA-23} start codon. This insertion element was submitted to the IS Database (http://www-is.biotoul.fr/) and termed IS*Acra1*. IS*Acra1* is a 732 bp element that encodes a predicted 220 amino acid transposase that is flanked by 15 bp inverted repeats and a 7 bp target site duplication. The element belongs to the IS*1595* family and the transposase showed 47% amino acid identity to IS*Ftu3* from *Francisella tularensis*. The bla_{OXA-23} gene and putative promoter region was amplified and cloned into shuttle plasmid pWH1266 and transformed into carbapenem-susceptible *A. baumannii* ATCC 17978 and *A. radioresistens* SH164, leading to carbapenem resistance (Table 1).

We found a single nucleotide difference between the isolates in *gyrA*, leading to a Ser-83→Phe amino acid substitution in isolate A1474. In *A. baumannii*, Ser-83 in GyrA is the most commonly altered amino acid associated with low-level fluoroquinolone resistance (typically ciprofloxacin MICs of 4–32 mg/L).[10] We did not detect any difference in *parC* between the two isolates. We have previously found efflux to be associated with the development of fluoroquinolone resistance.[9] To test for an efflux phenotype, agar dilution MICs were performed with the following commonly effluxed substrates: tetracycline, chloramphenicol, rifampicin, gentamicin, erythromycin and the dye rhodamine 6-G. Agar dilution MICs did not show any difference in MICs between isolates F1244 and A1474 against these substrates, suggesting efflux was not selected (see Table S2 available as Supplementary data at *JAC* Online).

In conclusion, this study demonstrates that carbapenem resistance was mediated through overexpression of the intrinsic bla_{OXA-23} and was associated with the novel IS*Acra1*.

Table 1. Carbapenem MICs determined by Etest in *A. radioresistens* isolates F1244 and A1474, control strains *A. radioresistens* SH164, *A. baumannii* ATCC 17978, and their respective bla_{OXA-23} transformants, and fluoroquinolone agar dilution MICs and the amino acid substitution in GyrA for *A. radioresistens* isolates F1244 and A1474

	MIC (mg/L)					
Strain	IPM	MEM	CIP	LVX	MXF	GyrA
F1244	>32	>32	0.25	0.25	0.25	Ser-83
A1474	>32	>32	32	8	2	Ser-83Phe
A. radioresistens SH164	0.38	0.38				
A. baumannii ATCC 17978	0.25	0.25				
A. radioresistens SH164 (pOXA-23)	>32	32				
A. baumannii ATCC 17978 (pOXA-23)	>32	>32				

IPM, imipenem; MEM, meropenem; CIP, ciprofloxacin; LVX, levofloxacin; MXF, moxifloxacin.

Fluoroquinolone resistance developed during ciprofloxacin therapy and was associated with a *gyrA* mutation. These data show for the first time the ability of *A. radioresistens* to develop fluoroquinolone resistance during therapy. Additionally, IS*Acra1* has the potential to spread OXA-23-mediated carbapenem resistance in *A. radioresistens*.

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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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Supplementary data

Table S1. Primers used in this study

Primer	Target/purpose	Sequence 5′–3′
OXA-23-up	amplify and sequence bla_{OXA-23}	CGTCTGAAAGCAGGACAACA
OXA-23-down	and surrounding area	ACCGCAATATACCGAGTTGC
OXA-23-clone	cloning IS*Acra1*- bla_{OXA-23}	TTAAATAATATTCAGCTGTT
OXA-23-F[a]	sequencing of bla_{OXA-23}	ATTTCTGACCGCATTTCCAT
OXA-23-R[a]		GGTTAGTTGGCCCCCTTAAA
gyrA_F_radio	amplify and sequence *gyrA*	TTGCCAGATGTGAGAGATGG
gyrA_R_radio	QRDR	GTTGGCATGACCTGAGGAAT
parC_F_radio	amplify and sequence *parC*	AAAACCGCTCAGTAGCAGAA
parC_R_radio	QRDR	CCCTGCCCAAGTTCAGATAA
OXA-RT-F	bla_{OXA-23} RT-PCR	CGGATTGGAGAACCAGAAAA
OXA-RT-R		GCAGAAAGCTTCATGGCTTC
rpoB_F	*rpoB* RT-PCR	TCTAACGGCGGTGGAAATAC
rpoB_R		GCTCATCACCCGTAGAAAGC

[a] bla_{OXA-23} primers previously published in; Woodford N, Ellington MJ, Coelho JM *et al.* Multiplex PCR for genes encoding prevalent OXA carbapenemases in *Acinetobacter* spp. *Int J Antimicrob Agents* 2006; **27**: 351-3.

Table S2. Agar dilution MICs for fluoroquinolones and commonly effluxed antibiotics and substrates, and the amino acid substitution in GyrA for *A. radioresistens* isolates F1244 and A1474.

		MIC (mg/L)								
Isolate	GyrA	CIP	LVX	MXF	TET	CHL	RIF	GEN	ERY	R6G
F1244	Ser-83	0.25	0.25	0.25	2	64	2	0.25	32	64
A1474	Phe-83	32	8	2	2	64	2	0.25	32	64

CIP, ciprofloxacin; LVX, levofloxacin; MXF, moxifloxacin; TET, tetracycline; CHL, chloramphenicol; RIF, rifampicin; GEN, gentamicin; ERY, erythromycin; R6G, rhodamine 6G

2.3.2 Insertion sequence IS*18* mediates overexpression of *bla*$_{\text{OXA-257}}$ in a carbapenem-resistant *Acinetobacter bereziniae* isolate

A. bereziniae harbors the intrinsic OXA-228-like, which is usually expressed at low level and thus does not confer carbapenem resistance. However, overexpression of *bla*$_{\text{OXA-228-like}}$ has recently been detected in a clinical *A. bereziniae* isolate from France, which was associated with reduced carbapenem susceptibility.[132] Increased *bla*$_{\text{OXA}}$ expression was due to alterations in the predicted promoter sequences, while no adjacent IS element was detected.

In the following publication the first case of IS-mediated *bla*$_{\text{OXA-228-like}}$ overexpression in *A. bereziniae* is reported. The insertion sequence IS*18* was detected adjacent to a novel OXA-228 variant, OXA-257, in a carbapenem-resistant *A. bereziniae* isolate from Germany. Sequence analysis of the *bla*$_{\text{OXA-257}}$ upstream region revealed the presence of two putative promoters, partially provided by IS*18*. Cloning and transfer of *bla*$_{\text{OXA-257}}$ and the putative promoters conferred carbapenem resistance in *A. baumannii* ATCC 17978. This highlights the potential of the OXA-228 subclass to confer carbapenem-resistance outside of *A. bereziniae*. Furthermore association with an IS element, usually associated with *A. baumannii*, indicates possible mobilization of the gene which would support its dissemination in the genus *Acinetobacter*.

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Insertion sequence IS*18* mediates overexpression of $bla_{OXA-257}$ in a carbapenem-resistant *Acinetobacter bereziniae* isolate

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Keywords: carbapenemase, qRT–PCR, *rpoB* sequencing

Sir,

Acinetobacter bereziniae, previously known as *Acinetobacter* genomic species 10, has been isolated primarily from clinical specimens and the hospital environment and more rarely from various other sources, including vegetables, soil and animals.[1,2] Antibiotic resistance is rarely reported in this species. Over the last decade, carbapenem resistance in *Acinetobacter* spp., mainly *Acinetobacter baumannii*, has emerged as a threat in hospitals around the world.[2] The most widespread mechanism resulting in carbapenem resistance in *Acinetobacter* spp. is mediated through carbapenem-hydrolysing class D β-lactamases, also known as oxacillinases. The overexpression of bla_{OXA} genes is often associated with insertion sequences (IS) located upstream and providing strong promoters. Carbapenem resistance in *A. bereziniae* has previously been associated with the metallo-β-lactamases IMP, SIM and VIM or overexpression of OXA-229, a variant of the intrinsic OXA-228-like, which was mediated by a mutated promoter.[3] To date, OXA-228-like has not been associated with an IS.

In the present study, we investigated a carbapenem-resistant *Acinetobacter* strain isolated from the bronchial secretions of a patient in Germany in 2012. Isolate KH243 was initially identified as *Acinetobacter guillouiae* by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. However, *rpoB* sequencing revealed 100% similarity to the *A. bereziniae* type strain CIP 70.12 (accession no. DQ207475).[1,4] By Etest, carbapenem MICs were 12 and >32 mg/L for imipenem and meropenem, respectively. Multiplex PCR for OXA subclasses that are associated with carbapenem resistance in *Acinetobacter* spp. (OXA-51, OXA-23, OXA-40, OXA-58, OXA-143 and OXA-235) was negative.[5,6] Based on published *A. bereziniae* sequences,[3] primers were designed to amplify and sequence the intrinsic bla_{OXA} and its surrounding region from isolate KH243 (Table 1). PCR revealed an unexpected large amplicon of ~2.1 kb. Sequencing of the purified PCR product by primer walking identified IS*18* 40 bp upstream of a novel $bla_{OXA-228}$ variant, which was numbered $bla_{OXA-257}$ by the Lahey β-lactamase database (http://www.lahey.org/Studies/) and was submitted to GenBank. OXA-257 possessed six amino acid differences compared with OXA-228. The IS*18*::$bla_{OXA-257}$ nucleotide sequence reported in this paper has been submitted to EMBL/GenBank under accession number KC567681.

The IS*18* insertion element encoded a transposase that harboured eight amino acid changes compared with the IS*18* sequence available in the IS database (http://www-is.biotoul.fr/). IS*18* was flanked by a 3 bp target site duplication (TTT) and 26 bp imperfect inverted repeats. In *Acinetobacter* spp., IS are often located upstream of bla_{OXA} genes and, by providing strong promoters, lead to overexpression of the OXA, resulting in carbapenem resistance.[7] For example, the intrinsic $bla_{OXA-51-like}$ and the acquired $bla_{OXA-58-like}$ in *A. baumannii* are often associated with IS*Aba1* and IS*Aba3*, respectively.[7] IS*18* has also been associated with $bla_{OXA-58-like}$.[8] Other IS elements include IS*Acra1*, which was recently identified and overexpressed bla_{OXA-23} in a carbapenem-resistant *Acinetobacter radioresistens* isolate.[9]

Two predicted promoters were found upstream of $bla_{OXA-257}$ with both −35 boxes located within the right inverted repeat of IS*18*. One was a hybrid promoter based on those previously described in *A. bereziniae* isolates Nec ($bla_{OXA-229}$) and Baz ($bla_{OXA-228}$).[3] The −35 and −10 boxes were identical to the −35 box in Nec and the −10 box in Baz, respectively, and were

Table 1. Primers used in this study

Primer	Target/purpose	5′–3′ sequence	Reference
$bla_{OXA-228-like}$_F1	sequencing and $bla_{OXA-228-like}$ standard curve	GCTAAAGTTTCTGCTGAGGA	this study
$bla_{OXA-228-like}$_R1		CCAGTTACCCCCAATAAACT	
$bla_{OXA-228-like}$_F2	$bla_{OXA-228-like}$ qRT–PCR	TAGTTTGGCATTTTCAGGTT	this study
$bla_{OXA-228-like}$_R2		AGCTTGAGTCTGTGCTTGAT	
IS_F	cloning primer (used in combination with $bla_{OXA-228-like}$_R1)	GGATTTACCAGTACATTTATC	this study
*rpoB*_beriF1	*A. bereziniae rpoB* standard curve	CGTATTGTGCGTTTATTGAA	this study
*rpoB*_beriR1		AAGTGGTTCAACTTGAGCAT	
*rpoB*_beriF2	*A. bereziniae rpoB* qRT–PCR	CGAGATCGATGAGAAGTTTG	this study
*rpoB*_beriR2		GACGACCCGCCATCTTAT	

separated by 22 bp instead of 17 bp (see Figure S1, available as Supplementary data at *JAC* Online). An additional promoter was predicted by Softberry BPROM promoter prediction and consisted of the −35 box of IS*18* and a −10 box separated by 13 bp (Figure S1, available as Supplementary data at *JAC* Online).[10] Interestingly, the TTCAAT −35 box identical to that from Baz ($bla_{OXA-228}$) was adjacent to the left inverted repeat.

OXA-228-like expression in isolate KH243 was compared with that in carbapenem-susceptible *A. bereziniae* isolate G3-59 by semi-quantitative RT–PCR (qRT–PCR) using *rpoB* as the reference gene. The primers used for qRT–PCR are shown in Table 1. Three independent experiments were performed using freshly prepared RNA and cDNA and revealed a 56-fold (±3.84) overexpression of $bla_{OXA-228-like}$ in isolate KH243 compared with that in G3-59. To investigate the potential to mediate carbapenem resistance, IS*18*::$bla_{OXA-257}$ was cloned into the shuttle vector pWH1266, but we were unable to transfer this into *A. bereziniae* G3-59. However, the construct was successfully transferred into *A. baumannii* ATCC 17978 by electroporation, as previously described for *Pseudomonas aeruginosa*.[11] Expression of $bla_{OXA-257}$ in *A. baumannii* ATCC 17978 raised both imipenem and meropenem MICs from 0.25 to >32 mg/L, demonstrating that IS*18*::$bla_{OXA-257}$ is able to confer carbapenem resistance.

In conclusion, this study has detected an IS upstream of the intrinsic bla_{OXA} in *A. bereziniae*, a phenomenon that has not been described in this species so far. IS*18* conferred overexpression of OXA-257, which mediated carbapenem resistance in *A. bereziniae* and *A. baumannii*. Moreover, because IS*18* has previously been described adjacent to acquired bla_{OXA} in *A. baumannii*,[8] these data suggest a potential for dissemination of OXA-257 in the genus *Acinetobacter*.

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Transparency declarations

None to declare.

Supplementary data

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

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Supplementary data

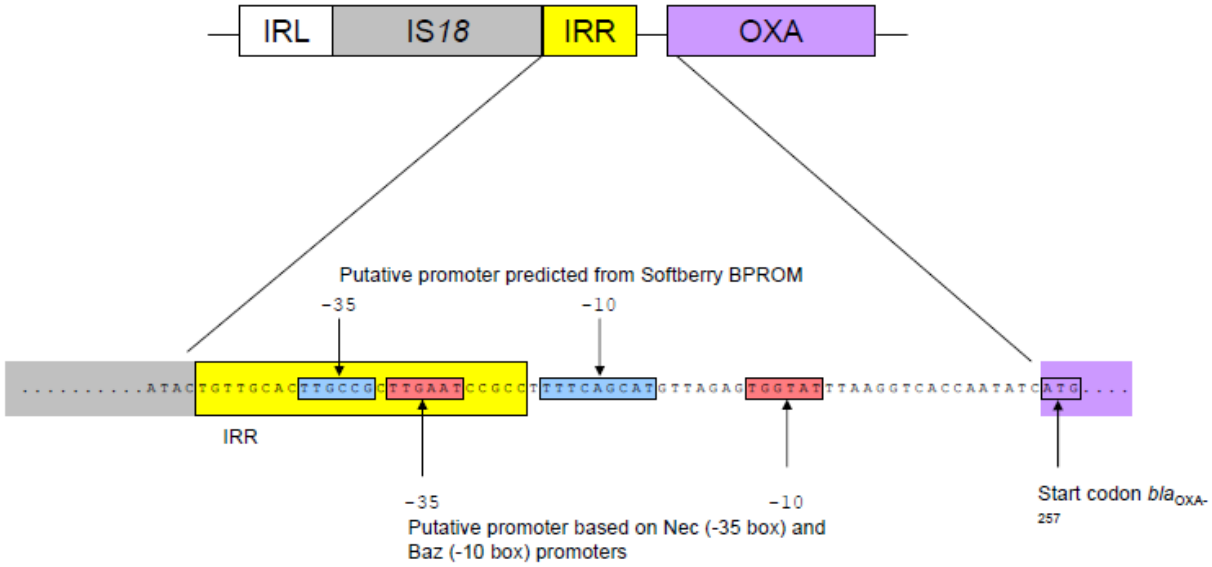

Figure S1. Orientation of IS*18* and predicted promoter sequences upstream of $bla_{\text{OXA-257}}$. Inside the right inverted repeat (IRR, coloured yellow) are two –35 boxes. Blue shading shows a predicted –35 box and –10 box (the –35 box is also described in IS*18*). Red shading shows a hybrid promoter based on those described in *Acinetobacter bereziniae* Nec and Baz.

2.4 Distribution and novel insights into acquired carbapenem-hydrolysing OXA in *Acinetobacter* spp.

2.4.1 Worldwide dissemination of acquired carbapenem-hydrolysing class D β-lactamases in *Acinetobacter* spp. other than *Acinetobacter baumannii*

In the literature, there are reports of carbapenem-hydrolysing class D β-lactamases in non-*baumannii Acinetobacter* spp. These include various subclasses of metallo-β-lactamases and oxacillinases.[133-137] Similar to *A. baumannii*, carbapenem resistance is more often associated with acquired oxacillinases than metallo-β-lactamases.

In the following publication the dissemination of acquired OXA in 453 non-*baumannii Acinetobacter* spp. isolates of worldwide origin was investigated. Oxacillinases were detected in 23 isolates by OXA-multiplex PCR, with a high rate (47.8%) of false-positive *bla*$_{OXA}$ detection. This indicates that the multiplex PCR is not a reliable tool for identification of *bla*$_{OXA}$ subclasses in non-*baumannii Acinetobacter* spp. *bla*$_{OXA}$ were mainly present in carbapenem-resistant *Acinetobacter* isolates, adjacent to IS*Aba1* (*bla*$_{OXA-23}$) and IS*Aba3* (*bla*$_{OXA-58}$). Partial sequencing of IS*Aba3* upstream of *bla*$_{OXA-58}$ revealed no difference between carbapenem-resistant and carbapenem-susceptible isolates, suggesting the presence of additional strain-dependant carbapenem-resistance determinants. For the first time OXA-23 in *Acinetobacter* genomic species 14TU/13BJ, OXA-255 (a novel OXA-143 variant; see 2.4.3) in *A. pittii* and OXA-23 together with OXA-58 in *A. pittii* were detected. In addition, acquired OXA were for the first time detected in *Acinetobacter* spp. from South Africa and North America.

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Short communication

Worldwide dissemination of acquired carbapenem-hydrolysing class D β-lactamases in *Acinetobacter* spp. other than *Acinetobacter baumannii*

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ABSTRACT

The aim of this study was to identify acquired OXA-type carbapenemases in *Acinetobacter* spp. other than *Acinetobacter baumannii*. From a total of 453 carbapenem-susceptible and -resistant *Acinetobacter* isolates collected worldwide, 23 were positive for bla_{OXA} genes by multiplex PCR. These isolates were identified as *Acinetobacter pittii* ($n = 18$), *Acinetobacter nosocomialis* ($n = 2$), *Acinetobacter junii* ($n = 1$) and *Acinetobacter* genomic species 14TU/13BJ ($n = 2$). The bla_{OXA} genes and associated insertion sequence (IS) elements were sequenced by primer walking. In 11 of these isolates, sequencing of the PCR products revealed that they were false-positive for bla_{OXA}. The remaining 12 isolates, originating from Europe, Asia, South America, North America and South Africa, harboured OXA-23 ($n = 4$), OXA-58 ($n = 5$), OXA-40-like ($n = 1$) and OXA-143-like ($n = 1$); one *A. pittii* isolate harboured both OXA-23 and OXA-58. IS elements were associated with bla_{OXA} in 10 isolates. OXA multiplex PCR showed a high degree of false-positive results (47.8%), indicating that detection of bla_{OXA} in non-*baumannii* *Acinetobacter* spp. should be confirmed using additional methods.

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2.4.2 OXA-235, a novel class D β-lactamase involved in resistance to carbapenems in *Acinetobacter baumannii*

A novel subclass of acquired CHDL in *A. baumannii* was identified and characterized. Detection of OXA-235-like in carbapenem-resistant *A. baumannii* isolates highlights the potential of this species to acquire novel resistance determinants. The novel subclass was identified in ten carbapenem-resistant *A. baumannii* isolates that originated from the USA and Mexico. The majority of isolates harbored OXA-235, while two single isolate harbored the amino acid variants OXA-236 and OXA-237, respectively. The subclass shows the highest amino acid identity with OXA-134a (85%), a member of the intrinsic subclass of *Acinetobacter lwoffii*,[138] which should not be confused with OXA-134 from *A. radioresistens* (see 2.3.1 and 3.2). OXA-235-like genes were flanked by two IS*Aba1* and were mainly encoded on plasmids. In *Acinetobacter baylyi*, a well-established model organism of the genus, and *A. baumannii* ATCC 17978, OXA-235 mediated reduced carbapenem susceptibility but not carbapenem resistance. However, some of the clinical isolates harbored multiple $bla_{\text{OXA-235-like}}$ copies which probably affected the overall bla_{OXA} expression and may partly explain MIC differences between the parent strains and the transformants. The ability of OXA-235 to alter carbapenem susceptibility and its association with IS*Aba1* suggests further spread in the genus *Acinetobacter*. To facilitate monitoring of worldwide OXA-235-like prevalence, inclusion of $bla_{\text{OXA-235-like}}$ primers in the OXA-multiplex PCR was established.

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OXA-235, a Novel Class D β-Lactamase Involved in Resistance to Carbapenems in *Acinetobacter baumannii*

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We investigated the mechanism of carbapenem resistance in 10 *Acinetobacter baumannii* strains isolated from the United States and Mexico between 2005 and 2009. The detection of known metallo-β-lactamase or carbapenem-hydrolyzing oxacillinase (OXA) genes by PCR was negative. The presence of plasmid-encoded carbapenem resistance genes was investigated by transformation of *A. baumannii* ATCC 17978. Shotgun cloning experiments and sequencing were performed, followed by the expression of a novel β-lactamase in *A. baumannii*. Three novel OXA enzymes were identified, OXA-235 in 8 isolates and the amino acid variants OXA-236 (Glu173-Val) and OXA-237 (Asp208-Gly) in 1 isolate each. The deduced amino acid sequences shared 85% identity with OXA-134, 54% to 57% identities with the acquired OXA-23, OXA-24, OXA-58, and OXA-143, and 56% identity with the intrinsic OXA-51 and, thus, represent a novel subclass of OXA. The expression of OXA-235 in *A. baumannii* led to reduced carbapenem susceptibility, while cephalosporin MICs were unaffected. Genetic analysis revealed that $bla_{OXA-235}$, $bla_{OXA-236}$, and $bla_{OXA-237}$ were bracketed between two IS*Aba1* insertion sequences. In addition, the presence of these acquired β-lactamase genes might result from a transposition-mediated mechanism. This highlights the propensity of *A. baumannii* to acquire multiple carbapenem resistance determinants.

Acinetobacter baumannii is a nosocomial pathogen that is characterized by its innate and acquired antimicrobial resistance (1–3). Of major concern is the increased incidence of carbapenem resistance, which has risen dramatically over the last decade (1, 4, 5). *A. baumannii* carbapenem resistance is most frequently mediated through the presence of a carbapenemase (6). Metalloenzymes, such as VIM, IMP, and NDM, are still rare in this species, with the majority of reported carbapenem resistance mediated through the action of carbapenem-hydrolyzing class D β-lactamases (CHDLs) (2, 7–9). The CHDLs, also called oxacillinases (OXA), exhibit weak hydrolysis of carbapenems. However, the genes encoding OXA are often associated with insertion sequences that provide strong promoters, leading to overexpression and carbapenem resistance (7).

There are currently five subclasses of OXA associated with *A. baumannii*; the intrinsic chromosomal OXA-51-like, of which there are over 70 variants, and the acquired OXA-23-like, OXA-24 (OXA-40-like), OXA-58-like, and OXA-143-like (9). Acquired OXA are found both chromosomally and on plasmids and can be detected by multiplex PCR (10, 11). Here, we describe a novel OXA subclass identified in *A. baumannii* strains that could not be detected by previous PCR methods.

MATERIALS AND METHODS

Bacterial strains and antimicrobial susceptibility. Nine *A. baumannii* clinical isolates from the United States and one isolate from Mexico were included. Dates and places of isolation are shown in Table 1. Species identification was confirmed by *gyrB* multiplex PCR (12). All isolates were tested for their susceptibility to imipenem and meropenem by Etest (bioMérieux, Nürtingen, Germany), following the manufacturer's instructions. Isolates were typed by repetitive-sequence-based PCR (DiversiLab rep-PCR) as previously described and assigned to international clones using our in-house database (8, 13).

PCR experiments. Detection of the genes encoding known acquired CHDLs (bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-51}, bla_{OXA-58}, and $bla_{OXA-143}$) and metallo-β-lactamases (bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{SPM}, bla_{SIM}, and bla_{NDM}) was performed as previously described (10, 11, 14, 15). The $bla_{OXA-51-like}$ gene was amplified and sequenced using primers OXA69A and OXA69B, and the presence of the insertion sequence IS*Aba1* upstream from it was investigated by PCR (7, 16).

Plasmid analysis and cloning. To determine if carbapenem resistance was plasmid encoded, plasmid DNA was extracted from all the *A. baumannii* isolates listed in Table 1 using a commercial kit (Qiagen, Hilden, Germany) and used to transform electrocompetent *A. baumannii* ATCC 17978 as previously described for *Pseudomonas aeruginosa* (17). Selection was performed with ticarcillin at 100 μg/ml. Reduced carbapenem susceptibility of transformants was tested by disc diffusion.

Plasmids that successfully conferred reduced carbapenem susceptibility when transformed in ATCC 17978 were used for shotgun cloning. Briefly, plasmid DNA was extracted, digested with the restriction enzyme EcoRI, ligated into EcoRI-cut pBBR1MCS (18), and used to transform *Escherichia coli* NEB5α (New England BioLabs, Germany). Transformants were selected on LB agar plates containing 25 μg/ml ticarcillin, and the recombinant plasmid pOXA-235 was retained for further analysis. The size of plasmid inserts was determined by restriction analysis. Replicon typing of plasmids was performed as previously described (19).

To confirm the plasmid/chromosomal location of $bla_{OXA-235-like}$, DNA-DNA hybridization was performed using total DNA preparations (Qiagen) and the hybridized DNA separated by electrophoresis and transferred onto Hybond N+ membranes. The location of $bla_{OXA-235-like}$ was assessed by hybridization of digoxigenin (DIG)-labeled probes (Roche,

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TABLE 1 Acquired OXA and strain information

Strain	Date of isolation (mo-yr)	Type of specimen	Location where isolate was recovered	Acquired OXA	Bacterial location of acquired bla_{OXA} gene	OXA-51-like variant	International clone[a]
BMBF 255	06-2005	Wound	USA[c]	236	Chromosome and plasmid	241	Unc
AF 684	03-2007	TS[b]	California	237	Plasmid	66	2
AF 670	06-2007	Blood	Arizona	235	Chromosome and plasmid	242	Unc
AF 401	07-2007	Small intestine	Mexico[d]	235	Chromosome and plasmid	65	5
AF 667	10-2007	Wound	Arizona	235	Chromosome and plasmid	242	Unc
AF 785	10-2007	TS	Nevada	235	Chromosome	66	2
AF 678	12-2007	Sputum	California	235	Chromosome	66	2
AF 707	01-2008	Wound	Illinois	235	Chromosome	66	2
AF 673	03-2008	Sputum	Arizona	235	Chromosome	66	2
AF 875	11-2009	Sputum	Utah	235	Chromosome	66	2

[a] As determined by rep-PCR and bla_{OXA-51} typing. Unc, unclustered.
[b] TS, tracheal secretion.
[c] State not known.
[d] Isolate recovered from unknown location in Mexico.

Mannheim, Germany) specific for $bla_{OXA-235-like}$. Chromosomal location was shown by colocalization with a $bla_{OXA-51-like}$ probe.

Novel $bla_{OXA-235}$ was cloned into the shuttle vector pWH1266 with its native promoter. Additionally, $bla_{OXA-235}$ and bla_{OXA-24} were cloned into shuttle vector pETRA, expressed using the CTX-M14 promoter, and transformed into *Acinetobacter baylyi* and *A. baumannii* ATCC 17978 for antibiotic susceptibility testing (20).

DNA sequencing. Recombinant strain *E. coli* NEB5α (pOXA-235) was extracted and sequenced by primer walking. A novel bla_{OXA} was identified by BLAST query. Primers specific for the new bla_{OXA} (forward, 5′-CAAGCCATGCAAGCTTCT-3′, and reverse, 5′-GCTGGACTTGAG GATCAAAG-3′) were designed and used to screen the remaining 9 isolates. To confirm sequences of novel bla_{OXA} variants, PCR and sequencing reactions were repeated using Phusion hot-start high-fidelity DNA polymerase (Thermo Fisher Scientific, Schwerte, Germany). Products were purified using the QIAquick PCR purification kit (Qiagen) and sequenced. Novel sequences were assigned numbers by the Lahey β-lactamase database (http://www.lahey.org/Studies/) and submitted to GenBank.

β-Lactamase purification. To purify OXA-235, the $bla_{OXA-235}$ gene was cloned into the pGEX-6P-1 vector with the following primers: 5′-AA AAGAATTCTTGCCTGTTTCAAATTCG-3′ (forward) and 5′-AAAAGT CGACTTACCCTTCAGCTTTCGG-3′ (reverse), which generated the restriction sites EcoRI and SalI, respectively. Similarly, bla_{OXA-24} that was previously cloned into the same vector was used as a comparator CHDL for kinetic studies (21). The constructs were transformed into *E. coli* BL21 and produced a fusion protein between glutathione *S*-transferase (GST) and the OXA-235/OXA-24 enzyme. The β-lactamase was purified to homogeneity according to the manufacturer's instructions for the GST gene fusion system (Amersham Pharmacia Biotech Europe GmbH). After cleavage of GST from OXA-235/OXA-24, the purified protein (≥99% pure) appeared in SDS-PAGE as a single band (data not shown).

Determination of kinetic parameters. Ampicillin and oxacillin powders were purchased from Sigma Chemical Co. (St. Louis, MO). Imipenem and meropenem powders were gifts from Merck (Whitehouse Station, NJ) and AstraZeneca (London, United Kingdom), respectively. Nitrocefin was obtained from Unipath Oxoid (Basingstoke, Hants, United Kingdom), and isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Roche (Basel, Switzerland). Hydrolysis of the antibiotics by the OXA-235 β-lactamase was monitored by recording the variation in absorbance resulting from the opening of the β-lactam ring under the following conditions: the antibiotic extinction coefficients for nitrocefin, ampicillin, oxacillin, imipenem, and meropenem were +15,000, −820, +440, −9,000, and −6,500 M^{-1} cm^{-1}, respectively, and the wavelengths used were 260 nm for oxacillin, 482 nm for nitrocefin, 300 nm for imipenem and meropenem, and 235 nm for ampicillin. The tests were repeated three times in phosphate-buffered saline (PBS) with 20 mg BSA/liter at 25°C. The kinetic parameters for nitrocefin were determined from the initial rates by Hanes-Woolf linearization of the Henri-Michaelis-Menten equation. For the other antibiotics, the K_m value was measured as the K_i in a competition experiment, with nitrocefin as the reporter substrate (22). The k_{cat} values were then obtained by monitoring hydrolysis of the antibiotic at a concentration of >10 times the K_m.

Multiplex PCR to detect $bla_{OXA-235-like}$. In order to detect $bla_{OXA-235-like}$ by PCR, we designed primers to amplify $bla_{OXA-235}$, $bla_{OXA-236}$, and $bla_{OXA-237}$. Primer pair OXA-235_F (5′-TTGTTGCCTT TACTTAGTTGC-3′) and OXA-235_R (5′-CAAAATTTTAAGACGGAT CG-3′) were tested by addition to the existing OXA multiplex PCR and amplified using the published parameters (10, 11). The multiplex PCR was tested against the 10 $bla_{OXA-235-like}$ isolates listed in Table 1, as well as previously characterized *A. baumannii* isolates possessing $bla_{OXA-51-like}$ and the acquired $bla_{OXA-23-like}$, $bla_{OXA-24-like}$, $bla_{OXA-58-like}$, and $bla_{OXA-143-like}$, as previously described (10).

Nucleotide sequence accession numbers. The nucleotide sequences of the novel bla_{OXA} variants reported in this paper have been submitted to the EMBL/GenBank database under accession numbers JQ820240 (OXA-235), JQ820242 (OXA-236), JQ820241 (OXA-237), JX025021 (OXA-241), and JX025022 (OXA-242).

RESULTS

Antibiotic susceptibility, plasmid analysis, and PCR experiments. The 10 isolates were confirmed as carbapenem-resistant *A. baumannii*, recording imipenem and meropenem MICs of ≥16 μg/ml. By PCR, they were negative for known CHDLs and metallocarbapenemases, and IS*Aba1* was not adjacent to $bla_{OXA-51-like}$. Sequencing of $bla_{OXA-51-like}$ revealed that six isolates possessed OXA-66, which is characteristic of international clone 2 (IC2) (23). Typing by rep-PCR revealed that five of the IC2 isolates showed ≥99% similarity in their rep-PCR fingerprints, which suggests clonal spread of a strain into Utah, Illinois, California, and Nevada (see Fig. S1 in the supplemental material). Two novel OXA-51-like enzymes were identified in unclustered isolates, OXA-241 and OXA-242 (Table 1). Strains AF667 and AF670 were 98.9% identical by rep-PCR and did not cluster with any isolate from our database.

Four isolates yielded plasmids that successfully transformed *A. baumannii* ATCC 17978 to reduced carbapenem susceptibility; these transformants exhibited phenotypic heterogeneous resistance that was characterized by a reduced inhibition zone compared to the inhibition zone of the untransformed control, within

```
                          10                  20        30        40        50        60        70        80
DBL                       |                   |         |         |         |         |         |         |
OXA-235    ...MKTLILLPLLSCLSLTACS...LPVSNSSSQITSTQSIQTIAKLFDQAQSSGVLVIQRGPHLQVYGNDLSRAHTEYIPASTFKILNALIGL
OXA-134    ...---I--F-------G-----.....----SLP-...QSI-T-A--S---------------DQQV-------N--N---V-------M-------
OXA-24     .MKKFI-PIFSISILV--S---SIKTKSEDNFHIS.-Q-HEKA-KSY--E--TQ--II-KE-KN-ST---A-A--NK--V-------M-------
OXA-143    .MKKFI-PI-SISTL--VS---SIQTKFEDTFHTS.NQ-HEKA-KSY--E--TQ--II-KK-KNIST---N-T------V-------M-------
OXA-51     ...-NIKT--LIT-AIFIS---PYIVTANPNH-ASK-DEKAEK-KN--NEVHTT------Q-QTQ-S-----A--S---V-------M-------
OXA-23     ...-NKYFTCYVVAS-F-SG-TVQHNLIN.ETPSQIVQGHN-V-HQY--EKNT-------TDKKINL---A----N---V-------M-------
OXA-58     MKLL-I-S-VC-SISIGAC-EHSMSRAKTSTIP-VNNSIID-NVQA--NEISADA-F-TYD-QNIKK--TH-D--K-A----------A-----

                    90        100       110       120       130       140       150 160       170       180
DBL                 |         |         |         |         |         |         |   |         |         |
OXA-235    QHGKATTNEIFKWDGKKRSFAAWEKDMTLGQAMQASAVPVYQELARRIGLELMQQEVQRIRFGNQQIGQHIDNFWLVGPLKITPEQEVEFASAL
OXA-134    --------------------T-----------------------------------------Q---------QV----------V--K---Q-V---
OXA-24     ENH---------------TYPM--------E--AL------------T------K--K-VN---TN--TQV-------------V---N--DD-
OXA-143    ENH----T-----------YPM--------D--AL------------T--D---K--K-VG---MN--TQV-------------I---N--DDF
OXA-51     E-H----T-V-----Q--L-PE--------D--KA--I----D----------SK--K-VGY--AD--TQV-------------Q--AQ--YK-
OXA-23     ENQ-TDI------K-E----T---------E--KL---------------D---K--K--G---AE---QV----------V--I-----V-Q-
OXA-58     ENH---ST--------P-F-K--D--F---E-----T------------PS---S-L---GY--M---TEV-Q---K---T---I---K-VYD-

                    190       200       210       220 230             240       250       260       270
DBL                 |         |         |         |   |               |         |         |         |
OXA-235    AQEQLAFDPQVQQQVKAMLLLQERQDYRLYAKSGWGMDVEPQVGWLTGWIETPQDEIVAFSLNMQMQSNMDPAIRLKILQQALAELALYPKAEG
OXA-134    -R-----------------F----KA----V-----------------V----A-----------NGI------E---------G-------
OXA-24     -HNR-P-KLET-EE--K---IK-VNGSKI---------G-T----------V-QANGKKIP----LE-KEG-SGS--NE-TYKS-EN-GII.....
OXA-143    -NNR-P-KLET-EE--K---IK-FNGSKI---------T----------V-KSNG-K------IE-KQG-PGS--NE-TYKS-EN-GII.....
OXA-51     -NKT-P-S-K--DE-QS--FIE-KNGNKI--------W--D----------VVQ--GN-------LE-KKGIPSSV-KE-TYKS-EQ-GIL.....
OXA-23     -HT--P-SEK--AN--N----E-SNG-KIFG-T--A--IK----------V-Q-DGK----A----E-R-E-PAS--NEL-MKS-KQ-NII.....
OXA-58     --G---P-K-E------E--YVERRGEN---------A-D-----YV-FV-KADGQV---A-----KAGD-I-L-KQLSLDV-DK-GVFHYL..
```

FIG 1 Alignment of the OXA-235 amino acid sequence with those of OXA-23, OXA-24, OXA-51, OXA-58, OXA-134, and OXA-143. Conserved residues are shaded. Dashes represent conserved amino acids. β-Lactamases are numbered according to the DBL numbering system (24).

which individual colonies were visible growing up to the imipenem disc. We did not detect cotransfer of other antibiotic resistance determinants, with the transformants showing no difference in their MICs to tetracycline, gentamicin, chloramphenicol, ciprofloxacin, or minocycline compared to the MICs for the untransformed parent strain (data not shown). The remaining 6 isolates that did not transform ATCC 17978 were presumed to have a chromosome-located resistance mechanism.

Identification of the OXA-235, -236, and -237 CHDLs. The transforming plasmids were used for shotgun cloning. EcoRI-restricted plasmid DNA from *A. baumannii* AF401 cloned into pBBR1MCS, followed by expression in *E. coli*, gave *E. coli*(pOXA-235). Analysis of shotgun-cloned plasmid DNA revealed a 2.5-kb insert which was sequenced by primer walking. DNA sequence analysis of plasmid pOXA-235 identified an 831-bp open reading frame (ORF) that encoded a novel class D β-lactamase of 276 amino acids (Fig. 1), which was assigned as OXA-235 by the β-lactamase database. Within the deduced protein sequence, a serine-threonine-phenylalanine-lysine tetrad (STFK) was found (24). A KSG element (positions 216 to 218) was present, as found in OXA-24, OXA-51, OXA-58, and OXA-143 CHDLs, whereas a KTG motif is present in the OXA-23 enzyme and in most class D β-lactamases lacking any carbapenemase activity. The class D β-lactamase structural element FGN at positions 70 to 73 (DBL numbering) was conserved in OXA-235, as has been found in all of the CHDL sequences identified in *A. baumannii*, with the exception of

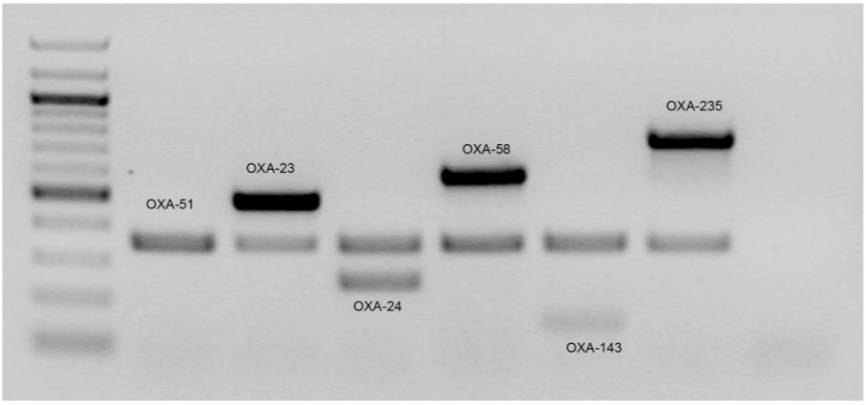

FIG 2 OXA-type multiplex PCR with additional OXA-235-like primers. The *Acinetobacter baumannii* isolates contain alleles encoding the intrinsic OXA-51-like (lanes 2 to 7) and the acquired OXA-23-like, OXA-24-like, OXA-58-like, OXA-143-like (lanes 3 to 6), and OXA-235 (lane 7). The molecular size marker (lane 1) is a 100-bp ladder (NEB, Frankfurt am Main, Germany).

TABLE 2 MICs of *A. baylyi* and *A. baumannii* ATCC 17978 transformed with pETRA::OXA-24 and pETRA::OXA-235

	MIC (μg/ml) of indicated strain transformed with:					
	pETRA[a]		OXA-24		OXA-235	
Antibiotic	>*A. baylyi*	*A. baumannii* 17978	*A. baylyi*	*A. baumannii* 17978	*A. baylyi*	*A. baumannii* 17978
Imipenem	0.125	0.25	32	>32	1	3
Meropenem	0.25	0.5	32	>32	2	4
Ampicillin	8	256	>256	>256	>256	>256
Cefoxitin	24	>256	24	>256	16	>256
Ceftazidime	4	6	4	6	4	6
Cefotaxime	6	16	8	16	8	16
Piperacillin	6	24	256	>256	48	>256
TZP[b]	6	16	256	>256	256	256

[a] pETRA, plasmid alone as negative control.
[b] TZP, piperacillin-tazobactam.

those of the OXA-51 and OXA-58 subclasses (25). Also, OXA-235 showed key residues for carbapenem hydrolysis, F or Y at 112 and Met-223, previously identified in OXA-24, which define a tunnel-like entrance for carbapenems to the active site (21).

The deduced amino acid sequence showed the greatest homology to OXA-134 (85% identity), an intrinsic oxacillinase of *A. lwoffii* (26). The amino acid identities with the acquired CDHLs OXA-23, OXA-24, OXA-58, and OXA-143 were 54% to 57%, and the amino acid identity with OXA-51 was 56% (Fig. 1). Primer walking also revealed that $bla_{\text{OXA-235}}$ was bracketed by two copies of IS*Aba1*. Primers were designed to amplify $bla_{\text{OXA-235}}$, and these were used to screen the remaining 9 isolates. All isolates tested positive for the presence of $bla_{\text{OXA-235}}$ and IS*Aba1* bracketing the gene. The PCR products were subsequently sequenced. Seven isolates were found to harbor OXA-235. Two novel variants of OXA-235 were also identified, OXA-236 (Glu173-Val) and OXA-237 (Asp208-Gly) (Table 1). Multiplex PCR with primer pair OXA-235_F/OXA-235_R amplified a 768-bp fragment that was easily distinguishable from the other bla_{OXA} within the current OXA multiplex PCR (Fig. 2).

DNA-DNA hybridization confirmed a plasmid location in 4 strains; however, in three of these strains, a second positive signal was visible that corresponded to genomic DNA (colocalized with $bla_{\text{OXA-51-like}}$). A 5th strain (BMBF 255) showed a positive signal for chromosomal and plasmid location of $bla_{\text{OXA-235-like}}$, but this plasmid failed to transform in ATCC 17978. PCR of extracted plasmid DNA confirmed these results. Two $bla_{\text{OXA-235}}$-positive plasmids were typeable using the method described by Bertini et al. (19), belonging to replicon groups 2 and 6 (originating from strains AF 401 and AF 678, respectively).

Transformation of $bla_{\text{OXA-235}}$ into *A. baylyi* and *A. baumannii* ATCC 17978 led to reduced carbapenem susceptibility (Table 2). Cephalosporin MICs were unaffected. Similarly, transformation of *A. baumannii* ATCC 17978 with pWH1266-OXA-235 resulted in raising imipenem and meropenem MICs from 0.125 to 6 μg/ml and from 0.19 to 16 μg/ml, respectively (data not shown). As seen with transformation with native plasmids, the transformants exhibited phenotypic heterogeneous resistance.

Kinetic determination of k_{cat}, K_m, and k_{cat}/K_m against oxacillin, nitrocefin, ampicillin, meropenem, and imipenem was carried out (Table 3). The results of the kinetic analysis show that OXA-24 has an approximately 1,000-times-lower k_{cat}/K_m to oxacillin than to OXA-235, which indicates that OXA-235 has more oxacillinase activity than OXA-24. Conversely, there was a higher activity of OXA-24 with respect to carbapenems, with this enzyme having around 5 times more catalytic efficacy than OXA-235 for meropenem and imipenem. The kinetic results are consistent with the MIC data (Table 2). Despite the low catalytic activity of OXA-235, the enzyme exhibits sufficient hydrolysis to reduce carbapenem susceptibility when it is expressed in shuttle plasmid pETRA or pWH1266 in *A. baylyi* and *A. baumanii*.

DISCUSSION

Three novel carbapenem resistance determinants that were undetected using established PCRs have been identified in *A. baumannii*. These CHDLs, OXA-235, OXA-236, and OXA-237, were identified in isolates that originated primarily from the United States, but one isolate was recovered in Mexico. Thus, there are similarities to OXA-143-like, which has so far only been reported in Brazil (27, 28), and contrasts with OXA-23, OXA-24, and OXA-58, which, although first identified in Europe (Scotland, Spain, and France, respectively) (25, 29, 30), have now been described glob-

TABLE 3 Kinetic data for purified OXA-235 and OXA-24[a] β-lactamases

	k_{cat} (s^{-1}) [mean (±SD)]		K_m (μM) [mean (±SD)]		k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)	
Antibiotic	OXA-24	OXA-235	OXA-24	OXA-235	OXA-24	OXA-235
Nitrocefin	NT[b]	65.64 (±7)	NT	53 (±7)	NT	1.23
Oxacillin	0.18 (±0.09)	110 (±27)	1,272 (±222)	818 (±139)	1.41×10^{-4}	0.13
Ampicillin	223 (±92.9)	0.73 (±0.015)	80 (±15)	85 (±21)	2.7	0.0085
Meropenem	0.37 (±0.1)	0.117 (±0.023)	0.0099 (±0.0014)	0.0159 (±0.0027)	37.37	7.35
Imipenem	1 (±0.47)	0.066 (±0.01)	0.58 (±0.084)	0.19 (±0.043)	1.72	0.34

[a] Data for OXA-24 are from reference 21.
[b] NT, not tested.

ally (8). Because OXA-236 and OXA-237 were identified in single isolates while OXA-235 was identified in eight isolates, we propose that the subclass be termed OXA-235-like.

We found OXA-235-like variants encoded on plasmids isolated from multiple geographical regions, and they were able to transform into laboratory strains and reduce carbapenem susceptibility. Sequencing of $bla_{\text{OXA-51-like}}$ revealed that the majority of isolates possess OXA-66, which is associated with IC2, the most widespread *A. baumannii* lineage (8, 31). This strongly suggests that there is potential for further spread of OXA-235-like. Indeed, we found five OXA-235 isolates that showed ≥99% similarity by rep-PCR, suggesting clonal spread, as well as two variants of this enzyme, disseminated in 5 U.S. states and Mexico. This contrasts with OXA-143, which we found in isolates that did not cluster with the international clones, and may in part explain why this OXA has thus far been confined to Brazil (8). Additionally, the genes encoding OXA-235-like were undetected using established PCR methods, so it is possible that they have been overlooked in other studies. For example, isolate BMBF 255 (OXA-236) was part of a previous study into the global spread of carbapenem-resistant *A. baumannii* (8), but we were unable to identify the mechanism of resistance at that time, and it was only identified after designing primers for $bla_{\text{OXA-235}}$.

OXA-235 hydrolyzed penicillins and carbapenems but did not show activity against expanded-spectrum cephalosporins, as observed with other CHDLs (25, 27, 30, 32). Similar to the latter enzymes, OXA-235 shows high affinity for the carbapenems but with low rates of hydrolysis (low k_{cat}). Despite this weak hydrolysis, it is very likely that OXA-235 contributes significantly to imipenem and meropenem resistance, as demonstrated previously with OXA-23, OXA-24, OXA-58, and OXA-143, especially as $bla_{\text{OXA-235-like}}$ is associated with IS*Aba1*, which has been shown to promote overexpression of OXA (7).

The origin of acquired CHDLs associated with *A. baumannii* is not known, but recent evidence has emerged that OXA-23 is intrinsic to *Acinetobacter radioresistens* and has been mobilized, possibly by IS*Aba1* (33). Other *Acinetobacter* species appear to have their own intrinsic OXA but, on the whole, are rarely associated with carbapenem resistance, despite their ability to hydrolyze carbapenems, possibly because expression is low (26, 34). As well as OXA-23, the intrinsic OXA-51-like of *A. baumannii* is associated with carbapenem resistance but only when overexpressed (IS*Aba1* associated) (7). This reinforces the role of IS*Aba1* and its importance in the genome plasticity of *A. baumannii* (35).

In summary, OXA-235, OXA-236, and OXA-237 are the first representatives of a novel subclass of CHDLs whose prevalence remains to be determined. It may, indeed, be quite prevalent, since resistance to carbapenems in *A. baumannii* has not always been associated with known carbapenemases and previously designed primers could not detect this novel gene.

ACKNOWLEDGMENTS

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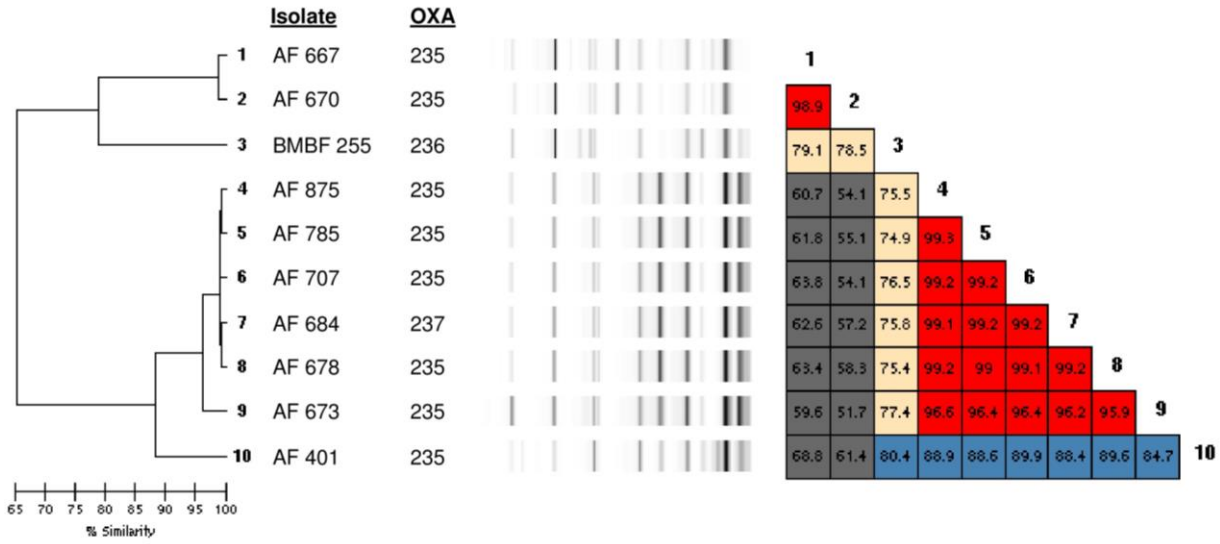
Supplementary data



Supplemental figure S1. Dendrogram and Ziggurat figure showing rep-PCR fingerprints of OXA-235-like positive *A. baumannii*.

2.4.3 Characterization of *bla*$_{\text{OXA-143}}$ variants in *Acinetobacter baumannii* and *Acinetobacter pittii*

To date, the acquired OXA-143 subclass has mainly been detected in carbapenem-resistant *A. baumannii* from Brazil and compromises two designated amino acid variants (OXA-143 and OXA-231).[110, 139-141] However, little information is given on the genetic environment of *bla*$_{\text{OXA-143-like}}$.

The following publication reports *bla*$_{\text{OXA-143-like}}$ detection in two carbapenem-resistant *A. baumannii* isolates from Brazil and Honduras, as well as a carbapenem-susceptible *A. pittii* isolate from the USA. Sequencing identified the OXA-143 variants OXA-231 and OXA-253 in the *A. baumannii* isolates, as well as OXA-255 in the *A. pittii* isolate. Furthermore, a similar genetic context was discovered for *bla*$_{\text{OXA-231}}$ compared to the published sequence of *bla*$_{\text{OXA-143}}$, while the genetic environment of the other two variants differed. For the first time the *bla*$_{\text{OXA-143-like}}$ transcription initiation site was determined, which led to the identification of putative promoter sequences. Regardless of differences in the predicted promoter sequences, all OXA-143 variants conferred carbapenem-resistance in the *A. baumannii* reference strain ATCC 17978. In contrast to the clinical isolate, OXA-255 also conferred carbapenem-resistance in the *A. pittii* reference strain SH024, which was associated with *bla*$_{\text{OXA-255}}$ expression. Identification of OXA-255 in *A. pittii* highlights the potential of this subclass to spread in non-*baumannii Acinetobacter* species. Interestingly, Girlich *et al.* recently reported presence of OXA-253 in *A. baumannii* from Brazil, which indicates distribution of this OXA-143 variant in the Western Hemisphere.[142]

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Characterization of $bla_{OXA-143}$ Variants in *Acinetobacter baumannii* and *Acinetobacter pittii*

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The acquired carbapenem-hydrolyzing oxacillinase (OXA) OXA-143 has thus far been detected only in *Acinetobacter baumannii* isolates from Brazil. The aim of this study was to characterize three OXA-143 variants: OXA-231 and OXA-253 from carbapenem-resistant *A. baumannii* isolates and OXA-255 in a carbapenem-susceptible *Acinetobacter pittii* isolate originating from Brazil, Honduras, and the United States, respectively. The 5′ rapid amplification of cDNA ends (RACE) technique identified the same transcription initiation site for all $bla_{OXA-143-like}$ genes and revealed differences in the putative promoter regions. However, all cloned OXA-143 variants conferred carbapenem resistance on *A. baumannii* ATCC 17978 and OXA-255 conferred carbapenem resistance on *A. pittii* SH024, which was correlated with $bla_{OXA-255}$ gene expression. This is the first description of OXA-143-like outside *A. baumannii*. Detection of OXA-143-like in the United States and Honduras indicates its dissemination through the American continent.

Acinetobacter baumannii and *Acinetobacter pittii* are members of the "*A. baumannii* group," which, together with *A. nosocomialis*, comprise three phenotypically similar clinically relevant *Acinetobacter* species (1, 2). *A. baumannii* and *A. pittii* cause nosocomial infections and are associated with clinical outbreaks (3–5). While *A. pittii* is frequently found on both intact and diseased human skin and mucous membranes, it is prevalent on general wards and is usually susceptible to carbapenems. *A. baumannii* mainly affects patients in intensive care (6, 7). Carbapenems are considered the drugs of choice to treat infections caused by multidrug-resistant (MDR) *A. baumannii*. However, over the last decade carbapenem resistance has increased in *A. baumannii*, compromising available treatment options. The most common carbapenem resistance determinants in *Acinetobacter* spp. are carbapenem-hydrolyzing oxacillinases (OXA). *A. baumannii* isolates harbor the intrinsic OXA-51, of which >80 variants have been identified, and five groups of acquired OXA (OXA-23, -40, -58, -143, and -235) (8, 9). Of these, only OXA-23, OXA-40, and OXA-58 have been detected in other *Acinetobacter* species. For example, $bla_{OXA-23-like}$, $bla_{OXA-40-like}$, and $bla_{OXA-58-like}$ have been described for carbapenem-nonsusceptible *A. pittii* isolates from China, Colombia, France, Germany, and the Irish Republic (10–13). bla_{OXA} genes are often associated with insertion sequences (IS) that mediate their mobility and overexpression, thereby leading to carbapenem resistance. However, bla_{OXA-40} and $bla_{OXA-143}$ seem to be exceptions to this (14). OXA-143 was first identified in 2009 in a carbapenem-resistant *A. baumannii* isolate and can be detected by multiplex PCR (15, 16). To date, OXA-143 has been reported only for *A. baumannii* isolates from certain states in Brazil (15, 17). However, outside Brazil, most bla_{OXA} screening is performed using the OXA multiplex PCR described by Woodford et al., which does not include $bla_{OXA-143-like}$ primers (18).

The aim of this study was to characterize $bla_{OXA-143}$ variants in two *A. baumannii* isolates and one *A. pittii* isolate.

MATERIALS AND METHODS

Bacterial strains, species identification, carbapenem susceptibility testing, and *A. baumannii* molecular typing. Carbapenem-resistant *A. baumannii* isolates AF81 and AF260 as well as carbapenem-susceptible *A. pittii* isolate AF726 were initially identified as $bla_{OXA-143-like}$ positive by multiplex PCR (Table 1) (16). AF81 originated from Brazil, AF260 originated from Honduras, and AF726 originated from the state of Indiana in the United States. Imipenem and meropenem susceptibility of *Acinetobacter* isolates and transformants was determined by Etest (bioMérieux, Nürtingen, Germany) according to standard protocols. To confirm species identification, *gyrB* multiplex PCR and *rpoB* sequencing were performed, as described previously (1). Isolates AF81 and AF260 were typed by repetitive sequence-based PCR (rep-PCR) (19).

PCR, sequencing, and cloning. The presence of bla_{OXA} genes was confirmed by multiplex PCR as described previously (8). Primers used for sequencing and cloning are shown in Table S1 in the supplemental material. $bla_{OXA-51-like}$ sequencing of isolates AF81 and AF260 was performed using primers OXA-69A and OXA-69B (20). $bla_{OXA-143-like}$ of isolate AF81 was amplified using primer pair OXA-231_F and OXA-231_R, cloned into pCR4-TOPO for sequencing (Invitrogen, Karlsruhe, Germany), and transferred into chemically competent *Escherichia coli* DH5α cells (New England BioLabs, Frankfurt am Main, Germany) according to the manufacturer's instructions. To sequence $bla_{OXA-143-like}$ from isolate AF260, total DNA was restricted by EcoRV endonuclease and shotgun cloned into pBBR1MCS, as previously described (21). $bla_{OXA-143-like}$-containing inserts of pBBR1MCS and pCR4-TOPO were amplified by PCR and sequenced by primer walking. To sequence $bla_{OXA-143-like}$ of AF726, total DNA was restricted by EcoRI endonuclease, self-ligated using Quick ligase (New England BioLabs), amplified by inverse PCR, and sequenced by primer walking. The $bla_{OXA-143}$ variants were numbered by the Lahey β-lactamase database (http://www.lahey.org/Studies/).

Assessment of $bla_{OXA-143-like}$ transferability. To determine the transferability of $bla_{OXA-143}$ variants, plasmids isolated from AF81, AF260, and AF726 were used for transformation. Electroporation was performed with

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TABLE 1 Characterization of clinical isolates AF81, AF260, and AF726[a]

Isolate	Country of origin	Date of isolation (mo/day/yr)	Carbapenem MIC (μg/ml)		Species identification	OXA-MPLX PCR	OXA-51 variant and rep-PCR type	OXA-143 variant	Replicon type and plasmid transfer
			IPM	MEM					
AF81	Brazil	01/04/2008	>32	>32	*A. baumannii*	51, 143	OXA-51, unclustered	231	GR 19, transferable
AF260	Honduras	02/01/2008	>32	>32	*A. baumannii*	51, 143	OXA-65, IC5	253	GR 12, transferable
AF726	USA (Indiana)	08/29/2007	0.75	1	*A. pittii*	143	ND	255	ND

[a] IPM, imipenem; MEM, meropenem; ND, not detected; MPLX, multiplex; IC, international clone; GR, group.

reference strains *A. baumannii* ATCC 17978 (plasmids of AF81 and AF260) and *A. pittii* SH024 (plasmid of AF726). Selection of *A. baumannii* transformants was performed on Mueller-Hinton agar (Oxoid, Wesel, Germany) supplemented with ticarcillin (150 μg/ml). Selection of *A. pittii* transformants was performed on Mueller-Hinton agar supplemented with 25, 40, 60, or 80 μg/ml of ticarcillin. The presence of $bla_{OXA-143-like}$ in the transformants was confirmed by PCR. In addition, plasmid replicon typing was performed with the clinical isolates, the reference strains, and the OXA transformants, as previously described (22).

Effect of OXA-143 variants on carbapenem susceptibility. To further characterize the impact of the three $bla_{OXA-143-like}$ variants on carbapenem susceptibility, the genes were amplified using primers listed in Table S1 in the supplemental material, cloned into the shuttle vector pWH1266, and transferred into *A. baumannii* reference strain ATCC 17978 by electroporation (23). Transformants were selected on Luria-Bertani agar (Oxoid) supplemented with 30 μg/ml of tetracycline. In addition, $bla_{OXA-143-like}$ of isolate AF726 was transferred into the *A. pittii* reference strain SH024 using the same selective medium.

Determination of $bla_{OXA-143-like}$ transcriptional initiation sites and gene expression. To identify the transcriptional start site of the three $bla_{OXA-143}$ variants, 5′ rapid amplification of cDNA ends (RACE) was performed using the 5′ RACE system for rapid amplification of cDNA ends, version 2.0 (Invitrogen). Primers used for 5′ RACE are shown in Table S1 in the supplemental material. Total RNA was prepared using the Qiagen RNeasy kit (Qiagen, Hilden, Germany). Amplicons of dC-tailed cDNA were purified and sequenced. In order to analyze differences in carbapenem susceptibility, $bla_{OXA-143-like}$ expression in the *A. pittii* strains was investigated based on independent experiments. Quantitative reverse transcription-PCR (qRT-PCR) was performed as described previously (8). $bla_{OXA-143-like}$ expression in the *A. pittii* transformant was compared to gene expression in AF726 and was normalized against expression of the *rpoB* reference gene. Standard curves for $bla_{OXA-143-like}$ and *rpoB* were included. Primers used for standard curves and qRT-PCR are shown in Table S1 in the supplemental material.

Nucleotide sequence accession numbers. The $bla_{OXA-231}$, $bla_{OXA-253}$, and $bla_{OXA-255}$ nucleotide sequences are available in GenBank under the following accession numbers: JQ326200 ($bla_{OXA-231}$), KC479324 ($bla_{OXA-253}$), and KC479325 ($bla_{OXA-255}$).

RESULTS AND DISCUSSION

Species identification, bla_{OXA} detection, OXA-51-like sequencing, and *A. baumannii* typing. *gyrB* multiplex PCR and *rpoB* sequencing confirmed AF81 and AF260 as *A. baumannii* and AF726 as *A. pittii*. Multiplex PCR confirmed the presence of $bla_{OXA-143-like}$ in all three isolates, as well as the presence of $bla_{OXA-51-like}$ in the two *A. baumannii* isolates. Sequencing of $bla_{OXA-51-like}$ identified bla_{OXA-51} and bla_{OXA-65} in isolates AF81 and AF260, respectively, which were not associated with IS*Aba1* (Table 1). Rep-PCR identified AF260 as international clone 5 (24), while AF81 did not cluster with any of the international clones. However, AF81 shared 98.5% similarity to *A. baumannii* 135040, the strain in which OXA-143 was first identified (15).

Identification of OXA-143-like-encoding genes. To amplify and sequence $bla_{OXA-143-like}$, flanking primers were designed based on $bla_{OXA-143}$ (GenBank accession no. GQ861437), which amplified the gene in AF81 but failed to amplify the gene in the other two isolates. Therefore, shotgun cloning was performed, and we cloned from AF260 an approximately 10-kb insert into pBBR1MCS, which was partially sequenced. Inverse PCR of AF726 revealed the presence of an approximately 3.5-kb amplicon which was also partially sequenced. Based on these sequences, $bla_{OXA-143-like}$ flanking primers specific for AF260 and AF726 were designed and used for cloning into pWH1266 (see Table S1 in the supplemental material). $bla_{OXA-143-like}$ sequencing revealed three OXA-143 variants which were assigned as OXA-231 (AF81), OXA-253 (AF260), and OXA-255 (AF726) by the Lahey β-lactamase database (Table 1). OXA-231 possessed one amino acid substitution compared to OXA-143 (D224→A) (Fig. 1A) and has been recently detected in another *A. baumannii* isolate from Brazil (25). OXA-253 shared 94% amino acid identity with OXA-143 (17 amino acid substitutions), while OXA-255 shared 92% amino acid identity with OXA-143 (21 amino acid substitutions). High similarity of OXA-253 and OXA-255 was also seen with OXA-182 (17 and 22 amino acid substitutions, respectively [Fig. 1A]). OXA-182 was detected in South Korea using $bla_{OXA-143-like}$ primers (26). Although OXA-253 and OXA-255 share similar amino acid identity with OXA-182 and OXA-143, they differ in their numbers of identical and positive (including conservative substitutions) amino acids. OXA-253 shares 258 of 275 identical amino acids with both enzymes, while the number of positive amino acids it shares with OXA-182 is higher than it shares with OXA-143 (266 and 262, respectively). In contrast, OXA-255 shares more identical amino acids with OXA-143 than OXA-182 (254 and 253, respectively) but fewer positive amino acids (261 and 264, respectively). This can be visualized in a phylogenetic tree of OXA variants (Fig. 1B). Thus, the OXA-143-like group shows large variations suggesting an ancient lineage, similar to OXA-51-like (27, 28).

$bla_{OXA-143-like}$ transferability. Clinical plasmids containing $bla_{OXA-231}$ and $bla_{OXA-253}$ were transferable into ATCC 17978. Replicon typing of ATCC 17978 transformants revealed that $bla_{OXA-231}$ and $bla_{OXA-253}$ were encoded on plasmids that harbored group (GR) 19 and GR 12 replicase genes, respectively (Table 1). Despite repeated attempts to transfer $bla_{OXA-255}$ and selection of transformants using ticarcillin concentrations as low as 25 μg/ml, the gene was not transferrable to either *A. pittii* or *A. baumannii* reference strains using plasmid preparations from AF726. Furthermore, plasmid replicon typing did not identify a known replicon in AF726. However, sequencing of $bla_{OXA-255}$ flanking regions identified sequences bracketing the $bla_{OXA-255}$ as plasmid sequences, which might indicate that this gene was initially plas-

(A)

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OXA-143   1 MKKFILPILSISTLLSVSACSSIQTKFEDTFHTSNQQHEKAIKSYFDEAQTQGVIIIKKGKNISTYGNNLTRAHTEYVPASTFKMLNALIGLENHKATTT 100
OXA-231     ----------------------------------------------------------------------------------------------------
OXA-253     --------F---I---L-------------SDI-D--QG-------------------E-----------A----------------------------
OXA-182     --------F---I---L---------------I---K--------------------E-----S-----V--------------------------N
OXA-255     --------F-------L----T--N---K-SDI-D----------------------E----RI-----V----------------------------

OXA-143 101 EIFKWDGKKRSYPMWEKDMTLGDAMALSAVPVYQELARRTGLDLMQKEVKRVGFGNMNIGTQVDNFWLVGPLKITPIQEVNFADDFANNRLPFKLETQEE 200
OXA-231     ----------------------------------------------------------------------------------------------------
OXA-253     ----------------------------------------------------------------------------------------L-------------
OXA-182     --------------------E-----------D----I--N-----------------------I---------------L-------------
OXA-255     ------------------------------------------------------S-----N--------------A-----L-------------

OXA-143 201 VKKMLLIKEFNGSKIYAKSGWGMDVTPQVGWLTGWVEKSNGEKVAFSLNIEMKQGMPGSIRNEITYKSLENLGII 275
OXA-231     ----------------------A-------------------------------------------------
OXA-253     ---------V---------------I-----------------P----L------S-----------------
OXA-182     ---------V---------------S-----------------S----------S-----------------
OXA-255     ---------V-----------------------------------P----L------S-----------------
```

(B)

FIG 1 (A) Alignment of OXA-143-like amino acid sequences. Variants are sorted according to their amino acid identity with OXA-143. OXA-231 is closely related to OXA-143, with only one amino acid change at position 224. (B) Phylogenetic tree of OXA variants. This neighbor-joining tree was generated using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

mid located and subsequently integrated into the chromosome (see below).

Genetic environment of $bla_{\text{OXA-143-like}}$. Alignment of bla_{OXA} and the surrounding sequences revealed high similarity of $bla_{\text{OXA-231}}$ flanking regions compared to $bla_{\text{OXA-143}}$ (GenBank accession no. GQ861437). The degrees of similarity were 98% for 193 bp upstream of $bla_{\text{OXA-231}}$ and 99% for 198 bp downstream of the gene. Accordingly, the start codon of a replicase gene was detected downstream of $bla_{\text{OXA-231}}$ (see Fig. S2 in the supplemental material). Flanking regions of the other two *bla* genes were not conserved, which explains why we were initially unable to amplify the whole genes from AF260 and AF726.

Downstream of $bla_{\text{OXA-255}}$, a putative peptidase gene was detected which showed 77% similarity to a peptidase encoded on *A. baumannii* plasmid p3ABSDF (GenBank accession no. CU468233) (see Fig. S2 in the supplemental material). Upstream of the $bla_{\text{OXA-255}}$ gene, an open reading frame coding for 136 amino acids of a putative TonB-dependent receptor plug domain was detected. Conserved domains of the plug superfamily function as gates within channel-forming TonB-dependent receptors, which are important for the iron uptake in Gram-negative bacteria (29). A BLAST search revealed 51% amino acid identity with a hypothetical protein previously described for *Acinetobacter* species NIPH1867 (locus tag WP_005210788), containing a TonB-dependent siderophore receptor domain.

In contrast, the sequence upstream of $bla_{\text{OXA-253}}$ showed 91% similarity with the sequence upstream of $bla_{\text{OXA-40-like}}$ in *A. baumannii* plasmid pAC92 (GenBank accession no. JN982952), containing a putative inner membrane protein and an XerC/D recombination site (see Fig. S2 in the supplemental material). Analysis of the partial protein sequence revealed only one amino acid difference compared to another *A. baumannii* membrane protein (locus tag WP_000465837). The XerC/D site (5′-ACTTCGTATA ATATCCATTATGTTAAAT-3′) was located 74 bp upstream of $bla_{\text{OXA-253}}$. In addition, another putative XerC/D recombination site (5′-ATATTGTATAACCTATATTATGTTATTT-3′) was identified 111 bp downstream of the gene. XerC/D recombination sites are often associated with $bla_{\text{OXA-40-like}}$ genes. However, our results indicate that the three OXA-143 variants described in this report might have evolved from different progenitors. This might also include OXA-182, as a putative transposase gene has been detected downstream of the *bla* gene, which is not part of the flanking regions of the other variants. Interestingly, the available upstream sequence of $bla_{\text{OXA-182}}$ (20 bp) is 100% identical with the same region upstream of $bla_{\text{OXA-143}}$, which further indicates relatedness of this OXA to the OXA-143 group.

Impact of OXA-231, OXA-253, and OXA-255 on carbapenem susceptibility. Cloning of $bla_{\text{OXA-143-like}}$ into pWH1266 and transfer into *A. baumannii* ATCC 17978 conferred carbapenem resistance. Imipenem and meropenem MICs increased from 0.25 μg/ml in the reference strain to >32 μg/ml in all transformants (see Table S3 in the supplemental material). Furthermore, OXA-255conferred carbapenem resistance on *A. pittii* SH024, with imipenem and meropenem MICs increasing from 0.25 and 0.5 μg/ml to 16 and >32 μg/ml, respectively (see Table S3 in the supplemental material).

Identification of $bla_{\text{OXA-143-like}}$ transcription initiation sites and $bla_{\text{OXA-255}}$ expression. In order to identify the transcriptional initiation site of $bla_{\text{OXA-143-like}}$ and deduce the promoter region, 5′ RACE was performed. We identified the same transcriptional ini-

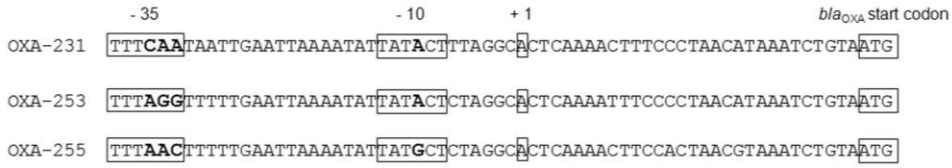

FIG 2 Alignment of $bla_{OXA-143-like}$ predicted promoter sequences. The start codon, the transcription initiation site, and the −10 and −35 boxes are boxed. Differences in the −10 and −35 boxes are marked by bold type.

tiation site for all $bla_{OXA-143-like}$ genes 30 bp upstream of the start codon (Fig. 2). Six base pairs upstream of the transcriptional initiation site, the same −10 box was identified in AF81 and AF260 (TATACT), while a substitution was present in AF726 (TATG CT). Interestingly, sequence alignment of the $bla_{OXA-231}$ and $bla_{OXA-143}$ upstream regions revealed the presence of the same putative promoters. The spacer region between the −10 and −35 boxes had an optimal size of 17 bp. Putative −35 boxes harbored 3 nucleotide differences (Fig. 2). Based on carbapenem MICs of ATCC 17978 transformants harboring recombinant pWH1266, OXA-143-like variants and their different promoter sequences did not seem to significantly influence carbapenem susceptibility (see Table S3 in the supplemental material). However, because OXA-255 did not confer carbapenem resistance on the clinical isolate AF726, while OXA-255-transformed ATCC 17978 and *A. pittii* SH024 were carbapenem resistant, we investigated expression of $bla_{OXA-255}$ in *A. pittii*. Expression analysis revealed that although $bla_{OXA-255}$ was expressed in AF726, it was overexpressed 24-fold in the SH024 transformant, which correlated with carbapenem resistance. This suggests that the $bla_{OXA-255}$ promoter is functioning and that there may be other regulatory mechanisms that affect its overexpression in the clinical isolate.

Conclusion. To date, the OXA-143 subclass has predominantly been described as occurring in carbapenem-resistant *A. baumannii* isolates from Brazil (17, 25, 30, 31). This study constitutes the first detection of OXA-143 variants in Honduras and the United States and indicates the spread of this carbapenemase in the Western Hemisphere. The occurrence of OXA-255 conferring carbapenem resistance on *A. baumannii* and on *A. pittii* highlights the potential of this OXA to spread within the genus *Acinetobacter*.

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Supplementary data

Table S1: Primers used in this study.

Primer	Target/Purpose	5'-3' sequence
OXA-69A	$bla_{\text{OXA-51-like}}$ sequencing	CTAATAATTGATCTACTCAAG (20)
OXA-69B		CCAGTGGATGGATGGATAGATTATC (20)
OXA-231_F	Cloning of $bla_{\text{OXA-231}}$ into pCR4-TOPO and pWH1266	TCCACTTTCAAAATACCTTC
OXA-231_R		TGCTATTCGTATTGCTGATA
OXA-253_F	Cloning of $bla_{\text{OXA-253}}$ and $bla_{\text{OXA-255}}$ into pWH1266	AGAAGGGTTTTAGGTTTTTGA
OXA-255_F		CATCTCGGTAAACAGTCGAT
OXA-253/255_R		TTCGAATTTGAAGTGTTTAGA
OXA-143_standard1	$bla_{\text{OXA-143-like}}$ for standard curve	CAGCAACATGAAAAAGCCATT
OXA-143_standard2		CAGGCATTCCTTGCTTCATT
OXA-143_RT1	$bla_{\text{OXA-255}}$ qRT-PCR	ACAACTGAGATTTTCAAATGG
OXA-143_RT2		TTCTTGATATACAGGAACTGC
OXA-143_RACE1	5´-RACE: cDNA synthesis	ATATACAGGAACTGCTGAAA
OXA-143_RACE2	5´-RACE: amplification of dC-tailed cDNA	GGTACCGTAGTGGATTTCAG
OXA-143_RACE3	5´-RACE: nested amplification of dC-tailed cDNA	TTGATGCAGGGACATATTCT

Supplementary data continued

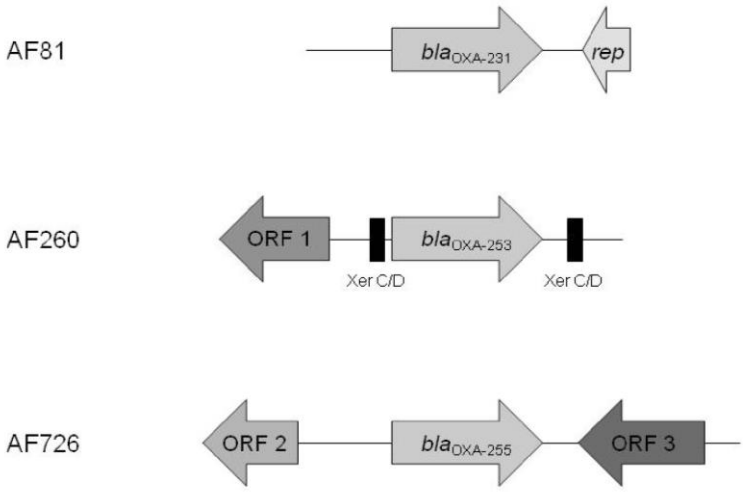

Figure S2: Schematic composition of $bla_{OXA-143-like}$ flanking regions. *rep*, partial replicase gene; ORF1, partial putative inner membrane protein; XerC/D, recombination sites; ORF2, partial putative TonB-dependent receptor; ORF3, full-length putative peptidase.

Supplementary data continued

Table S3: Carbapenem susceptibility of clinical *Acinetobacter* isolates AF81, AF260 and AF726, *A. baumannii* ATCC 17978, *A. pittii* SH024 and transformants harboring $bla_{\text{OXA-143-like}}$-encoding clinical plasmids and recombinant pWH1266.

Strain	Carbapenem MIC [µg/ml]	
	IPM	MEM
AF81 (OXA-231)	>32	>32
AF260 (OXA-253)	>32	>32
AF726 (OXA-255)	0.75	1
ATCC 17978	0.25	0.25
SH024	0.25	0.5
ATCC 17978 (pOXA-231)	>32	>32
ATCC 17978 (pOXA-253)	>32	>32
ATCC 17978 (pWH1266::$bla_{\text{OXA-231}}$)	>32	>32
ATCC 17978 (pWH1266::$bla_{\text{OXA-253}}$)	>32	>32
ATCC 17978 (pWH1266::$bla_{\text{OXA-255}}$)	>32	>32
SH024 (pWH1266::$bla_{\text{OXA-255}}$)	16	>32

IPM, imipenem; MEM, meropenem

Unpublished results II

Previous studies have reported an effect of certain physiological conditions on antimicrobial susceptibility in Gram-negative bacteria. For example, 8 mM sodium-salicylate and pH 6.0 have been reported to affect fluoroquinolone susceptibility and induce expression of the porin OmpC in *Serratia marcescens*.[143, 144] In addition, 4-32 mM sodium-salicylate has been reported to decrease porin expression in *P. aeruginosa*, which can be associated with carbapenem resistance.[145, 146] Interestingly, an effect of physiological conditions on antimicrobial susceptibility has also been reported in *A. baumannii*. For example, it has been shown that pH 5.8 alters fluoroquinolone susceptibility in clinical *A. baumannii* isolates.[147] Other studies reported altered porin expression in the presence of 16 mM sodium-salicylate or 200 mM NaCl, which can be associated with carbapenem resistance.[116, 148] For example, Hood *et al.* have reported that gene expression of the porins CarO and the 33-36 kDa Omp in ATCC 17978 is reduced in the presence of NaCl.[148] However, when we analyzed the outer membrane protein profiles of ATCC 17978 grown in Mueller-Hinton broth under different physiological conditions (200 mM NaCl, 16 mM sodium-salicylate, pH 5.8) we could not detect significant changes compared to standard conditions (pH 7.2) (data not shown). Interestingly, it is also reported that the majority of OXA in *A. baumannii* are inhibited *in vitro* in the presence of NaCl.[110, 149-151] However, kinetic data on OXA-23 are missing and data on OXA-40 are contradictory. For example, one study reported complete inhibition of OXA-40 by 1 mM NaCl, while a different study reported half maximal inhibition in the presence of 3 M NaCl.[150, 152] However, the inhibitory effect of NaCl on purified OXA enzymes has led some researchers to screen for the presence of OXA by performing carbapenem MICs in media supplemented with 200 mM NaCl.[94, 153, 154] A 4-fold reduction of the carbapenem MIC in the presence of NaCl was considered to be significant for OXA inhibition. The susceptibility of each transformant was compared to the respective recipient. The cloned OXA genes were *bla*$_{\text{OXA-219}}$ (*bla*$_{\text{OXA-51}}$ variant; see 2.2.2), *bla*$_{\text{OXA-23}}$, *bla*$_{\text{OXA-40}}$, *bla*$_{\text{OXA-143}}$, *bla*$_{\text{OXA-58}}$ and *bla*$_{\text{OXA-164}}$ (*bla*$_{\text{OXA-58}}$ variant)[155]. Carbapenem susceptibility testing was performed under standard testing conditions (Mueller-Hinton agar, pH 7.2; incubation at 37°C), as well as Mueller-Hinton agar supplemented with 16 mM sodium-salicylate, 200 mM NaCl and pH 5.8. The results are summarized in Table 6.

Table 6: The effect of different physiological conditions on carbapenem susceptibility in *A. baumannii* ATCC 17978 and ATCC 19606 and their transformants harboring pWH1266::bla_{OXA} recombinant plasmids. Significant MIC reductions (≥ 4-fold) under certain conditions are highlighted in bold and red color.

OXA (ATCC strain)	Imipenem MIC [mg/L]				Meropenem MIC [mg/L]			
	MHA	pH 5.8	NaCl	sodium-salicylate	MHA	pH 5.8	NaCl	sodium-salicylate
17978	0.25	0.5	0.25	0.25	1	2	0.5	1
19606	0.25	0.5	0.25	0.5	1	1	1	1
219 (51-like) (17978)	32	**8**	16	**8**	128	**32**	64	64
219 (51-like) (19606)	16	8	8	8	64	32	64	64
40 (17978)	128	64	64	64	512	**128**	**128**	**128**
40 (19606)	64	32	32	64	256	**64**	**64**	128
23 (17978)	32	32	32	32	256	**32**	**64**	**32**
23 (19606)	64	32	64	32	256	128	**64**	128
143 (17978)	128	64	64	**32**	>512	**256**	**256**	**256**
143 (19606)	64	64	32	64	512	256	256	**128**
58 (17978)	32	**8**	16	32	64	**8**	**8**	32
58 (19606)	64	**8**	32	**16**	64	**8**	32	32
164 (58-like) (17978)	32	**8**	16	16	32	**8**	**8**	16
164 (58-like) (19606)	32	**8**	16	16	32	**8**	**8**	**8**

MIC, minimal inhibitory concentration; MHA, Mueller-Hinton agar

Carbapenem susceptibility of the reference strains was not affected under the different physiological conditions (Table 6). Interestingly, the effect of the tested conditions on carbapenem susceptibility of OXA-23, OXA-40, OXA-143 and OXA-219 was dependent on the strain. For example, MICs of OXA-219 in ATCC 17978 were reduced under low pH (imipenem and meropenem) and sodium-salicylate (imipenem), whereas MICs in ATCC 19606 were unaffected.

Both OXA-40 transformants showed reduced meropenem susceptibility under low pH and presence of NaCl, while no change for imipenem was detected. Interestingly, all OXA-58-like transformants revealed a reduction of the carbapenem MICs under pH 5.8 (Table 6).

I presented preliminary data of this work as part of an oral presentation at the 21th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID)/27th International Congress of Chemotherapy (ICC) in Milan, Italy, in 2011.[156] Data of Table 6 were part of a poster at the 9th International Symposium on the Biology of *Acinetobacter* in Cologne, Germany, in 2013.[157]

3. Concluding discussion

Currently, carbapenems are considered as the antibiotics of choice to treat infections with MDR Gram-negative bacteria, including *A. baumannii*. Alarmingly, carbapenem-resistance rates in *A. baumannii* are rising and carbapenem-resistance determinants in non-*baumannii Acinetobacter* species are increasingly reported, which highlights the need for novel treatment strategies.[99, 135, 136, 158] The dissemination of carbapenem-hydrolysing β-lactamase genes is supported by their genetic alterability which is based on mutations and association with mobile genetic structures, like plasmids, transposons and IS elements.

The work presented in this thesis highlights the diversity of carbapenem-hydrolysing enzymes in the genus *Acinetobacter* and gives novel insights into their genetic context and dissemination. Table 7 and Table 8 summarize novel OXA variants and genetic compositions associated with carbapenem resistance in *Acinetobacter* spp., which were identified over a three-year period.

Table 7: Novel OXA variants identified in *Acinetobacter* spp.

OXA subclass	Variant*	Species	Reference
OXA-51-like	OXA-113a (nucleotide variant of OXA-113), OXA-200, OXA-201, OXA-202, OXA-219, OXA-223, OXA-241, OXA-242, OXA-387, OXA-391	*A. baumannii*	Higgins *et al.*[111, 159] accession no. OXA-387: KJ173478 accession no. OXA-391: KJ427797
OXA-23-like	OXA-225	*A. baumannii*	accession no.: JN638887
OXA-143-like	OXA-231, OXA-253, OXA-255	*A. baumannii* and *A. pittii*	Zander *et al.*[160]
OXA-235-like	OXA-235, OXA-236, OXA-237	*A. baumannii*	Higgins *et al.*[111]
OXA-228-like	OXA-257	*A. bereziniae*	Zander *et al.*[161]

*If not mentioned otherwise the variation is based on the amino acid sequence.

Table 8: Novel insights into the genetic composition of carbapenemase genes in *Acinetobacter* spp.

bla gene	Genetic context	Species	Reference
$bla_{\text{NDM-1}}$	composite transposon bracketed by IS*Aba125*; chromosomal location	*A. baumannii*	Pfeifer *et al*.[162]
$bla_{\text{OXA-23}}$	upstream inserted IS*Acra1*	*A. radioresistens*	Higgins *et al*.[163]
$bla_{\text{OXA-143-like}}$	$bla_{\text{OXA-231}}$: downstream located replicase gene $bla_{\text{OXA-253}}$: bracketed by XerC/D recombination sites; upstream located inner membrane protein gene $bla_{\text{OXA-255}}$: bracketed by TonB-dependent receptor gene and peptidase gene	*A. baumannii* and *A. pittii*	Zander *et al*.[160]
$bla_{\text{OXA-257}}$	upstream inserted IS*18*	*A. bereziniae*	Zander et al.[161]

bla, β-lactamase gene; IS, insertion sequence

These results underline the rapid evolution of carbapenem-resistance genes and their potential for transmission in the genus *Acinetobacter*.

3.1 IS*Aba125*-associated $bla_{\text{NDM-1}}$ in *Acinetobacter* spp.

Due to their potential to act against the whole class of β-lactams (with exception of monobactams), metallo-β-lactamases are of great concern in the clinic. Compared to CHDL, metallo-β-lactamases are rarely reported in *Acinetobacter* spp. Since the identification of NDM-1 in 2009, eight amino acid variants have been identified (NDM-1 to NDM-8), which mainly emerged in *E. coli*, *K. pneumoniae* and *P. aeruginosa* isolates with a wide distribution in India. However, NDM-1 and NDM-2 have also been detected in MDR *A. baumannii* from different parts of the world, including Algeria, China, Egypt, Germany, India, Israel and Oman.[164] Recently, NDM-1-producing MDR *A. pittii, A. johnsonnii* and *A. lwoffii* from China were reported, highlighting distribution of the subclass within the genus *Acinetobacter*.[165-167]

The NDM-1-producing *A. baumannii* 161/07 from Germany (see 2.1) was isolated in 2007 prior to the isolation of the *K. pneumonia* and *E. coli* strains the gene was first identified in.[119, 162] Although India is proposed to be the origin of NDM-1, *A. baumannii* 161/07 was isolated from a patient repatriated from Serbia without a connection to India, which also suggests the Balkan as a potential origin of NDM-1. In *A. baumannii* 161/07 *bla*$_{\text{NDM-1}}$ was encoded on a composite transposon, which was flanked by two copies of IS*Aba125* and had inserted into a chromosomal MFS symporter. The *trpF* and *groS-groL-insE* regions have been frequently detected in the genetic environment of *bla*$_{\text{NDM-1}}$ in different genera, suggesting the same genetic origin of these regions.[168] Recently Poirel *et al.* identified the same transposon in two *A. baumannii* isolates and assigned it as Tn*125*, which is proposed to be the major driving force for NDM distribution in *A. baumannii*.[169] In contrast to *A. baumannii* 167/07, the transposon had inserted into chromosomal genes of unknown function, which highlights the presence of multiple insertion sites for Tn*125* on the *A. baumannii* genome. Furthermore, almost identical transposons have been recently identified on plasmids in carbapenem-resistant *A. lwoffii*,[168] which suggests mobilization from the *A. baumannii* genome. The transposons in *A. lwoffii* were located next to an aminoglycoside resistance gene (*aphA6*). The same gene was also detected in *A. baumannii* 161/07, where it was flanked by IS*Aba125*. Interestingly, *aphA6* seems to be involved in the genesis of *bla*$_{\text{NDM-1}}$. Toleman *et al.* have recently shown that *bla*$_{\text{NDM-1}}$ is a chimeric gene composed of the first 19 bp of *aphA6* and a preexisting metallo-β-lactamase gene.[170] It was proposed that the in-frame fusion of both genes resulted in beneficial changes in gene expression and the structure of the enzyme.[170]

To date, NDM and other metallo-β-lactamase subclasses are still rarely detected in *A. baumannii* and might not emerge as a global threat for human health. However, the dissemination of NDM variants in MDR *Acinetobacter* spp. should be regarded with concern. As with other carbapenem-resistant *A. baumannii* isolates NDM-expressing isolates often retain only susceptibility to colistin or tigecycline, which highlights the need for new treatment strategies. Like CHDLs, metalloenzymes are stable against β-lactamase inhibitors, whereby a combination with β-lactam agents would be ineffective against NDM-producing bacteria. Currently, available agents that target metallo-β-lactamases are missing. However, putative inhibitors such as sulfur-containing compounds and dicarboxylates with zinc-binding properties have been recently reported, which highlights the potential for the development of novel treatment options.[171]

3.2 The diversity of OXA carbapenemases in *Acinetobacter* spp.

The genus *Acinetobacter* harbors a wide spectrum of carbapenem-hydrolysing OXA enzymes. This includes acquired and intrinsic OXA subclasses. Between 2008 and 2014 multiple intrinsic OXA subclasses have been identified in *Acinetobacter* species other than *A. baumannii*, which are summarized in Table 9.[93, 112, 105, 132, 138, 172]

Table 9: Intrinsic OXA subclasses and the respective number of variants identified in non-*baumannii* *Acinetobacter* species.

Intrinsic OXA subclass	*Acinetobacter* species	Number of amino acid variants
23	*A. radioresistens*	6
134a[#]	*A. lwoffii* and *A. schindleri*	10
211	*A. johnsonii*	7
214	*A. haemolyticus*	5
213	*A. calcoaceticus*, *A. pittii* and AGS `between 1 and 3´	27
228	*A. bereziniae* and *A. guillouiae*	10
266	*A. venetianus*	1
279	*A. parvus* and AGS 6	3
286	AGS 14TU/13BJ, AGS 16, AGS 17, Taxon 19[*], Taxon 21[*] and Taxon 22[*]	10
294	AGS 14BJ and Taxon 20[*]	4
296	Taxon 96[*]	1
299	*A. bouvetii*	1
308	*A. gerneri*	1

[#]OXA-134a is not identical to the OXA-23 variant OXA-134 in *A. radioresistens*; [*]putative novel species[105]; AGS, *Acinetobacter* genomic species

The intrinsic OXA-134a subclass from *A. lwoffii* (accession no.: HQ122933) is not related to the OXA-23 variant OXA-134 in *A. radioresistens* (accession no.: FJ195387) (see 2.3.1). The terms OXA-134 and OXA-134a, which are used in this thesis, correspond to the respective GenBank submissions. However, it should be noted that the number 134 was assigned on the basis of the *A. lwoffii* sequence (http://www.lahey.org/Studies/).

The diversity of naturally occurring OXA subclasses in *Acinetobacter* spp. may indicate a broad range of silent carbapenem-resistance determinants in the *Acinetobacter* genus, which remains to be confirmed. However, in *A. baumannii* IS-mediated overexpression of the intrinsic *bla*$_{\text{OXA-51-like}}$ is the most common carbapenem-resistance mechanism in the absence of acquired OXA. In addition, we have identified a similar carbapenem-resistance mechanism in *A. radioresistance* and *A. bereziniae*.[161, 163] This shows that carbapenem resistance due to IS-mediated overexpression of intrinsic *bla*$_{\text{OXA}}$ is not limited to *A. baumannii*. Moreover, the ability of IS elements to change their genetic location in addition to the variety of IS elements which have already been identified in *Acinetobacter* spp., indicates an almost unlimited potential for *bla*$_{\text{OXA}}$ overexpression. The insertion sites of a certain IS element upstream of a specific gene seem to be located at the same position. For example, IS*Aba1* usually inserts 9 bp upstream of the *ampC* start codon in *A. baumannii*.[173] Furthermore, sequences available at GenBank reveal that adjacent to *bla*$_{\text{OXA-51-like}}$ IS*Aba1* is usually located 7 bp upstream of the start codon. In *A. radioresistens* IS*Acra1* inserted into ATATAA, which was located 62 bp upstream of *bla*$_{\text{OXA-23}}$ (see 2.3.1).[163] In contrast *A. bereziniae* IS*18* inserted into TTT, which was located 37 bp upstream of the *bla*$_{\text{OXA-257}}$ start codon (see 2.3.2). To analyze the presence of the respective insertion sites adjacent to *bla*$_{\text{OXA-23-like}}$ in *A. radioresistens* and *bla*$_{\text{OXA-228-like}}$ in *A. bereziniae*, available sequences from each species at GenBank were aligned. This revealed the presence of the same insertion site for IS*Acra1* adjacent to *bla*$_{\text{OXA-23-like}}$ in *A. radioresistens* and for IS*18* adjacent to *bla*$_{\text{OXA-228-like}}$ in *A. bereziniae*, respectively. The location of the IS*Acra1* insertion site varied between 62 and 64 bp upstream of the OXA gene. It remains unknown, if these differences in the location of the insertion site significantly alter the promoter and thereby the effect on *bla*$_{\text{OXA-23-like}}$ expression.

As chromosomally encoded OXA subclasses are often species-specific, PCR amplification using subclass-specific primers is thought to be reliable for species identification. For example, the OXA-multiplex PCR described by Woodford *et al.* can aid in the identification of *A. baumannii*.[122] Furthermore, detection of OXA-134a-like has been proposed for *A. lwoffii* and *A. schindleri* identification.[174] However, presence of these intrinsic OXA in other *Acinetobacter* species indicates *bla*$_{\text{OXA}}$ mobilization, which seems to be associated with IS elements. For example, in *A. radioresistens bla*$_{\text{OXA-23-like}}$ is adjacent to an ATPase gene.

This gene is also commonly adjacent to acquired *bla*$_{\text{OXA-23-like}}$ (e.g. in Tn*2006*), whereby a former mobilization together with the intrinsic gene from *A. radioresistens* seems likely.[112] In addition to *A. radioresistens*, the intrinsic *bla*$_{\text{OXA-51-like}}$ from *A. baumannii* has been mobilized. Recently, Chen *et al.* detected presence of two IS*Aba1*-*bla*$_{\text{OXA-51-like}}$ copies in *A. baumannii*, one located on the chromosome and one located on a plasmid.[175] Similar to the *A. baumannii* chromosome, *yncA* was located adjacent to *bla*$_{\text{OXA-51-like}}$ on the plasmids. In addition, Lee *et al.* reported the emergence of plasmid-associated IS*Aba1*-*bla*$_{\text{OXA-51-like}}$-*yncA* in carbapenem-resistant *A. nosocomialis* and *Acinetobacter* genomic species `close to 13TU´, indicating dissemination of OXA-51-like in non-*baumannii Acinetobacter* species. The mobilization of intrinsic OXA from *A. radioresistens* and *A. baumannii* highlights the potential of other intrinsic OXA genes to be mobilized and disseminate outside of their parent species. Interestingly, the insertion sequence IS*18*, which mediated overexpression of *bla*$_{\text{OXA-257}}$ in *A. bereziniae* KH243, has already been detected in other *Acinetobacter* species. For example, in *A. baumannii* and *A. nosocomialis* IS*18* has been associated with *bla*$_{\text{OXA-58-like}}$ and the aminoglycoside-resistance gene *aac(6')-Ij*, respectively.[176, 177] Presence of IS*18* in these species highlights the existence of potential insertion sites for IS*18* transposition, whereby emergence of IS*18*-*bla*$_{\text{OXA-257}}$ in *A. nosocomialis* and *A. baumannii* in the foreseeable future seems likely. Based on these data, potential mobilization of intrinsic *bla*$_{\text{OXA}}$ in the genus *Acinetobacter* strongly suggests that detection of these genes should not be considered as an unambiguous method for species identification.[178]

In addition to OXA-23-like and OXA-51-like, four acquired subclasses of carbapenem-hydrolysing oxacillinases have been identified in *Acinetobacter* spp.; OXA-40-like, OXA-58-like, OXA-143-like and the novel OXA-235-like (see 2.4.2).[111] In contrast to OXA-51-like and OXA-23-like, the origin of other acquired OXA subclasses remains unknown. Acquired OXA are globally detected in carbapenem-resistant *A. baumannii*, with OXA-23-like being the most prevalent[67], and are increasingly reported in non-*baumannii Acinetobacter* spp. For example, subclasses OXA-23 and OXA-58 are widespread in carbapenem-resistant non-*baumannii Acinetobacter* spp. originating mainly from Asia and Europe, with a high distribution in *A. pittii* and *A. nosocomialis*.[130, 158, 179-181]

The recent detection of OXA-23 in *A. nosocomialis* from South Africa, as well as OXA-58 in *A. junii* from Australia indicate worldwide distribution of both subclasses in *Acinetobacter* species.[158, 182] To date, OXA-235-like has only been detected in *A. baumannii* from Mexico and the USA, which is based on the ten isolates the subclass was first identified in.[111]

Compared to OXA-23-like and OXA-58-like, subclasses OXA-40 and OXA-143 are less frequently reported in *Acinetobacter* spp. Regarding worldwide dissemination, OXA-40 variants are prevalent in carbapenem-resistant *Acinetobacter* spp. from Europe, Asia and South America, with a wide distribution in *A. baumannii* and *A. pittii*.[133, 183, 184] Interestingly, OXA-72 seems to be the most prevalent OXA-40 variant detected in both species.[133, 158, 184] Recent reports also indicate the emergence of the OXA-40 subclass in *A. baylyi*, *A. calcoaceticus*, *A. haemolyticus* and *A. junii*.[185-187] In contrast to the other acquired OXA subclasses in *Acinetobacter* spp., the presence of IS elements upstream of *bla*$_{\text{OXA-40-like}}$ and *bla*$_{\text{OXA-143-like}}$ is unusual. For example, since its first description in 2000, association of *bla*$_{\text{OXA-40-like}}$ with an IS element has only been detected once, while so far no IS element has been detected upstream of *bla*$_{\text{OXA-143-like}}$.[150, 160, 183] As explained before, IS elements can be involved in the mobilization of *bla*$_{\text{OXA}}$, which may explain why OXA-40 and OXA-143 subclasses are less frequent. Furthermore, IS elements can provide full-length or partial promoters (see 1.4). Partial promoter regions (e.g. a -35 region) can generate a hybrid promoter with the natural *bla*$_{\text{OXA}}$ promoter (e.g. a -10 region). As IS elements commonly confer *bla*$_{\text{OXA}}$ overexpression it seems obvious that promoters provided by IS elements are stronger than the natural *bla*$_{\text{OXA}}$ promoters. Interestingly, alignment of the three *bla*$_{\text{OXA-143-like}}$ predicted promoter regions with *bla*$_{\text{OXA-40-like}}$ sequences in *A. baumannii* and *A. haemolyticus* revealed high similarity (Figure 13). The associated *bla*$_{\text{OXA-40-like}}$ sequences shown in Figure 13 are based on six sequences which were available at GenBank. Compared to OXA-143-like, a nucleotide substitution was identified at position 28 upstream of the *bla*$_{\text{OXA-40-like}}$ start codons.

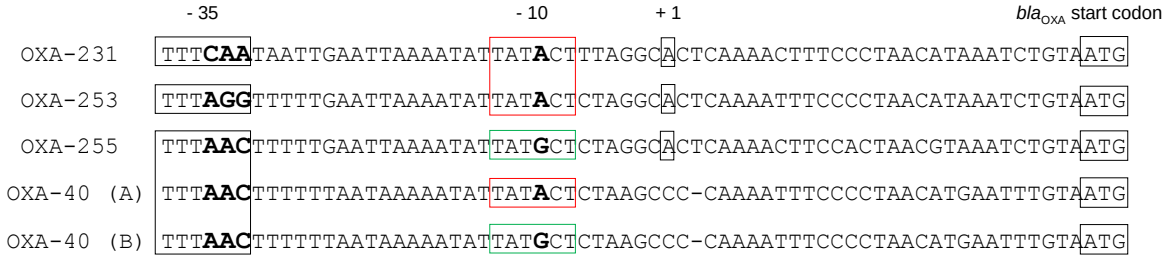

Figure 13: Alignment of predicted *bla*$_{\text{OXA-143-like}}$ promoter regions with sequences upstream of *bla*$_{\text{OXA-40-like}}$. The *bla*$_{\text{OXA}}$ start codon, *bla*$_{\text{OXA-143-like}}$ transcription initiation sites and predicted -35 and -10 regions are highlighted with boxes. (A) Sequence upstream of *bla*$_{\text{OXA-40}}$ in *A. baumannii* and *A. haemolyticus*; (B) Sequence upstream of *bla*$_{\text{OXA-40}}$ in *A. baumannii* (identical to sequences adjacent to *bla*$_{\text{OXA-72}}$ and *bla*$_{\text{OXA-160}}$); –, nucleotide deletion compared to sequences upstream of *bla*$_{\text{OXA-143-like}}$.

The promoter sequences that were predicted for *bla*$_{\text{OXA-143-like}}$ were also present upstream the OXA-40-like genes. The predicted -35 region from *bla*$_{\text{OXA-255}}$ was detected in all sequences (Figure 13). Furthermore, the predicted -10 region from *bla*$_{\text{OXA-231}}$ and *bla*$_{\text{OXA-253}}$ was present upstream of *bla*$_{\text{OXA-40}}$ in *A. baumannii* and *A. haemolyticus* (accession no.: AY228470, JN982951 and JN982952), while the predicted -10 region from *bla*$_{\text{OXA-255}}$ was present upstream of *bla*$_{\text{OXA-40}}$, *bla*$_{\text{OXA-72}}$ and *bla*$_{\text{OXA-160}}$ in three *A. baumannii* sequences (accession no.: GU199038, GU199039 and GU199040). In contrast to OXA-40-like and OXA-143-like, comparison of other *bla*$_{\text{OXA}}$ upstream regions in *A. baumannii* did not reveal such a similarity between two different subclasses. To date, transcription initiation sites of *bla*$_{\text{OXA-40-like}}$ have not been identified. However, as OXA-40-like and OXA-143-like are usually not associated with IS elements, the similarity between their upstream regions suggests the presence of stronger natural promoters compared to other acquired OXA subclasses in *Acinetobacter* spp.

3.3 The alterability of carbapenem susceptibility in *A. baumannii*

Previous studies have shown that physiological conditions, like low pH and presence of NaCl or sodium-salicylate can affect carbapenem-resistance determinants in *A. baumannii*.[116, 143, 147, 148] Based on these results one might speculate that because the physiological conditions at the site of an infection in the human body are variable, this might impact upon antimicrobial susceptibility. For example, in contrast to standard susceptibility testing methods, the pH in infected body fluids and tissues is often lower than pH 7.2.[147] However, our results indicate that the effect of the tested physiological conditions on carbapenem susceptibility in *A. baumannii* is mainly strain-dependent (see 2.4, Unpublished results II). With the exception of OXA-58-like transformants there was no consistent reduction of carbapenem MICs under pH 5.8, 200 mM NaCl or 16 mM sodium-salicylate. MICs were not reduced to the wildtype level, which suggests that the transformants retained some OXA activity. It should be noted that although some OXA enzymes are inhibited by NaCl, this does not seem to influence carbapenem susceptibility *in vivo*, which could be due to strain-dependent regulatory mechanisms. Therefore carbapenem MICs in the presence of NaCl is not a reliable method to detect OXAs. Interestingly, pH 5.8 reduced carbapenem susceptibility in all OXA-58-like transformants from a resistant level to a non-susceptible level. This might indicate a potential selective advantage for *A. baumannii* in expressing OXA-58 and OXA-164 under pH 5.8 in the presence of carbapenems, however this remains to be determined.

It has already been shown that carbapenem resistance can rapidly develop within the same strain. For example, we investigated a conversion of OXA-66 into OXA-82 in outbreak-related *A. baumannii* isolates, which was associated with the presence of IS*Aba1* and carbapenem resistance (see 2.2.3).[188] Interestingly, the intrinsic OXA-51-like from *A. baumannii* exhibits the largest number of amino acid variants within a single subclass. Since the first identification of OXA-51 in 2005, currently 106 variants have been identified (http://www.lahey.org/Studies/).[99, 189] The correlation between OXA-51 variants and international clonal lineages in *A. baumannii* indicates the evolution of an ancestral OXA-51-like within these lineages (see 2.2.2).

As mutations can randomly occur during DNA-replication, single *A. baumannii* cells within a population might not always harbor the same OXA-51-like. Regarding the diversity of OXA-51 variants within a single IC lineage, it could be proposed that some OXA-51 variants may be more beneficial for the bacterium than others. For example, in the absence of acquired OXA, carbapenem resistance seems to be associated with certain OXA-51 variants and the presence of IS*Aba1*. This might indicate that more ancestral OXA-51-like are not as efficient in carbapenem hydrolysis as their closely related variants, which remains to be confirmed by enzyme kinetics. Using an isogenic background we have shown that overexpression of OXA-82, OXA-109 or OXA-201 confers carbapenem resistance in *A. baumannii* ATCC 17978, while the OXA-66-expressing transformant remained susceptible (see 2.2, Unpublished results I). But if overexpressed OXA-82 and OXA-109 already confer carbapenem-resistance, the question comes up, what might be the advantage of a conversion into OXA-201? We have already shown that pH 5.8 can reduce carbapenem susceptibility in *A. baumannii* from a resistant to a non-susceptible phenotype. Therefore it might be possible that, compared to the OXA-201-expressing transformant, carbapenem susceptibility of OXA-82- or OXA-109-expressing transformants could be reduced under a physiological condition that we have not yet tested. Preliminary carbapenem susceptibility testing in the presence of 200 mM NaCl, 16 mM sodium-salicylate, pH 5.8 and incubation in the presence of CO_2 did not reveal significant differences between the OXA transformants with regards to their MICs versus carbapenems (data not shown). However, as carbapenems are not the sole substrates for CHDL it is also possible that the OXA-201-expressing transformant shows higher MICs in the presence of other β-lactam agents, which remains to be elucidated.

4. Summary

Acinetobacter baumannii is a hospital-acquired pathogen which causes global concern in health-care facilities, because this Gram-negative bacterium can easily thrive in the hospital environment and infections can be difficult to treat. Widespread multidrug-resistance (MDR) and the potential for epidemic spread contribute to the global success of *A. baumannii*. Currently the drugs of choice to treat infections with MDR *A. baumannii* are carbapenems but resistance against these β-lactams is rising, which dramatically reduces available treatment options.

The aim of this PhD project was to investigate carbapenem-resistance mechanisms in *A. baumannii* and non-*baumannii Acinetobacter* spp. This thesis analyses carbapenem-hydrolyzing β-lactamases in *Acinetobacter* spp. and gives novel insights into their dissemination and genetic context. In addition to the analysis of the metallo-β-lactamase NDM-1 in *A. baumannii*, the work was mainly focused on carbapenem-hydrolysing class D β-lactamases (CHDL), including intrinsic and acquired oxacillinases. IS elements were frequently associated with carbapenemase genes, involved in their overexpression, disruption and potential mobilization. Eighteen novel OXA genes, an OXA subclass (OXA-235-like) and an IS element (IS*Acra1*) were identified. Expansion of the OXA-multiplex PCR with the addition of an OXA-235-like primer pair provides an advanced method to monitor CHDL distribution in *Acinetobacter* spp. The results presented here demonstrate that using the OXA-multiplex PCR for OXA-51-like detection as the sole method to identify *A. baumannii* is not reliable.

OXA-51-like sequencing and rep-PCR-based DiversiLab revealed correlation of $bla_{\text{OXA-51}}$ variants with *A. baumannii* international clonal lineages IC1 to IC8. In the absence of acquired OXA, IS*Aba1* was associated with OXA-51 variants in carbapenem-resistant *A. baumannii*. In addition, outbreak-related *A. baumannii* isolates revealed a conversion of the intrinsic OXA-66 into OXA-82, which was associated with presence of IS*Aba1* and carbapenem resistance. These results suggested that it might be beneficial for *A. baumannii* to harbor one OXA-51 variant over another.

This question was investigated on the basis of an isogenic background using *A. baumannii* ATCC 17978 transformants overexpressing four closely related OXA-51-like of the IC2 lineage; OXA-66, OXA-82, OXA-109 or OXA-201, respectively. OXA-66 converts into OXA-201 by two amino acid substitutions (L167V and P130Q), with intermediates hypothesized to be either OXA-82 (L167V) or OXA-109 (P130Q). While IS*Aba1*-mediated overexpression of OXA-82, OXA-109 and OXA-201 conferred carbapenem resistance in ATCC 17978, the OXA-66-expressing transformant remained carbapenem-susceptible. This indicates that carbapenem resistance due to IS*Aba1*-mediated $bla_{\text{OXA-51-like}}$ in *A. baumannii* is dependent on the OXA-51 variant.

For the first time carbapenem resistance in *A. radioresistens* and *A. bereziniae* mediated through overexpression of their intrinsic bla_{OXA} associated with an IS element was observed. As IS-mediated overexpression of the intrinsic OXA-51-like is the most prevalent carbapenem-resistance mechanism in *A. baumannii* in the absence of acquired OXA, the potential of IS*Acra1*-mediated $bla_{\text{OXA-23}}$ overexpression and IS*18*-mediated $bla_{\text{OXA-257}}$ overexpression to spread in the genus *Acinetobacter* was discussed.

Novel insights into the dissemination and genetic composition of acquired OXA in *Acinetobacter* spp. were observed. For the first time OXA-23 was detected in *Acinetobacter* genomic species 14TU/13BJ and OXA-143, as well as co-expressed OXA-23 and OXA-58 were identified in *A. pittii* isolates, which highlighted the spread of acquired OXA in the genus *Acinetobacter*. In addition to Europe and Asia, detection of OXA-23 in non-*baumannii* *Acinetobacter* spp. from South Africa indicated its global dissemination. For the first time, emergence of OXA-143-like in *A. baumannii* and *A. pittii* outside of Brazil and South Korea was observed. 5´-RACE identified the transcription initiation site of $bla_{\text{OXA-143-like}}$ and allowed prediction of putative promoter regions. Alignment of sequences upstream of $bla_{\text{OXA-40-like}}$ revealed the presence of the same putative promoter regions, which is unusual between different OXA-subclasses.

Alterability of carbapenem susceptibility in *A. baumannii* was observed under different physiological conditions. The effect of NaCl and sodium-salicylate was mainly strain-dependent, while pH 5.8 indicated a potential selective advantage of OXA-23-, OXA-40- or OXA-143-expressing *A. baumannii* compared to OXA-58-like-expressing *A. baumannii*. In contrast to former publications these results revealed that carbapenem susceptibility testing in the presence and absence of 200 mM NaCl is not reliable for OXA detection in *A. baumannii*.

Taken together this thesis illustrates the diversity and variability of carbapenem-hydrolyzing β-lactamases in the genus *Acinetobacter*. The common association of IS elements with carbapenemase genes highlighted their influence on genome plasticity. Based on the presented data it is tempting to hypothesize that carbapenem resistance will further disseminate in the genus *Acinetobacter* in the foreseeable future.

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