Diversity of regulatory systems and their contribution to colistin and tigecycline resistance in *Acinetobacter baumannii*

Dissertation

zur

Erlangung des Doktorgrades (Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

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Bonn 2021

Angefertigt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Rheinischen Friedrich-Wilhelms-Universität Bonn

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Promotionsdatum: 14. Oktober 2021

Erscheinungsjahr: 2021

Teile dieser Arbeit wurden bereits veröffentlicht:

Gerson S, Nowak J, Zander E, Ertel J, Wen Y, Krut O, Siefert H, Higgins PG. Diversity of mutations in regulatory genes of resistance-nodulation-cell division efflux pumps in association with tigecycline resistance in *Acinetobacter baumannii*. J Antimicrob Chemother. 2018;73:1501–8. doi:10.1093/jac/dky083.

Gerson S, Betts JW, Lucaßen K, Nodari CS, Wille J, Josten M, Göttig S, Nowak J, Stefanik D, Roca I, Vila J, Cisneros JM, La Ragione R, Seifert H, Higgins PG. Investigation of Novel *pmrB* and *eptA* Mutations in Isogenic *Acinetobacter baumannii* Isolates Associated with Colistin Resistance and Increased Virulence In Vivo. Antimicrob Agents Chemother. 2019;63:e01586-18. doi:10.1128/AAC.01586-18.

Gerson S, Lucaßen K, Wille J, Nodari CS, Stefanik D, Nowak J, Wille T, Betts JW, Roca I, Vila J, Cisneros JM, Seifert H, Higgins PG. Diversity of amino acid substitutions in PmrCAB associated with colistin resistance in clinical *Acinetobacter baumannii* isolates. Int J Antimicrob Agents. 2020;55(3):105862. doi:10.1016/j.ijantimicag.2019.105862.

Danksagung

Ein großer Dank gilt meinem Doktorvater Prof. Dr. Harald Seifert für die Unterstützung und Betreuung während meiner Zeit in seinem Labor, die Beurteilung meiner Dissertation und die Möglichkeit meine Ergebnisse auf verschiedenen Konferenzen präsentieren zu dürfen.

Herzlichen Dank an Prof. Dr. Uwe Deppenmeier für die freundliche Übernahme des Zweitgutachtens und die Bereitstellung eines Plasmids.

A special thanks to Dr. Paul Higgins for all the help, being an excellent mentor and for constructive and challenging discussions. Thank you, for making this thesis possible!

Einen herzlichen Dank an alle Kollegen der AG Seifert/Higgins und des IMMIH, aber insbesondere an Kai Lucaßen, Dr. Carolina S. Nodari, Julia Wille, Dr. Esther Wohlfarth, Yvonne Stelzer und Alex Wunderlich für ihre stete Hilfsbereitschaft, neue Ideen und gute Gespräche sowie für ihre Unterstützung bei verschiedenen Experimenten und Veröffentlichungen.

I would like to thank Dr. Jonathan Betts for conducting the *in vivo* assays and the willingness to discuss my work.

Vielen Dank an Michaele Josten sowie das Team der *Mass Spectrometry Core Facility* um Dr. Marc Sylvester für die Unterstützung und die Durchführung der Massenspektrometrie.

Des Weiteren möchte ich mich bei allen Mitgliedern der DFG Forschergruppe FOR2251 für ihre Hilfe, interessante Meetings und großartige Gespräche bedanken, insbesondere aber auch Prof. Dr. Beate Averhoff für ein Plasmid und Dr. Stephan Göttig für *A. baumannii* Isolate.

Der größte Dank aber gilt meinen Freunden, meiner Familie und besonders meinen Eltern. Ich möchte mich von Herzen für ihre Liebe und ihren Glauben an mich, ihre immerwährende Unterstützung und aufbauende Worte bedanken. Ohne sie wäre diese Arbeit nicht möglich gewesen.

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Abbreviation index

AAS	Amino acid substitution
ABC	Adenosine-triphosphate (ATP)-binding cassette
Ade	Acinetobacter drug efflux
AFLP	High-resolution amplified fragment length polymorphism
APS	Ammonium persulfate
ARDRA	Amplified ribosomal DNA restriction analysis
ATP	Adenosine-triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CCA	Coliforms chromogenic agar
CDS	Coding sequences
CFU	Colony forming unit
cgMLST	Core genome multilocus sequence typing
CLSI	Clinical and Laboratory Standards Institute
EDTA	Ethylenediaminetetraacetic acid
ESBL	Extended spectrum β-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FA	Formaldehyde agarose
HAMP	Histidine kinase, adenylyl cyclase, methyl-accepting chemotaxis protein and phosphatase
HATPase_c	Histidine kinase-like ATPases
HGT	Horizontal gene transfer
HisKA	Histidine kinase A (phosphoacceptor) domain
HK	Histidine kinase
IC	International clones
ICU	Intensive care unit
IS	Insertion sequence
IS*Aba*	Insertion sequence *A. baumannii*
L-Ara4N	4-amino-4-deoxy-1-arabinose
LBA	Luria-Bertani Agar
LPS	Lipopolysaccharide
MALDI-TOF MS	Matrix-assisted laser desorption ionization-time of flight mass spectrometry
MATE	Multidrug and toxic compound extrusion
MDR	Multidrug-resistant
MFP	Membrane fusion protein
MFS	Major facilitator superfamily
MHA	Mueller-Hinton agar
MIC	Minimal inhibitory concentration
MLST	Multilocus sequence typing
MOPS	3-[N-morpholino]propanesulfonic acid
MRSA	Methicillin-resistant *Staphylococcus aureus*
NCBI	National Center for Biotechnology Information
OD	Optical density
OM(P)	Outer membrane (protein)
PACE	Proteobacterial antimicrobial compound efflux
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PDR	Pandrug-resistant
PEtN	Phosphoethanolamine
PFGE	Pulsed-field gel electrophoresis
qRT-PCR	Semi-quantitative real-time polymerase chain reaction
REC	Regulatory/receiver domain
rep-PCR	Repetitive extragenic palindromic sequence-based PCR
RND	resistance-nodulation-cell division
rpm	Rounds per minute
RR	Response regulator
RT	Room temperature
SBA	Sheep blood agar
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis
SMR	Small multidrug resistance
S.O.C.	Super optimal broth with catabolite repression
Spp.	species
SRA	Sequence read archive
ST	Sequence type
TAE	Tris-acetate EDTA
TCS	Two-component system
TEMED	Tetramethylethylenediamine
TMD	Transmembrane domain
VAP	Ventilator-associated pneumonia
VRE	Vancomycin-resistant enterococci
WGS	Whole genome sequencing

1. Introduction

In February 2017 a list of drug-resistant bacteria, the so-called “priority pathogens”, was published by the World Health Organisation [1]. These pathogens pose great threat to human health and the development of new antimicrobial drugs is of utmost importance to fight infections caused by these bacteria. Since the discovery of the first naturally occurring antibiotic, the β-lactam penicillin, by Alexander Fleming in 1928 [2], the discovery and development of novel antimicrobial agents has increased constantly over the last decades, providing the clinician with a wide range of treatment options. However, the frequency of bacteria which have evolved to survive exposure to antimicrobials has been on the rise ever since, diminishing the available treatment options of bacterial infections. The Clinical and Laboratory Standards Institute (CLSI) defines antimicrobial resistance as the ability of a bacterium to not be inhibited by the usually applied concentrations of the antimicrobial agent and specific microbial resistance mechanisms are likely [3]. *Staphylococcus aureus* was the first bacterium found to be resistant to penicillin in 1941 [4]. Since then, the number of resistant bacteria increased constantly. Nowadays, multidrug-resistant bacteria are common and even pandrug-resistant pathogens, which are resistant to all currently available antimicrobial agents, are emerging. A reason for this considerable increase of resistance is the remarkable ability of bacteria to acquire or upregulate resistance determinants [5]. Moreover, the spread of resistance determinants is relatively easy among a bacterial species, but also possible among other bacteria. The so-called “ESKAPE organisms” are a group of bacteria, which are most prevalent in hospitals and increasingly resistant to antimicrobial agents. These organisms include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* species [6]. The World Health Organisation declared carbapenem-resistant *A. baumannii* as “number one” on the priority list of pathogens [1], which indicates the persistence of this bacterium and its success as a globally distributed pathogen. But what is it, that makes this bacterium so dangerous for global health? This thesis focuses on antibiotic resistance mechanisms in *A. baumannii*, with emphasis on the antibiotics of last resort - tigecycline and colistin - and their potential effects on virulence and pathogenicity of the bacterium.

1.1. The genus *Acinetobacter*

Acinetobacter baumannii is the most prominent member of the genus *Acinetobacter*, which belongs to the subclass *γ-Proteobacteria* and *Moraxellaceae* family [7]. In general, bacteria of the genus *Acinetobacter* are defined as Gram-negative, strictly aerobic, mesophilic, oxidase-negative, catalase-positive, glucose non-fermenting, non-fastidious, indole- and nitrate-negative with a G + C content of 40-50% [7–9]. *Acinetobacter* spp. are coccoid to coccobacillary cells of about 1.5 µm in length (Figure 1.1 A) and are metabolically versatile [9].

The first *Acinetobacter* strain was isolated from a soil sample by Beijerinck in 1911 and designated "*Micrococcus calcoaceticus*", but was later re-named as *Acinetobacter calcoaceticus*, after Brisou and Prevot proposed the current genus name in 1954 [10, 11]. The name "*Acinetobacter*" originates from the Greek ακινετοσ (akinetos) which means non-motile. However, several studies observed motility of *Acinetobacter* species, specifically of *A. baumannii*. The bacterium has two different forms of motility, the surface-associated motility, when grown on semi-solid media (agarose) in the absence of light (Figure 1.1 B), and twitching motility with the help of type IV pili [12–14]. On solid blood agar plates, *A. baumannii* appears as greyish to white colonies with a smooth or mucoid form (Figure 1.1 C).

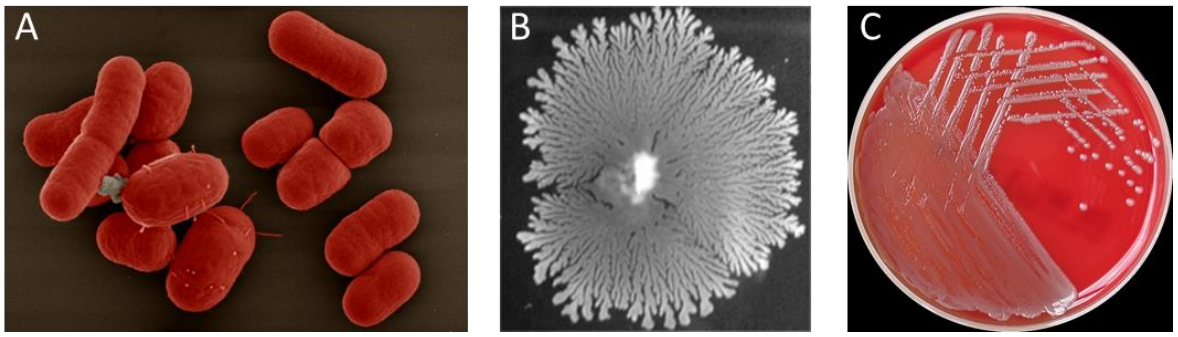


Figure 1.1: Morphological appearance of *A. baumannii*
(A) Scanning electron microscopy picture of *A. baumannii* cells. Taken from Holland and Laue, RKI [15]. (B) *A. baumannii* spreading over the surface of a semi-solid agar plate. Taken from Wilharm, RKI [16]. (C) Appearance of *A. baumannii* on a blood agar plate Taken from Jakubů [17].

At the time of writing, the genus *Acinetobacter* comprises 68 species, 6 of which carry provisional designations such as *Acinetobacter* genomic sp. 16 or *Acinetobacter* taxon 21 [18]. More familiar species include *A. baumannii*, *A. nosocomialis*, *A. calcoaceticus*, *A. pittii* and *A. seifertii*. *Acinetobacter* spp. can be found ubiquitously in nature and have been isolated e.g. from soil and water samples [19], plants such as rice, tobacco and lettuce [20–22] and from animals (e. g. horses and birds [23, 24]). Moreover, *Acinetobacter* spp. are commensal inhabitants of the human skin in about 43% of healthy individuals and in 75% of hospitalised patients [25]. *A. lwoffii* was found most frequently, followed by *A. johnsonii* and *A. junii* as part of the normal skin flora [25]. In 25% of healthy individuals *A. johnsonii* and *A. guillouiae* have also been isolated from faeces [26]. Although, *A. baumannii* has been detected on human skin

or in faeces [26, 27] and can be found as an environmental organism [8, 24], its natural habitat remains unknown. The majority of known *Acinetobacter* species are non-pathogenic environmental organisms, but for example *A. baumannii*, *A. nosocomialis* and *A. pittii* can cause serious infections in humans [9].

Members of the genus *Acinetobacter* share similar genetic and phenotypic properties, which often make them difficult to differentiate. For example, *A. baumannii*, *A. calcoaceticus*, *A. nosocomialis*, *A. pittii* and *A. seifertii* are closely related and often referred to as the "*A. calcoaceticus* - *A. baumannii* complex" [28–30]. There are several methods available for the identification of *Acinetobacter* species, but some are very time consuming and therefore not suitable for diagnostic laboratories. Those include DNA-DNA hybridisation, the phenotypic identification scheme by Bouvet and Grimont [31], amplified 16S rRNA gene restriction analysis (ARDRA) [32], and high-resolution amplified fragment length polymorphism (AFLP) [33]. In routine diagnostics, the semi-automated MALDI-TOF mass spectrometry is often used for species identification and is based on the generation of species-specific mass spectra, which are compared to online databases [34]. Another semi-automated system, the Vitek2™ system (bioMérieux), can be used both for species identification and antimicrobial susceptibility testing [35]. Due to the high phenotypical similarity, the Vitek2™ system is not able to differentiate between species of the *A. calcoaceticus* - *A. baumannii* complex. PCR-based methods are more simple and rapid methods for the identification of *Acinetobacter* species. The partial sequencing of two variable and species-specific regions of the RNA polymerase β-subunit *rpoB* [36] and the *gyrB* multiplex PCR, which uses species-specific primer amplifying a partial region of *gyrB* [37], are reliable and fast methods for *Acinetobacter* species identification.

1.2. *Acinetobacter baumannii* – a successful pathogen

A. baumannii is a serious nosocomial pathogen causing a wide range of infections. The most common clinical manifestations of *A. baumannii* are ventilator-associated pneumonia (VAP), bloodstream and urinary tract infections, skin and soft tissue infections and post-neurosurgical meningitis [8]. They occur most often in patients suffering from critical injuries and diseases in the intensive care unit (ICU) with indwelling devices or patients that have a compromised immune system. There are several other risk factors contributing to hospital-acquired *A. baumannii* infections, which include major surgery, trauma or burns, premature birth, mechanical ventilation, invasive procedures and foreign devices like catheters [38]. Moreover, community-acquired pneumonia has been reported in tropical regions like Asia and Australia, although these infections are still rare and often associated with comorbidities, like diabetes mellitus or with excessive alcohol consumption and heavy smoking [39].

A. baumannii is a very persistent pathogen and therefore of major concern for the healthcare system. The bacterium has a high desiccation tolerance with isolates reported to survive up

to 4 months on dry surfaces [40, 41]. Moreover, *A. baumannii* can form biofilms on abiotic surfaces including medical devices like mechanical ventilators and catheters (Figure 1.2) [42]. Biofilm forming *A. baumannii* isolates have been reported to survive twice as long on surfaces compared to other isolates and were involved mainly with device-associated infections [43, 44].

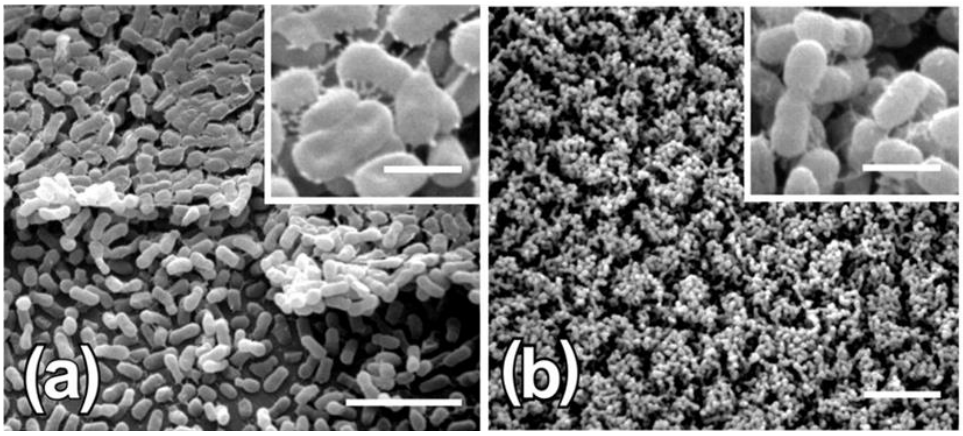


Figure 1.2: Biofilm formation by *A. baumannii*
Scanning electron microscopy pictures of *A. baumannii* ATCC 19606 cells forming a biofilm (a) above and (b) below the liquid-air interface. Taken from Tomaras *et al.* [45].

The clinical relevance of *A. baumannii* infections is still the subject of discussion [8], since high mortality rates could be a consequence of comorbidities and infections occur mainly in patients with severe underlying disease. Nevertheless, there are various virulence factors contributing to infections and persistence in the human host and hospital environment (Table 1.1) [14, 46, 47]. Several virulence factors are involved in bacterial attachment to surfaces (or epithelial cells), biofilm development and its maintenance. Loehfelm *et al.* identified a homologue of the staphylococcal biofilm-associated protein (Bap) in *A. baumannii*, which is a cell surface protein and directly involved in biofilm formation and maintaining mature biofilm architecture [48]. Further investigation of the Bap protein showed that it is required for adherence to human epithelial cells, which is an important step in host colonization and infection [49]. Moreover, the *csu*-operon (*csuA/BABCDE*) was described to be important in bacterial attachment and biofilm formation as well as pili production and assembly [45]. Furthermore, the polysaccharide poly-β-(1-6)-*N*-acetylglucosamine (PNAG), which is encoded by the operon *pgaABCD*, is necessary for the integrity and thickness of biofilms and may play a role in cell adherence [50]. In addition, the outer membrane protein OmpA is described to be a key virulence factor of *A. baumannii* [46, 47]. Although OmpA is not essential for biofilm formation, it is associated with thickness of biofilms on polystyrene, and attachment to a range of eukaryotic cells [47]. Another virulence factor are glycoconjugates, which are involved in the first defence against stresses (e.g. desiccation) and the host immune system [14, 46]. Important glycoconjugates include the capsular polysaccharide, which mediates

resistance to killing by the host complement system [51], glycosylated proteins and peptidoglycan as well as the lipopolysaccharide (LPS) and its anchor lipid A [14, 46]. To survive in the human host or hospital environment, *A. baumannii* possesses micronutrient acquisition systems, including high-affinity iron chelating molecules called siderophores (e.g. acinetobactin) [52], zinc chelating proteins and manganese import systems [14]. Moreover, *A. baumannii* possesses several protein secretion systems for the export of effector proteins, bacterial competition and adhesion [14, 46]. Further virulence factors that have been described in *A. baumannii* are listed in Table 1.1.

Table 1.1:Virulence factors of *A. baumannii*

Virulence factor	Feature	Reference
Biofilm-associated protein Bap	Biofilm formation, adherence to epithelial cells	[48, 49]
CsuA/BABCDE chaperone pili assembly system	Bacterial attachment, biofilm formation, pili production and assembly	[45]
Outer membrane protein OmpA-	Thickness of biofilms on polystyrene, attachment to eukaryotic cells	[46, 47]
pgaABCD operon	Integrity and thickness of biofilms, cell adhesion	[50]
Capsule	Resistance to complement-mediated killing	[51]
Glycoconjugates	Evasion of immune system, modification for resistance against antimicrobial agents	[14, 46, 51]
Micronutrient acquisition systems	Iron acquisition siderophores (e. g. acinetobactin), zinc chelating proteins (e. g. calprotectin), manganese import systems	[14, 52, 53]
Adhesion *Acinetobacter* autotransporter Ata	Adhesion to host extracellular matrices and basal membranes	[54]
Protein secretion systems	Adhesion, bacterial competition, export of effector proteins	[14, 46]
Phospholipases A, C and D	Host cell invasion	[55]
Penicillin-binding protein 7/8 (PBP-7/8)	Peptidoglycan biosynthesis, cellular stability	[56]
Outer membrane vesicles	Delivery of virulence factors, transfer of genetic material	[47]

Due to its long survival on dry surfaces and ability to form biofilms, the environmental contamination in hospital settings is a major problem for infection control. *A. baumannii* can be transmitted easily from patient to patient or from hospital personnel to patient, if it colonizes surfaces like the hospital bed, bed curtains, pillows and equipment (e.g. ventilators, infusion pumps, etc.) [57]. There have also been reports of *A. baumannii* contaminating the hands of healthcare workers, which promotes both the intra- and inter-hospital spread of the bacterium [58]. However, the spread is not restricted to the hospital, but can also occur

between healthcare settings within a city or even on a national and international scale, most commonly via the transfer of colonized patients [8].

Because of the easy spread and various transmission routes, outbreaks of *A. baumannii* infections are reported with increasing frequency [59]. Initially, three *A. baumannii* clones have been found to be predominant in outbreaks worldwide and were designated international clones (IC) 1-3 (formerly European clones I-III) [60]. Later, five further clonal lineages (IC4-8) were identified, indicating the rapid and concerning appearance of new epidemic clones [61]. Epidemic clones of *A. baumannii* were identified by multilocus sequence typing (MLST), repetitive extragenic palindromic sequence-based PCR (rep-PCR) and pulse-field gel electrophoresis (PFGE) [62–64]. Recently, a whole-genome sequence-based typing method has been developed, which defined the core genome of *A. baumannii* for the use in a core genome MLST (cgMLST) scheme and is highly discriminatory between clonal lineages and well-suited to investigate outbreaks [65]. The most widespread clonal lineage is IC2, which can be found worldwide. IC1 and IC3 isolates have been detected in Europe, South Africa, and the USA, while IC4 isolates were reported in Europe, India, South America, and the USA. Isolates belonging to the clonal lineages IC5, IC6, IC7 and IC8 can be found nearly exclusively in Europe and South America [61]. However, *A. baumannii* isolates not related to any of the known clonal lineages are detected worldwide indicating the potential for emergence of new epidemic clones [61].

1.2.1. Genome plasticity and horizontal gene transfer in *A. baumannii*

Generally, the genome of *A. baumannii* displays overall high consistency and only recently clonal expansion, e.g. into epidemic clonal lineages like IC2, is taking place, which is most likely associated with selective pressure caused by antimicrobial agents [9, 66]. Due to the high number of whole-genome sequence data for *A. baumannii*, it was possible to describe the core and accessory (or pan) genome of the bacterium. The core genome of *A. baumannii* was estimated to be about 2200 coding sequences (CDSs), which corresponds to 60% of the average genome content of all *A. baumannii* strains, and includes genes of metabolic and general cellular function and of hypothetical proteins [67]. The core genome is relatively stable and is also used for distinguishing between clonal lineages and to delineate outbreaks using a total of 2390 CDSs [65]. In contrast, the accessory genome of *A. baumannii* is very large with over 8800 CDSs and can undergo rapid evolution, which highlights the ability of *A. baumannii* to acquire genetic material including antimicrobial resistance determinants [9, 67]. There is evidence that the acquisition of antimicrobial resistance determinants is the main reason for intra-clonal diversity and the evolutionary success of the international clonal lineages of *A. baumannii* [66]. This is mainly due to the ability of *A. baumannii* to integrate genetic material coming from other organisms, either of the same species or of other genera. The process of horizontal gene transfer (HGT) includes bacterial transformation, transduction and conjugation, respectively [68]. Bacterial transformation describes the active uptake of free

exogenous DNA. Clinical *A. baumannii* isolates are often naturally competent and able to take up exogenous DNA in liquid cultures [69] and while moving on wet surfaces [70]. Taglia *et al*. showed that *A. baumannii* increased its natural transformation frequency upon the addition of Ca^{2+} or albumin and that natural transformation in *A. baumannii* is dependent on the pH of the environment [71]. This indicates an enhancement of natural competence in the human host and the danger of rapid and easy acquisition of antimicrobial resistance determinants. An example of an antimicrobial resistance gene transferred via natural transformation competence is the New Delhi metallo-β-lactamase 1 (NDM-1), which was first discovered in *Klebsiella pneumoniae* and was later transferred to *A. baumannii* isolates [72]. Another mechanism of HGT is bacterial conjugation, which is a transfer of DNA from one cell to another using a conjugation pilus [68]. Towner *et al.* reported that *A. baumannii* might share a common pool of intrinsic plasmids, which harbour a general system for plasmid mobilization and transfer, indicating the potential of *A. baumannii* for very flexible reorganization of its plasmidome [73]. Another reason for the high genetic diversity among *A. baumannii* clones is the presence of mobile genetic elements such as transposons, insertion sequences (IS), integrons and antimicrobial resistance islands. Resistance islands like AbaR1 or AbaR25, which carry diverse broad host-range mobile elements (transposons, integrons, IS-elements) and large gene cassettes, including several antimicrobial resistance determinants originating from *Acinetobacter* or other genera like *Pseudomonas* or *Escherichia*, promote the genetic exchange among different species and the uptake of resistance genes [74]. In contrast to the very large resistance islands (up to 120 kb), IS-elements are very small (< 2.5 kb) but abundant mobile genetic elements. They consist of a transposase gene flanked by two 15 bp short inverted repeats [75]. Moreover, during transposition and insertion processes, target site duplications of up to 9 bp are created on both sides of the IS-element, which are termed direct repeats [75]. Besides the transfer of genetic material, e.g. by bracketing a resistance gene, IS-elements are also involved in the insertional inactivation of genes like the outer membrane porin CarO [76]. Furthermore, IS-elements often harbour strong promoter sequences or provide the -35 region to generate hybrid promoters with -10 regions located on the bacterial chromosome to facilitate in the differential overexpression of genes [75, 77, 78]. IS*Aba1* is the most common IS-element in *A. baumannii* and is often associated with overexpression of antimicrobial resistance determinants like β-lactamases (e.g. bla_{AmpC}) [77, 78].

1.3. Antimicrobial resistance mechanisms in *A. baumannii*

Another reason for the success of *A. baumannii* as a global pathogen is its ability to up-regulate and acquire antimicrobial resistance determinants against multiple antimicrobial classes [79]. Multidrug-resistant (MDR) isolates, which are resistant to three or more antimicrobial classes, are frequently found in association with hospital-acquired infections. Even pandrug-resistant (PDR) strains, which are no longer susceptible to all available antibiotics, are emerging [80].

Antimicrobial agents either inhibit the growth of bacteria (bacteriostatic) or cause cell death (bactericidal). Target sites for antimicrobial agents include the bacterial cell wall synthesis, cell membrane, DNA and RNA synthesis, protein synthesis and the bacterial metabolism. According to their mode of action and their chemical properties, antimicrobials are grouped into different classes. The most important antimicrobial classes**,** which are active against Gram-negative bacteria, are aminoglycosides (e.g. amikacin and gentamicin), tetracyclines (e.g. tetracycline and minocycline) and glycylcyclines (tigecycline), macrolides (e.g. erythromycin and azithromycin), fluoroquinolones (e.g. ciprofloxacin and levofloxacin), ß-lactams (e.g. penicillin and ampicillin, cephalosporins like cefotaxime and cefepime and carbapenems like imipenem and meropenem), sulphonamides (e.g. sulfamethoxazole) and polymyxins (colistin, polymyxin B) [81]. Moreover, there are several antimicrobial agents, which are not grouped into any of the mentioned classes (e.g. chloramphenicol, rifampicin, trimethoprim) [81].
Over the last decades, *A. baumannii* has acquired diverse resistance mechanisms to overcome all known antimicrobial drug classes. In general, one differentiates between intrinsic (natural) and acquired resistance caused by the uptake of plasmids or transposons harbouring resistance determinants or by chromosomal mutations [82]. The major mechanisms of resistance in *A. baumannii* include reduced accumulation of the antimicrobial (decreased uptake, or increased efflux of the drug), inactivation of the antimicrobial by hydrolysis or modification and reduction of drug affinity by alteration or overproduction of the target [83]. An overview of the different antimicrobial resistance mechanisms in *A. baumannii* is depicted in Table 1.2.

Resistance to β-lactam antibiotics and carbapenems is mainly mediated by β-lactamases. The β-lactamases (*bla*) genes can be chromosomally or plasmid encoded and are found in large numbers in both Gram-positive and -negative bacteria. These enzymes hydrolyse the amide bond in the β-lactam ring and are classified according to their mode of action. They are often named after their drug target (e. g. carbapenemases, oxacillinase and cephalosporinases). Using the Ambler classification scheme, β-lactamases are grouped into four classes (A-D). Class A, C and D contain serine-β-lactamases, which need a serine residue in their active centre, and class B consists of metallo-β-lactamases using zinc ions in their active site [84]. *A. baumannii* encodes two intrinsic β-lactamases, a class C AmpC-type β-lactamase, which contributes to cephalosporin but not carbapenem resistance, and a class D β-lactamase of the OXA-51 subgroup (OXA-51-like), which can be associated with carbapenem resistance [85]. Both β-lactamases are expressed at low basal levels and can strongly affect the susceptibility to β-lactams/carbapenems only if associated with IS-elements (e.g. IS*Aba1*) upstream of bla_{AmpC} or $bla_{OXA\text{-}51}$ [78, 93].

Table 1.2: Overview of non efflux-mediated resistance mechanisms in *A. baumannii*

Antimicrobial class	Resistance determinant	Mechanism	Reference
β-lactams	β-lactamases (e. g. AmpC, OXA-51 and OXA-23)	hydrolysation of β-lactam ring	[83, 85]
	Porins (e. g. CarO)	reduced drug uptake	[76, 83, 85, 86]
	Penicillin-binding proteins (PBPs)	alternative target with lower binding affinity	[87]
Aminoglycosides	Phosphotransferases, acetyltransferases and adenyltransferases (e.g. aph(3')-*Ia*, AAC(6')*I-ad* and aadA1)	drug modification	[83]
	RNA-methylase ArmA	target site modification	[8, 83]
Fluoroquinolones	Specific mutations in *gyrAB* and *parCE*	target site modification	[83, 88]
	Pentapeptide repeat protein *qnr*-like genes	target site protection	[89, 90]
	Acetyltransferase aac(6')*Ib-cr*	drug modification	
Tetracyclines	Ribosomal protection proteins *tet(M), tet(O)*	target site protection	[83]
	Flavin-dependent monooxygenase *tet(X)*	drug modification	[91]
Rifampicin	Mutations in *rpoB*	target site modification	[83, 92]
	ADP-ribosylating transferase Arr-2	drug modification	
Chloramphenicol	Chloramphenicol acetyltransferases *cat*-genes	drug modification	
Macrolides	Phosphorylases *mph*-genes	drug modification	
Sulphonamides	Dihydropteroate synthases *sul1* and *sul2*	alternative target with lower binding affinity	
Trimethoprim	Dihydrofolate reductase *dfr*-genes	alternative target with lower binding affinity	

Beside the intrinsically encoded β-lactamases, *A. baumannii* can acquire a wide variety of β-lactamases, including narrow- and extended-spectrum (e.g. TEM-1, OXA-20, PER-1, KPC, CTX-M and VEB-1) as well as carbapenem-hydrolysing class D β-lactamases [83, 85]. The first carbapenem-hydrolysing β-lactamases discovered in *A. baumannii* were the group of OXA-23 and OXA-23-like enzymes, which are the most widespread acquired oxacillinases in *A. baumannii* [94]. Other important class D β-lactamases in *A. baumannii* include the group of OXA-40/OXA-24-like, OXA-58-like, OXA-143-like and OXA-235-like [83, 85, 95, 96]. Moreover, some *A. baumannii* isolates can harbour metallo-β-lactamases like IMP-like, VIM-like, SIM-like and NDM-like [72, 83, 85].

Besides hydrolysing enzymes, other non-enzymatic mechanisms contribute to β-lactam and specifically carbapenem resistance. While the modification and overproduction of penicillin-binding proteins (PBPs) has been reported only rarely, a more common mechanism is the reduced uptake of the drug through the outer membrane [85]. Due to their outer membrane, Gram-negatives are intrinsically resistant to some antimicrobial classes (e. g. glycopeptides like vancomycin and lincosamides like clindamycin), as the outer membrane forms a barrier preventing the drugs from entering the cell. Other drugs like e. g. carbapenems, tetracyclines and fluoroquinolones utilize non-specific outer membrane proteins (OMPs) (also called porins) for cell entry. However, the loss, reduction or replacement of porins in the outer membrane as well as alteration of porin function by specific mutations contribute to antimicrobial resistance [86]. Mussi *et al.* reported that the insertional inactivation of the porin CarO by IS*Aba125* is associated with imipenem and meropenem resistance in *A. baumannii* [76]. Moreover, the loss of the 33-36 kDa OMP was associated with carbapenem resistance and imipenem susceptibility could be modified by altering the amino acid composition of the porin OprD in *A. baumannii* [85].

Aminoglycoside resistance in *A. baumannii* is mainly mediated by drug-modifying enzymes like phosphotransferases (e.g. *aph(3')-Ia*), acetyltransferases (e.g. *aac(6')I-ad*) and adenyltransferases (e.g. *aadA1*) [83]. Besides modifying enzymes, *A. baumannii* utilizes a plasmid-mediated RNA-methyltransferase (ArmA), which methylates 16S rRNA, the target of aminoglycosides, thereby reducing the binding affinity of the drug [8, 83]. Another example for target modification is the mechanism leading to reduction in quinolone susceptibility. Specific mutations in the so-called Quinolone-Resistance-Determining-Region (QRDR) lower the affinity of the drugs to their target sites gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parA* and *parC).* An example is the amino acid substitutions S83L in GyrA [88]. Furthermore, plasmid-mediated quinolone resistance, which is based on target site protection by e.g. *qnrA* or enzymatic modification of the drug by aac(6')*Ib-cr*, has only rarely been detected in *A. baumannii* isolates [89, 90]. The antimicrobial agent rifampicin targets the bacterial RNA polymerase and blocks transcription. Resistance occurs by chromosomal mutations in the RNA-polymerase β-subunit gene *rpoB* or by enzymatic inactivation of rifampicin by the *arr-2* gene encoding an ADP-ribosylating transferase [83].

1.3.1. Multidrug efflux pumps

Besides the above-mentioned resistance mechanisms, *A. baumannii* intrinsically encodes several different efflux pumps, which have a wide variety of substrates including most antimicrobial classes (Table 1.3). The natural function of efflux pumps is suggested to be the transport of toxic environmental substrates (e.g. bile salts) or metabolic by-products of the bacterium rather than the efflux of antimicrobial agents [97, 98]. Moreover, efflux pumps work synergistically with the low permeability of the outer membrane to reduce uptake and accumulation of toxic compounds or antimicrobial agents [86, 99]. There are six different

families of efflux pumps, which differ in their substrate spectrum, structure, and energy source (Table 1.3, Figure 1.3). The adenosine-triphosphate (ATP)-binding cassette (ABC) superfamily of efflux pumps is characterised by their usage of ATP as energy source, while all other efflux pump families are drug-ion antiporters, i.e. they utilize H^+ or Na^+ ions as energy source (Figure 1.3) [97, 100]. All efflux pump families are located in the bacterial inner membrane, while only the RND-type efflux pumps also span the periplasmic space and outer membrane (Figure 1.3) [101].

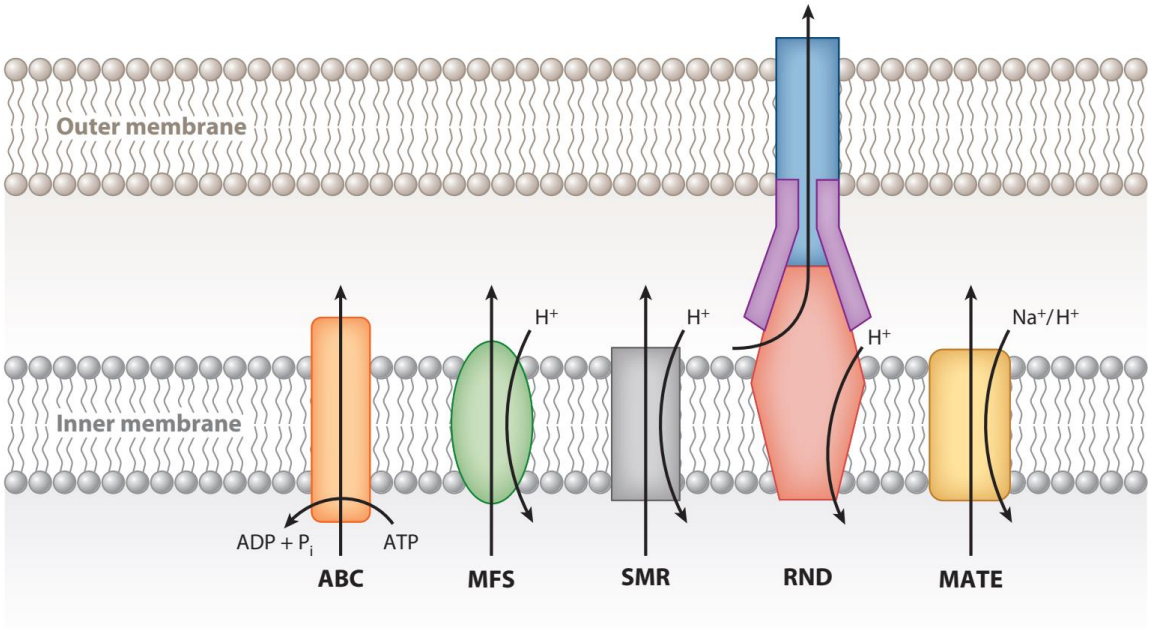


Figure 1.3: Efflux pumps in *A. baumannii*

Overview of the five bacterial efflux pumps families. The ATP-binding cassette superfamily (ABC), the major facilitator superfamily (MFS), the small multidrug resistance (SMR) superfamily and the multidrug and toxic compound extrusion (MATE) superfamily pump their substrates from the intracellular space over the inner membrane into the periplasmic space. The resistance-nodulation-cell division (RND) efflux pump is the only pump spanning also the outer membrane for export of substrates from the periplasm into the extracellular space. Except for the ABC superfamily, which utilizes ATP as energy source, all efflux pumps use an H^+-/Na^+-substrate antiport mechanism. Taken from Delmar *et al.* [101].

Efflux pumps of the ABC-type are mainly responsible for protein export, uptake of a wide range of molecules and efflux of toxic metal ions [102]. They are only rarely involved in the transport of antimicrobial substances and thus in the acquisition of resistance in Gram-negative bacteria [100]. Exceptions are ABC-type efflux pumps encoded by the *msr*-genes and the *macAB*, which have been described to be associated with macrolide resistance in *Pseudomonas* spp. and *Escherichia coli*, respectively [102, 103]. The MacB pump has also been described in *A. baumannii* [104].

Table 1.3: Overview of multidrug-efflux pumps in *A. baumannii*

Family	Efflux pump	Substrate	Reference
ABC	MacB	Macrolides	[104]
MATE	AbeM	Norfloxacin, ofloxacin, ciprofloxacin, gentamicin, kanamycin, erythromycin, chloramphenicol, trimethoprim, dyes, and detergents	[106]
MFS	CmlA	Phenicols	[74, 100]
	CraA	Chloramphenicol, thiamphenicol, florfenicol, dyes, disinfectants, and detergents	[107, 108]
	AmvA	Ciprofloxacin, erythromycin, minocycline, norfloxacin, novobiocin, dyes, disinfectants, and detergents	[109]
	ErmAB	Colistin	[110]
	Tet(A), Tet(B)	Tetracycline and minocycline	[100]
PACE	AceI	Synthetic biocides (e. g. chlorhexidine)	[105]
RND	AdeABC	Aminoglycosides, β-lactams, tetracyclines, tigecycline, fluoroquinolones, macrolides, chloramphenicol, trimethoprim, dyes, detergents, and biocides	[111, 112]
	AdeIJK	Chloramphenicol, β-lactams, tetracyclines, tigecycline, macrolides, fluoroquinolones, trimethoprim, rifampicin, fusidic acid, novobiocin, dyes, detergents, and biocides	[112, 113]
	AdeFGH	Chloramphenicol, fluoroquinolones, trimethoprim, tetracyclines, tigecycline, sulphonamides, detergents, and dyes	[112, 114]
	5 putative RND-type efflux pumps	Erythromycin, ertapenem, amikacin, gentamicin, rifampicin, tobramycin, ceftriaxone, ethidium bromide	[115–117]
SMR	AbeS	Erythromycin, novobiocin, amikacin, chloramphenicol, ciprofloxacin, norfloxacin, dyes and detergents	[118]

Furthermore, the *Acinetobacter* chlorhexidine efflux protein I (AceI) of the Proteobacterial Antimicrobial Compound Efflux (PACE) family was described to export only chlorhexidine and other synthetic biocides [105]. Therefore, members of the PACE-family are associated with biocide resistance rather than antimicrobial resistance in *A. baumannii* and other genera like *Pseudomonas* and *Escherichia* [105].

The major facilitator superfamily (MFS) is the largest and most substrate-diverse (e.g. amino acids, peptides, nucleotides, drugs, sugars and ions) family of efflux pumps in prokaryotes [119, 120]. MFS pumps usually are substrate-specific monomers, which consist of 12 transmembrane α-helices connected by hydrophilic loops and forming a pore spanning the inner membrane, and a cytoplasmic N- and C-terminus [119, 120]. Antimicrobial substrates are macrolides, aminoglycosides, tetracyclines and chloramphenicol [120]. One of the best studied examples of MFS pumps are the tetracycline efflux pumps Tet(A) and Tet(B). While

Tet(A) is able to export tetracycline, Tet(B) pumps out both tetracycline and minocycline and is far more prevalent in *A. baumannii* [100, 121]. Another example of an MFS pump is the chloramphenicol resistance *Acinetobacter* (CraA) efflux pump, a homologue of the MdfA efflux pump in *E. coli* [107]. CraA was suggested to be responsible for the intrinsic chloramphenicol resistance of *A. baumannii* as its sole substrate and only recently a broader substrate spectrum of CraA has been revealed (Table 1.3) [107, 108]. The chloramphenicol resistance gene (*cmlA*) also encodes for an MFS efflux pump, which confers resistance to chloramphenicol and was identified as part of the large resistance island AbaR1 in *A. baumannii* [74, 100]. A fourth MFS pump described in *A. baumannii* is the AmvA pump, which extrudes a multitude of different substrates including ciprofloxacin, erythromycin, minocycline, norfloxacin, and novobiocin as well as detergents, disinfectants, and dyes (Table 1.3) [109]. Although AmvA displays a wide spectrum of substrates, it contributes only marginally to antimicrobial resistance but significantly to resistance against disinfectants, detergents and dyes indicating the pump prefers biocides as substrate [109].

Efflux pumps belonging to the multidrug and toxic compound extrusion (MATE) family are the only efflux pumps utilizing not only the proton-motif-force but also sodium ions (Na^+) as energy source (Figure 1.3). Generally, MATE efflux pumps have a broad spectrum of substrates, but are incapable of extruding compounds carrying negative charges [122]. The structure of MATE efflux pumps is similar to that of MFS transporters as it consists of 12 transmembrane helices. They are organised in a “V” shape, with six transmembrane helices in each arm of the “V”, which separates the protein into an N- and a C-domain [122]. *A. baumannii* harbours the MATE efflux pump AbeM, which has been described as a proton-driven efflux pump and contributes to resistance against fluoroquinolones like ciprofloxacin, aminoglycosides, chloramphenicol, and erythromycin as well as various dyes and detergents [106].

Another example of a multidrug efflux pump in *A. baumannii* is AbeS, which belongs to the family of small multidrug resistance (SMR) efflux pumps. This group consists of small proteins, about 12 kDA in size and 100 to 140 amino acids in length, which span the membrane with four transmembrane regions forming hydrophilic loops [123]. Using the proton-motive-force, the SMR pumps are able to transport various quaternary ammonium and other lipophilic compounds including antimicrobial agents [123]. SMR efflux pumps are a very diverse family of transporters and confer resistance to β-lactams, cephalosporins, and aminoglycosides [123]. AbeS specifically contributes to resistance against erythromycin, novobiocin, aminoglycosides, fluoroquinolones, detergents, and dyes in *A. baumannii* [118].

The most important multidrug efflux pumps in bacteria belong to the resistance-nodulation-cell division (RND) family. These pumps function by an H^+-antiporter system and have a diverse physiological role in many bacteria. Besides an association with biofilm formation, surface

motility, virulence and pathogenicity (e.g. by exporting virulence factors like adhesins, toxins or proteins important for colonization and infection) [98, 124, 125], RND-type efflux pumps have also a very broad substrate specificity. Many classes of antimicrobials are substrates of the RND-type efflux pumps including tetracyclines, aminoglycosides, macrolides, chloramphenicol, rifampicin and fluoroquinolones, so that they contribute to multidrug resistance in many bacteria [112]. In addition, the RND-type efflux pumps can also extrude various biocides, detergents, antiseptics, heavy metals and dyes (e.g. ethidium bromide) [112]. Resistance to these substrates is associated with the overexpression of RND-type efflux pumps in Gram-negative bacteria like *E. coli* [126] and is mainly due to mutations in or disruption of regulatory genes (see also Chapter 1.3.2). However, overexpression of RND-type efflux pumps is an energy-consuming process and essential metabolites could be transported out of the cells, so that bacterial growth can be negatively affected [127]. Therefore, the expression of RND-pumps is usually tightly regulated by local and global regulators as well as two-component systems (TCS) (see also Chapter 1.3.4). One of the best studied RND-type efflux pumps are the AcrAB-TolC system in *E. coli* and the MexAB-OprM system in *P. aeruginosa* [128, 129]. AcrAB-TolC serves as a model of a bacterial RND-type efflux pump. Its structure and function are described in the following section.

RND-type efflux pumps are tripartite transporter systems, which consist of a transporter protein spanning the cytoplasmic membrane, a periplasmic membrane fusion protein (MFP), and an outer membrane protein (OMP) channelling substrates directly out of the bacterial cell (Figure 1.4 A) [130]. The complete system contains an AcrA hexamer as well as an AcrB and TolC trimer, respectively [130, 131]. Each monomer of the transporter protein AcrB consists of a large polypeptide chain with 12 transmembrane domains (TMD), which are separated by large periplasmic domains between helix 1 and 2 as well as helix 7 and 8 [130]. Deletion of one monomer in the trimeric complex leads to inactivation of the pump, which indicates an essential role of the trimer in the pumping mechanism [130, 132]. The MFP AcrA has an overall flexible and elongated shape anchoring the membrane-bound proteins AcrB and TolC. AcrA contains a β-barrel domain, a central lipoyl domain, an α-helix hairpin, which interacts with TolC, and a membrane-proximal domain, which contains the C- and N-terminal part of the protein and interacts with AcrB close to the cytoplasmic membrane (Figure 1.4 B) [131, 132]. Moreover, the AcrA hexamer forms a sealed complex around TolC and AcrB, preventing substrates from leaving the pump and its channel during the transport process [133]. The OMP TolC trimer forms a 12-stranded β-barrel, which spans the outer membrane forming a pore to channel substrates out of the cell, and an α-helical domain containing 12 α-helical hairpin structures reaching into the periplasm (Figure 1.4 C) [131, 132]. Each monomer of TolC provides four strands of the β-barrel [131].

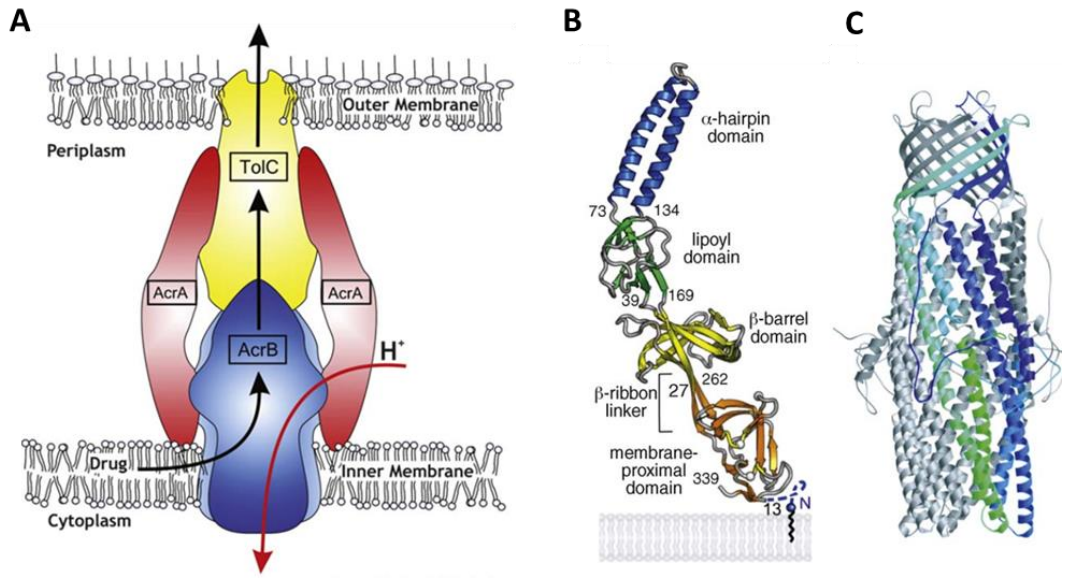


Figure 1.4: Schematic structure of RND-type efflux pump AcrAB-TolC in *E. coli* and protein structures of the MexAB-OprM pump in *P. aeruginosa*

(A) The AcrAB-TolC efflux pump is a tripartite system with the transporter protein AcrB spanning the inner membrane, the periplasmic membrane fusion protein (MFP) AcrA and the outer membrane protein (OMP) TolC, which forms a channel directly out of the cell. The pump utilizes the proton-motive-force for the substrate transport. (B) Protein structure of MexA containing four different domains with an overall flexible structure. (C) Protein structure of the OprM trimer forming a 12-stranded β-barrel pore in the outer membrane and α-helical hairpin structures in the periplasm. Taken from Blair and Piddock [132].

Furthermore, the small protein AcrZ (49 amino acids) has been identified as a binding partner of AcrB and seems to be involved in allosteric modulation and therefore substrate specificity of the pump [134]. The amino acid residues involved in AcrB-AcrZ binding are conserved, which suggests that binding partners like AcrZ might also exists for other RND-type efflux pumps as well [132]. It is unknown if an ArcZ-homologue is available in *A. baumannii*. The genes encoding an RND-type efflux pump are usually chromosomal and organized in a single operon, which often also includes the regulatory gene or TCS. However, the OMP can also be encoded elsewhere or missing, as it can also be recruited from other RND-efflux pump systems. An example is the MexXY system in *P. aeruginosa*, which recruits OprM encoded in the *mexAB-oprM* operon [98].

To understand the transport process of AcrB, it is necessary to further analyse the protein structure of the pump (Figure 1.5). The periplasmic domain of AcrB contains the TolC docking domain and the porter (pore) domain [135, 136]. The TolC docking domain forms a funnel-like structure with a central pore, which is located in the porter (pore) domain of AcrB. It consists of three α-helices and leads to a central cavity and further to a transmembrane hole formed by a ring-like arrangement of the TMD of AcrB [135, 136]. The central pore is very small and does not allow the passage of substrates, therefore a conformational change is necessary to transport substrates through the pore. A “three-step rotating mechanism” of AcrB has been

proposed by Murakami *et al.* and Seeger *et al*., which involves three different conformational states for each AcrB monomer: the access or loose (L) state, the binding or tight (T) state and the extrusion or open (O) state (Figure 1.6) [135–137]. In the access or loose state, the central cavity of the AcrB trimer is open to the periplasm and substrates can enter the pump. During the binding or tight state, a hydrophobic substrate binding pocket is created. The substrate can enter through an opening near the cytoplasmic membrane and travel through an uptake channel to the binding pocket.

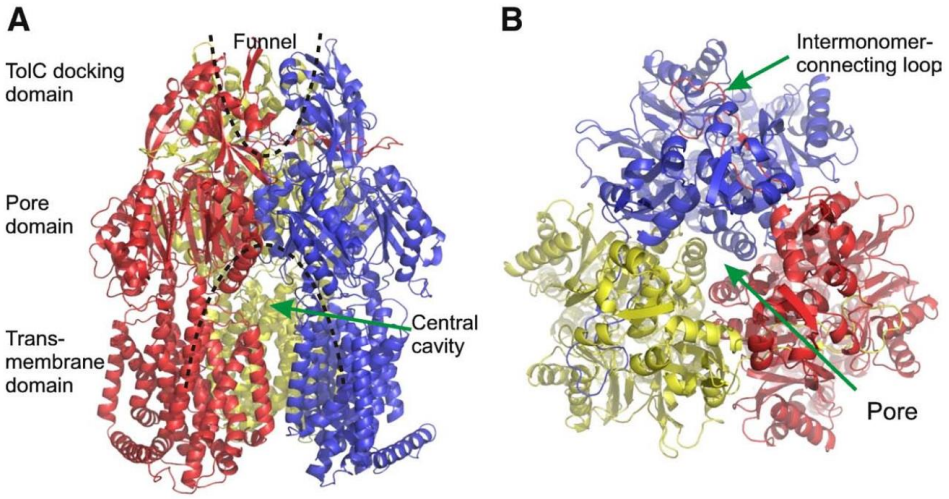


Figure 1.5: Structural overview of the AcrB trimer

(A) Each AcrB monomer consists of a transmembrane domain, a pore domain and a TolC docking domain. The TolC docking domain forms a funnel with a central pore located in the pore domain. The central pore leads to a central cavity and a transmembrane hole. (B) Central pore of the AcrB trimer. The monomers are connected by intermonomer-connecting loops. Taken from Pos [135].

In the extrusion or open state, the opening to the periplasm is closed and the exit for the substrate through the central cavity and pore to the funnel in the TolC docking domain is opened. Due to shrinkage of the binding pocket, the substrate is pushed out into the funnel towards TolC and the AcrB monomer returns to the access or loose state [135–137]. Conformational changes and the movement of the substrate is promoted by the proton-motive force and residues D407, D408, K940 in AcrB have been described to be essential for proton translocation [137]. Besides the rotation of AcrB, TolC has to be opened to channel the substrates out of the cell, which is mediated by AcrA [138]. AcrA recruits TolC and conformational changes in AcrA are suggested to stimulate TolC opening towards AcrB. Afterwards TolC remains in a constitutively open state to allow constant exit of substrates [138]. It is still controversial, if only AcrA interacts with TolC or if there is a direct connection between AcrB and TolC [131, 138].

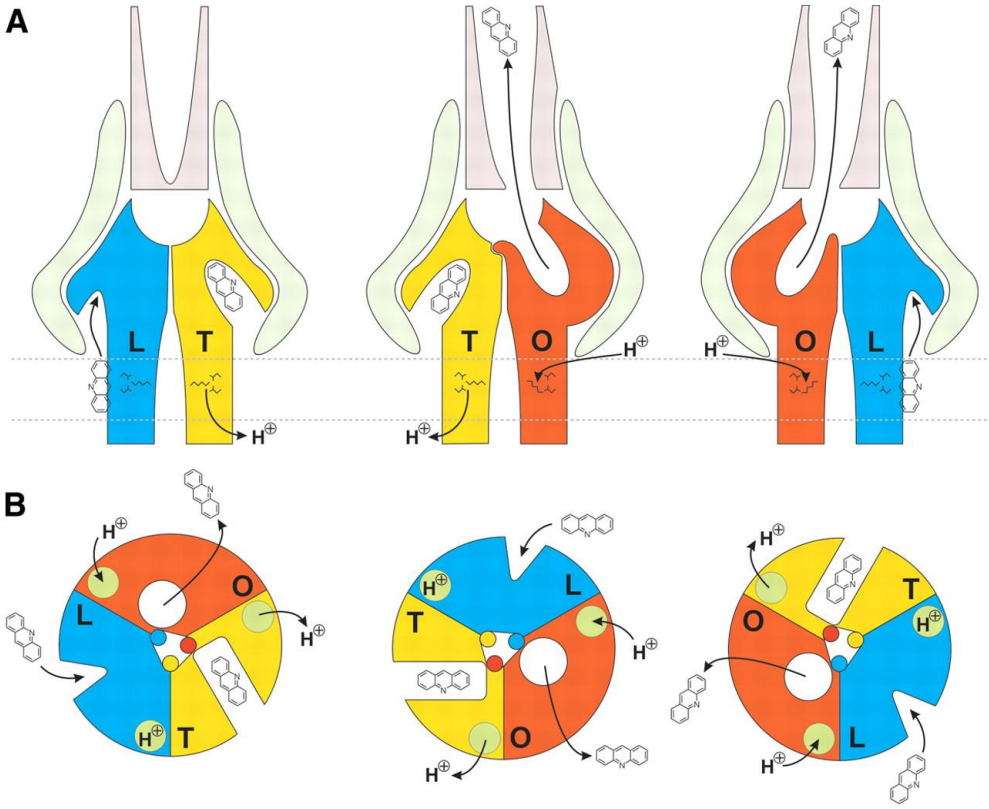


Figure 1.6: Schematic overview of AcrB conformations and its pumping mechanism
The three AcrB monomers each have a different conformational state during the rotational transport mechanism: loose (L, blue), tight (T, yellow) and open (O, red). During the loose state, substrates can enter the pump. In T conformation, a substrate binding pocket is created. In the open state, the opening to the periplasm is closed and the exit for the substrate through the central cavity is opened. The substrate is pushed out into the funnel and the AcrB monomer returns to the access or loose state. The conformational changes are promoted by the proton-motive force. Taken from Seeger *et al*. [136].

As many as eight different RND-type efflux pumps are encoded in *A. baumannii*. The best studied examples are the *Acinetobacter* drug efflux (Ade) pumps AdeABC, AdeIJK and AdeFGH [111, 113, 114]. Moreover, five as-yet uncharacterised RND-type efflux pumps have been described in *A. baumannii* [115]. Overall, RND-type efflux pumps are widely distributed in *A. baumannii*. Nowak *et al*. reported AdeJ, AdeG and three of the uncharacterised RND pumps were present in all tested *A. baumannii* isolates, while AdeB was detected in 97% of isolates [115].

The AdeABC system was first described by Magnet *et al*. as the first RND-efflux pump in *A. baumannii*, primarily in association with aminoglycoside resistance. However, AdeABC effluxes a broad range of substrates including hydrophobic, hydrophilic, amphiphilic, neutral, and positively charged molecules. Moreover, many antibiotics, dyes, detergents, and biocides are transported by AdeABC, e.g. aminoglycosides, tetracyclines, ethidium bromide, and chlorhexidine (Table 1.3) [111, 112]. The pump consists of the MFP AdeA, the transporter AdeB and the OMP AdeC. It has been shown, that AdeC is not essential for a multidrug resistant phenotype and can probably be recruited from other RND-type efflux

systems [139, 140]. The pump and its regulatory system are chromosomally encoded by the *adeRSABC* operon, whereby *adeRS* encodes the TCS AdeRS and is transcribed in reverse orientation [111, 139]. AdeRS consists of the sensor kinase AdeS and the response regulator AdeR and positively regulates the expression of *adeABC*. Inactivation of AdeRS has been shown to increase susceptibility in *A. baumannii* [139, 141], while mutations in *adeRS* have been described to result in *adeB* overexpression and an MDR phenotype [142, 143]. For example, Yoon *et al.* reported that the amino acid substitutions N125K, R152K, H189Y, I252S, and G336S in AdeS and P56S, L192R, and E219A in AdeR are associated with *adeABC* overexpression and multidrug resistance in *A. baumannii*, indicating the diversity of mutations in these regulatory genes [142].

The second RND-type efflux pump in *A. baumannii* is the AdeIJK pump, which consists of the MFP AdeI, the transpoter AdeJ and the OMP AdeK. The pump is encoded by the *adeIJK* operon and regulated by the TetR-like transcription regulator AdeN [113, 144]. AdeN functions as a transcriptional repressor, thereby negatively regulating *adeIJK* expression. The gene is located 813 kbp upstream of the *adeIJK* operon and while it is constitutively expressed, it does not regulate its own expression [144]. Generally, AdeIJK displays a similar substrate range as AdeABC, which includes chloramphenicol, fluoroquinolones, fusidic acid, macrolides, novobiocin, rifampicin, tetracyclines, tigecycline, and trimethoprim as well as biocides, detergents, and dyes, but not aminoglycosides (Table 1.3) [112, 113, 144]. Therefore, a synergistic effect of AdeIJK and AdeABC has been proposed [113]. This hypothesis has been further supported by Sugawara and Nikaido, who investigated the expression and substrate range of *adeIJK*, *adeABC* and *acrAB-tolC* in *E. coli* and revealed an overall similar substrate range for the three pumps, but efficiency and changes in minimal inhibitory concentrations (MICs) differed between the pumps [145]. Moreover, a reduction or inhibition of bacterial growth upon increased *adeIJK* expression was observed, suggesting that the overexpression of the pump is toxic for *A. baumannii* [113, 145]. A similar effect was described for *adeABC* overexpression in *E. coli* [145] and transcriptomics revealed the level of *adeIJK* overexpression always to be lower than that of *adeABC* [146]. This suggests that RND efflux pumps in general can be expressed only to a certain threshold, which might be lower for *adeIJK* compared to *adeABC* in *A. baumannii*. This moderate overexpression of *adeIJK* might also account for the low-level resistance observed in *A. baumannii* [113, 145].

The third RND-type efflux pump in *A. baumannii* consists of the MFP AdeF, the transporter AdeG and the OMP AdeH. The pump is encoded by the *adeFGH* operon, which is controlled by the LysR transcription regulator *adeL* encoded in opposite direction upstream of the operon [114]. It is suggested that AdeFGH does not contribute to intrinsic resistance in *A. baumannii* as it is not constitutively expressed in clinical isolates [114]. Nevertheless, the amino acid substitutions V139G in AdeL for example can lead to the overexpression of *adeFGH*, which is associated with an MDR phenotype [114]. Substrates of AdeFGH include chloramphenicol,

fluoroquinolones, sulphonamides, tetracyclines, tigecycline, trimethoprim, detergents and dyes, but not aminoglycosides or β-lactams (Table 1.3) [112, 114].

The other five RND-type efflux pumps are still largely uncharacterised and not much is known about their substrate spectrum. The five transporter proteins are between 1011 and 1216 amino acids in length and share 23 – 40% amino acid identity with AdeB [115]. Only in one case were genes encoding an MFP and OMP also detected adjacent to the RND transporter encoding gene, while no MFP and OMP, or only the MFP were found for the other four RND-type efflux pumps [115]. Moreover, a regulator has been found upstream of the genes encoding the RND transporter only for one RND-type pump [115]. This pump has also been further characterised by Tipton *et al*. and was designated ArpA (MFP, ACICU_RS18260), ArpB (transporter, ACICU_RS18255), and ArpR (TetR transcription regulator, ACICU_RS18265) [116]. ArpAB was shown to play a role in *A. baumannii* phase variation from opaque to translucent colonies, surface motility and susceptibility to aminoglycosides [116]. Furthermore, ArpR is, similar to AdeN, a transcriptional repressor and also involved in the switch from opaque to translucent colonies [116]. Another one of the putative RND-type efflux pumps lacking both MFP and OMP has been investigated by Srinivasan *et al*. and was designated AbeD (ACICU_RS14485) [117]. Possible substrates of AbeD are amikacin, ceftriaxone, ertapenem, erythromycin, gentamicin, rifampicin, tobramycin as well as ethidium bromide. Moreover, the pump seems to contribute to oxidative stress tolerance, motility, fitness and virulence in *A. baumannii* [117]. These studies indicate that RND-type efflux pumps might play other physiological roles in *A. baumannii* besides the extrusion of antimicrobial agents.

RND efflux pumps contribute strongly to an MDR phenotype in *A. baumannii*, but they always work in synergy with other resistance mechanisms as well as with reduced influx into the bacterial cell [86, 99, 147, 148]. Although β-lactam antibiotics have been shown to be a substrate of AdeABC and AdeIKL [111, 113, 145], the association of efflux with carbapenem resistance is still controversial in *A. baumannii*. While some studies described no correlation of the overexpression of RND efflux pumps with carbapenem resistance [148], others showed a drop in carbapenem MICs after exposure to an efflux pump inhibitor [149]. However, in most cases a synergistic effect of β-lactamases, alteration of outer membrane pores (reduced influx) as well as efflux seem to contribute to carbapenem resistance in *A. baumannii* [147, 150].

1.3.2. Tigecycline resistance mechanisms

Tigecycline (formerly GAR-936) belongs to the antibiotic class of glycylcyclines, which are structurally related to tetracycline antibiotics. After the discovery of GAR-936 by Petersen *et al*. in 1993 [151], it was approved for clinical use in 2005 as Tygacil® (Wyeth Pharmaceuticals) in treatments of complicated intra-abdominal infections as well as complicated skin and soft tissue infections [152]. Tigecycline displays only minor side effects like nausea and diarrhoea

and is active against a wide range of Gram-positive and Gram-negative, aerobic and anaerobic bacteria, including MDR pathogens like methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant enterococci (VRE) and ESBL-producing *Enterobacterales* [152]. Tigecycline enters the bacterial cell either by passive diffusion through the cell wall and cell membrane or by active transport [152]. Once in the cytosol, the molecule binds reversibly to the 30S subunit of the ribosome. It interferes with binding of the aminoacyl-tRNA to the ribosomal A site, which inhibits protein biosynthesis and thereby acts bacteriostatically [153, 154]. Tigecycline differs by the addition of a *t*-butylglyclamido side chain to the D ring of the tetracycline nucleus from other tetracyclines like minocycline (Figure 1.7). Due to its structural difference tigecycline attaches to its target site in the bacterial ribosome in a different orientation and with a five-time higher binding affinity compared to other tetracyclines [153]. Moreover, the large side chain provides several advantages including increased lipid solubility, steric hindrance for evasion of tetracycline efflux pumps Tet(A-E) and Tet(K) and increased affinity to overcome resistance mechanisms due to the ribosomal modification by Tet(M) and Tet(O) [152, 153]. Therefore, tigecycline can evade common tetracycline resistance mechanisms. In addition, it displays excellent tissue penetration and has minor side effects, so that it was considered to be a promising antimicrobial agent in treatment of complicated infections e.g. with carbapenem-resistant *A. baumannii* [152].

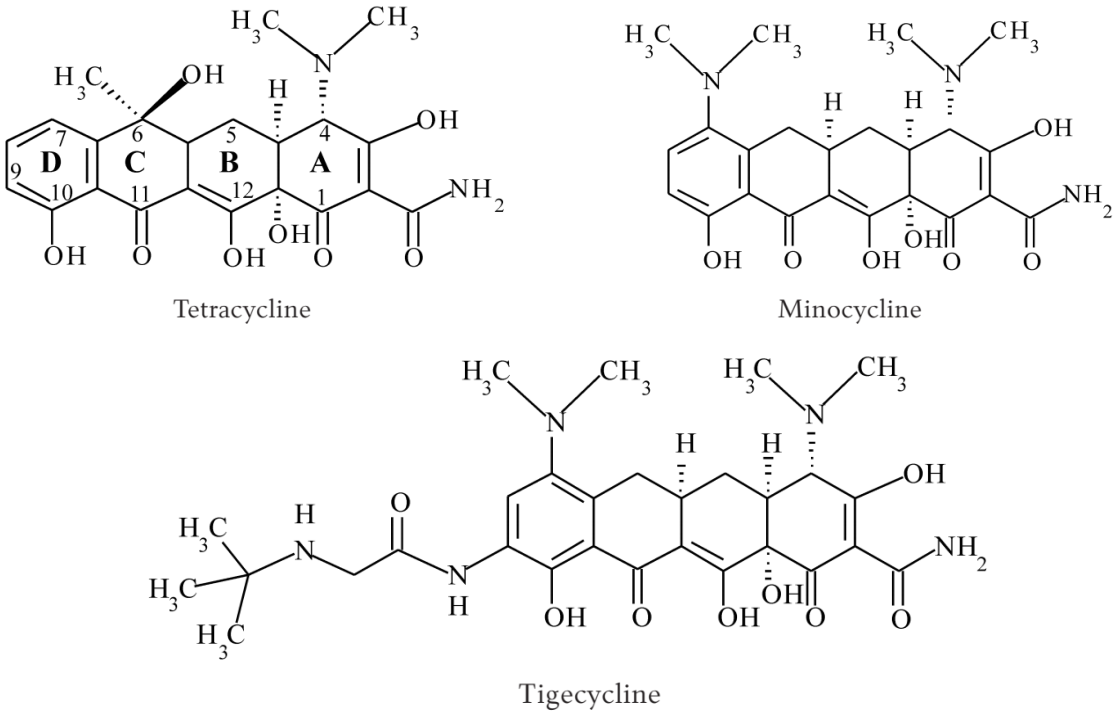


Figure 1.7: Chemical structure of tigecycline

Tigecycline differs from tetracycline or minocycline by the long *t*-butylglycylamido side chain at position 9 of the tetracycline nucleus D ring. Taken from Seputiene *et al.* [152].

However, tigecycline is prone to oxidation and therefore relatively unstable, which makes it difficult to administer and causes problems in susceptibility testing methods [155]. New formulations of tigecycline containing oxygen-reducing agents might counteract these problems in the clinical settings [156]. Moreover, tigecycline is not approved for the therapy of infections usually caused by *A. baumannii*, e.g. bloodstream infections or VAP, so that clinical data on tigecycline therapy is insufficient. In addition, *A. baumannii* displays variations in tigecycline MICs depending on the testing method used. For example, tigecycline MICs determined by Etest were found to be significantly higher compared to MICs determined by broth microdilution or automated systems such as Vitek2^{TM} [157]. Due to these difficulties, there are no breakpoints available for the definition of tigecycline susceptibility or resistance in *A. baumannii*.

A. baumannii has evolved a variety of mechanisms to overcome the action of tigecycline and resistant isolates are increasingly reported [158]. The main tigecycline resistance mechanism is the overexpression of RND-type efflux pumps [149]. Overexpression of RND-efflux pumps is often caused by mutations in the regulatory genes. For example, a mutation in *adeR* leading to the amino acid substitution D20N was shown to cause *adeABC* overexpression in a carbapenem-resistant *A. baumannii* isolate [143]. The D20N substitution was further characterized by Nowak *et al*. and revealed reduced susceptibility to six antimicrobial classes including tigecycline through *adeB* overexpression [159]. Several other amino acid substitutions in AdeRS have been described to be associated with *adeABC* overexpression and tigecycline resistance in both clinical and laboratory-derived *A. baumannii* isolates, which indicates the high genetic diversity of *adeRS* [139, 160, 161]. Furthermore, the insertion of IS*Aba1* in *adeS* was described to be associated with overexpression of *adeABC* and tigecycline resistance in *A. baumannii* [162–164]. Sun *et al.* suggested IS*Aba1* provides a strong promoter for the constitutive expression of a truncated *adeS*, which leads to overexpression of *adeABC* [163]. Moreover, the MFS-pump Tet(A) works in synergy with the RND-type efflux pumps AdeABC and AdeIJK and appears to be necessary for high level tigecycline resistance in *A. baumannii* [165].

Besides the overexpression of RND-type efflux pumps due to mutations in the regulators, several other tigecycline resistance mechanisms have been described. A frameshift mutation in *plsC*, encoding a 1-acyl-sn-glycerol-3-phosphate acyltransferase involved in phospholipid biosynthesis and cellular permeability has been described to lead to an up to 3-fold decrease in tigecycline susceptibility [166]. A mutation in this gene is suggested to decrease the membrane permeability for tigecycline, thereby reducing the bacterial susceptibility [166]. Chen *et al.* reported the association of a deletion mutation in the tigecycline-related-methyltransferase (*trm*) gene with tigecycline resistance in *A. baumannii* [167]. The gene codes for a S-adenosyl-L-methionine (SAM)-dependent methyltransferase and a mutation leading to a truncated protein was shown to increase tigecycline MICs [167]. Furthermore,

tigecycline is a substrate of the enzyme TetX, which is a flavin-dependent monooxygenase and modifies first and second generation tetracyclines [91]. Tigecycline modification leads to a weakened tigecycline substitute, which is not capable to efficiently bind its target thereby decreasing susceptibility [91]. In addition, several *tet(X)* variants have been reported (*tet(X1)* to *tet(X6)*) in Gram-negative pathogens including *Acinetobacter* spp., which can be plasmid-borne facilitating the easy spread among different bacterial species [168–170]. Finally, a frameshift mutation in *abrp* coding for a peptidase was associated with reduced susceptibility to chloramphenicol, doxycycline, fosfomycin, tigecycline and tetracycline, and is involved in changes of the membrane potential and permeability [171].

1.3.3. Resistance to polymyxin antimicrobials

Polymyxins are a group of polypeptide antibiotics, which are characterized by a heptapeptide ring consisting mainly of 2,4-diaminobutyric acid (Dab), and a fatty acid attached to the three amino acid side chain (Figure 1.8) [172]. Polymyxins are decapeptides, which are positively charged under physiological pH and harbour only two variable amino acid positions in the heptapeptide ring and one variable position in the peptide side chain. Differences in the amino acid content and fatty acid composition of the peptide affect the biological and antimicrobial activity [172]. Polymyxins are exclusively bactericidal against Gram-negative bacteria, but only polymyxin B and E (colistin) have been used therapeutically in clinical settings. Both polymyxins differ by one amino acid in the heptapeptide ring. While colistin harbours a D-leucine, polymyxin B has a D-phenylalanine in the sixth position of the amino acid chain (Figure 1.8). Colistin is non-ribosomally produced by *Paenibacillus polymyxa* (formerly *Bacillus polymyxa* var. *colistinus*) and was first isolated in 1949 [173]. It consists of a mixture of closely related components, the only difference being the fatty acid attached to the polypeptide. Colistin A and colistin B carry the fatty acids (S)-6-methyloctanoic acid and (S)-6-methylheptanoic acid, respectively (Figure 1.9) [174]. Two forms of colistin are available for clinical use, colistin sulfate and colistin methanesulfate, which differ in their potency and chemical structure. Moreover, colistin sulfate is administered orally and topically and is both more potent and toxic, while colistin methanesulfate is used predominantly intravenously and intramuscularly [175]. Recently, the CLSI and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) warned about problems in susceptibility testing using commercially available products as several errors in MICs were detected and bacteria were falsely categorized as colistin-resistant or susceptible [176]. Microbroth dilution is currently the only method recommended for colistin susceptibility testing [176].

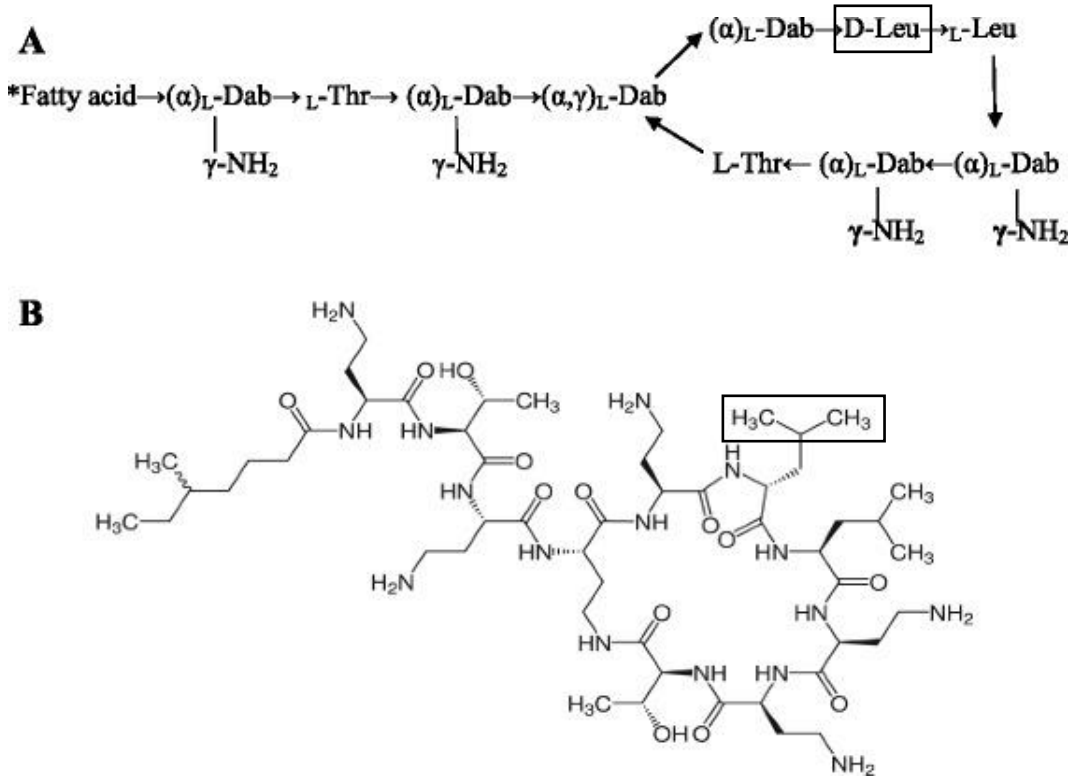


Figure 1.8: Chemical structure of colistin

(A) Descriptive structure of colistin. Fatty acid: (S)-6-methyloctanoic acid for colistin A, (S)-6-methylheptanoic acid for colistin B. Thr = threonine, Leu = leucine, Dab = α,γ-diaminobutyric acid. (B) Chemical structure of colistin. Marked by the black rectangle is the amino acid D-leucine, which is exchanged for D-phenylalanine in polymyxin B. Modified from Gurjar *et al.* [177].

Colistin has been extensively used as therapeutic agent for many years in Japan, the United States and Europe, but due to its high nephrotoxicity and the advent of various less toxic antimicrobial compounds with antipseudomonal activity, the clinical use of colistin was discontinued in the 1980s [175, 178]. However, the increasing frequency of MDR pathogens like *A. baumannii*, *K. pneumoniae* and *P. aeruginosa* and the paucity of new antimicrobial agents with activity against them led to the reintroduction of colistin as a therapeutic agent in the 1990s [175]. There is also some evidence that the reported nephrotoxicity of colistin is manageable with careful dosing and critical care service [179], so that the antimicrobial agent is now used as a last-line antibiotic for otherwise untreatable infections caused by MDR bacteria. Unfortunately, colistin resistance is increasingly reported among *A. baumannii* [180]. Another concerning fact is the emergence of heteroresistance in *A. baumannii* isolates, which describes the existence of colistin-resistant subpopulations within an otherwise colistin-susceptible bacterial population [181]. Heteroresistance occurs often after colistin treatment and contributes to the regrowth and development of colistin resistance [181].

The main targets of colistin are the bacterial cell envelope and specifically the lipopolysaccharide (LPS) of the outer membrane. The cell envelope of Gram-negative bacteria consists of an inner and outer membrane, and sandwiched between them is the periplasmic

space with a thin layer of peptidoglycan (Figure 1.9 A) [182]. The asymmetrical outer membrane forms an intrinsic barrier against numerous compounds and antibiotics [86, 183]. In general, its main components are phospholipids, different types of proteins such as lipoproteins and porins, and LPS, which consists of its hydrophobic anchor lipid A, a core oligosaccharide and a polysaccharide (O-antigen) [184]. LPS and specifically the O-antigen differ greatly among Gram-negative bacteria, and they contribute to the evasion of and resistance to components of the host immune system [185]. *A. baumannii* lacks the O-antigen and instead harbours an extended core oligosaccharide, which is comprised of an oligomer of sugars and two 3-deoxy-D-manno-oct-2-ulsonic acid (Kdo) residues [183]. Although the acetylation patterns of lipid A can differ among bacterial species, the lipid A of *A. baumannii* is similar to that found in *E. coli* and consists of two glucosamine molecules, which are phosphorylated at the 1' and 4' position and acetylated with 3-hydroxymyristic acids (Figure 1.9 B) [86, 183]. Both acetylation and phosphorylation of lipid A give the outer membrane an overall hydrophobic and anionic character. The major lipid A species found in *A. baumannii* is hepta-acetylated lipid A, which can be further modified by the addition of e.g. phosphoethanolamine (Figure 1.9 B) [186, 187]. The negative charge of the outer membrane provides a target for cationic antimicrobial peptides like colistin.

Colistin binds to LPS and specifically to lipid A by electrostatic interaction, displacing the divalent cations Mg^{2+} and Ca^{2+} [175]. These cations normally stabilize the LPS by linking phosphate groups of LPS together, and their displacement leads to the insertion of the fatty acid chain of colistin into the outer membrane [175, 182]. The integrity of the outer membrane is disrupted and colistin can enter the periplasmic space, where it causes the same pore formation in the inner membrane leading to membrane leakage, disruption of the proton motive force and subsequent cell death [183]. Due to high structural similarity, a similar mode of action and cross-resistance between colistin and polymyxin B has been reported [174].

Resistance to colistin can be either intrinsic (e.g. in *Brucella* spp., *Proteus* spp. and *Serratia* spp.) or acquired by the complete loss of LPS or by modification of lipid A [188]. *A. baumannii* is the only bacterium known to utilize the loss of LPS by inactivating lipid A biosynthesis as a resistance mechanism [183]. Usually, components of the LPS biosynthesis are essential for cell survival and the absence of these genes would cause cell death. *A. baumannii* has found a way to tolerate mutations in the first three genes (*lpxA*, *lpxC* and *lpxD*) of the lipid A biosynthesis pathway to use the loss of LPS as a resistance mechanism [189]. Inactivation of these genes can be either by mutations causing premature termination or by the insertion of IS-elements like IS*Aba11* [189, 190]. However, loss of LPS alters the physical and chemical characteristics of the cell envelope, which also affects the bacterial physiology and virulence [183].

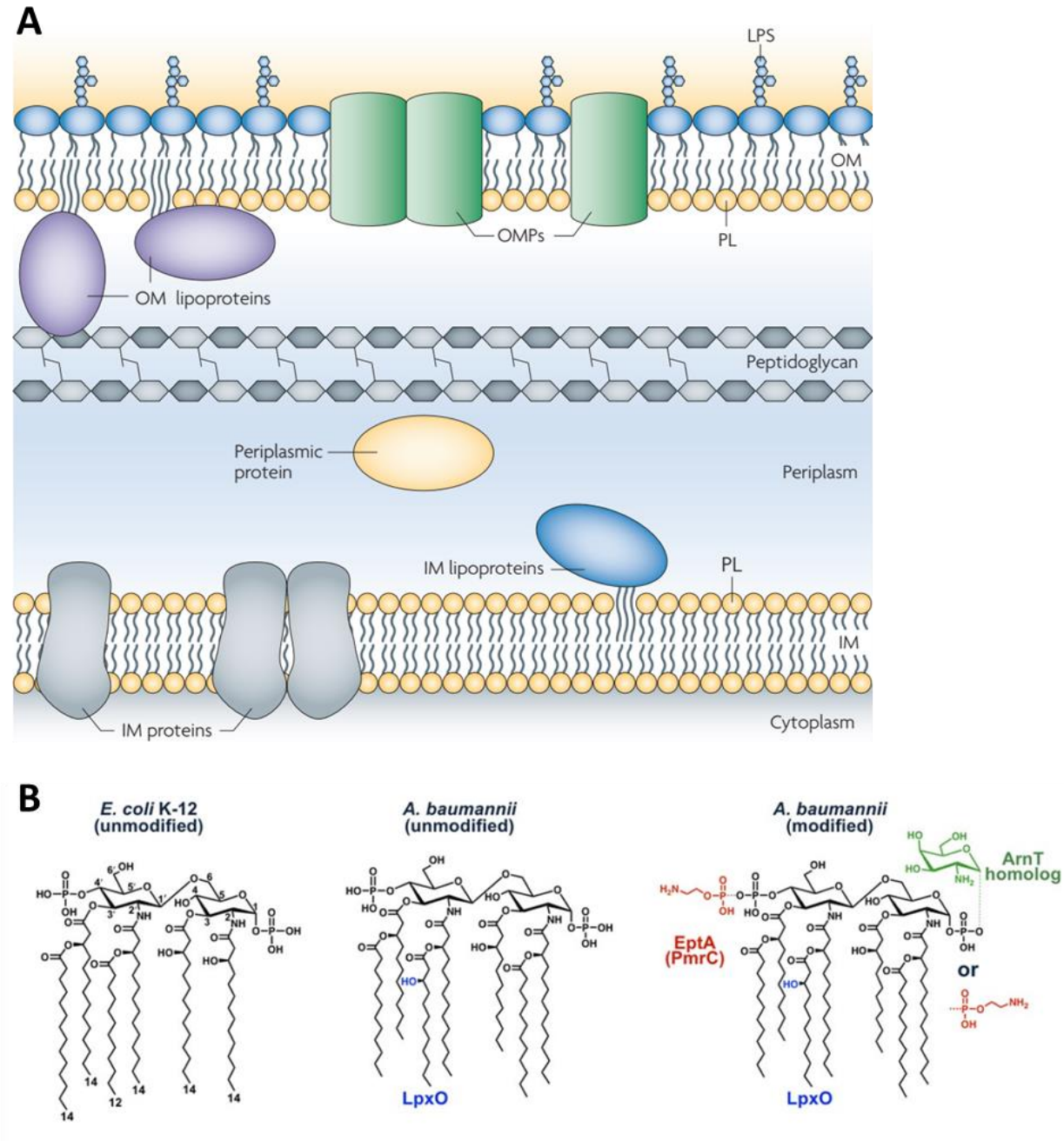


Figure 1.9: Schematic overview of the cell envelope of Gram-negative bacteria and the lipid A structures of *E. coli* and *A. baumannii*

(A) Gram-negative bacteria have a cell envelope made up of two membranes. The inner membrane (IM) is a phospholipid (PL) bilayer containing lipoproteins and integral proteins spanning the membrane (e.g. efflux pumps). The outer membrane (OM) forms an intrinsic barrier around the cell and is anchored to a thin peptidoglycan layer in the periplasm, which separates the two membranes. The OM consists of phospholipids and LPS as well as porins and lipoproteins. Taken from Ruiz *et al* . [182]. (B) The unmodified lipid A from *A. baumannii* is similar to that of *E. coli* with the exception of an additional acyl chain and hydroxyl group most likely due to a LpxO-homologue (blue). The hepta-acylated lipid A from *A. baumannii* can be modified in two positions by the addition of either phosphoethanolamine by EptA (PmrC) (red) or galactosamine by an ArnT-homologue (NaxD) (green). Taken from Powers and Trent [183].

Another resistance mechanism is the modification of lipid A by the addition of 4-amino-4-deoxy-1-arabinose (L-Ara4N), phosphoethanolamine (PEtN) or galactosamine (GalN) (Figure 1.9 B) [191, 192]. These modifications lower the net negative charge of the cell envelope, which affects the binding of the positively charged colistin to lipid A and prevents the disruption of the cell membrane [175, 193]. Lipid A modification as a mechanism of resistance is a complex process and has been extensively studied in many Gram-negative pathogens (reviewed in [193]). In general, mutations in the regulatory TCSs *phoPQ* and *pmrAB* lead to overexpression of genes responsible for lipid A modifications (e.g. *arnBCADTEF* for L-Ara4N addition and *pmrC* for PEtN addition) [193]. Moreover, some bacteria harbour additional regulators, which negatively regulate PhoPQ (e.g. MgrB) and mutations in these regulators can cause constitutive activation of PhoPQ [193]. *A. baumannii* is lacking the biosynthesis genes for L-Ara4N but it chromosomally encodes *pmrCAB*. The PEtN-transferase PmrC is responsible for PEtN addition to lipid A and is strictly regulated by the TCS PmrAB [191, 194, 195]. Several different amino acid substitutions have been described in PmrA and PmrB in association with colistin resistance in *A. baumannii* isolates [191, 194–196]. Moreover, *A. baumannii* often encodes one or more *pmrC*-homologues (termed *eptA*) on its chromosome, which are located near mobile elements (integrase or prophage) indicating their horizontal acquisition and might also contribute to colistin resistance [196]. Recently, Deveson Lucas *et al.* predicted the negative regulation of *eptA* by the global regulator H-NS [197]. The disruption of H-NS by IS*Aba125* has been shown to increase *eptA* expression leading to lipid A modification by PEtN addition and reduction of colistin susceptibility in *A. baumannii* [197]. Furthermore, the addition of galactosamine to lipid A was also reported to contribute to colistin resistance in *A. baumannii* [192]. This modification is mediated by the deacetylase NaxD, which is also controlled by the PmrAB TCS [198]. Although lipid A modification leads to high- and low-level colistin resistance in *A. baumannii*, the resistant phenotype has been shown to revert to its susceptible state upon colistin withdrawal, either by changing back to an unmutated *pmrAB* version or by compensatory mutations [199].

In 2015, a plasmid-mediated colistin resistance determinant named *mcr-1* was reported in *E. coli* originating from animal and human samples as well as food in China [200]. The gene *mcr-1* encodes for a phosphoethanolamine transferase, which mediates the addition of PEtN to lipid A, and was associated with an IS-element indicating easy and rapid spread of *mcr-1* [200]. Nowadays, several *mcr-1* homologues (*mcr-2* to *mcr-8.2*) have been observed worldwide in many Gram-negative pathogens including *K. pneumoniae* and *Salmonella* spp. [201, 202]. Only the *mcr-1* homologue *mcr-4.3* has recently been detected in *A. baumannii* [203]. Furthermore, the contribution of efflux pumps to colistin resistance has been suggested. The MFS pump ErmAB was described to be associated with reduced colistin susceptibility in *A. baumannii* and *in silico* interaction studies proposed colistin to be a substrate of RND-type efflux pumps [110, 204].

1.3.4. Regulation of resistance mechanisms – two-component systems

Two-component systems (TCSs) are common regulators in bacteria, which are usually involved in the adaptive response of bacteria to a wide range of environmental stresses, growth conditions and stimuli. Moreover, TCSs play a role in important processes like bacterial metabolism, cell development, motility and virulence [205, 206]. Since TCSs are found ubiquitously in bacteria, they are suggested as potential targets for the development of novel antimicrobial agents [207]. The TCSs consist of a membrane-bound histidine kinase (HK), which senses the external stimuli via its extracellular sensor domain, and a response regulator (RR), which modulates gene expression as response to the signal [208]. Both the HK and the RR are very versatile and adapted to the signal they are responding to. Conserved domains of HKs include the sensor (input) domain, which senses the external signal, and the transmitter domain, which transduces the signal to the RR by phosphorylation. The sensor domain is usually formed by an extracellular loop between two membrane-spanning domains [206, 209]. The transmitter domain contains a catalytic ATP-binding (CA or HATPase_c) domain and the dimerization and phosphorylation (DHp or HisKA-domain), which contains the histidine residue important for phosphorylation and signal transduction [206]. In some HKs, the histidine residue is located in a histidine phosphotransfer (HPt) domain, which is separated from the dimerization domain [206]. Moreover, many HKs also contain a histidine kinase, adenylyl cyclase, methyl-accepting chemotaxis protein, and phosphatase or short HAMP-domain, which serves as a linker region between the sensor and transmitter domains and is involved in conformational changes of the protein as well as mediating phosphorylation of the histidine residue [206, 210]. The RRs also carry conserved domains, namely the regulatory or receiver (REC) domain, which contains the aspartate residue for the phosphoryl transfer, and the effector or DNA-binding domain, which is very variable to ensure a wide range of responses [206]. Upon sensing an external stimulus, the CA domain of the HK binds ATP and phosphorylates the histidine residue in the HisKA-domain (Figure 1.10) [206, 208]. The phosphoryl group is subsequently transferred to the aspartate residue in the receiver domain of the RR, which causes a conformational change in the protein, affecting the DNA-binding affinity of the effector domain and modulating gene expression (Figure 1.10) [206, 208].

TCSs can be local or global regulators of gene expression and the wide variety of different responses ensures the adaptation of the bacterium in many different environments. This thesis focuses on the TCSs AdeRS and PmrAB, which are both local regulators of antimicrobial resistance mechanisms in *A. baumannii*. The external stimulus sensed by the HKs AdeS or PmrB is diverse and can include ions, pH, temperature, osmolarity, oxygen or the contact with host cells [205]. While PmrB is most likely sensing the presence of cations (Fe^{3+}, Mg^{2+} or Ca^{2+}) in the periplasm or the presence of cationic peptides like colistin, which has been described for the global TCS PhoPQ regulating PmrAB activity in *Salmonella enterica* [205], the signal activating AdeS is still unknown. Some suggest the substrate itself could be the external signal activating AdeRS, while others propose activation due to solutes in the periplasm or an

association with light exposure, the environmental temperature or iron content [141, 211]. Furthermore, the RR AdeR recognizes and binds a 10 bp direct-repeat (AAGTGTGGAGNAAGTGTGGAG) in the intercistronic region between *adeR* and *adeABC* to regulate gene expression [212, 213], while the RR PmrA binds to a so-called PmrA-Box, a conserved DNA motif (CTTAAG repeat) within the promoter of the target gene in *S. enterica* [214].

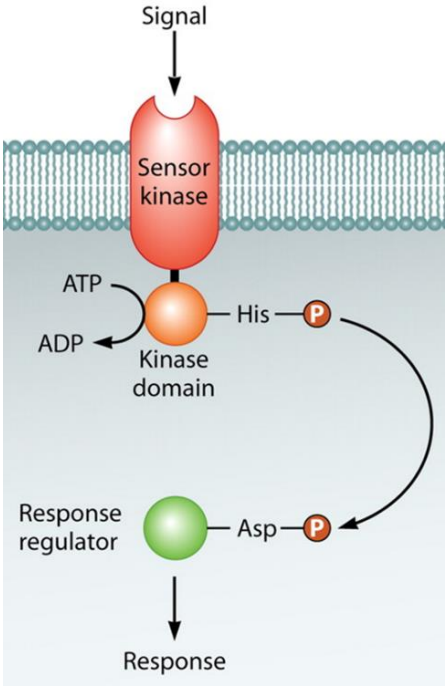


Figure 1.10: Signal transduction in two-component systems
Upon sensing the external stimulus, the conserved histidine residue of the sensor kinase is phosphorylated. The phosphoryl group is subsequently transferred to the receiver domain of the response regulator, which modulates gene expression. Taken from Bretl *et al*. [215].

The regulation of efflux pump expression can be quite complex in some bacteria. For example, the RND-type efflux pump AcrAB-TolC in *E. coli* is directly regulated by AcrR, which negatively regulates the expression of the pump [216]. However, AcrR seems to be only a secondary regulator, as global regulators like the *marRAB* operon, the TCS *SoxRS* and *rob* positively activate *acrAB* transcription [216, 217]. The activators MarA, SoxS and Rob are regulated by their repressors MarR and SoxR, respectively, and in addition also control the transcription of *micF* that down-regulates the expression of outer membrane channels contributing to reduced influx of antimicrobials [217]. Not much information is available on global regulators and their interaction with the regulatory genes of RND-efflux pumps (*adeRS*, *adeN* or *adeL*) in *A. baumannii*. Lin *et al*. reported the TCS BaeSR to positively regulate *adeABC* expression and contribute to tigecycline resistance, which might suggest BaeSR is a global regulator of AdeABC [218]. Moreover, the global regulator SoxR contributes to multidrug resistance in *Enterobacterales* and overexpression of *soxR* resulted in decreased expression of the efflux pumps *abeS*, *adeM*, *adeJ,* and *adeG* but not *adeB* and *craA* in *A. baumannii* [219].

Similar to RND-type efflux pumps, the regulation of colistin resistance can be very diverse and complicated in Gram-negative bacteria [193]. For example, PmrA regulates the expression of up to 20 different genes, which modify LPS or are important for cell homeostasis and metabolism, and further regulators are involved in the activation and regulation of PmrAB in *S. enterica* [214]. In *A. baumannii,* only PmrAB and the putative global regulator H-NS have been described to contribute to colistin resistance [197] and it remains to be elucidated, if PmrA regulates genes apart from *pmrC* in *A. baumannii*.

1.4. Aim of this study

This thesis focuses on tigecycline and colistin resistant clinical *A. baumannii* isolates. To elucidate the mechanism(s) of tigecycline and colistin resistance, the aim was to identify and characterise isogenic pairs of *A. baumannii* isolates, which would allow the analysis of resistance mechanisms in very similar genetic backgrounds. Whole-genome sequencing was used for the identification of resistance determinants and novel mutations associated with either tigecycline or colistin resistance. Moreover, whole-genome sequencing data allowed for the analysis of the resistome as well as the prevalence of mutations or insertion sequences in the isolate collection.

1) Tigecycline resistance

This thesis focuses on RND-type efflux pumps and especially the efflux pump regulators AdeRS, AdeN and AdeL. It was aimed to identify novel mutations in the regulatory genes by investigating their natural variance. The analysis of gene expression and susceptibility data revealed the association of the mutations with efflux pump expression and tigecycline resistance. Moreover, the aim was to further elucidate the role of insertion elements with efflux pump expression and tigecycline resistance as well as to determine their prevalence in *A. baumannii* isolates. Results were compared to susceptible isolates, *A. baumannii* reference strains, and *adeRS* and *adeN* knockout strains.

2) Colistin resistance

The aim was to investigate the mechanisms leading to colistin resistance in *A. baumannii* isolates by focusing on the *pmrCAB* operon and identifying novel mutations in *pmrB*. Their correlation with colistin susceptibility, *pmrC* expression, and lipid A modification was investigated and further analysed by the complementation of a *pmrAB* knockout strain and *A. baumannii* reference strains with mutated *pmrAB* variants. Moreover, it was aimed to further characterise the role of the *pmrC*-homologue *eptA* in colistin resistance and to investigate the prevalence of *pmrCAB* mutations in clinical isolates. The fitness and virulence of colistin-resistant isolates was investigated in an *in vivo* model.

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals and buffers

All chemicals and ready-to-use buffers were obtained from AppliChem (Darmstadt, Germany), Sigma-Aldrich and Merck (Darmstadt, Germany) or Carl Roth (Karlsruhe, Germany) unless stated otherwise or presented in Table 2.1. All self-made buffers were prepared using demineralized water and sterilized by autoclaving or filtration if necessary.

Table 2.1: Chemicals and buffers

Chemical/ Buffer	Manufacturer
5-Chloro-2-mercaptobenzothiazole (CMBT)	Sigma-Aldrich, Darmstadt, Germany
100 bp and 1 kb DNA ladder	New England BioLabs, Frankfurt, Germany
DNA Gel loading dye	New England Biolabs
Ethanol 96% absolute	Th. Geyer, Hamburg, Germany
RNA Protect Bacteria Reagent	Qiagen, Hilden, Germany
Midori Green Advanced	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
Phosphate buffered saline (PBS)	Biochrom GmbH, Berlin, Germany
BBL™ Mueller Hinton II Agar	BD Clonetech, Heidelberg, Germany
BBL™ Mueller Hinton II Broth	BD Clonetech
10x Tris/glycine/SDS (SDS-PAGE running buffer)	BioRad, Munich, Germany
1.5 M Tris-HCL, pH 8.8	BioRad
0.65 M Tris-HCL, pH 6.8	BioRad
Coliforms Chromogenic Agar (CCA)	Carl Roth, Karlsruhe, Germany
10x Tris/glycine/SDS (SDS-PAGE running buffer)	BioRad
1.5 M Tris-HCL, pH 8.8	BioRad
0.65 M Tris-HCL, pH 6.8	BioRad

2.1.2. Antimicrobial agents

Antimicrobial powder for determination of the minimal inhibitory concentration (MIC) and preparation of selective agar plates are listed in Table 2.2. Antimicrobial discs for disc diffusion assays were obtained from Oxoid (Wesel, Germany) (Table 2.3).

Table 2.2: Antimicrobial powder

Antimicrobial class	Antimicrobial	Abbreviation	Manufacturer
Aminoglycoside	Amikacin sulfate	AMK	Molekula, Newcastle upon Tyne, UK
	Gentamicin sulfate	GEN	Sigma-Aldrich, Steinheim, Germany
	Kanamycin	KAN	Molekula
Carbapenem	Meropenem	MEM	Molekula
Fluoroquinolone	Ciprofloxacin	CIP	Bayer Pharma AG, Berlin, Germany
	Levofloxacin	LEV	Sanofi Aventis, Frankfurt, Germany
Glycylcycline	Tigecycline	TGC	Molekula
Macrolide	Azithromycin	AZI	Pfizer Pharma GmbH, Münster, Germany
	Erythromycin	ERY	AppliChem, Darmstadt, Germany
Penicillin	Ampicillin sodium salt	AMP	Sigma-Aldrich and Molekula
Polymyxin	Colistin	COL	Molekula
Tetracycline	Minocycline hydrochloride	MIN	Molekula
	Tetracycline hydrochloride	TET	Sigma-Aldrich
Other	Chloramphenicol	CHL	Serva, Heidelberg, Germany
	Rifampicin	RIF	Molekula

Table 2.3: Antimicrobial discs

Antimicrobial disc (Abbreviation)	Concentration [µg]	Antimicrobial disc (Abbreviation)	Concentration [µg]
Amikacin (AK)	30	Ertapenem (ETP)	10
Ampicillin (AMP)	10	Gentamicin (CN)	10
Ampicillin/sulbactam (SAM)	20	Imipenem (IPM)	10
Cefepime (FEP)	30	Kanamycin (KAN)	1000
Cefepime/clavulanic acid (CPM+CV)	30+10	Meropenem (MEM)	10
Cefotaxime (CTX)	5	Moxifloxacin (MXF)	5
Cefotaxime/clavulanic acid (CTX+CV)	30+10	Piperacillin (PRL)	30
Ceftazidime (CAZ)	10	Piperacillin/tazobactam (TZP)	36
Ceftazidime/clavulanic acid (CAZ+CV)	30+10	Tobramycin (TOB)	10
Cefuroxime (CXM)	30	Trimethoprim/ sulfamethoxazole (SXT)	25
Ciprofloxacin (CIP)	5		

2.1.3. Consumables

All consumables used in this study are listed in Table 2.4.

Table 2.4: Consumables

Consumable	Manufacturer
96-well PCR plate white (DNA-, DNase- and RNase-free)	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
96-well plate (transparent U-shaped cell culture microplate)	TPP Techno Plastic Products AG, Trasadingen, Switzerland
Nucleon Delta Surface 96-well plate (black)	Thermo Fisher Scientific, Schwerte, Germany
Electroporation cuvettes, 0.2 cm gap	BioRad, Munich, Germany
Criterion cassettes, 18-well 1.0 mm	BioRad
Micronaut S colistin plates	Merlin Diganostika, Bornheim, Germany
Sterile filter Biosart® 100 Monitor (0.45 µm grey/white)	Sartorius AG, Göttingen, Germany

2.1.4. Equipment

All equipment used in this thesis is listed in Table 2.5.

Table 2.5: Equipment

Equipment	Manufacturer
BioPhotometer Spectrophotometer	Eppendorf, Hamburg, Germany
Bruker Biflex III MALDI-TOF mass spectrometer	Bruker Daltonics, Bremen, Germany
Disc Dispenser	Oxoid, Wesel, Germany
Gel Doc XR+ System	BioRad, Munich, Germany
Gene Pulser II electroporation system	BioRad
LightCycler® 480	Roche, Mannheim, Germany
MiSeq Sequencer	Illumina GmbH, Munich, Germany
Multipoint inoculator	Mast Laboratories, Liverpool, United Kingdom
NanoDrop 2000 Spectrophotometer	Thermo Fisher Scientific, Schwerte, Germany
Plate Reader Infinite M1000	Tecan, Crailsheim, Germany
Power Supply Power Pac 300	BioRad
Qubit®2-0 Fluorometer	Invitrogen, Thermo Fisher Scientific, Schwerte, Germany
Speed Vac™ SPD111V centrifuge	Savant™, Thermo Fisher Scientific
Thermal cycler C1000 Touch®	BioRad
Thermal cycler MyCycler™	BioRad
Thermal cycler Professional Trio Biometra	Analytik Jena, Jena, Germany
Thermomixer comfort	Eppendorf
VITEK DensiCheck	bioMérieux, Nürtingen, Germany
Criterion™ vertical electrophoresis chamber	BioRad

2.1.5. Culture media

Commercially available sheep blood agar (SBA) and Mueller-Hinton agar (MHA) plates were obtained from Oxoid (Wesel, Germany). S.O.C. (Super optimal broth with catabolite repression) medium for transformation of *E. coli* was obtained from ClonTech, Takara Bio Europe SAS (Saint-Germain-en-Laye, France) or Sigma-Aldrich (Steinheim, Germany). Self-made culture media and agar were prepared in demineralised water and are listed in Table 2.6. Except for the Coliforms Chromogenic Agar, all media were autoclaved for 20 min at 120°C and 100 kPa.

Table 2.6: Self-made culture media and agar

Self-made culture media and agar	Abbreviation	Recipe (1 L)
Luria-Bertani Agar	LBA	10 g tryptone 10 g NaCl 5 g yeast extract 16 g agar
Luria-Bertani Broth	LB	10 g tryptone 10 g NaCl 5 g yeast extract
Mueller-Hinton Agar	MHA	38 g BBL™ Mueller Hinton II agar (BD Clonetech, Heidelberg, Germany)
Mueller-Hinton Broth	MHB	22 g BBL™ cation-adjusted Mueller Hinton II broth (BD Clonetech, Heidelberg, Germany)
Coliforms Chromogenic Agar	CCA	26.4 g CCA (Carl Roth, Karlsruhe, Germany)

2.1.6. Master Mix, enzymes, and kits

In Table 2.7 all enzymes, kits and master mix used in this study are presented.

Table 2.7: Enzymes, Master Mix and Kits

	Name	Manufacturer
Enzyme	Antarctic phosphatase	New England BioLabs, Frankfurt, Germany
	Endonucleases (PstI-HF, ScaI-HF)	New England BioLabs
	Lysozyme	Sigma-Aldrich, Steinheim, Germany
	T4 polynucleotide kinase	New England BioLabs
	Quick Ligase	New England BioLabs,
Master Mix	Q5® High-Fidelity 2x Master Mix	New England BioLabs,
	2xTaq PCR Master Mix	Qiagen, Hilden, Germany
Kits	In-Fusion® HD EcoDry™ Cloning Kit	ClonTech, Takara Bio Europe SAS, Saint-Germain-en-Laye, France
	RNeasy Mini Kit	Qiagen
	RNase-Free DNase Set	Qiagen
	QIAquick PCR Purification Kit	Qiagen
	MagAttract HMW DNA Kit	Qiagen
	QIAquick Gel Extraction Kit	Qiagen
	QIAprep Spin Miniprep Kit	Qiagen
	QuantiTect Reverse Transcription Kit	Qiagen
	QuantiFast SYBR Green PCR Kit	Qiagen
	NucleoSpin Extract II PCR clean-up Kit	Macherey-Nagel, Düren, Germany
	Nextera XT Library Prep Kit	Illumina GmbH, Munich, Germany
	Qubit dsDNA BR assay	Thermo Fisher Scientific GmbH, Schwerte, Germany
	MiSeq Reagent Kit v2	Illumina GmbH
	MiSeq Reagent Kit v3	Illumina GmbH

2.1.7. Bacterial reference strains and *A. baumannii* clinical isolates

2.1.7.1. Bacterial strains and competent cells

In Table 2.8 all bacterial strains as well as the commercially available competent cells used for cloning and transformation experiments are listed.

Table 2.8: Bacterial reference strains and competent cells

Bacterium	Strain	Experiment	Manufacturer/ Reference
E. coli	ATCC 25922	Reference strain	[3]
	NEB5α	chemically competent cells	New England BioLabs, Frankfurt, Germany
	Stellar™	chemically competent cells	Clontech, Takara Bio Europe SAS, Saint-Germain-en-Laye, France
	K12 HB101 pRK2013	Helper strain for triparental conjugation, harbouring the plasmid pRK2013	Kindly provided by Prof. Dr. Uwe Deppenmeier (University of Bonn, Germany)
Staphylococcus aureus	ATCC 29213	Reference strain	[3]
Pseudomonas aeruginosa	ATCC 27853	Reference strain	[3]
A. baumannii	ACICU	Reference strain	[220]
	AYE	Reference strain	[221]
	ATCC 19606	Reference strain; wild type strain for gene knockouts	[31, 222]
	ATCC 17978	Reference strain	[11]
	Δ*adeN*	Gene knockout of *adeN*	This thesis
	Δ*pmrAB*	Gene knockout of *pmrAB*	
	Δ*adeRS*	Gene knockout of *adeRS*	Kindly provided by Kai Lucaßen (University of Cologne) [223]

2.1.7.2. Clinical isolates and isolate pairs

Clinical A*. baumannii* isolates were obtained from the MagicBullet clinical study [27, 224], which was carried out between 2012 and 2015 collecting samples from 15 hospitals in Greece, Italy and Spain. The isolates were recovered from respiratory tract or stool samples obtained from patients with VAP. Furthermore, two *A. baumannii* isolates were obtained from a wound and a respiratory tract sample originating from a patient hospitalized in Frankfurt am Main, Germany, who had suffered a stroke. Sixty-five of the *A. baumannii* isolates were considered the first respiratory isolate per patient and have already been described by Nowak *et al.* [27], while 12 isolates were additionally chosen for investigation in so-called isolate pairs. In Table 2.9 all clinical *A. baumannii* isolates are listed.

Table 2.9: Clinical *A. baumannii* isolates

The *A. baumannii* isolates were either obtained from patients in Greece, Italy, and Spain during the MagicBullet clinical trial or from patients in Frankfurt am Main, Germany. Listed are the patient ID, the sample type, hospital, and country as well as the date of isolation. MB, MagicBullet, R, respiratory tract sample, S, stool sample, W, wound swab.

Isolate	Patient ID	Sample type	Hospital	City, country	Date of isolation
MB-R-1	01-001	R	Hospital Virgen del Rocío	Seville, Spain	10.05.2012
MB-R-2	01-001	R	Hospital Virgen del Rocío	Seville, Spain	13.05.2012
MB-R-6	01-002	R	Hospital Virgen del Rocío	Seville, Spain	04.06.2012
MB-R-7	01-002	R	Hospital Virgen del Rocío	Seville, Spain	12.06.2012
MB-R-10	01-004	R	Hospital Virgen del Rocío	Seville, Spain	26.06.2012
MB-R-12	01-016	R	Hospital Virgen del Rocío	Sevilla, Spain	22.10.2012
MB-R-17	02-010	R	Hospital Universitario La Fe	Valencia, Spain	28.07.2012
MB-R-18	02-012	R	Hospital Universitario La Fe	Valencia, Spain	05.10.2012
MB-R-23	09-018	R	Complexo Hospitalario Universitario de A Coruña	A Coruña, Spain	05.11.2012
MB-R-45	04-036	R	Hospital Universitario de Ciudad Real	Ciudad Real, Spain	22.01.2013
MB-R-53	01-041	R	Hospital Virgen del Rocío	Seville, Spain	07.02.2013
MB-R-61	04-051	R	Hospital Universitario de Ciudad Real	Ciudad Real, Spain	20.03.2013
MB-R-62	09-061	R	Complexo Hospitalario Universitario de A Coruña	A Coruña, Spain	15.03.2013
MB-R-64	14-053	R	Policlínico Universitario A. Gemelli	Rome, Italy	18.03.2013
MB-R-65	14-091	R	Policlinico Universitario A. Gemelli	Rome, Italy	25.07.2013
MB-R-67	14-095	R	Policlinico Universitario A. Gemelli	Rome, Italy	25.07.2013
MB-R-71	21-072	R	University Hospital ATTIKON	Athens, Greece	15.04.2013
MB-R-75	21-078	R	University Hospital ATTIKON	Athens, Greece	20.05.2013
MB-R-79	21-079	R	University Hospital ATTIKON	Athens, Greece	02.06.2013
MB-R-80	21-090	R	University Hospital ATTIKON	Athens, Greece	10.07.2013
MB-R-81	04-108	R	Hospital Universitario de Ciudad Real	Ciudad Real, Spain	18.10.2013
MB-R-83	14-111	R	Policlinico Universitario A. Gemelli	Rome, Italy	14.11.2013
MB-R-85	18-121	R	University of Napoli Federico II	Naples, Italy	20.11.2013
MB-R-89	21-106	R	University Hospital ATTIKON	Athens, Greece	25.10.2013
MB-R-90	21-107	R	University Hospital ATTIKON	Athens, Greece	18.11.2013
MB-R-91	21-126	R	University Hospital ATTIKON	Athens, Greece	18.11.2013
MB-R-93	22-113	R	University Hospital Evangelismos	Athens, Greece	07.11.2013
MB-R-94	22-124	R	University Hospital Evangelismos	Athens, Greece	29.11.2013
MB-R-96	22-130	R	University Hospital Evangelismos	Athens, Greece	14.12.2013
MB-R-97	25-131	R	University Hospital of Ioannina	Ipirus, Greece	18.12.2013
MB-R-98	27-098	R	Sotiria University Hospital	Athens, Greece	12.09.2013
MB-R-100	30-127	R	General Hospital of Larissa	Larissa, Greece	07.12.2013

Table 2.9: continued

Isolate	Patient ID	Sample type	Hospital	City, country	Date of isolation
MB-R-104	30-132	R	General Hospital of Larissa	Larissa, Greece	15.01.2014
MB-R-105	06-154	R	Hospital Universitario Doctor Peset	Valencia, Spain	16.03.2014
MB-R-109	14-137	R	Policlínico Universitario A. Gemelli	Rome, Italy	20.02.2014
MB-R-110	14-142	R	Policlínico Universitario A. Gemelli	Rome, Italy	17.02.2014
MB-R-112	14-151	R	Policlínico Universitario A. Gemelli	Rome, Italy	21.02.2014
MB-R-114	21-103	R	University Hospital ATTIKON	Athens, Greece	11.10.2013
MB-R-116	21-139	R	University Hospital ATTIKON	Athens, Greece	06.02.2014
MB-R-119	21-140	R	University Hospital ATTIKON	Athens, Greece	06.02.2014
MB-R-120	21-141	R	University Hospital ATTIKON	Athens, Greece	06.02.2014
MB-R-123	21-157	R	University Hospital ATTIKON	Athens, Greece	16.04.2014
MB-R-125	21-158	R	University Hospital ATTIKON	Athens, Greece	17.04.2014
MB-R-126	21-159	R	University Hospital ATTIKON	Athens, Greece	16.05.2014
MB-R-127	21-164	R	University Hospital ATTIKON	Athens, Greece	20.05.2014
MB-R-129	22-148	R	University Hospital Evangelismos	Athens, Greece	17.02.2014
MB-R-130	22-155	R	University Hospital Evangelismos	Athens, Greece	11.04.2014
MB-R-131	22-168	R	University Hospital Evangelismos	Athens, Greece	06.05.2014
MB-R-132	30-156	R	General Hospital of Larissa	Larissa, Greece	24.03.2014
MB-R-133	14-171	R	Policlinico Universitario A. Gemelli	Rome, Italy	06.21.2014
MB-R-135	21-173	R	University Hospital ATTIKON	Athens, Greece	07.02.2014
MB-R-138	30-174	R	General Hospital of Larissa	Larissa, Greece	19.06.2014
MB-R-141	18-179	R	University of Napoli Federico II	Naples, Italy	07.20.2014
MB-R-143	14-180	R	Policlínico Universitario A. Gemelli	Rome, Italy	08.05.2014
MB-R-146	28-182	R	University Hospital of Larissa	Athens, Greece	30.08.2014
MB-R-148	04-188	R	Hospital Universitario de Ciudad Real	Ciudad Real, Spain	29.10.2014
MB-R-149	28-189	R	General Hospital of Larissa	Larissa, Greece	10.06.2014
MB-R-151	14-204	R	Policlínico Universitario A. Gemelli	Rome, Italy	02.02.2015
MB-R-152	14-210	R	Policlinico Universitario A. Gemelli	Rome, Italy	03.03.2015
MB-R-153	21-205	R	University Hospital ATTIKON	Athens, Greece	02.03.2015
MB-R-157	15-229	R	University of Napoli Federico II	Naples, Italy	08.06.2015
MB-R-158	21-176	R	University Hospital ATTIKON	Athens, Greece	30.07.2014
MB-R-159	21-216	R	University Hospital ATTIKON	Athens, Greece	17.04.2015
MB-R-160	21-221	R	University Hospital ATTIKON	Athens, Greece	29.04.2015
MB-R-161	29-222	R	Papanikolaou General Hospital	Thessaloniki, Greece	09.04.2015
MB-R-164	30-230	R	General Hospital of Larissa	Larissa, Greece	22.06.2015
MB-R-165	30-233	R	General Hospital of Larissa	Larissa, Greece	21.09.2015
MB-S-5	01-001	S	Hospital Virgen del Rocío	Seville, Spain	24.05.2012
MB-S-6	01-001	S	Hospital Virgen del Rocío	Seville, Spain	24.05.2012
MB-S-23	01-016	S	Hospital Virgen del Rocío	Seville, Spain	25.10.2012

Table 2.9: continued

Isolate	Patient ID	Sample type	Hospital	City, country	Date of isolation
MB-S-43	01-002	S	Hospital Virgen del Rocío	Seville, Spain	19.06.2012
MB-S-177	01-041	S	Hospital Virgen del Rocío	Seville, Spain	04.02.2013
MB-S-271	02-077	S	Hospital Universitario La Fe	Valencia, Spain	17.05.2013
MB-S-273	02-077	S	Hospital Universitario La Fe	Valencia, Spain	21.05.2013
MB-S-1044	22-168	S	Evangelismos Hospital	Athens, Greece	10.05.2014
SG3161	KBT6804357	W	University Hospital Frankfurt am Main	Frankfurt a. M., Germany	03.2016
SG3166	KBT6804357	R	University Hospital Frankfurt am Main	Frankfurt a. M., Germany	03.2016

To search for tigecycline resistance mechanisms, several additional *A. baumannii* isolates were chosen for comparison to the MagicBullet clinical isolates (Table 2.10). The so called ABC-isolates were collected as part of the Tigecycline Evaluation Surveillance Trial (TEST) and have been described previously by Lucaßen *et al*. [225, 226].

Table 2.10: Additional *A. baumannii* isolates (ABC-isolates)

The *A. baumannii* isolates were obtained from patients in Italy, Brazil and Pakistan during the TEST study [225].

Isolate No. (ABC No.)	Country (City)	Year of collection	Clonal lineage	Oxford ST	Pasteur ST	Tigecycline MIC [mg/L]
1168335 (ABC061)	Italy (Bari)	2014	IC2	2	281	0.5
1189384 (ACB068)	Italy (Naples)	2014	IC2	2	281	0.5
1232447 (ABC085)	Italy (Rome)	2015	IC2	2	218	0.5
1381753 (ABC193)	Italy (Salerno)	2016	IC2	2	1839 or 218[a]	0.5
1474297 (ABC246)	Italy (Rome)	2016	IC2	2	218 or 2164[a]	0.25
1308973 (ABC163)	Brazil (Porto Alegre)	2015	IC1	1	1604 or 231[a]	0.5
1454407 (ABC212)	Pakistan (Peschawar)	2016	IC1	1106	1932 or 1955[a]	0.5

[a] multiple perfect hits found for the allele Oxf_gdhB using the MLST Finder

2.1.8. Plasmids and constructs

All plasmids used, and constructs generated in this thesis are listed in Table 2.11. For all plasmids and constructs kanamycin was used as the selective antibiotic marker, in concentrations between 10 – 50 mg/L. Selected plasmid maps are depicted in Appendix 5.

Table 2.11: Plasmids and constructs

Plasmid/Construct	Experiment	Reference
pJN17/04	Shuttle plasmid for *A. baumannii*	[159]
pBIISK-sacBkanR[a]	Suicide plasmid for creation of markerless gene knockouts	[55]
pRK2013[b]	Plasmid of *E. coli* helper strain for triparental conjugation	
pJN17/04::*adeN*-19606	Complementation of *A. baumannii* Δ*adeN* with *adeN* (including putative promoter region) of different *A. baumannii* isolates	This thesis
pJN17/04::*adeN*-S271		
pJN17/04::*adeN*-R71		
pJN17/04::*adeN*-R110		
pJN17/04::*adeN*-R131		
pJN17/04::*adeN*-R133		
pJN17/04::*adeN*-R161		
pJN17/04::*adeN*I-S273	Complementation of *A. baumannii* Δ*adeN* with truncated *adeN* upstream of IS (including putative *adeN* promoter region)	
pJN17/04::*adeN*I-S1044		
pJN17/04::*adeN*II-S273	Complementation of *A. baumannii* Δ*adeN* with truncated *adeN* downstream of IS (including putative promoter region in IS)	
pJN17/04::*adeN*II-S1044		
pJN17/04::*pmrAB*-19606	Complementation of *A. baumannii* Δ*pmrAB* with *pmrAB* (including putative promoter region) of different *A. baumannii* isolates	
pJN17/04::*pmrAB*-R2		
pJN17/04::*pmrAB*-S6		
pJN17/04::*pmrAB*-R90		
pJN17/04::*pmrAB*-R119		
pBIISK-sacBkanR::*pmrAB*-up/downstream	Suicide plasmid containing the up- and downstream regions of *pmrAB* for creation of a markerless knockout	
pBIISK-sacBkanR::*adeN*-up/downstream	Suicide plasmid containing the up- and downstream regions of *adeN* for creation of a markerless knockout	

[a] kindly provided by Prof. Dr. Beate Averhoff, University of Frankfurt, Germany
[b] kindly provided by Prof. Dr. Uwe Deppenmeier, University of Bonn, Germany

2.1.9. Oligonucleotides

All PCR primers were designed using Primer3 software and are listed in Table 2.12.

Table 2.12: Oligonucleotides

Primer	Sequence (5' to 3')	Experiment	Reference
H48	GCGAGGAGCACATTTCCTAA	*pmrCAB* amplification and sequencing	[194]
H49	TGTAGTCACTCACGATGCTGAA		
H50	TTAAAGTTACATCTTGCTTTGCC		
H51	GGCAAAGCAAGATGTAACTTTAA		
H52	TCGATGAAATTCTAGATACTCAAATG	qRT-PCR of *pmrB*	
H53	CCCAAATATCGATAAACAGATCTTC		
H54	TTGAAGCAGATCCGTCAAAG	*pmrCAB* amplification and sequencing	
H55	TGCACCCAAATTTAAACCATC		
H56	CCGACTTGTGATACGAATGC		
H57	ATGACAAAAATCTTGATGATTGAAGAT	*pmrCAB* amplification and sequencing	[195]
H58	TTATGATTGCCCCAAACGGTAG		
H59	GTGCATTATTCATTAAAAAAAC		

Table 2.12: continued

Primer	Sequence (5' to 3')	Experiment	Reference
H60	TCACGCTCTTGTTTCATGTA	*pmrCAB* amplification and sequencing	[195]
H61	GGTTCGTGAAGCTTTCG		
H62	CCTAAATCGATTTCTTTTTG		
H63	ATGTTTAATCTCATTATAGCCA		
H64	TTAGTTTACATGGGCACAA		
H65	GGTTGTTATTGAAGAAAGTAT		
H66	TCAATCCAAGTCACTTGGTAAC		
H69	ACTTCGGTAATAAAGATGGCT	Primer flanking IS-element in *adeN*	This thesis
H70	TGAGATATGAGTAAGGCTTGAG		
H71	GAACGATGCTGCTCAAAACT	Confirmation of *adeG* mutation in isolate MB-S-177	
H72	CCGGCACTATTACGGGTTTT		
H75	TTCCCCAAAACTATGCTCTACTT	Amplification and sequencing of *adeN* (including the putative promoter region)	[227]
H76	GTTGTTGGCTGGGTGGAAG		
H79	AGAAGCATGGACGACTCTCA	Amplification of *adeN* and truncated IS-element	This thesis
H80	GTCAGTTGCACTTGGTCGAA		[228]
J3	TGCCCTTTCTACAAACAGCT	Sanger sequencing of *spoT*	This thesis
J4	ATCATATCCGCCGGCCATTA		
J5	GACGGGACATTTGGTTCTCC		
J6	ATTGCACCACCAACCGTAAC	Confirmation of *adeJ* mutation in isolate MB-R-119	[229]
J7	ACGTTGTTCAACTGCTTCGG		This thesis
J8	TCAGTTGGGCATGATTTAATACA	Amplification and sequencing of H-NS	
J9	AGCAGAATCGCTTGGCTTT		
J14	TCATTTGGCTTAATACATGGTCTG	Amplification and sequencing of *pmrCAB*	[194]
J15	GGTGGAATGGGTCAATAACG	qRT-PCR of *pmrA*	[191]
J16	GGTGTTGCTGCTCTTTGACG		[194]
J41	GCATGAATGAAATGGCCGAT	Amplification and sequencing of *spoT*	This thesis
J42	TGACGCCATTGTAAAAGATCT		
J44	GATTATCGCCGTGAAATGTTT	Amplification and sequencing of *adeN* and truncated IS*Aba125*	This thesis
J45	CGTAACACCCTAACCAGAAA	Confirmation of *adeN* knockout	
J48	AGCATGCTTGAAATAACACG		
J49	GGAAAGCCACGTTGTGTCTC	Amplification of *sacB*/*kanR* cassette in pBIISK-sacBkanR	[55]
J52	GCTCTGCCAGTGTTACAACC		
J69	ACCTGAACACATTGCCTTTT	qRT-PCR of *adeN* downstream IS-element (with H70)	This thesis
J70	CAGTTTCATTTGATGCTCGA	Flanking region of PstI restriction site in pBIISK-*sacBkanR*	
J71	CGTATGTTGTGTGGAATTGT		
J72	AGAGCATCGAATTCCTCGTATGCCAGGTATTGATG	Amplification of *adeN* up- and downstream regions for Δ*adeN*	
J73	CTGTTGTTGGCTGGGAAAATTTAATAACGACGGCT		
J74	TCGTTATTAAATTTTCCCAGCCAACAACAGATAAT		
J75	GTGGATCCCCCGGGCTAAGCATTGGTCAGAAGAGG		
J76	AGAGCATCGAATTCCTCTACTTTAGCGTATGGTGC	Amplification of *pmrAB* up- and downstream regions for Δ*pmrAB*	[230]
J77	AAACAAGAGCTTACGGTTCTAGGCTCGCTTTAGTT		
J78	AAGCGAGCCTAGAACCGTAAGCTCTTGTTTCACTT		
J79	GTGGATCCCCCGGGCGGATTTTAATTGTCGGCTGG		
K12	CCGCATAATAGGTAGCAACA	Amplification and sequencing of *pmrC* including putative promoter region	This thesis
K25	AGTGTGGAGTAAGTGTGGAGA	Amplification and sequencing of *adeRS*	[227]
K28	AAACTTGCTCAATACGACGG		This thesis

Table 2.12: continued

Primer	Sequence (5' to 3')	Experiment	Reference
K40	GCGGTATAGAACGTCGTTTA	qRT-PCR *adeN* upstream of IS-element	
K41	TGAACCACCAGCATGATTAA		
K42	AAAGCGTTTTATTGTGCCAA	qRT-PCR *adeS* upstream of IS-element	
K43	CGGCGGAGTGAATTCTATTA		
K44	TGTTTGACTTTAAGGCGGTA	qRT-PCR *adeS* downstream of IS-element	
K45	CGCAATATACAGGAGTGGAA		
K58	GCCACTCATCGCAGTAAACCGACTTGTGATACGAA	Amplification of *pmrAB* for In-Fusion cloning into pJN17/04	[230]
K59	ATGAATTACAACAGTTAGGTTTCATGATGGGTTGG		
L37	AGCGTGATACATTTGAAGGT	Alternative primer *pmrC*	This thesis
L38	GTTATTGACCCATTCCACCT		
L39	GATCGCGTTGAACAATATCA	qRT-PCR *pmrC* alternative	[230]
L40	GCAATACAACTAACCGTGGA		
L53	CAGACCATGTATTAAGCCAGA	Amplification of *pmrAB* alternative	This thesis
L54	TTTGGGCAGTGAACATTTTC		
L55	CCTTTTGCGATATTTGCCTT	qRT-PCR *pmrB* alternative	
L56	ATCAATGGTAGGCAAAAGCT		
M26	CAAGGACGTATGCAACAAGT	qRT-PCR *adeB* alternative	This thesis
M27	CTAATTGACCGCTTGAACCC		
M45	AGGCGAGGTTAAACTTATCC	Amplification and sequencing of *pmrCAB* alternative	
M46	TAACTTATGGACAGGTTGGG		
M47	ACAACCTAAGATGACACTGA		
M80	CAAGGAGCCATCGTCATATA	Amplification and sequencing of *eptA*	[230]
M81	ATGGTGTTTATCCGGGTTAC		
N1	CATAAGAAGGCTCCGAAGAA	Amplification and sequencing of *eptA*	[230]
N2	TGTCATCATAACATTCGCCA		
N3	TGTTGCTACCTATTATGCGG	Specific primers for qRT-PCR of *pmrC* (N3/N4) and *eptA* (N4/N5)	
N4	ATTTTGAATTTGGTCGGGTG		
N5	TCAGTTCTTTTCTTAGGGGC		
N6	GAGCATTTCAACTGGGGATA	Amplification and sequencing of *eptA*	
N7	TCTTATTGGTTACTAAGCTCA		This thesis
JE23	TTTGAGAAGCACACGGTCAC	Flanking region of ScaI restriction site in plasmid pJN17/04	[231]
JE24	TCATCCTGCCCTTATGTTCC		
A7	AGGCATGAGTGTTATTCGGG	Amplification and sequencing of *adeRS*	[227]
JE37	AAGTAAAATAGCTTTAAAACGATCCA		
B8	CACGAATGCAGAAGTTG	Amplification and sequencing of IS*Aba1*	[232]
B9	CGACGAATACTATGACAC		
C65	GAGTCTAATGGCGGTGGTTC	qRT-PCR of *rpoB*	[233]
C66	ATTGCTTCATCTGCTGGTTG		
C25	GAATAAGGCACCGCAACAAT	qRT-PCR of *adeB*	[143]
C26	TTTCGCAATCAGTTGTTCCA		
A48	GTATGAATTGATGCTGC	Amplification of *adeB*	[149]
A49	CACTCGTAGCCAATACC		
C19	GCGAATGGACGTATGGTTCT	qRT-PCR of *adeJ*	[143]
C20	CATTGCTTTCATGGCATCAC		
D2	AATCCAGCCTTTTTCAATCG	Detection of the *adeRSABC* operon	[148]
D12	GCATTTTTGACGGAAACCTC		[143]
D13	TTAGTCACGGCGACCTCTCT		

2.1.10. Software and bioinformatic tools

All software and bioinformatic tools used in this study are listed in Table 2.13.

Table 2.13: Bioinformatic tools and software used for sequence data analysis

Bioinformatic tool/ Software	Manufacturer/Provider	Experiment	Reference
Velvet assembler (version 1.1.04).	Part of Ridom SeqSphere+ software	*De novo* assembly of draft genomes	
Ridom SeqSphere+ v.7.0.3	Ridom GmbH, Münster, Germany	cgMLST, allele calling	
Artemis	https://www.sanger.ac.uk/science/tools/artemi	Visualization and nucleotide/ amino acid sequence extraction	[234]
ResFinder	https://cge.cbs.dtu.dk/services/ResFinder/	Resistome analysis	[235]
MLST Finder	https://cge.cbs.dtu.dk/services/MLST/	Extraction of MLST types	[236]
PubMLST	https://pubmlst.org/abaumannii/	Extraction of MLST types	[237]
IS-Mapper	https://github.com/jhawkey/IS_mapper	Search for insertion sequences in the genome	[238]
ISfinder	http://www-is.biotoul.fr	Identification of IS-elements	[239]
Conserved domain database (CDD)	https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml	Prediction of e.g. transmembrane domains	[240]
ContigExpress®	Thermo Fisher Scientific, Schwerte, Germany	DNA sequence analysis and editing	
Vector NTI Advanced®	Thermo Fisher Scientific, Schwerte, Germany	DNA sequence analysis, creation of plasmid maps, *in silico* cloning, primer design	
FlexAnalysis™ MALDI-TOF software	Bruker Daltonics, Bremen, Germany	Analysis of MALDI-TOF mass spectra	
Primer3	http://primer3.ut.ee/	Primer design	

2.2. Methods

2.2.1. Selection of *A. baumannii* isolates for the investigation of resistance mechanisms

In total, 77 *A. baumannii* isolates were investigated in this thesis. Sixty-five isolates were previously described by Nowak *et al.* [27] and are referred to as first respiratory isolate per patient. Additional isolates were chosen for the investigation of resistance mechanisms in so-called isolate pairs. An isolate pair is defined as a pair of isogenic *A. baumannii* isolates from the same patient, which were collected on multiple occasions during a patients' hospital course from respiratory, stool and wound samples. Moreover, the pairs demonstrated a phenotype shift in tigecycline MICs by at least one dilution step and in colistin MICs by at least four dilution steps, i.e. the initial isolate from an earlier time point displayed a low MICs of either tigecycline or colistin, while the isolate from a later time point showed a high MIC of the same antibiotic. In addition, isolates representing isolate pairs were required to demonstrate less than ten allele differences in both the core and accessory genomes by WGS (Chapter 2.2.8.2) [227, 230].

2.2.2. Bacterial growth conditions and plasmid maintenance

Bacterial strains were routinely grown in LB broth at 37°C and 220 rpm on an orbital shaker or on SBA plates at 37°C. For plasmid maintenance and selection, *E. coli* and *A. baumannii* transformants were grown on LBA plates supplemented with appropriate concentrations of kanamycin (10-50 mg/L). When necessary, the optical density (OD) of the bacterial culture was measured at 600 nm using the BioPhotometer.

2.2.3. Growth kinetics

Growth kinetics were performed in triplicates as described previously by Nowak *et al*. [159]. Briefly, a McFarland suspension of 0.5, which was measured using the VITEK DensiCheck, generated from a fresh overnight culture was used to inoculate 10 ml MH broth 1:100. The cultures were incubated for 6 h at 37°C and 220 rpm on an orbital shaker and every hour a sample was taken. Samples were serially diluted in cold saline (0.45% NaCl) and 0.1 ml were plated onto MHA plates. The colony forming units (cfu)/ml were determined after overnight incubation at 37°C using the following formula: cfu/mL = cfu * 0.1 mL / dilution.

Furthermore, growth curves were conducted overnight using the Infinite M1000 plate reader. Overnight cultures of the bacterial strains were diluted in fresh LB medium to reach an OD_{600} of 0.2. Afterwards, 50 µl of the cell suspension were added to 50 µl LB medium in a transparent 96-well plate. The OD was measured at 600 nm every 15 min for up to 16 h at 37°C using the Infinite M1000 plate reader. The growth rate, µ and doubling time, td were calculated as follows: $\mu = (\ln(x_2) - \ln(x_1)) / (t_2 - t_1)$ with $x = OD_{600}$ at time point t and $td = \ln(2) / \mu$.

2.2.4. Antimicrobial susceptibility testing

Three different methods were used for the determination of the minimal inhibitory concentration (MIC) and *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *S. aureus*

ATCC 29213 were used as control strains. For defining the MIC, the EUCAST resistance breakpoints for *A. baumannii* were used [241]. While the colistin breakpoints for *A. baumannii* are defined as susceptible, MIC ≤ 2 mg/L, and resistant, MIC ≥ 4 mg/L [241], there are no such breakpoints for tigecycline. Therefore, all *A. baumannii* isolates analysed in this thesis were not separated as tigecycline-susceptible or tigecycline-resistant, but rather sorted in groups of isolates displaying low or high tigecycline MICs. The EUCAST breakpoints for *Enterobacterales* were used as tentative guideline to define low and high tigecycline MICs for the *A. baumannii* isolates [241]: low MIC, i.e. isolates with tigecycline MICs of ≤ 2 mg/L, high MIC, i.e. isolates with tigecycline MICs of ≥ 4 mg/L.

2.2.4.1. Agar dilution

To determine the antimicrobial susceptibility of the isolate pairs, agar dilution was performed following the current Clinical Laboratory Standards Institute (CLSI) guidelines [3]. In brief, bacteria were grown in 10 ml LB broth overnight and diluted 1:10 in 0.85% saline solution. The antimicrobial agents (Table 2.2) were two-fold serial diluted and fresh MHA was supplemented with the antimicrobial according to the CLSI guidelines [3]. The plates were inoculated with the diluted bacterial cultures using a multipoint inoculator. The MIC was determined after overnight incubation at 37°C. The MIC determination of isolate pairs was repeated at least three times independently.

2.2.4.2. Disc diffusion

For disc diffusion assays, the bacteria were grown on SBA overnight at 37°C. Single colonies were used to inoculate a cell suspension in 0.85% saline solution with a McFarland of 0.5, which was measured using the VITEK DensiCheck. The bacterial suspension was plated onto MHA and discs containing specific concentrations of the antimicrobial agents (Table 2.3) were placed onto the plate using a disc dispenser. After overnight incubation at 37°C, the zone of inhibition was measured and the EUCAST resistance breakpoints were used for defining resistance [241].

2.2.4.3. Microbroth dilution

Susceptibility to colistin was determined by microbroth dilution using pre-manufactured microtiter plates (Micronaut S colistin plates). The MICs were determined in cation-adjusted MHB according to the international standard ISO 20776-1 [242]. Microbroth dilution was repeated at least three times independently for *A. baumannii* isolate pairs.

2.2.5. DNA and RNA purification

Genomic DNA was purified using the MagAttract HMW DNA Kit and plasmid DNA was purified using the QIAprep Spin Miniprep Kit according to manufacturer's instructions. For both the genomic DNA and the plasmid DNA extraction, the bacteria were grown in 10 ml LB broth overnight at 37°C and between 0.5 to 2 ml were used for the DNA extraction.

Total RNA was purified from a bacterial culture, which was grown till the mid-log phase (OD_{600} 0.7 - 0.9). 500 µl bacterial culture were mixed with 1 ml RNA Protect Bacteria Reagent, incubated for 5 min at RT and centrifuged at 8000 rpm for 12 min. The supernatant was removed and if necessary, the pellet was frozen at -20°C for storage. Total RNA was extracted using the RNeasy Mini Kit as recommended by the manufacturer. An additional digestion of genomic DNA was included using the DNase kit. RNA quality was confirmed by RNA gel electrophoresis and RNA concentration was measured using the NanoDrop 2000 spectrophotometer. 1 µg RNA were subjected to cDNA synthesis, which was performed using the QuantiTect Reverse Transcription Kit according to manufacturer's instructions.

2.2.6. PCR-based methods

2.2.6.1. PCR amplification

Standard PCR amplification was done using crude cell lysate, purified genomic DNA, or plasmid DNA as template. For crude cell lysis, one bacterial colony was resuspended in 100 µl ultrapure water and incubated at 99°C for 5-10 min. Afterwards, the cell suspension was cooled on ice and centrifuged at 13000 rpm for 1 min. The supernatant was used as template in the PCR reaction. The 2xTaq PCR Master Mix or the Q5® High-Fidelity 2x Master Mix was used for PCR amplification. The PCR reactions were prepared as depicted in Table 2.14 and the PCR conditions are shown in Table 2.15.

Table 2.14: Reaction mixture for PCR amplification

2xTaq PCR Master Mix	Q5® High-Fidelity 2x Master Mix
12.5 µl Taq PCR Master Mix (2x)	25 µl Q5® High-Fidelity Master Mix (2x)
0.5 µl primer 1 (10 pmol/µl)	2.5 µl primer 1 (10 pmol/µl)
0.5 µl primer 2 (10 pmol/µl)	2.5 µl primer 2 (10 pmol/µl)
1 µl DNA template	1 µl DNA template
10.5 µl RNase-free H_2O	19 µl RNase-free H_2O
25 µl final volume	50 µl final volume

Table 2.15: PCR conditions

	2xTaq PCR Master Mix		Q5® High-Fidelity 2x Master Mix	
Step	**Temperature**	**Time**	**Temperature**	**Time**
Initial denaturation	94°C	3 min	98°C	30 sec
Denaturation	94°C	30 sec	98°C	10 sec
Annealing	55-65°C*	30 sec	55-65°C*	20 sec
Extension	72°C	1 min/1 kb	72°C	30 sec/kb
Final elongation	72°C	10 min	72°C	2 min
Storage	10°C	∞	10°C	∞
No. of cycles	25-35		25-35	

*dependent on primer sequence

To amplify genes with high G-C content or using primers containing tails for InFusion cloning (Chapter 2.2.11.4), a two-step PCR was performed. Here, the annealing temperature was increased after 5-10 cycles to improve primer binding and to decrease the probability of unspecific amplicons (Table 2.16).
If necessary, PCR products were purified using the QIAquick PCR Purification Kit or the NucleoSpin Extract II PCR clean-up kit according to the manufacturers' instructions.

Table 2.16: Two-step PCR conditions

	2xTaq PCR Master Mix		Q5® High-Fidelity 2x Master Mix	
Step	**Temperature**	**Time**	**Temperature**	**Time**
Initial denaturation	94°C	3 min	98°C	30 sec
Denaturation	94°C	30 sec	98°C	10 sec
Annealing	45-55°C*	30 sec	45-55°C*	20 sec
Extension	72°C	1 min/1 kb	72°C	30 sec/kb
No. of cycles	5-10	5-10		
Denaturation	94°C	30 sec	98°C	10 sec
Annealing	55-70°C**	30 sec	55-70°C**	20 sec
Extension	72°C	1 min/1 kb	72°C	30 sec/kb
Final elongation	72°C	10 min	72°C	2 min
Storage	10°C	∞	10°C	∞
No. of cycles	20-30		20-30	

*dependent on primer sequence, but at least 5°C below second annealing temperature
** dependent on primer sequence, but at least 5°C above first annealing temperature

2.2.6.2. Semi-quantitative reverse transcription PCR

Gene expression was analysed by semi-quantitative reverse transcription PCR (qRT-PCR). Total RNA and cDNA were prepared as described in Chapter 2.2.5. For qRT-PCR, standard curves for each measured gene were prepared. For this purpose, the respective gene was amplified by PCR using the Q5® High-Fidelity 2x MasterMix and the primers listed in Table 2.17. After purification and quantification of the amplicon, a serial dilution of the amplicon was prepared in ultrapure water for the standard curve. The amount of the unknown target was always within the range of the standard curve, which ranged from 10^1 - 10^7 molecules/µl in all experiments. The qRT-PCR reaction was set up using the QuantiFast SYBR Green PCR Kit and gene-specific primers, which are presented in Table 2.17. Both the reaction mixture and the qRT-PCR conditions for measurement of transcripts were set up as recommended by the manufacturer. As reference the *rpoB* gene was used, which encodes the β-subunit of the RNA polymerase. The qRT-PCR experiments were done in triplicates using freshly prepared RNA and cDNA and were repeated independently at least three times. The LightCycler® 480 was used for measurement of transcripts. The relative expression of the respective gene was compared between isolates representing an isolate pair or to *A. baumannii* reference or knockout strains and statistical analysis was done performing unpaired t-test taking the recorded absolute values.

Table 2.17: Oligonucleotides used for qRT-PCR

Primer	Sequence (5' to 3')	Gene (amplicon size in bp)	Reference
C65	GAGTCTAATGGCGGTGGTTC	*rpoB* (110)	[233]
C66	ATTGCTTCATCTGCTGGTTG		
J15	GGTGGAATGGGTCAATAACG	*pmrA* (141)	[191]
J16	GGTGTTGCTGCTCTTTGACG		[194]
H52	TCGATGAAATTCTAGATACTCAAATG	*pmrB* (132)	[194]
H53	CCCAAATATCGATAAACAGATCTTC		
L55	CCTTTTGCGATATTTGCCTT	*pmrB* alternative (150)	This thesis
L56	ATCAATGGTAGGCAAAAGCT		
N3	TGTTGCTACCTATTATGCGG	*pmrC* (148)	[230]
N4	ATTTTGAATTTGGTCGGGTG		
L39	GATCGCGTTGAACAATATCA	*pmrC* alternative (151)	
L40	GCAATACAACTAACCGTGGA		
N4	ATTTTGAATTTGGTCGGGTG	*eptA* (183)	
N5	TCAGTTCTTTTCTTAGGGGC		
C25	GAATAAGGCACCGCAACAAT	*adeB* (124)	[143]
C26	TTTCGCAATCAGTTGTTCCA		
M26	CAAGGACGTATGCAACAAGT	*adeB* alternative (103)	This thesis
M27	CTAATTGACCGCTTGAACCC		
C19	GCGAATGGACGTATGGTTCT	*adeJ* (113)	[143]
C20	CATTGCTTTCATGGCATCAC		
K40	GCGGTATAGAACGTCGTTTA	*adeN* upstream IS (110)	This thesis
K41	TGAACCACCAGCATGATTAA		
H70	TGAGATATGAGTAAGGCTTGAG	*adeN* downstream IS (132)	
J69	ACCTGAACACATTGCCTTTT		
K42	AAAGCGTTTTATTGTGCCAA	*adeS* upstream IS (104)	
K43	CGGCGGAGTGAATTCTATTA		
K44	TGTTTGACTTTAAGGCGGTA	*adeS* downstream IS (111)	
K45	CGCAATATACAGGAGTGGAA		

2.2.7. Gel electrophoresis

For DNA gel electrophoresis, a 0.5 – 2% agarose gel was prepared with 1x TAE buffer containing 10 µg ethidium bromide or Midori Green. The PCR amplicon or plasmid DNA was mixed with 6x DNA loading dye and electrophoresis was performed in 1x TAE buffer at 100 V for 30 - 40 min. Afterwards, DNA was visualized using the Gel Doc XR+ System.

For quality control of RNA, RNA gel electrophoresis was performed using a formaldehyde agarose (FA) gel. The gel and buffers were prepared according to the recommendation given by the RNeasy Mini Kit manufacturer and are presented in Table 2.18. In brief, after dissolving the agarose by heating, the FA gel was cooled to 65°C and formaldehyde and ethidium bromide were added. The gel was equilibrated in 1x FA gel running buffer for 30 min prior to loading the samples. RNA samples were mixed 1:5 with 5x RNA loading buffer, incubated for 5 min at 65°C in a thermomixer and snap cooled on ice. Approximated 15 µl of sample were

loaded onto the gel and electrophoresis was performed at 60 V for 1 - 1.5 h. Afterwards, RNA was stained with ethidium bromide and visualized using the Gel Doc XR+ System.

Table 2.18: Formaldehyde agarose gel electrophoresis buffer

Buffer	Recipe
10x FA gel buffer (1 L)	200 mM 3-[N-morpholino]propanesulfonic acid (MOPS) 50 mM sodium acetate 10 mM EDTA pH to 7.0 with NaOH
1x FA gel running buffer (1 L)	100 mL 10x FA gel buffer 20 mL 37% (12.3 M) formaldehyde 880 mL RNase-free water
5x RNA loading buffer (10 mL)	16 µl saturated aqueous bromphenol blue solution 80 µl 500 mM EDTA, pH 8.0 720 µl 37% (12.3 M) formaldehyde 2 mL 100% glycerol 3.084 mL formamide 4 mL 10x FA gel buffer 100 µl RNase-free water
1.2% FA gel	0.6 g agarose 5 mL 10x FA gel buffer 45 mL RNase-free water 900 µl 37% (12.3 M) formaldehyde 0.5 µl of a 10 mg/mL ethidium bromide stock solution

2.2.8. Sequencing techniques and data analysis

2.2.8.1. Sanger sequencing

Sanger sequencing was performed by LGC Genomics (Berlin, Germany). The samples were prepared as LGC Genomics recommended and primers are listed in Table 2.12. Sequence data was further analysed using the ContigExpress software.

2.2.8.2. Whole-genome sequencing

All respiratory *A. baumannii* isolates, isolate pairs and knockouts were subjected to whole-genome sequencing (WGS) and analysed as previously described [65]. Briefly, genomic DNA was extracted using the MagAttract HMW DNA Kit and quantified using the Qubit dsDNA BR assay in the Qubit®2-0 Fluorometer. Sequencing libraries were produced using the Nextera XT Library Prep Kit for a 250bp paired-end sequencing run using an Illumina MiSeq sequencer and the MiSeq Reagent kit v2 or v3. The resulting FASTQ files were quality trimmed and assembled *de novo* as previously described [65].

2.2.9. Sequence data analysis

The Ridom SeqSphere+ v.7.0.3 software was used to assemble draft genomes *de novo* using the Velvet assembler (version 1.1.04), to align the assembled draft genomes to the *A. baumannii* reference strain ACICU, to determine differences in specific open reading frames in each draft genome sequence as well as for core genome MLST (cgMLST). The cgMLST

analysis was performed as previously described [65]. Assembled draft genomes were further analysed using various bioinformatic tools listed in Table 2.13. Since differences like amino acid substitutions can also represent genetic polymorphisms associated with e.g. different clonal lineages, it is necessary to include susceptible *A. baumannii* isolates as well as other reference strains in the analysis of antimicrobial resistance determinants. Besides *A. baumannii* ACICU (IC2), the reference strains *A. baumannii* AYE (IC1), ATCC 17978 and ATCC 19606 were included in the analysis. For colistin, all amino acid substitutions found in the four reference strains or in colistin-susceptible isolates were determined to be polymorphisms and excluded. The analysis of tigecycline resistance mechanisms is more complicated since tigecycline breakpoints are not available for *A. baumannii* and isolates cannot be grouped into tigecycline-susceptible or -resistant. Instead, the isolates were sorted by low and high tigecycline MICs (see Chapter 2.2.4). However, the majority of *A. baumannii* isolates in this thesis displayed tigecycline MICs ≥ 2 mg/L and isolates for comparison in the analysis of antimicrobial resistance determinants are lacking. Therefore, additional *A. baumannii* isolates (ABC-isolates) with tigecycline MICs ≤ 0.5 mg/L were chosen and included in the analyses described above. All amino acid substitutions found in isolates with MICs ≤ 1 mg/L (corresponding to the tigecycline MIC distribution of the four reference strains and the ABC-isolates) were determined to be polymorphisms and excluded.

The algorithm followed for this analysis was also described in Gerson *et al*. [227, 230, 243] and is further depicted in Figure 2.1. A list of ORFs of special interest is given in Appendix 1. Of note, the assembly of colistin resistance determinants *pmrCAB* using short read technology is challenging because of the presence of one or more *pmrC*-homologues (termed *eptA*) in the bacterial chromosome [244]. Therefore, all incomplete *pmrC* and *eptA* sequences were confirmed by Sanger Sequencing using the primers listed in Table 2.12.

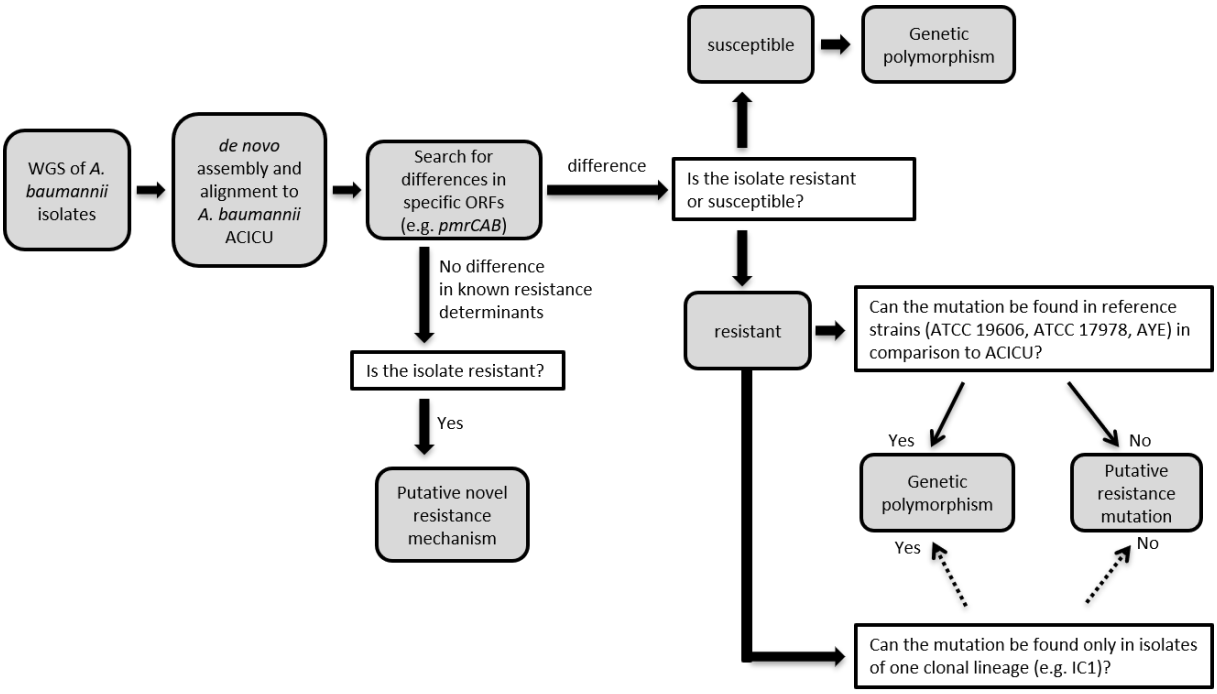


Figure 2.1: Schematic overview of the algorithm followed for the identification of novel mutations associated with antimicrobial resistance

WGS of *A. baumannii* isolates was followed by the *de novo* assembly and alignment of the draft genomes to the genome of *A. baumannii* ACICU as reference strain. Alleles known to be associated with tigecycline or colistin resistance were compared between the *A. baumannii* isolates and the reference strain ACICU. If a difference was detected in a susceptible isolate, the difference was considered to be a genetic polymorphism. All differences found in resistant isolates but not in any other reference strain, were considered to be associated with resistance and declared to be a putative resistance mutation. Moreover, if a difference was detected only in isolates belonging to e.g. the clonal lineage IC1, it was assumed to be a genetic polymorphism specific for this clonal lineage, although further investigation is required in these cases. Adapted from [243].

2.2.10. Nucleotide sequence accession numbers

All raw reads generated by WGS were submitted to the Sequencing Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra/) of the National Center for Biotechnology Information (NCBI) under the BioProject accession numbers PRJNA431710 and PRJNA482774 as well as the SRA accession numbers SRP131448 (https://www.ncbi.nlm.nih.gov/sra/SRP131448) and SRP155178 (https://www.ncbi.nlm.nih.gov/sra/SRP155178). A detailed list of BioSample accession numbers is provided in Appendix 2.

2.2.11. Cloning

2.2.11.1. Target genes

To analyse the effect of gene mutations on gene expression or lipid A modification, the target genes were cloned into the shuttle vector pJN17/04 and introduced into *A. baumannii* reference or knockout strains. The target genes were amplified including their putative promoter regions, i.e. approximately 200-300 bp upstream of the gene were also amplified. The target genes were *pmrA* (locus tag ACICU_RS14980), *pmrB* (ACICU_RS14975), and *adeN* (ACICU_RS10555) with or without part of an insertion sequence. In all cases, the genes were amplified, purified and inserted into the vector either by blunt-end ligation or In-Fusion cloning (Chapter 2.2.11.4). Examples of plasmid maps are depicted in Appendix 5.

2.2.11.2. Restriction digests of plasmid-DNA

Plasmid DNA was linearized using the endonucleases ScaI-HF and PstI-HF. The reaction mixtures contained 3 µl endonuclease, 7 µl of CutSmart buffer (NEB) and 1 µg plasmid DNA. Ultrapure water was added to reach a total volume of 70 µl. The reaction digest was incubated at 37°C for 1 h and if necessary, heat inactivation at 65°C for 20 min was performed. Depending on the cloning method used, the linearized plasmid was purified using the NucleoSpin Extract II PCR clean-up kit or further processed for blunt end ligation. To control if the digest was successful, 5-10 µl of the digested DNA were applied to an agarose gel and gel electrophoresis was performed as described in Chapter 2.2.7.

2.2.11.3. Blunt-end ligation

For blunt-end ligation of a single insert and a linearized vector, the vector was dephosphorylated using Antarctic phosphatase, which was added directly to the endonuclease reaction mixture. The amplified and purified target gene was phosphorylated using the T4 Polynucleotide Kinase. Afterwards, ligation was performed using the Quick Ligase Kit. All enzymatic reactions are listed in Table 2.19. Afterwards, 5 µl of the ligation mixture were used to transform chemically competent *E. coli* cells.

Table 2.19: Enzymatic reactions for blunt-end ligations

	Reaction set up	Incubation conditions
Dephosphorylation	1 µl Antarctic phosphatase 2 µl Antarctic phosphatase buffer	15 min, 37°C 5 min, 65°C
Phosphorylation	x µl insert (purified PCR product) 3 µl T4 polynucleotide kinase buffer 1 µl T4 polynucleotide kinase fill up with ultrapure water 30µl total volume	30 min, 37°C 20 min, 65°C
Ligation	50 ng linearized/dephosphorylated plasmid 1 µl phosphorylated insert 1 µl quick ligase 10 µl Quick ligase buffer x µl ultrapure water 20 µl total volume	10-15 min, RT

2.2.11.4. In-fusion cloning

The In-Fusion cloning method is a fast method for cloning of one or more DNA-fragments into a vector and is based on recombination of complementary overlaps. The In-Fusion® HD EcoDry™ Cloning Kit was used according to manufacturer's instructions, which provides a recombinase recognizing and fusing together the overlapping ends of vector and insert. Plasmids were linearized as described above and purified using the NucleoSpin Extract II PCR clean-up kit. Inserts were amplified by PCR using primers containing tails for creation of the overlapping regions. All primers are listed in Table 2.12.

2.2.12. Transformation experiments

2.2.12.1. Transformation of competent *E. coli* cells

Transformation of plasmids into chemically competent *E. coli* NEB5α or Stellar© cells was done by heat shock. The commercially available competent *E. coli* cells were mixed with up to 5 µl of ligation or InFusion reaction mixture and incubated for 30 min on ice. Afterwards, the cells were incubated for exactly 45 seconds at 42°C and directly cooled on ice for 1-2 min. Cells were recovered in pre-warmed S.O.C. medium for 1 hour at 37°C before plating in appropriate dilutions onto LBA supplemented with selective antibiotics.

2.2.12.2. Transformation of *A. baumannii*

Transformation of *A. baumannii* cells was done by electroporation or triparental conjugation.

For electroporation, electro-competent *A. baumannii* cells had to be prepared. *A. baumannii* was grown in 10 – 50 mL LB broth overnight. Bacterial cells were centrifuged at 13000 rpm for 3 min and washed three times with 0.3 M sucrose at RT or 10% glycerol on ice. The cells were resuspended in 100 µl 0.3 M sucrose or 10% glycerol, mixed with 50-100 ng of plasmid DNA and transferred into a 2 mm gap electroporation cuvette. Electroporation of *A. baumannii* was performed at 25 µF, 200 Ω and 2.0-2.5 kV using the Gene Pulser II system. Afterwards, cells

were recovered in 900 µL pre-warmed LB medium and incubated for 1h at 37°C and 220 rpm on an orbital shaker. Afterwards the cells were plated on LBA supplemented with the appropriate selective antibiotic.

Moreover, triparental conjugation was performed [245]. This method is based on conjugational mating of the donor strain and the acceptor strain using the helper strain *E. coli* K12 HB101 harbouring the plasmid pRK2013, which provides the necessary *tra*-genes for the transfer of the donor plasmid. The *E. coli* helper strain was kindly provided by Prof. Dr. Uwe Deppenmeier (University of Bonn, Germany).
In this thesis, plasmid transfer from an *E. coli* donor strain to an *A. baumannii* acceptor strain was proposed. Both the *E. coli* helper plasmid pRK2013 and the donor strain plasmid pJN17/04 carry a kanamycin resistance cassette. Therefore, an additional selective antibiotic had to be used. Analysis of the antimicrobial susceptibility of the acceptor *A. baumannii* by disc diffusion revealed that *A. baumannii* was also resistant to ampicillin, while both the *E. coli* donor (Stellar© cells) and *E. coli* helper strain were susceptible. Different antimicrobial concentrations were tested, and best results were obtained using both kanamycin (10 mg/L) and ampicillin (10 mg/L) for selection of transformants. Additionally, coliforms chromogenic agar (CCA) was used to differentiate between *E. coli* and *A. baumannii*. While *E. coli* appears purple or blue on CCA plates, *A. baumannii* colonies appear white. Only white colonies growing on CCA supplemented with kanamycin/ampicillin are suggested to be correct transformants.
For conjugation, the donor, the acceptor, and *E. coli* helper strains were grown in LB broth till mid-log phase (OD_{600} 0.7 – 0.9). 1-2 mL of the bacterial cultures were centrifuged at 5300 g and 4°C for 5 min. After washing of the cells with cold PBS the pellet was resuspended in 1 mL PBS. 0.5 mL of each bacterial suspension was mixed 1:1:1 in a new tube and 400 µl of the mixed bacterial suspension was pipetted onto a sterile filter. The filter was placed onto MHA plates and incubated at 37°C overnight. On the next day, the filter was carefully removed from the agar plate and transferred to a falcon tube. The bacterial lawn was put into solution by washing the filter with PBS. Serial dilutions of the cell suspension were plated onto CCA plates supplemented with 10 mg/L kanamycin/ampicillin and incubated at 37°C for 24-48 h. White colonies were subcultured on LBA containing again 10 mg/L kanamycin/ampicillin.

Successful transformation by either electroporation or triparental mating was confirmed by PCR and subsequent Sanger sequencing of the insert and its flanking regions.

2.2.13. Markerless gene knockout

The creation of a markerless gene knockout was done using reference strain *A. baumannii* ATCC 19606 following the protocol of Stahl *et al.* [55]. The method is based on recombination of up- and downstream regions of the gene of interest on the chromosome. During recombination of these regions, the target gene is relocated onto the plasmid pBIISK-

sacBkanR. The plasmid pBIISK-sacBkanR harbours the *sacB* gene, which encodes levansucrase. This enzyme hydrolyses sucrose to the polysaccharide levan, which is toxic to Gram-negative bacteria [246]. Therefore, the selection on LBA supplemented with sucrose ensures that the plasmid is lost by the cells. The chances of selecting the knockout or the wild type *A. baumannii* ATCC 19606 are 50%, respectively.

The plasmid pBIISK-sacBkanR was kindly provided by Prof. Dr. Beate Averhoff (University of Frankfurt, Germany). It was linearized using the endonuclease PstI and the up- and downstream regions (1400 - 2000 bp) of the gene of interest were fused into the linearized vector using the In-Fusion® HD EcoDry™ Cloning Kit. Primers are listed in Table 2.20. The plasmid was transformed into *E. coli* HST08 Stellar© cells by heat shock and transformants were selected on LBA plates supplemented with 50 mg/L kanamycin. Positive transformants were confirmed by PCR and the plasmid-DNA was isolated using the QIAprep Spin Miniprep Kit. Next, the plasmid was transformed into *A. baumannii* ATCC 19606 by electroporation and transformants were selected on LBA supplemented with 50 mg/L kanamycin. Afterwards, counter-selection was performed, first overnight in LB broth containing 10% sucrose at 37°C and 220 rpm shaking, followed by serial dilution of the overnight culture and plating onto LBA plates supplemented with 10% sucrose. Only the wild type or knockout strains, which have lost the plasmid, are able to grow on sucrose. Successful *A. baumannii* knockouts were confirmed by PCR screening, Sanger sequencing and/or WGS. All primers used for the selection and confirmation of knockouts are listed in Table 2.20.

Table 2.20: Primers for creation of a markerless gene knockout in *A. baumannii* ATCC 19606

Primer	Sequence (5' to 3')	Gene (amplicon size in bp)	Reference
J72	AGAGCATCGAATTCCTCGTATGCCAGGTATTGATG	Upstream region *adeN* (1605)	This thesis
J73	CTGTTGTTGGCTGGGAAAATTTAATAACGACGGCT		
J74	TCGTTATTAAATTTTCCCAGCCAACAACAGATAAT	Downstream region *adeN* (1425)	
J75	GTGGATCCCCCGGGCTAAGCATTGGTCAGAAGAGG		
J76	AGAGCATCGAATTCCTCTACTTTAGCGTATGGTGC	Upstream region *pmrAB* (1990)	[230]
J77	AAACAAGAGCTTACGGTTCTAGGCTCGCTTTAGTT		
J78	AAGCGAGCCTAGAACCGTAAGCTCTTGTTTCACTT	Downstream region *pmrAB* (1720)	
J79	GTGGATCCCCCGGGCGGATTTTAATTGTCGGCTGG		
J70	CAGTTTCATTTGATGCTCGA	Flanking region of PstI restriction site in plasmid pBIISK-sacBkanR	This thesis
J71	CGTATGTTGTGTGGAATTGT		
J49	GGAAAGCCACGTTGTGTCTC	Amplification of the sacBkanR cassette for confirmation of plasmid loss in knockout strains	[55]
J52	GCTCTGCCAGTGTTACAACC		
J45	CGTAACACCCTAACCAGAAA	Confirmation of *adeN* knockout	This thesis
J48	AGCATGCTTGAAATAACACG		
H48	GCGAGGAGCACATTTCCTAA	Confirmation of *pmrAB* knockout	[194]
H56	CCGACTTGTGATACGAATGC		

2.2.14. Ethidium bromide accumulation assay

The accumulation of ethidium bromide in *A. baumannii* cells was measured as described by Nowak *et al.* [159]. Briefly, bacterial cells were grown in LB medium till the mid-log phase was reached (OD_{600} 0.7 - 0.9). The cells were centrifuged at 4000 g and 4°C for 5 min and washed twice with potassium phosphate buffer (50 mM potassium phosphate buffer, 1 mM magnesium sulfate (MgSO4), pH 7.4). The cells were adjusted to an OD_{600} of 20, transferred into a black 96-well plate and supplemented with 0.2% (w/v) glucose. A final concentration of 10 µM ethidium bromide was used and the fluorescence was measured every 10 sec for 30 min using the Infinite M1000 PRO plate reader (λexcite 530nm, λemit 600nm). The experiment was conducted at least three times independently.

2.2.15. Analysis of Lipid A composition

2.2.15.1. LPS and Lipid A extraction

For LPS extraction, *A. baumannii* strains were cultured in 250 ml LB medium at 37°C and 220 rpm. To see if the composition of lipid A varied in different growth phases, the bacteria were cultured overnight or until the mid-log phase was reached (OD_{600} of 0.7 - 0.9). The cultures were centrifuged at 4000 g for 15 min and the cell pellet was washed twice with phosphate buffered saline (PBS).

Approximately 200-400 mg of the cell pellet was subjected to LPS extraction following the Tri-Reagent method as described by Yi and Hackett [247] with some minor modifications. The cell pellet was resuspended in 400 µl Tri-Reagent and incubated at RT for 20 min. After incubation, 20 µl of chloroform/mg cells was added. The mixture was strongly vortexed and incubated for an additional 10 min at RT. After a centrifugation step at 12000 g for 10 min, the aqueous phase was transferred into a new tube and fresh chloroform as well as 300 µl ultrapure water were added to the organic phase, followed by an additional incubation and centrifugation step. To ensure complete removal of LPS, this step was repeated twice. For purification of the collected aqueous phase, 500 µl chloroform were added and the incubation and centrifugation steps were repeated. Afterwards, the aqueous phase was transferred into a new tube and the volume was reduced to approximately 200 µl in a speed vac. Depending on the experiment, LPS was further subjected to purification by the cold ethanol magnesium precipitation method [248] for SDS-PAGE analysis or lipid A was further isolated from LPS by mild acid hydrolysis as described by Yi and Hackett [247] for mass spectrometry.

2.2.15.2. Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

For quality control, the LPS samples were visualized on an SDS-PAGE gel. For this purpose, the LPS was subjected to the cold ethanol magnesium precipitation method [248]. LPS was resuspended in 500 µl ice cold 95% ethanol containing 0.375 M magnesium chloride and centrifuged at 12000 g for 15 min. The pellet was dissolved in 200 µl distilled water. About 15-20 µl of LPS were mixed with 2-5 µl 10x Laemmli buffer and incubated at 95°C for 5 min. After cooling the samples on ice, the samples were loaded onto a 16% SDS-PAGE gel. Gel

composition and buffer recipes are shown in Table 2.21. The gel was freshly poured using commercially available Criterion™ empty gel cassettes. Gel electrophoresis was performed in 1 x TGS running buffer using a Criterion™ vertical electrophoresis chamber at 100 V for 15 min and 150-200 V for another 30-60 min.

Table 2.21: SDS-PAGE gel and buffers

Gel or buffer	Composition
Stacking gel	2.5 mL 0.65 M Tris-HCl, pH 6.8 100 µl 10 % SDS 1 ml acrylamide solution (40%) 100 µl 10 % APS 10 µl TEMED 6.1 ml H_2O
16 % Separation gel	2.5 ml 1.5 M Tris-HCl, pH 8.8 100 µl 10 % SDS 4 ml acrylamide/bisacrylamide (37:5:1) 150 µl 10 % APS 15 µl TEMED 3.5 ml H_2O
10x Tris/glycine/SDS (TGS) (SDS-PAGE running buffer)	25 mM Tris 192 mM glycine 0.1% SDS pH 8.3
10x Laemmli buffer	0.06 M Tris-HCl pH 6.8 2 % SDS 25 % glycerol 0.2 % saturated bromphenol blue solution 10 % β-mercaptoethanol

To visualize extracted LPS or Lipid A, the SDS-PAGE gel was stained using silver staining according to Blum *et al.* [249]. This method is based on silver ions binding to proteins or lipids and is especially sensitive (detection limit 0.1 – 1 ng). All buffers used for silver staining were prepared fresh and are listed in Table 2.22. The gel was incubated in fixation solution for 20 min. Afterwards, the gel was washed with 50% methanol for 10 min and again with ultrapure water for 10 min. To increase resolution and sensitivity, the gel was incubated in thiosulfate solution for 1 min. The gel was washed twice in ultrapure water (1 min) and incubated in a silver nitrate solution for 20 min in the fridge. The solution was removed, and the developer solution was added and incubated till the LPS or Lipid A was visible on the gel. Directly afterwards the stop solution was added, and the gel was washed with ultrapure water. Pictures were taken using the Gel Doc XR+ System.

Table 2.22: Silver staining buffer

Buffer	Recipe
Fixation solution (1 L)	500 ml methanol 120 ml acetic acid 0.5 ml 37 % formaldehyde 380 ml ultrapure water
50 % methanol (100 ml)	50 ml methanol 50 ml ultrapure water
0.02 % thiosulfate solution (100 ml)	0.02 g thiosulfate dissolved in 100 ml ultrapure water
0.1 % silver nitrate solution (50 ml) (sensitive to light and heat)	0.1 g silver nitrate ($AgNO_3$) dissolved in 50 ml ultrapure water
Developer solution (100 ml)	3 g sodium carbonate (Na_2CO_3) 50 µl 37% formaldehyde pinch of thiosulfate dissolved in 100 ml ultrapure water
Stop solution (300 ml)	15 ml acetic acid 285 ml ultrapure water

2.2.15.3. Mass spectrometry analysis

To analyse the lipid A composition using mass spectrometry, lipid A was isolated by mild acid hydrolysis [247]. Crude LPS was resuspended in 500 µl 1% SDS in 10 mM sodium acetate (pH 4.5). After the sample was dissolved completely by incubation in an ultrasound bath, the mixture was boiled at 100°C for 1 h and afterwards dried in a speed vac. The mixture was then washed with water and acidified ethanol, centrifuged at 200 g for 10 min and again washed twice with 95% ethanol. After centrifugation, the lipid A pellet was dried overnight at RT and subjected to mass spectrometry measurements.

The mass spectrometry analysis was done using a Bruker Biflex III MALDI-TOF mass spectrometer as described previously [230]. Briefly, the crude lipid A extract was resolved in chloroform:methanol:H_2O in a ratio of 4:4:1 [v/v]. Afterwards, it was subjected to an ultrasound bath for 20 min followed by a centrifugation step at 13000 rpm for 2 min. 1 µl of the sample was mixed 1:1 with the matrix (5-Chloro-2-mercaptobenzothiazole and ammonium-EDTA), spotted directly onto the MALDI target plate and air-dried. Mass spectra were measured using negative ion mode within a mass range of 500–3,000 Da and 300 shots were accumulated for each mass spectrum [230, 250]. Furthermore, some samples were also sent to the Mass spectrometry Core Facility, Institute of Biochemistry and Molecular Biology (University of Bonn, Germany), where MALDI-TOF mass spectrometry was performed as described above and the data for lipid A composition analysis was provided. Data analysis was done using flexAnalysis software (Table 2.13).

2.2.16. *In vivo* virulence in *Galleria mellonella*

The *in vivo* virulence of *A. baumannii* isolates was conducted by Jonathan W. Betts (University of Surrey, Great Britain) using the *G. mellonella* invertebrate model (TruLarv TM, Biosystems Technology, Exeter, United Kingdom) as previously described [230, 251]. In total, 5 isolate pairs were tested: tigecycline isolate pair 1 (MB-R-2, MB-S-5) and the colistin isolate pairs 1-4. Briefly, 10 larvae were infected with each *A. baumannii* isolate by injecting 10^5 cfu/larvae into the left proleg, while PBS was used in the control injections. The larvae were incubated aerobically at 37 °C. The survival (live/dead) at 0, 24, 48, 72 and 96 h post infection as well as the melanisation scores as an indicator of morbidity were recorded [252]. All assays were performed in triplicates and results were pooled for analysis. Statistical analysis was carried out using unpaired t-test at 96 h [251].

3. Results

3.1. Description and characterisation of *A. baumannii* isolates and knockout strains

3.1.1. Characterisation of *A. baumannii* isolate pairs

In total, eight isolate pairs were identified as having the same isogenic background as demonstrated by cgMLST (<10 allele differences) and displayed an increase in tigecycline or colistin MICs by at least one dilution step.

3.1.1.1. Tigecycline isolate pairs

Four isolate pairs were identified to investigate tigecycline resistance. Isolates representing an isolate pair were collected from the same patient and the isolates with low tigecycline MICs (≤ 2 mg/L) preceded the isolates with high tigecycline MICs (≥ 4 mg/L) (Table 3.1). Two isolate pairs were recovered from patients in Seville, Spain, one isolate pair from Valencia, Spain and one from Athens, Greece. Except for the isolates from Greece (IC1), all isolates belonged to the most widespread clonal lineage IC2. Isolate pair 1 and 2 displayed a shift in tigecycline MICs from low (MIC 1 and 2 mg/L) to high (MIC 16 mg/L), respectively. The tigecycline MICs of isolate pair 3 and 4 differed by one dilution step (shift from 2 mg/L to 4 mg/L). The difference in tigecycline MICs was seen consistently in three independent experiments in all isolate pairs. Moreover, isolate pair 4 was the only isolate pair recovered during tigecycline therapy.

Table 3.1: *A. baumannii* isolate pairs for the investigation of tigecycline resistance mechanisms
Four isogenic pairs of clinical *A. baumannii* isolates were chosen to investigate tigecycline resistance mechanisms. Tigecycline MICs were determined by agar dilution, clonal lineages were determined by cgMLST and the Oxford MLST type was determined using pubMLST. F, sample recovered at the end of the patient's antibiotic treatment, S, stool sample, R, respiratory tract sample. Adapted from [227].

Isolate pair No.	**1**		**2**		**3**		**4**	
Isolate	**MB-R-2**	**MB-S-5**	**MB-R-7**	**MB-S-43**	**MB-S-271**	**MB-S-273**	**MB-R-131**[a]	**MB-S-1044**
Patient	01-001		01-002		02-077		22-168	
Antibiotic treatment[b]	Colistin		Colistin		Colistin		Colistin	
Year of isolation	2012		2012		2013		2014	
Date and day[c] **of isolation**	May 13	May 24	June 12	June 19	May 17	May 21	May 6	May 10
	3	F	8	F	3	F	0	F
Sample type	R	S	R	S	S	S	R	S
Origin	Sevilla, Spain		Sevilla, Spain		Valencia, Spain		Athens, Greece	
Clonal lineage	IC2		IC2		IC2		IC1	
Oxford MLST type	ST-208		ST-208		ST-1117		ST-1567	
MIC [mg/L]	1	16	2	16	2	4	2	4
Locus tag differences	1		3		2		1	

[a] first respiratory isolate of the patient, [b] the antibiotic treatment during the patient's hospital stay, [c] hospital day after enrolment into the clinical trial

3.1.1.2. Colistin isolate pairs

Another four isolate pairs were selected to investigate colistin resistance (Table 3.2). The isolates were either from the same patient or from different patients within the same hospital. Two isolate pairs were collected from patients in Seville, Spain and one isolate pair from a hospital in Athens, Greece. These isolates belonged to the clonal lineage IC2. The fourth isolate pair originated from Frankfurt, Germany and belonged to the clonal lineage IC4. Except for isolate pair 2, all isolates were recovered during colistin therapy and the isolates exhibited an increase in colistin MICs by at least four dilutions steps as determined by broth microdilution. Except for isolate pair 3, all isolate pairs displayed an increase in colistin MICs from 1 or 2 mg/L to 256 mg/L. Both isolates of isolate pair 3 were colistin-resistant, but their colistin MICs still differed by at least four dilution steps (32 vs. ≥ 256 mg/L). Isolate pair 1 was recovered from one patient and isolate MB-R-2 preceded the isolate MB-S-6. Isolate pairs 2 and 3 were obtained from different patients but the isolates with low colistin MICs preceded the isolates with high colistin MICs. Isolate pair 4 was recovered from one patient who was infected with both isolates at the same time when transferred to the hospital. This isolate pair exhibited a

shift in colistin MICs from 1 to 256 mg/L. All MIC differences were seen consistently in three independent experiments.

Table 3.2: *A. baumannii* isolate pairs for the investigation of colistin resistance mechanisms
Four isogenic pairs of clinical *A. baumannii* isolates were chosen to investigate colistin resistance mechanisms. Colistin MICs were determined by broth microdilution, clonal lineages were determined by cgMLST and the Oxford MLST type was determined using pubMLST. F, sample recovered at the end of the patient's antibiotic treatment, S, stool sample, R, respiratory tract sample, W, wound sample. Adapted from [230].

Isolate pair No.	**1**		**2**		**3**		**4**	
Isolate	**MB-R-2**	**MB-S-6**	**MB-S-23**	**MB-S-177**	**MB-R-90[a]**	**MB-R-119[a]**	**SG3161**	**SG3166**
Patient	01-001		01-016	01-041	21-107	21-140	KBT6804357	
Antibiotic treatment[b]	Colistin		Meropenem		Colistin		Colistin	
Year of isolation	2012		2012	2013	2013	2014	2016	
Date and day[c] of isolation	May 13	May 24	Oct. 25	Feb. 04	Nov. 18	Feb. 06	March	
	3	F	8	F	3	F	not known	
Sample type	R	S	S	S	R	R	W	R
Origin	Sevilla, Spain		Sevilla, Spain		Athens, Greece		Frankfurt, Germany	
Clonal lineage	IC2		IC2		IC2		IC4	
Oxford MLST type	ST-208		ST-1114		ST-451		ST-236	
MIC [mg/L]	2	256	2	256	32	>256	1	256
Locus tag differences	4		9		5		1	

[a] first respiratory isolate of the patient, [b] the antibiotic treatment during the patient's hospital stay, [c] hospital day after enrolment into the clinical trial

3.1.1.3. Analysis of antimicrobial susceptiblity, resistomes and growth of isolate pairs

The susceptibility of the eight isolate pairs to an additional 12 antimicrobial agents besides tigecycline and colistin was determined (Table 3.3). Since all isolates were resistant to at least nine different antimicrobials from six different antimicrobial classes, the isolates were considered to have an MDR phenotype. Comparing the MICs of colistin susceptible and resistant isolates representing a colistin isolate pair, the MICs were overall very similar. In contrast, significant differences in MICs were observed for isolates comprising a tigecycline isolate pair. In some cases, a 4-fold increase in MICs was observed (e.g. minocycline and gentamicin) and the phenotype was changed from susceptible to resistant to amikacin in tigecycline isolate pair 2, which suggests increased efflux activity (Table 3.3).

Resistome analysis by ResFinder revealed the presence of various resistance determinants in the eight isolate pairs (Figure 3.1). The resistome of isolates representing a tigecycline isolate pair was identical, so that an association of the resistance determinants with tigecycline resistance and reduced antimicrobial susceptibility was excluded. In contrast, the acquired resistomes of colistin isolate pairs were identical only in isolate pair 1 (MB-R-2, MB-S-6) and 3 (MB-R-90, MB-R-119). Isolate pair 2 differed in aminoglycoside and sulphonamide resistance determinants due to the presence of resistance genes *aadA1*, *aac(3')-Ia* and *sul1* in isolate MB-S-23. In isolate pair 4, the isolate SG3166 carried additional aminoglycoside (*strA*, *strB*) and tetracycline (tet(B)) resistance determinants compared to isolate SG3161. None of the differences in the acquired resistome affected the phenotype of the isolates (Table 3.3).

The growth curves of all isolate pairs were analysed and are depicted in Appendix 6. No significant differences were revealed between isolates representing an isolate pair. Nevertheless, the colistin-resistant isolates MB-R-119 and SG2166 had slightly reduced growth compared to the isolates with lower colistin MICs, while isolate MB-S-177 had slightly elevated growth compared to MB-S-23.

Table 3.3: MICs of different antimicrobial agents of the isolate pairs

The MICs of twelve different antimicrobial agents [mg/L] were determined by agar dilution. Colistin MICs were determined either by agar dilution (tigecycline isolate pairs) or microbroth dilution (colistin isolate pairs). Adapted from [227, 230].

	Tigecycline isolate pairs							
	Isolate pair #1		**Isolate pair #2**		**Isolate pair #3**		**Isolate pair #4**	
Antimicrobial	**MB-R-2**	**MB-S-5**	**MB-R-7**	**MB-S-43**	**MB-S-271**	**MB-S-273**	**MB-R-131**	**MB-S-1044**
Tigecycline	1	16	2	16	2	4	2	4
Tetracycline	≥256	≥256	≥256	≥256	≥256	≥256	8	8
Minocycline	8	16	4	16	4	32	2	2
Ciprofloxacin	64	≥256	64	≥256	128	≥256	64	128
Levofloxacin	8	64	16	64	8	16	8	32
Amikacin	64	≥256	8	≥256	16	16	≥256	≥256
Gentamicin	16	128	8	128	≥256	≥256	≥256	≥256
Chloramphenicol	128	≥256	128	≥256	≥256	≥256	≥256	≥256
Azithromycin	64	128	32	128	32	32	128	128
Erythromycin	32	128	32	128	64	64	64	64
Meropenem	8	8	8	8	128	≥256	64	64
Rifampicin	128	128	64	128	8	8	4	4
Colistin[a]	2	4	2	2	2	2	32	≥256
	Colistin isolate pairs							
	Isolate pair #1		**Isolate pair #2**		**Isolate pair #3**		**Isolate pair #4**	
Antimicrobial	**MB-R-2**	**MB-S-6**	**MB-S-23**	**MB-S-177**	**MB-R-90**	**MB-R-119**	**SG3161**	**SG3166**
Tigecycline	1	4	32	4	8	8	1	2
Tetracycline	≥256	≥256	64	32	≥256	≥256	≥256	≥256
Minocycline	8	8	4	4	32	32	8	8
Ciprofloxacin	64	64	≥256	128	≥256	≥256	≥256	128
Levofloxacin	8	8	128	16	32	128	32	32
Amikacin	64	64	≥256	≥256	≥256	≥256	64	128
Gentamicin	16	16	≥256	8	≥256	≥256	4	2
Chloramphenicol	128	128	≥256	≥256	≥256	≥256	128	128
Azithromycin	64	32	128	32	32	32	32	16
Erythromycin	32	32	64	32	32	32	64	32
Meropenem	16	16	32	64	128	64	64	16
Rifampicin	128	128	4	8	8	4	4	4
Colistin	2	256	2	256	32	≥256	1	256

[a] except for MB-R-131, colistin MICs were determined by agar dilution, which is not the reference method for colistin. Results should be treated with caution.

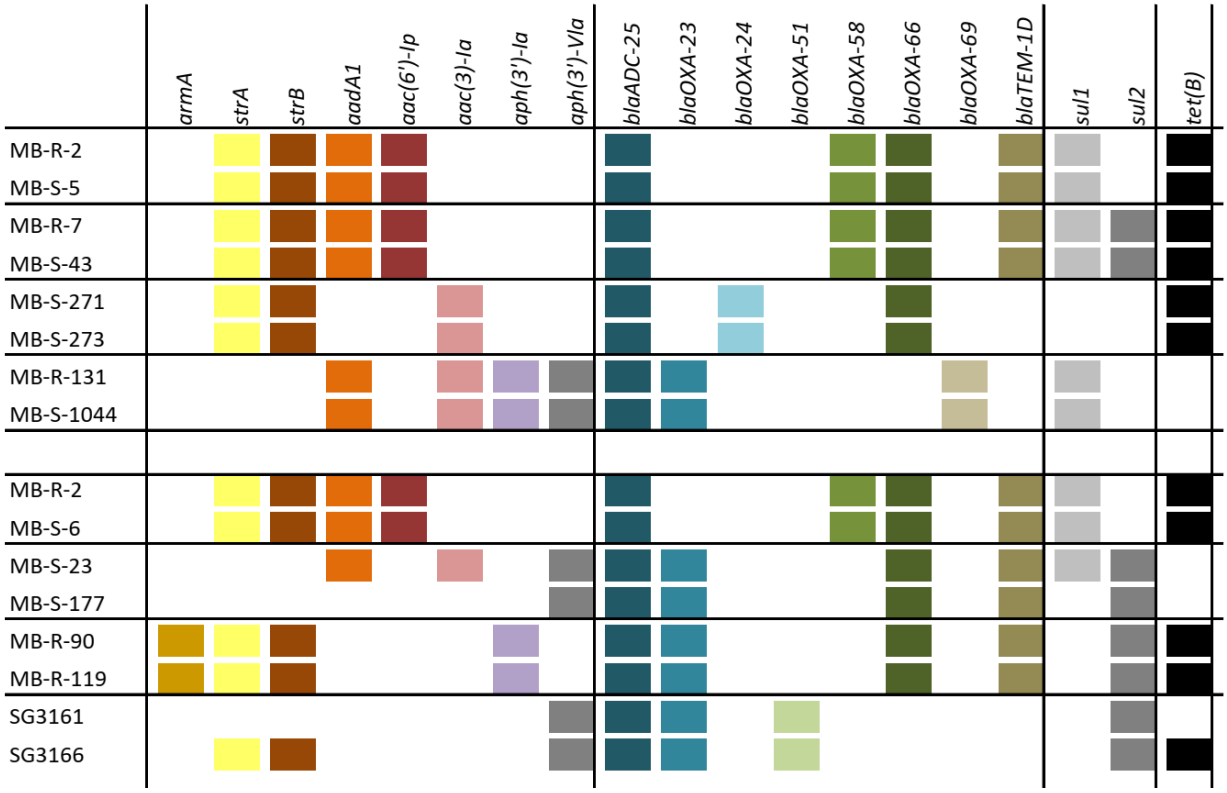


Figure 3.1: Antibiotic resistance determinants detected in *A. baumannii* isolate pairs
Resistomes were determined by ResFinder. Colours indicate the presence of the resistance determinant in the draft genomes of the isolate. Adapted from [227, 230].

3.1.2. Characterisation of first respiratory *A. baumannii* isolates per patient

In addition to the eight isolate pairs, the first respiratory isolate per patient collected during the MagicBullet clinical trial were included in this study (65 *A. baumannii* isolates) [27]. An overview about their origin, clonal lineages as well as tigecycline and colistin MICs is depicted in Table 3.4. Most respiratory isolates belonged to the international clonal lineage IC2 (n = 54), while five isolates belonged to IC1 based on cgMLST. Previously, six isolates have been categorized as not related to any of the known clonal lineages [227], however an additional investigation by cgMLST revealed 3 isolates to be variants of IC2. In total, ten isolates displayed low tigecycline MICs (MICs ≤ 2 mg/L), while all other isolates showed tigecycline MICs ranging from 4 to 16 mg/L. For colistin, 33 isolates were identified as susceptible and 32 as resistant. More details for all isolates are given in Appendix 2 and Appendix 4.

Table 3.4: Overview of 65 first respiratory *A. baumannii* isolates.

The isolates were obtained from Greece, Italy and Spain during the MagicBullet clinical trial. Clonal lineages were determined by cgMLST. Tigecycline MICs were determined by agar dilution, while colistin susceptibility was tested by microbroth dilution. UN = not related to any of the known clonal lineages. Adapted from [227].

Country		Greece	Italy	Spain	Total
No. of cities (hospitals)		4 (7)	2 (3)	4 (5)	10(15)
No. of patients		37	14	14	65
Clonal lineages (no. of isolates)		IC1 (5), IC2 (32)	IC2 (13), UN (1)	IC2 (12), UN (2)	IC1 (5), IC2 (57), UN (3)
Tigecycline MICs [mg/L]	≤ 2	6 (16.2%)	1 (7.1%)	3 (21.4%)	10 (15.4%)
	≥ 4	31 (83.8%)	13 (92.9%)	11 (78.6%)	55 (84.6%)
Colistin MICs [mg/L]	≤ 2	15 (40.5%)	8 (57.1%)	10 (71.4%)	33 (50.8%)
	≥ 4	22 (59.5%)	6 (42.9%)	4 (28.6%)	32 (49.2%)

Resistome analysis revealed a large variety of resistance determinants in the isolates (Figure 3.2). In total, 15 different aminoglycoside resistance determinants, 13 β-lactamases and 9 resistance genes associated with e.g. sulphonamide, tetracycline and macrolide resistance were detected. More than 50% of isolates carried the aminoglycoside resistance genes *armA*, *strA*, *strB*, *aph(3')-Ia* and *aph(3')-VIa*, while *aac(3)-IIa* and *aac(6')-Ian* were detected only in isolates not related to any known clonal lineage (Figure 3.2 A). All isolates carried the intrinsic OXA-51 or OXA-51-like (OXA-66, -69, -82, -387 and -391) β-lactamase. Moreover, an *ampC*-like gene (bla_{ADC-25}) was detected in all but one isolate and more than 50% of isolates harboured the bla_{OXA-23} and the bla_{TEM-1D} gene (Figure 3.2 B). Several other resistance determinants were found in the isolates, including *sul2* (sulphonamide resistance) and *tet(B)* (tetracycline resistance), which were present in more than 50% of isolates (Figure 3.2 C). An overview of all resistance determinants is also presented in Appendix 3.

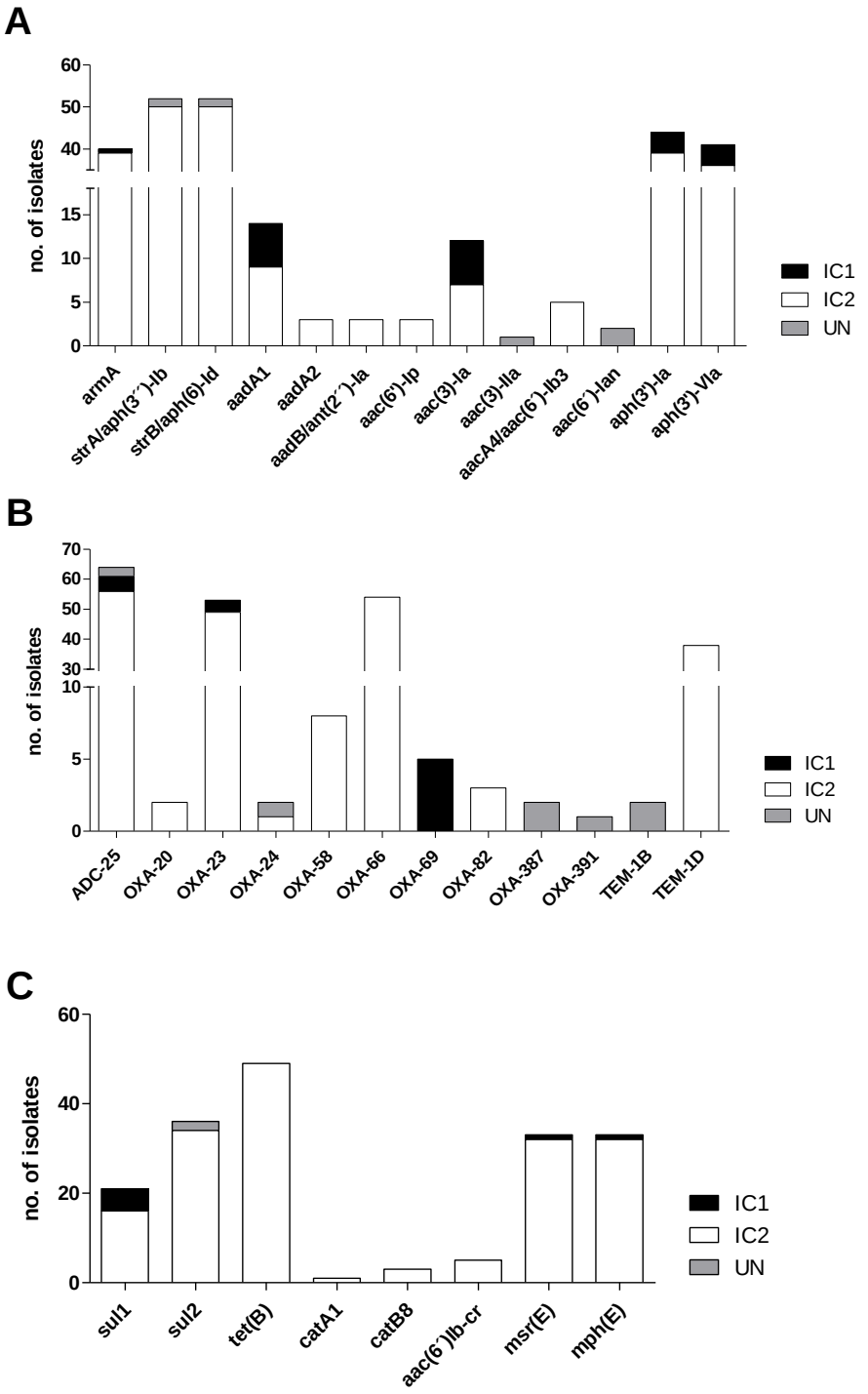


Figure 3.2: Overview of acquired resistance determinants in *A. baumannii* isolates

The resistome of *A. baumannii* isolates was determined by ResFinder (settings: 60% minimum length, 90% identity threshold). Represented are all resistance determinants detected in the first respiratory *A. baumannii* isolate per patient (n = 65). The resistance determinants were sorted according to the clonal lineage IC1, IC2 and unrelated to any known clonal lineage (UN). (A) aminoglycoside resistance determinants, (B) β-lactamases, (C) other resistance determinants: sulphonamides (*sul1*, *sul2*), phenicols (*catA1*, *catB8*), tetracycline (*tet(B)*), fluoroquinolones (*aac(6')Ib-cr*) and macrolides (*msr(E)*, *mph(E)*).

The prevalence of different efflux pumps was investigated in the 65 *A. baumannii* isolates and the results are summarised in Table 3.5. The majority of isolates contained at least 12 different efflux pumps and two efflux pumps (CmlA, Tet(A)) were not detected in any isolate. AdeB was absent in four isolates, while two uncharacterised RND-type efflux pumps (RND1 and RND3) were missing in 2 and 10 isolates, respectively. Moreover, 49 isolates contained Tet(B), while the MFS-pumps ErmB and ErmB-like II were missing in one and two isolates, respectively.

Table 3.5: Prevalence of efflux pumps in *A. baumannii* isolates
The different efflux pumps were detected in the draft genomes by PCR or by a BLASTn search.

Efflux pump family	Efflux pump transporter	Total no. of isolates (%)
RND	AdeB	61 (93.8)
	AdeJ	65 (100)
	AdeG	65 (100)
	AbeD	65 (100)
	ArpB	65 (100)
	RND1 (ACICU_RS00750)	63 (96.9)
	RND2 (ACICU_RS15300)	65 (100)
	RND3 (ACICU_RS17095)	55 (84.6)
MATE	AbeM	65 (100)
SMR	AbeS	65 (100)
ABC	MacB	65 (100)
PACE	AceI	65 (100)
MFS	CraA	65 (100)
	AmvA	65 (100)
	CmlA	0
	ErmB	64 (98.5)
	ErmB-like I	65 (100)
	ErmB-like II	63 (96.9)
	Tet(A)	0
	Tet(B)	49 (75.4)

3.1.3. Characterisation of *A. baumannii* knockout strains

To investigate the tigecycline and colistin resistance mechanisms, *adeN* and *pmrAB* gene knockout strains were created using the reference strain *A. baumannii* ATCC 19606 as wild type. In addition, an *adeRS* knockout strain has been created by Kai Lucaßen (University of Cologne) [223].

Analysis of gene expression by qRT-PCR revealed a significant increase of *adeJ* expression in the *adeN* knockout strain compared to the wild type strain *A. baumannii* ATCC 19606 (Figure 3.3 A). Since *adeN* is a transcriptional repressor, an increase of expression was expected after deletion of the gene. In contrast, the expression of *adeB* and *pmrC* was strongly decreased in the *adeRS* and *pmrAB* knockout strains, respectively (Figure 3.3 A). Therefore, all knockout strains seem to be suitable as controls in comparison to clinical *A. baumannii* isolates in qRT- PCR experiments. Moreover, the growth curves of all three knockout strains were

analysed in comparison to the wildtype strain ATCC 19606 (Figure 3.3 B). The growth rates were similar between the isolates indicating that the absence of *adeN*, *adeRS* or *pmrAB* has no effect on bacterial growth.

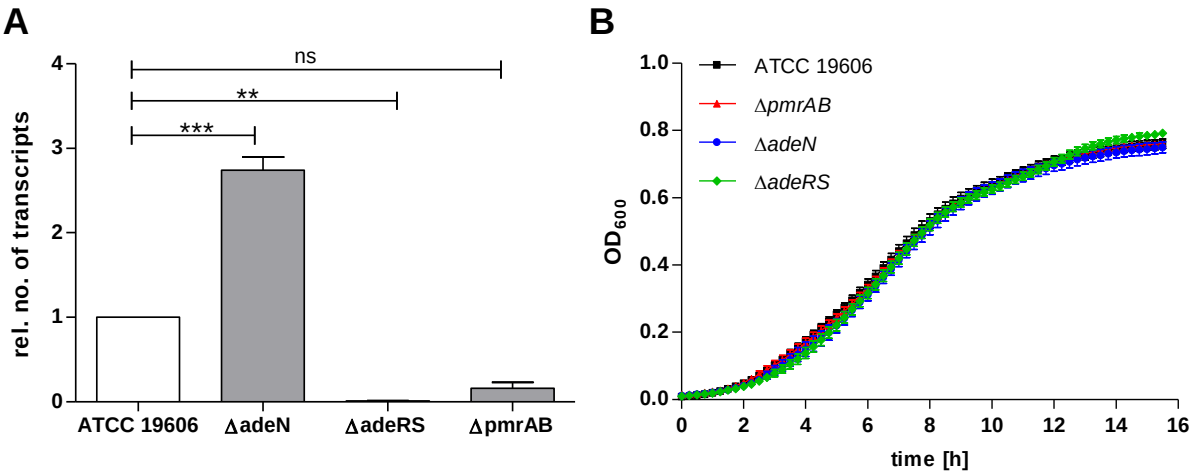


Figure 3.3: Relative gene expression and growth curves of *A. baumannii* knockout strains

(A) Relative gene expression was determined by qRT-PCR. Depicted are the expression of the RND-type efflux pumps *adeJ* and *adeB* in knockout strains Δ*adeN* and Δ*adeRS* and of the acetyltransferase *pmrC* in knockout strain Δ*pmrAB,* respectively. The number of transcripts was normalized to the expression of the reference gene *rpoB* and compared to the wild type strain *A. baumannii* ATCC 19606, which was set as 1. Results are represented as mean ± SEM. Statistical analysis was done by performing an unpaired t-test using the recorded absolute values. *** p <0.001, ** p<0.01, * p<0.05, ns = not significant. (B) Growth curves of the knockout strains Δ*adeN* (blue), Δ*adeRS* (green) and Δ*pmrAB* (red) compared to the wild type strain *A. baumannii* ATCC 19606 (black). Growth curves were conducted overnight using the Infinite plate reader. Represented is mean ± SEM of three independent experiments.

Resistome analysis by ResFinder revealed the presence of both an AmpC-like (ADC-25) and OXA-51-like (OXA-98) β-lactamase as well as the sulphonamide resistance determinant *sul2* in the reference strain ATCC 19606 and the three knockout strains.

To analyse the phenotype of the *adeJ*, *adeB* and *pmrC* knockouts, the MICs of different antimicrobial agents were determined (Table 3.6). While the deletion of *pmrAB* did not change the MICs of any antimicrobial agent except for meropenem, the MICs of ciprofloxacin, erythromycin, levofloxacin, minocycline, rifampicin, tetracycline, and tigecycline were increased by one dilution step in Δ*adeN* compared to the wild type *A. baumannii* ATCC 19606. The MICs of the *adeRS* knockout were previously determined and decreased for all antimicrobials tested compared to the wild type ATCC 19606, which indicates that a loss of the efflux pump AdeRSABC significantly reduces the antimicrobial resistance of *A. baumannii* [223].

Table 3.6: MICs of different antimicrobial agents of *A. baumannii* knockout strains

The MICs [mg/L] of twelve different antimicrobial agents were determined by agar dilution and in case of colistin by microbroth dilution in three independent experiments. The MICs of the *adeRS* knockout were previously determined and kindly provided by Kai Lucaßen (University of Cologne) [223]. n.d. = not determined.

	MIC [mg/L]			
Antimicrobial	**ATCC 19606**	**Δ*adeN***	**Δ*pmrAB***	**Δ*adeRS***
Tigecycline	1	2	1	0.25
Tetracycline	8	16	8	2
Minocycline	0.25	0.5	0.25	0.125
Ciprofloxacin	1	2	1	n.d.
Levofloxacin	0.5	1	0.5	0.25
Amikacin	8	8	8	2
Gentamicin	16	16	16	1
Azithromycin	32	32	32	8
Erythromycin	32	64	32	8
Chloramphenicol	≥256	≥256	≥256	128
Colistin	1	n.d.	1	n.d.
Meropenem	0.5	0.5	0.25	0.25
Rifampicin	4	8	4	2

3.2. Tigecycline resistance in *A. baumannii*

Part of the results in Chapter 3.2 have been published in Gerson *et al*. [227].

3.2.1. Investigation of tigecycline resistance mechanisms in isolate pairs

3.2.1.1. Insertion sequences in regulatory genes of the RND-type efflux pumps

Analysis of the draft genome assemblies of the isolate pairs and analysis by IS-Mapper revealed the presence of insertion sequences in regulatory genes of RND-type efflux pumps in isolates with higher tigecycline MICs (MIC ≥ 4 mg/L) (Figure 3.4). IS*Aba1* was inserted in *adeS* in the isolates MB-S-5 and MB-S-43. The insertion site differed by two nucleotides indicating independent insertion events, and the insertion occurred in reverse orientation in both isolates (Figure 3.4 A). In the other two isolates with higher tigecycline MICs, *adeN* was disrupted by different insertion sequences. In isolate MB-S-273, IS*Aba1* was inserted in *adeN*, while the gene was disrupted by IS*Aba125* in MB-S-1044. Both insertions occurred in forward orientation (Figure 3.4 B).

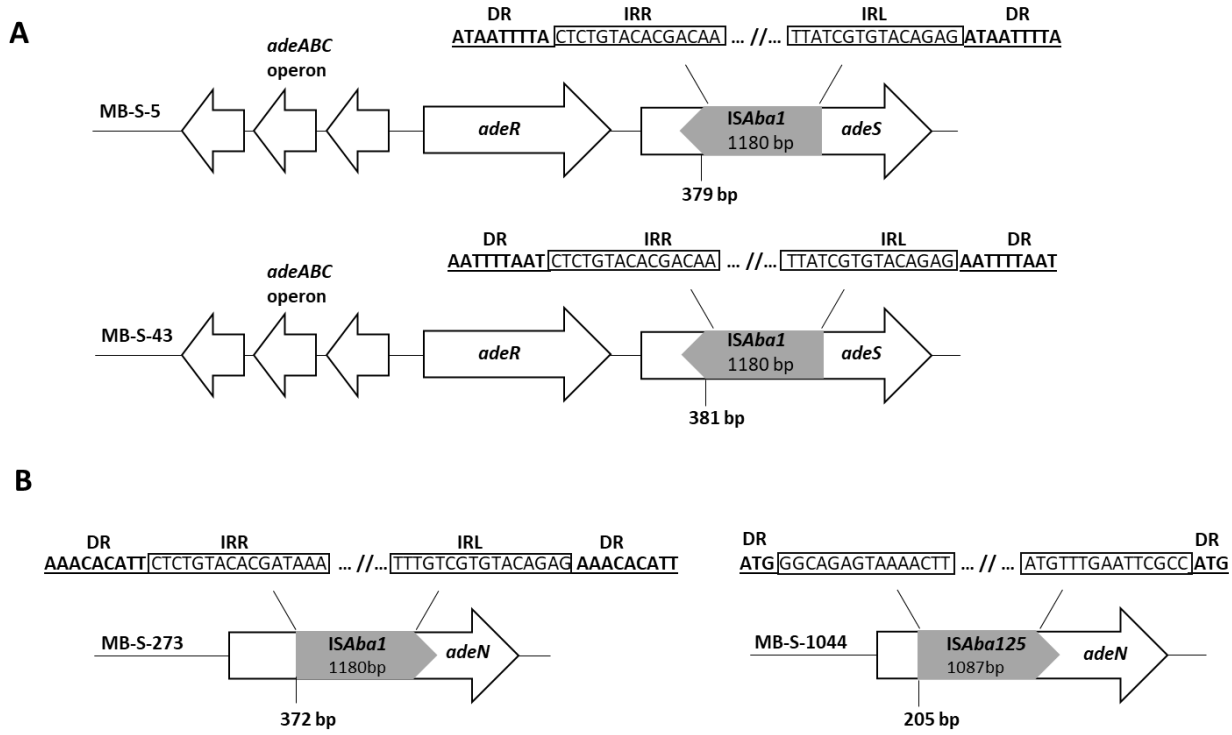


Figure 3.4: Insertion sequences in *adeS* and *adeN* in *A. baumannii* isolates

The insertion of IS*Aba1* or IS*Aba125* was detected using IS-Mapper and by PCR. (A) Insertion of IS*Aba1* in *adeS* in isolates MB-S-5 and MB-S-43. The insertion site differed by two nucleotides and was in reverse orientation. (B) Insertion of IS*Aba1* and IS*Aba125* in *adeN* in forward orientation in isolates MB-S-273 and MB-S-1044. The insertion site is given in base pair. DR = direct repeat. IRR = inverted repeat right. IRL = inverted repeat left. Modified from [227].

Moreover, IS*Aba1* was inserted in reverse orientation in the intergenic region between *adeS* and *adeR* in isolate pair 3 (Appendix 8). Since the insertion occurred in both isolate MB-S-271 and MB-S-273, an association with reduced tigecycline MICs was excluded in this isolate pair.

3.2.1.2. Increased expression of RND-type efflux pumps and increased efflux activity

To investigate the effect of *adeS* and *adeN* disruption, the gene expression of the respective RND-type efflux pumps *adeB* and *adeJ* was measured by qRT-PCR (Figure 3.5). The insertion of IS*Aba1* in *adeS* was associated with a significant increase of *adeB* expression in both isolates ($p \leq 0.01$). The number of *adeB* transcripts was 45-fold higher in isolate MB-S-5 and 35-fold higher in MB-S-43 compared to the isolates with lower tigecycline MICs (Figure 3.5 A). In contrast, the disruption of *adeN* was associated with a 6-fold and 2-fold increased expression *adeJ* in the isolates MB-S-273 and MB-S-1044, respectively (Figure 3.5 B).

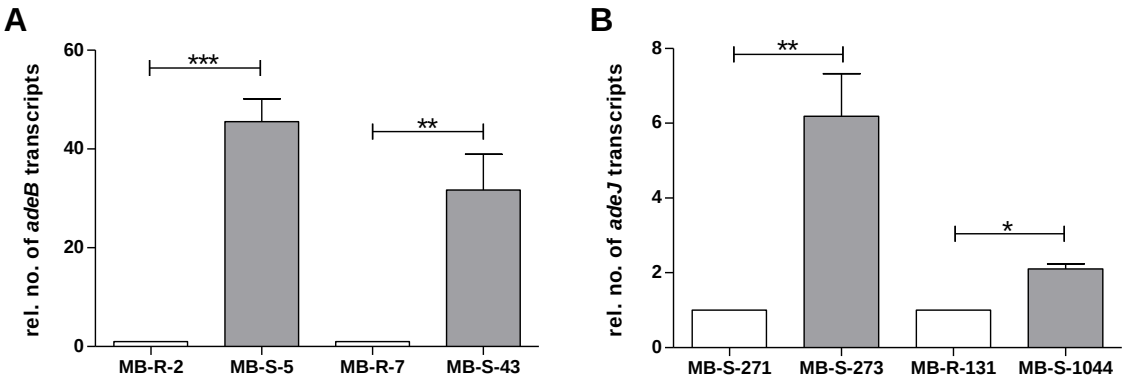


Figure 3.5: Relative expression of *adeB* and *adeJ* in the four isolate pairs

Relative expression of the RND-type efflux pumps *adeB* and *adeJ* was determined by qRT-PCR. (A) Expression of *adeB* of isolate pair 1 (MB-R-2, MB-S-5) and isolate pair 2 (MB-R-7, MB-S-43). (B) Expression of *adeJ* of isolate pair 3 (MB-S-271, MB-S-273) and isolate pair 4 (MB-R-131, MB-S-1044). The number of transcripts was normalized to the expression of the reference gene *rpoB* and compared between isolates with low tigecycline MICs and isolates with high tigecycline MICs. Results are represented as mean ± SEM. Statistical analysis was done by performing an unpaired t-test using the recorded absolute values. *** p <0.001, ** p<0.01, * p<0.05. Modified from [227].

An increased expression of *adeB* is often associated with increased efflux of the respective substrates out of the cells. The efflux activity can be measured by determining the accumulation of ethidium bromide, a substrate of *adeB*, using a fluorometric assay. Due to the higher efflux activity the accumulation of ethidium bromide in the bacterial cell is reduced and the fluorescence signal is lower. In both isolates with elevated *adeB* expression, the accumulation of ethidium bromide was reduced indicating increased efflux activity in both isolates MB-S-5 and MB-S-43 (Figure 3.6).

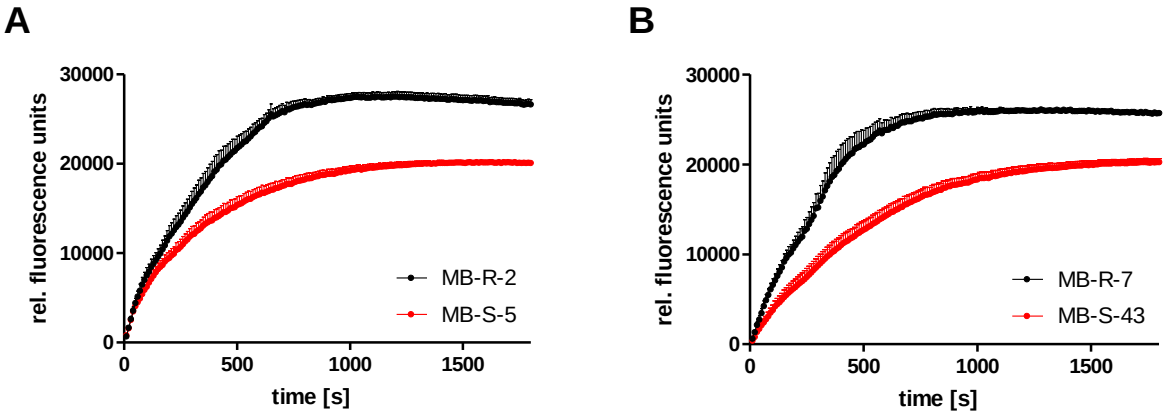


Figure 3.6: Ethidium bromide accumulation in isolates with disrupted *adeS*

The fluorescence intensity was recorded after ethidium bromide was added to the bacterial cultures of isolate pair 1 (MB-R-2, MB-S-5) (A) and isolate pair 2 (MB-R-7, MB-S-43) (B). Fluorescence was measured every 10 seconds at excitation (530 nm) and emission (600 nm), respectively. Represented are mean ± SEM of three independent experiments.

3.2.1.3. Reduced susceptibility in isolate pairs and increased virulence

Since a disruption of the regulatory genes *adeS* and *adeN* led to an increased expression of *adeB* and *adeJ*, respectively, the susceptibility to other antimicrobial agents was tested (Table 3.3, Figure 3.7). Increased *adeB* expression was associated with elevated MICs of antibiotics from at least six different antimicrobial classes, including aminoglycosides (amikacin, gentamicin), tetracyclines (minocycline), fluoroquinolones (ciprofloxacin, levofloxacin), macrolides (azithromycin, erythromycin), chloramphenicol and rifampicin (Figure 3.7). In contrast, elevated *adeJ* expression was associated with an increase in the MICs of minocycline, ciprofloxacin, levofloxacin and meropenem and therefore with a reduction of susceptibility to three different antimicrobial classes. No significant changes in MICs of other antimicrobial agents were observed for isolate pair 3 (MB-S-271, MB-S-273) and isolate pair 4 (MB-R-131, MB-S-1044) (Table 3.3).

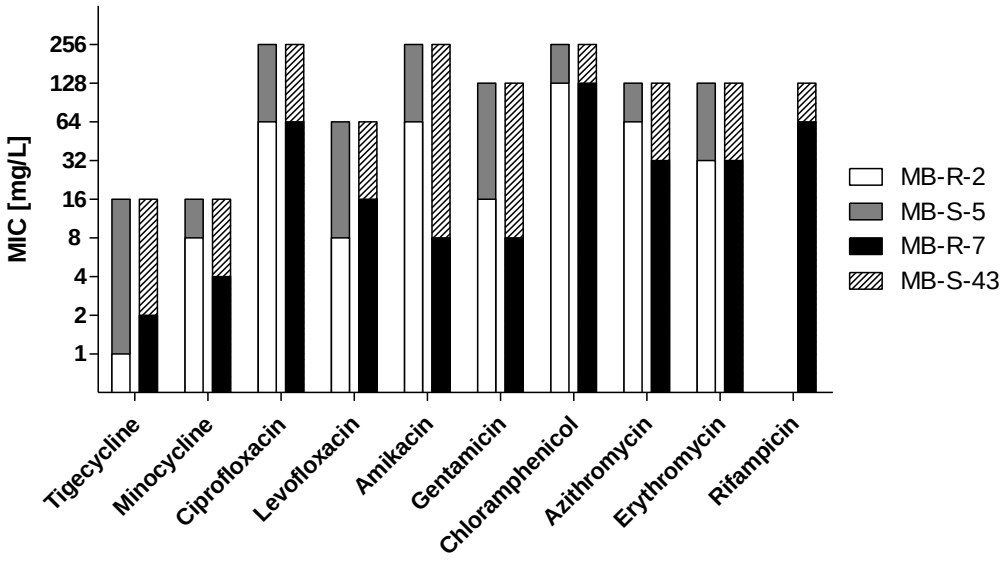


Figure 3.7: Overview of MICs changes in isolates with *adeS* disruption by IS*Aba1*

MICs were determined by agar dilution. Depicted are only antimicrobials for which a change in MICs was observed in the two isolate pairs, i.e. the rifampicin MICs were identical in isolate MB-R-2 and MB-S-5 (isolate pair 1).

Moreover, the virulence of isolate pair 1 (MB-R-2, MB-S-5) was analysed by Jonathan W. Betts (University of Surrey, Great Britain) using the *in vivo G. mellonella* infection model. The larvae were infected with the *A. baumannii* isolates MB-R-2 and MB-S-5 and survival as well as melanisation was assessed (Figure 3.8). The isolate with higher tigecycline MICs (MB-S-5) displayed significantly increased virulence compared to the isolate MB-R-2 (Figure 3.8 A). The larvae infected with the isolate MB-S-5 displayed a survival rate of 10% after 96 h, while 40% of larvae survived an infection with the isolate MB-R-2 ($p \leq 0.02$) (Figure 3.8 A). Similar results were obtained for the melanisation as an indicator of morbidity. The melanisation score was

relatively high for both isolates, but it was significantly higher in larvae infected with the isolate MB-S-5 over the complete time curse ($p \leq 0.018$) (Figure 3.8 B).

In summary, the disruption of *adeS* by IS*Aba1* was associated with a significant increase of *adeB* expression and efflux activity as well as a reduction of susceptibility to six different antimicrobial classes and increased virulence in *G. mellonella*. In comparison, the disruption of *adeN* by IS*Aba1* or IS*Aba125* was associated with elevated *adeJ* expression (lower increase compared to *adeB* expression upon the disruption of *adeS*) and elevation of MICs to three different antimicrobial classes.

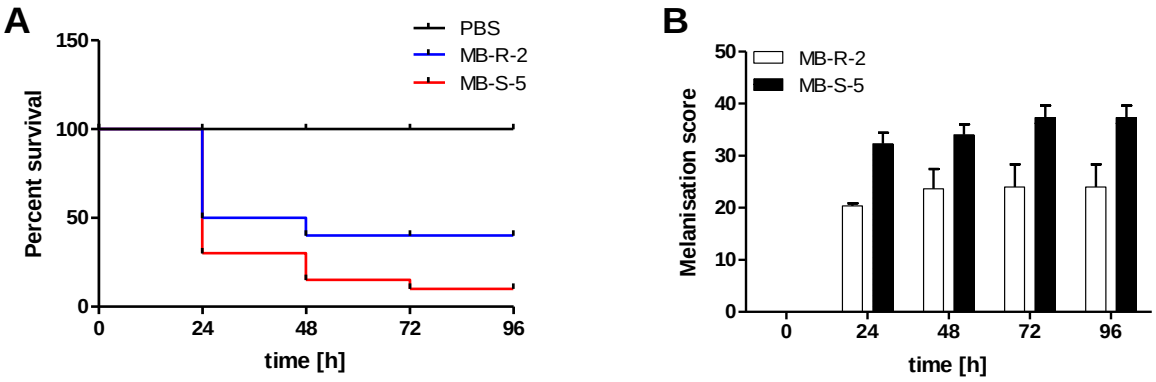


Figure 3.8: Virulence of *A. baumannii* isolate pair in *G. mellonella*
(A) Survival of *G. mellonella* infected with isolate pair 1 (MB-R-2, MB-S-5) using 10^5 cfu/larvae over 96 h. The PBS control is depicted in black, isolate MB-R-2 in blue and MB-S-5 in red. (B) Melanisation observed over 96 h in *G. mellonella* infected with isolate pair 1 (MB-R-2 in white, MB-S-5 in black).

3.2.1.4. Investigation of truncated *adeS* and *adeN*

Sun *et al*. reported that the truncated *adeS* part downstream of IS*Aba1* is overexpressed under the control of a hybrid promoter, which is provided by IS*Aba1* [163]. To determine the expression of the truncated *adeS*, qRT-PCR was performed using primers that anneal to the *adeS* regions up- and downstream of the insertion sequence IS*Aba1* in isolate pair 1 (MB-R-2, MB-S-5) and isolate pair 2 (MB-R-7, MB-S-43), respectively (Figure 3.9 A, B). In both isolates displaying higher tigecycline MICs, the truncated *adeS* upstream of IS*Aba1* was expressed less compared to the isolates with lower tigecycline MICs (Figure 3.9 A). In contrast, the expression of the truncated *adeS* downstream of IS*Aba1* was significantly increased in both MB-S-5 and MB-S-43 (Figure 3.9 B). These results indicate that IS*Aba1* provides a promoter for the overexpression of the truncated *adeS* downstream of IS*Aba1*. Cloning experiments of the respective truncated *adeS* forms should provide insight into the regulatory ability of both *adeS* forms. Unfortunately, cloning experiments of both parts of *adeS* into shuttle vectors and into the *adeRS* knockout strain failed consistently, although the experiment was performed and optimised numerous times.

Similar experiments were conducted for the isolate pairs 3 (MB-S-271, MB-S-273) and 4 (MB-R-131, MB-S-1044) with disrupted *adeN*. In contrast, analysis of gene expression by qRT-PCR revealed unchanged expression of *adeN* upstream of the insertion sequences and significantly reduced expression of *adeN* downstream of IS*Aba1* or IS*Aba125* (Figure 3.9 C, D).

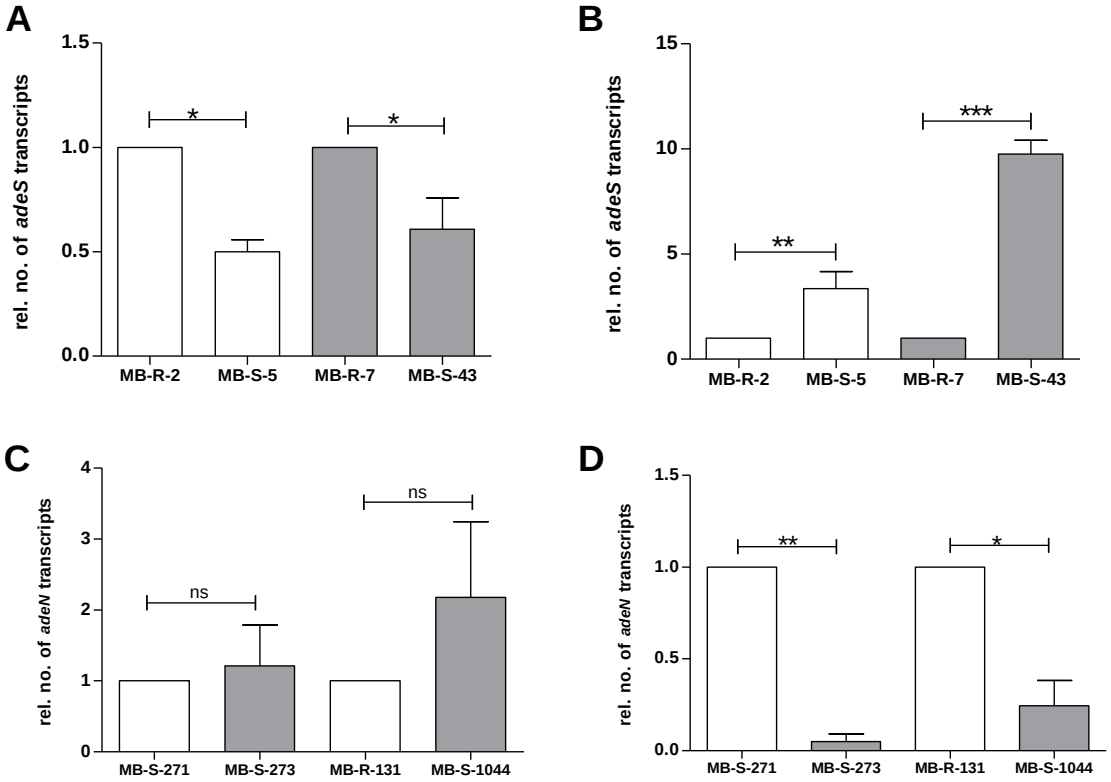


Figure 3.9: Relative expression of *adeS* and *adeN* up- and downstream of insertion sequences
Relative expression of truncated *adeS* and *adeN* in isolate pairs was determined by qRT-PCR. (A) Expression of *adeS* upstream of IS*Aba1*. (B) Expression of *adeS* downstream of IS*Aba1*. (C) Expression of *adeN* upstream of insertion sequences. (D) Expression of *adeN* downstream of insertion sequences. The number of transcripts was normalized to the expression of the reference gene *rpoB* and compared between isolates with low tigecycline MICs and isolates with high tigecycline MICs. Results are represented as mean ± SEM. Statistical analysis was done by performing an unpaired t-test using the recorded absolute values. *** p <0.001, ** p<0.01, * p<0.05, ns = not significant.

Cloning experiments showed that the complementation of the *adeN* knockout strain with the truncated *adeN* from MB-S-273 and MB-S-1044 or with the wild type *adeN* from the isolate MB-S-271 and MB-R-131 did not result in a change of tigecycline MICs (MIC 2 mg/L for all transformants). These results indicate insertional inactivation of *adeN* is most likely not compensated by overexpression of a truncated *adeN*.

3.2.1.5. Other resistance determinants in *A. baumannii* isolate pairs

To exclude the involvement of other resistance determinants or mutations in the reduction of antimicrobial susceptibility in the isolate pairs, the resistome as well as various other efflux pumps and chromosomal genes were analysed. All other genes of the three RND-type efflux

pumps and the five uncharacterised RND-type efflux pumps were identical in isolates representing an isolate pair. Furthermore, no differences in the putative promoter regions of *adeRSABC*, *adeN-IJK* and *adeLFGH* were detected comparing the regions between isolates of an isolate pair or to the reference strain *A. baumannii* ACICU. *A. baumannii* also contains other efflux pumps of the MATE-type (*abeM*), the SMR-type (*abeS*), the MFS-type (*craA*, *amvA, ermAB*), the ABC-type (*macB*) and the PACE-type (*aceI*). The amino acid sequences of all efflux pumps were compared between isolates of an isolate pair and revealed no differences.

Furthermore, the differences in MICs of the fluoroquinolones ciprofloxacin and levofloxacin were not due to *gyrA/B* or *parC/E* since a comparison of these genes between isolates of an isolate pair revealed no differences. Therefore, an association of these genes with changes in antimicrobial susceptibility was excluded and changes in MICs were most likely caused by increased efflux.

Other genes have also been described in association with tigecycline resistance. First, the flavin-dependent monooxygenase TetX and its variants and the MFS pump Tet(A) [91, 165]. These resistance determinants were not detected in any of the *A. baumannii* isolates by ResFinder or through sequence analysis. Besides Tet(A) and TetX, the genes *trm, plsC* and *abrP* were reported to be associated with tigecycline resistance [166, 167, 171]. While *plsC* and *abrP* were identical in isolates representing an isolate pair, the analysis of *trm* revealed the deletion of one nucleotide (position 1077) in both isolates of isolate pair 4 (MB-R-131, MB-S-1044). The frameshift mutation has been previously described to be associated with tigecycline resistance. Since a nucleotide deletion (at position 267 or 1092) occurred also in the *A. baumannii* ABC-isolates, which displayed low tigecycline MICs ranging from 0.25 to 0.5 mg/L, an association with tigecycline resistance is unlikely. In addition, the regulators SoxR and the TCSs BaeSR have been reported in association with tigecycline resistance [218, 219]. The genes encoding for these regulators were identical in isolates representing an isolate pair, so that an association with tigecycline resistance was also excluded.

Furthermore, a closer look at other allele differences detected in the core and accessory genomes of the four isolate pairs was taken. In total, less than 10 allele differences were observed since this was a requirement for the selection of isolates as isogenic pairs. Most of them included hypothetical proteins, proteins with unknown function or proteins, which were assumed to not be associated with efflux pumps or their regulation (Table 3.7).

Table 3.7: Allele differences detected in *A. baumannii* isolate pairs

Draft genomes of *A. baumannii* isolates were aligned to the genome of reference strain ACICU and alleles were compared within an isolate pair to detect differences in the core and accessory genome using SeqSphere. Missing open reading frames were not considered.

Isolate pair #	Allele differences (locus tag)	Annotation	Amino acid difference
1	ACICU_RS15320	Uncharacterized conserved protein	I116S
2	ACICU_RS05250	Hypothetical protein	I221V
	ACICU_RS05255	Hypothetical protein	Q64H
	ACICU_RS09100	Hypothetical protein	N121D
3	ACICU_RS03350	FilE protein	3 amino acid deletion
	ACICU_RS11150	Hypothetical protein	L30V
4	ACICU_RS04105	Hypothetical protein	Y212F

3.2.2. Investigation of resistance mechanisms in other clinical *A. baumannii* isolates

The investigation of isolate pairs revealed that tigecycline resistance/increased tigecycline MICs was associated with the disruption of *adeS* or *adeN,* and there was an increase in RND-type efflux pump expression and reduction in susceptibility to different antimicrobial agents. To determine the prevalence of these insertion sequences and other tigecycline resistance mechanisms in *A. baumannii*, 65 *A. baumannii* isolates from a wide geographical region were investigated. Since only 2 isolates displayed tigecycline MICs ≤ 1 mg/L, the *A. baumannii* ABC-isolates (MIC range, 0.25 - 0.5 mg/L) were additionally used for comparison. All isolates were analysed by WGS and screened specifically for differences in the RND-type efflux pump regulators *adeRS, adeL* and *adeN*.

3.2.2.1. Prevalence of insertion sequences in regulators of RND-type efflux pumps

The regulatory genes *adeRS*, *adeN,* and *adeL* were undisrupted in isolates with low tigecycline MICs (MIC ≤ 2 mg/L). Moreover, no insertion sequences were detected in *adeR* or *adeL* in any isolate. In 23 isolates with high tigecycline MICs (MIC ≥ 4 mg/L), disruption of *adeS* or *adeN* was detected (Table 3.8).

Table 3.8: Insertion sequences and other differences detected in RND-type efflux pump regulators in tigecycline-resistant *A. baumannii* isolates

Insertion sequences were detected using IS-Mapper, WGS data or by PCR and Sanger sequencing. Modified from [227].

	Number of isolates	
IS-element	***adeS***	***adeN***
IS*Aba1*	3	18
IS*Aba27*	0	1
IS*Aba125*	0	1
IS*Aba1* insertion in intergenic region of *adeRS*	1	0

IS*Aba1* was inserted in reverse orientation in *adeS* in three isolates (MB-R-148, MB-R-160, MB-R-164). While the insertion site in MB-R-148 and MB-R-164 was the same as found in isolate MB-S-5 (isolate pair 1), the insertion sites differed by one nucleotide in isolate MB-R-160 (Appendix 8). Moreover, the insertion in *adeS* was associated with amino acid substitutions in AdeR and ArpR and a tigecycline MIC of 16 mg/L. In 20 isolates, *adeN* was disrupted by insertion sequences and the MICs were 2- to 4-fold lower compared to isolates with disrupted *adeS* (Figure 3.10). In most isolates *adeN* was disrupted by IS*Aba1* (n=18). Only in two isolates, IS*Aba1* was inserted in reverse orientation in *adeN* (MB-R-12, MB-R-53) and a total of four different insertion sites were detected (Appendix 7). Besides IS*Aba1*, IS*Aba27* (MB-R-18) and IS*Aba125* (MB-R-105) were each found in *adeN* in one isolate, respectively (Table 3.8, Appendix 7). The insertion of IS-elements in *adeN* was associated with amino acid substitutions in one or more RND-type efflux pump regulators in 17 isolates (MIC range, 4 - 8 mg/L). In the isolate with IS*Aba125* disrupting *adeN*, IS*Aba1* was additionally inserted in reverse orientation between *adeR* and *adeS* without disrupting one of the genes (MIC 8 mg/L) (Table 3.8, Appendix 8 C). Moreover, IS*Aba1* disrupted *adeA* in one isolate (Appendix 8 B), which might indicate that AdeABC is not functional in this isolate and a different tigecycline mechanism is utilized (MIC 4 mg/L).

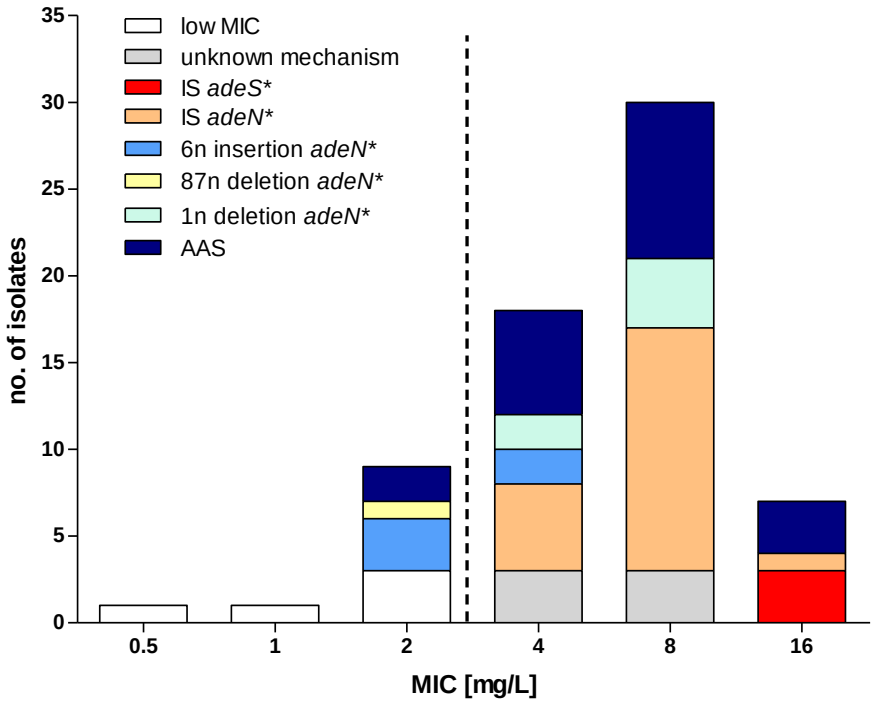


Figure 3.10: Overview of tigecycline MICs and differences in RND-type efflux pump regulators
Depicted are tigecycline MICs in comparison to the differences detected in the RND-type efflux pump regulators *adeS*, *adeN, adeL* and *arpR* in 65 *A. baumannii* isolates. The dashed line separates isolates displaying low and high tigecycline MICs. AAS = amino acid substitution, IS = insertion sequence, n = nucleotide, * = these differences appeared in combination with amino acid substitutions in one or more of the other RND-type efflux pump regulators. Adapted from [227].

The disruption of *adeS* was detected in isolates MB-R-148, MB-R-160 and MB-R-164. The analysis of *adeB* expression is challenging in these isolates because they are not part of an isogenic pair of isolates and an *A. baumannii* isolate with low tigecycline MICs and similar genetic background is missing. Instead, the reference strain *A. baumannii* ATCC 19606 and the *adeRS* knockout, were used for comparison of *adeB* expression. Analysis by qRT-PCR revealed a significantly increased expression of *adeB* in the three isolates (Figure 3.11 A). While isolates MB-R-148 and MB-R-164 displayed a 4-fold increased expression, the number of *adeB* transcripts was about 10-times higher in isolate MB-R-160 compared to the *adeRS* knockout strain and the reference strain *A. baumannii* ATCC 19606 (Figure 3.11 A). A disruption of *adeN* was detected in 20 isolates and two isolates were randomly selected for analysis of *adeJ* expression by qRT-PCR (Figure 3.11 B). In isolate MB-R-18 *adeN* was disrupted by IS*Aba27* and in isolate MB-R-105 IS*Aba125* was inserted in *adeN*. In both isolates, a significant increase in *adeJ* expression was observed compared to *A. baumannii* ATCC 19606. Moreover, the increase in *adeJ* expression was comparable between both isolates and Δ*adeN*.

In summary, the disruption of *adeN* by insertion sequences is more frequent than the disruption of *adeS* in clinical *A. baumannii* isolates. However, the increase in *adeB* expression due to the disrupted of *adeS* seems to be higher in comparison to the increase in *adeJ* expression due to insertions in *adeN*.

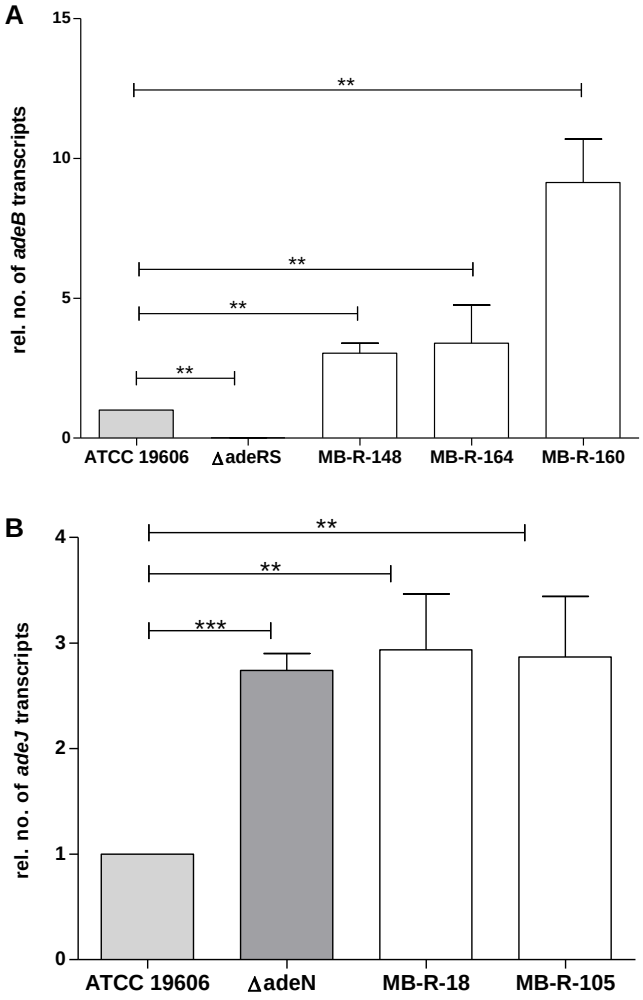


Figure 3.11: Relative expression of *adeB* and *adeJ* in isolates with disrupted *adeS* and *adeN*
Relative expression of the RND-type efflux pumps *adeB* (A) and *adeJ* (B) was determined by qRT-PCR. The number of transcripts was normalized to the expression of the reference gene *rpoB* and compared between isolates with disrupted *adeS* or disrupted *adeN*, the reference strain ATCC 19606 and the *adeRS* or *adeN* knockout strain. Results are represented as mean ± SEM. Statistical analysis was done by performing an unpaired t-test using the recorded absolute values. *** $p<0.001$, ** $p<0.01$.

3.2.2.2. Other differences in the regulatory genes of the RND-type efflux pumps

In 23 *A. baumannii* isolates, tigecycline resistance was associated with insertion sequences, while in 40 *A. baumannii* isolates with high tigecycline MICs (MIC ≥ 4 mg/L), no insertion elements were detected in the regulatory genes. Therefore, other resistance mechanisms are suggested in these isolates (e.g. small nucleotide deletions or point mutations).

Interestingly, one isolate with low tigecycline MIC (MB-R-67) and two isolates with high tigecycline MICs (MB-R-17 and MB-R-18) contained only *adeRSAB*, with *adeC* neither detectable in the draft genomes nor by PCR (Table 3.9). Moreover, *adeS* or *adeR* were absent in another four isolates (MIC range, 2 - 4 mg/L). Further analysis revealed that in three isolates

adeC and *adeB* were at least partially present, while *adeA* was not detectable (MB-R-75, MB-R-79, MB-R-89). In one isolate the whole *adeRSABC* operon could not be found in the draft genome or by PCR (MB-R-161). Instead, differences in *adeN* were detected in the four isolates (Table 3.9). One of the four isolates harboured a large 87-nucleotide deletion (position 236-323) in *adeN* (MIC 2 mg/L), while the other three isolates carried a 6-nucleotide insertion in *adeN* (Table 3.9, Figure 3.12). This insertion was detected in a total of five isolates (MIC range, 2 - 4 mg/L) (Table 3.9,Figure 3.10). In another six isolates with high tigecycline MICs (MIC range, 4 - 8 mg/L), the deletion of either nucleotide 45 (n = 2) or nucleotide 62 (n = 4) leading to a frameshift mutation and subsequently a premature stop codon was observed (Table 3.9, Figure 3.12). Furthermore, in three isolates, a point mutation at position 592 (G→T) creating a premature stop codon was detected in *adeN.* Although originally thought to be associated with elevated tigecycline MICs [227], the premature stop codon, 87-nucleotide deletion, and the 6-nucleotide insertion in *adeN* were also found in the in isolates displaying low tigecycline MICs of ≤ 2 mg/L. Therefore, an association with tigecycline resistance is unlikely.

Table 3.9: Genetic modifications detected in *A. baumannii* isolates.

Genetic modifications in RND-type efflux pumps detected by WGS data or by PCR and Sanger sequencing in *A. baumannii* isolates with tigecycline MICs ≥ 2 mg/L. Modified from [227].

Genetic modification	Number of isolates	TGC MIC [mg/L]
adeRSABC missing or truncated	6	2-8
1-nucleotide deletion in *adeN*	6	4-8
6-nucleotide insertion in *adeN*	5	2-4
premature stop codon in *adeN*	3	8
87-nucleotide deletion in *adeN*	1	2

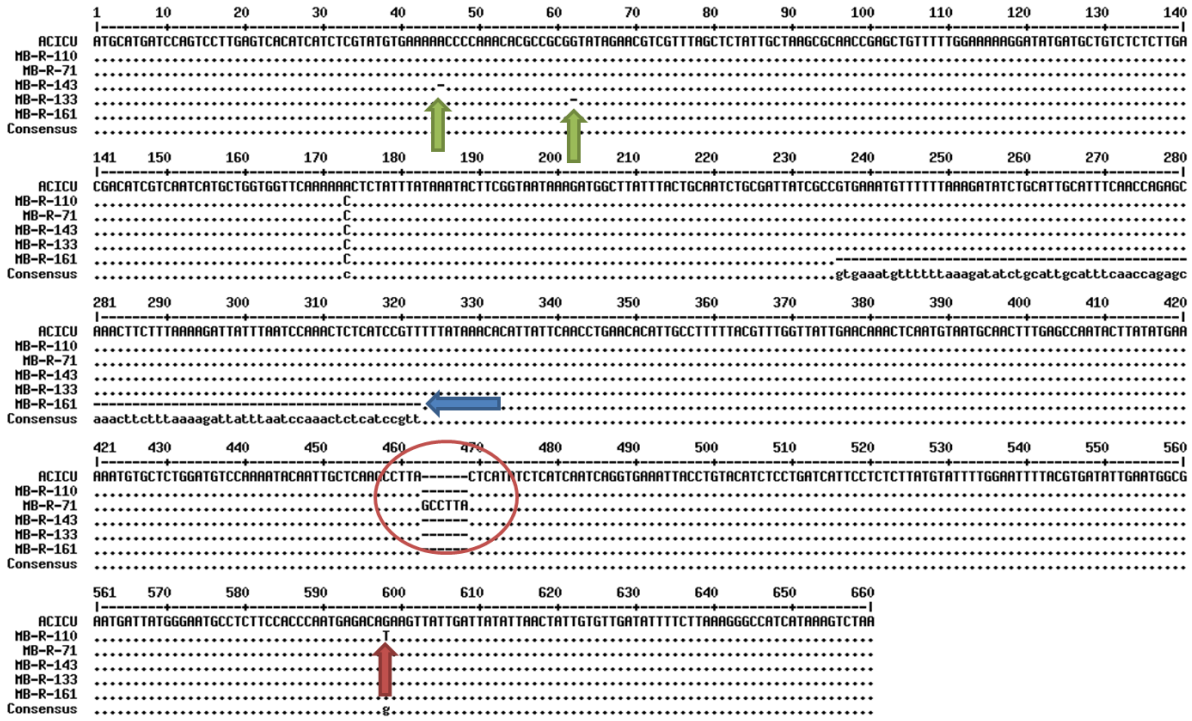


Figure 3.12: Alignment of *adeN* from *A. baumannii* isolates

Alignment of the nucleotide sequence of *adeN* from different *A. baumannii* isolates. Isolate MB-R-71 with a 6-nucleotide insertion (red circle), MB-R-110 with a premature stop codon due to a single nucleotide exchange (red arrow), MB-R-143 and MB-R-133 with a one nucleotide deletion leading to a frameshift and premature stop codon (green arrows) and MB-R-161 with an 87-nucleotide deletion (blue arrow).

For every difference that was detected in *adeN*, one isolate was selected for analysis of gene expression of *adeJ* by qRT-PCR. Since the deletion of one nucleotide resulted in a frameshift mutation and subsequently in a premature stop codon regardless of the position of the deleted nucleotide, only one representative isolate was chosen for qRT-PCR. The expression of *adeJ* was measured in isolate MB-R-71 (6 nucleotide insertion), MB-R-110 (premature stop codon), MB-R-143 (one nucleotide deletion) and MB-R-161 (87 nucleotide deletion) and compared to the expression of *adeJ* in *A. baumannii* ATCC 19606 and the *adeN* knockout strain (Figure 3.13). In all isolates, *adeJ* expression was slightly increased compared to the reference strain ATCC 19606, but only in isolates MB-R-71 and MB-R-161, the expression of *adeJ* was significantly higher (Figure 3.13). Interestingly, *adeJ* expression was lower in all isolates compared to the expression measured in the *adeN* knockout strain, indicating that the complete loss of the repressor *adeN* has greater impact on *adeJ* expression compared to mutations in or truncation of the gene.

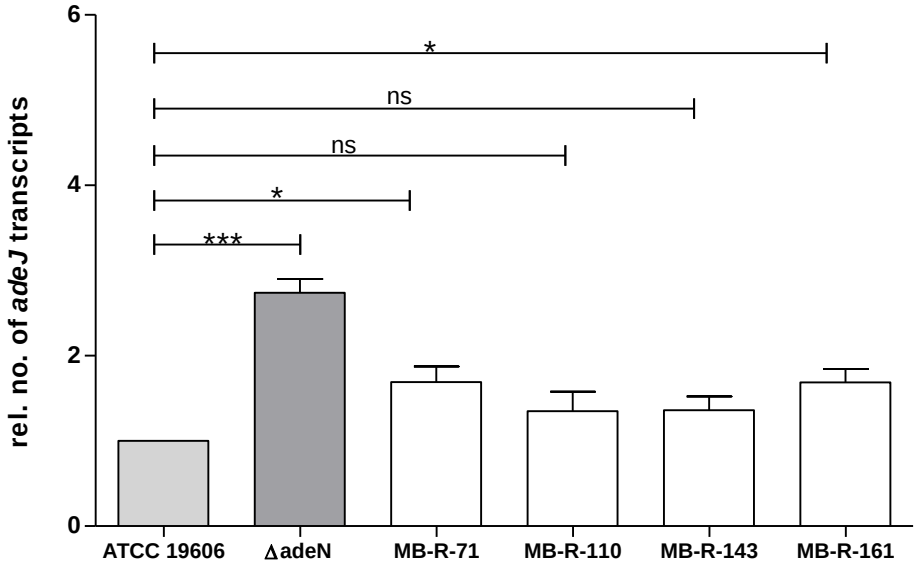


Figure 3.13: Relative expression of *adeJ* in isolates with mutations in *adeN*

Relative expression of the RND-type efflux pumps *adeJ* was determined by qRT-PCR. The number of transcripts was normalized to the expression of the reference gene *rpoB* and compared between isolates with mutations in *adeN* and the reference strain *A. baumannii* ATCC 19606 as well as the *adeN* knockout strain (Δ*adeN*). Results are represented as mean ± SEM. Statistical analysis was done by performing an unpaired t-test using the recorded absolute values. *** p<0.001, ** p<0.01, * p<0.05, ns = not significant.

Moreover, *adeN* from isolates MB-R-71 (6-nucleotide insertion), MB-R-110 (premature stop codon), MB-R-133 (one nucleotide deletion) and MB-R-161 (87-nucleotide deletion) were cloned into the plasmid pJN17/04 and introduced into the *adeN* knockout strain. The determination of tigecycline MICs revealed no significant changes in tigecycline MICs ranging between 1 and 2 mg/L for each transformant (Table 3.10).

Table 3.10: Tigecycline MICs for the *adeN* knockout complemented with different *adeN* variants

The *adeN* knockout was complemented with *adeN* variants from different clinical isolates and the tigecycline MICs were determined by agar dilution.

Strain	Plasmid	*adeN* difference	TGC MIC [mg/L]
A. baumannii ATCC 19606			1
A. baumannii Δ*adeN*			2
A. baumannii Δ*adeN*	pJN17/04 (empty vector)		2
A. baumannii Δ*adeN*	pJN17/04::*adeN*-19606	wild type	1
A. baumannii Δ*adeN*	pJN17/04::*adeN*-R71	6-nucleotide insertion	1
A. baumannii Δ*adeN*	pJN17/04::*adeN*-R110	premature stop codon	2
A. baumannii Δ*adeN*	pJN17/04::*adeN*-R133	1-nucleotide deletion	2
A. baumannii Δ*adeN*	pJN17/04::*adeN*-R161	87-nucleotide deletion	2

3.2.2.3. Amino acid substitutions detected in the regulators of the RND-type efflux pumps

Point mutations in ORFs can lead to amino acids substitutions, therefore the amino acid sequences of AdeRS, AdeN and AdeL of the 65 isolates were compared to that of the reference strain *A. baumannii* ACICU. A large number of polymorphisms were detected (e.g. the AdeR substitutions I120V, V136A and L241P were also observed in the reference strains or *A. baumannii* isolates with low tigecycline MICs such as the ABC-isolates). Nevertheless, some amino acid substitutions in AdeRS, AdeN, AdeL and ArpR were found in isolates with high tigecycline MICs, which are listed in Table 3.11.

Table 3.11: Amino acid substitutions in RND-type efflux pump regulators.

Amino acid substitutions were determined by comparing the amino acid sequences of RND-type efflux pump regulators AdeS, AdeN, AdeL and ArpR with those of *A. baumannii* reference strains and isolates with low tigecycline MICs. Modified from [227].

AdeR	No. of isolates	AdeS	No. of isolates	AdeN	No. of isolates	AdeL	No. of isolates	ArpR	No. of isolates
D26N	2	D167N, S354P	2	H170Y	2	I37L	6	N130D[b]	41
V119I	2	A325T	2	G213V	3				
D21V, D26N	17	I62M[a]	3	D181N[a]	1				
N115K	1	Q339K[b], V137F	1	F108L[c]	1				
G25S, D26N, E147K	2	Q339K[b]	4						
G25S, D26N	3								

[a] published in Gerson *et al*. [227] but excluded after re-analysis with *A. baumannii* ABC-isolates
[b] amino acid substitution found both in isolates with low and high tigecycline MICs
[c] amino acid substitution found only in one isolate with low tigecycline MIC of 2 mg/L

In AdeL, only the amino acid substitution I37L (n=6) was associated with elevated tigecycline MICs (MIC range, 4 - 8 mg/L), while in AdeN the substitutions H170Y (n=2), F108L (n=1) and G213V (n=3) were found. Six different substitutions were found either as a single substitution per isolate or in combinations in AdeR (D21V, G25S, D26N, N115K, V119I, E147K) in 27 isolates. In AdeS the amino acid substitutions A325T and the combination of substitutions D167N and S354P were found in two isolates, respectively. Moreover, the substitution Q339K was found as single amino acid change in four isolates or in combination with the substitution V137F in one isolate. Of note, all isolates carrying the substitution Q339K displayed tigecycline MICs ranging from 1 to 16 mg/L and belonged to the clonal lineage IC1 suggesting the substitution is specific for this clonal lineage and is not associated with tigecycline resistance. Moreover, one isolate carrying the substitution Q339K had an additional deletion of one nucleotide at position 30 in *adeS* (MIC 1 mg/L). Besides the well characterised RND-type efflux pumps AdeABC, AdeIJK and AdeFGH, *A. baumannii* harbours up to five additional RND-type efflux pumps one of which is called ArpAB. This pump is regulated by the TetR-like transcription

regulator ArpR and the amino acid change N130D was observed in ArpR in 41 isolates (MIC range, 2 - 16 mg/L). Generally, amino acid substitutions we associated with tigecycline MICs ranging from 2 to 16 mg/L (Figure 3.10). In 18 isolates with high tigecycline MICs (MIC ≥ 4 mg/L), the only difference detected were amino acid substitutions, i.e. no insertion elements or other mutations were detectable in the RND-type efflux pump regulators. Interestingly, most amino acid substitutions in AdeR were found in the receiver domain and near important residues involved in the pocket formed around a magnesium ion (Figure 3.14 A). In AdeS, an amino acid substitution was detected in each of the important protein domains, namely in the HAMP domain (V137F), the HisKA domain (D167N), and the HATPase domain (A325T, Q339K, S354P) (Figure 3.14 B).

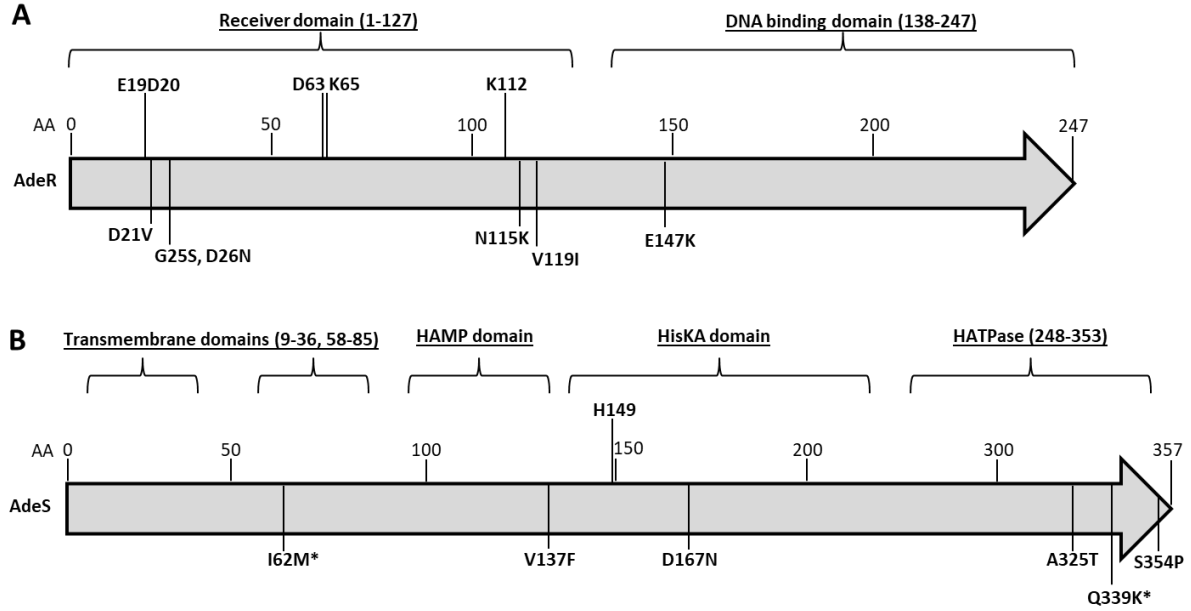


Figure 3.14: Schematic overview of amino acid substitutions in RND-type efflux pump regulators AdeR and AdeS

All amino acid substitutions detected in *A. baumannii* isolates with tigecycline MICs ≥ 2 mg/L are mapped. (A) Substitutions found in the response regulator AdeR. Depicted are important residues E19, D20, K65, and K112, which are involved in pocket formation around a magnesium ion and the phosphorylation site K63 [213]. (B) Substitutions detected in the histidine kinase AdeS. Depicted are the transmembrane and HATPase domain and the histidine residue at position 149, which is the site of autophosphorylation [139]. AA = amino acid. * = AdeS substitution has been published previously but was excluded after re-analysis. Adapted from [227].

3.2.2.4. Efflux pumps and other tigecycline resistance determinants in *A. baumannii* isolates

The association of *trm* with tigecycline resistance has already been excluded during the investigation of isolate pairs, while the resistance determinants Tet(A) and Tet(X) or their variants were not detected in any of the *A. baumannii* isolates. Furthermore, amino acid substitutions were detected in PlsC and AbrP, but all substitutions were also found in the reference strains or isolates with low tigecycline MIC (e.g. ABC-isolates) and therefore were

considered to be genetic polymorphisms. Moreover, several amino acid substitutions were observed in AdeAB, AdeFGH, AdeJ, AbeM, and the uncharacterised RND-type efflux pumps (Table 3.12, Appendix 4). Of note, the majority of amino acid substitutions in efflux pumps were found in two isolates (MB-R-17, MB-R-18) not belonging to any of the known clonal lineages, suggesting that the amino acid differences are genetic polymorphisms. Furthermore, the regulators SoxR, MarR and the TCS BaeSR were also analysed and revealed no differences. However, SoxR was not detectable in the draft genome of one isolate (MB-R-97), which was also observed for ABC-isolates.

Table 3.12: Overview of amino acid substitutions in efflux pumps in *A. baumannii* isolates

Amino acid differences were detected by comparing draft genomes of the isolates with the reference strain ACICU as well as with reference strains AYE, ATCC 19606, ATCC 17978 and isolates with low tigecycline MICs.

Protein	Amino acid difference	Tigecycline MIC [mg/L]	No. of isolates
AdeA	T325A[a]	4-8	2
	L6Q	4	1
AdeB	S1036T[a]	4-8	2
	P327S	4-8	2
AdeF	T45P[a]	4-8	2
AdeG	A14S[a]	4-8	2
	V748I, T749R	16	1
	P1052S	4	1
AdeH	A25V, D446V, S481N[a]	4-8	2
AdeJ	V158I	8	1
AbeD	S1030T, T1031S[a]	4-8	2
ArpA	V291I[a]	4-8	2
RND1 (ACICU_RS00750)	V17I	4-8	6
	I561V	2-16	46
RND2 (ACICU_RS15300)	T866N	8-16	2
	N674S[a]	4-8	2
RND3 (ACICU_RS17095)	I141V, V157I, S331F, G522S, P654S[a]	4-8	2
RND3-MFP (ACICU_RS17100)	E58D, E153D, G274R[a]	4-8	2
RND3-OMP (ACICU_RS17105)	A97V, P466S, A469T[a]	4-8	2
AbeM	V86I[a]	4-8	2

[a] amino acid substitution detected in isolates MB-R-17 and MB-R-18

In summary, 11 *A. baumannii* isolates with tigecycline MICs between 4 and 8 mg/L (MB-R-6, MB-R-10, MB-R-79, MB-R-89, MB-R-97, MB-R-110, MB-R-130, MB-R-151, MB-R-152, MB-R-157, MB-R-165) carried none of the above-mentioned mutations or differences in efflux pumps and their regulators. However, it is important to point out, that some isolates harboured amino acid substitutions or other differences in the RND-type efflux pump regulators, which were detected both in isolates displaying low tigecycline MICs of 2 mg/L or high tigecycline MICs of ≥ 4 mg/L (e.g. N130D in ArpR, 6-nucleotide insertion in *adeN*). It is suggested that there are different and hitherto unknown resistance mechanism(s) at play and further investigation is required to elucidate the tigecycline resistance mechanism in these isolates.

3.3. Colistin resistance in *A. baumannii*

Part of the results have been published in Gerson *et al*. [230].

3.3.1. Investigation of colistin resistance mechanisms in *A. baumannii* isolate pairs

3.3.1.1. Differences in PmrCAB and other colistin resistance determinants

Differences in the *pmrCAB* operon were found in the four isolates with high colistin MICs by comparing the draft genomes between isolates representing an isolate pair. These genetic differences led to the amino acid substitutions S17R, A28V, and I232T, as well as the four amino acid deletion ΔL9-G12 in PmrB (Table 3.13). Both PmrA and PmrC were identical in isolates representing an isolate pair. A *pmrC*-homologue (termed *eptA*) was detected by WGS and PCR in both isolates of isolate pairs 1, 2, and 3, but was absent in isolate pair 4, A*. baumannii* ATCC 19606 and the knockout strain Δ*pmrAB*. In the colistin-resistant isolate MB-S-177 of isolate pair 2, the amino acid substitution R127L was detected in EptA (Table 3.13). Moreover, IS*Aba1* was found upstream of *eptA* in both isolates of isolate pair 2. The insertion occurred in reverse orientation and the same insertion site, but a point mutation (A1091T) was detected near the putative promoter region in IS*Aba1* in the colistin-resistant isolate MB-S-177.

Table 3.13: Overview of amino acid substitutions detected in *A. baumannii* isolate pairs
Amino acid sequences of PmrCAB and EptA were compared between isolates representing an isolate pair. Colistin MICs were determined by microbroth dilution.

#	Isolate	Colistin MIC [mg/L]	PmrB substitution	EptA substitution
1	MB-R-2	2	ΔL9-G12	
	MB-S-6	256		
2	MB-S-23	2	I232T	R127L
	MB-S-177	256		
3	MB-R-90	32	A28V	
	MB-R-119	>256		
4	SG3161	1	S17R	
	SG3166	256		

Besides mutations in the *pmrCAB* operon, other genes (e.g. *hns*, genes of lipid A biosynthesis) have been described to be associated with colistin resistance. However, no mutations in or disruption of the global transcription regulator H-NS or genes involved in LPS/lipid A biosynthesis were detected in the isolate pairs, therefore these gene products were considered not to be associated with colistin resistance in the isolates. Furthermore, the plasmid-mediated resistance determinant *mcr-1* or its homologues were not present in any isolate. Two amino acid substitutions in the RND-type efflux pumps AdeG and AdeJ were detected in the colistin-resistant isolates of isolate pair 2 and 3 (Table 3.14), while no other differences associated with colistin resistance were found in any other efflux pump known to date in *A. baumannii* including the *ermAB*-like efflux pumps. In addition, allele differences in

hypothetical or uncharacterised proteins, proteins of the bacterial metabolism, two putative transcription regulators, proteins involved in DNA replication and a permease were detected in the isolate pairs except for isolate pair 4 (Table 3.14).

Table 3.14: Allele differences in *A. baumannii* isolate pairs

The allele differences were determined using cgMLST and amino acid sequences and were compared between isolates representing an isolate pair. Taken from [230].

Isolate pair	Allele	Annotation	Amino acid difference
1	ACICU_RS01665	Hypothetical protein	G242S
	ACICU_RS15320	Uncharacterized conserved protein	I116S
	ACICU_RS15345	Predicted permease	G229R
2	ACICU_RS04295	Elongation factor	I469N, D471V
	ACICU_RS07545	Uncharacterized protein	Premature stop codon
	ACICU_RS07770	Putative transcription regulator	P174T
	ACICU_RS08730	Chorismate synthase	K141N
	ACICU_RS11465	Hydroxyethylthiazole kinase	Premature stop codon
	ACICU_RS11970	Putative TetR transcription regulator	K188N
	ACICU_RS12530	RND-type transporter AdeG	T749R
	ACICU_RS16885	Guanosine polyphosphate pyrophospho-hydrolases/synthetase (SpoT)	G575V
3	ACICU_RS00010	Type IIA topoisomerase	E479D
	ACICU_RS12175	Gamma-aminobutyrate permease	S455T
	ACICU_RS14125	DNA helicase	Truncated protein in MB-R-90
	ACICU_RS14905	RND-type transporter AdeJ	V158I
4	No allele differences besides in *pmrB* detected		

3.3.1.2. Increased expression of *pmrCAB* and *eptA*

The effect of the PmrB amino acid deletion and substitutions on the expression of *pmrC* was investigated by qRT-PCR. The relative expression of *pmrC* was compared between isolates within an isolate pair (Figure 3.15). The expression of *pmrC* was significantly increased in the colistin-resistant isolates MB-S-6 and SG3166 (Figure 3.15 A). The expression of *pmrC* was approximately 13-times higher in isolate MB-S-6 and about 10-fold increased in isolate SG3166 compared to the colistin-susceptible isolates. Although both isolates of isolate pair 3 were considered colistin-resistant, the expression of *pmrC* was about 4-times higher in isolate MB-R-119 compared to MB-R-90 (Figure 3.15 A). In contrast, a different resistance mechanism is suggested in the colistin-resistant isolate MB-S-177, because *pmrC* expression was significantly decreased.

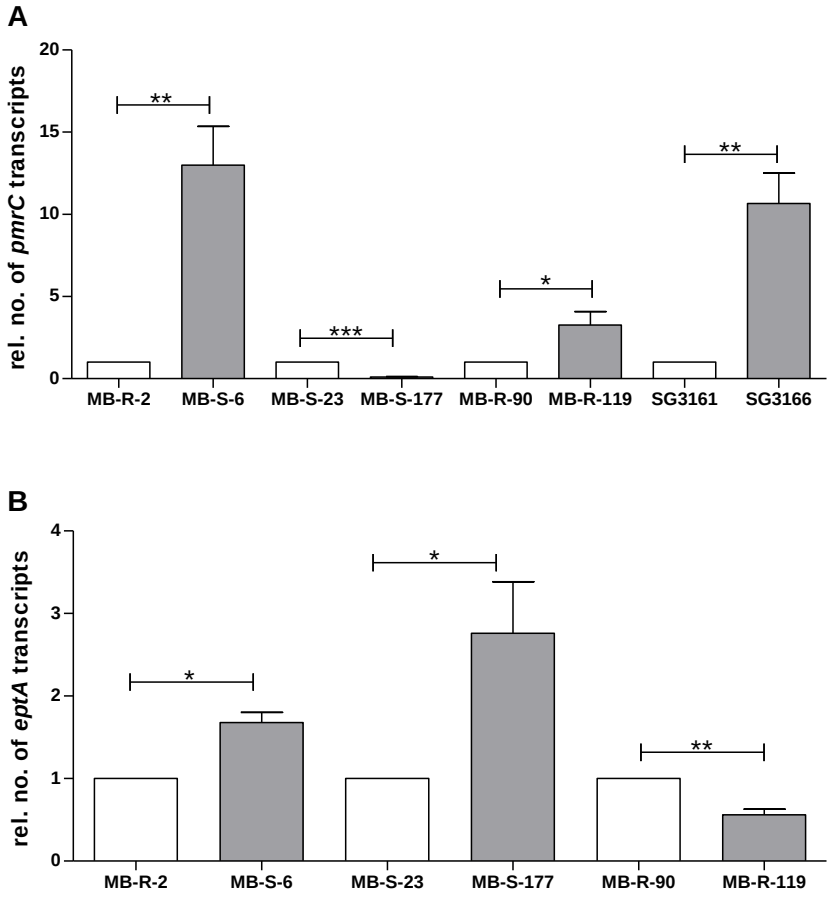


Figure 3.15: Relative expression of *pmrC* and *eptA* in isolate pairs

Relative expression of *pmrC* (A) and *eptA* (B) was determined by qRT-PCR. After the normalization to the expression of the reference gene *rpoB*, the relative number of transcripts of isolates with low colistin MIC (white) was related to isolates with high colistin MIC (grey). Results are represented as mean ± SEM. Statistical analysis was done performing unpaired t-test using the recorded absolute values. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Adapted from [230].

Furthermore, the expression of the *pmrC*-homologue *eptA* was analysed by qRT-PCR (Figure 3.15 B). The expression of *eptA* was about twice as high in MB-S-6 compared to the colistin-susceptible isolate. In contrast, *eptA* expression was significantly reduced in MB-R-119 compared to MB-R-90. Surprisingly, *eptA* expression was significantly higher in MB-S-177, although in both isolates IS*Aba1* was detected upstream of *eptA* in reverse direction. These findings might indicate an association between point mutations in IS*Aba1* and *eptA* mutations with increased *eptA* expression and colistin resistance.

3.3.1.3. Modification of lipid A

Increased expression of *pmrC* or *eptA* is associated with the addition of PEtN to lipid A. To investigate this, LPS and lipid A were extracted from mid-log phase and overnight cultures of the isolate pairs and the reference strain *A. baumannii* ATCC 19606 and compared. Subsequent analysis by MALDI-TOF revealed no significant differences between the mid-log

phase and the overnight cultures, since similar mass spectra were obtained for both growth phases. Major lipid A species were detected in the reference strain ATCC 19606 and the isolate pairs including hepta-acylated lipid A (m/z 1910), hepta-acylated lipid A with shorter fatty acid chains (m/z 1883), hexa-acylated lipid A (m/z 1728), penta-acylated lipid A lacking one fatty acid moiety (m/z 1530) and tetra-acylated lipid A (m/z 1404) (Table 3.15, Figure 3.16). The addition of PEtN (m/z 124) to hepta-acylated lipid A (m/z 1910) is shown by the mass at m/z 2034 (+1 PEtN) and m/z 2157 (+2 PEtN). Only in isolate pair 1, the addition of a single PEtN molecule was observed exclusively in the colistin-resistant isolate MB-S-6 (Figure 3.16 B). In contrast, the mass at m/z 2034 was detected in both the colistin-susceptible and -resistant isolate of isolate pairs 2 and 4 (Figure 3.16 C, E). These findings were rather unexpected and might indicate a different resistance mechanism in these isolates or that PEtN addition to lipid A is a strain-specific colistin resistance mechanism. Moreover, single PEtN addition was detected in both isolates of isolate pair 3, thus the relatively high colistin MICs were probably not due to PEtN addition alone (Figure 3.16 D).

Table 3.15: Overview of lipid A species detected in *A. baumannii* isolate pairs

Mass spectra were measured by MALDI-TOF mass spectrometry using negative ion mode within a mass range of 500-3000 Da. Represented are the major lipid A species: hepta-acylated lipid A (m/z 1910), hepta-acylated lipid A with shorter fatty acid chains (m/z 1883), hexa-acylated lipid A (m/z 1728), penta-acylated lipid A lacking one fatty acid moiety (m/z 1530) and tetra-acylated lipid A (m/z 1404). Masses of lipid A modified with PEtN (m/z 124) are highlighted in light grey.

		Isolate pair #1		Isolate pair #2		Isolate pair #3		Isolate pair #4	
	ATCC 19606	MB-R-2	MB-S-6	MB-S-23	MB-S-177	MB-R-90	MB-R-119	SG3161	SG3166
Detected mass [m/z]	1404.133	1404.111	1404.238	1404.366	1403.332	1403.996	1404.010	1404.023	1404.007
	1530.086	1530.153				1529.960	1529.836	1529.939	1530.815
	1728.320	1728.426	1728.449	1728.643	1728.592	1728.174	1728.107	1728.140	1728.177
					1852.681	1852.206	1851.137	1851.155	1851.188
	1882.474	1882.628	1883.622	1883.825	1883.770	1882.330	1882.260	1883.281	1882.330
	1910.542	1910.703	1910.694	1910.892	1910.855	1910.377	1910.288	1910.316	1910.354
			2006.661	2006.799	2005.898	2005.351	2006.290	2005.312	2006.360
			2034.763	2034.919	2034.942	2034.413	2034.325	2034.357	2034.489
							2157.357		

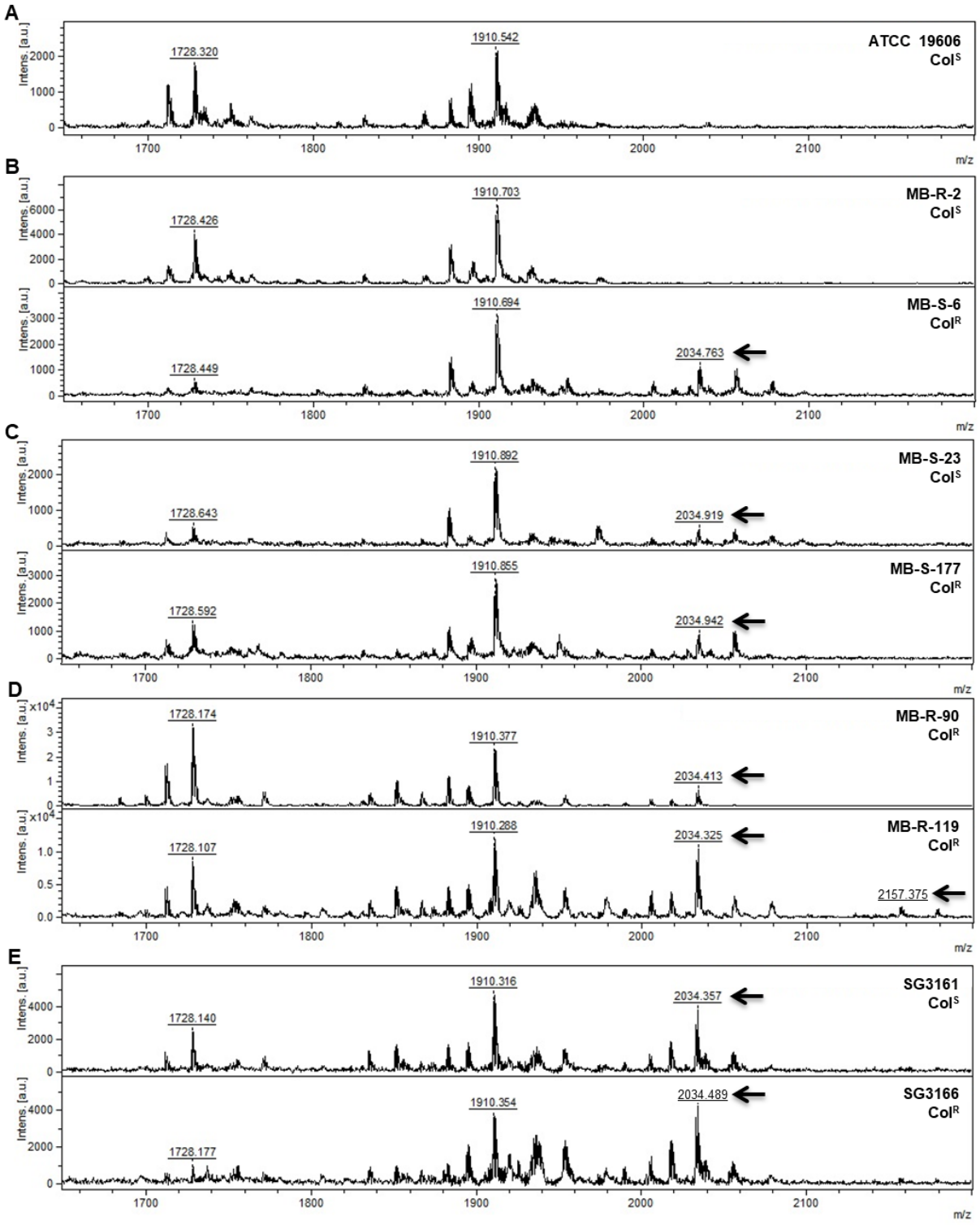


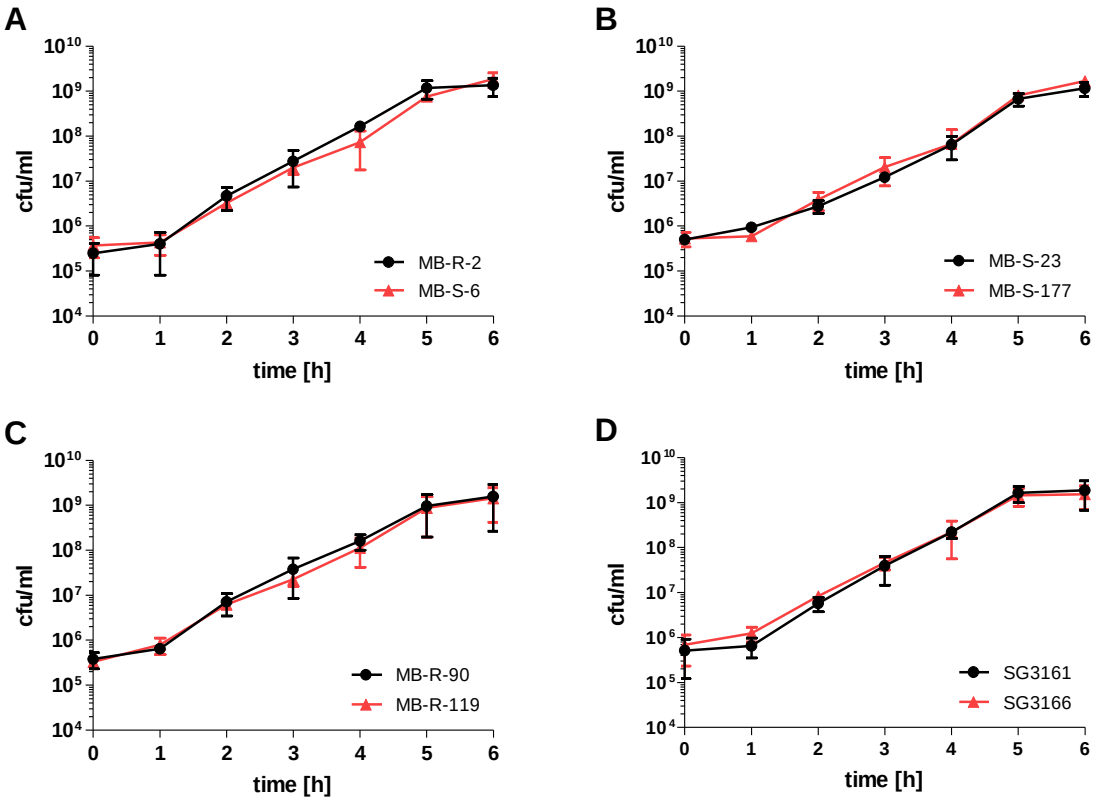
Figure 3.16: Mass spectra of lipid A in *A. baumannii* isolate pairs and reference strain *A. baumannii* ATCC 19606

Lipid A was extracted from overnight cultures of *A. baumannii* isolates. The mass spectra are represented in the range of m/z 1650 to 2200 and masses of important lipid A species are given in the spectra. (A) *A. baumannii* ATCC 19606, (B) isolate pair 1 (MB-R-2, MB-S-6), (C) isolate pair 2 (MB-S-23, MB-S-177), (D) isolate pair 3 (MB-R-90, MB-R-119), (E) isolate pair 4 (SG3161, SG3166). Arrow = PEtN addition indicated by a mass at m/z 2034 and 2157. Col^S = colistin-susceptible, Col^R = colistin-resistant. Adapted from [230].

Of note, the addition of two PEtN molecules (m/z 2157) was observed only in the isolate MB-R-119. Furthermore, single PEtN addition to hexa-acylated lipid A (m/z 1728) is shown by a mass at m/z 1852 and was observed for the colistin-resistant isolate MB-S-177 as well as for both isolates of isolate pairs 3 and 4 (Table 3.15). The addition of PEtN to hepta-acetylated lipid A carrying shorter fatty acid chains (m/z 1883) is indicated by a mass at m/z 2006 and was revealed for both isolates of isolate pairs 2, 3, and 4 as well as for isolate MB-S-6 (Table 3.15). The modification of lipid A by galactosamine, which is represented by a mass at m/z 2071, was not detected in any isolates.

3.3.1.4. Unchanged fitness and increased virulence in isolates with high colistin MICs

Since mutations in *pmrB* have been described to negatively affect bacterial fitness and virulence [253, 254], both the growth of colistin-resistant isolates and their virulence in the *G. mellonella* model of infection were investigated. Similar growth curves were obtained within an isolate pair, i.e. no differences in growth were detected comparing the isolates with low colistin MIC to those displaying high colistin MICs (Figure 3.17). The growth rates and doubling times were calculated for all isolates and although isolates with higher colistin MIC displayed also slightly longer doubling times, no significant differences were detected for the cfu/mL (Figure 3.17). Therefore, the development of colistin resistance did not appear to have a significant fitness cost.



	MB-R-2	MB-S-6	MB-S-23	MB-S-177	MB-R-90	MB-R-119	SG3161	SG3166
Growth rate [h^{-1}]	0.22	0.20	0.20	0.21	0.29	0.25	0.28	0.22
Doubling time [h]	3.1	3.4	3.4	3.3	2.4	2.8	2.5	3.1

Figure 3.17: Growth curves of *A. baumannii* isolate pairs.
Growth curves were conducted by inoculating MH broth with the respective *A. baumannii* isolate and taking a sample every hour over the course of 6 hours. Dilutions were plated onto MHA and the number of cfu/mL was calculated. (A) Isolate pair 1 (MB-R-2, MB-S-6), (B) isolate pair 2 (MB-S-23 MB-S-177), (C) isolate pair 3 (MB-R-90, MB-R-119), (D) isolate pair 4 (SG3161, SG3166). Colistin-susceptible isolates are shown in black, colistin-resistant isolate in red. Results are represented as mean cfu/ml ± SEM from three independent experiments. Adapted from [230].

The virulence of the isolate pairs was investigated by Jonathan W. Betts (University of Surrey, Great Britain) using the *in vivo G. mellonella* model of infection. The larvae were infected with the *A. baumannii* isolates and survival as well as melanisation were assessed (Figure 3.18, Figure 3.19). Overall, colistin-resistant isolates exhibited increased virulence compared to susceptible isolates. The survival rate of the larvae infected with the colistin-susceptible isolate MB-R-2 was 40% after 48 h, while no larvae survived when infected with the colistin-resistant isolate MB-S-6 ($p \leq 0.021$) (Figure 3.18 A). In isolate pair 2, the survival rate of larvae infected with MB-S-177 was also lower (30%) after 96 h compared to larvae infected with MB-S-23 (60%) ($p \leq 0.04$) (Figure 3.18 B). The virulence of isolate pair 3 differed, although both isolates were colistin-resistant. An infection with MB-R-90 led to a 50% survival of larvae, while

the survival rate of larvae infected with MB-R-119 was only 20% after 96 h ($p \leq 0.04$) (Figure 3.18 C). Comparing the isolates of isolate pair 4, no significant differences in survival rates were detected after 96 h (30%), although a higher percentage of larvae were killed in the first 24 h when infected with the colistin-resistant isolate SG3166 ($p \leq 0.17$) (Figure 3.18 D).

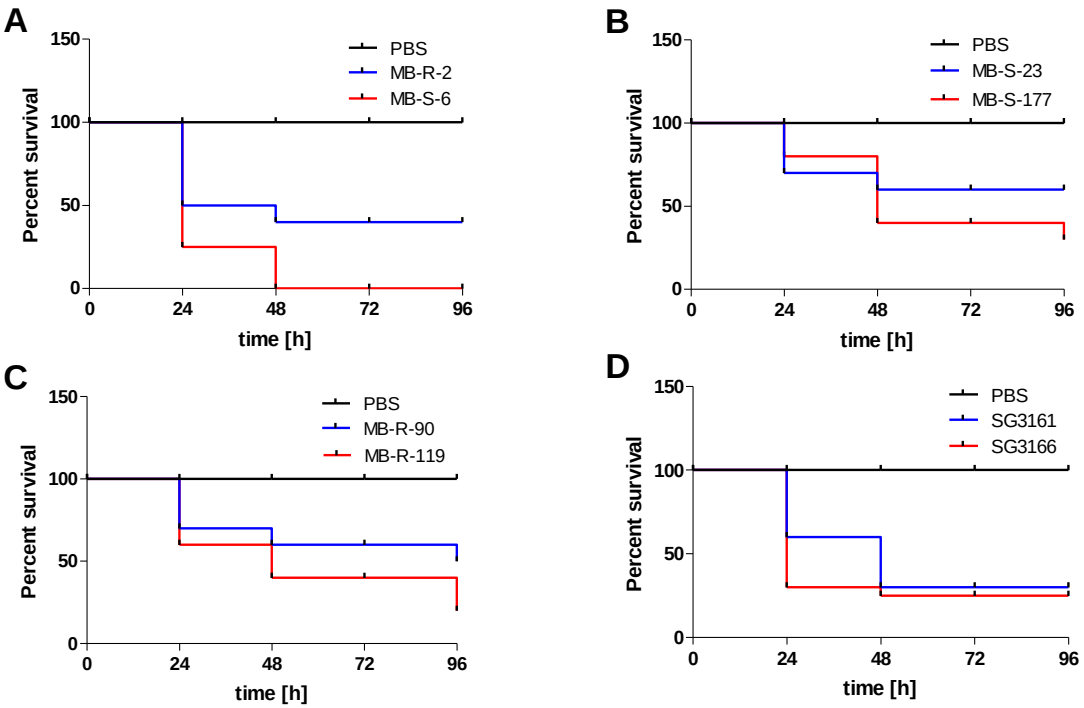


Figure 3.18: Survival curves for *G. mellonella* infected with different *A. baumannii* isolates

G. mellonella was infected with the four isolate pairs using 10^5 cfu/larvae and survival was observed over 96 h. Isolates with low colistin MIC are represented in blue, isolates with high colistin MIC in red and the PBS control in black. Illustrated are isolate pair 1-4 (A-D). Adapted from [230].

All *A. baumannii* isolates induced high levels of melanisation, but even higher melanisation scores were observed in larvae infected with isolates with elevated colistin MICs (Figure 3.19). An increased melanisation score was observed over the entire time course (24 h – 96 h) for larvae infected with the colistin-resistant isolate MB-S-6 (Figure 3.19 A). The rates of melanisation were unchanged or decreased after 24 h but was increased after 48 – 96 h in larvae infected with MB-S-177 and MB-R-119 (Figure 3.19 B, C). In contrast, an infection with isolate pair 4 revealed lower melanisation scores for SG3161 after 24 h and similarly constant melanisation scores between 48 and 96 h for both isolates, indicating no significant differences in morbidity (Figure 3.19 D). Both the decreased survival rates and the elevated melanisation scores indicate a greater virulence in the colistin-resistant *A. baumannii* isolates MB-S-6, MB-S-117 and MB-R-119.

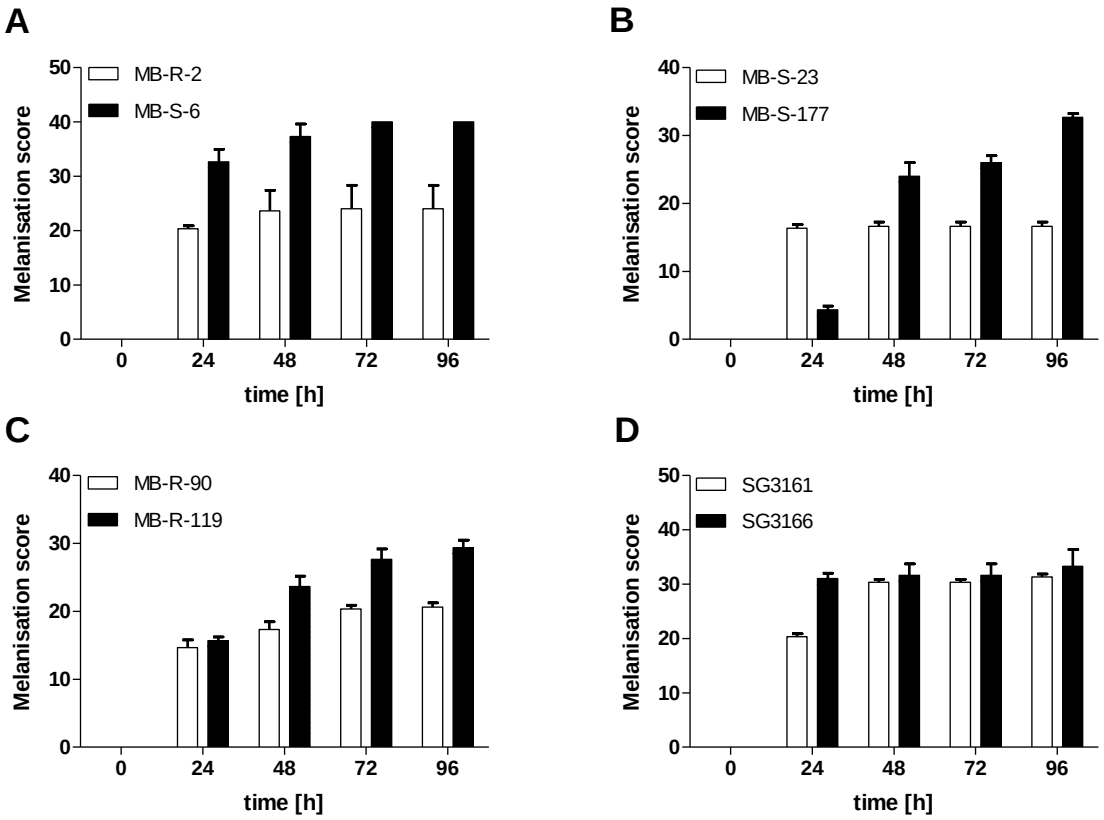


Figure 3.19: Melanisation scores (morbidity) of *G. mellonella* infected with *A. baumannii*

G. mellonella was infected with *A. baumannii* isolates using 105 cfu/larvae and melanisation was observed over 96 h. The PBS control had a melanisation score of 0. A higher melanisation score indicates greater morbidity. Isolates with low colistin MICs are represented in white, isolates with high colistin MIC in black. Depicted are isolate pairs 1-4 (A-D). Adapted from [230].

3.3.1.5. Introduction of *pmrAB*-variants into *A. baumannii* Δ*pmrAB*

A *pmrAB* knockout strain (Δ*pmrAB*) was created to further analyse the association of *pmrB* mutations with colistin resistance. It was planned to introduce the *pmrAB* variants from the isolate pairs into the knockout strain as well as *A. baumannii* reference strains ATCC 19606 and ATCC 17978. Although attempted, modified, and optimised numerous times, most of the cloning and transformation experiments were unsuccessful. Only the *pmrAB* variants from isolate pairs 1 and 3 as well as from the reference strain ATCC 19606 as control were cloned successfully into the plasmid pJN17/04. An introduction of these plasmids into Δ*pmrAB* and the *A. baumannii* reference strains ATCC 19606 and ATCC 17978 was difficult, e.g. pJN17/04::*pmrAB*-R90 was transferred successfully only into *A. baumannii* ATCC 17978. Surprisingly, the colistin MICs of Δ*pmrAB* was 1 mg/L regardless of the *pmrAB* variant introduced (Table 3.16). In contrast, the colistin MICs of *A. baumannii* ATCC 19606 decreased from 1 mg/L to 0.25 or 0.5 mg/L after the introduction of *pmrAB* from isolate pair 1 or MB-R-119, respectively. Moreover, the colistin MICs were unchanged or decreased for one dilution step in *A. baumannii* ATCC 17978 and its transformants, while an increase of one

dilution step was observed after the introduction of *pmrAB* from MB-S-6. Nevertheless, only slight changes in colistin MICs were observed and all wild type strains and their transformants were still considered colistin-susceptible.

Table 3.16: Colistin MICs of *A. baumannii* strains complemented with *pmrAB* variants
Colistin MICs were determined by microbroth dilution in three indepentend experiments. Adapted from [230].

Strain	Plasmid	Colistin MIC [mg/L]
A. baumannii ATCC 19606 (wild type)		1
A. baumannii ATCC 19606	pJN17/04::*pmrAB*-R2	0.25
A. baumannii ATCC 19606	pJN17/04::*pmrAB*-S6	0.25
A. baumannii ATCC 19606	pJN17/04::*pmrAB*-R119	0.5
A. baumannii ATCC 19606 Δ*pmrAB* (knockout)		1
A. baumannii ATCC 19606 Δ*pmrAB*	pJN17/04 (empty vector)	1
A. baumannii ATCC 19606 Δ*pmrAB*	pJN17/04::*pmrAB*-19606	1
A. baumannii ATCC 19606 Δ*pmrAB*	pJN17/04::*pmrAB*-R2	1
A. baumannii ATCC 19606 Δ*pmrAB*	pJN17/04::*pmrAB*-S6	1
A. baumannii ATCC 19606 Δ*pmrAB*	pJN17/04::*pmrAB*-R119	1
A. baumannii ATCC 17978 (wild type)		0.5
A. baumannii ATCC 17978	pJN17/04::*pmrAB*-R2	0.5
A. baumannii ATCC 17978	pJN17/04::*pmrAB*-S6	1
A. baumannii ATCC 17978	pJN17/04::*pmrAB*-R90	0.25
A. baumannii ATCC 17978	pJN17/04::*pmrAB*-R119	0.5

Since the phenotype (colistin-susceptible) was not affected by the complementation of the knockout or reference strains with the different *pmrAB* variants, the expression of the genes on the plasmid and *pmrC* in Δ*pmrAB* and the reference strains was investigated. Gene expression was analysed by qRT-PCR and large numbers of *pmrA* and *pmrB* transcripts were detected in all transformants compared to the *pmrAB* knockout strain (Figure 3.20), so that expression of plasmid-encoded *pmrAB* was likely.

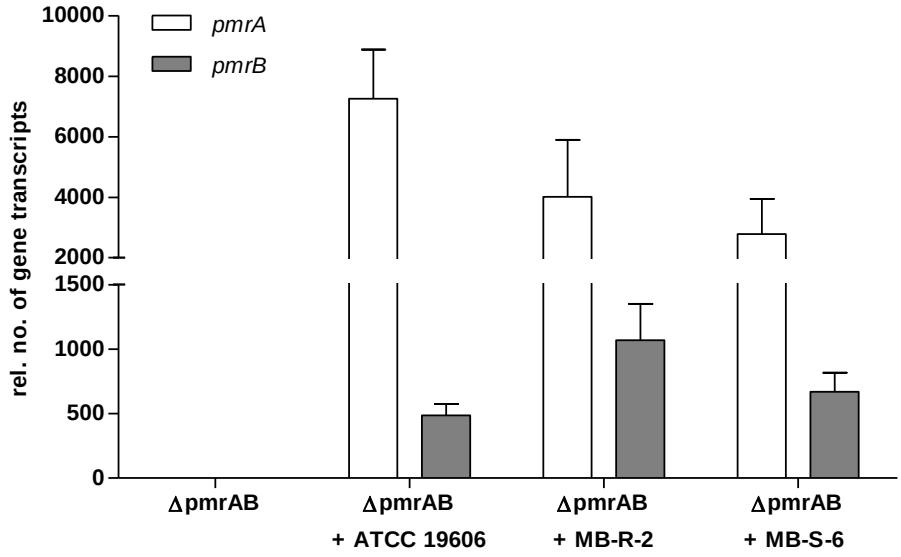


Figure 3.20: Relative expression of *pmrAB* on the plasmid pJN17/04

The relative expression of *pmrA* (white) and *pmrB* (grey) was determined by qRT-PCR. After the normalisation to the expression of the reference gene *rpoB*, the relative number of transcripts of the transformants carrying different *pmrAB* variants was related to the expression of *pmrAB* of the knockout strain Δ*pmrAB*. Results are represented as mean ± SEM.

Moreover, the expression of *pmrC* did not differ in Δ*pmrAB* complemented with *pmrAB* from MB-R-2 or *A. baumannii* ATCC 19606 but was significantly increased in Δ*pmrAB* complemented with *pmrAB* from MB-S-6 (Figure 3.21 A). This indicates that the short amino acid deletion (ΔL9-G12) in PmrB is indeed associated with differential *pmrC* expression. A basal expression of *pmrC* is indicated by a slightly increased *pmrC* expression in the *A. baumannii* wild type strain ATCC 19606 compared to Δ*pmrAB* (Figure 3.21 A). Similar results were obtained for the *A. baumannii* reference strains ATCC 19606 and ATCC 17978 complemented with *pmrAB* variants from isolate pair 1 and 3 (Figure 3.21 B, C). The introduction of *pmrAB* from MB-S-6 and MB-R-119 led to a significant increase of *pmrC* expression in both reference strains. Moreover, significant elevated *pmrC* expression was also observed when comparing the number of transcripts within isolate pairs. These results suggest an association of *pmrB* mutations with increased *pmrC* expression, although colistin MICs did not differ.

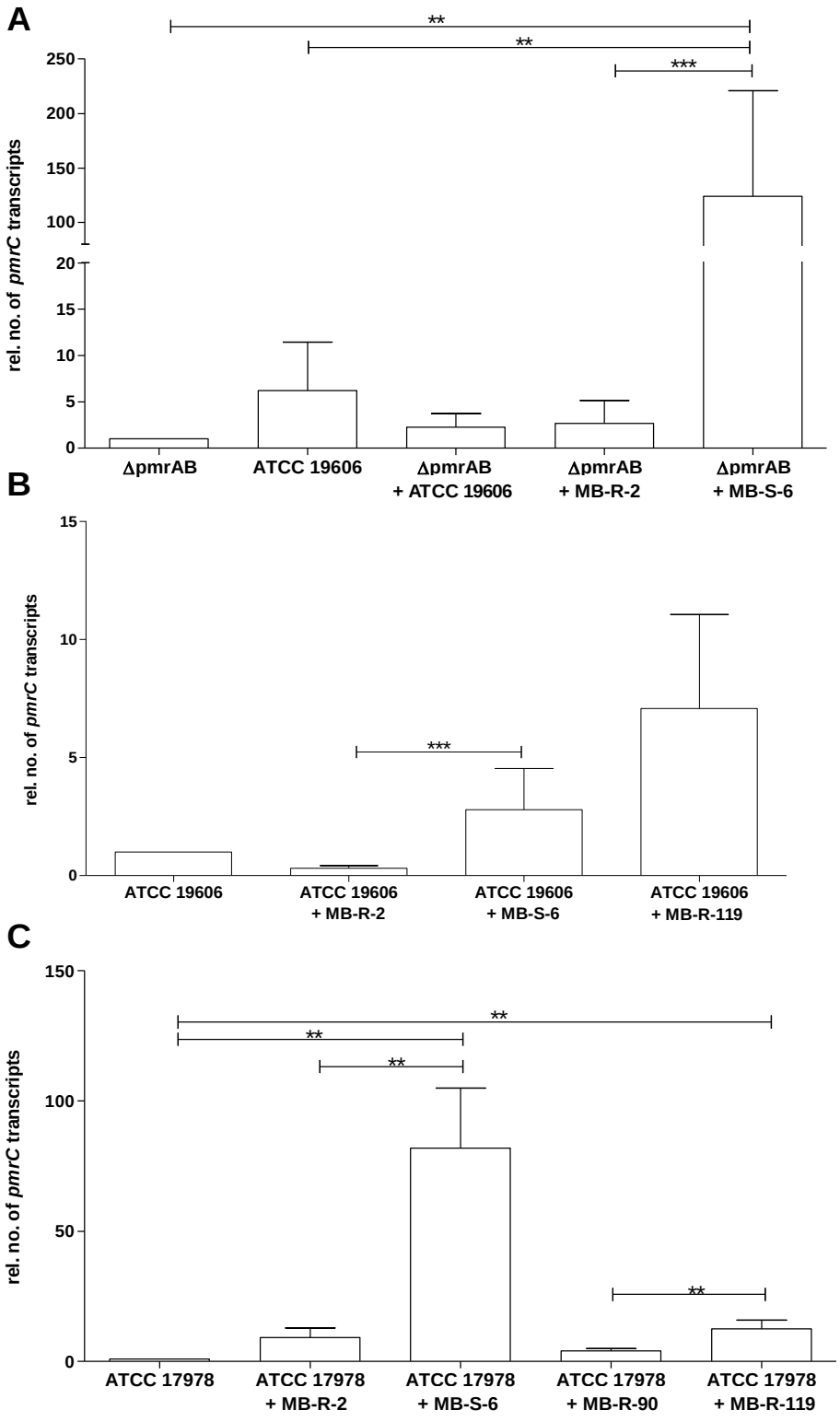


Figure 3.21: Relative *pmrC* expression of *A. baumannii* strains complemented with *pmrAB*-variants

The number of *pmrC* transcripts of *A. baumannii* Δ*pmrAB* (A) [230], ATCC 19606 (B) and ATCC 17978 (C) harbouring different *pmrAB* variants (from ATCC 19606, MB-R-2, MB-S-6, MB-R-90 and MB-R-119) was related to the respective wild type strain or the knockout (Δ*pmrAB*). The number of transcripts was normalized to the expression of the reference gene *rpoB* and the wild type strain or the *pmrAB* knockout were set as 1. Results are represented as mean ± SEM. Statistical analysis was done performing unpaired t-test using the recorded absolute values. *** $p < 0.001$, ** $p < 0.01$.

Although *pmrC* was overexpressed in Δ*pmrAB* harbouring pJN17/04::*pmrAB*-S6, the colistin MICs were not increased. Therefore, the modification of lipid A with PEtN was analysed by mass spectrometry (Figure 3.22). Overall similar mass spectra as already described for the four isolates pairs were observed for all strains harbouring different *pmrAB* variants, which included masses at m/z 1404, 1530, 1728 and 1910. The modification of lipid A by PEtN (m/z 2034) was detected only in Δ*pmrAB* complemented with pJN17/04::*pmrAB*-S6 (Figure 3.22, Table 3.17), which indicates that the *pmrB* mutation (ΔL9-G12) resulted both in increased *pmrC* expression and subsequent lipid A modification. It is therefore surprising that the colistin MICs did not change and a different hitherto unknown factor contributing to resistance or strain-dependency are suggested.

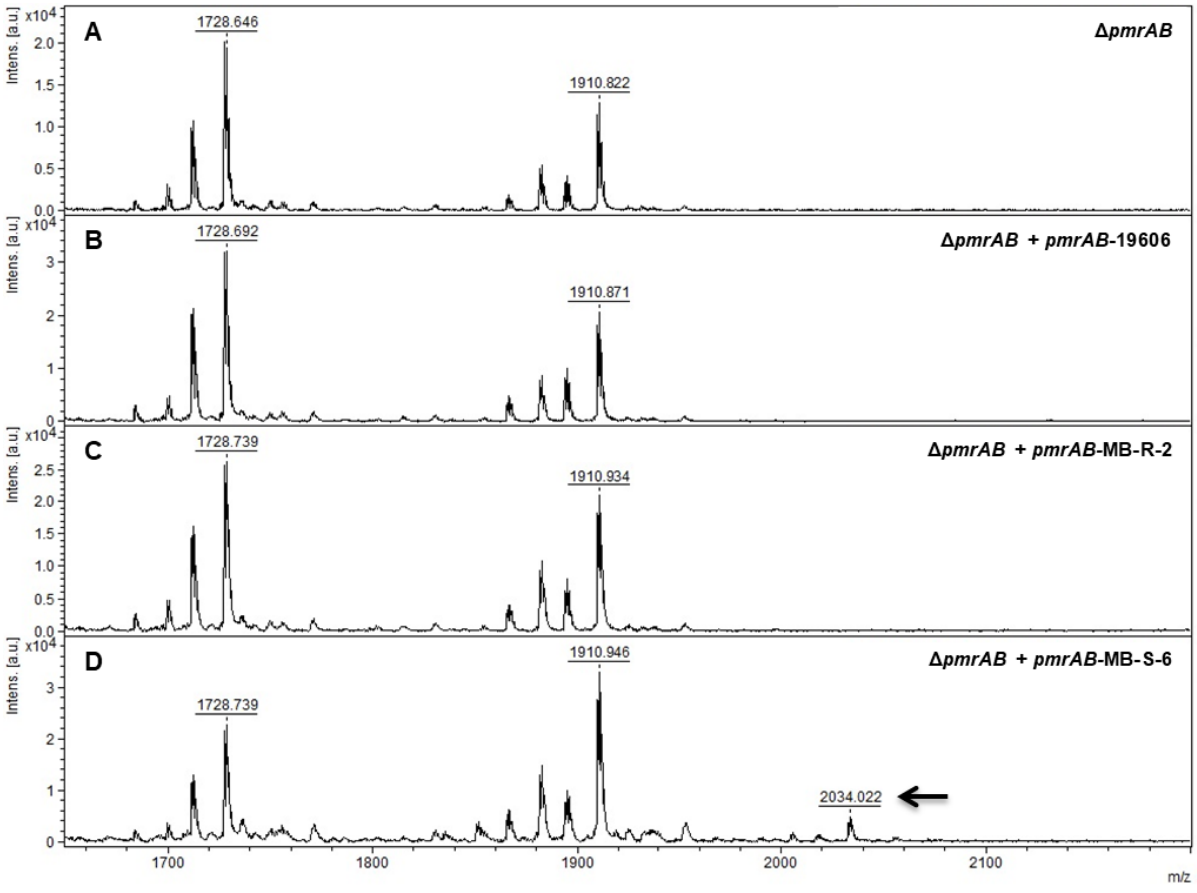


Figure 3.22: Mass spectra of lipid A extracted from *A. baumannii* Δ*pmrAB* strains

Represented are mass spectra in the range of m/z 1650 to 2200 and masses of important lipid A species are given in the spectra. The knockout strain *A. baumannii* Δ*pmrAB* was complemented with *pmrAB* variants originating from *A. baumannii* ATCC 19606 as well as isolates MB-R-2 and MB-S-6. Arrow = PEtN addition indicated by a mass at m/z 2034. Adapted from [230].

Table 3.17: Overview of lipid A species detected in the knockout strain Δ*pmrAB*

Mass spectra were measured by MALDI-TOF mass spectrometry using negative ion mode within a mass range of 500-3000 Da. Represented are the major lipid A species: hepta-acylated lipid A (m/z 1910), hepta-acylated lipid A with shorter fatty acid chains (m/z 1883), hexa-acylated lipid A (m/z 1728), penta-acylated lipid A lacking one fatty acid moiety (m/z 1530) and tetra-acylated lipid A (m/z 1404). Masses of lipid A modified with PEtN (m/z 124) are highlighted in light grey.

	ATCC 19606	**Δ*pmrAB***	**Δ*pmrAB* + *pmrAB*-19606**	**Δ*pmrAB* + *pmrAB*-MB-R-2**	**Δ*pmrAB* + *pmrAB*-MB-S-6**
Detected mass [m/z]	1404.133	1404.104	1404.019	1404.091	1404.143
	1530.086	1530.089	1530.080	1530.060	
	1728.320	1728.646	1728.692	1728.739	1728.739
					1851.525
	1882.474	1882.430	1882.315	1882.394	1882.448
	1910.542	1910.822	1910.871	1910.934	1910.946
					2005.559
					2034.022

3.3.2. Differences in PmrCAB and EptA in respiratory *A. baumannii* isolates

Part of the results have been published in Gerson *et al*. [243].

To investigate the occurrence of *pmrCAB* and *eptA* mutations as well as IS-elements associated with colistin resistance the 65 respiratory *A. baumannii* isolates were again analysed regarding their colistin resistance mechanisms. In total, 32 isolates were colistin-resistant, corresponding to nearly 50% of tested isolates. As already mentioned, the isolates were analysed by WGS and their genomes were screened for differences in *pmrCAB* and *eptA*, as well as other colistin resistance determinants (e. g. LPS biosynthesis genes).

In total, compared to the ACICU reference strain, 6 amino acid substitutions were observed in PmrA in 4 isolates, 25 amino acid substitutions in PmrB in 7 isolates and 69 amino acid substitutions in PmrC in 21 isolates (Table 3.18). All amino acid substitutions were unique to colistin-resistant isolates. The majority of these PmrCAB differences were found in the two colistin-resistant isolates MB-R-93 and MB-R-96 (Table 3.19). Both isolates were recovered in Athens, Greece, represented the clonal lineage IC2 and displayed colistin MICs of ≥ 256 mg/L. Although both MB-R-93 and MB-R-96 belong to the clonal lineage IC2, their PmrCAB sequence shared high similarity with PmrCAB obtained from the IC4 isolate SG3161 (Appendix 15-17).

Table 3.18: Overview of PmrCAB and other allele differences detected in colistin-resistant *A. baumannii* isolates

Amino acid differences were detected by comparison of the PmrCAB amino acid sequences of the isolates with *A. baumannii* reference strain ACICU and AYE as well as colistin-susceptible isolates belonging to the clonal lineages IC1 and IC2. Adapted from [243].

Isolate	Colistin MIC [mg/L]	Amino acid substitutions			Other allele differences
		PmrA	PmrB	PmrC	
MB-R-10	8		T42P		
MB-R-17	8		P170L		
MB-R-53	64				EptA
MB-R-83	8				EptA
MB-R-90	32			R125P	
MB-R-91	4			R125P	
MB-R-94	8			R125P	
MB-R-98	4			R125P	
MB-R-110	16				ZndP, permease transporter
MB-R-114	8			R125P	
MB-R-116	128		L153F	R125P	
MB-R-119	≥ 256		A28V	R125P	
MB-R-120	64		L153F	R125P	
MB-R-123	4			R125P	
MB-R-125	4			R125P	
MB-R-126	4			R125P	
MB-R-127	8			R125P	
MB-R-129	8			R125P	
MB-R-130	16			R125P	
MB-R-131	32			R125P	
MB-R-141	≥ 256		S128R	R125P	EptA
MB-R-143	≥ 256		S128R	R125P	EptA
MB-R-148	≥ 256	G54E			
MB-R-152	8				ZndP, permease transporter
MB-R-153	8			R125P	
MB-R-157	8				ZndP, permease transporter
MB-R-158	4			R125P	
MB-R-159	4			R125P	
MB-R-160	4			R125P	
MB-R-164	≥ 256	G54E			

Table 3.19: Amino acid substitutions in PmrCAB in two *A. baumannii* isolates

Amino acid differences were detected by comparison of the PmrCAB amino acid sequences of the two *A. baumannii* isolates MB-R-93 and MB-R-96 with the reference strain ACICU and AYE as well as colistin-susceptible isolates belonging to the clonal lineages IC1 and IC2. Adapted from [243].

	Amino Acid Substitutions
PmrA	I18T, T44N, E133Q, S134G, T168S
PmrB	A28T, K45Q, H54Y, F65L, K67R, I76L, H86N, L93F, E99Q, Q110E, I112V, R165S, P170S, K179M, E210D, R211S, E320D, I322V, H362N, Y363F
PmrC	N3S, T7K, L20V, H36K, Q37K, T40L, F45L, V47I, I50T, L57V, L60V, V67A, I85V, M110I, V111A, S116A, V118A, I122L, V127G, I135V, F146L, Q148S, V151I, S152L, R153P, F160L, L162I, V163A, A164L, S165V, V150G, T175A, S199T, L203V, H208R, I220K, Q223E, R230Q, V231T, N234D, I240V, D269N, F271L, V297A, N345D, K348Q, Y357L, T371S, A373P, D377K, I382V, V387M, A401G, Q419P, T420A, L423I, I447V, K449N, L454F, L469M, A477S, V486I, E494D, S495G, H499N, S508N, L527M, H547N

Since BLAST analysis revealed low amino acid sequence identity with PmrCAB originating from other *Acinetobacter* species or Gram-negative pathogens, homologous recombination of *pmrCAB* across different *A. baumannii* clonal lineages is suggested. Of note, it is likely that not all amino acid substitutions will be responsible for colistin resistance. A comparison of the PmrCAB sequence between the two IC2 isolates MB-R-93 and MB-R-96 and the colistin-susceptible IC4 isolate SG3161 (Chapter 3.3.1.1) revealed only three amino acid differences (S134G in PmrA, P170S and R211S in PmrB) unique for the colistin-resistant isolates MB-R-93 and MB-R-96 (Table 3.19).

3.3.2.1. Amino acid substitutions in PmrCAB

The amino acid substitution G54E was found in receiver domain of PmrA in the two colistin-resistant isolates MB-R-148 and MB-R-164. Both isolates displayed high colistin MICs of ≥ 256 mg/L (Table 3.18, Figure 3.25) and the analysis of *pmrC* expression in MB-R-148 revealed an over 50-fold increased expression of *pmrC* compared to the colistin-susceptible *A. baumannii* reference strain ATCC 19606 and Δ*pmrAB* (Figure 3.23). This result indicates an association of the PmrA substitution G54E with *pmrC* overexpression and colistin resistance.

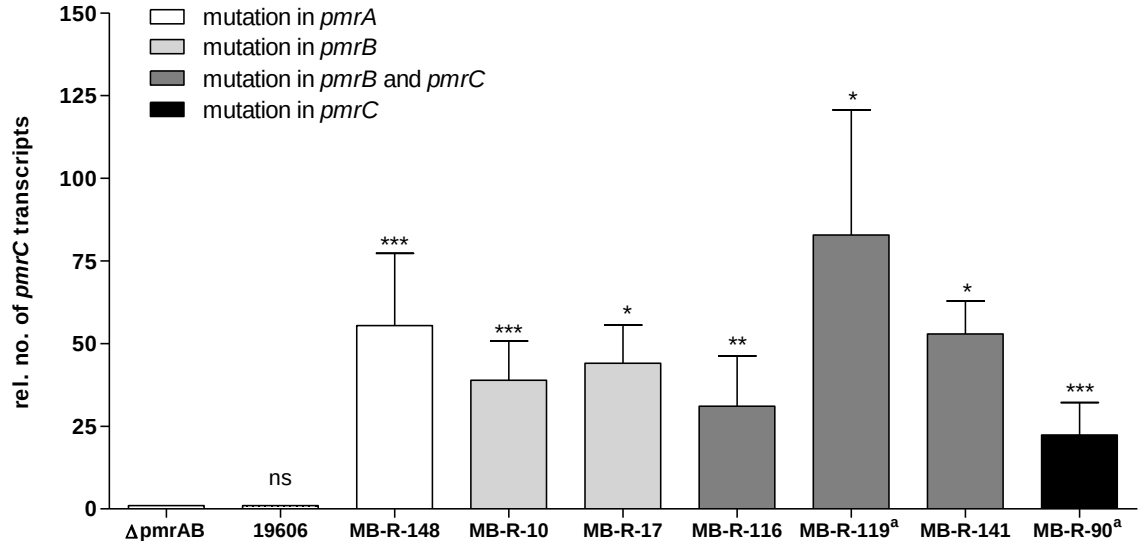


Figure 3.23: Relative *pmrC* expression determined by qRT-PCR

The colistin-susceptible *pmrAB* knockout (Δ*pmrAB*) was used for comparison to *A. baumannii* ATCC 19606 and isolates harbouring *pmrCAB* mutations. After being normalised to the expression of the reference gene *rpoB,* the *pmrAB* knockout was set to 1. Results are represented as mean ± SEM. Statistical analysis was done by performing an unpaired t test using the recorded absolute values. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. ns, not significant. [a], expression analysis was also done in Chapter 3.3.1.2. Adapted from [243].

In or near the periplasmic and the transmembrane domains of PmrB the five amino acid substitutions A28V, T42P, S128R, L153F and P170L were detected in seven colistin-resistant isolates with colistin MICs ranging from 8 to ≥ 256 mg/L (Table 3.18, Figure 3.24, Figure 3.25). The expression of *pmrC* was analysed by qRT-PCR in chosen isolates (MB-R-105, MB-R-17, MB-R-116, MB-R-119, MB-R-141) and *pmrC* expression was about 30 to 80 times higher compared to the *A. baumannii* reference strain ATCC 19606 and Δ*pmrAB* (Figure 3.23). Moreover, the PmrB substitutions A28V, S128R and L153F were also associated with the substitution R125P in PmrC (Table 3.18). While single mutations in either *pmrB* or *pmrC* contributed to elevated colistin MICs ranging from 4 to 32 mg/L, a combination of mutations resulted in very high colistin MICs of 64 to ≥ 256 mg/L (Table 3.18, Figure 3.25). Nevertheless, no significant difference in *pmrC* expression was detected comparing isolates with single mutations in *pmrB* or *pmrC* and isolates harbouring a combination of mutations in both genes (Figure 3.23).

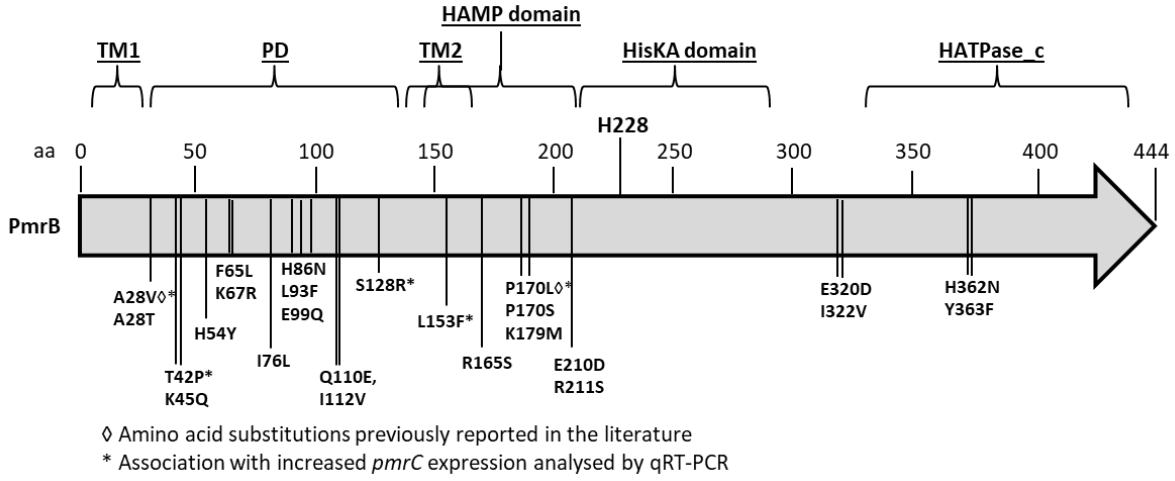


Figure 3.24: Overview of amino acid substitutions in the histidine kinase PmrB

Amino acid substitutions were found by comparing the amino acid sequences of colistin-resistant isolates with *A. baumannii* reference strains ACICU, ATCC 19606, ATCC 17978 and AYE as well as colistin-susceptible isolates. Putative domains of the histidine kinase are given [193]. aa = amino acid, TM1 = first transmembrane domain (aa 10-29), PD = periplasmic domain (aa 29-142), TM2 = second transmembrane domain (aa 142-164), HAMP domain = Histidine kinases, adenylyl cyclases, methyl-binding proteins, and phosphatases domain (aa 145-214), HisKA domain = Histidine kinase A domain (aa 218-280), HATPase_c domain = (aa 326-437), H228 = putative histidine residue involved in autophosphorylation and transfer of the phosphoryl group to PmrA. Adapted from [243].

In PmrC, the amino acid substitution R125P was found in 21 unrelated colistin-resistant isolates (Table 3.18). The substitution was associated with colistin MICs ranging from 4 to ≥ 256 mg/L, although colistin MICs of ≥ 64 mg/L were observed only in isolates harbouring a combination of PmrB and PmrC substitutions (Figure 3.25, Table 3.18). Isolate MB-R-90 was chosen as an example of an isolate carrying the PmrC substitution R125P. The expression of *pmrC* was significantly increased in MB-R-90 compared to the *A. baumannii* reference strain ATCC 19606 and the *pmrAB* knockout, indicating an association of *pmrC* mutations with colistin resistance (Figure 3.23).

To investigate the effect of *pmrB* mutations on the phenotype of colistin-susceptible *A. baumannii* strains, *pmrAB* of isolate MB-R-119 (A28V in PmrB) was introduced into the *A. baumannii* reference strains ATCC 19606 and ATCC 17978 (Chapter 3.3.1.5). In both reference strains an increase in *pmrC* expression was observed following the introduction of pJN17/04::*pmrAB*- 119 (Figure 3.21), but no changes in colistin MICs were observed, all strains remained susceptible (MIC 0.25 – 1 mg/L) (Table 3.16). These results suggest an association of the PmrB substitution A28V with increased *pmrC* expression, but not necessarily with a change in phenotype from susceptible to resistant.

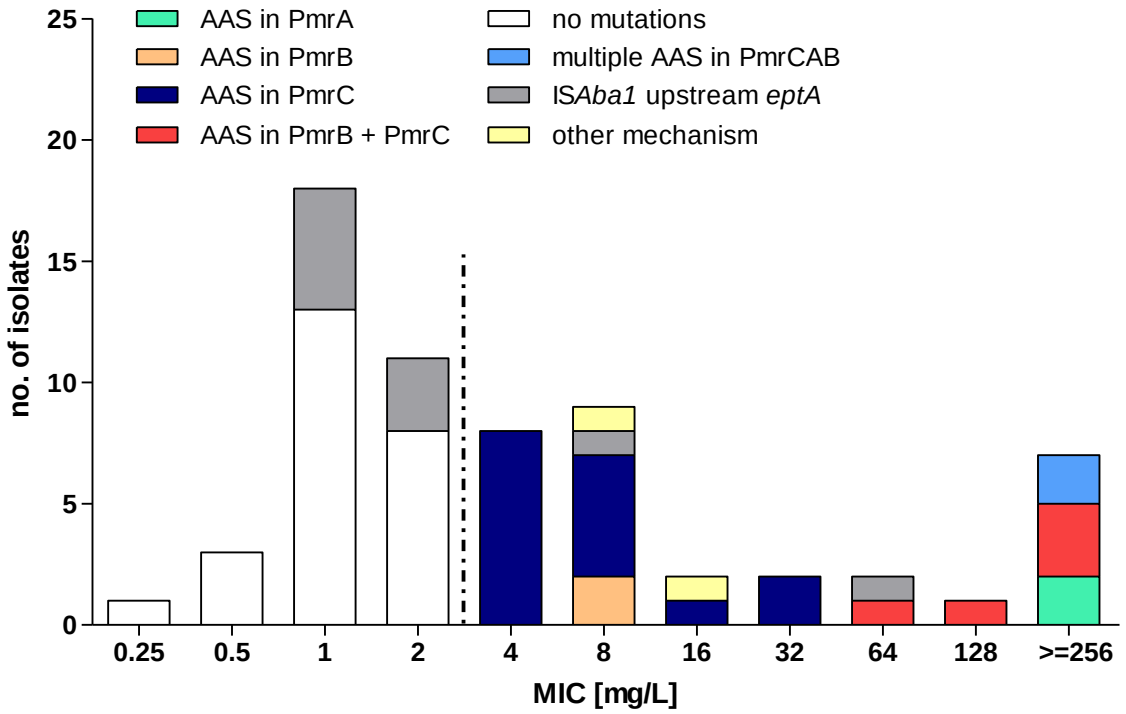


Figure 3.25: Overview of colistin MICs in comparison to differences detected in PmrCAB and EptA
Differences were detected by comparing the amino acid sequences of PmrCAB or EptA between isolates or with *A. baumannii* reference strains. IS*Aba1* was identified by PCR and Sanger sequencing. The dotted line is separating colistin-susceptible and -resistant isolates. AAS = Amino acid substitution. Adapted from [243].

3.3.2.2. Differences in the *pmrC*-homologue *eptA*

The *pmrC*-homologue *eptA* was present in 29 colistin-susceptible (45.3%) and 27 colistin-resistant isolates (40.6%). Interestingly, *eptA* was only found in isolates belonging to the clonal lineage IC2 and shared high sequence homology (Appendix 18). Amino acid differences in EptA were detected only in one colistin-resistant isolate (MB-R-53), which was recovered from the same patient as isolate pair 2 (Chapter 3.3.1). Besides the amino acid substitution in EptA, IS*Aba1* was also detected upstream of *eptA* in combination with a point mutation (A→T) in position 1091 near the promoter region of IS*Aba1* in isolate MB-R- 53. Furthermore, the insertion sequence IS*Aba1* was upstream of *eptA* in reverse orientation and exactly at the same insertion site in eight colistin-susceptible isolates (MIC 1-2 mg/L) and one other colistin-resistant isolate (MB-R-83) (Table 3.20). Interestingly, the two isolates MB-R-141 and MB-R-143, bearing both the PmrB and PmrC substitutions S128R and R125P, respectively, also presented an IS*Aba125* insertion sequence that disrupted the *eptA* gene (Table 3.20). Therefore, and due to the presence of IS*Aba1* upstream of *eptA* both in colistin-susceptible and -resistant isolates an association of *eptA* with colistin resistance is unlikely.

Table 3.20: Overview of differences in EptA in *A. baumannii* isolates

Amino acid differences were detected by comparing the amino acid sequences of EptA with *A. baumannii* reference strains ACICU and AYE. Differences in the genome and presence of IS*Aba1* were detected by comparing the nucleic acid sequences with the *A. baumannii* reference strains ACICU and AYE as well as by PCR and Sanger sequencing.

Isolate	Colistin MIC [mg/L]	*eptA* difference
MB-R-12	2	IS*Aba1* upstream in reverse orientation
MB-R-23	1	IS*Aba1* upstream in reverse orientation
MB-R-53	64	Amino acid substitution R127L; IS*Aba1* upstream in reverse orientation and harbouring a point mutation (A→T in position 1091)
MB-R-62	2	IS*Aba1* upstream in reverse orientation
MB-R-64	1	IS*Aba1* upstream in reverse orientation
MB-R-81	1	IS*Aba1* upstream in reverse orientation
MB-R-83	8	IS*Aba1* upstream in reverse orientation
MB-R-135	1	IS*Aba1* upstream in reverse orientation
MB-R-138	2	IS*Aba1* upstream in reverse orientation
MB-R-141	≥256	Disruption by IS*Aba125*
MB-R-143	≥256	Disruption by IS*Aba125*
MB-R-151	1	IS*Aba1* upstream in reverse orientation

Nevertheless, both a colistin-susceptible isolate (MB-R-12) and the colistin-resistant isolates MB-R-2 (isolate pair 1) and MB-R-53 were selected to compare the expression of *eptA* by qRT-PCR (Figure 3.26). The expression of *eptA* was significantly increased in isolate MB-R-53 compared to the colistin-susceptible isolates MB-R-2 and MB-R-12. Moreover, the expression of *eptA* was similar in isolates MB-R-2 and MB-R-12, although IS*Aba1* was detected upstream of *eptA* in MB-R-12. These results indicate that the presence of IS*Aba1* upstream of *eptA* is not associated with increased *eptA* expression and colistin resistance unless additional mutations are present in IS*Aba1* and/or *eptA*.

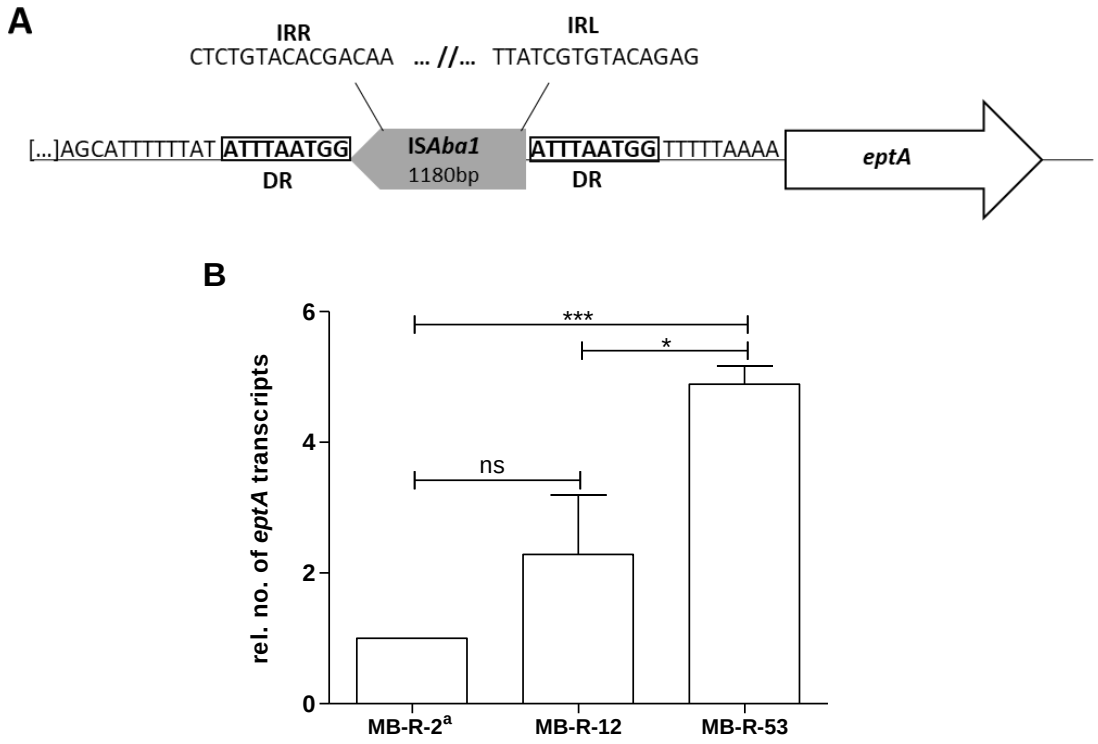


Figure 3.26: Schematic overview of IS*Aba1* insertion upstream *eptA* and relative *eptA* expression determined by qRT-PCR

(A) Schematic overview of IS*Aba1* insertion upstream of *eptA*. IRR = inverted repeat right. IRL = inverted repeat left. DR = direct repeat. (B) The colistin-susceptible *A. baumannii* isolate MB-R-2 (isolate pair 1) and MB-R-12 (IS*Aba1* upstream *eptA*) were used for comparison to the colistin-resistant isolate MB-R-53. After being normalised to the expression of the reference gene *rpoB,* the isolate MB-R-2 was set to 1. Results are represented as mean ± SEM. Statistical analysis was done by performing an unpaired t test using the recorded absolute values. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. ns, not significant. [a], expression analysis was done in Chapter 3.3.1.2. Modified from [243].

3.3.2.3. Other allele differences detected in colistin-resistant *A. baumannii* isolates

In total, four colistin-resistant isolates with a still unknown colistin resistance mechanism remain (MB-R-83, MB-R-110, MB-R-152, MB-R-157). In these four isolates, no amino acid differences were detected in PmrCAB or EptA. Only in isolate MB-R-83 IS*Aba1* was detected upstream of *eptA*, which is probably not associated with colistin resistance (Chapter 3.3.2.2). The expression of *pmrC* and *eptA* was analysed by qRT-PCR in isolates MB-R-110, MB-R-152 and MB-R-157 and revealed no differences compared to *A. baumannii* Δ*pmrAB* (Figure 3.27), indicating a different colistin resistance mechanism in these isolates.

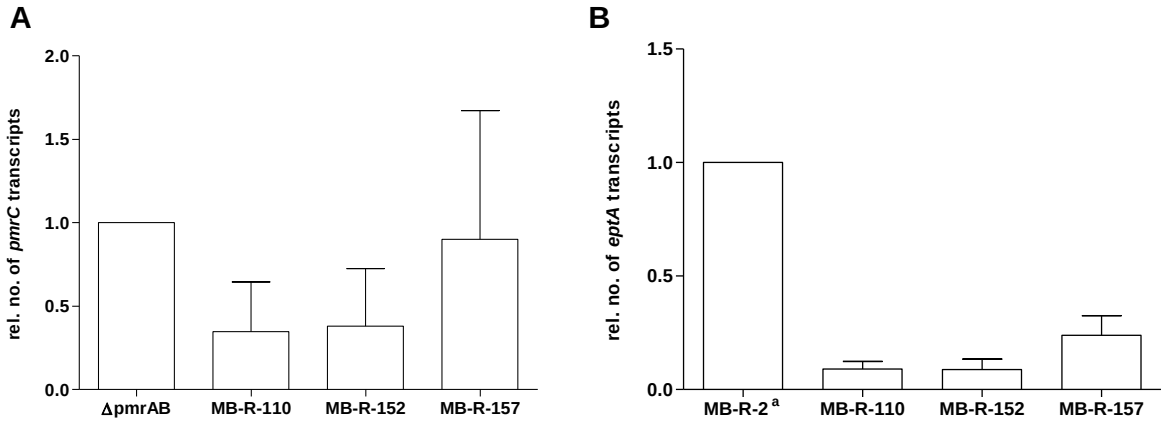


Figure 3.27: Relative *pmrC* and *eptA* expression in colistin-resistant *A. baumannii* isolates
The expression of *pmrC* (A) and *eptA* (B) was measured in the colistin-resistant *A. baumannii* isolates MB-R-110, MB- R- 152 and MB-R-157 and compared to the *pmrAB* knockout strain (Δ*pmrAB*) or the colistin-susceptible isolate MB-R-2. After being normalised to the expression of the reference gene *rpoB,* the isolate MB-R-2 and the *pmrAB* knockout were set to 1. Results are represented as mean ± SEM. [a], expression analysis was done in Chapter 3.3.2.2.

To investigate the resistance mechanism in the four isolates, alleles known to be associated with colistin resistance (e. g. *hns*, genes involved in lipid A biosynthesis or *emrAB* and *emrB*- like efflux pumps) were analysed. No disruption of or mutations in genes involved in lipid A biosynthesis or the global transcription regulator H-NS were detected. Moreover, neither the resistance gene *mcr-1* nor its variants were found in any colistin-resistant isolate. Instead in all four isolates, differences in efflux pumps genes were detected. All allele differences found in MB-R-83 were also detected in colistin-susceptible isolates and therefore not associated with colistin-resistance (Table 3.21, Appendix 4). The colistin resistance mechanism remains to be elucidated in isolate MB-R-83, although the presence of a potentially novel resistance mechanism is suggested in this isolate. In isolates MB-R-110, MB-R-152 and MB-R-157 the amino acid substitutions A529T in ZndP (ACICU_RS10195) and G106S in a putative permease transporter (ACICU_RS15345) were observed (Table 3.21, Appendix 4). These differences were found exclusively in the three isolates, which might indicate an association with colistin resistance. However, the allele differences could also be simply a result of genetic polymorphisms, since all three isolates are strongly different from other IC2 isolates as determined by cgMLST (Appendix 9). Therefore, the three isolates were previously considered not related to any known clonal lineage [227]. Further investigation is required to elucidate their association with colistin resistance.

Table 3.21: Overview of allele differences in colistin-resistant *A. baumannii* isolates

Allele differences were detected by comparing amino acid sequences with colistin-susceptible isolates and the *A. baumannii* reference strains ACICU, AYE, ATCC 19606 and ATCC 17978.

Isolate	Colistin MIC [mg/L]	Allele difference
MB-R-83	8	*adeN*: IS*Aba1* insertion AdeL: I37L RND1 (ACICU_RS00750): V17I, I561V IS*Aba1* upstream *eptA*
MB-R-110	16	ZndP (A529T) permease transporter (G106S)
MB-R-152	8	ZndP (A529T) permease transporter (G106S)
MB-R-157	8	ZndP (A529T) permease transporter (G106S)

4. Discussion

A. baumannii is a successful pathogen of global importance and a major threat to human health-care due to its persistence and desiccation tolerance in hospital settings, its propensity of epidemic spread, and its antimicrobial resistance determinants. The bacterium has evolved numerous different resistance mechanisms against most of the clinically available antimicrobial agents, leaving colistin and tigecycline often as a last option in the treatment of infections with MDR *A. baumannii*. However, resistance against both tigecycline and colistin is increasing and pandrug-resistant *A. baumannii* isolates are reported more frequently [27, 80]. It is therefore important to elucidate the mechanisms of tigecycline and colistin resistance, not only for surveillance purposes, but also to enable strategies to overcome resistance, and to aid in the identification of potential new targets for antimicrobial drug development.

This study investigated colistin and tigecycline resistance mechanism in *A. baumannii* isolates, which were recovered as part of a large multicentre study involving patients from 15 hospitals in Greece, Italy and Spain and thus originated from a wide geographical region. A previous study has shown that many of these isolates are multidrug- or even pandrug-resistant [27]. Generally, the prevalence of antimicrobial resistance is higher in Southern and Eastern Europe compared to Northern Europe [255]. In 2018, the EARS-NET surveillance report showed that 81.3% of *A. baumannii* isolates in Greece, 44.4% in Spain and 75.7% in Italy were resistant to carbapenems, fluoroquinolones and aminoglycosides, respectively. In comparison, the resistance rates for the three antimicrobials were less than 5% in Northern Europe (e.g. 2.2% in Germany) [256]. Therefore, the high incidence of multidrug- and pandrug-resistant *A. baumannii* isolates in the MagicBullet clinical trial is not surprising. The MDR phenotype of the isolates is partly explained by the presence of a high number of different resistance determinants. While all isolates had intrinsic resistance determinants such as for example the OXA-51-like β-lactamase, they additionally harboured at least two acquired resistance determinants, and more than 50% of isolates harboured 10 to 15 resistance determinants. There is evidence that the acquisition of antimicrobial resistance determinants is mainly due to horizontal gene transfer and the presence of mobile genetic elements (e.g. insertion sequences or integrons), which are frequently found in *A. baumannii* [66, 257]. *A. baumannii* is not only naturally competent [70], but also harbours traces of genes originating from prophages as well as a pool of intrinsic plasmids [73, 196]. This indicates a large machinery for horizontal gene transfer and the ability to rapidly acquire genetic material including antimicrobial resistance determinants. Genetic divergence in *A. baumannii* clonal lineages is further promoted by genome wide homologous recombination, which has been reported e.g. for genes encoding cell surface molecules [258]. All these increase the genetic variability of *A. baumannii* belonging to different clonal lineages. An example is the sequence variability observed for the RND-efflux pump AdeB in *A. baumannii* [259], which makes it especially

difficult to decide if mutations are a cause of resistance or simply a genetic variance. Indeed, Yoon *et al.* identified some *adeS* mutations as genetic polymorphisms, which were not associated with *adeABC* overexpression and antimicrobial resistance [142]. To exclude genetic polymorphisms in the work presented in this thesis, eight isolate pairs were chosen for the investigation of resistance mechanisms in this study. Isolate pairs have the advantage of an identical or very similar isogenic background [227, 230], so that typical genetic variations seen in epidemiologically unrelated strains can be excluded and any genetic difference is most likely a result of antimicrobial selection pressure. Similar approaches have been conducted by Hornsey *et al.* for tigecycline resistance mechanisms and Dahdouh *et al.*, Durante-Mangoni *et al.* and Beceiro *et al.* for the investigation of colistin resistance in *A. baumannii* [195, 260–262]. Furthermore, whole genome sequencing was used for the identification of novel mutations associated with resistance. This method is a powerful tool as it not only provides information about the phylogeny of *A. baumannii* but also about the presence of resistance determinants, virulence and pathogenicity factors as well as the natural variance of these genes or their regulators [263]. An identification scheme for genetic differences associated with antimicrobial resistance was created and allowed the detection of several novel mutations in the regulatory genes (e. g. *adeRS* and *pmrAB*) as well as genes previously reported to be associated with tigecycline and colistin resistance, respectively. However, this thesis also highlights the difficulties to correlate a genotype with a clinical phenotype as discrepancies were found between the genetic mutations and the resulting phenotype [263].

4.1. Tigecycline resistance in *A. baumannii*

The antimicrobial agent tigecycline is approved for the treatment of complicated skin and soft tissue infections as well as complicated intra-abdominal infections [152], both of which are only rarely caused by *A. baumannii*. However, the alarming increase of carbapenem-resistant *A. baumannii* isolates in the last decades has made both tigecycline and colistin antimicrobial agents of last resort with *in vitro* activity against carbapenem-resistant isolates. Although tigecycline displays several advantages such as minor side effects, ability to evade tetracycline resistance mechanisms and good tissue penetration [152, 153], its clinical application is under discussion due to limited efficacy and increasing resistance of many pathogens [264]. Moreover, tigecycline is prone to oxidation, which causes problems in application of the drug and susceptibility testing [155]. Indeed, major variations in tigecycline MICs were observed depending on the antimicrobial testing method used [157, 264, 265]. In several studies, a significantly higher number of *A. baumannii* isolates displaying tigecycline MICs ≥ 4 mg/L was reported using Etest compared to other testing method like broth microdilution or semi-automated systems like Vitek2™ or BD Phoenix™ [157, 264, 266]. It remains to be elucidated if the same applies to other testing methods based on the diffusion of the antimicrobial agent through agar (e.g., disc diffusion and agar dilution). In fact, discrepancies became apparent comparing the tigecycline MICs of the MagicBullet isolates using different methods. The MIC

results obtained by agar dilution in this thesis were approximately two-fold higher compared to the results obtained by broth microdilution by Seifert *et al*. (Appendix 2) [267, 268]. Looking at the MIC data obtained by broth microdilution a total of 60 isolates displayed low tigecycline MICs of ≤ 2 mg/L and only 5 isolates showed higher tigecycline MICs of ≥ 4 mg/L. This is a considerable difference to the results obtained with agar dilution and might suggest that MIC variations as described for Etest also apply to agar dilution and that results obtained with this method should be treated with caution at least regarding clinical decision making in patients infected with *A. baumannii* [264]. However, the analysis of draft genome data revealed several amino acid substitutions and other differences in the regulatory genes of RND-type efflux pumps which are suggested to be associated with elevated tigecycline MICs (Chapter 4.1.2). Moreover, another major problem when working with tigecycline is the lack of tigecycline breakpoints for *Acinetobacter* spp. Generally, breakpoints are used to define susceptibility of a bacterial population, which directly correlates with the likelihood of therapeutic success in clinical settings [269]. Since clinical data about therapeutic success or failure is lacking and tigecycline is usually only used in severe disease with limited alternative treatment options, neither EUCAST nor CLSI have defined tigecycline breakpoints for *Acinetobacter* spp. The tigecycline breakpoints defined for *Enterobacterales* are sometimes used as a surrogate, which is not based on scientific or clinical data and has previously been discussed critically [265]. Looking at the tigecycline MIC distribution of wild type *A. baumannii* isolates most isolates (>2000 isolates) display MICs between 0.064 and 2 mg/L [270]. Using both the wild type distribution and the breakpoints for *Enterobacterales* defined by EUCAST (version 8.1, 2018, susceptible = MIC ≤ 1 mg/L, intermediate = MIC 2 mg/L, resistant = MIC ≥ 4 mg/L) [241] as a tentative guideline, a distinction for *A. baumannii* can be made between isolates with low tigecycline MICs (≤ 2 mg/L) and high MICs (≥ 4 mg/L). Of note, the tigecycline breakpoints for *Enterobacterales* have recently been revised by EUCAST (version 10.0, 2020) and susceptibility is now defined as MIC ≤ 0.5 mg/L [269].

The absence of tigecycline breakpoints for *A. baumannii* is a problem not only for diagnostic laboratories trying to find a treatment option leading to therapeutic success in clinical settings. It is also a problem for the analysis of resistance determinants in this thesis, which is based on the comparison of resistant versus susceptible isolates or reference strains (both for finding genetic differences as well as for excluding genetic polymorphisms). While in all isolates representing isolate pairs (defined as a pair of isogenic isolates displaying a shift in their tigecycline antimicrobial susceptible (MIC) phenotype and with less than ten allele differences in their genome) differences in their genotype allowed for an analysis of tigecycline resistance mechanisms, the analysis of the collection of 65 *A. baumannii* isolates is mainly based on the comparison to reference strains and susceptible isolates. Looking at the isolates with the lowest tigecycline MICs, MB-R-67 (MIC 0.5 mg/L) and MB-R-94 (MIC 1 mg/L), a major problem becomes apperent. While the majority of the MagicBullet isolates belonged to the clonal lineage IC2, MB-R-67 was not related to any known clonal lineage and MB-R-94 belonged to

the clonal lineage IC1. Both isolates carried many allele differences (e. g. in *adeS*, *adeB* and *adeL*) compared to *A. baumannii* ACICU and all other IC1 isolates in this study carried the same allele differences as observed in MB-R-94. Although it remains to be elucidated, if these allele differences are associated with tigecycline resistance, it is plausible to assume that they are rather clonal lineage-specific polymorphisms or a result of low genetic relatedness. Such a natural sequence variance has for example been reported in *adeB* in isolates belonging to the clonal lineages IC1, IC2 and IC3 [259]. To include some more isolates for comparison, several additional *A. baumannii* isolates with low tigecycline MICs ≤ 0.5 mg/L from a worldwide *A. baumannii* collection (ABC-isolates) were included in the analysis. The ABC-isolates together with MB-R-67 and MB-R-94 provide an isolate pool of different clonal lineages (IC1, IC2 and not related to any known lineage) displaying a tigecycline MIC range between 0.25 and 1 mg/L, which makes them suitable for comparison to isolates displaying higher tigecycline MICs.

In this study, a large number of isolates displayed high tigecycline MICs (MIC ≥ 4 mg/L) (84.6%). Since there are no tigecycline resistance breakpoints available for *A. baumannii* and MICs can vary depending on the methodology, it is difficult to obtain and compare the prevalence of tigecycline resistance in Europe or worldwide.

4.1.1. Insertion sequences associated with RND-type efflux pump overexpression

Tigecycline resistance in *A. baumannii* is often associated with the overexpression of RND-efflux pumps due to mutations in the two-component system *adeRS*. Besides amino acid substitutions, other mechanism leading to the overexpression of RND-type efflux pumps have been described in the literature, e.g. the insertion of IS*Aba1* into *adeS* [163, 271]. Indeed, the analysis of four isolate pairs revealed the disruption of *adeS* and *adeN* by insertion sequences IS*Aba1* or IS*Aba125* in isolates with high tigecycline MICs ≥ 4 mg/L. In addition, disruption of *adeS* by IS*Aba1* was detected in three respiratory *A. baumannii* isolates and IS*Aba1*, IS*Aba27* and IS*Aba125* were inserted in *adeN* in a total of 20 isolates. Generally, IS-elements are found abundantly in the genome and plasmidome of *A. baumannii* and other *Acinetobacter* spp. [75, 257, 272]. According to the Reference Centre for Bacterial Insertion Sequences (ISfinder) there are currently 59 different insertion sequences known in *A. baumannii* [239], whereby IS*Aba1* is one of the most common IS-elements [75, 272, 273]. Some insertion sequences like IS*Aba1* recognise more than one specific insertion site, which fits well with the observations of different insertion sites for IS*Aba1* and IS*Aba125* in this thesis [75, 272]. Moreover, insertion sequences are often associated with a multidrug-resistant phenotype in *A. baumannii* either by insertional inactivation or by the alteration of gene expression. For example, the insertion of IS*Aba125* in *carO* resulting in carbapenem resistance and disruption of *lpxA* or *lpxC* by IS*Aba11* was associated with colistin resistance [76, 190]. Furthermore, both IS*Aba1* and IS*Aba125* are able to provide a promoter for overexpression of antimicrobial resistance determinants [78, 93, 232, 274]. When inserted in reverse orientation, IS*Aba1* or IS*Aba125*

provide the -35 region for the generation of a hybrid promotor [75, 78]. In this thesis, both insertional inactivation of *adeN* by IS*Aba1*, IS*Aba125,* and IS*Aba27* as well as the overexpression of *adeS* due to insertion of IS*Aba1* were observed.

The disruption of *adeS* was associated with an increase in *adeB* expression, increased efflux activity, and decreased susceptibility to at least six different antimicrobial classes. The disruption of *adeS* by IS*Aba1* has been described previously in *A. baumannii* and was associated with *adeABC* overexpression and elevated MICs of tigecycline and other antimicrobials [162–164]. Expression analysis of the truncated *adeS* fragments up- and downstream of the IS-element revealed significantly increased expression of *adeS* downstream of IS*Aba1* in both isolates with high tigecycline MICs of isolate pair 1 and 2. This result fits to the observations by Sun *et al.* suggesting that IS*Aba1* provides a strong promoter for the constitutive expression of a truncated *adeS*, which subsequently leads to overexpression of *adeABC* [163].

The disruption of *adeN* was associated with elevated *adeJ* expression and increased MICs to at least three antimicrobial classes. Since the TetR-like transcription regulator *adeN* is a transcriptional repressor, disruption of *adeN* by IS-elements abolishes its function, which subsequently leads to the overexpression of *adeIJK* and has already been shown *in vitro* [144]. Analysis of the truncated *adeN* up- and downstream of the IS-elements revealed unchanged or decreased expression of the truncated *adeN* compared to the intact gene. Moreover, complementation of the *adeN* knockout with truncated *adeN* from the isolates with high tigecycline MICs did not result in significant MIC changes. These findings indicate that IS*Aba1* or IS*Aba125* in forward orientation do not provide a promoter and that *adeJ* overexpression is a result of insertional inactivation of the transcriptional repressor *adeN* [144]. Interestingly, the expression of *adeJ* was significantly lower compared to *adeB* expression due to *adeS* disruption and the increase in cross-resistance is less significant in isolates with *adeN* disruption. This suggests that *adeB* overexpression has a higher impact on antimicrobial resistance including to tigecycline, in clinical settings. This is not entirely unexpected since overexpression of *adeJ* has been reported to be toxic for *A. baumannii* and might explain lower *adeJ* expression levels and tigecycline MICs [113]. Moreover, tigecycline is a substrate for all three RND-efflux pumps, but AdeABC has been reported to have a greater effect on tigecycline susceptibility compared to AdeIJK and AdeFGH [161]. The overexpression of RND-type efflux pumps as a mechanism of tigecycline resistance is not restricted to *A. baumannii.* The insertion of an IS-element upstream of a putative efflux pump operon was described to be associated with tigecycline non-susceptibility in *K. pneumoniae* and the overexpression of efflux pumps as tigecycline resistance mechanism has further been reported in *A. nosocomialis* and other Gram-negative pathogens [125, 158, 275–277].

The insertion of IS-elements was not restricted to *adeS* or *adeN* in this thesis. IS*Aba1* was also detected in *adeA* in reverse orientation. It remains to be elucidated how a disruption of *adeA* affects the expression of *adeABC* or efflux pump assembly and function. While a missing

membrane fusion protein/anchor for the membrane-bound AdeB and AdeC might abolish the functionality of the pump itself, the insertion of IS*Aba1* in reverse orientation could also lead to overexpression of *adeBC*. Furthermore, IS*Aba1* was detected in reverse orientation in the intergenic region of *adeR* and *adeS* in this study, which could lead to overexpression of *adeS* and subsequently of *adeB*. Lopes *et al.* reported the insertion of IS*Aba1* and other IS-elements like IS*Aba16* in *adeR* and the intergenic region between *adeR* and *adeA* in *A. baumannii* isolates [278]. Although these insertions of IS-elements in or adjacent to RND-type efflux pump genes are not necessarily associated with antimicrobial resistance, *adeRSABC* seems to be a frequent target site for the insertion of various IS-elements. The insertion of IS-elements is not always deleterious but can also represent a rapid response to various stresses and can have an effect on virulence and pathogenicity as well as antimicrobial resistance of *A. baumannii* [273, 279–281]. For example, Wright *et al.* reported the association of IS-elements with the adaptive response to oxidative stress in *A. baumannii* [281]. Moreover, the insertion of IS*Aba1* in *adeN* was described to not only elevate *adeJ* expression but also to increase the virulence and pathogenicity of *A. baumannii* [282, 283]. More than 30% of *A. baumannii* isolates in this study carried an insertion sequence in *adeN,* which might suggest that they are more virulent compared to isolates with intact *adeN*. In addition, the virulence of isolate MB-S-5 of isolate pair 1 was significantly increased in a *G. mellonella* infection model, indicating a potential contribution of increased efflux activity and *adeS* disruption with *A. baumannii* virulence. This is not surprising as RND-type efflux pumps have a diverse physiological role and are associated with e.g. biofilm formation and virulence [98, 124, 284], while AdeRS is directly or indirectly involved in the regulation of up to 579 genes in *A. baumannii* including genes required for virulence [279]. However, an investigation of two isolate pairs recovered pre- and post-tigecycline therapy revealed an increase in virulence for the isolate with higher tigecycline MICs in one pair, but this isolate also carried mutations in genes involved in biofilm formation and DNA mismatch repair indicating that besides increased efflux activity other factors might affect virulence [285]. Indeed, *A. baumannii* possesses a large variety of different virulence factors, which could contribute to the increased virulence in isolate pair 1 in the *Galleria* model [14, 46, 47].

Since the disruption of *adeS* and *adeN* by insertion sequences has been associated with the overexpression of efflux pumps, antimicrobial resistance and virulence in *A. baumannii*, both genes and the presence of insertion sequences might be useful as potential markers of MDR and virulence of *A. baumannii* isolates [282, 283]. Although the prediction of insertion sites is difficult, the presence and/or location of IS-elements in the bacterial genome could also serve as potential marker to predict the phenotype of the bacterium. IS-elements are able to create genomic variation with large effects on gene function [273, 281] and *A. baumannii* seems to utilize this effect frequently. This correlates with its high amount of insertion sequences within the genome, which is about 40% higher compared to *K. pneumoniae* [273].

4.1.2. Amino acid substitutions and other differences in RND-type efflux pump regulators

The overexpression of RND-type pumps can lead to an increase in antimicrobial resistance and bacterial virulence, which could explain why mutations in *adeRS* and other regulators of RND-type efflux pumps resulting in amino acid substitutions are frequently observed in clinical *A. baumannii* isolates and why there is a high sequence variance in efflux pump genes [139, 142, 143, 160, 284, 286]. For example, Huys *et al.* showed a high sequence variability of *adeB*, which is not necessarily a result of antimicrobial pressure but may indicate natural sequence variance, and described the potential to use *adeB* as a target for sequence-based typing of *A. baumannii* [259]. It is important to keep in mind that gene mutations leading to amino acid substitutions could also be a result of a simple genetic polymorphism and might not be associated with antimicrobial resistance [149]. The amino acid substitution A94V in AdeS has been reported as such a polymorphism in *A. baumannii* isolates belonging to clonal lineage IC1 [142] and a high number of polymorphisms were also revealed in all regulatory genes (e.g. N58T in AdeN, I120V and V136A in AdeR) in this thesis. Nevertheless, various amino acid substitutions in association with elevated tigecycline MICs were identified in the RND-pump regulatory proteins AdeL, AdeN and AdeRS.

Generally, mutations in specific domains of two component systems like AdeRS are known to influence protein function and are associated with antimicrobial resistance [139, 159, 287]. For example, Marchand *et al.* described the amino acid substitutions T153M in AdeS in the HisKA domain, which contains the histidine residue important for the autophosphorylation of AdeS [139]. A defect in the phosphatase activity of AdeS as a result of the substitution was suggested resulting in constitutive expression of *adeABC* and resistance [139]. In this thesis, the amino acid substitution D167N in AdeS was observed, which is also located near the histidine residue and therefore might result in a similar defect in the phosphatase activity of AdeS, leading to antimicrobial resistance. The amino acid substitution in one of the two transmembrane domains of AdeS (I62M) has also been described by Hornsey *et al.*, who suggested that the substitution might affect signal transduction and thereby susceptibility to tigecycline [233]. However, the substitution I62M was also detected in ABC-isolates with low tigecycline MICs indicating the substitution is not associated with high tigecycline MICs ≥ 4 mg/L. Moreover, another four amino acid substitutions were found in the HAMP domain (V137F) and the HATPase domain (A325T, Q339K, S357P) in this study. While the HAMP-domain is involved in conformational changes and phosphorylation of AdeS, the HATPase domain is responsible for ATP binding [206, 210]. Changes in amino acids in these two domains might change protein function considerably and thus could lead to increased *adeABC* expression. Many more amino acid substitutions in AdeS have been described in the literature [114, 142, 284, 286]. Although experimental proof is often lacking, the high number of AdeS substitutions suggest it could be a common mechanism of *adeB* overexpression and antimicrobial resistance in *A. baumannii*. Of note, the amino acid substitution Q339K was

excluded as genetic polymorphism and detected only in isolates belonging to clonal lineage IC1 in this study. The same substitution has recently also been found in an *A. baumannii* isolate from Germany, belonging to the clonal lineage IC1 and having a tigecycline MIC of 2 mg/L [226]. These findings indicate that the substitution is probably a clonal lineage-specific polymorphism and not linked to high tigecycline MICs ≥ 4 mg/L.

Interestingly, the highest number of amino acid substitutions was detected in AdeR in this thesis, which indicates that changing the signal reception and thereby altering the DNA binding properties of AdeR is an important mechanism of RND-pump overexpression and resistance in *A. baumannii*. Most AdeR substitutions in this thesis and in the literature have been described to be located in the receiver domain. For example, the amino acid substitution D20N in AdeR has been demonstrated to be the cause of *adeABC* overexpression and reduced susceptibility to six antimicrobial classes including tigecycline in a carbapenem-resistant *A. baumannii* isolate [143, 159]. Furthermore, the substitution P116L in AdeR is close to the conserved residue at position 112, which was suggested to affect the ability of the receiver domain to control the DNA-binding of AdeR leading to constitutive expression of *adeABC* [139]. Moreover, the crystal structure of AdeR revealed important amino acid residues that are associated with activation as well as DNA-binding and dimerization of AdeR [213]. Wen *et al.* described the binding of magnesium ions by a pocket, which is formed by the phosphorylation site D63 together with residues G19, D20 and L112 [213]. Of note, the majority of AdeR substitutions in this thesis (D21V, G25S, D26N, N115K and V119I) were located near or in the pocket and were found as single substitutions or in combination in 25 isolates. Since some additional amino acid substitutions were described in this region in the literature (e.g. D58V, P116L, D20N, A91V and P56S) [139, 142, 233, 286], a hotspot for mutations centred around the magnesium binding pocket of AdeR is indicated in *A. baumannii* isolates. Similar hotspots were also identified by Xu *et al.* in regions encoding the DNA-binding domains of AdeR and in the HAMP and HATPase domains of AdeS [271].

Compared to AdeRS, amino acid substitutions in AdeL have rarely been reported in association with efflux pump overexpression, antimicrobial resistance and/ or elevated tigecycline MICs [114, 142]. In this thesis, the substitution I37L was detected in AdeL, which is located in the helix-turn-helix domain, which is involved in sequence-specific DNA binding in LysR transcription regulators [288]. Therefore, amino acid changes in this domain could alter the DNA binding properties of AdeL and thus affect *adeFGH* expression.

Generally, AdeN is a TetR-like transcriptional repressor, whose protein structure consists of nine helices comprising the DNA-binding domain as well as the ligand binding domain [289]. Although it is still unclear how different mutations and changes in *adeN* affect protein function, at least the large nucleotide deletion, the frameshift, and the insertion are suggested to abolish protein function and lead to an increase in *adeJ* expression. This is supported by the findings of Rosenfeld *et al*., who reported a similar frameshift mutation and revealed an increase of *adeJ* expression comparable to the one observed in this thesis [144]. Interestingly,

the premature stop codon and the 6-nucleotide insertion, which was previously reported to be associated with elevated tigecycline MICs [227], was also detected in the ABC-isolates displaying low tigecycline MICs. Therefore, an association of a truncated *adeN* due to a premature stop codon with elevated tigecycline MICs was excluded. Furthermore, the large 87-nucleotide deletion and the 6-nucleotide insertion in *adeN* were also detected in isolates displaying low tigecycline MICs of 2 mg/L, which indicates that both differences in *adeN* have to be excluded as polymorphisms similar to the premature stop codon. These finding are rather surprising since the *adeJ* expression in all isolates carrying *adeN* differences is comparable and tigecycline MICs in the *adeN* knockout complemented with the truncated *adeN* and *adeN* carrying an insertion are unchanged (MIC 1 - 2 mg/L). However, the premature stop codon resulted in a shortening of AdeN by 17 amino acids at the C-terminus (Appendix 13), which is distal of important protein domains of AdeN and therefore might explain the low impact on *adeJ* expression and tigecycline MICs. Moreover, complete loss of *adeN* seems to have a greater effect than *adeN* mutations on the *adeJ* expression profile since *adeJ* expression was highest in the *adeN* knockout. It is possible that a truncated or changed AdeN protein is still able to bind DNA and affect gene expression to a certain extent, although much less compared to the wildtype AdeN. Moreover, complementation of the *adeN* knockout with the different *adeN* variants resulted in unchanged tigecycline MICs compared to the *A. baumannii* reference strain ATCC 19606 or the *adeN* knockout indicating a potential association of the *adeN* differences with elevated tigecycline MICs. It is suggested that isolate with tigecycline MIC of 2 mg/L could be a transition state with mutations or other differences in the RND-type efflux pump regulators that do not result in a considerable increase but rather in small MIC elevations. Furthermore, three amino acid substitutions were detected in AdeN (F108L, H170Y, G213V), though the substitution F108L is likely a result of the large 87-nucleotide deletion in *adeN* in isolate MB-R-161. A study of single point mutations in the tetracycline repressor protein TetR could show that a simple change of amino acids in position 17 was able to completely switch protein function [290]. Usually, tetracycline binds to TetR, which causes a conformational change, alters the DNA binding affinity of TetR and induces gene transcription. The amino acid substitution changed the protein assembly and structural organisation of TetR and instead of inducing gene expression, tetracycline is now considered a co-repressor negatively regulating gene expression [290]. Therefore, the observed amino acid substitutions in AdeN, a TetR-like regulator, might also strongly affect protein function by reverting the function of AdeN from a repressor to an inducer of gene transcription.

An interesting fact is that most differences in the RND-type efflux pump regulators occurred in combinations, i.e. insertions or deletions in *adeN* or IS-elements in *adeS* always occurred together with amino acid substitutions in one of the regulators (e. g. AdeR) (Figure 3.10). Moreover, high tigecycline MICs of 8 mg/L or more were present only in isolates carrying such

combinations of mutations/ differences in the regulators. This suggests an additive effect of mutations, which has also been observed for colistin resistance (see Chapter 4.2) [291].

In four *A. baumannii* isolates, the *adeRSABC* operon was missing completely or partially. Previous studies have shown that the eight RND-type efflux pumps are not always present in *A. baumannii* clinical isolates [115, 140]. Nemec *et al.* reported that in about 20% of *A. baumannii* isolates *adeRSABC* was not detectable using a PCR-based approach; the genes were missing to varying degrees, i.e. in some isolates only *adeC* was missing, while other isolates contained only *adeS* or *adeRSA* [140]. Moreover, Nowak *et al.* investigated the prevalence of RND-type efflux pumps in *A. baumannii* and showed that *adeB*, RND1 (ACICU_RS00750) and RND3 (ACICU_RS17095) are only found in 56 to 97% of isolates [115]. These observations fit well with the results described in this thesis. While it is likely that the absence of *adeC* is compensated by the presence of other outer membrane pores such as *adeK* or *adeH*, and a multidrug-resistant phenotype is not affected, the loss of *adeRS* or *adeB* is associated with a significant change in the expression of more than 500 genes in *A. baumannii* including a decreased expression of *adeABC* and an increase in antimicrobial susceptibility [279]. Moreover, it is likely that the bacterial fitness and virulence is altered, since *adeRS* play a role in many different physiological functions [279, 280].

Besides the well characterised RND-type efflux pumps AdeRSABC, AdeN-IJK and AdeLFGH, *A. baumannii* can harbour up to five additional RND-type efflux pumps in its genome. Only for the RND-type efflux pump ArpAB, a TetR-like transcription regulator ArpR has been characterised [116, 231]. The amino acid substitution N130D was found in 41 *A. baumannii* isolates. Similar to AdeN, ArpR has been proposed to be a transcriptional repressor of ArpAB and amino acid changes might strongly affect protein function, which has already been described for the tetracycline repressor protein TetR [290]. However, ArpAB has not yet been associated with antimicrobial resistance in *A. baumannii*. Moreover, the amino acid substitution was also detected in isolates with low tigecycline MICs of 2 mg/L, so that it remains to be investigated if it could affect tigecycline susceptibility.

4.1.3. Other allele differences associated with tigecycline resistance

Besides efflux several other tigecycline resistance mechanisms have been described in the literature, all of which involve enzymes, for example the monooxygenase Tet(x) [91]. Only in recent years has Tet(x) been found more frequently as a tigecycline resistance mechanism in Gram-negative bacteria [168–170], while the other mechanisms involving *abrp*, *plsC* and *trm* have been reported rarely [166, 167, 171]. This fits well to the observations made in this study, since all amino acid substitutions found in AbrP and PlsC were considered polymorphisms The acetyltransferase PlsC is involved in phospholipid and bacterial cell membrane biosynthesis and a frameshift mutation has been shown to result in an increase in tigecycline MICs [166]. A similar frameshift mutation was also found in this study, but was also found in isolates with low tigecycline MICs and thus excluded.

Besides the mutations found in the regulators of RND-type efflux pumps, several other amino acid substitutions were detected in AdeAB, AdeFGH, AdeJ, AbeM, and the uncharacterised RND-type efflux pumps. Since no additional differences were detected in the regulators of the efflux pump AdeABC, AdeIJK, AdeGFH in these isolates, it is likely that the respective efflux pumps are not overexpressed. However, further investigation is required to determine if amino acid differences might change the substrate spectrum of the transporter proteins. Amino acid changes in certain positions of the RND-type efflux pumps AcrB and OqxB in *E. coli* have been reported to affect the substrate binding efficiency of the pumps, which led to a reduction or increase in the MICs of the respective antimicrobial [292, 293]. Therefore, it is possible that similar changes have occured in this study (e.g. for AdeB), but further investigation is required to elucidate the effect of the amino acid substitutions in the various pumps and their association with elevated tigecycline MICs. Furthermore, it is also important to point out that most amino acid substitutions in efflux pumps were detected in two isolates (MB-R-17, MB-R-18), which do not belong to any of the known clonal lineages and harbour many allele differences compared to the *A. baumannii* reference strain ACICU or other isolates. Therefore, it is questionable if the difference found in the efflux pumps are associated with elevated tigecycline MICs or natural variance observed frequently in isolates of different clonal lineages.

If all genetic differences detected in isolates with low tigecycline MICs of ≤ 2 mg/L are excluded as polymorphisms and thus were not associated with tigecycline resistance, a total of 11 *A. baumannii* isolates with tigecycline MICs between 4 and 8 mg/L remain in this study, which did not carry any differences in alleles known to be associated with tigecycline resistance: MB-R-6, MB-R-10, MB-R-79, MB-R-89, MB-R-97, MB-R-110, MB-R-130, MB-R-151, MB-R-152, MB-R-157, MB-R-165. Three of the isolates carried the premature stop codon in *adeN* (MB-R-110, MB-R-152, MB-R-157), two isolates had a 6-nucleotide insertion in *adeN* (MB-R-79, MB-R-89), one isolate harboured the amino acid substitution I62M in AdeS (MB-R-151), and five isolates showed amino acid substitutions in regulators of RND-type efflux pumps (MB-R-6, MB-R-10, MB-R-97, MB-R-130, MB-R-165). Some of the allele differences have previously been reported to be associated with tigecycline resistance [227, 233] but were excluded in the present thesis by the comparison to the *A. baumannii* ABC-isolates or other isolates with low tigecycline MICs (MIC ≤ 2 mg/L). However, it is suggested that isolates with an MIC of 2 mg/L could display a transition state, i.e. incremental changes occur that increase the tigecycline MICs slightly and in small steps. This is not necessarily a problem in clinical settings as the infection with such isolates might still be treatable, but it could eventually pave the way for further increases of tigecycline MICs, especially considering the fact that accumulation of mutations leading to higher tigecycline MICs ≥ 8 mg/L was observed in this study. Nevertheless, some of the above-mentioned isolates are suggested to utilize a different and hitherto unknown tigecycline resistance mechanism. This is not an uncommon

phenomenon, and several other studies were also unable to elucidate all the mechanisms leading to tigecycline resistance in *A. baumannii* isolates. For example, Xu *et al*. investigated the mechanisms leading to increased tigecycline MICs in 24 *A. baumannii* isolates and while many isolates harboured mutations or IS-elements in *adeRS* and *adeN,* six isolates seem to harbour a novel, yet unknown tigecycline resistance mechanism [271]. The hypothesis and later the discovery of novel resistance mechanisms has recently been shown by the investigation of *mcr-1* in colistin resistance in *A. baumannii* (see Chapter 4.2). *Mcr-1* was unknown, because nobody was looking for such a specific resistance mechanism, but as soon as it was identified *mcr-1* or its variants were detected in many different isolates, bacterial species and even in isolates from old studies recovered long before the discovery of *mcr-1* [202]. This indicates that resistance mechanisms are often discovered by accident, since in most cases investigators only have vague ideas what the new mechanism might involve. Even though the presence of several novel resistance mechanisms is highly likely, unknown resistance mechanisms are difficult to publish and reports about isolates with unidentified tigecycline resistance mechanisms are rare [271]. Moreover, RND-type efflux pumps and other efflux pumps contribute to antimicrobial resistance in *A. baumannii,* but for high level resistance or a multidrug-resistant phenotype, a synergy between efflux and other resistance mechanism is often required [86, 99, 147, 148]. Chopra *et al.* compared the protein expression profile of an MDR *A. baumannii* isolate with the reference strain ATCC 17978 and reported that complex changes in proteomics are associated with a MDR phenotype [294]. This study indicates that besides influx or efflux of the drug, many more proteins and thus mechanisms might be involved in antimicrobial resistance both in general and specifically for tigecycline resistance. Another factor favouring the development of new and unknown tigecycline resistance mechanisms is that tigecycline-resistant isolates can occur in patients that had not been exposed to the drug, since the development of tigecycline resistance independently of selective pressure was observed for most of the isolate pairs in this study and several more examples have been reported in the literature [143, 295].

4.2. Colistin resistance in *A. baumannii*

The prevalence of colistin resistance in *A. baumannii* was relatively low compared to the resistance rates of other antimicrobial agents worldwide, making colistin a favourable choice as last resort antimicrobial agent in treatments of infections with MDR *A. baumannii*. For example, the SENTRY antimicrobial surveillance study tested nearly 4700 *A. baumannii* isolates between 2006 and 2009 and reported 0.9% of colistin-resistant isolates, while 40.3%, 66.9% and 51.4% of isolates were resistant to imipenem, ciprofloxacin and amikacin, respectively [296]. Nevertheless, colistin resistance is increasing steadily in the last decade. The EARS-Net study described a colistin resistance rate of about 4.0% in *A. baumannii* isolates collected from 30 European countries in 2016, whereby most colistin-resistant isolates (70.7%) originated from Greece and Italy, although it was stated that the results should be

treated with caution e.g. due to the small number of tested isolates [255]. Similar results were described in studies in Greece reporting colistin resistance rates of 21.1% in 2014, 27.3% in 2015 and 32.8% between 2015 and 2017, respectively [291, 297, 298]. Furthermore, in 2009 a study in Spain described 40.6% of *A. baumannii* isolates to be colistin-resistant [299]. Therefore, the high prevalence of nearly 50% colistin-resistant isolates in the collection of 65 *A. baumannii* isolates in this study is not surprising and indicates an alarmingly rapid increase of colistin resistance in Southern Europe [27, 227, 243]. Besides *A. baumannii* other Gram-negative pathogens more frequently display colistin non-susceptibility, e.g. a study in Greece reported an increase in colistin resistance in *K. pneumoniae* isolates from 0% in 2002 to 26.9% in 2016 [300]. When talking about resistance rates, it is important to keep in mind that increased frequency of colistin resistance might also be a result of outbreaks caused by clonally related strains, distorting the resistance rates. Moreover, the determination of colistin MICs is not as straightforward as for other antimicrobial agents like for example carbapenems because of the poor diffusion of colistin into agar and its cationic properties making agar-based antimicrobial testing unreliable [301]. Moreover, the presence of colistin heteroresistant *A. baumannii* isolates may be undetectable with certain susceptibility testing methods and their occurrence could falsify the results. In the last two decades, several studies compared colistin MIC determination methods in terms of reliability and reproducibility (e.g. [301–303]). While Turlej-Rogacka *et al.* reported the superiority of agar dilution for the determination of colistin MICs [303], another study recommended the broth dilution method or automated systems such as Vitek2™ [302]. Nowadays, the broth microdilution is considered the gold standard for the determination of colistin MICs, but due to the different testing methods and their advantages and drawbacks, the true colistin resistance rates in *A. baumannii* may vary. Nevertheless, the increase in colistin resistance in *Enterobacterales* in the last years can at least partly be explained by the emergence of plasmid-borne colistin resistance determinants like *mcr-1* and its variants, which facilitate a rapid spread among Gram-negative pathogens [200, 201, 203], and partly by the increasing frequency of multidrug resistant isolates, leaving colistin as antimicrobial therapy of last resort and resulting in increased use of the antimicrobial agent. However, *A. baumannii* developed colistin resistance through other mechanisms, since neither *mcr-1* and its variants nor changes in the LPS/lipid A biosynthesis genes were detected in any of the isolates in this study. Instead, differences in the *pmrCAB* operon and *eptA* were observed in the isolate pairs as well as in the 65 clinical isolates.

4.2.1. Colistin resistance associated with differences in the *pmrCAB* operon

Generally, colistin resistance is mainly associated with mutations in the *pmrCAB* operon leading to the upregulation of *pmrC* expression, which modifies lipid A by PEtN addition. In the literature, many different amino acid substitutions or short amino acid deletions have been described in PmrA and PmrB, both in clinical and in laboratory-derived *A. baumannii*

isolates and other Gram-negative bacteria including *K. pneumoniae*, *P. aeruginosa* or *S. enterica* [191, 193–196, 304]. Similar to the two-component system AdeRS associated with efflux-mediated tigecycline resistance in *A. baumannii*, mutations in some domains of *pmrAB* are suggested to affect protein function by changing signal transduction or autophosphorylation [196, 206, 208, 253]. Interestingly, most amino acid substitutions in PmrCAB were found in only two colistin-resistant *A. baumannii* isolates from Greece representing the IC2 lineage (MB-R-93 and MB-R-96), which shared high *pmrCAB* sequence similarity with isolates belonging to the clonal lineage IC4. *A. baumannii* is naturally competent and has the ability to perform genome-wide homologous recombination [258]. Therefore, it is possible that *A. baumannii* utilizes homologous recombination of *pmrCAB* across different clonal lineages and acquire mutations, which could result in reduced colistin susceptibility. Lesho *et al.* reported the presence of similar *pmrA* and *pmrC* variants (compared to *pmrA* and *pmrC* described in the two isolates MB-R-93 and MB-R-96 in this thesis) in clinical *A. baumannii* isolates, although it was proposed these variants were acquired by evolutionary mechanisms rather than by horizontal gene transfer [196]. Looking at the large number of amino acid substitutions in the two colistin-resistant isolates, it is likely that most of them are genetic polymorphisms and only the substitutions S134G in PmrA (which is not located in one of the important protein domains known for PmrA) as well as P170S and R211S in PmrB are associated with colistin resistance. Both PmrB substitutions are located in the HAMP domain, which is involved in the phosphorylation of the histidine residue [206, 210], and many more substitutions have been reported in this region in the literature (e.g. L208F) [191, 193]. Especially P170S is likely to be associated with colistin resistance, since it is at the same position as the substitutions P170Q and P170L, which were also detected in isolate MB-R-17. Both substitutions have been previously reported to be associated with colistin resistance and increased expression of *pmrC* in *A. baumannii* [191, 230, 254].

Only a few amino acid substitutions have been described in PmrA in the literature, of which nearly all were located in the receiver domain of the protein (e.g. E8D and P102H) [193, 194, 196]. The receiver domain contains the conserved aspartate residue (Asp52), which receives the phosphate upon autophosphorylation of the histidine kinase PmrB. The binding of phosphate affects gene transcription by enhancing the binding of PmrA to its DNA recognition site [206, 208]. Mutations in this protein domain are suggested to influence DNA binding and transcriptional regulation, which subsequently might lead to an increase in the expression of *pmrC* and thus colistin resistance. While no amino acid substitutions in PmrA were found in the four isolate pairs, the substitution G54E was detected in the two colistin-resistant isolates MB-R-148 and MB-R-164. It is adjacent to the conserved aspartate residue in the receiver domain, which therefore might strongly affect the phosphorylation of PmrA, protein function, and the expression level of *pmrC*. This finding is at least partly confirmed by the significant increase of *pmrC* expression in isolate MB-R-148. Of note, the substitution G54E has been

previously observed in a colistin-resistant *A. baumannii* isolate, but in combination with the PmrC substitution R109H [291].

The histidine kinase PmrB shows higher sequence variability and a higher mutational frequency compared to PmrA or PmrC in *A. baumannii* and also in other Gram-negative pathogens [193], which might indicate a higher importance of *pmrB* mutations in colistin resistance. Most of the amino acid substitutions, which were described in the literature and this thesis, were located in important protein domains of the histidine kinase [193]. Since several enzymatic functions are combined in PmrB (e.g. autophosphorylation, phosphotransfer), the different domains of the protein interact with each other and mutations in these regions can strongly affect protein function (e.g. an amino acid change could stabilise the interactions between the domains or destabilise the protein structure) [206]. By comparing isogenic isolates, the three single amino acid substitutions S17R, A28V and I232T as well as the four-amino acid deletion ΔL9-G12 were detected in PmrB in the colistin-resistant isolates. The substitutions S17R, A28V and the small deletion ΔL9-G12 are located in the first transmembrane domain of PmrB, which is involved in sensing and transducing extracellular stimuli [193]. The substitution S17R has been described previously by Lesho *et al*. [196] and similar amino acid substitutions in this domain have been reported in *A. baumannii* (e.g. T13N, S14L) and other Gram-negative pathogens (e.g. V15I in *P. aeruginosa*, ΔR14 in *K. pneumoniae*) [193–195]. Besides the already mentioned PmrB substitutions P170L and R211S, the amino acid changes T42P, L153F and S128R were also found in other colistin-resistant *A. baumannii* isolates in this study. While T42P and S128R are located in the periplasmic domain involved in sensing the extracellular stimuli, L153F is part of the second transmembrane domain. All of the mentioned PmrB substitutions are therefore suggested to affect signal transduction and autophosphorylation. Interestingly, the periplasmic and the transmembrane domains in PmrB seem to be the mutational hotspot, since most amino acid changes were observed in these regions. These domains are responsible for sensing external stimuli and protein signalling [206, 208], which indicates that signal transduction is preferably changed in association with colistin resistance.

In the literature, mutations in *pmrAB* are often found to be the cause for increased *pmrC* expression, the modification of lipid A and therefore colistin resistance [191, 193–195]. Surprisingly, this rule could not be applied to all the isolate pairs harbouring *pmrB* mutations. Apart from isolate pair 2, all isolates harbouring *pmrB* mutations also displayed increased *pmrC* expression, but only isolate pair 1 (MB-R-2, MB-S-6) displayed the expected results of increased *pmrC* expression in combination with the modification of lipid A. Moreover, introduction of *pmrAB* carrying the deletion ΔL9-G12 into the *pmrAB* knockout strain resulted in elevated *pmrC* expression and the addition of PEtN to lipid A but did not change colistin MICs. Similar results were observed for the introduction of the PmrB variant carrying the A28V substitution into the *A. baumannii* reference strains ATCC 17978 and ATCC 19606. Therefore,

mutations in *pmrB* are linked with the upregulation of *pmrC* and modification of lipid A, but not necessarily with a phenotype shift from colistin-susceptible to -resistant or even a change in MICs. Instead, these data suggest that colistin resistance is a more complex mechanism than previously suspected in *A. baumannii* and that other, yet unknown factors might be involved [305]. Further evidence for this suggestion is given by the results of the other isolate pairs. In isolate pair 3 (MB-R-90, MB-R-119) both isolates were colistin-resistant. Nevertheless, isolate MB-R-119 carrying the amino acid substitution A28V in PmrB displayed higher colistin MICs, increased *pmrC* expression compared to the other isolate and the addition of more than one PEtN molecule to lipid A, which indicates an additive effect of *pmrB* mutations. In isolate pair 2 (MB-S-23, MB-S-177), the substitution I232T in PmrB did not affect the expression of *pmrC*, which indicates that the mutation did not influence the activity of the histidine kinase and thus is most likely not associated with colistin resistance. Interestingly, Lesho *et al.* described an amino acid change from threonine to isoleucine in the same position (T232I), which was associated with an increase in *pmrC* expression and colistin resistance [196]. Furthermore, the modification of lipid A by PEtN addition was detected in both the colistin-susceptible and the -resistant isolate in isolate pair 2 (MB-S-23, MB-S-177) and isolate pair 4 (SG3161, SG3166). Although this can be partially explained by the involvement of *eptA*, it strongly indicates that PEtN additions appear to be less crucial for a colistin-resistant phenotype as initially thought. These surprising findings have not been described in the literature before and strongly suggest the involvement of another factor in colistin resistance.
Amino acid changes in PmrB are also found in combination with other amino acid substitutions for example in PmrC (e. g. R125P), which might have an additive effect on colistin resistance. Similar results have been observed in other clinical *A. baumannii* isolates harbouring a combination of different *pmr* mutations [291]. This is supported by the fact that elevated colistin MICs up to 32 mg/L were observed in isolates harbouring single mutations (Figure 3.25), while much higher colistin MICs up to ≥ 256 mg/L were found in isolates carrying a combination of mutations in for example both *pmrB* and *pmrC*. The potential of mutation accumulation as a mechanism for increased antimicrobial resistance is suggested [193], although the expression of *pmrC* was not significantly different due to accumulation of more than one mutation.

Only the substitution R125P was detected in PmrC in several colistin-resistant isolates in this thesis. This amino acid change has also previously been described and is one of only two PmrC substitutions (together with R109H) reported in association with colistin resistance in the literature [291, 306]. The substitution R125P was found in a multidrug-resistant *A. baumannii* isolate which was obtained from a veterinary urine sample and carried additional mutations in *pmrA* and *pmrB* [306]. Since *pmrC* expression was increased in the tested isolate carrying the R125P substitution, an association with colistin resistance is suggested. However, PmrC substitutions seem to have a lesser impact on colistin resistance compared to amino acid

changes in PmrA or PmrB. This is supported by the observation of relatively low colistin MICs (≤ 32 mg/L) in isolates harbouring only the PmrC mutation compared to isolates carrying a combination of PmrB and PmrC substitutions (≥ 64 mg/L). Moreover, an association of PmrC substitutions with colistin resistance has rarely been reported in the literature [291, 306], indicating it to be a less common colistin resistance mechanism in *A. baumannii*. The exact mechanism of colistin resistance due to PmrC substitutions remains to be elucidated, although an alteration of protein function or activity is likely. Conserved amino acid residues and protein domains of phosphoethanolamine transferases have been described in *E. coli* and *Neisseria meningitidis*, including Glu114, which is predicted to bind PEtN in *N. meningitidis* [307, 308]. The substitution R125P is not part of these conserved amino acid residues, but position 125 is near the conserved glutamic acid residue (Glu114) and therefore a change in amino acids in this region might influence protein catalysis. However, it is important to point out that phosphoethanolamine transferases of *N. meningitidis* and *A. baumannii* share only about 30 to 50% amino acid identity.

4.2.2. The role of the *pmrC*-homologue *eptA* in colistin resistance

Although novel *pmrCAB* mutations were identified in some colistin-resistant *A. baumannii* isolates and the four isolate pairs, several isolates remain which do not carry a *pmrCAB* mutation and/or which showed increased *pmrC* expression and PEtN addition independently of *pmrCAB*. In these isolates, an involvement of the *pmrC*-homologue *eptA* with colistin resistance was suggested. The *eptA* nucleotide sequence shares high homology with *pmrC* (Appendix 18), which makes a differentiation between these genes as well as genome assemblies challenging [244]. A PCR-based approach was used to confirm the presence of *eptA* in the draft genomes of the *A. baumannii* isolates. However, it is difficult to say if a single or multiple copies of *eptA* are present in the genomes, even though it is known that the presence of more than one *eptA* in *A. baumannii* and other bacterial species is possible [196, 307]. So far not much is known about *eptA* and its function in *A. baumannii*. Lesho *et al.* were among the first to describe the presence of *eptA* in clinical *A. baumannii* isolates [196]. Deveson Lucas *et al.* investigated the regulation and function of *eptA* in *A. baumannii* and its association with colistin resistance [197]. It was shown that increased *eptA* expression contributes to colistin resistance, but it seems to be a less frequent colistin resistance mechanism compared to *pmrCAB* [197]. Moreover, *eptA* was only detected in IC2 isolates in this thesis, which indicates that the presence of *eptA* might be lineage-dependent. Similar observations have been made by Trebosc *et al.* who also analysed the overexpression of *eptA* as colistin resistance mechanism in *A. baumannii* [309]. Additional studies showed that EptA is highly conserved within a species but not in comparison with other Gram-negative bacteria [307]. Moreover, it was suggested that *eptA* is acquired by horizontal gene transfer, since the genetic regions surrounding *eptA* seem to originate from prophages or integrases [196, 197]. This is further supported by the fact that insertion sequences like IS*Aba1* and IS*Aba125* were detected in

proximity of or disrupting *eptA* in this thesis as well as in the literature [309]. The presence of insertion sequences might suggest the possible dissemination of *eptA* as has been observed for *mcr-1* and its variants or other antimicrobial resistance determinants like β-lactamases [72, 78, 203]. For example, a BLAST analysis revealed nearly 92% identity of EptA with a phosphoethanolamine transferases from *K. pneumoniae*.

IS*Aba1* was detected upstream of *eptA* in reverse orientation in several isolates (e. g. both isolates of isolate pair 2, MB-R-53 and MB-R-12). When IS*Aba1* is inserted upstream of a gene in reverse orientation, it was shown to influence the expression pattern of genes by providing a strong promoter for the overexpression of various antimicrobial resistance determinants in *A. baumannii* (e.g. bla_{OXA}) [75, 78, 94, 163]. This suggests an association of IS*Aba1* with increased *eptA* expression and subsequent colistin resistance. However, the insertion sequence was detected in both colistin-susceptible and -resistant isolates and the *eptA* expression levels were relatively unchanged regardless of IS*Aba1* present upstream of *eptA*. These findings suggest that EptA is not involved in colistin resistance in the majority of the *A. baumannii* isolates in this study, which contradicts observations of Trebosc *et al.* and Potron *et al.* describing IS*Aba1-eptA* expression as a colistin resistance mechanism in clinical *A. baumannii* isolates [309, 310]. A different story is presented by isolate pair 2, where IS*Aba1-eptA* expression might explain the addition of PEtN to lipid A in both isolates. However, the colistin-resistant isolate MB-R-177 harboured additionally the combination of a point mutation (A1091T) in IS*Aba1* as well as the EptA substitution R127L and displayed significantly increased *eptA* expression. The same combination of mutations was detected in isolate MB-R-53, which was recovered from the same patient as isolate pair 2 and is thus clonally related. Only in these two isolates an association of *eptA* (in combination with point mutations in IS*Aba1* and *eptA*) with colistin resistance was suggested. Similar to the amino acid substitution R125P in PmrC, the amino acid change in EptA has the possibility to alter protein function as it is near the conserved Glu114 residue, which is predicted to bind PEtN in *N. meningitidis* [307, 308]. Moreover, the point mutation in IS*Aba1* is located near the promoter region, which might affect the strength of the promoter and thus could increase *eptA* expression, which was observed for isolate MB-R-53 compared to isolates without this point mutation in the insertion sequence. Nevertheless, this combination of mutations in *eptA* and IS*Aba1* is probably quite rare and was not detected in any other isolate. Taking a look at the other three isolate pairs, no other mutations were found in *eptA*. Nevertheless, an increased *eptA* expression was observed in the colistin-resistant isolate of isolate pair 1, while it was reduced in isolate MB-R-119 of isolate pair 3. These findings might indicate the involvement of another factor impacting on the regulation of *eptA* expression. For example, disruption of the global transcription regulator H-NS has been described to result in increased *eptA* expression [197]. Since H-NS was intact in all isolates in this study and since no additional changes were found in EptA, it is plausible to assume that the contribution of the *pmrC*-homologue to colistin resistance was negligible in all isolates except for the two isolates MB-S-177 and MB-R-53, and

that *pmrCAB* mutations seem to be the most common colistin resistance mechanism in *A. baumannii*. Nevertheless, the role of *eptA* in colistin resistance and maybe also the dissemination of *eptA* as resistance determinant among different *A. baumannii* isolates remains to be further elucidated.

4.2.3. Fitness and virulence in colistin-resistant isolates

Colistin resistance in *A. baumannii* has been correlated with reduced fitness and impaired virulence in several studies [253, 254, 311, 312], while others could not detect a noticeable change in the growth characteristics or virulence properties of colistin-resistant *A. baumannii* isolates [261, 262]. This difference can be explained mostly by the mechanism of resistance [313]. For example, modification of the lipid A biosynthesis or the complete loss of LPS due to mutations in or the disruption of *lpxA*, *lpxC* and *lpxD* affects the bacterial physiology and virulence by altering the cell envelope [183]. For example, LPS deficient *A. baumannii* display reduced fitness and virulence *in vivo* [314], which suggests LPS loss is a resistance mechanism only found in laboratory strains. Since LPS stabilises the outer membrane, which forms an intrinsic barrier against numerous compounds and antibiotics [86, 183], and contributes to the resistance to components of the host immune system [185], a retention of fitness or virulence due to the modifications or the complete loss of LPS is plausible. In contrast, mutations in *pmrCAB* seem to have a minimal or no effect on fitness and virulence of *A. baumannii* isolates [313]. This fits well to the observations in this thesis, since the four isolate pairs displayed relatively unchanged fitness and even increased virulence in the *G. mellonella* model. Moreover, colistin-resistant *A. baumannii* isolates led to high melanisation scores, which is an indicator for an immune response of the host (*G. mellonella*). The virulence of other *A. baumannii* isolates in this study remains to be investigated, but due to their *pmrCAB*-mediated colistin resistance, an increase in virulence and unchanged fitness is suggested. Similar observations have been made in *Salmonella* spp., where an increased virulence was accomplished by altering the innate immune recognition and adaptive response in the host during an infection. In particular, PmrAB-mediated LPS modifications have been proposed to alter the Toll-like receptor–LPS interaction and neutrophil recruitment in the host, as well as to reduce the susceptibility to bacterial killing by antibacterial peptides [315]. The *G. mellonella* larvae naturally produce a large number of different antibacterial peptides as part of their innate immunity and the increase in virulence of the colistin-resistant isolates might be due to an additional resistance to these antibacterial peptides [316]. Resistance to these peptides would reduce the effectiveness of the animal's innate immunity and thus result in increased pathogenicity of the *A. baumannii* isolates [230, 316]. Furthermore, *A. baumannii* harbours a variety of virulence factors, including glycoconjugates, which include LPS and its anchor lipid A, and which are responsible for the first defence against e.g. desiccation stress or the host immune system [14, 46]. Therefore, *pmrAB*-mediated lipid A modification may contribute to changes in the virulence of *A. baumannii* not only in *in vivo* models, but also in

the human host. LPS derived from *A. baumannii* has been shown to stimulate innate immune responses in humans via Toll-like receptor 4 signalling [46, 317]. Moreover, Beceiro *et al.* hypothesise that the presence of tetra-acylated lipid A species (m/z 1404) in colistin-resistant *A. baumannii* isolates might lead to an reduced inflammatory response in the host, because this particular species of lipid A is associated with reduced activation of the human immune system [195]. Tetra-acylated lipid A was found in all isolate pairs, which further underlines their potentially increased virulence and better qualification to survive in the human host. However, it cannot be ruled out that other yet unknown virulence factors, are involved in the increased virulence of colistin-resistant isolates. Since *pmrAB* belongs to the two-component systems, which are usually involved in a wide range of adaptive responses of the bacteria to its environment as well as cellular processes including virulence [205, 206], mutations in *pmrAB* could have an additional effect on the differential gene expression of other virulence factors apart from lipid A/LPS. Future studies should investigate differences in the genome regarding virulence factors and their expression in the isolate pairs or other colistin-resistant isolates from this study. The fact that alterations in *pmrAB* not only might lead to colistin resistance but also to increased virulence of *A. baumannii* isolates is of major concern for clinical settings.

4.2.4. Efflux pumps and other factors associated with colistin resistance

Several novel *pmrCAB* mutations have been found in *A. baumannii* isolates and are suggested to be associated with colistin resistance. Future studies might elucidate the effect of each *pmr* mutation on *pmrC* expression and lipid A modification, i.e. if they contribute to a colistin-resistant phenotype. Nevertheless, lipid A modification by PEtN seems to be less important for colistin resistance and the current simple rule of *pmrAB* mutations leading to *pmrC* overexpression, lipid A modification and increased colistin MICs cannot be applied to all *A. baumannii* isolates. An explanation could be that *pmrCAB* mutations have a strain-specific effect [196, 305]. Such strain-specificity has already been shown in *A. baumannii*, namely with efflux and carbapenem resistance [230, 279, 318]. Furthermore, Park *et al.* described the overexpression of *pmrCAB* in colistin-resistant *A. baumannii* independent of *pmrAB* mutations, which indicates the involvement of other yet unknown factors contributing to colistin resistance [304]. Some studies showed the involvement of environmental factors like magnesium, iron and pH to have an influence on colistin resistance. For example, Adams *et al.* proposed that an acidic pH and ferric ions could induce colistin resistance [194]. However, the expression of *pmrA* was not always increased, which indicates the involvement of other regulatory factors yet to be discovered [194]. Similar observations have been made in *S. enterica*, where low magnesium, high iron levels, and a low pH can have an impact on colistin resistance by affecting the two-component systems PhoPQ and PmrAB [315, 319]. Such environmental factors have not been tested in this study. Future studies with the isolate pairs or the different *pmrCAB* mutations might give further insight into the involvement of

such environmental factors and if they are the missing piece in the complexity of colistin resistance in *A. baumannii*.

To find other factors that potentially contribute to colistin resistance, allele differences were identified and further analysed in the isolate pairs and in the four *A. baumannii* isolates, which do not harbour a difference in any of the known colistin resistance determinants (MB-R-83, MB-R-110, MB-R-152 and MB-R-157). While the colistin resistance mechanism remains to be elucidated in isolate MB-R-83, amino acid changes were detected in a putative zinc-dependant peptidase ZndP and a putative permease transporter in the other three isolates. Both proteins have previously been described in colistin-resistant *A. baumannii* isolates [244]. When linked to the phospholipase PldA, ZndP is suggested to have a role in outer membrane processing [244], but further investigation is required to elucidate its association with colistin resistance. Although these differences were found exclusively in these three isolates, they could also be genetic polymorphisms. All three isolates(MB-R-110, MB-R-152 and MB-R-157) display unidentified tigecycline as well as colistin resistance mechanisms and were considered not to be related to any known clonal lineage in a previous study, so they are genetically quite different from other IC2 isolates in cgMLST and suitable isolates for comparison are missing [227]. Therefore, further investigation is required to elucidate the association of the allele differences with antimicrobial resistance.

As already mentioned above, several amino acid substitutions were detected in various efflux pumps, which include for example AdeB, AdeG, AdeJ, or the regulator AdeN. The contribution of efflux pumps to colistin resistance is still under debate. The mode of action of colistin is different from other antimicrobial agents because it is not required to enter the bacterial cell. Instead, colistin binds to the lipid A of the outer membrane and displaces divalent cations, which disrupts the integrity of the outer membrane and upon entering the periplasmic space also of the inner membrane [175, 182, 183]. As soon as colistin has disrupted the inner membrane to enter the cell and be a substrate of efflux pumps, cell death is probably no longer avoidable, which makes an effect of inner membrane efflux pumps (e. g. efflux pumps of the MFS or MATE family) on colistin susceptibility questionable. If colistin has entered the periplasmic space, only RND-type efflux pumps could pump out colistin to avoid the disruption of the inner membrane. However, no correlation between increased expression of RND-type efflux pumps (due to e. g. mutations in *adeRS* or IS-elements in *adeN*) was observed in this study. Most amino acid substitutions in efflux pumps found in colistin-resistant isolates, were in the transporter protein itself, which might influence the substrate spectrum and substrate binding of the transporter [293]. Nevertheless, an association of efflux and colistin resistance or heteroresistance has been observed in several studies and in different Gram-negative pathogens. For example, it was recently shown that colistin resistance is a result of a synergistic action of the RND-type efflux pump MexXY/OprM and membrane impermeability by L-Ara4N addition to LPS in *P. aeruginosa* [320]. Machado *et al.* described that the efflux

pump genes *adeB*, *adeJ*, *adeG*, *craA*, *amvA*, *abeS* and *abeM* were overexpressed in clinical isolates upon colistin exposure [321], and Lin *et al.* reported the association of the ErmAB efflux pump with colistin resistance in *A. baumannii* [110]. Moreover, a contribution of ABC efflux pumps and RND-type efflux pumps to the resistance to various different cationic antimicrobial peptides (CAMPs) has been reported for various Gram-positive and Gram-negative bacteria (reviewed in [322]). It remains to be elucidated if colistin is indeed a substrate of efflux pumps and the active efflux of the peptide confers resistance. Colistin or the subsequent cellular stress could also be recognized as external signals by two-component systems (e. g. *adeRS*) regulating the expression of efflux pumps, so that an increase in efflux activity is only an indirect result of colistin exposure.

Besides mutations in *pmrB*, several allele differences were detected in isolate pairs 1 to 3. Two allele differences were detected in putative transcription regulators, which could be involved in the regulation of *eptA* or *pmrCAB*. In other bacteria, colistin resistance is associated with and controlled by a complex network of different genes and regulators (global and local). For example, other Gram-negative pathogens like *S. enterica* or *K. pneumoniae* do not rely solely on the *pmrCAB*-operon for the modification of lipid A, but also use the *arn*-operon, which is responsible for the addition of L-Ara4N to LPS [193]. Moreover, an additional two-component system (PhoPQ) and other local and global regulators like MgrB are also involved in the regulation and activation of PmrAB, which regulates not only *pmrC* and the *arn*-operon, but additional genes involved in the modification of LPS [193, 214]. Since such a complex regulatory network controlling gene expression and PmrAB activation is present in other bacteria, it is possible that such a network also exists in *A. baumannii* and that there are other genes involved in the modification of LPS yet to be identified.

One amino acid substitution (G575V) was found in the guanosine polyphosphate pyrophosphohydrolase/synthase (SpoT) in the colistin-resistant isolate or isolate pair 2 (MB-S-177). SpoT is involved in the purine metabolism und synthesis of guanosine tetraphosphate/pentaphosphate ((p)ppGpp), which has a diverse physiological role in bacteria. It serves as so called alarmone in the stringent stress response and is also involved in the regulation of bacterial growth, virulence, survival during host invasion, antibiotic resistance, and persistence (review in [323]). Depending on the cellular levels of (p)ppGpp the tolerance to various antimicrobial agents like e.g. vancomycin, penicillin or other β-lactams is affected [324, 325]. Moreover, only recently Jung *et al.* have suggested that (p)ppGpp is also involved in the regulation of efflux pumps in *A. baumannii* [326]. In most bacterial species (p)ppGpp is synthesized by the enzymes RelA and SpoT, which naturally occur in several different alleles [325]. For example, the *spoT1* allele carries two different mutations, is frequently found in *E. coli* K12 strains, and is responsible for the overproduction of (p)ppGpp [325]. Similarly, the amino acid substitution in SpoT (G575V) observed in this study is suggested to change the basal level of (p)ppGpp in the bacterial cell, which might induce

stringent response processes and consequently also cellular changes (e.g. in the consistency of the cell envelope and LPS) that could promote the formation of colistin-resistant cells. An amino acid substitution in the same position in SpoT has previously been reported in an *E. coli* strain and is located in the C-terminal part of the protein, which has been proposed to regulate the hydrolase and synthetase activity of SpoT, thereby regulating the basal levels of (p)ppGpp [325, 327]. Moreover, a *relA* ortholog has been identified in *A. baumannii* and was shown to increase the formation of persister cells tolerant to colistin and rifampicin, and the virulence in *G. mellonella* [328], which might also indicate an involvement of *spoT* in colistin tolerance in *A. baumannii*. In addition, (p)ppGpp has been shown to negatively regulate *hns* expression in *E. coli* [329]. Therefore, a contribution of (p)ppGpp in combination with downregulation of H-NS as additional factors to increased colistin MICs in *A. baumannii* is imaginable. An example for this colistin resistance mechanism might be isolate pair 2, where elevated colistin MICs were associated with increased *eptA* expression. Since H-NS was suggested to negatively regulate *eptA* expression [197], the increase in *eptA* expression could be caused by downregulation of H-NS due to increased (p)ppGpp levels, which might be a result of the amino acid substitution G575V in SpoT in isolate MB-S-177. Nevertheless, further work is required to investigate the effect of the SpoT substitution on cellular (p)ppGpp levels and its role in colistin resistance in *A. baumannii*.

5. Summary and conclusion

A. baumannii is a nosocomial pathogen, which causes a wide range of serious infections including ventilator-associated pneumonia (VAP) and bloodstream infections in patients with a compromised immune system and critically ill patients in the intensive care unit. The high desiccation tolerance, the ability to form biofilms, and multiple other virulence factors contribute to the persistence and easy spread of *A. baumannii* in the hospital environment. Furthermore, *A. baumannii* can acquire and upregulate antimicrobial resistance determinants to overcome all known antimicrobial drugs, including the antimicrobial agents of last resort tigecycline and colistin, so that multidrug- and even pandrug-resistant isolates have emerged. For these reasons, the World Health Organisation declared *A. baumannii* as "number one priority organism", which pose a great threat to human health and for which the development of new antimicrobial agents is urgently needed.

This thesis focuses on the investigation of tigecycline and colistin resistance mechanisms in clinical multidrug-resistant *A. baumannii* isolates collected as part of the MagicBullet clinical trial from patients with VAP in Greece, Italy, and Spain. Several *A. baumannii* isolate pairs were identified, which are defined as a pair of isogenic isolates from the same patient or hospital, displaying a shift in their tigecycline or colistin resistance phenotype and having a very similar genetic background. This enabled the identification and exclusion of typical genetic variations seen in epidemiologically unrelated isolate in this thesis. In some isolate pairs in this study, the mutations and subsequent resistance occurred independently of selective pressure, which underlines the need for fast identification of resistance mechanisms. For this purpose, an identification scheme for genetic differences associated with tigecycline and colistin resistance was created in this thesis, which is mainly based on whole-genome sequencing of isolates and the comparison of draft genomes to *A. baumannii* reference strains and a collection of susceptible *A. baumannii* isolates. This genomic approach resulted in the identification of several novel mutations in tigecycline and colistin resistance determinants, as well as a more in-depth analysis of the bacterial resistome and the prevalence of mutations and/or resistance mechanisms. Further analysis of these mutations and resistance mechanisms was conducted by measuring gene expression, creating gene knockouts, using an *in vivo* infection model, and mass spectrometry.

In the first part of this thesis, tigecycline resistance mechanisms were investigated with a focus on the resistance-nodulation-cell division (RND) efflux pumps and especially the efflux pump regulators AdeRS, AdeN and AdeL. The analysis of isolate pairs revealed the frequent disruption of *adeS* and *adeN* by the insertion sequences IS*Aba1* or IS*Aba125*. The disruption of *adeS* was associated with a significant increase in *adeB* expression, efflux activity, and virulence in the *Galleria mellonella* infection model as well as a reduced susceptibility to several antimicrobial classes. In comparison, the disruption of *adeN* by either IS*Aba1* or

IS*Aba125* was associated with elevated *adeJ* expression (lower compared to the increase in *adeB* expression upon *adeS* disruption) and increased minimal inhibitory concentrations (MICs) to three different antimicrobial classes. These results suggest that the disruption of *adeS* has a greater impact on resistance to other antimicrobial agents and may therefore impact in clinical settings. The analysis of a collection of respiratory *A. baumannii* isolates in this thesis revealed a high prevalence of insertion sequences in the RND-type efflux pumps and their regulators indicating these genes could be a frequent target site for various insertion sequences and thus might serve as potential marker to predict the antimicrobial resistance phenotype of the bacterium. Furthermore, three different nucleotide deletions, a nucleotide insertion, and a premature stop codon were identified in *adeN*, while several point mutations leading to amino acid substitutions in *adeRS*, *adeN* and *adeL* were also found to be associated with elevated tigecycline MICs in this thesis.

In the second part of this thesis, colistin resistance mechanisms were analysed in *A. baumannii*, focusing on the *pmrCAB* operon and the *pmrC*-homologue *eptA*, which are known to be involved in the modification of lipid A in the bacterial outer membrane. The analysis of isolate pairs revealed novel mutations in *pmrB*, which were associated with increased colistin MICs, elevated *pmrC* expression, unchanged fitness, and increased virulence in the *G. mellonella* infection model. In this thesis, evidence was found that *pmrB* mutations are associated with an increase in *pmrC* expression, but not necessarily with lipid A modification by the addition of phosphoethanolamine (PEtN) or changes in colistin MICs. These results suggest that the addition of PEtN alone does not confer high level colistin resistance and that other yet unknown factors seem to play a role in colistin resistance. The analysis of the collection of respiratory *A. baumannii* isolates also revealed several novel mutations in the *pmrCAB* operon, which were associated with elevated colistin MICs and increased *pmrC* expression. Moreover, first indications were found in this study that *A. baumannii* uses homologous recombination of the *pmrCAB* operon across different clonal lineages to acquire mutations. The role of the *pmrC*-homologue *eptA* in colistin resistance was investigated and the results indicate that the presence of *eptA* is lineage-dependent and often associated with insertion sequences in the proximity of the gene. In this thesis, no association of *eptA* overexpression, PEtN addition and elevated colistin MICs was found, which suggest the involvement of *eptA* with colistin resistance in *A. baumannii* might be strain-specific and negligible in this study. Moreover, the overexpression of *eptA* independent of mutations and insertion sequences was observed in this thesis, which indicates the involvement of other yet unknown *eptA* regulators. Instead, this study found *pmrCAB* mutations and subsequent overexpression of *pmrC* seem to be the most common colistin resistance mechanism in *A. baumannii*, although the results suggest that colistin resistance is a much more complex mechanism in *A. baumannii*.

Combining the results of both parts of this thesis, several findings were made in both tigecycline and colistin resistance mechanisms in *A. baumannii.* First, there is evidence that both *adeR* and *pmrB* harbour mutational hotspots, which might serve as genetic marker for the prediction of the bacterial antimicrobial resistance and virulence phenotype. Second, a combination of different mutations in the two-component systems *adeRS* or *pmrAB* often resulted in increased tigecycline or colistin MICs, respectively, which suggests an additive effect of mutations. Finally, mutations in *adeRS* or *pmrAB* can increase bacterial virulence and pathogenicity in the *Galleria* model, which is concerning considering the frequency of mutations or insertion sequences in *adeRS* or *pmrAB* observed in this study. Nevertheless, no correlation between increased expression of RND-type efflux pumps and colistin resistance was observed and further investigation is required to elucidate the role of efflux pumps in colistin resistance.

In conclusion, tigecycline and colistin resistance mechanisms are diverse and multifactorial, with several factors often working in tandem to enable resistance against antimicrobial agents. Both *pmrAB* and *adeRS* are among the most frequently mutated genes in this study, which indicates the importance of both two-component systems in the development of antimicrobial resistance in *A. baumannii*. The use of a genomic approach highlights the importance and potential of whole-genome sequencing as a tool in the study of antimicrobial resistance in clinically relevant pathogens, but also revealed that a genotype does not always correlate with the predicted phenotype. Additional factors (e.g. environmental factors), clonal lineage specific polymorphisms or natural genetic variance need to be considered when investigating novel mutations and resistance mechanisms, respectively. Besides efflux and lipid A modification, many more proteins and thus mechanisms might be involved in tigecycline and colistin resistance in *A. baumannii*, since several isolates remain that harbour an unidentified colistin or tigecycline resistance mechanism. Unidentified resistance mechanisms are - though rarely published - a common occurrence, which provides examples and isolates where the established model does not fit and opens opportunities for further research. Overall, this study opens a window into the complexity of antibiotic resistance mechanisms in *A. baumannii,* but future work is needed to understand them more comprehensively.

6. References

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7. Appendix

Appendix 1: Overview of genes and locus tags

Family	Gene	Operon	Specification	Locus tag or NCBI accession no.	Reference
RND-type efflux pumps	*adeR*	*adeRSABC*	TCS	ACICU_RS09210	[111, 139]
	adeS			ACICU_RS09215	
	adeA		MFP	ACICU_RS09205	
	adeB		transporter	ACICU_RS09200	
	adeC		OMP	ACICU_RS09195	
	adeN	*adeN-IJK*	regulator	ACICU_RS10555	[113, 144]
	adeI		MFP	ACICU_RS14900	
	adeJ		transporter	ACICU_RS14905	
	adeK		OMP	ACICU_RS14910	
	adeL	*adeLFGH*	regulator	ACICU_RS12520	[114]
	adeF		MFP	ACICU_RS12525	
	adeG		transporter	ACICU_RS12530	
	adeH		OMP	ACICU_RS12535	
Other RND-type efflux pumps	*abeD*		transporter	ACICU_RS14485	[115, 117]
	arpB	*arpRAB*	transporter	ACICU_RS18255	[115, 116]
	arpA		MFP	ACICU_RS18260	
	arpR		regulator	ACICU_RS18265	
	RND1		Transporter	ACICU_RS00750	[115]
	RND2-MFP		MFP	ACICU_RS15295	
	RND2		Transporter	ACICU_RS15300	
	RND3		Transporter	ACICU_RS17095	
	RND3-MFP		MFP	ACICU_RS17100	
	RND3-OMP		OMP	ACICU_RS17105	
MATE efflux pump	*abeM*		transporter (efflux pumps)	ACICU_RS02135	[106]
SMR efflux pump	*abeS*			ACICU_RS12495	[118]
MFS efflux pump	*craA*			ACICU_RS16745	[107, 108]
	amvA			ACICU_RS11265	[109]
	cmlA			ABAYE3640	[100]
	ermA	*ermAB*		ACICU_RS09320	[110]
	ermB			ACICU_RS09315	
	ermB-like I			ACICU_RS03860	
	ermB-like II			ACICU_RS09500	
	tet(A)			ABAYE3597	[100, 165]
	tet(B)			ABAYE3637	
PACE efflux pump	*aceI*			ACICU_RS11295	[105]
ABC efflux pump	*macB*			ACICU_RS02850	[104]

Appendix 1: continued

Family	Gene	Operon	Specification	Locus tag or NCBI accession no.	Reference
(Global) transcription regulators	*hns*		regulator	ACICU_RS01520	[197]
	marR		regulator	ACICU_RS09930	
	soxR		regulator	ACICU_RS06735	[219]
	baeS	*baeSR*	TCS	ACICU_RS15620	[218]
	baeR			ACICU_RS15615	
(Putative) colistin resistance determinants	*pmrA*	*pmrCAB*	TCS	ACICU_RS14980	[191,195, 330]
	pmrB			ACICU_RS14975	
	pmrC		enzyme	ACICU_RS14985	
	eptA		enzyme	DMO12_03360	[196]
	naxD		enzyme	ACICU_RS14295	[198]
	spoT		enzyme	ACICU_RS16885	
	zndP		enzyme	ACICU_RS10195	[244]
	vacJ			ACICU_RS03260	
	135			ACICU_RS17740	
	pheS			ACICU_RS03150	
	pldA			ACICU_RS10190	
	ttg2C			ACICU_RS16520	
	mcr-1 and variants		enzyme	NCBI BioProject PRJNA313047	
(Putative) tigecycline resistance determinants	*abrP*		enzyme	ACICU_RS03385	[171]
	trm		enzyme	ACICU_RS15495	[167]
	plsC		enzyme	ACICU_RS04375	[166]
	tet(X) and variants		enzyme	NCBI BioProject PRJNA313047	
LPS biosynthesis	*lpsB*		enzymes	ACICU_RS02330	[244]
	lpxA			ACICU_RS10485	
	lpxB			ACICU_RS08570	
	lpxC			ACICU_RS17675	
	lpxD			ACICU_RS10495	
	lpxH			ACICU_RS11535	
	lpxK			ACICU_RS08105	
	lpxL			ACICU_RS02335	
	lpxM			ACICU_RS14235	
	kdtA/waaA			ACICU_RS17585	

Appendix 2: Overview of epidemiological strain types and antimicrobial susceptibility testing data of *A. baumannii* isolates. Tigecycline MICs obtained with broth microdilution were previously published by Seifert *et al*. [267,268]. AD = agar dilution, BMD = broth microdilution, n.d. = not determined.

Isolate	Clonal lineage	Oxford MLST	Pasteur MLST	Tigecycline AD MIC [mg/L]	Tigecycline BMD MIC [mg/L]	Colistin MIC [mg/L]	NCBI sample name	NCBI BioSample accession no.
MB-R-1	IC2	ST-208	ST-745	2	1	2	MB_1	SAMN08398901
MB-R-2	IC2	ST-208	ST-745	1	n.d.	2	MB_2	SAMN08398902
MB-R-6	IC2	ST-208	ST-745	4	1	2	MB_6	SAMN08398903
MB-R-7	IC2	ST-208	ST-745	2	n.d.	2[a]	MB_7	SAMN08398904
MB-R-10	IC2	ST-208	ST-745	4	1	8	MB_10	SAMN08398905
MB-R-12	IC2	ST-1114	ST-2	8	8	2	MB_12	SAMN08398906
MB-R-17	UN	ST-1566	ST-537	8	2	8	MB_17	SAMN08398907
MB-R-18	UN	ST-1556	ST-537	4	1	1	MB_18	SAMN08398908
MB-R-23	IC2	ST-218	ST-2	2	0.25	1	MB_23	SAMN08398909
MB-R-45	IC2	ST-208	ST-2	4	1	0.25	MB_45	SAMN08398910
MB-R-53	IC2	ST-1114	ST-2	16	2	64	MB_53	SAMN08398911
MB-R-61	IC2	ST-208	ST-2	8	1	0.5	MB_61	SAMN08398912
MB-R-62	IC2	ST-218	ST-2	2	0.5	2	MB_62	SAMN08398913
MB-R-64	IC2	ST-451	ST-2	8	2	1	MB_64	SAMN08398914
MB-R-65	IC2	ST-451	ST-2	4	1	2	MB_65	SAMN08398915
MB-R-67	UN	ST-1568	ST-1066	0.5	0.125	2	MB_67	SAMN08398916
MB-R-71	IC2	ST-436	ST-2	2	0.5	1	MB_71	SAMN08398917
MB-R-75	IC2	ST-436	ST-2	2	0.5	1	MB_75	SAMN08398918
MB-R-79	IC2	ST-436	ST-2	4	1	1	MB_79	SAMN08398919
MB-R-80	IC2	ST-436	ST-2	2	0.5	1	MB_80	SAMN08398920
MB-R-81	IC2	ST-208	ST-2	8	0.5	1	MB_81	SAMN08398921
MB-R-83	IC2	ST-1569	ST-1067	8	2	8	MB_83	SAMN08398922
MB-R-85	IC2	ST-451	ST-2	4	1	2	MB_85	SAMN08398923
MB-R-89	IC2	ST-436	ST-2	4	1	1	MB_89	SAMN08398924
MB-R-90	IC2	ST-451	ST-2	8	2	32	MB_90	SAMN08398925
MB-R-91	IC2	ST-451	ST-2	8	2	4	MB_91	SAMN08398926
MB-R-93	IC2	ST-452	ST-2	4	1	>256	MB_93	SAMN08398927
MB-R-94	IC1	ST-1567	ST-1	1	0,125	8	MB_94	SAMN08398928
MB-R-96	IC2	ST-452	ST-2	8	1	>256	MB_96	SAMN08398929
MB-R-97	IC1	ST-1567	ST-1	4	0,5	1	MB_97	SAMN08398930
MB-R-98	IC2	ST-436	ST-2	8	1	4	MB_98	SAMN08398931
MB-R-100	IC2	ST-436	ST-2	8	2	2	MB_100	SAMN08398932
MB-R-104	IC2	ST-436	ST-2	4	1	2	MB_104	SAMN08398933
MB-R-105	IC2	ST-208	ST-2	8	2	1	MB_105	SAMN08398934
MB-R-109	IC2	ST-436	ST-2	16	4	0,5	MB_109	SAMN08398935
MB-R-110	IC2	ST-281	ST-2	8	2	16	MB_110	SAMN08398936
MB-R-112	IC2	ST-436	ST-2	16	4	1	MB_112	SAMN08398937
MB-R-114	IC2	ST-451	ST-2	8	2	8	MB_114	SAMN08398938
MB-R-116	IC2	ST-451	ST-2	8	2	128	MB_116	SAMN08398939
MB-R-119	IC2	ST-451	ST-2	8	2	256	MB_119	SAMN08398940
MB-R-120	IC2	ST-451	ST-2	8	2	64	MB_120	SAMN08398941

Appendix 2: continued

Isolate	Clonal lineage	Oxford MLST	Pasteur MLST	Tigecycline AD MIC [mg/L]	Tigecycline BMD MIC [mg/L]	Colistin MIC [mg/L]	NCBI Sample name	NCBI BioSample accession no.
MB-R-123	IC2	ST-451	ST-2	8	2	4	MB_123	SAMN08398942
MB-R-125	IC2	ST-436	ST-2	8	2	4	MB_125	SAMN08398943
MB-R-126	IC2	ST-451	ST-2	8	2	4	MB_126	SAMN08398944
MB-R-127	IC2	ST-451	ST-2	8	2	8	MB_127	SAMN08398945
MB-R-129	IC1	ST-1567	ST-1	16	4	8	MB_129	SAMN08398946
MB-R-130	IC1	ST-1567	ST-1	4	1	16	MB_130	SAMN08398947
MB-R-131	IC1	ST-1567	ST-1	2	1	32	MB_131	SAMN08398948
MB-R-132	IC2	ST-208	ST-2	4	1	2	MB_132	SAMN08398949
MB-R-133	IC2	ST-208	ST-2	4	1	1	MB_133	SAMN08398950
MB-R-135	IC2	ST-451	ST-187	8	1	1	MB_135	SAMN08398951
MB-R-138	IC2	ST-451	ST-2	4	1	2	MB_138	SAMN08398952
MB-R-141	IC2	ST-425	ST-2	8	2	≥256	MB_141	SAMN08398953
MB-R-143	IC2	ST-425	ST-2	8	2	256	MB_143	SAMN08398954
MB-R-146	IC2	ST-436	ST-2	8	0,5	2	MB_146	SAMN08398955
MB-R-148	IC2	ST-425	ST-2	16	2	≥256	MB_148	SAMN08398956
MB-R-149	IC2	ST-436	ST-2	4	1	1	MB_149	SAMN08398957
MB-R-151	IC2	ST-218	ST-2	4	1	1	MB_151	SAMN08398958
MB-R-152	IC2	ST-281	ST-2	8	1	8	MB_152	SAMN08398959
MB-R-153	IC2	ST-425	ST-2	8	1	8	MB_153	SAMN08398960
MB-R-157	IC2	ST-281	ST-632	8	1	8	MB_157	SAMN08398961
MB-R-158	IC2	ST-425	ST-2	8	2	4	MB_158	SAMN08398962
MB-R-159	IC2	ST-425	ST-2	8	2	4	MB_159	SAMN08398963
MB-R-160	IC2	ST-425	ST-2	16	4	4	MB_160	SAMN08398964
MB-R-161	IC2	ST-436	ST-2	2	0,5	1	MB_161	SAMN08398965
MB-R-164	IC2	ST-425	ST-2	16	2	≥256	MB_164	SAMN08398966
MB-R-165	IC2	ST-195	ST-2	4	1	0,5	MB_165	SAMN08398967
MB-S-5	IC2	ST-208	ST-745	16	n.d.	4[a]	MB_5	SAMN08398968
MB-S-6	IC2	ST-208	ST-745	2	n.d.	256	MB_6C	SAMN09714552
MB-S-23	IC2	ST-1114	ST-2	16	n.d.	2	MB_23C	SAMN09714553
MB-S-43	IC2	ST-208	ST-745	16	n.d.	2[a]	MB_43	SAMN08398969
MB-S-177	IC2	ST-1114	ST-2	4	n.d.	256	MB_177	SAMN09714554
MB-S-271	IC2	ST-1117	ST-2	2	n.d.	2[a]	MB_271	SAMN08398970
MB-S-273	IC2	ST-1117	ST-2	4	n.d.	2[a]	MB_273	SAMN08398971
MB-S-1044	IC1	ST-1567	ST-1	4	n.d.	≥256[a]	MB_1044	SAMN08398972
SG3161	IC4	ST-236	ST-15	1	n.d.	1	SG3161	SAMN09714555
SG3166	IC4	ST-236	ST-15	2	n.d.	256	SG3166	SAMN09714556

[a] MICs were determined by agar dilution, which is not the reference method for colistin, and results should be regarded with caution

Appendix 3: Overview of antimicrobial resistance determinants detected in *A. baumannii* isolates

Antimicrobial class	Gene	Total number of isolates (%)	No. of isolates representing different clonal lineages		
			IC2	IC1	UN
Aminoglycosides	*armA*	40 (61.5%)	39	1	0
	strA/aph(3´´)-Ib	52 (80%)	50	0	2
	strB/aph(6)-Id	52 (80%)	50	0	2
	aadA1	14 (21.5%)	9	5	0
	aadA2	3 (4.6%)	3	0	0
	aadB/ant(2´´)-Ia	3 (4.6%)	3	0	0
	aac(6')-Ip	3 (4.6%)	3	0	0
	aac(3)-Ia	12 (18.5%)	7	5	0
	aac(3)-IIa	1 (1.5%)	0	0	1
	aacA4/aac(6´)-Ib3	5 (7.7%)	5	0	0
	aac(6´)-Ian	2 (3.1%)	0	0	2
	aph(3')-Ia	44 (67.7%)	39	5	0
	aph(3')-VIa	41 (63.1%)	36	5	0
β-lactams	bla_{ADC-25}	64 (98.5%)	56	5	3
	bla_{OXA-20}	2 (3.1%)	2	0	0
	bla_{OXA-23}	53 81.5%)	49	4	0
	bla_{OXA-24}	2 (3.1%)	1	0	1
	bla_{OXA-58}	8 (12.3%)	8	0	0
	bla_{OXA-66}	54 (83.1%)	54	0	0
	bla_{OXA-69}	5 (7.7%)	0	5	0
	bla_{OXA-82}	3 (4.6%)	3	0	0
	$bla_{OXA-387}$	2 (3.1%)	0	0	2
	$bla_{OXA-391}$	1 (1.5%)	0	0	1
	bla_{TEM-1B}	2 (3.1%)	0	0	2
	bla_{TEM-1D}	38 (58.5%)	38	0	0
Sulphonamides	*sul1*	21 (32.3%)	16	5	0
	sul2	36 (55.4%)	34	0	2
Tetracyclines	*tet(B)*	49 (75.4%)	49	0	0
Phenicol	*catA1*	1 (1.5%)	1	0	0
	catB8	3 (4.6%)	3	0	0
Fluoroquinolones	*aac(6´)Ib-cr*	5 (7.7%)	5	0	0
Macrolides	*msr(E)*	33 (50.8%)	32	1	0
	mph(E)	33 (50.8%)	32	1	0

Appendix 4: Overview of allele differences associated with tigecycline and colistin resistance in *A. baumannii* isolates

Isolate	TGC MIC [mg/L]	COL MIC [mg/L]	Allele differences
MB-R-1	2	2	ArpR: N130D RND1 (ACICU_RS00750): I561V
MB-R-6	4	2	ArpR: N130D RND1 (ACICU_RS00750): I561V
MB-R-10	4	8	ArpR: N130D RND1 (ACICU_RS00750): I561V PmrB: T42P
MB-R-12	8	2	*adeN*: IS*Aba1* insertion RND2 (ACICU_RS15300): T866N RND3-MFP (ACICU_RS17100): A193S, T285I, M348I, V385I IS*Aba1* upstream *eptA*
MB-R-17	8	8	AdeS: D167N, S354P; AdeA: T325A; AdeB: S1036T; AdeC missing AdeN: H170Y AdeF: T45P, AdeG: A14S; AdeH: A25V, D446V, S481N AbeD (ACICU_RS14485): S1030T, T1031S ArpA: V291I, AbeM: V86I RND2 (ACICU_RS15300): N674S RND3 (ACICU_RS17095): I141V, V157I, S331F, G522S, P654S RND3-MFP (ACICU_RS17100): E58D, E153D, G274R RND3-OMP (ACICU_RS17105): A97V, P466S, A469T PmrB: P170L
MB-R-18	4	1	AdeS: D167N, S354P; AdeA: T325A; AdeB: S1036T; AdeC missing AdeN: H170Y, IS*Aba27* insertion AdeF: T45P, AdeG: A14S; AdeH: A25V, D446V, S481N AbeD (ACICU_RS14485): S1030T, T1031S ArpA: V291I, AbeM: V86I RND2 (ACICU_RS15300): N674S RND3 (ACICU_RS17095): I141V, V157I, S331F, G522S, P654S RND3-MFP (ACICU_RS17100): E58D, E153D, G274R RND3-OMP (ACICU_RS17105): A97V, P466S, A469T
MB-R-23	2	1	IS*Aba1* upstream *eptA*
MB-R-45	4	0.25	*adeN*: 1 nucleotide deletion (position 62) ArpR: N130D RND1 (ACICU_RS00750): I561V
MB-R-53	16	64	*adeN*: IS*Aba1* insertion AdeG: V748I, T749R RND2 (ACICU_RS15300): T866N RND3-MFP (ACICU_RS17100): A193S, T285I, M348I, V385I IS*Aba1* upstream *eptA* (point mutation A→T in position 1091) EptA: R127L SpoT: G575V, elongation factor (ACICU_RS04295): I469N, D471V chorismate synthase (ACICU_RS08730): K142N hydroxyethylthiazole kinase (ACICU_RS11465): premature stop codon
MB-R-61	8	0.5	*adeN*: 1 nucleotide deletion (position 62) ArpR: N130D RND1 (ACICU_RS00750): I561V
MB-R-62	2	2	IS*Aba1* upstream *eptA*

Appendix 4: continued

Isolate	TGC MIC [mg/L]	COL MIC [mg/L]	Allele differences
MB-R-64	8	1	*adeN*: IS*Aba1* insertion AdeL: I37L RND1 (ACICU_RS00750): V17I IS*Aba1* upstream *eptA*
MB-R-65	4	2	*adeN*: IS*Aba1* insertion AdeL: I37L RND1 (ACICU_RS00750): V17I
MB-R-67	0.5	2	non identified
MB-R-71	2	1	*adeN*: six-nucleotide insertion (GCCTTA in position 463) ArpR: N130D RND1 (ACICU_RS00750): I561V
MB-R-75	2	1	*adeRSABC*: missing *adeN*: six-nucleotide insertion (GCCTTA in position 463) ArpR: N130D RND1 (ACICU_RS00750): I561V
MB-R-79	4	1	*adeRSABC*: missing *adeN*: six-nucleotide insertion (GCCTTA in position 463) ArpR: N130D RND1 (ACICU_RS00750): I561V
MB-R-80	2	1	*adeN*: six-nucleotide insertion (GCCTTA in position 463) ArpR: N130D RND1 (ACICU_RS00750): I561V
MB-R-81	8	1	*adeN*: 1 nucleotide deletion (position 62) ArpR: N130D RND1 (ACICU_RS00750): I561V IS*Aba1* upstream *eptA*
MB-R-83	8	8	*adeN*: IS*Aba1* insertion AdeL: I37L RND1 (ACICU_RS00750): V17I, I561V IS*Aba1* upstream *eptA*
MB-R-85	4	2	AdeA: L6Q *adeN*: IS*Aba1* insertion AdeL: I37L RND1 (ACICU_RS00750): V17I, I561V
MB-R-89	4	1	*adeRSAB*: missing *adeN*: six-nucleotide insertion (GCCTTA in position 463) ArpR: N130D RND1 (ACICU_RS00750): I561V
MB-R-90	8	32	AdeR: D21V, D26N *adeN*: IS*Aba1* insertion ArpR: N130D RND1 (ACICU_RS00750): I561V PmrC: R125P
MB-R-91	8	4	AdeR: D21V, D26N *adeN*: IS*Aba1* insertion ArpR: N130D RND1 (ACICU_RS00750): I561V PmrC: R125P
MB-R-93	4	≥256	AdeR: V119I; AdeS: A325T; AdeB: P327S RND1 (ACICU_RS00750): I561V PmrCAB: multiple amino acid substitutions (please refer to Table 3.19)

Appendix 4: continued

Isolate	TGC MIC [mg/L]	COL MIC [mg/L]	Allele differences
MB-R-94	1	8	AdeB: 2 nucleotide deletion, AdeS: Q339K RND1 (ACICU_RS00750): I561V PmrC: R125P
MB-R-96	8	≥256	AdeR: V119I; AdeS: A325T; AdeB: P327S PmrCAB: multiple amino acid substitutions (please refer to Table 3.19)
MB-R-97	4	1	SoxR missing AdeS: Q339K
MB-R-98	8	4	AdeR: D21V, D26N ArpR: N130D RND1 (ACICU_RS00750): I561V PmrC: R125P *hns, naxD, vacJ* missing
MB-R-100	8	2	AdeR: G25S, D26N, E147K ArpR: N130D RND1 (ACICU_RS00750): I561V *hns* missing
MB-R-104	4	2	AdeR: G25S, D26N, E147K ArpR: N130D RND1 (ACICU_RS00750): I561V
MB-R-105	8	1	*adeRS*: IS*Aba1* insertion between *adeR* and *adeS* *adeN*: IS*Aba125* insertion ArpR: N130D RND1 (ACICU_RS00750): I561V
MB-R-109	16	0.5	AdeR: D26N ArpR: N130D RND1 (ACICU_RS00750): I561V
MB-R-110	8	16	ZndP: A529T putative permease transporter (ACICU_RS15345): G106S
MB-R-112	16	1	AdeR: D26N ArpR: N130D RND1 (ACICU_RS00750): I561V
MB-R-114	8	8	AdeR: D21V, D26N *adeN*: IS*Aba1* insertion ArpR: N130D RND1 (ACICU_RS00750): I561V PmrC: R125P
MB-R-116	8	128	AdeR: D21V, D26N *adeN*: IS*Aba1* insertion ArpR: N130D RND1 (ACICU_RS00750): I561V PmrB: L153F, PmrC: R125P
MB-R-119	8	256	AdeR: D21V, D26N *adeN*: IS*Aba1* insertion; AdeJ: V158I ArpR: N130D RND1 (ACICU_RS00750): I561V PmrB: A28V, PmrC: R125P
MB-R-120	8	64	AdeR: D21V, D26N *adeN*: IS*Aba1* insertion ArpR: N130D RND1 (ACICU_RS00750): I561V PmrB: L153F, PmrC: R125P

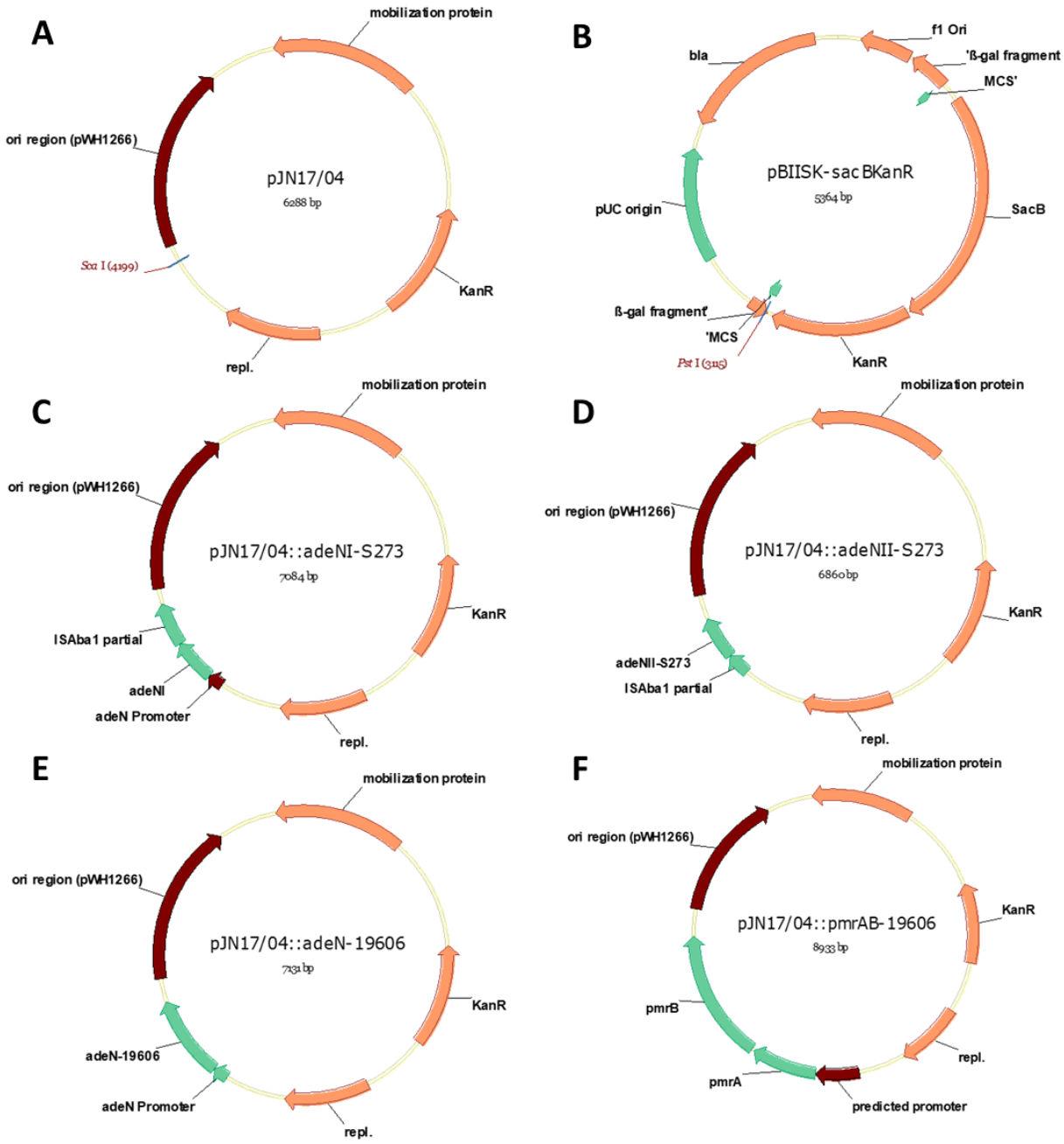
Appendix 4: continued

Isolate	TGC MIC [mg/L]	COL MIC [mg/L]	Allele differences
MB-R-123	8	4	AdeR: D21V, D26N *adeN*: IS*Aba1* insertion ArpR: N130D RND1 (ACICU_RS00750): I561V PmrC: R125P *vacJ* missing
MB-R-125	8	4	AdeR: D21V, D26N ArpR: N130D RND1 (ACICU_RS00750): I561V PmrC: R125P
MB-R-126	8	4	AdeR: D21V, D26N *adeN*: IS*Aba1* insertion ArpR: N130D RND1 (ACICU_RS00750): I561V PmrC: R125P
MB-R-127	8	8	AdeR: D21V, D26N *adeN*: IS*Aba1* insertion ArpR: N130D RND1 (ACICU_RS00750): I561V PmrC: R125P
MB-R-129	16	8	AdeS: V137F AdeS: Q339K PmrC: R125P
MB-R-130	4	16	AdeS: Q339K PmrC: R125P
MB-R-131	2	32	AdeS: Q339K PmrC: R125P
MB-R-132	4	2	*adeN*: IS*Aba1* insertion ArpR: N130D RND1 (ACICU_RS00750): I561V
MB-R-133	4	1	*adeN*: 1 nucleotide deletion (position 62) ArpR: N130D RND1 (ACICU_RS00750): I561V
MB-R-135	8	1	*adeN*: IS*Aba1* insertion AdeL: I37L RND1 (ACICU_RS00750): V17I, I561V IS*Aba1* upstream *eptA*
MB-R-138	4	2	*adeN*: IS*Aba1* insertion AdeL: I37L RND1 (ACICU_RS00750): V17I, I561V IS*Aba1* upstream *eptA*
MB-R-146	8	2	AdeR: N115K ArpR: N130D RND1 (ACICU_RS00750): I561V
MB-R-148	16	≥256	AdeR: G25S, D26N; *adeS*: IS*Aba1* insertion ArpR: N130D RND1 (ACICU_RS00750): I561V PmrA: G54E
MB-R-149	4	1	AdeR: G25S, D26N AdeG: P1052S ArpR: N130D RND1 (ACICU_RS00750): I561V

Appendix 4: continued

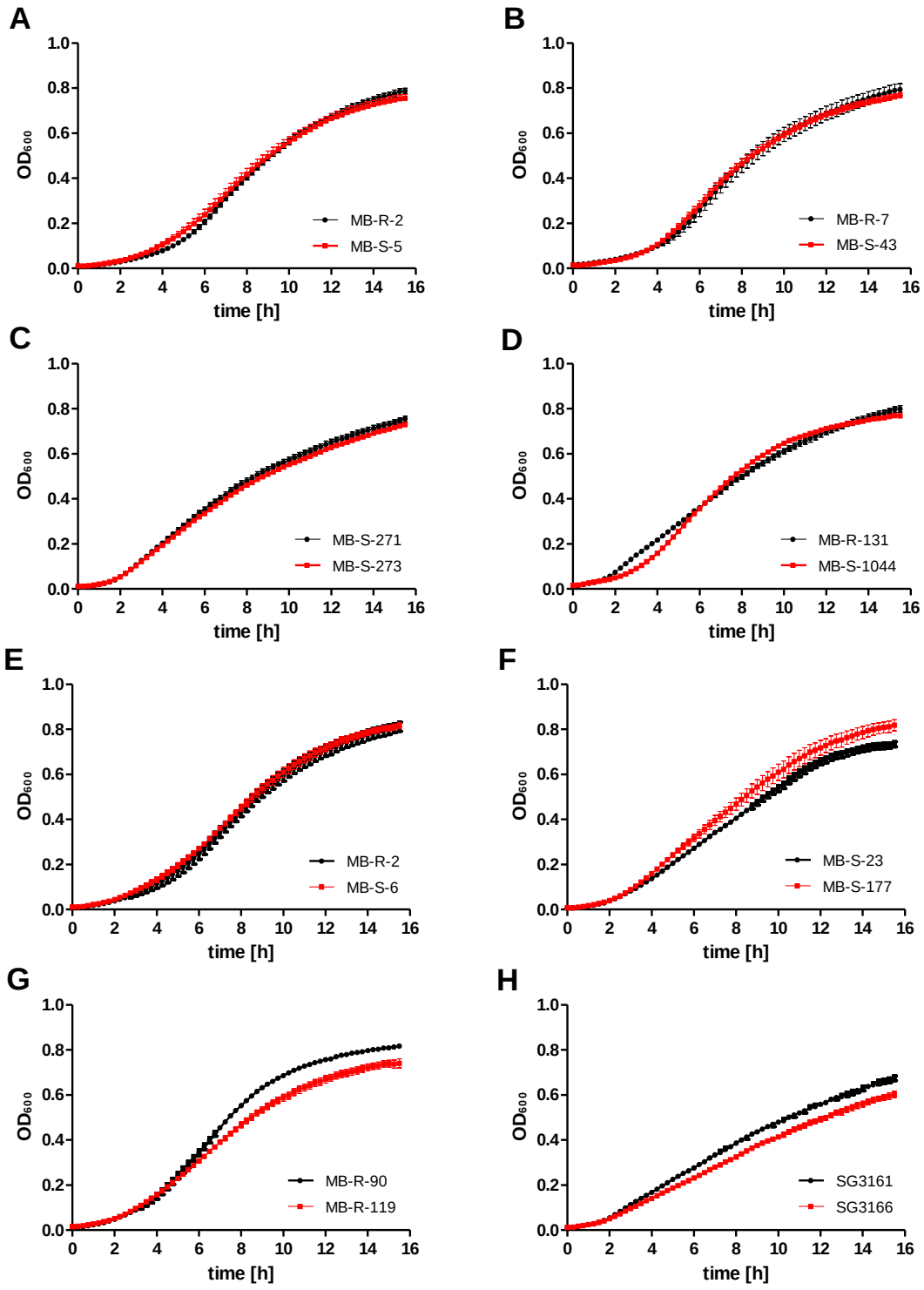
Isolate	TGC MIC [mg/L]	COL MIC [mg/L]	Allele differences
MB-R-151	4	1	IS*Aba1* upstream *eptA*
MB-R-152	8	8	ZndP: A529T putative permease transporter (ACICU_RS15345): G106S
MB-R-153	8	8	AdeR: D21V, D26N ArpR: N130D RND1 (ACICU_RS00750): I561V PmrC: R125P
MB-R-157	8	8	ZndP: A529T putative permease transporter (ACICU_RS15345): G106S
MB-R-158	8	4	AdeR: D21V, D26N AdeN: G213V ArpR: N130D RND1 (ACICU_RS00750): I561V PmrC: R125P
MB-R-159	8	4	AdeR: D21V, D26N AdeN: G213V ArpR: N130D RND1 (ACICU_RS00750): I561V PmrC: R125P *hns*: 1 nucleotide deletion in position 84
MB-R-160	16	4	AdeR: D21V, D26N *adeS*: IS*Aba1* insertion AdeN: G213V ArpR: N130D RND1 (ACICU_RS00750): I561V PmrC: R125P
MB-R-161	2	1	*adeRSABC*: missing *adeN*: 87 nucleotide deletion, F108L ArpR: N130D RND1 (ACICU_RS00750): I561V
MB-R-164	16	≥256	AdeR: G25S, D26N; *adeS*: IS*Aba1* insertion ArpR: N130D RND1 (ACICU_RS00750): I561V PmrA: G54E
MB-R-165	4	0.5	disruption of *adeA* by IS*Aba1* ArpR: N130D RND1 (ACICU_RS00750): I561V IS*Aba1* upstream *pmrCAB*

Appendix 5: Plasmid maps of the shuttle vector pJN17/04, the suicide plasmid pBIISK-sacBkanR and examples of gene constructs



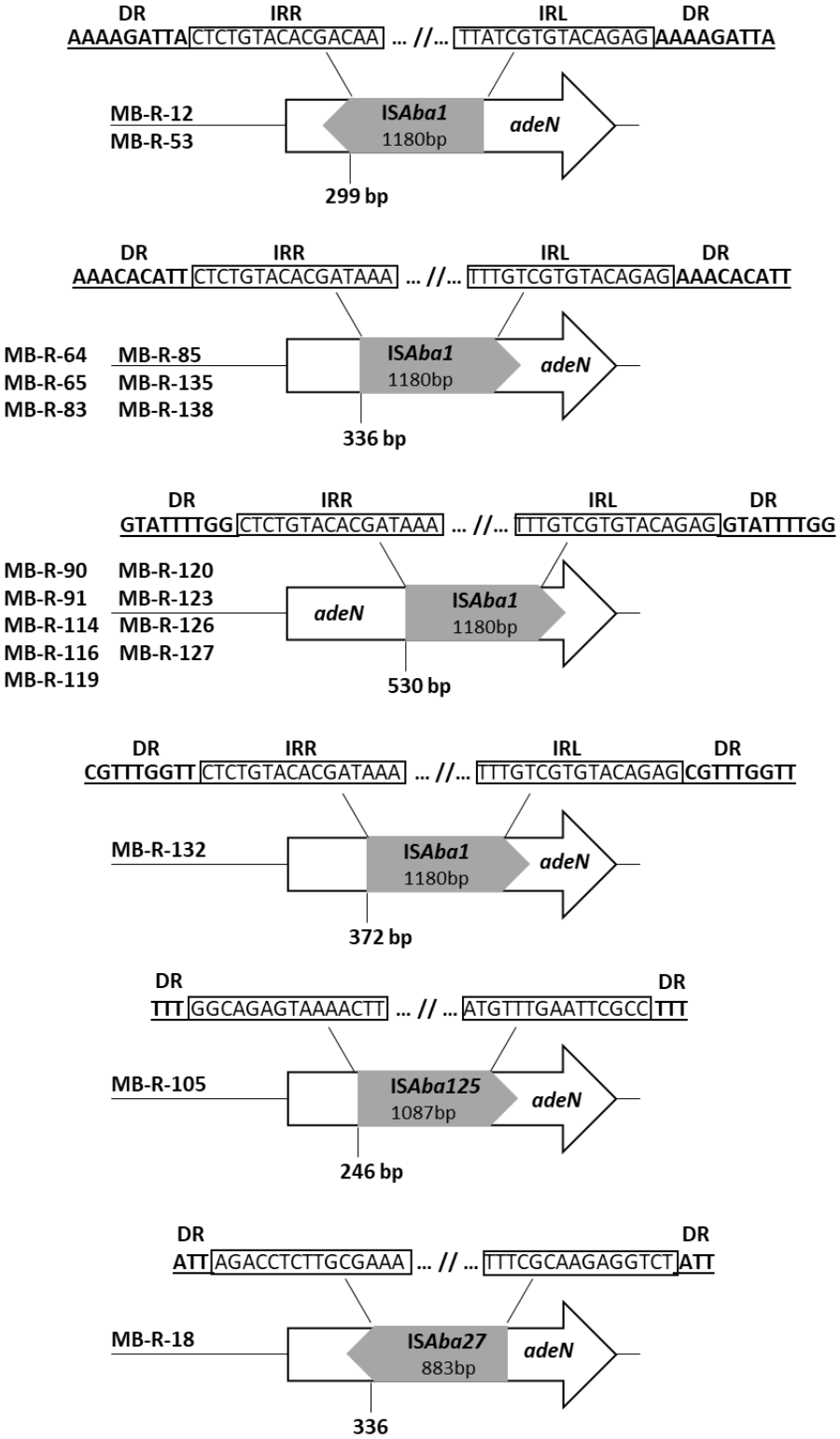
Depicted are plasmid cards of (A) the shuttle vector pJN17/04 [159] and (B) the suicide plasmid pBIISK-sacBkanR used for creating a markerless knockout [55]. The shuttle vector pJN17/04 also contains the truncated *adeN* upstream or downstream of an IS-element (C, D) or the complete genes *adeN* and *pmrAB* (both from *A. baumannii* ATCC 19606) (E, F). Moreover, different *adeN* and *pmrAB* variants originating from various MagicBullet isolates were introduced into pJN17/04 (plasmid cards not shown).

Appendix 6: Growth curves of *A. baumannii* isolates pairs

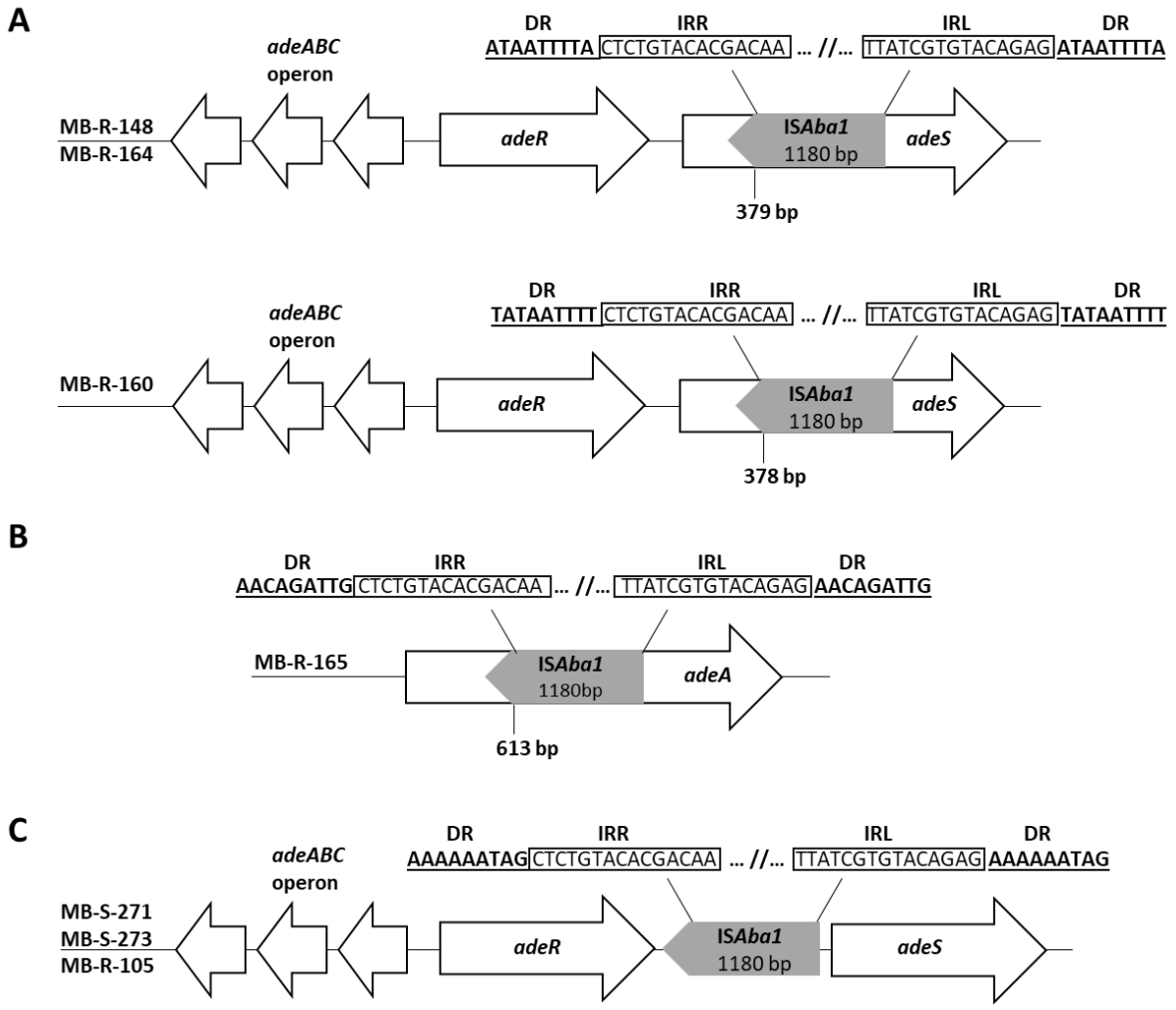


Growth curves were conducted using the Infinite M1000 plate reader. Given are the tigecycline isolate pairs 1-4 (A-D) and colistin isolate pairs 1-4 (E-H). Represented as mean ± SEM of three independent experiments.

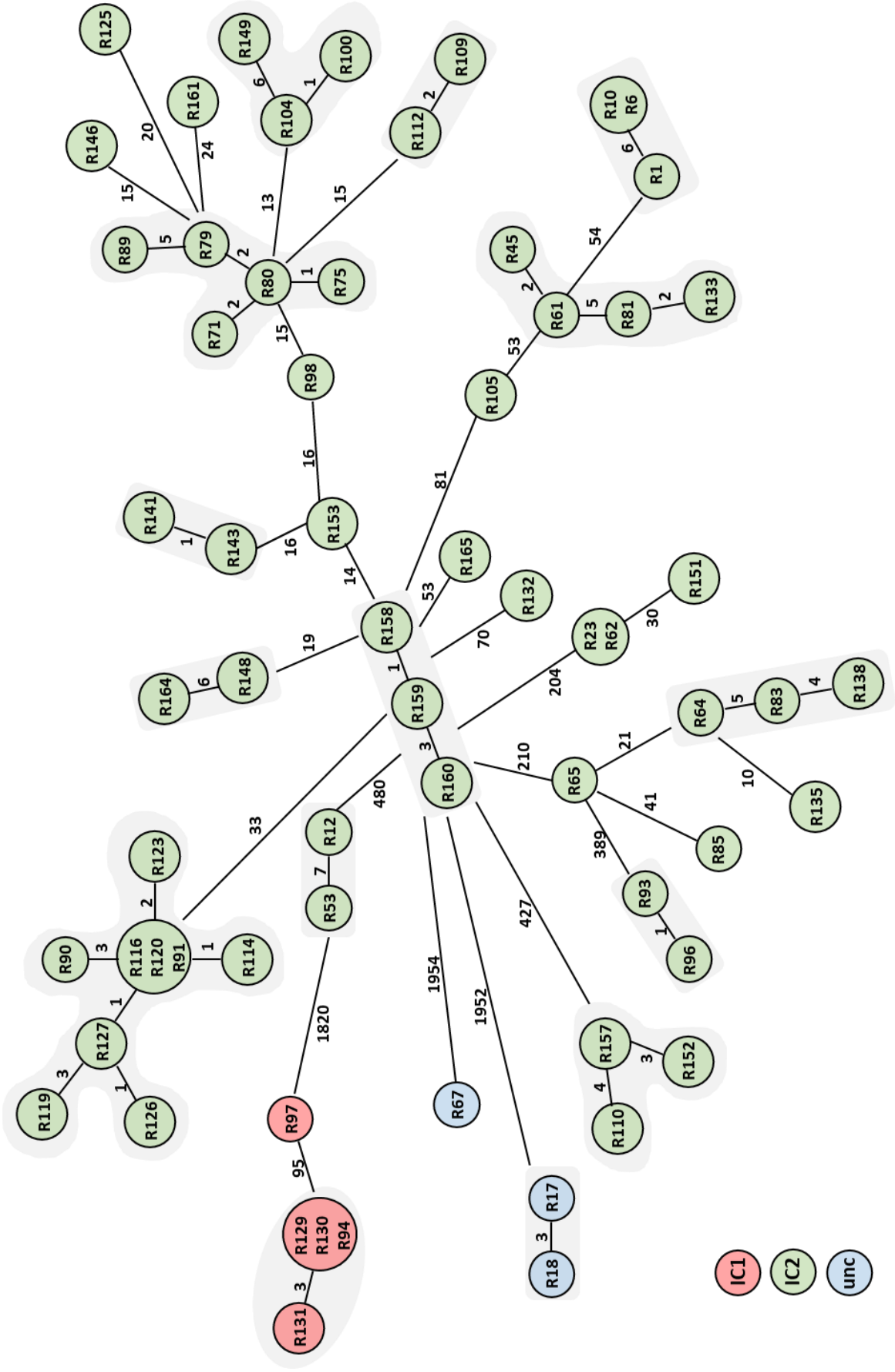
Appendix 7: Overview of insertion sequences in *adeN* observed in *A. baumannii* isolates



Appendix 8: Overview of insertion sequences in *adeS* (A), *adeA* (B) and in the intergenic region between *adeR* and *adeS* (C) observed in *A. baumannii* isolates



Appendix 9: cgMLST tree of 65 respiratory *A. baumannii* isolates.



Appendix 10: Amino acid sequence alignment of AdeR.

A. baumannii reference and comparison strains: ACICU, AYE, ATCC 19606, ATCC 17978
A. baumannii isolates with low tigecycline MICs: MB-R-67, ABC163
A. baumannii isolates with high tigecycline MICs: MB-R-17, MB-R-93, MB-R-100, MB-R-109, MB-R-143, MB-R-146, MB-R-148

```
           1                                                  50                          80
ACICU      MFDHSFSFDC QDKVILVVED DYDIGDIIEN YLKREGMSVI RAMNGKQAIE LHASQPIDLI LLDIKLPELN GWEVLNKIRQ
AYE        MFDHSFSFDC QDKVILVVED DYDIGDIIEN YLKREGMSVI RAMNGKQAIE LHASQPIDLI LLDIKLPELN GWEVLNKIRQ
ATCC 19606 MFDHSFSFDC QDKVILVVED DYDIGDIIEN YLKREGMSVI RAMNGKQAIE LHASQPIDLI LLDIKLPELN GWEVLNKIRQ
ATCC 17978 MFDHSFSFDC QDKVILVVED DYDIGDIIEN YLKREGMSVI RAMNGKQAIE LHASQPIDLI LLDIKLPELN GWEVLNKIRQ
MB-R-67    MFDHSFSFDC QDKILVVED DYDIGDIIEN YLKREGMSVI RAMNGKQAIE LHASQPIDLI LLDIKLPELN GWEVLNKIRQ
ABC163     MFDHSFSFDC QDKVILVVED DYDIGDIIEN YLKREGMSVI RAMNGKQAIE LHASQPIDLI LLDIKLPELN GWEVLNKIRQ
MB-R-17    MFDHSFSFDC QDKVILVVED DYDIGDIIEN YLKREGMSVI RAMNGKQAIE LHASQPIDLI LLDIKLPELN GWEVLNKIRQ
MB-R-93    MFDHSFSFDC QDKVILVVED DYDIGDIIEN YLKREGMSVI RAMNGKQAIE LHASQPIDLI LLDIKLPELN GWEVLNKIRQ
MB-R-100   MFDHSFSFDC QDKVILVVED DYDSNDIIEN YLKREGMSVI RAMNGKQAIE LHASQPIDLI LLDIKLPELN GWEVLNKIRQ
MB-R-109   MFDHSFSFDC QDKVILVVED DYDINDIIEN YLKREGMSVI RAMNGKQAIE LHASQPIDLI LLDIKLPELN GWEVLNKIRQ
MB-R-143   MFDHSFSFDC QDKVILVVED VYDINDIIEN YLKREGMSVI RAMNGKQAIE LHASQPIDLI LLDIKLPELN GWEVLNKIRQ
MB-R-146   MFDHSFSFDC QDKVILVVED DYDIGDIIEN YLKREGMSVI RAMNGKQAIE LHASQPIDLI LLDIKLPELN GWEVLNKIRQ
MB-R-148   MFDHSFSFDC QDKVILVVED DYDSNDIIEN YLKREGMSVI RAMNGKQAIE LHASQPIDLI LLDIKLPELN GWEVLNKIRQ

           81               100                                                150        160
ACICU      KAQTPVIMLT ALDQDIDKVM ALRIGADDFV VKPFNPNEVI ARVQAVLRRT QFANKVTNKN KLYKNIEIDT DTHSVYIHSE
AYE        KAQTPVIMLT ALDQDIDKVM ALRIGADDFV VKPFNPNEVV ARVQAVLRRT QFANKATNKN KLYKNIEIDT DTHSVYIHSE
ATCC 19606 KAQTPVIMLT ALDQDIDKVM ALRIGADDFV VKPFNPNEVV ARVQAVLRRT QFANKATNKN KLYKNIEIDT DTHSVYIHSE
ATCC 17978 KAQTPVIMLT ALDQDIDKVM ALRIGADDFV VKPFNPNEVV ARVQAVLRRT QFANKATNKN KLYKNIEIDT DTHSVYIHSE
MB-R-67    KAQTPVIMLT ALDQDIDKVM ALRIGADDFV VKPFNPNEVV ARVQAVLRRT QFANKATNKN KLYKNIEIDT DTHSVYIHSE
ABC163     KAQTPVIMLT ALDQDIDKVM ALRIGADDFV VKPFNPNEVV ARRQAVLRRT QFANKATNKN KLYKNIEIDT DTHSVYIHSE
MB-R-17    KAQTPVIMLT ALDQDIDKVM ALRIGADDFV VKPFNPNEVV ARVQAVLRRT QFANKATNKN KLYKNIEIDT DTHSVYIHSE
MB-R-93    KAQTPVIMLT ALDQDIDKVM ALRIGADDFV VKPFNPNEVI ARVQAVLRRT QFANKVTNKN KLYKNIEIDT DTHSVYIHSE
MB-R-100   KAQTPVIMLT ALDQDIDKVM ALRIGADDFV VKPFNPNEVI ARVQAVLRRT QFANKVTNKN KLYKNKEIDT DTHSVYIHSE
MB-R-109   KAQTPVIMLT ALDQDIDKVM ALRIGADDFV VKPFNPNEVI ARVQAVLRRT QFANKVTNKN KLYKNIEIDT DTHSVYIHSE
MB-R-143   KAQTPVIMLT ALDQDIDKVM ALRIGADDFV VKPFNPNEVI ARVQAVLRRT QFANKVTNKN KLYKNIEIDT DTHSVYIHSE
MB-R-146   KAQTPVIMLT ALDQDIDKVM ALRIGADDFV VKPFKPNEVI ARVQAVLRRT QFANKVTNKN KLYKNIEIDT DTHSVYIHSE
MB-R-148   KAQTPVIMLT ALDQDIDKVM ALRIGADDFV VKPFNPNEVI ARVQAVLRRT QFANKVTNKN KLYKNIEIDT DTHSVYIHSE

           161                                                200                         247
ACICU      NKKILLNLTL TEYKIISFMI DQPHKVFTRG ELMNHCMNDS DALERTVDSH VSKLRKKLEE QGIFQMLINV RGVGYRLDNP LAVKDDA
AYE        NKKILLNLTL TEYKIISFMI DQPHKVFTRG ELMNHCMNDS DALERTVDSH VSKLRKKLEE QGIFQMLINV RGVGYRLDNP LAVKDDA
ATCC 19606 NKKILLNLTL TEYKIISFMI DQPHKVFTRG ELMNHCMNDS DALERTVDSH VSKLRKKLEE QGIFQMLINV RGVGYRLDNP PAVKDDA
ATCC 17978 NKKILLNLTL TEYKIISFMI DQPHKVFTRG ELMNHCMNDS DALERTVDSH VSKLRKKLEE QGIFQMLINV RGVGYRLDNP LAVKDDA
MB-R-67    NKKILLNLTL TEYKIISFMI DQPHKVFTRG ELMNHCMNDS DALERTVDSH VSKLRKKLEE QGIFQMLINV RGVGYRLDNP LAIKDDA
ABC163     NKKILLNLTL TEYKIISFMI DQPHKVFTRG ELMNHCMNDS DALERTVDSH VSKLRKKLEE QGIFQMLINV RGVGYRLDNP LAVKDDA
MB-R-17    NKKILLNLTL TEYKIISFMI DQPHKVFTRG ELMNHCMNDS DALERTVDSH VSKLRKKLEE QGIFQMLINV RGVGYRLDNP LAIKDDA
MB-R-93    NKKILLNLTL TEYKIISFMI DQPHKVFTRG ELMNHCMNDS DALERTVDSH VSKLRKKLEE QGIFQMLINV RGVGYRLDNP LAVKDDA
MB-R-100   NKKILLNLTL TEYKIISFMI DQPHKVFTRG ELMNHCMNDS DALERTVDSH VSKLRKKLEE QGIFQMLINV RGVGYRLDNP LAVKDDA
MB-R-109   NKKILLNLTL TEYKIISFMI DQPHKVFTRG ELMNHCMNDS DALERTVDSH VSKLRKKLEE QGIFQMLINV RGVGYRLDNP LAVKDDA
MB-R-143   NKKILLNLTL TEYKIISFMI DQPHKVFTRG ELMNHCMNDS DALERTVDSH VSKLRKKLEE QGIFQMLINV RGVGYRLDNP LAVKDDA
MB-R-146   NKKILLNLTL TEYKIISFMI DQPHKVFTRG ELMNHCMNDS DALERTVDSH VSKLRKKLEE QGIFQMLINV RGVGYRLDNP LAVKDDA
MB-R-148   NKKILLNLTL TEYKIISFMI DQPHKVFTRG ELMNHCMNDS DALERTVDSH VSKLRKKLEE QGIFQMLINV RGVGYRLDNP LAVKDDA
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Appendix 11: Amino acid sequence alignment of AdeS.

A. baumannii reference and comparison strains: ACICU, AYE, ATCC 1906, ATCC 17978
A. baumannii isolates with low tigecycline MICs: MB-R-67, MB-R-94, ABC163, ABC212, ABC085
A. baumannii isolates with high tigecycline MICs: MB-R-17, MB-R-93, MB-R-151, MB-R-129

```
          1                                                  50                                                  80
ACICU     MKSKLGISKQ LFIALTIVNL SVTLFSVVLG YVIYNYAIEK GWISLSSFQQ EDWTSFHFVD WIWLATVIFC GCIISLVIGM
AYE       ..........  .........I  ..........  I.........  ..........  ..........  ..........  ..........
ATCC 19606 ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
ATCC 17978 ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
MB-R-67   ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
MB-R-94   ..........  .........I  -.........  I.........  ..........  ..........  ..........  ..........
ABC163    ..........  .........I  ..........  I.........  ..........  ..........  ..........  ..........
ABC212    ..........  .........I  ..........  I.........  ..........  ..........  ..........  ..........
ABC085    ..........  ..........  ..........  ..........  ..........  ..........  .M........  ..........
MB-R-17   ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
MB-R-93   ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
MB-R-151  ..........  ..........  ..........  ..........  ..........  ..........  .M........  ..........
MB-R-129  ..........  .........I  ..........  I.........  ..........  ..........  ..........  ..........

          81                100                                                                 150        160
ACICU     RLAKRFIVPI NFLAEAAKKI SHGDLSARAY DNRIHSAEMS ELLYNFNDMA QKLEVSVKNA QVWNAAIAHE LRTPITILQG
AYE       ..........  .V........  ..........  ..........  ..........  ..........  ..........  ..........
ATCC 19606 ..........  ..........  ..........  ..........  ..........  ..........  ..........  .A........
ATCC 17978 ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
MB-R-67   ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
MB-R-94   ..........  .V........  ..........  ..........  ..........  ..........  ..........  ..........
ABC163    ..........  .V......N.  ..........  ..........  ..........  ..........  ..........  .M........
ABC212    ..........  ..........  ..........  ..........  .K........  ..........  ..........  ..........
ABC085    ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
MB-R-17   ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
MB-R-93   ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
MB-R-151  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
MB-R-129  ..........  .V........  ..........  ..........  ..........  ..........  .F........  ..........

          161                                 200                                                 240
ACICU     RLQGIIDGVF KPDEVLFKSL LNQVEVLSHL VEDLRTLSLV ENQQLRLNYE LFDFKAVVEK VLKAFEDRLD QAKLVPELDL
AYE       ..........  ..........  ..G.......  ..........  ..........  ..L.......  ..........  ..........
ATCC 19606 ..........  ..........  ..G.......  ..........  ..........  ..L.......  ..........  ..........
ATCC 17978 ..........  .L........  ..G.......  ..........  ..........  ..L.......  ..........  ..........
MB-R-67   ..........  ..........  ..G.......  ..........  ..........  ..........  ..........  .....I....
MB-R-94   ..........  ..........  ..G.......  ..........  ..........  ..L.......  ..........  ..........
ABC163    ..........  ..........  ..G.......  ..........  ..........  ..L.......  ..........  ..........
ABC212    ..........  ..........  ..G.......  ..........  ..........  ..L.......  ..........  ..........
ABC085    ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
MB-R-17   ..N.......  ..........  ..G.......  ..........  ..........  ..........  ..........  ..........
MB-R-93   ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
MB-R-151  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
MB-R-129  ..........  ..........  ..G.......  ..........  ..........  ..L.......  ..........  ..........

          241  250                                                          300                 320
ACICU     TSTPVYCDRR RIEQVLIALI DNAIRYSHAG KLKISSEVVS QNWILKIEDE GPGIATEFQD DLFKPFFRLE ESRNKEFGGT
AYE       ..........  ..........  ..........  ..........  ..........  ..........  .R........  ..........
ATCC 19606 ..........  ..S.......  ..........  ..........  ..........  ..........  .R........  ..........
ATCC 17978 ..........  ..........  ..........  ..........  ..........  ..........  .R.Y......  ..........
MB-R-67   ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
MB-R-94   ..........  ..........  ..........  ..........  ..........  ..........  .R........  ..........
ABC163    ..........  ..........  ..........  ..........  ..........  ..........  .R........  ..........
ABC212    ..........  ..........  ..........  ..........  ..........  ..........  .R........  ..........
ABC085    ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
MB-R-17   ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
MB-R-93   ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
MB-R-151  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
MB-R-129  ..........  ..........  ..........  ..........  ..........  ..........  .R........  ..........

          321                         350          361
ACICU     GLGLAVVHAI IVALKGTIQY SNQGSKSIFT IKISMNN
AYE       ...........  ...........  ...V.......  ...GHEEMG
ATCC 19606 ...........  .V.........  ...V.......  ...........
ATCC 17978 ...........  ...........  ...V.......  ...GHEEIG
MB-R-67   ...........  .V.........  ...V.......  ...........
MB-R-94   ...........  ...........  ...V.......  ...GHEEMG
ABC163    ...........  ...........  ...V.......  ...GHEEMG
ABC212    ...........  ...........  ...V.......  ...GHEEMG
ABC085    ...........  ...........  ...........  ...........
MB-R-17   ...........  ...........  ...V.......  ...P.......
MB-R-93   ...T.......  ...........  ...........  ...........
MB-R-151  ...........  ...........  ...........  ...........
MB-R-129  ...........  ...........  ...V.......  ...GHEEMG
```

Appendix 12: Amino acid sequence alignment of AdeL.

A. baumannii reference and comparison strains: ACICU, AYE, ATCC 1906, ATCC 17978
A. baumannii isolates with low tigecycline MICs: MB-R-67, ABC212
A. baumannii isolates with high tigecycline MICs: MB-R-64

```
           1                                                  50                                                  80
ACICU      MDLFHAMRVF NKVVETNSFS LAADSLGLPR ASVTTTIQAL EKHLQVRLLN RTTRKISLTP DGAVYYDRTA RILADVADIE
AYE        VDLFHAMRVF NKVVETNSFS LAADSLGLPR ASVTTTIQAL EKHLQVRLLN RTTRKISLTP DGAVYYDRTA RILADVADIE
ATCC 19606 VDLFHAMRVF NKVVETNSFS LAADSLGLPR ASVTTTIQAL EKHLQVRLLN RTTRKISLTP DGAVYYDRTA RILADVADIE
ATCC 17978 ~~~~~~MRVF NKVVETNSFS LAADSLGLPR ASVTTTIQAL EKHLQVRLLN RTTRKISLTP DGAVYYDRTA RILADVADIE
MB-R-67    VDLFHAMRVF NKVVETNSFS LAADSLGLPR ASVTTTIQAL EKHLQVRLLN RTTRKISLTP DGAVYYDRTA RILADVADIE
ABC212     VDLFHAMRVF NKVVETNSFS LAADSLGLPR ASVTTTIQAL EKHLQVRLLN RTTRKISLTP DGAVYYDRTA RILADVADIE
MB-R-64    VDLFHAMRVF NKVVETNSFS LAADSLGLPR ASVTTLIQAL EKHLQVRLLN RTTRKISLTP DGAVYYDRTA RILADVADIE

           81                100                                                                 150         160
ACICU      SSFHDAERGP RGQLRIDVPV SIGRLILIPR LRDFHARYPD IDLVIGLNDR PVDLVGEAVD CAIRVGELKD SSLIARRIGT
AYE        SSFHDAERGP RGQLRIDVPV SIGRLILIPR LRDFHARYPD IDLVIGLNDR PVDLVGEAVD CAIRVGELKD SSLIARRIGT
ATCC 19606 SSFHDAERGP RGQLRIDVPV SIGRLILIPR LRDFHARYPD IDLVIGLNDR PVDLVGEAVD CAIRVGELKD SSLIARRIGT
ATCC 17978 SSFHDAERGP RGQLRIDVPV SIGRLILIPR LRDFHARYPD IDLVIGLNDR PVDLVGEAVD CAIRVGELKD SSLIARRIGT
MB-R-67    SSFHDAERGP RGQLRIDVPV SIGRLILIPR LRDFHARYPD IDLVIGLNDR PVDLVGEAVD CAIRVGELKD SSLIARRIGT
ABC212     SSFHDAERGP RGQLRIDVPV SIGRLILIPR LRVFHARYPD IDLVIGLNDR PVDLVGEAVD CAIRVGELKD SSLIARRIGT
MB-R-64    SSFHDAERGP RGQLRIDVPV SIGRLILIPR LRDFHARYPD IDLVIGLNDR PVDLVGEAVD CAIRVGELKD SSLIARRIGT

           161                                  200                                                 240
ACICU      FQCATAASPI YLEKYGEPTS IEDLQKNHKA IHFFSSRTGR NFDWDFVVDD LIKSVSVRGR VSVNDGDAYI DLALQGFGII
AYE        FQCATAASPI YLEKYGEPTS IEDLQKNHKA IHFFSSRTGR NFDWDFVVDD LIKSVSVRGR VSVNDGDAYI DLALQGFGII
ATCC 19606 FQCATAASPI YLEKYGEPTS IEDLQKNHKA IHFFSSRTGR NFDWDFVVDD LIKSVSVRGR VSVNDGDAYI DLALQGFGII
ATCC 17978 FQCATAASPI YLEKYGEPTS IEDLQKNHKA IHFFSSRTGR NFDWDFVVDD LIKSVSVRGR VSVNDGDAYI DLALQGFGII
MB-R-67    FQCATAASPI YLEKYGEPTS IEDLQKNHKA IHFFSSRTGR NFDWDFVVDD LIKSVSVRGR VSVNDGDAYI DLALQGFGII
ABC212     FQCATAASPI YLEKYGEPTS IEDLQKNHKA IHFFSSRTGR NFDWDFVVDD LIKSVSVRGR VSVNDGDAYI DLALQGFGII
MB-R-64    FQCATAASPI YLEKYGEPTS IEDLQKNHKA IHFFSSRTGR NFDWDFVVDD LIKSVSVRGR VSVNDGDAYI DLALQGFGII

           241  250                                                 300                 320
ACICU      QGPRYMLTNH LESGLLKEVL PQWTPAPMPI SAVYLQNRHL SLKVKVFVDW VAELFAGCPL LGGTALPFDQ KCEFACDKET
AYE        QGPRYMLTNH LESGLLKEVL RQWTPAPMPI SAVYLQNRHL SLKVKVFVDW VAELFAGCPL LGGTALPFDQ KCEFACDKET
ATCC 19606 QGPRYMLTNH LESGLLKEVL PQWTPAPMPI SAVYLQNRHL SLKVKVFVDW VAELFAGCPL LGGTALPFDQ KCEFACDKET
ATCC 17978 QGPRYMLTNH LESGLLKEVL PQWTPAPMPI SAVYLQNRHL SLKVKVFVDW VAELFAGCPL LGGTALPFDQ KCEFACDKET
MB-R-67    QGPRYMLTNH LESGLLKEVL PQWTPAPMPI SAVYLQNRHL SLKVKVFVDW VAELFAGCPL LGGTALPFDQ KCEFACDKET
ABC212     QGPRYMLTNH LESGLLKEVL RQWTPAPMPI SAVYLQNRHL SLKVKVFVDW VAELFAGCPL LGGTALPFDQ KCEFACDKET
MB-R-64    QGPRYMLTNH LESGLLKEVL PQWTPAPMPI SAVYLQNRHL SLKVKVFVDW VAELFAGCPL LGGTALPFDQ KCEFACDKET

           321                  343
ACICU      GHEYTIRTLV EQHNIAEAYT LKT
AYE        GHEYTIRTLV EQHNIAEAYT LKT
ATCC 19606 GHEYTIRTLV EQHNIAEAYT LKT
ATCC 17978 GHEYTIRTLV EQHNIAEAYT LKT
MB-R-67    GHEYTIRTLV EQHNIAERV  LKT
ABC212     GHEYTIRTLV EQHNIAEAYT LKT
MB-R-64    GHEYTIRTLV EQHNIAEAYT LKT
```

Appendix 13: Amino acid sequence alignment of AdeN.

A. baumannii reference and comparison strains: ACICU, AYE, ATCC 1906, ATCC 17978
A. baumannii isolates with low tigecycline MICs: MB-R-67, ABC061, ABC085
A. baumannii isolates with high tigecycline MICs: MB-R-17, MB-R-151, MB-R-158, MB-R-71, MB-R-110, MB-R-161

```
           1                                                  50                          80
ACICU      MHDPVLESHH LVCEKPQTRR GIERRLALLL SATELFLEKG YDAVSLDDIV NHAGGSKNSI YKYFGNKDGL FTAICDYRRE
AYE        ..........  ..........  ..........  ..........  ..........  ..........  T.......  ..........  ..........
ATCC 19606 ..........  ..........  ..........  ..........  ..........  ..........  T.......  ..........  ..........
ATCC 17978 ..........  ..........  ..........  ..........  ..........  ..........  T.......  ..........  ..........
MB-R-67    ..........  ..........  ..........  ..........  ..........  ..........  T.......  ..........  ..........
ABC061     ..........  ..........  ..........  ..........  ..........  ..........  T.......  ..........  ..........
ABC085     ..........  ..........  ..........  ..........  ..........  ..........  T.......  ..........  ..........
MB-R-17    ..........  ..........  ..........  ..........  ..........  ..........  T.......  ..........  ..........
MB-R-151   ..........  ..........  ..........  ..........  ..........  ..........  T.......  ..........  ..........
MB-R-158   ..........  ..........  ..........  ..........  ..........  ..........  T.......  ..........  ..........
MB-R-71    ..........  ..........  ..........  ..........  ..........  ..........  T.......  ..........  ..........
MB-R-110   ..........  ..........  ..........  ..........  ..........  ..........  T.......  ..........  ..........
MB-R-161   ..........  ..........  ..........  ..........  ..........  ..........  T.......  ..........  --

           81                100                                                 150        160
ACICU      MFFKDICIAF QPEQTSLKDY LIQTLIRFYK HIIQPEHIAF LRLVIEQTQC NATLSQYLYE KCALDVQNTI AQA--LLISH
AYE        ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..--......
ATCC 19606 ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..--......
ATCC 17978 ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..--......
MB-R-67    ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..--......
ABC061     ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..--......
ABC085     ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..--......
MB-R-17    ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..--......
MB-R-151   ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..--......
MB-R-158   ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..--......
MB-R-71    ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..LA......
MB-R-110   ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..--......
MB-R-161   ----------  ----------  -------L..  ..........  ..........  ..........  ..........  ..--......

           161                                 200                 217
ACICU      QSGEITCTSP DHSSLMYFGI LRDIEWRMIM GMPLPPNETE VIDYINYCVD IFLKGHHKV
AYE        ..........  ..........  ..........  ..........  ..........  .........
ATCC 19606 ..........  ..........  ..........  ...M......  ..........  .........
ATCC 17978 ..........  ..........  ..........  ..........  ..........  .........
MB-R-67    ..........  ..........  ..........  ..........  ..........  .........
ABC061     ..........  ..........  ..........  .+........  ..........  .........
ABC085     ..........  ..N.......  ..........  ..........  ..........  .........
MB-R-17    ..........  .Y........  ..........  ..........  ..........  .........
MB-R-151   ..........  ..N.......  ..........  ..........  ..........  .........
MB-R-158   ..........  ..........  ..........  ..........  .....V....  .........
MB-R-71    ..........  ..........  ..........  ..........  ..........  .........
MB-R-110   ..........  ..........  ..........  .+........  ..........  .........
MB-R-161   ..........  ..........  ..........  ..........  ..........  .........
```

Appendix 14: Amino acid alignment of ArpR.

A. baumannii reference and comparison strains: ACICU, AYE, ATCC 1906, ATCC 17978
A. baumannii isolates with low tigecycline MICs: MB-R-67, ABC163
A. baumannii isolates with high tigecycline MICs: MB-R-45, MB-R-53

```
           1                                                  50                                 80
ACICU      MQTVNQSGRP KDLEKRARIL QAAKAIFLKS GYHGTSMNLI AQEAGVTKLT VYNHFQDKAN LFICAITETC EETLGTKPFE
AYE        MQTVNQSGRP KDLEKRARIL QAAKAIFLKS GYHGTSMNLI AQEAGVTKLT VYNHFQDKAN LFICAITETC EETLGTKPFE
ATCC 19606 VQTVNQSGRP KDLEKRARIL QAAKAIFLKS GYHGTSMNLI AQEAGVTKLT VYNHFQDKAN LFICAITETC EETLGTKPFE
ATCC 17978 MQTVNQSGRP KDLEKRARIL QAAKAIFLKS GYHGTSMNLI AQEAGVTKLT VYNHFQDKAN LFICAITETC EETLGTKPFE
MB-R-67    VQTVNQSGRP KDLEKRARIL QAAKAIFLKS GYHGTSMNLI AQEAGVTKLT VYNHFQDKAN LFICAITETC EETLGTKPFE
ABC163     VQTVNQSGRP EDLEKRARIL QAAKAIFLKS GYHGTSMNLI AQEAGVTKLT VYNHFQDKAN LFICAITETC EETLGTKPFE
MB-R-45    VQTVNQSGRP KDLEKRARIL QAAKAIFLKS GYHGTSMNLI AQEAGVTKLT VYNHFQDKAN LFICAITETC EETLGTKPFE
MB-R-53    VQTVNQSGRP KDLEKRARIL QAAKAIFLKS GYHGTSMNLI AQEAGVTKLT VYNHFQDKAN LFICAITETC EETLGTKPFE

           81                100                                                 150        160
ACICU      LDASADFYQT LYIVCSRALQ IIYSPEALKL EHVLFELAAE QSPLALQFFN ASHTRLQNQL VEFFQKAAQL GFIQADDPLY
AYE        LDASADFYQT LMVCSRALQ IIYSPEALKL EHVLFELAAE QSPLALQFFN ASHTRLQNQL VEFFQKAAQL GFIQADDPLY
ATCC 19606 LDASADFYQT LMVCSRALQ IIYSPEALKL EHVLFELAAE QSPLALQFFN ASHTRLQNQL VEFFQKAAQL GFIQADDPLY
ATCC 17978 LDASADFYQT LYIVCSRALQ IIYSPEALKL EHVLFELAAE QSPLALQFFN ASHTRLQNQL VEFFQKAAQL GFIQADDPLY
MB-R-67    LDASADFYQT LMVCSRALQ IIYSPEALKL EHVLFELAAE QSPLALQFFN ASHTRLQNQL VEFFQKAAQL GFIQADDPLY
ABC163     LDASADFYQT LMVCSRALQ IIYSPEALKL EHVLFELAAE QSPLALQFFN ASHTRLQNQL VEFFQKAAQL GFIQADDPLY
MB-R-45    LDASADFYQT LMVCSRALQ IIYSPEALKL EHVLFELAAE QSPLALQFD ASHTRLQNQL VEFFQKAAQL GFIQADDPLY
MB-R-53    LDASADFYQT LMVCSRALQ IIYSPEALKL EHVLFELAAE QSPLALQFFN ASHTRLQNQL VEFFQKAAQL GFIQADDPLY

           161                                  200     207
ACICU      QTELLLTLLL GVRHHKVLLR IIPVPNAQEI DRFIRDAIDL FLLRYRH
AYE        QTELLLTLLL GVRHHKVLLG IIPVPTDQEI DRFIRDAIDL FLLKYRH
ATCC 19606 QTELLLTLLL GVRHHKVLLG IIPVPTDQEI DRFIRDAIDL FLLKYRH
ATCC 17978 QTELLLTLLL GVRHHKVLLR IIPVPNAQEI DRFIHDAIDL FLLRYRH
MB-R-67    QTELLLTLLL GVRHHKVLLR IIPVPNAQEI DRFIRDAIDL FLLKYRH
ABC163     QTELLLTLLL GVRHHKVLLG IIPVPTDQEI DRFIRDAIDL FLLKYRH
MB-R-45    QTELLLTLLL GVRHHKVLLR IIPVPNAQEI DRFIRDAIDL FLLRYRH
MB-R-53    QTELLLTLLL GVRHHKVLLG IIPVPTDQEI DRFIRDAIDL FLLKYRH
```

Appendix 15: Amino acid sequence alignment of PmrA.

A. baumannii reference and comparison strains: ACICU, AYE, ATCC 1906, ATCC 17978
Colistin-susceptible *A. baumannii* isolates: SG3161 (IC4), MB-R-1, MB-R-132
Colistin-resistant *A. baumannii* isolates: MB-R-93, MB-R-148

```
           1                                                  50                                 80
ACICU      MTKILMIEDD FMIAESTITL LQYHQFEVEW VNNGLDGLAQ LAKTKFDLIL LDLGLPMMDG MQVLKQIRQR AATPVLIISA
AYE        MTKILMIEDD FMIAESTITL LQYHQFEVEW VNNGLDGLAQ LAKTKFDLIL LDLGLPMMDG MQVLKQIRQR AATPVLIISA
ATCC 19606 MTKILMIEDD FMIAESTITL LQYHQFEVEW VNNGLDGLAQ LAKTKFDLIL LDLGLPMMDG MQVLKQIRQR AATPVLIISA
ATCC 17978 MTKILMIEDD FMIAESTITL LQYHQFEVEW VNNGLDGLAQ LAKTKFDLIL LDLGLPMMDG MQVLKQIRQR AATPVLIISA
SG3161     MTKILMIEDD FMIAEST TL LQYHQFEVEW VNNGLDGLAQ LAN KFDLIL LDLGLPMMDG MQVLKQIRQR AATPVLIISA
MB-R-1     MTKILMIEDD FMIAESTITL LQYHQFEVEW VNNGLDGLAQ LAKTKFDLIL LDLGLPMMDG MQVLKQIRQR AATPVLIISA
MB-R-132   MTKILMIEDD VMIAESTITL LQYHQFEVEW VNNGLDGLAQ LAKTKFDLIL LDLGLPMMDG MQVLKQIRQR AATPVLIISA
MB-R-93    MTKILMIEDD FMIAEST TL LQYHQFEVEW VNNGLDGLAQ LAN KFDLIL LDLGLPMMDG MQVLKQIRQR AATPVLIISA
MB-R-148   MTKILMIEDD FMIAESTITL LQYHQFEVEW VNNGLDGLAQ LAKTKFDLIL LDLELPMMDG MQVLKQIRQR AATPVLIISA

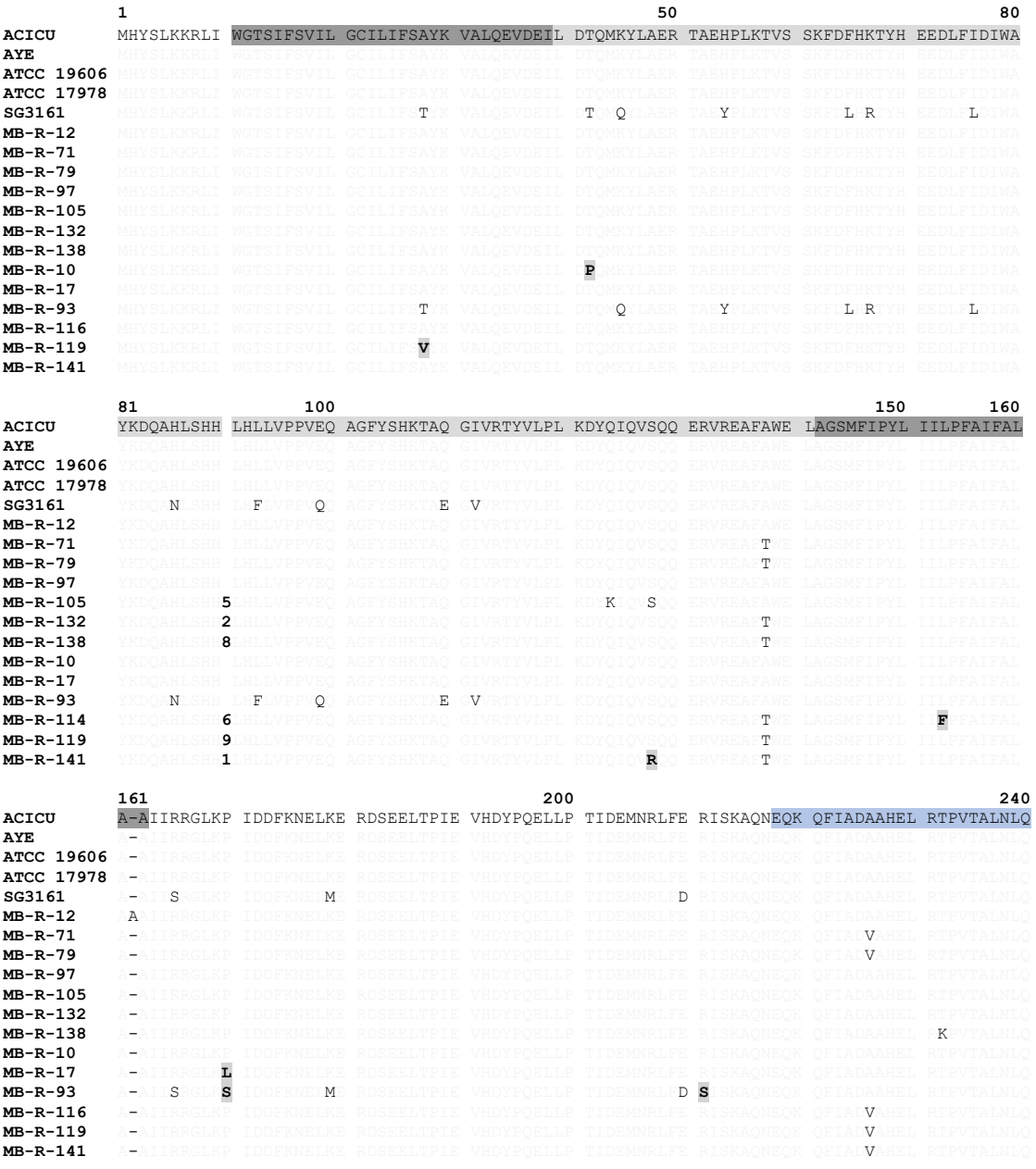
           81                100                                                 150        160
ACICU      RDQLQNRVDG LNLGADDYLI KPYEFDELLA RIHALLRRSG VEAQLASQDQ LLESGDLVLN VEQHIATFKG QRIDLSNREW
AYE        RDQLQNRVDG LNLGADDYLI KPYEFDELLA RIHALLRRSG VEAQLASQDQ LLESGDLVLN VEQHIATFKG QRIDLSNREW
ATCC 19606 RDQLQNRVDG LNLGADDYLI KPYEFDELLA RIHALLRRSG VEAQLASQDQ LLESGDLVLN VEQHIATFKG QRIDLSNREW
ATCC 17978 RDQLQNRVDG LNLGADDYLI KPYEFDELLA RIHALLRRSG VEAQLASQDQ LLESGDLVLN VEQHIATFKG QRIDLSNREW
SG3161     RDQLQNRVDG LNLGADDYLI KPYEFDELLA RIHALLRRSG VEAQLASQDQ LL SGDLVLN VEQHIATFKG QRIDLSNREW
MB-R-1     RDQLQNRVDG LNLGADDYLI KPYEFDELLA RIHALLRRSG VEAQLASQDQ LLESGDLVLN VEQHIATFKG QRIDLSNREW
MB-R-132   RDQLQNRVDG LNLGADDYLI KPYEFDELLA RIHALLRRSG VEAQLASQDQ LLESGDLVLN VEQHIATFKG QRIDLSNREW
MB-R-93    RDQLQNRVDG LNLGADDYLI KPYEFDELLA RIHALLRRSG VEAQLASQDQ LL GDLVLN VEQHIATFKG QRIDLSNREW
MB-R-148   RDQLQNRVDG LNLGADDYLI KPYEFDELLA RIHALLRRSG VEAQLASQDQ LLESGDLVLN VEQHIATFKG QRIDLSNREW

           161                                200                       224
ACICU      AILIPLMTHP NKIFSKANLE DKLYDFDSDV TSNTIEVYVH HLRAKLGKDF IRTIRGLGYR LGQS
AYE        AILIPLMTHP NKIFSKANLE DKLYDFDSDV TSNTIEVYVH HLRAKLGKDF IRTIRGLGYR LGQS
ATCC 19606 AILIPLMTHP NKIFSKANLE DKLYDFDSDV TSNTIEVYVH HLRAKLGKDF IRTIRGLGYR LGQS
ATCC 17978 AILIPLMTHP NKIFSKANLE DKLYDFDSDV TSNTIEVYVH HLRAKLGKDF IRTIRGLGYR LGQS
SG3161     AILIPLM HP NKIFSKANLE DKLYDFDSDV TSNTIEVYVH HLRAKLGKDF IRTIRGLGYR LGQS
MB-R-1     AILIPLMTHP NKIFSKANLE DKLYDFDSDV TSNTIEVYVH HLRAKLGKDF IRTIRGLGYR LGQS
MB-R-132   AILIPLMTHP NKIFSKANLE DKLYDFDSDV TSNTIEVYVH HLRAKLGKDF IRTIRGLGYR LGQS
MB-R-93    AILIPLM HP NKIFSKANLE DKLYDFDSDV TSNTIEVYVH HLRAKLGKDF IRTIRGLGYR LGQS
MB-R-148   AILIPLMTHP NKIFSKANLE DKLYDFDSDV TSNTIEVYVH HLRAKLGKDF IRTIRGLGYR LGQS
```

Appendix 16: Amino acid sequence alignment of PmrB.

Important protein domains are shown: transmembrane domains (dark grey); periplasmic domain (light grey); HisKA domain (blue); and HATPase_c domain (orange) [193].

A. baumannii reference and comparison strains: ACICU, AYE, ATCC 1906, ATCC 17978
Colistin-susceptible *A. baumannii* isolates: SG3161 (IC4), MB-R-12, MB-R-71, MB-R-79, MB-R-97, MB-R-105, MB-R-132, MB-R-138
Colistin-resistant *A. baumannii* isolates: MB-R-10, MB-R-17, MB-R-93, MB-R-116, MB-R-119, MB-R-141



Appendix 16: continued

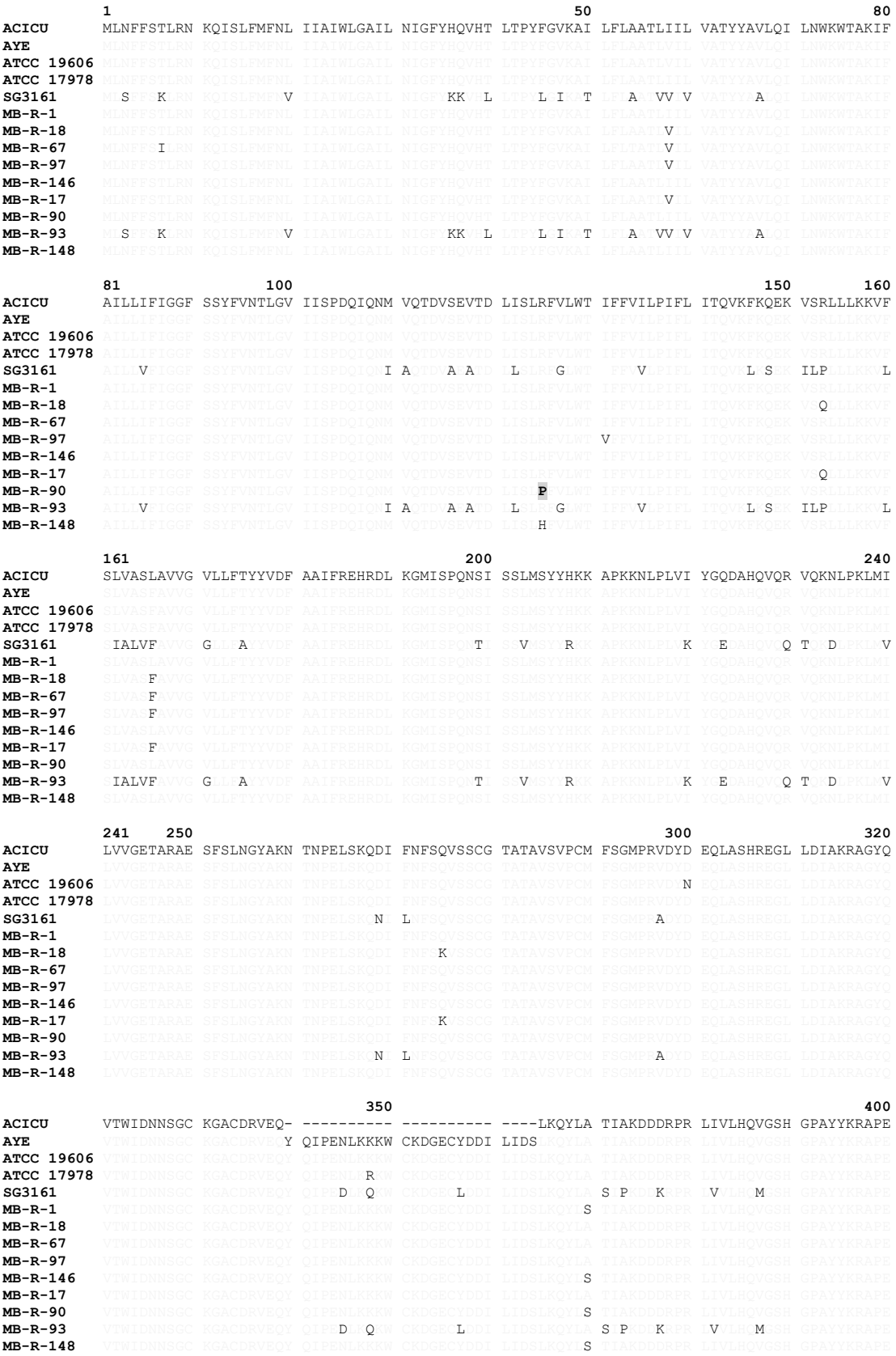
```
           241    250                                                  300                  320
ACICU      TKILLSQFPE HESLQNLSKG LARIQHLVTQ LLALAKQDVT LSMVEPTGYF QLNDVALNCV EQLVNLAMQK EIDLGFVRNE
AYE        TKILLSQFPE HESLQNLSKG LARIQHLVTQ LLALAKQDVT LSMVEPTGYF QLNDVALNCV EQLVNLAMQK EIDLGFVRNE
ATCC 19606 TKILLSQFPE HESLQNLSKG LARIQHLVTQ LLALAKQDVT LSMVEPTGYF QLNDVALNCV EQLVNLAMQK EIDLGFVRNE
ATCC 17978 TKILLSQFPE HESLQNLSKG LARIQHLVTQ LLALAKQDVT LSMVEPTGYF QLNDVALNCV EQLVNLAMQK EIDLGFVRNE
SG3161     TKILLSQFPE HESLQNLSKG LARIQHLVTQ LLALAKQDVT LSMVEPTGYF QLNDVALNCV EQLVNLAMQK EIDLGFVRND
MB-R-12    TKILLSQFPE HESLQNLSKG LARIQHLVTQ LLALAKQDVT LSMVEPTGYF QLNDVALNCV EQLVNLAMQK EIDLGFVRNE
MB-R-71    TKILLSQFPE HESLQNLSKG LARIQHLVTQ LLALAKQDVT LSMVEPTGYF QLNDVALNCV EQLVNLAMQK EIDLGFVRNE
MB-R-79    TKILLSQFPE HESLQNLSKG LARIQHLVTQ LLALAKQDVT LSMVEPTGYF QLNDVALNCV EQLVNLAMQK EIDLGFVRNE
MB-R-97    TKILLSQFPE HESLQNLSKG LARIQHLVTQ LLALAKQDVT LSMVEPTGYF QLNDVALNCV EQLVNLAMQK EIDLGFVRNE
MB-R-105   TKILLSQFPE HESLQNLSKG LARIQHLVTQ LLALAKQDVT LSMVEPTGYF QLNDVALNCV EQLVNLAMQK EIDLGFVRNE
MB-R-132   TKILLSQFPE HESLQNLSKG LARIQHLVTQ LLALAKQDVT LSMVEPTGYF QLNDVALNCV EQLVNLAMQK EIDLGFVRNE
MB-R-138   TKILLSQFPE HESLQNLSKG LARIQHLVTQ LLALAKQDVT LSMVEPTGYF QLNDVALNCV EQLVNLAMQK EIDLGFVRNE
MB-R-10    TKILLSQFPE HESLQNLSKG LARIQHLVTQ LLALAKQDVT LSMVEPTGYF QLNDVALNCV EQLVNLAMQK EIDLGFVRNE
MB-R-17    TKILLSQFPE HESLQNLSKG LARIQHLVTQ LLALAKQDVT LSMVEPTGYF QLNDVALNCV EQLVNLAMQK EIDLGFVRNE
MB-R-93    TKILLSQFPE HESLQNLSKG LARIQHLVTQ LLALAKQDVT LSMVEPTGYF QLNDVALNCV EQLVNLAMQK EIDLGFVRND
MB-R-116   TKILLSQFPE HESLQNLSKG LARIQHLVTQ LLALAKQDVT LSMVEPTGYF QLNDVALNCV EQLVNLAMQK EIDLGFVRNE
MB-R-119   TKILLSQFPE HESLQNLSKG LARIQHLVTQ LLALAKQDVT LSMVEPTGYF QLNDVALNCV EQLVNLAMQK EIDLGFVRNE
MB-R-141   TKILLSQFPE HESLQNLSKG LARIQHLVTQ LLALAKQDVT LSMVEPTGYF QLNDVALNCV EQLVNLAMQK EIDLGFVRNE

           321                              350                                                  400
ACICU      PIEMHSIEPT VHSIIFNLID NAIKYTPHQG VINISVYTDP DHYACIQIED SGAGIDPENY DKVLKRFYRV HHHLEVGSGL
AYE        PIEMHSIEPT VHSIIFNLID NAIKYTPHQG VINISVYTDQ DHYACIQIED SGAGIDPENY DKVLKRFYRV HHHLEVGSGL
ATCC 19606 PIEMHSIEPT VHSIIFNLID NAIKYTPHQG VINISVYTDP DHYACIQIED SGAGIDPENY DKVLKRFYRV HHHLEVGSGL
ATCC 17978 PIEMHSIEPT VHSIIFNLID NAIKYTPHQG VINISVYTDP DHYACIQIED SGAGIDPENY DKVLKRFYRV HHHLEVGSGL
SG3161      VEMHSIEPT VHSIIFNLID NAIKYTPHQG VINISVYTDP DNFACIQIED SGAGIDPENY DKVLKRFYRV HHHLEVGSGL
MB-R-12    PIEMHSIEPT VHSIIFNLID NAIKYTPHQG VINISVYTDP DHYACIQIED SGAGIDPENY DKVLKRFYRV HHHLEVGSGL
MB-R-71    PIEMHSIEPT VHSIIFNLID NAIKYTPHQG VINISVYTDP DHYACIQIED SGAGIDPENY DKVLKRFYRV HHHLEVGSGL
MB-R-79    PIEMHSIEPT VHSIIFNLID NAIKYTPHQG VINISVYTDP DHYACIQIED SGAGIDPENY DKVLKRFYRV DHHLEVGSGL
MB-R-97    PIEMHSIEPT VHSIIFNLID NAIKYTPHQG VINISVYTDQ DHYACIQIED SGAGIDPENY DKVLKRFYRV HHHLEVGSGL
MB-R-105   PIEMHSIEPT VHSIIFNLID NAIKYTPHQG VINISVYTDP DHYACIQIED SGAGIDPENY DKVLKRFYRV HHHLEVGSGL
MB-R-132   PIEMHSIEPT VHSIIFNLID NAIKYTPHQG VINISVYTDP DHYACIQIED SGAGIDPENY DKVLKRNYRV HHHLEVGSGL
MB-R-138   PIEMHSIEPT VHSIIFNLID NAIKYTPHQG VINISVYTDP DHYACIQIED SGAGIDPENY DKVLKRFYRV HHHLEVGSGL
MB-R-10    PIEMHSIEPT VHSIIFNLID NAIKYTPHQG VINISVYTDP DHYACIQIED SGAGIDPENY DKVLKRFYRV HHHLEVGSGL
MB-R-17    PIEMHSIEPT VHSIIFNLID NAIKYTPHQG VINISVYTDP DHYACIQIED SGAGIDPENY DKVLKRFYRV HHHLEVGSGL
MB-R-93     VEMHSIEPT VHSIIFNLID NAIKYTPHQG VINISVYTDP DNFACIQIED SGAGIDPENY DKVLKRFYRV HHHLEVGSGL
MB-R-116   PIEMHSIEPT VHSIIFNLID NAIKYTPHQG VINISVYTDP DHYACIQIED SGAGIDPENY DKVLKRFYRV HHHLEVGSGL
MB-R-119   PIEMHSIEPT VHSIIFNLID NAIKYTPHQG VINISVYTDP DHYACIQIED SGAGIDPENY DKVLKRFYRV HHHLEVGSGL
MB-R-141   PIEMHSIEPT VHSIIFNLID NAIKYTPHQG VINISVYTDP DHYACIQIED SGAGIDPENY DKVLKRFYRV HHHLEVGSGL

           401                                  444
ACICU      GLSIVDRATQ RLGGTLTLDK SLELGGLSVL VKLPKVLHLH ETRV
AYE        GLSIVDRATQ RLGGTLTLDK SLELGGLSVL VKLPKVLHLH ETRA
ATCC 19606 GLSIVDRATQ RLGGTLTLDK SLELGGLSVL VKLPKVLHLN ETRA
ATCC 17978 GLSIVDRATQ RLGGTLTLDK SLELGGLSVL VKLPKVLHLH ETRA
SG3161     GLSIVDRATQ RLGGTLTLDK SLELGGLSVL VKLPKVLHLH ETRA
MB-R-12    GLSIVDRATQ RLGGTLTLDK SLELGGLSVL VKLPKVLHLH ETRV
MB-R-71    GLSIVDRATQ RLGGTLTLDK SLELGGLSVL VKLPKVLHLH ETRV
MB-R-79    GLSIVDRATQ RLGGTLTLDK SLELGGLSVL VKLPKVLHLH ETRV
MB-R-97    GLSIVDRATQ RLGGTLTLDK SLELGGLSVL VKLPKVLHLH ETRA
MB-R-105   GLSIVDRATQ RLGGTLTLDK SLELGGLSVL VKLPKVLHLH ETRV
MB-R-132   GLSIVDRATQ RLGGTLTLDK SLELGGLSVL VKLPKVLHLH ETRV
MB-R-138   GLSIVDRATQ RLGGTLTLDK SLELGGLSVL VKLPKVLHLH ETRV
MB-R-10    GLSIVDRATQ RLGGTLTLDK SLELGGLSVL VKLPKVLHLH ETRV
MB-R-17    GLSIVDRATQ RLGGTLTLDK SLELGGLSVL VKLPKVLHLH ETRA
MB-R-93    GLSIVDRATQ RLGGTLTLDK SLELGGLSVL VKLPKVLHLH ETRA
MB-R-116   GLSIVDRATQ RLGGTLTLDK SLELGGLSVL VKLPKVLHLH ETRV
MB-R-119   GLSIVDRATQ RLGGTLTLDK SLELGGLSVL VKLPKVLHLH ETRV
MB-R-141   GLSIVDRATQ RLGGTLTLDK SLELGGLSVL VKLPKVLHLH ETRV
```

Appendix 17: Amino acid sequence alignment of PmrC.

A. baumannii reference and comparison strains: ACICU, AYE, ATCC 1906, ATCC 17978
Colistin-susceptible *A. baumannii* isolates: SG3161 (IC4), MB-R-1, MB-R-18, MB-R-67, MB-R-97, MB-R-146
Colistin-resistant *A. baumannii* isolates: MB-R-17, MB-R-90, MB-R-93, MB-R-148



Appendix 17: continued

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           401                                                 450                                                 480
ACICU      AYQPFKPTCD TNAIQGCSQT ELLNSYDNTI VYTDHVLSQM INTLKEISKY QTGLWYLSDH GESTGEHGLY LHGSPYAIAP
AYE        AYQPFKPTCD TNAIQGCSQT ELLNSYDNTI VYTDHVLSQM INTLKEISKY QTGLWYLSDH GESTGEHGLY LHGSPYAIAP
ATCC 19606 AYQPFKPTCD TNAIQGCSQT ELLNSYDNTI VYTDHVLSQM INTLKEISKY QTGLWYLSDH GESTGEHGLY LHGSPYAIAP
ATCC 17978 AYQPFKPTCD TNAIQGCSQT ELLNSYDNTI VYTDHVLSQM INTLKEISKY QTGLWYLSDH GESTGEHGLY LHGSPYAIAP
SG3161     G QPFKP CD TNAIQGC PA I SYDNTI VYTDHVLSQM INTLKE V N QTG F WYLSDH GESTGEH M LHGSP S AP
MB-R-1     AYQPFKPTCD TNAIQGCSQT ELLNSYDNTI VYTDHVLSQM INTLKEISKY QTGLWYLSDH GESTGEHGLY LHGSPYAIAP
MB-R-18    AYQPFKPTCD TNAIQGCSQT ELLNSYDNTI VYTD I SQM INTLKEISKY QTGLWYLSDH GESTGEHGLY LHGSPYAIAP
MB-R-67    AYQPFKPTCD TNAIQGCSQT ELLNSYDNTI VYTDHVLSQM INTLKEISKY QTGLWYLSDH GESTGEHGLY LHGSPYAIAP
MB-R-97    AYQPFKPTCD TNAIQGCSQT ELLNSYDNTI VYTDHVLSQM INTLKEISKY QTGLWYLSDH GESTGEHGLY LHGSPYAIAP
MB-R-146   AYQPFKPTCD TNAIQGCSQT ELLNSYDNTI VYTDHVLSQM INTLKEISKY QTGLWYLSDH GESTGEHGLY LHGSPYAIAP
MB-R-17    AYQPFKPTCD TNAIQGCSQT ELLNSYDNTI VYTD I SQM INTLKEISKY QTGLWYLSDH GESTGEHGLY LHGSPYAIAP
MB-R-90    AYQPFKPTCD TNAIQGCSQT ELLNSYDNTI VYTDHVLSQM INTLKEISKY QTGLWYLSDH GESTGEHGLY LHGSPYAIAP
MB-R-93    G QPFKPTCD TNAIQGC PA I SYDNTI VYTDHVLSQM INTLKE V N QTG F WYLSDH GESTGEH M LHGSP S AP
MB-R-148   AYQPFKPTCD TNAIQGCSQT ELLNSYDNTI VYTDHVLSQM INTLKEISKY QTGLWYLSDH GESTGEHGLY LHGSPYAIAP

           481              500                                                 549
ACICU      SQQTHVPMIM WFSESWKQHN LAQVNCLSQQ TKQKLSQDNL FPSLLSLLDV TTQVINPQLD MLHSCAHVN
AYE        SQQTHVPMIM WFSESWK R LAQVNCLSQQ TKQKLSQDNL FPSLLSLLDV K QVINPQLD MLHSCAHVN
ATCC 19606 SQQTHVPMIM WFSESWKQHN LAQVNCLSQQ TKQKLSQDNL FPSLLSLLDV K QVINPQLD MLHSCAHVN
ATCC 17978 SQQTHVPMIM WFSESWKQHN LAQVNCLSQQ TKQKLSQDNL FPSLLSLLDV K QVINPQLD MLHSCAHVN
SG3161     SQQTH I PMIM WFS DG K N LAQVNC N Q TKQKLSQDNL FPSLLS M LDV K QVINPQLD MLHSCA N VN
MB-R-1     SQQTHVPMIM WFSESWKQHN LAQVNCLSQQ TKQKLSQDNL FPSLLSLLDV TTQVINPQLD MLHSCAHVN
MB-R-18    SQQTHVPMIM WFSESWKQHN LAQVNCLSQQ TKQ N LSQDNL FPSLLSLLDV K QVINPQLD MLHSCAHVN
MB-R-67    SQQTHVPMIM WFSESWKQHN LAQVNCLSQQ TKQKLSQDNL FPSLLSLLDV K QVINPQLD MLHSCAHVN
MB-R-97    SQQTHVPMIM WFSESWK R LAQVNCLSQQ TKQKLSQDNL FPSLLSLLDV K QVINPQLD MLHSCAHVN
MB-R-146   SQQTHVPMIM WFSESWKQHN LAQVNCLSQQ TKQKLSQDNL FPSLLSLLDV TTQVINPQLD MLHSCAHVN
MB-R-17    SQQTHVPMIM WFSESWKQHN LAQVNCLSQQ TKQ N LSQDNL FPSLLSLLDV K QVINPQLD MLHSCAHVN
MB-R-90    SQQTHVPMIM WFSESWKQHN LAQVNCLSQQ TKQKLSQDNL FPSLLSLLDV TTQVINPQLD MLHSCAHVN
MB-R-93    SQQTH I PMIM WFS DG K N LAQVNC N Q TKQKLSQDNL FPSLLS M LDV K QVINPQLD MLHSCA N VN
MB-R-148   SQQTHVPMIM WFSESWKQHN LAQVNCLSQQ TKQKLSQDNL FPSLLSLLDV TTQVINPQLD MLHSCAHVN
```

Appendix 18: Amino acid sequence alignment of EptA.

A. baumannii reference and comparison strain: ACICU, AYE (PmrC)
Colistin-susceptible *A. baumannii* isolates: SG3161 (IC4), MB-R-1, MB-R-12, MB-R-81
Colistin-resistant *A. baumannii* isolate: MB-R-53

```
           1                                                       50                                     90
ACICU      MALNVFKFKK ICKDVTLLNF NLLLSIWLGL FLNIGFLKKI HQLTPYNGIK SVLFLGATLV ILIAAYNLIF QLINWKWTAK IFAILLIFIG
PmrC(AYE)   MLN FSTL  RN QIS FM     IIA   A I     YHQV  T    F V AI   A   I    V T YAVL QILNWKWTAK IFAILLIFIG
MB-R-1     MALNVFKFKK ICKDVTLLNF NLLLSIWLGL FLNIGFFKKI HQLTPYNGIK SVLFLGATLV ILIAAYNLIF QLINWKWTAK IFAILLIFIG
MB-R-12    MALNVFKFKK ICKDVTLLNF NLLLSIWLGL FLNIGFFKKI HQLTPYNGIK SVLFLGATLV ILIAAYNLIF QLINWKWTAK IFAILLIFIG
MB-R-81    MALNVFKFKK ICKDVTLLNF NLLLSIWLGL FLNIGFFKKI HQLTPYNGIK SVLFLGATLV ILIAAYNLIF QLINWKWTAK IFAILLIFIG
MB-R-53    MALNVFKFKK ICKDVTLLNF NLLLSIWLGL FLNIGFFKKI HQLTPYNGIK SVLFLGATLV ILIAAYNLIF QLINWKWTAK IFAILLIFIG

           91      100                                                    150                              180
ACICU      GFSSYFVNTL GVIISPDQIQ NMVQTDVSEF TDLISLRFVL WTVFFVILPI FLITQVKFKQ EKASRLLLKK VFSLVASFAV VGVLLFTYYV
PmrC(AYE)  GFSSYFVNTL GVIISPDQIQ NMVQTDVSEV TDLISLRFVL WTIFFVILPI FLITQVKFKQ EKVSRLLLKK VFSLVASLAV VGVLLFTYYV
MB-R-1     GFSSYFVNTL GVIISPDQIQ NMVQTDVSEF TDLISLRFVL WTVFFVILPI FLITQVKFKQ EKASRLLLKK VFSLVASFAV VGVLLFTYYV
MB-R-12    GFSSYFVNTL GVIISPDQIQ NMVQTDVSEF TDLISLRFVL WTVFFVILPI FLITQVKFKQ EKASRLLLKK VFSLVASFAV VGVLLFTYYV
MB-R-81    GFSSYFVNTL GVIISPDQIQ NMVQTDVSEV TDLISLRFVL WTVFFVILPI FLITQVKFKQ EKASRLLLKK VFSLVASFAV VGVLLFTYYV
MB-R-53    GFSSYFVNTL GVIISPDQIQ NMVQTDVSEF TDLISLLFVL WTVFFVILPI FLITQVKFKQ EKASRLLLKK VFSLVASFAV VGVLLFTYYV

           181              200                                                   250                  270
ACICU      DFAAIFREHR DLKGMISPQN SISSLMSYYH KKAPKKNLPL VIYGQDAHQV QQVQKNLPKL MILVVGETAR AESFSLNGYA KNTNPELSKQ
PmrC(AYE)  DFAAIFREHR DLKGMISPQN SISSLMSYYH KKAPKKNLPL VIYGQDAHQV QRVQKNLPKL MILVVGETAR AESFSLNGYA KNTNPELSKQ
MB-R-1     DFAAIFREHR DLKGMISPQN SISSLMSYYH KKAPKKNLPL VIYGQDAHQV QQVQKNLPKL MILVVGETAR AESFSLNGYA KNTNPELSKQ
MB-R-12    DFAAIFREHR DLKGMISPQN SISSLMSYYH KKAPKKNLPL VIYGQDAHQV QQVQKNLPKL MILVVGETAR AESFSLNGYA KNTNPELSKQ
MB-R-81    DFAAIFREHR DLKGMISPQN SISSLMSYYH KKAPKKNLPL VIYGQDAHQV QQVQKNLPKL MILVVGETAR AESFSLNGYA KNTNPELSKQ
MB-R-53    DFAAIFREHR DLKGMISPQN SISSLMSYYH KKAPKKNLPL VIYGQDAHQV QQVQKNLPKL MILVVGETAR AESFSLNGYA KNTNPELSKQ

           271                        300                                                350          360
ACICU      DIFNFSQVSS CGTATAVSVP CMFSGMPRVD YDEQLASHRE GLLDIAKRAG YQVTWIDNNS GCKGACDRVE QYQIPENLKK KWCKDGECYD
PmrC(AYE)  DIFNFSQVSS CGTATAVSVP CMFSGMPRVD YDEQLASHRE GLLDIAKRAG YQVTWIDNNS GCKGACDRVE QYQIPENLKK KWCKDGECYD
MB-R-1     DIFNFSQVSS CGTATAVSVP CMFSGMPRVD YDEQLASHRE GLLDIAKRAG YQVTWIDNNS GCKGACDRVE QYQIPENLKK KWCKDGECYD
MB-R-12    DIFNFSQVSS CGTATAVSVP CMFSGMPRVD YDEQLASHRE GLLDIAKRAG YQVTWIDNNS GCKGACDRVE QYQIPENLKK KWCKDGECYD
MB-R-81    DIFNFSQVSS CGTATAVSVP CMFSGMPRVD YDEQLASHRE GLLDIAKRAG YQVTWIDNNS GCKGACDRVE QYQIPENLKK KWCKDGECYD
MB-R-53    DIFNFSQVSS CGTATAVSVP CMFSGMPRVD YDEQLASHRE GLLDIAKRAG YQVTWIDNNS GCKGACDRVE QYQIPENLKK KWCKDGECYD

           361                                   400                                                  450
ACICU      DILIDSLKQY LATIAKDDDR PRLIVLHQVG SHGPAYYKRA PEAYQPFKPT CDTNAIQGCS QTELLNSYDN TIVYTDHVLS QMINTLKEIS
PmrC(AYE)  DILIDSLKQY LATIAKDDDR PRLIVLHQVG SHGPAYYKRA PEAYQPFKPT CDTNAIQGCS QTELLNSYDN TIVYTDHVLS QMINTLKEIS
MB-R-1     DILIDSLKQY LATIAKDDDR PRLIVLHQVG SHGPAYYKRA PEAYQPFKPT CDTNAIQGCS QTELLNSYDN TIVYTDHVLS QMINTLKEIS
MB-R-12    DILIDSLKQY LSTIAKDDDR PRLIVLHQVG SHGPAYYKRA PEAYQPFKPT CDTNAIQGCS QTELLNSYDN TIVYTDHVLS QMINTLKEIS
MB-R-81    DILIDSLKQY LATIAKDDDR PRLIVLHQVG SHGPAYYKRA PEAYQPFKPT CDTNAIQGCS QTELLNSYDN TIVYTDHVLS QMINTLKEIS
MB-R-53    DILIDSLKQY LATIAKDDDR PRLIVLHQVG SHGPAYYKRA PEAYQPFKPT CDTNAIQGCS QTELLNSYDN TIVYTDHVLS QMINTLKEIS

           451                                                 500                                    540
ACICU      KYQTGLWYLS DHGESTGEHG LYLHGSPYAI APSQQTHVPM IMWFSESWKQ HNLAQVNCLS QQTKQKLSQD NLFPSLLSLL DVKTQVVNNK
PmrC(AYE)  KYQTGLWYLS DHGESTGEHG LYLHGSPYAI APSQQTHVPM IMWFSESWKQ HNLAQVNCLS QQTKQKLSQD NLFPSLLSLL DVTTQVINPQ
MB-R-1     KYQTGLWYLS DHGESTGEHG LYLHGSPYAI APSQQTHVPM IMWFSESWKQ HNLAQVNCLS QQTKQKLSQD NLFPSLLSLL DVKTQVVNNK
MB-R-12    KYQTGLWYLS DHGESTGEHG LYLHGSPYAI APSQQTHVPM IMWFSESWKQ HNLAQVNCLS QQTKQKLSQD NLFPSLLSLL DVKTQVVNNK
MB-R-81    KYQTGLWYLS DHGESTGEHG LYLHGSPYAI APSQQTHVPM IMWFSESWKQ HNLAQVNCLS QQTKQKLSQD NLFPSLLSLL DVKTQVVNNK
MB-R-53    KYQTGLWYLS DHGESTGEHG LYLHGSPYAI APSQQTHVPM IMWFSESWKQ HNLAQVNCLS QQTKQKLSQD NLFPSLLSLL DVKTQVVNNK

           541    551
ACICU      LDMLSQCKX
PmrC(AYE)  LDMLHSCAHVN
MB-R-1     LDMLSQCKX
MB-R-12    LDMLSQCKX
MB-R-81    LDMLSQCKX
MB-R-53    LDMLSQCKX
```

8. List of publications

Publications

- Gerson S, Nowak J, Zander E, Ertel J, Wen Y, Krut O, Siefert H, Higgins PG. Diversity of mutations in regulatory genes of resistance-nodulation-cell division efflux pumps in association with tigecycline resistance in *Acinetobacter baumannii*. J Antimicrob Chemother. 2018;73:1501–8. doi:10.1093/jac/dky083.
- Gerson S, Betts JW, Lucaßen K, Nodari CS, Wille J, Josten M, Göttig S, Nowak J, Stefanik D, Roca I, Vila J, Cisneros JM, La Ragione R, Seifert H, Higgins PG. Investigation of Novel *pmrB* and *eptA* Mutations in Isogenic *Acinetobacter baumannii* Isolates Associated with Colistin Resistance and Increased Virulence In Vivo. Antimicrob Agents Chemother. 2019; 63:e01586-18. doi:10.1128/AAC.01586-18.
- Gerson S, Lucaßen K, Wille J, Nodari CS, Stefanik D, Nowak J, Wille T, Betts JW, Roca I, Vila J, Cisneros JM, Seifert H, Higgins PG. Diversity of amino acid substitutions in PmrCAB associated with colistin resistance in clinical *Acinetobacter baumannii* isolates. Int J Antimicrob Agents. 2020;55(3):105862. doi:10.1016/j.ijantimicag.2019.105862.
- Cerezales M, Biniossek L, Gerson S, Xanthopoulou K, Wille J, Wohlfarth E, Kaase M, Seifert H, Higgins PG. Novel multiplex PCRs for detection of the most prevalent carbapenemase genes in Gram-negative bacteria within Germany. J Med Microbiol. 2021;70(3). doi: 10.1099/jmm.0.001310
- Lucaßen K, Gerson S, Xanthopoulou K, Wille J, Wille T, Seifert H, Higgins PG. Comparison of the *A. baumannii* reference strains ATCC 17978 and ATCC 19606 in antimicrobial resistance mediated by the AdeABC efflux pump. 2021;65(8):e0057021. doi: 10.1128/AAC.00570-21

Oral Presentations

- Gerson S, Seifert H, Higgins PG. Investigation of colistin resistance in *Acinetobacter baumannii* from Spain and Greece isolated as part of the MagicBullet clinical trial. 2015. 67th Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie e.V. Münster, Germany.
- Gerson S, Freier L, Zander E, Nowak J, Seifert H, Higgins PG. Insertion sequences in the RND-type efflux pump regulatory genes *adeS* and *adeN* are associated with tigecycline resistance in *Acinetobacter baumannii*. 2016. 68th Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie e.V. Ulm, Germany.
- Gerson S, Nowak J, Wohlfarth E, Krut O, Seifert H, Higgins PG. Diversity of mutations in regulatory genes of resistance-nodulation-cell division efflux pumps in association with tigecycline resistance in *Acinetobacter baumannii*. 2017. 27th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID). Vienna, Austria.
- Gerson S, Ertel J, Stefanik D, Seifert H, Higgins PG. Novel *pmrAB* mutations detected in colistin-resistant *Acinetobacter baumannii* isolates. 2018. 28th European Congress of Clinical Microbiology and Infectious Diseases. Madrid, Spain.

(e)Poster

- Gerson S, Vinals MA, Seifert H, Higgins PG. Investigation of tigecycline and colistin resistance in *Acinetobacter baumannii* from Spain and Greece isolates as part of the MagicBullet clinical trial. 2015. 10th International Symposium on the Biology of Acinetobacter. Athens, Greece.
- Gerson S, Ertel J, Göttig S, Josten M, Seifert H, Higgins PG. Investigation of *pmrB* mutations and potential novel colistin resistance mechanisms in clinical *Acinetobacter baumannii* isolates. 2017. 11th International Symposium on the Biology of Acinetobacter. Seville, Spain.
- Gerson S, Nowak J, Zander E, Krut O, Seifert H, Higgins PG. Insertion sequences IS*Aba1* and IS*Aba125* are associated with tigecycline resistance in *Acinetobacter baumannii*. 2016. 26th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID). Amsterdam, Netherlands.