Acinetobacter baumannii RND efflux pump regulators and their impact on antimicrobial susceptibility

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Kai Lucaßen

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Berichterstatter: Prof. Dr. Harald Seifert

Prof. Dr. Karin Schnetz

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1 Introduction

The term ESKAPE organisms (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) was introduced in 2008 to summarize a group of bacterial organisms, which developed an exceptional significance, since their increasing prevalence and their ability to escape the action of antimicrobials became one of the most concerning challenges in healthcare.[1] Growing antimicrobial resistance and virulence enabled these organisms to cope with harmful external influences and favored their persistence throughout the hospital environment.[2, 3] Inappropriate use and overconsumption of antimicrobials in livestock, agriculture, and health-care facilitated the emergence and spread of resistant isolates resulting in the evolution of multi-drug resistant (MDR, non-susceptibility to one or more agents of at least three antimicrobial classes), extensive drug resistant (XDR, non-susceptibility to at least one agent in all but two or fewer antimicrobial classes) and even pan-drug resistant (PDR, non-susceptibility to all currently available agents in all antimicrobial categories) bacteria.[4-6] Finally, the loss of treatment options prompted the World Health Organization (WHO) in 2017 to call for novel antimicrobial agents against priority pathogens including the ESKAPE group. Within this scope, carbapenem-resistant *A. baumannii* was assigned priority level 1: critical.[7]

1.1 The genus *Acinetobacter*

The discovery of the genus *Acinetobacter* goes back to 1911 when Beijerinck cultivated an organism isolated from soil, which was initially named *Micrococcus calcoaceticus*.[8] In 1954, the term *Acinetobacter* was introduced to separate the non-motile organisms from the genus *Achromobacter*.[9] Advanced characterization eventually grouped several organisms under the genus *Acinetobacter*.[10] *Acinetobacter* spp. are Gram-negative, strictly aerobic, non-fermentative coccobacilli.[11] Because of their multiple habitats, they are considered as ubiquitous organisms, which have been isolated from water and soil, from food products, from animals and as a part of the physiological skin flora.[12-14] Remarkably, the carriage rate is particularly high among hospitalized patients, at up to 75%.[14] Currently the genus *Acinetobacter* comprises 72 validly named species.[15] Of these *Acinetobacter* spp., *Acinetobacter baumannii*, *Acinetobacter calcoaceticus*, *Acinetobacter dijkshoorniae*, *Acinetobacter nosocomialis*, *Acinetobacter pittii*, and *Acinetobacter seifertii* have a DNA-sequence homology of about 70% but cannot be phenotypically distinguished from each other in diagnostic settings using standard laboratory

based tests, therefore the species were combined in the *A. calcoaceticus-A. baumannii*-complex (ACBcomplex).[16]

1.2 Epidemiology of *A. baumannii*

Although natural habitats like avian populations and soil, as well as presence in domestic animals like cattle and pets have been discovered, *A. baumannii* is mainly associated with the healthcare setting.[17] Moreover, intensive care units (ICU) are known reservoirs for MDR isolates.[11] In particular, carbapenem-resistant *A. baumannii* is assigned a special role, as treatment options are severely limited. Following the rankings of the WHO from 2017, the U.S. Centers for Disease Control and Prevention has listed carbapenem-resistant *A. baumannii* as one of the urgent threats among antimicrobial resistant organisms in their most recent antibiotic threats report from 2019.[7, 18] According to the European Centre for Disease Prevention and Control, carbapenem-resistant *A. baumannii* causes sporadic hospital outbreaks in Germany, whereas it is already endemic in southern European countries like Portugal, Italy, and Greece (Figure 1).[19]

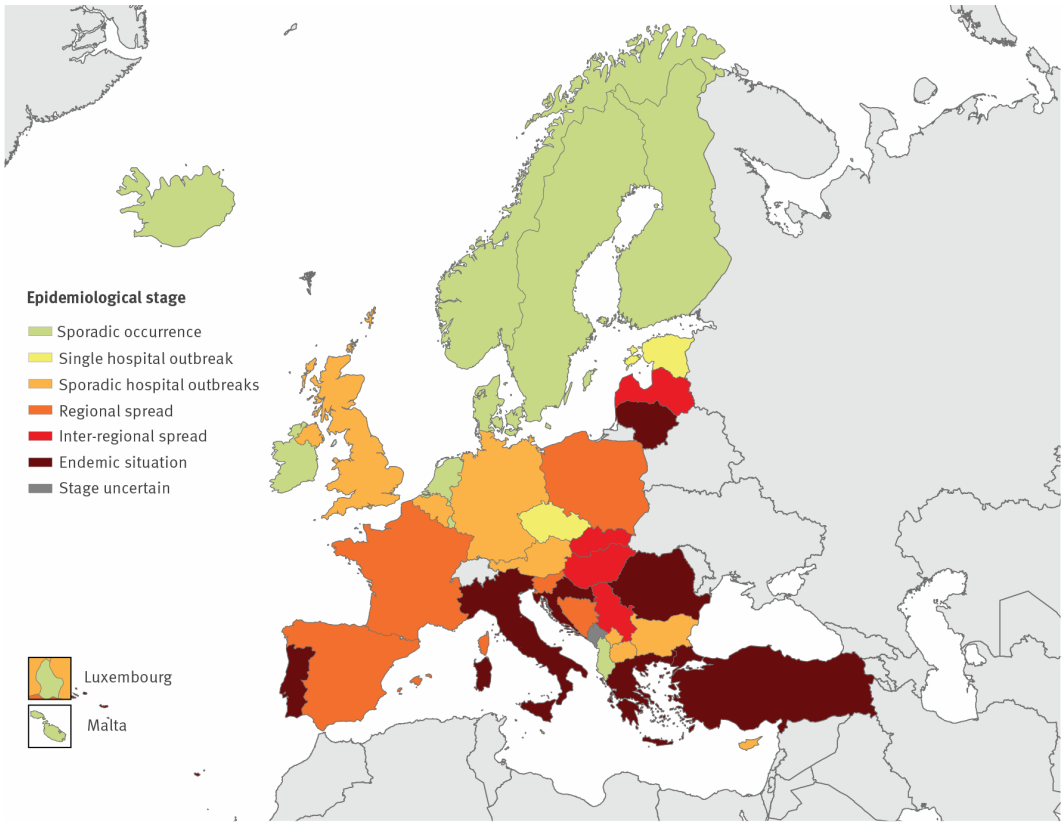

Figure 1. Epidemiological stage of carbapenem resistant *A. baumannii*.[19]

1.3 Pathogenicity and virulence in *A. baumannii*

Since *A. baumannii* is mainly recovered from specimens obtained from patients in intensive care units, it is associated with various nosocomial infections, especially among patients with severe comorbidities. The most common clinical manifestations associated with *A. baumannii* are ventilator-associated-pneumonia, bloodstream infections following invasive procedures, as well as wound and urinary tract infections, meningitis, and soft-tissue infections.[11] However, since colonization with *A. baumannii* is more likely than infection, it is generally considered as an organism of low pathogenicity. Nevertheless, the course of infection can be serious. *A. baumannii* possesses several properties that confer pathogenicity, including lipopolysaccharides (LPS) acting as ligands for Toll-like receptor 4,[20] capsular polysaccharides that protect bacterial cells from complement-mediated killing,[21] phospholipases C and D, which facilitate epithelial cell invasion and evasion of the host immune response,[22] outer membrane vesicles secreting outer membrane protein A,[23, 24] which induces adherence and invasion of epithelial cells as well as apoptosis in host cells,[25] and siderophore systems such as acinetobactin that scavenges iron.[26]

Adherence to abiotic surfaces like glass, polycarbonate, polystyrene, and stainless steel favors the emerge of biofilms even on medical equipment, as *A. baumannii* biofilms were detected on ventilation tubes, catheters, and consequently on artificial heart valves and orthopedic devices, as well within skin and soft tissue, wounds and occlusive dressings.[27-30] Biofilms are complex surface-associated cell communities, induced by primary binding of single cells which are influenced by the negative charge of the bacteria as well as the hydrophobicity of components of the cell surface.[31] This is followed by proliferation and accumulation. The cells encase themselves in an extracellular polysaccharide substance separated by fluid-filled channels, which results in the final formation of biofilms. An established biofilm has a complex three-dimensional architecture enclosed in a protecting matrix creating an optimal environment for exchange of genetic material (e.g. resistance plasmids) between cells.[32] An important step for propagation and persistence of the bacterial cell community is the ability of some cells to detach from biofilm regions and disperse, thereby forming biofilms in new environmental niches. Moreover, mechanisms of antibiotic resistance in a biofilm differ from the mechanisms observed in individual cells. Bacteria are less susceptible to antimicrobial agents when grown in a biofilm, because of poor agent penetration, as well as to other harmful conditions such as ultra-violet (UV) light, acid exposure, dehydration, and phagocytosis in comparison to planktonic growing cells.[33, 34] Analyses of mRNA expression have shown that the gene expression

pattern of biofilm forming *A. baumannii* cells is distinct from that of other modes of growth, including gene overexpression as well as genes expressed only in biofilms.[35]

However, on wet surfaces many *A. baumannii* isolates reveal a motile phenotype, which is associated with virulence.[36] *A. baumannii* motility is flagella-independent and driven by retraction of type IV pili.[37] These appendages emanating from the surface of the outer membrane generate motor forces by assembling and disassembling.[38] Furthermore, type IV pili are participating in processes such as adherence to surfaces and biofilm formation.[39] The dynamic nature of these filaments to elongate by polymerization and retract by depolymerization induce changes in the outer membrane and contributes to uptake of naked DNA from the extracellular environment, followed by transport across the cell envelope into the cytoplasm resulting in genetic transformation.[40] This mechanism, described as natural competence, facilitates the acquisition of novel resistance determinants by horizontal gene transfer.

Moreover, its high propensity to environmental persistence has enabled *A. baumannii* to thrive in the hospital environment facilitating its dissemination.[20, 41] Adaptation to stresses like desiccation was observed in clinical *A. baumannii* isolates, which still were viable after a period of about 100 days under dry conditions.[42, 43] The composition of the outer membrane and the capsule, especially the presence of capsular polysaccharides enables *A. baumannii* to retain water and prevent desiccation.[44, 45]

1.4 Antimicrobial resistance mechanisms in *A. baumannii*

The fundamental mechanism of action of antimicrobial agents is to bind to its bacterial target site and to interfere with cellular processes like cell wall synthesis, protein biosynthesis, or nucleic acid replication (Figure 2). In this way, the various classes of antimicrobials available cause a slowdown of bacterial cell growth (bacteriostatic action) or lead to bacterial cell death (bactericidal action).

A. baumannii has revealed a diverse set of intrinsic and acquired resistance mechanisms, which are based on enzymatic inactivation of the drug, alterations in the structure of antimicrobial target sites preventing binding of antimicrobial agents, and properties or devices that deny access of an adequate amount of the antimicrobial agent to its target site.[46]

Many resistance mechanisms act very specifically and accordingly confer resistance only to a single antimicrobial, or a single class of antimicrobial.[47] This applies, for example, in the case of enzymatic inactivation. Prominent representatives of this mechanism are β-lactamases,

which inactivate β-lactam agents by hydrolysis of the amide bond of the β-lactam ring. Thereby β-lactamases prevent the inhibition of peptidoglycan crosslinking, which would otherwise cause impaired cell wall synthesis resulting in cell lysis. According to their mode of action, β-lactamases are divided into serine- and metallo-β-lactamases, which either need a serine residue or one or two zinc ions in their active centre, respectively.[48] Every class of β-lactamases can be present in *A. baumannii*, either chromosomally encoded and/or encoded on plasmids.[49] As the β-lactam subclass of carbapenems is of particular importance for the treatment of *A. baumannii* infections, the emergence of carbapenem hydrolyzing β-lactamases (carbapenemases) is concerning.[50]

Furthermore, antimicrobial resistance can be caused by enzymatic modification of antimicrobials. This mechanism is based on the transfer of chemical groups like acyl-, phosphate-, nucleotidyl-, and ribitoyl groups.[51] Because they form large molecules with many hydroxyl and amide groups, aminoglycosides are particularly affected by these modifying enzymes. The antimicrobial action of aminoglycosides is caused by inhibition of protein synthesis induced by binding of aminoglycoside molecules to the 16S ribosomal RNA of the ribosomal 30S subunit. Drug modification inhibits binding to the ribosome and therefore decreases antimicrobial activity of the drug. Three main classes of aminoglycoside-modifying enzymes have been described: acetyltransferases, phosphotransferases and nucleotidyltransferases. These enzymes are highly diverse and rather specific to few agents.[52]

An additional mechanism of resistance is the alteration of antimicrobial targets. This mechanism is associated with resistance to compounds like quinolones and the subsequent fluoroquinolones. Quinolones inhibit DNA synthesis by binding to bacterial topoisomerases. Acquired quinolone resistance genes encode pentapeptide repeat proteins that bind to topoisomerases and thus protect them against binding of quinolones.[53] However, in *A. baumannii* it is amino acid substitutions within the topoisomerase genes *gyrA*, *gyrB*, *parC*, and *parE* that are responsible for fluoroquinolone resistance.[54]

Polymyxins like colistin are cyclic antimicrobial peptides used to treat infections caused by Gram-negative bacteria. Polymyxins bind to LPS and cause disruption of both cell membranes because of their hydrophobic tail.[55] Modifications of LPS affect the binding of colistin. In particular, the addition of phosphoethanolamine to lipid A caused by overexpression of *pmrC*, which is induced by mutations within the two-component regulatory-system PmrAB, is associated with colistin resistance.[56, 57]

As these mechanisms require specific adjustments of antimicrobial agents or targets, they are accordingly specific to single antimicrobials or antimicrobial classes. Contrary to this, mechanisms reducing the concentration of antimicrobials on their site of action affect a broad range of drugs. This can be established either by increased efflux activity or by reduced permeability. Due to rather small porins, which generally occur in relatively small numbers, the permeability of the *A. baumannii* outer membrane is strongly reduced compared to other Gram-negative organisms.[58] Accordingly, reduced expression of outer membrane porins (OMPs) like CarO or OprD has been shown to be associated with antimicrobial resistance in *A. baumannii*.[59, 60]

Efflux plays a major role in reduced susceptibility to tetracyclines and glycylcyclines. These antibiotics inhibit protein synthesis by binding to the 16S rRNA and inhibiting the binding of aminoacyl-tRNA to the mRNA-ribosome complex.[61] Tigecycline was developed to overcome the main mechanisms of tetracycline resistance, such as acquisition of specific efflux pumps like TetA.[62] However, *A. baumannii* tolerance to tigecycline is associated with overexpression of efflux pumps.[63, 64] Additionally, modifications of tigecycline induced by the mono-oxygenase TetX has been reported in various Gram-negative bacteria as a tigecycline resistance mechanism.[65]

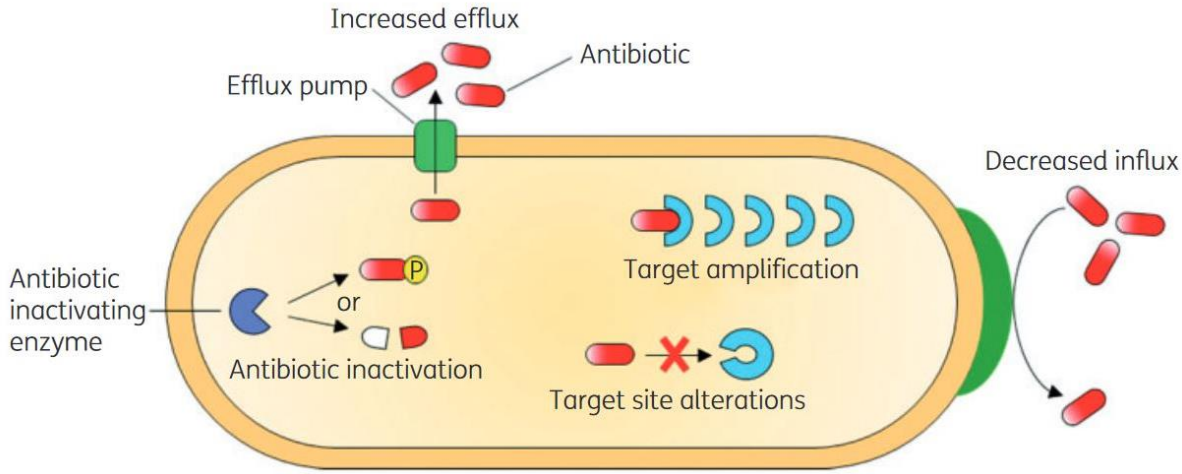

Figure 2. Antimicrobial resistance mechanisms. Bacterial strategies to reduce the harmful impact of antimicrobials: enzymatic antibiotic inactivation, modification of the antimicrobial target, target amplification, decreased antimicrobial influx, increased antimicrobial efflux. Reprinted with permission.[66]

1.5 Acquisition of antimicrobial resistance

The mechanisms of antimicrobial resistance are basically the result of the acquisition of antimicrobial resistance genes or mutations in existing genetic information.[67] The transmission of

nucleic acids is termed as horizontal gene transfer. This mechanism promotes the intra- and interspecies spread of antimicrobial resistance genes and is therefore of great importance for the emergence of MDR bacteria. Horizontal gene transfer takes place through conjugation, transduction, or transformation. Conjugation requires direct cell-to-cell contact by cell surface pili or adhesins and occurs unidirectionally. Accordingly, DNA, predominantly in the form of plasmids, is transferred from a donor to a recipient.[68] Transduction is a bacteriophage-dependent mechanism of transfer, whereas transformation describes the uptake of exogenous DNA, a process that has been observed in *A. baumannii* during motility (1.3).[40, 69]

Depending on their location, insertion sequences (IS) like IS*Aba1* can act as resistance determinants and are therefore also involved in the spread of antimicrobial resistance. IS are DNA sections, which contain a recombinase flanked by inverted repeats, and thus can be exchanged and spread between different genetic segments.[70] IS elements exhibit different forms of resistance conveyance. For example, a pair of IS elements can bracket a resistance gene and can be transmitted as a continuous transposon.[71] In addition, some IS elements can increase the expression of genes whose natural expression does not confer resistance. If an IS element harboring a potent promoter is inserted upstream of a potential resistance gene, overexpression and subsequent resistance occurs. This form of resistance acquisition has been observed in carbapenem-resistant *A. baumannii* isolates that revealed IS-induced overexpression of the intrinsic $bla_{\text{OXA-51}}$.[72] Furthermore, IS can insert themselves into genes, causing disruption and inactivation, and consequently affect the bacterial phenotype in terms of virulence and antimicrobial susceptibility.[73]

In addition to IS elements, spontaneous mutations play an important role in the resistance-correlated modification of existing genetic information. Desiccation and rehydration is associated with a substantial increase in mutation frequency, as these processes are associated with DNA lesions, such as alkylation, oxidation, cross-linking, base removal, and strand breaks.[74] In this regard, mutations altering promoter or regulator activity are connected to antimicrobial resistance.[56, 57, 75] Hence, DNA altering mechanisms are a potential trigger for the development of antimicrobial resistance.[76, 77]

1.6 Bacterial efflux pumps

Efflux pumps were first characterized in Gram-negative bacteria in 1993.[78] They are components of the bacterial membrane that secrete intracellular metabolic waste products and harmful compounds out of the cell into the extracellular environment.[79] More importantly, efflux pumps

increase bacterial tolerance to environmental conditions such as bile salts, detergents, dyes, heavy metals, organic pollutants, plant-produced compounds, and solvents.[80-85] Furthermore, efflux pumps are suggested to lower the intracellular concentration of quorum sensing inducer molecules and therefore modulate virulence and pathogenesis.[86] Since efflux pumps additionally enable bacteria to survive high antimicrobial concentrations, they contribute to the resistance phenotype. According to their amino acid composition and energy source, bacterial efflux pumps are categorized into six major groups, the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily, the multi-drug and toxic compound extrusion (MATE) family, the major facilitator superfamily (MFS), the small multi-drug resistance (SMR) family, the proteobacterial antimicrobial compound efflux (PACE) family, and the resistance nodulation-cell division (RND) family (Figure 3).[87-89] Although many efflux pumps are intrinsic to *A. baumannii* and therefore chromosomally encoded, several examples for acquired efflux pumps are known.[81, 90] Genes encoding for efflux pump proteins have been identified on mobile genetic elements, such as plasmids and transposons, or on resistance islands like AbaR1, which carries antimicrobial resistance associated efflux genes such as *tetA* and *cmlA*.[91]

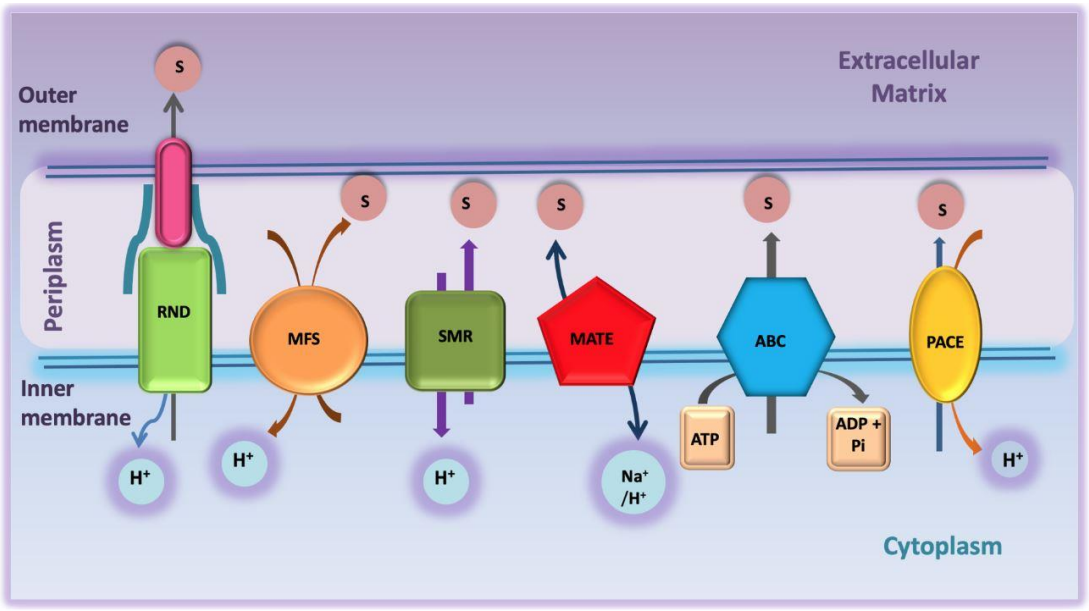

Figure 3. Schematic depiction of bacterial efflux families. The required energy for efflux activity provided by ATP hydrolysis (ABC family) or by ion antiport. The figure was adapted from Microbial Pathogenesis.[92]

1.6.1 RND-efflux pumps

Of the efflux families characterized so far, RND pumps have the highest clinical relevance. In particular, their broad substrate spectrum, including detergents, bile salts, and dyes and frequently used antimicrobials, as well as antiseptics and disinfectants used in health care, assigns

this class a significant role.[79, 93] RND-efflux pumps function as a tripartite complex containing an inner membrane transporter, an outer membrane channel or outer membrane factor (OMF), and a membrane fusion protein (MFP). The inner membrane transporter is essential for the function of the efflux system as it harbors three periplasmic binding pockets and consequently participates in substrate recognition, binding, and transport.[94] They are structured as an asymmetric homotrimer of which each monomer takes a different conformation corresponding to one of the three functional states (access, binding, and extrusion) of the transport cycle.[95, 96] These switch-like conformational changes of the transporter protomers are energized by the proton motive force across the cell membrane causing a functional rotation mechanism, which leads to an extrusion of the substrate through the distal part of the transporter into the outer membrane channel and consequently out of the bacterial cell.[97]

In *A. baumannii* three chromosomally encoded RND-type efflux pumps (Ade-*Acinetobacter* drug efflux) have been characterized: AdeABC, AdeIJK, and AdeFGH.

AdeABC efflux pumps

AdeABC was the first characterized RND efflux pump in *A. baumannii*.[98] AdeABC comprises the proteins AdeA (MFP), AdeB (transporter) and AdeC (OMF), which are encoded in an operon consisting of the genes *adeA*, *adeB*, and *adeC*. However, previous studies have reported that the role of the outer membrane factor AdeC is not crucial for the functionality of the pump. Furthermore, the recruitment of another outer membrane component instead of AdeC to form a functional tripartite complex and similarly serve the purpose, as seen in a study about MexXY-OprM efflux pumps in *P. aeruginosa*, was suggested.[99-101] AdeABC is polyspecific, and substrates included are aminoglycosides, fluoroquinolones, β-lactams, chloramphenicol, trimethoprim, erythromycin, tetracyclines, glycylcyclines and ethidium bromide.[100, 102, 103] Therefore, AdeABC is considered as the major contributing factor to MDR among RND efflux pumps. This is particularly true when AdeABC is overexpressed. Although carbapenems are reportedly substrates of the pump, the role of the AdeABC in carbapenem resistance is still under debate and studies are inconclusive.[104] Moreover, studies based on carbapenem susceptibility testing in the presence and absence of efflux pump inhibitors, to enlighten the role of AdeABC in carbapenem resistance, came to contradictory results.[59, 105-108] Carbapenem resistance is mainly associated with the presence or overproduction of carbapenemases, but some studies revealed a correlation of carbapenemases with overexpression of RND efflux pumps. A study on OXA-23 producing *A. baumannii* isolates reported that an *adeB* deleterious mutant

lost resistance to meropenem.[109] However, additional studies revealed that overexpression of AdeABC is proportional to the level of resistance in clinical isolates.[107, 110]

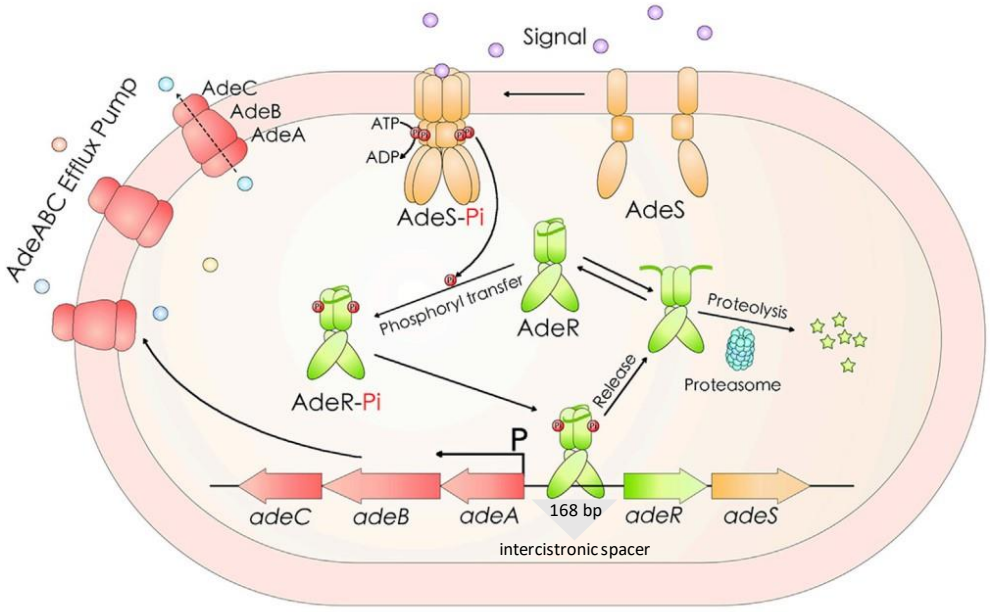

Figure 4. Regulation of AdeABC by the two-component regulatory system AdeRS. External signals cause autophosphorylation of AdeS followed by phosphorylation of AdeR. Functional AdeR binds to the specific DNA sequence within a 168 bp intercistronic spacer between the start codons of *adeA* and *adeR, w*hich are encoded in opposite directions, to initiate gene transcription. Adapted from Ouyang et al. 2021 with permission.[111]

The expression of AdeABC depends on the two-component regulatory system AdeRS, which is transcribed upstream, and in the opposite direction of AdeABC (Figure 4). Two-component regulatory systems are ubiquitous signal transduction proteins allowing cells to sense and respond to environmental stimuli.[112] The transmembrane sensor kinase undergoes conformational changes upon binding of an environmental stimulus causing ATP dependent autophosphorylation at a conserved histidine residue. Subsequently, the phosphate group is transferred to an aspartate residue of the corresponding response regulator, which then initiates expression of the respective gene.[113] With regard to AdeRS, AdeS represents the sensor histidine kinase, which comprises two transmembrane N-terminal helices linked by a extracellular sensor domain (residue 34 – 61), a histidine kinase, adenylyl cyclase, methyl-accepting chemotaxis protein and phosphatase (HAMP) domain (residue 84 – 138), a dimerization histidine phosphotransfer (DHp) domain (residue 146 – 204), and a C-terminal catalytic ATP binding domain (residue 204 – 357).[111] To exhibit full histidine kinase activity, AdeS forms a homo-hexamer. In the event of activation induced by external stimuli, ATP hydrolysis of the catalytic domain is followed by an autophosphorylation at the conserved phosphorylation site histidine 149. The

corresponding response regulator AdeR consists of a receiver domain (residue 1 – 127) and a DNA binding domain (residue 138 - 247) and is functional as a dimer. AdeR is activated by phosphoryl transfer from the AdeS histidine 149 residue to the AdeR phosphorylation site aspartate 63 within the receiver domain (Figure 5). Finally, the DNA binding domain recognizes a 10 bp direct-repeat DNA sequence (AAGTGTGGAGNAAGTGTGGAG) of a 168 bp intercistronic region between *adeR* and *adeABC* and acts as transcriptional activator.[114, 115] The external signal responsible for transcription activation has to date not been identified. Moreover, studies revealed that the number of *adeRS* transcripts is not crucial for regulation of *adeABC* expression, as no linear correlation between expression levels of *adeRS* and *adeB* was identified.[116] However, some mutations within *adeRS* are associated with increased *adeABC* expression and consequently antimicrobial resistance including amino acid substitutions in the receiver domain and the DNA binding domain of AdeR, as well as in the sensor domain, the HAMP domain, and the DHp domain of AdeS.[63, 64, 87, 100, 117, 118] Additionally, truncation of AdeS by IS*Aba1* insertion has been shown to increase *adeB* expression and reduce antimicrobial susceptibility.[63, 119, 120]

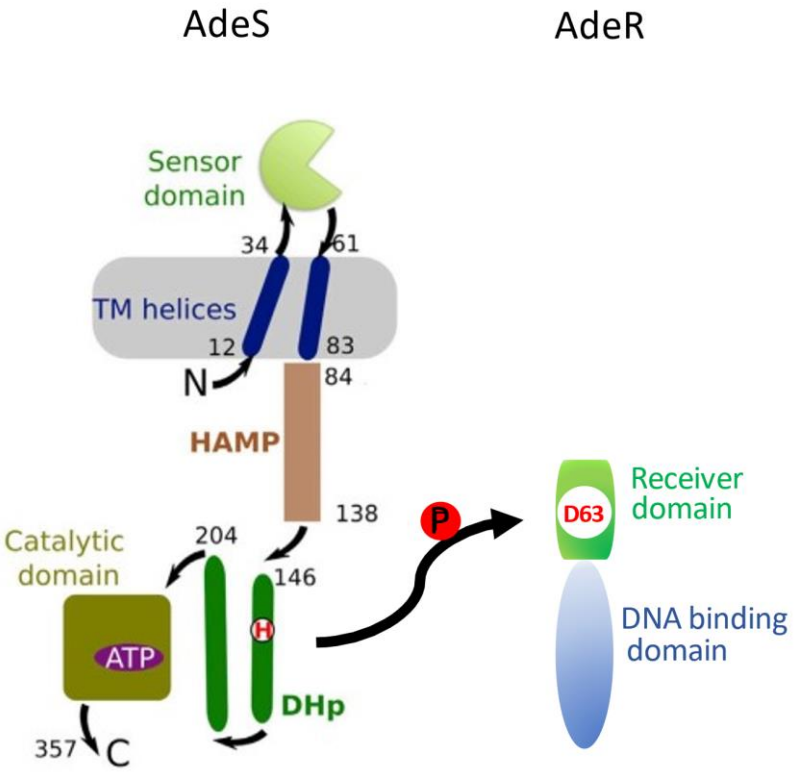

Figure 5. Schematic illustration of AdeS and AdeR activation. Upon external stimuli the AdeS catalytic domain induces autophosphorylation in the DHp domain at residue H149. Phosphoryl transfer to D63 of the receiver domain activates AdeR. Adapted from Ouyang et al. 2021 with permission.[111]

AdeIJK efflux pumps

An additional common RND efflux pump in *A. baumannii* is AdeIJK composed of AdeI (MFP), AdeJ (transporter), and AdeK (OMF).[67] This efflux pump has a comparably broad substrate specificity including a partial overlap with AdeABC.[102] Moreover, these two efflux pumps are found to show synergism for multiple antimicrobials. Knockouts of either AdeABC or AdeIJK led to increased antimicrobial susceptibility. Subsequently, the inactivation of both the efflux pumps showed a further increased susceptibility to antibiotics such as fluoroquinolones, tetracyclines, and tigecycline.[87, 121] AdeIJK is assumed to be constitutively expressed and to contribute to reduced susceptibility to β-lactams, such as ticarcillin, cephalosporins, and aztreonam, fluoroquinolones, tetracyclines, tigecycline, lincosamides, rifampin, chloramphenicol, cotrimoxazole, novobiocin, erythromycin, and fusidic acid. Additional substrates of AdeIJK are acridine, pyronine, safranine and sodium dodecyl sulfate.[87, 121] Overexpression of AdeIJK above a certain threshold has been shown to be toxic.[122] However, increased expression levels have been identified recently in clinical isolates associated with reduced antimicrobial susceptibility.[63]

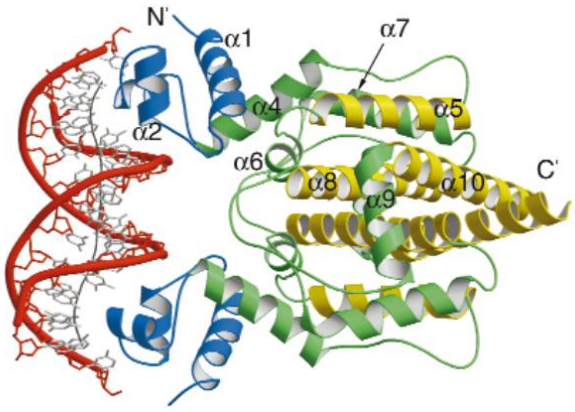

Figure 6. Structure of the TetR–DNA complex. Structures of homodimeric TetR. Blue: DNA-binding domains (helices α1 to α3). Green: helices affected by conformational changes upon binding of tetracycline (α4, α6, α9 and to some extent α7). Red and grey: DNA.[123]

The expression of *adeIJK* is regulated by constitutively expressed TetR-like repressor AdeN, which is encoded in a distance of 813 kb.[122] The TetR-family represents a group of widespread transcriptional repressors of bacterial multi-drug efflux genes.[124] TetR was first described as repressor of the MFS tetracycline resistance gene *tetA*.[125] Similar to AdeIJK, TetA is toxic when overexpressed. Therefore, TetR represses the transcription very tightly in the absence of tetracycline by binding to the operator upstream of *tetA*.[126] TetR harbors an N-terminal DNA-binding domain presented by helices α1 to α3 containing a helix-turn-helix motif (α2 and

α3).[127] Binding of tetracycline by hydrogen bonds to TetR residues 64 (α4), 82, 86 (α5) and 116 (α7) induces conformal changes in helices α4, α6, α7, and α9, causing dissociation of the repressor DNA complex and subsequently induces transcription.[123] Up to now, the detailed repression and dissociation mechanism of AdeN has not been characterized. However, it was shown that disruption by IS or deletion of *adeN* causes reduced antimicrobial susceptibility and elevated virulence in *A. baumannii*.[63, 128]

AdeFGH efflux pumps

The third RND efflux pump discovered in *A. baumannii* is AdeFGH, which also has the potential to affect antimicrobial susceptibility when overexpressed. Studies revealed that in strains missing AdeABC and AdeIJK, expression of AdeFGH was increased up to 300-fold but did not cause an MDR phenotype. On the other hand, a mutant exhibiting AdeFGH overexpression, in the presence of wildtype AdeABC and AdeIJK expression, revealed resistance to chloramphenicol, fluoroquinolones, and trimethoprim. Additionally, susceptibility to β-lactams, tetracyclines, tigecycline, and rifampin was decreased.[129] Other substrates of AdeFGH are nalidixic acid, ethidium bromide, erythromycin and sodium dodecyl sulphate (SDS). Furthermore, it has been suggested that AdeFGH driven extrusion of quorum sensing molecules into the environment induces biofilm formation in an *A. baumannii* population.[130]

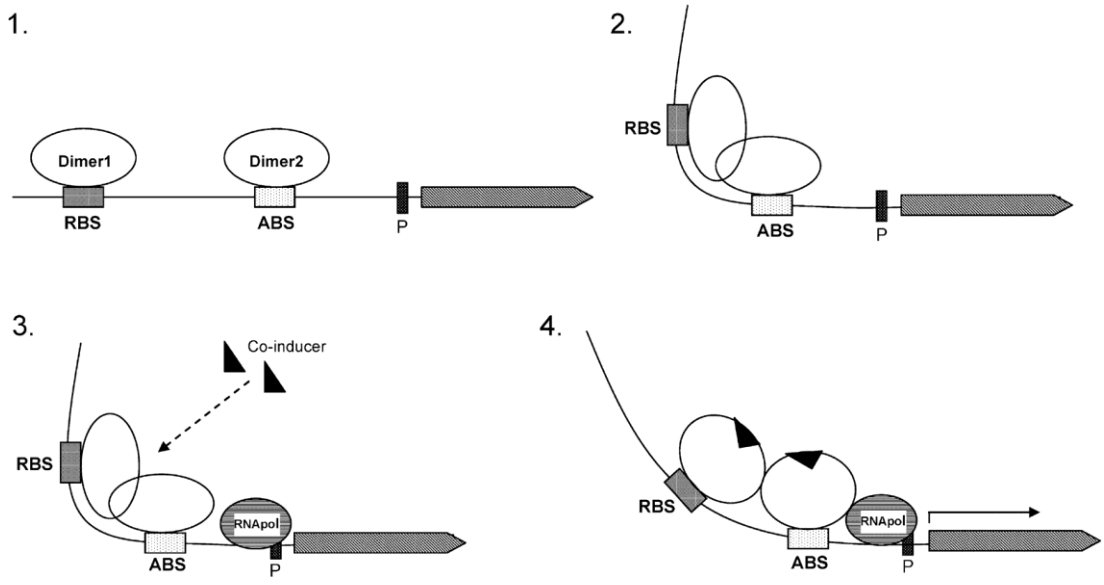

Figure 7. Schematic representation of LTTR gene regulation. (1) Each one LTTR dimer bound at the RBS and at the ABS. (2) Interaction between the two LTTR dimers causes formation of a tetrameric protein and DNA bending. (3) Binding of RNA polymerase at the promoter region of the target gene. Transcription is not induced in the absence of a co-inducer. (4) Co-inducer binding to the LTTR tetramer causes reduction of DNA bend. Consequently, the LTTR tetramer is brought into contact with the RNA polymerase at the promoter site of the target gene initiating its transcription.[131]

The expression of AdeFGH is controlled by the LysR-type transcriptional regulator (LTTR) AdeL, which is encoded directly upstream of *adeFGH* and transcribed in opposite direction. LTTR are the most common transcriptional regulators among prokaryotes. LTTR are autoregulated and consist of a conserved N-terminal helix-turn-helix DNA-binding domain connect by a flexible hinge to a less conserved C-terminal effector-binding domain, which is divided in two subdomains (RD1 and RD2). Transcription is initiated by each one LTTR dimer binding to a regulatory binding site (RBS) and the adjacent activation binding site (ABS), forming a tetramer. Subsequently, an effector-mediated structural change within the tetramer finally induces transcription by DNA-bending (Figure 7).[131] It has been shown that mutations within *adeL,* as the deletion of the last 11 C-terminal residues, as well as the amino acid substitutions V139G and T319K, can cause overexpression of AdeFGH and consequently reduce susceptibility to chloramphenicol, fluoroquinolones, tetracyclines, and tigecycline.[129]

1.6.2 MATE

Efflux pumps of the MATE superfamily can be found in eukaryotes and prokaryotes. These transporters form 12 transmembrane helices and depend on a Na^+/cation or proton gradient.[132] The chromosomally encoded proton antiporter AbeM is the most prevalent MATE transporter in *A. baumannii*. AbeM displays a wide range of substrates including antimicrobials such as fluoroquinolones, aminoglycosides, macrolides, and chloramphenicol.[133]

1.6.3 MFS

The major facilitator superfamily is ubiquitous in prokaryotes and eukaryotes. MFS pumps are gradient-dependent antiporters that usually are rather substrate specific. Examples of MFS efflux pumps in *A. baumannii* are AmvA, CmlA, CraA, FloR, TetA, and TetB. AmvA mainly contributes to efflux of substrates like detergents, disinfectants, and dyes. Additionally, it was shown that erythromycin belongs to its substrates, as inactivation of the *amvA* gene decreased the erythromycin minimum inhibitory concentration (MIC).[134] CmlA and FloR are encoded on the AbaR1 resistance island in *A. baumannii* and are associated with resistance to chloramphenicol and florfenicol, respectively.[91] The efflux activity of CraA is limited to chloramphenicol only and is assumed to contribute to intrinsic chloramphenicol resistance of *A. baumannii* as it is found in the majority of isolates.[135] TetA and TetB are acquired MFS pumps and the most prevalent tetracycline efflux pumps in *A. baumannii*.[136-138] TetA is mainly associated with tetracycline resistance only, but it was shown recently that some TetA variants may contribute

to efflux of tigecycline in synergy with RND pumps.[87, 139] TetB on the other hand, mediates resistance to tetracycline and minocycline.[87]

1.6.4 SMR

SMR efflux pumps are small dimers exhibiting different orientation for each monomer.[140] The chromosomally encoded SMR pump AbeS has been shown to efflux acriflavine, acridine orange, benzalkonium, deoxycholate, and SDS as well as conferring resistance to the antimicrobials chloramphenicol, fluoroquinolones, erythromycin, and novobiocin.[141] Another SMR efflux pump in *A. baumannii* is QacE which is associated with resistance to quaternary ammonium compounds and is encoded as part of the AbaR1 resistance island.[91, 142]

1.6.5 ABC

ABC transporters are one of the most common efflux pump types and are present in eukaryotes and prokaryotes. They represent the only transporter family using ATP hydrolysis as an energy source.[143, 144] ABC transporters contribute to efflux of amino acids, peptides, lipids, polysaccharides, and oligonucleotides as well as antimicrobials.[145, 146] The ABC transporter MacAB was first described in *E. coli* and is also the main ABC efflux pump in *A. baumannii*.[147] It is regulated by the two-component regulatory system BaeSR and consists of the membrane fusion protein MacA, the ABC transporter protein MacB, and the outer membrane protein TolC.[148] The main substrates of the MacAB-TolC complex are erythromycin and gramicidin.[149]

1.6.6 PACE

PACE family efflux pumps are the most recent type of efflux pumps identified in *A. baumannii.*[150] PACE efflux pumps are widespread in many Gram-negative bacteria and are mainly associated with increased tolerance to biocides used as disinfectants and antiseptics.[151] AceI was the first discovered PACE efflux pump and was identified in response to chlorhexidine, which is needed to induce formation of a functional AceI dimer.[89, 152]

1.7 Efflux pump inhibition

Efflux pump inhibitors (EPIs) are compounds that inhibit efflux pumps, reduce substrate export, and therefore induce intercellular accumulation of the corresponding compound. Consequently, EPIs have the potential to increase antimicrobial activity in MDR bacteria.[117, 121, 129] However, the use of EPIs as a combination therapy with antibiotics is extremely difficult, as many efflux pump families are also essential in eukaryotes and inhibition would have harmful

implications for patients. There are two main mechanisms of EPIs, either by direct binding to the efflux pump or by energy dissipation.[153] Binding of EPIs can be competitive on the substrates binding site, or non-competitive if binding of the inhibitor causes a decrease on the affinity of the pump towards its substrate.

Phenylalanine-arginine-β-naphthylamide (PAβN) was the first synthetic RND pump inhibitor increasing levofloxacin, erythromycin, and chloramphenicol but not tetracycline MICs in *P. aeruginosa* by binding to MexAB, MexCD, and MexEF pumps in a competitive manner.[154] Verapamil is usually used as in the treatment of hypertension and acts as ion channel blocker. It was found to inhibit MATE efflux in *Mycobacterium tuberculosis* by binding to the active site of the efflux protein.[155] 1-(1-napthylmethyl)-piperazine (NMP) was first described to inhibit the *E. coli* AcrAB-TolC RND efflux pump by inducing conformational changes by binding.[156] In *A. baumannii*, NMP was shown to increase susceptibility to fluoroquinolones and tigecycline.[157, 158]

Since efflux pumps depend on cellular energy, decoupling of energy sources is an effective way to impair efflux activity. This kind of inhibition does not require direct interaction between inhibitor and efflux pump. Carbonyl cyanide m-chlorophenyl-hydrazone (CCCP) is an ionophore that disrupts the proton motive force, which also inactivates bacterial metabolism. CCCP has been shown to restore ciprofloxacin susceptibility in *A. baumannii*.[159] However, its toxicity to mammalian cells prevents the clinical use of CCCP.[153]

The substance IITR08027 has been reported as a possible inhibiter against the MATE efflux pump AbeM in *A. baumannii*, as it restores fluoroquinolone susceptibility in resistant isolates by disrupting the proton gradient. Moreover, the compound is found to exhibit low cytotoxicity.[160]

Next to synthetic efflux pumps, several naturally derived efflux pump inhibitors have been described. Epigallocatechin 3-gallate (EGCG), reserpine, and polyamine agents are thought to hinder efflux by direct binding to the outer membrane channel or the pump section, respectively.[161, 162 163]

As nanoparticles are associated with antimicrobial activity, studies have been carried out to determine their role as efflux pump inhibitors. Tocopherol polyethylene glycol succinate-capped silver nanoparticles have been reported to inhibit efflux in *A. baumannii* by addressing the expression of RND efflux pump genes *adeB* and *adeJ*.[164] Furthermore, copper nanoparticle-

capped with N-lauryltyramine are implied to exhibit efflux inhibition, since they caused reduced ciprofloxacin and ethidium bromide MIC in *A. baumannii* and *E. coli*.[165]

An additional approach to inhibit efflux activity is the use of bacteriophages. Bacteriophages might become an alternative to antimicrobials and represent a possibility to overcome antimicrobial resistance. They bind to bacterial host cells at membrane proteins. Therefore, OMF of RND-type efflux pumps are potential receptors for bacteriophages, causing impaired efflux activity.[166]

1.8 Aim of the study

Efflux of *A. baumannii*, particularly that driven by pumps of the RND family, is of high clinical relevance because it mediates tolerance to a broad spectrum of potentially harmful substrates, including antimicrobials along with antiseptics and disinfectants widely applied in healthcare facilities. Therefore, gaining insight into regulatory mechanisms of RND efflux is of critical importance.

The aim of this thesis was to investigate the regulation of the AdeABC, AdeIJK, and the AdeFGH efflux pumps in *A. baumannii*. Specifically, the following topics were addressed:

- Identification of alterations within regulatory genes associated with increased RND efflux activity and determination of their respective prevalence based on a comprehensive collection of worldwide obtained isolates.
- Characterization of frequently identified alterations in terms of antimicrobial susceptibility, RND pump expression, and efflux activity, based on genetical modification of well characterized *A. baumannii* reference strains.
- Application of various reporter systems to investigate the expression of regulatory genes under different conditions of growth.

2 Experimental concepts

The results of this thesis are based on the step-by-step development and execution of a workflow algorithm enabling the identification and characterization of mutations within RND-type efflux regulatory genes. Additionally, experiments to investigate regulator expression were carried out. The methodological approach will be described below.

2.1 Identification of RND-type regulatory mutations

In order to identify mutations within RND efflux regulatory genes that may cause increased efflux pump expression, and thereby contribute to the antimicrobial resistance phenotype, a workflow based on the detection algorithm by Gerson et al. was used, which aimed to identify mutations contributing to colistin resistance.[56]

Tigecycline tolerance is a reliable indicator of increased efflux activity, as efflux is the main mechanism for increased tolerance to tigecycline in *A. baumannii*. Up to now, no tigecycline breakpoints for *A. baumannii* are available and therefore the tested isolates were categorized as exhibiting low (≤ 0.5 mg/l) or high (> 0.5 mg/l) tigecycline MICs based on the EUCAST breakpoints for Enterobacterales.[167]

The phenotypic classification of the *A. baumannii* isolates was combined with analysis of corresponding DNA sequences obtained by whole-genome sequencing (WGS). Therefore, total DNA from the bacterial isolates was extracted and used for short-read sequencing on an Illumina MiSeq platform. The obtained raw reads were *de novo* assembled with the Velvet assembler (version 1.1.04). The molecular epidemiology was investigated using a validated core genome MLST (cgMLST) scheme, using SeqSphere$^+$ version 7.0.4 software (Ridom, Münster, Germany).[168]

2.1.1 Initial assessment of genetic polymorphisms

Identification of alterations in the genes encoding RND-type efflux regulators associated with increased tigecycline MICs was performed by comparing DNA and amino acid sequences of isolates with high tigecycline MIC to low tigecycline MIC isolates of the same collection. DNA and amino acid sequences of RND-type efflux pump regulatory genes *adeRS, adeN* and *adeL* were analyzed using MultAlin.[169] Any alteration not identified exclusively in high tigecycline MIC isolates (for example also found in a reference strain or a low tigecycline MIC isolate), was classified as a genetic polymorphism not contributing to increased efflux activity and was

therefore excluded. Mutations within regulatory genes that only occurred in high tigecycline MIC isolates were assessed as putative resistance mutations and subsequently further analyzed. Furthermore, the bacterial genomes were screened for the most prevalent IS elements in *A. baumannii* using the bioinformatics tools IS-Finder and IS-Mapper.[170, 171]

2.2 Methodological approaches for analyzing putative resistance mutations

Characterization of amino acid substitutions suspected to increase the expression of RND efflux pumps was based on two different approaches. One way is the comparison of clinical isolate pairs, which were genetically identical apart from a single nucleotide exchange causing a relevant amino acid substitution within a RND efflux regulator. If no suitable isolate couple was available, characterization was carried out by genetic manipulation of reference strains, which is described in the following.

2.2.1 Genetic modification of *A. baumannii* reference strains

The wildtype regulator of *A. baumannii* reference strains ATCC 17978 and ATCC 19606 was deleted via markerless mutagenesis.[172] For recomplementation of knockout strains, the *adeRS* backbones of the *A. baumannii* reference strains ACICU and ATCC 17978 were fused to the shuttle vector pJN17/04.[64] The obtained *adeRS* shuttle plasmids were subjected to site-directed mutagenesis to exchange single nucleotides within the sequence of *adeRS* to induce the desired amino acid substitutions. Primers for PCR amplification of the plasmid including the corresponding nucleotide exchange were designed using the online tool NEBaseChanger.[173] Sanger sequencing (LGC Genomics GmbH Berlin, Germany) was used to confirm the correct nucleotide exchange. Subsequently, wildtype and modified *adeRS* shuttle vectors were transformed into *A. baumannii* ATCC 17978 Δ*adeRS*.

2.2.2 Characterization of putative resistance mutations

To analyze the impact of the specific regulator alterations, complementary isolate couples were compared in terms of antimicrobial susceptibility, expression of the RND efflux pump, and efflux activity.

Antimicrobial susceptibility testing

Susceptibility to tetracycline, gentamicin (Sigma–Aldrich, Steinheim, Germany), meropenem, amikacin, minocycline, rifampicin (Molekula, Newcastle upon Tyne, UK), levofloxacin (Sanofi Aventis, Frankfurt, Germany), ciprofloxacin (Bayer Pharma AG, Berlin, Germany),

azithromycin (Pfizer Pharma GmbH, Münster, Germany), chloramphenicol (Serva, Heidelberg, Germany) and erythromycin (AppliChem, Darmstadt, Germany) was tested by agar dilution following the current CLSI guidelines.[174] Minimal inhibitory concentrations for tigecycline (Molekula, Newcastle upon Tyne, UK) were determined by broth microdilution following the CLSI guidelines.[174]

Quantification of gene expression

Expression of *adeB* was evaluated by semi-quantitative real-time PCR (qRT-PCR) as described previously.[175] The DNA-directed RNA polymerase subunit beta gene *rpoB* was used as a reference gene, and its expression was quantified simultaneously with *adeB* expression. Statistical analysis was based on an unpaired t-test using the recorded absolute values.

Determination of efflux activity

The AdeABC substrate ethidium bromide was used to investigate efflux activity. Correspondingly, the fluorescence of prepared cell suspensions containing 10 µM ethidium bromide was measured in an Infinite M1000 PRO plate reader (Tecan, Crailsheim, Germany) every 15 s over a period of 30 min. The plate reader was set to an excitation wavelength of 530 nm and an emission wavelength of 600 nm. Accumulation studies were carried out with and without the proton motive force uncoupler CCCP (Sigma-Aldrich), used at a final concentration of 500 µM.

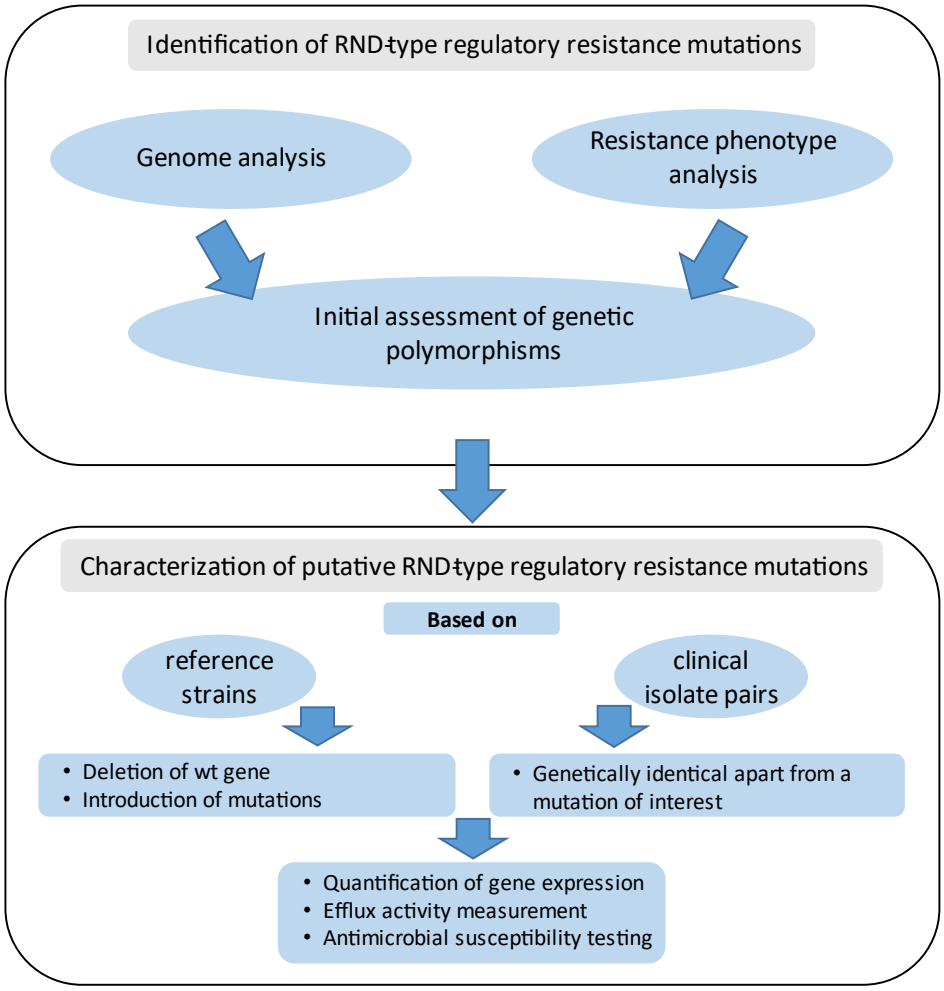

Figure 8. Schematic overview of the course of mutation analyses. Isolates were subjected to WGS and antimicrobial susceptibility testing. RND efflux pump and regulatory genes were compared between low and high tigecycline MIC isolates. Genetic alterations limited to high tigecycline MIC isolates were considered to be associated with increased RND efflux pump expression and therefore investigated further. This detailed characterization was either based on induction of the specific mutation in reference strains, or clinical isolate pairs, which were identical except from the mutation that was to be analyzed. Characterization was performed by determining the change in RND efflux pump expression, efflux activity, and antibiotic susceptibility.

2.3 Expression studies

Expression of RND efflux pumps or regulators was investigated by using different reporter systems, i.e. LacZ, luciferase, and GFP. The *A. baumannii* ATCC 17978 genome sequence was used for primer design for reporter plasmid generation. The designed primers amplify the upstream region of RND efflux pump or regulator operons containing the putative promoters. Primers were designed so that after the fusion of the target promoter-region and the reporter gene does not disturb the open reading frame of the respective promoterless reporter gene (*lacZ,*

luxCDABE, sfgfp). An empty vector control and a *carO* transformant was included as negative and positive control, respectively, for each reporter system.

LacZ

One reporter appointed in this way was the β-galactosidase gene *lacZ* of the reporter plasmid pIG14/09. For visualization of gene expression, the lactose analogue X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was used, which is hydrolyzed by β-galactosidase to galactose and 5-bromo-4-chloro-3-hydroxyindole. This by-product in turn forms dimers and is oxidized to the insoluble dark blue colored precipitate 5,5'-dibromo-4,4'-dichloro-indigo. The β-galactosidase activity was determined in combination with different growth media like Luria Bertani (LB) and Mueller Hinton (MH) agar plates and broth, as well as motility agarose, supplemented with X-gal (40 mg/ml). Correspondingly, expression of the respective genes of interest was indicated by formation of blue colonies.

GFP

Additionally, the green fluorescent protein (GFP) was used to investigate gene expression. Promoter regions were fused to the reporter plasmid pWH1266::*sfgfp*.[176] GFP is a small protein originally obtained from the jellyfish *Aequorea Victoria*, which emits a fluorescent signal when excited by UV light without the dependency of exogenous substrates. In this study, an advanced GFP version was used. Since the temperature factor affects the reliability of the original GFP reporters, a more robust GFP derivative was developed, which is called superfolder GFP (sfGFP). This protein exhibits increased thermal stability and better resistance to chemical denaturation compared to conventional GFP.[177] Detection of gene expression using this sfGFP reporter system requires detection of emission at 535 nm after excitation at 483 nm.

Luciferase

The luciferase reporter plasmid pLPV1Z was kindly provided by Massimiliano Lucidi (Department of Engineering, University Roma Tre, Rome, Italy).[178] The genes of the luciferase operon *luxCDABE* originate from *Xenorhabdus luminescens*. This bioluminescence reporter has the advantage that both the luciferase (encoded by *luxAB*) and the machinery responsible for synthesis of the long-chain aldehyde substrate that is essential in the bioluminescence reaction (encoded by *luxCDE*) are located on one operon and no additional substrate is required.[179] In this way the expression of luciferase complies with the genes to be investigated.

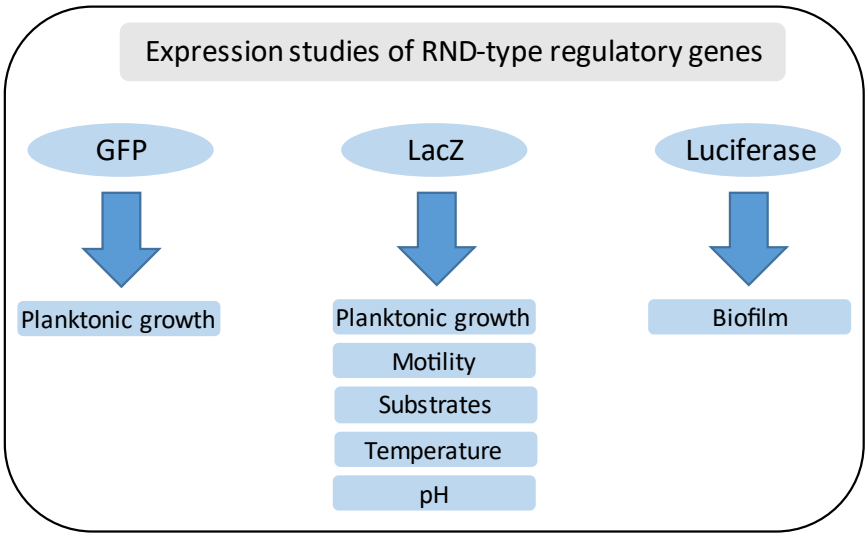

Figure 9. Reporters used to monitor RND efflux pump and regulator expression under different modes of growth.

2.4 Investigation of the motile phenotype in *A.baumannii*

Motility describes the movement-dependent spread of bacteria over wet surfaces. In *A. baumannii*, motility can exhibit different forms such as surface motility and twitching motility, which can be observed at the interphase between the bottom of the petri dish and the medium.[40] Motility experiments were carried out using a semi-solid growth medium composed of 0.5% agarose, 5 g/l tryptone, and 2.5 g/l NaCl.[37] Depending on the applied assay (gene expression analysis, antimicrobial susceptibility testing, and investigation of natural competence) additional supplements were added. If not stated otherwise, 2 µl of McFarland (McF) 0.5 bacterial suspensions were inoculated on the surface of the agarose or stab-inoculated. Motility was observed after 24 h incubation at 37 °C with increased humidity to prevent the medium from drying out.

3 Results

Since the present thesis constitutes a cumulative dissertation, some of the results are presented in the form of the corresponding prepared publications in the respective sections. The following provides a summary of the individual subchapters.

In the course of this work the regulatory aspect of increased RND efflux in *A. baumannii* was investigated. Therefore, a worldwide collection of clinical carbapenem-resistant *A. baumannii* isolates was analyzed for mutations causing increased tigecycline tolerance, which is an indicator for increased efflux activity (3.1). Subsequently, a selection of RND regulator amino acid substitutions were characterized in terms of their impact on RND efflux pump expression, efflux activity, and antimicrobial susceptibility (3.2. and 3.3). Additionally, studies to analyze the impact of the regulatory genes *adeN* and *adeL* were carried out based on genetic knockouts in *A. baumannii* ATCC 19606 (3.4). Moreover, expression of RND regulatory genes was determined under different growth conditions using reporter plasmids (3.5) and the relation between the two-component regulatory system AdeRS, motility, and natural competence was investigated (3.6).

3.1 Prevalence of RND efflux pump regulator variants associated with tigecycline resistance in carbapenem-resistant *A. baumannii* from a worldwide survey

Purpose of the study presented in the publication attached below, was to detect mechanisms causing reduced tigecycline susceptibility in 113 genetically unique carbapenem-resistant *A. baumannii* isolates obtained from a global collection between 2012 and 2016. The main focus was on RND-type efflux regulatory genes that were investigated for putative efflux increasing mutations. The most frequently identified mechanism associated with reduced tigecycline susceptibility was the disruption of the AdeIJK repressor *adeN*, either by IS elements or nucleotide deletions causing premature stop codons. However, less frequent amino acid substitutions and disruption by IS elements within the two-component regulatory system *adeRS*, which regulates expression of the AdeABC efflux pump, were found to correlate with comparatively higher tigecycline MICs. Furthermore, an altered version of *tviB*, which contributes to capsular polysaccharide synthesis, was identified. Other previously identified mechanisms, as for example the presence of *tet*(A) and *tet*(X), as well as mutations in putative resistance determinants *trm*, *plsC*, *rrf*, *msbA* and genes encoding 30S ribosomal proteins, were not identified in any isolate.

Our data provide the foundation for further investigation of regulator alterations and their contribution to increased efflux and reduced antimicrobial susceptibility.

My contribution to the following publication was conception of the analysis, antimicrobial susceptibility testing, sequencing data analysis, data interpretation and composing the manuscript. Preliminary data of this study were presented at the 29th ECCMID, Amsterdam, April 2019, and the 71st DGHM-Conference, Leipzig, March 2020.

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Prevalence of RND efflux pump regulator variants associated with tigecycline resistance in carbapenem-resistant *Acinetobacter baumannii* from a worldwide survey

Kai Lucaßen [1], Carina Müller[1,2], Julia Wille [1,2], Kyriaki Xanthopoulou [1,2], Meredith Hackel[3], Harald Seifert[1,2] and Paul G. Higgins [1,2]*

[1]*Institute for Medical Microbiology, Immunology and Hygiene, University of Cologne, Goldenfelsstrasse 19-21, 50935 Cologne, Germany;* [2]*German Center for Infection Research (DZIF), Partner Site Bonn-Cologne, Cologne, Germany;* [3]*International Health Management Associates, 2122 Palmer Drive, Schaumburg, IL 60173, USA*

*Corresponding author. E-mail: paul.higgins@uni-koeln.de

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Objectives: To determine the most common tigecycline resistance mechanisms in carbapenem-resistant *Acinetobacter baumannii* isolates obtained during the global Tigecycline Evaluation Surveillance Trial (TEST).

Methods: Tigecycline MICs were determined by broth microdilution. WGS was used to screen for the previously identified tigecycline resistance mechanisms, as well as mutations in resistance-nodulation-cell division (RND)-type efflux pump regulators.

Results: From a total 313 isolates, 113 genetically unique tigecycline-resistant isolates were analysed. The most frequent and worldwide distributed mechanism associated with tigecycline resistance was disruption of *adeN*, which encodes the repressor of the RND efflux pump AdeIJK, either by IS elements or nucleotide deletions causing premature stop codons. However, mutations leading to amino acid substitutions and disruption by IS elements within the two-component regulatory system *adeRS*, which regulates expression of the AdeABC efflux pump, correlate with higher tigecycline MICs, but these were found less frequently and were mainly restricted to Southern European countries. Furthermore, an altered version of *tviB* was identified in several tigecycline-resistant isolates that did not have putative resistance mutations within RND-type regulators. The resistance determinants *tet*(A) and *tet*(X), as well as resistance mutations in putative resistance determinants *trm*, *plsC*, *rrf*, *msbA* and genes encoding 30S ribosomal proteins, were not identified in any isolate.

Conclusions: The most prevalent tigecycline resistance mechanisms were caused by alterations in the regulators of RND-type efflux pumps. These data provide the basis for further characterization of regulator alterations and their contribution to increased efflux and tigecycline resistance, and also should be taken into account in drug discovery programmes to overcome the contribution of efflux pumps.

Introduction

The Gram-negative *Acinetobacter baumannii* has gained worldwide importance as one of the most problematic nosocomial pathogens causing ventilator-associated pneumonia, meningitis and wound, urinary tract and bloodstream infections, predominantly in patients in ICUs. Treatment of these *A. baumannii* infections is challenging because of their frequent resistance to a wide range of antimicrobial agents, such as aminoglycosides, fluoroquinolones and β-lactams, including carbapenems.[1] Hence, in particular against carbapenem-resistant isolates, only few available antimicrobials (including tigecycline and colistin) remain for the treatment of *A. baumannii* infections.

Many antimicrobial resistance determinants, such as those conferring resistance to β-lactams, folic acid pathway inhibitors, tetracycline, aminoglycosides, macrolides and chloramphenicol, are responsible for resistance to a single antimicrobial, or only a single class of antimicrobials, and are mostly acquired through horizontal gene transfer, e.g. bla_{TEM}, *sul1* or *tet*(B).[2] Concerning tigecycline resistance, the monooxygenase TetX has recently gained importance and has been reported in *Escherichia coli* and *A. baumannii*, among other organisms.[3–6] However, *tet*(X) is still a relatively rare resistance gene, which does not correlate with the high frequency of tigecycline-resistant *A. baumannii* isolates.[7] Foong *et al.*[8] described the contribution of the tetracycline

transporter TetA to reduced tigecycline susceptibility. Furthermore, mutations in the tigecycline-related methyltransferase (*trm*), the 1-acyl-sn-glycerol-3-phosphate acyltransferase (*plsC*), the 30S ribosomal subunit protein S10 (*rpsJ*), the ribosome recycling factor (*rrf*), the UDP-*N*-acetylglucosamine 6-dehydrogenase (*tviB*) and the lipid transporter ATP-binding/permease (*msbA*) genes are associated with tigecycline resistance.[9–13]

In contrast to acquired resistance determinants, intrinsic efflux-mediated antibiotic resistance, especially by the resistance-nodulation-cell division (RND) family, can contribute to reduced susceptibility simultaneously to numerous antimicrobial classes. RND-type efflux pumps are chromosomally encoded tripartite pumps consisting of an inner membrane spanning pump subunit and an outer membrane pore, which are joined together via a linker protein. RND transporters are intrinsic to all Gram-negative bacteria and, besides their ability to pump out antimicrobials (including tigecycline), their contribution to reducing intracellular concentrations of a diverse array of compounds, such as antiseptics, detergents, heavy metals and disinfectants, is of clinical relevance.[14–17]

In *A. baumannii*, there are three characterized RND efflux pumps, AdeABC, AdeFGH and AdeIJK, which are subject to different types of regulators: the two-component system AdeRS (AdeABC), the LysR-like transcriptional regulator AdeL (AdeFGH) or the TetR-like repressor AdeN (AdeIJK).[18] In particular, AdeABC and AdeIJK have been shown to affect antimicrobial susceptibility and to contribute to tigecycline resistance.[19,20] Additionally, it was shown that mutational hotspots in these regulators cause overexpression of the corresponding RND efflux pumps, causing antimicrobial resistance.[7,21]

Up to now, studies investigating RND-type efflux overexpression in correlation with mutations in regulatory genes in *Acinetobacter* spp. have been confined to a few geographical regions like Southern Europe or China.[7,22] The present study investigates the worldwide prevalence and contribution of alterations of RND-type efflux pump regulatory genes to tigecycline resistance in *A. baumannii* obtained from 47 countries as part of the worldwide Tigecycline Evaluation Surveillance Trial (TEST) and investigates other mechanisms behind elevated tigecycline MICs.[23]

Materials and methods

Strain collection

The investigated isolates were collected between 2012 and 2016 in 114 centres worldwide as part of the TEST study.[23] All included isolates were carbapenem resistant. The origins of the isolates are summarized for this study as Africa, Asia, Europe, North America and Latin America. No isolates were available from the Australian continent. The isolates were from clinical specimens obtained from the CNS, the cardiovascular system, intraabdominal sources, the urogenital tract and the respiratory tract, as well as from skin and skin structure and osteoarticular infections. Duplicate isolates obtained from the same patient were excluded.[24]

Antimicrobial susceptibility testing

Susceptibility to tigecycline (Molekula, Newcastle upon Tyne, UK) was determined by broth microdilution following CLSI guidelines using *Staphylococcus aureus* ATCC 29213 and *E. coli* ATCC 25922 as quality control strains.[25] Tigecycline resistance was defined as an MIC of >0.5 mg/L according to the EUCAST breakpoint for Enterobacterales, since no tigecycline breakpoint is available for *A. baumannii*.[26]

WGS

Total DNA from the bacterial isolates was extracted using the MagAttract HMW DNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and used for short-read sequencing. Sequencing libraries were prepared using a Nextera XT Library Prep Kit (Illumina GmbH, Munich, Germany) for a 250 bp paired-end sequencing run on an Illumina MiSeq platform. The raw sequencing reads generated in this project were submitted to the European Nucleotide Archive (https://www.ebi.ac.uk/ena/) under study accession number PRJEB27899.

Sequencing data analysis

The obtained raw reads were *de novo* assembled with the Velvet assembler (version 1.1.04). The molecular epidemiology was investigated using a validated core-genome MLST (cgMLST) scheme, using SeqSphere$^+$ version 7.0.4 software (Ridom, Münster, Germany).[27] Isolates that showed ≤10 allele differences and were provided by the same participating laboratory were considered potential duplicates and also excluded. The acquired resistome was analysed using ResFinder v.3.2. ISfinder and ISMapper were used to analyse the genome assemblies for IS elements.[28,29] DNA and amino acid sequences of RND-type efflux pump regulatory genes *adeRS*, *adeN* and *adeL*, as well as *plsC*, *trm*, *rrf*, *tvib*, *msbA* and genes encoding 30S ribosomal proteins S1–S21, were analysed using MultAlin.[30]

Detection of RND-type efflux overexpression contributing regulatory alterations

Identification of alterations in the genes encoding RND-type efflux regulators associated with tigecycline resistance was performed according to a modified version of a previously published approach, taking into account the change in tigecycline breakpoint values.[31] Briefly, DNA and amino acid sequences of tigecycline-resistant isolates included in this study were compared with those of susceptible isolates identified within the same collection. An alteration not identified exclusively in tigecycline-resistant isolates (for example also found in a reference strain or a tigecycline-susceptible clinical isolate) was treated as a genetic polymorphism not contributing to tigecycline resistance and was therefore excluded.

Results

Isolate selection and description

In total, 313 isolates were included in the present study, originating from Asia ($n = 88$), Africa ($n = 29$), Europe ($n = 67$), North America ($n = 53$) and Latin America ($n = 76$). Of these isolates, 148 were found to be tigecycline resistant, resulting in a worldwide prevalence of tigecycline resistance of 47.3% (Figure 1). After excluding genetically closely related isolates as determined by cgMLST, 113 unique tigecycline-resistant isolates from Asia ($n = 31$), Africa ($n = 7$), Europe ($n = 32$), North America ($n = 22$) and Latin America ($n = 21$) remained for further analysis. Most of these belonged to the international clonal lineage IC2 ($n = 84$). Further identified lineages were IC1 ($n = 9$), IC3 ($n = 2$) IC4 ($n = 1$), IC5 ($n = 9$), IC7 ($n = 1$) and IC8 ($n = 1$). In addition, five isolates were assigned as USA-clone-1, whereas one isolate did not cluster with any known IC.

The majority of tigecycline-resistant isolates had a tigecycline MIC of 2 mg/L ($n = 69$). Additionally, MICs of 1 mg/L ($n = 20$), 4 mg/L ($n = 19$) and 8 mg/L ($n = 5$) were determined for the resistant

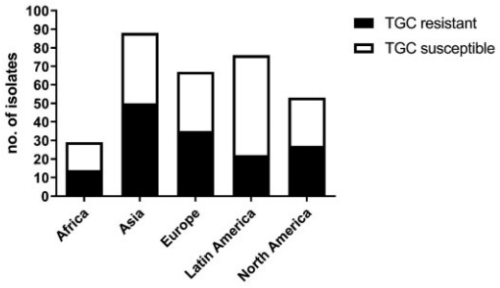

Figure 1. Geographical distribution of tigecycline-resistant and tigecycline-susceptible carbapenem-resistant *A. baumannii* isolates based on the current EUCAST breakpoint for Enterobacterales. TGC, tigecycline.

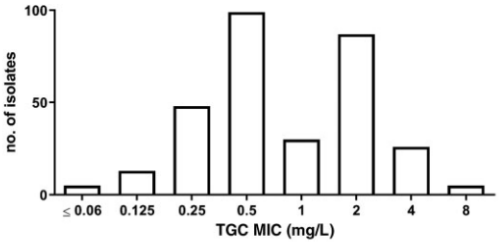

Figure 2. Tigecycline MIC distribution for 313 investigated carbapenem-resistant *A. baumannii* isolates. TGC, tigecycline.

isolates (Figure 2). Resistome analysis carried out with ResFinder revealed that none of the isolates investigated in this study carried the tigecycline resistance determinants *tet*(X) or *tet*(A).

Prevalence of tigecycline resistance-associated regulatory gene alterations

In our study, IS elements in *adeN* represent both the most common and most disseminated efflux alteration, since they were identified in African ($n = 3$), Asian ($n = 6$), European ($n = 6$), North American ($n = 6$) and Latin American isolates ($n = 2$) (Table 1 and Figure 3). Apart from a singleton which was not assigned to any IC, all these isolates belonged to IC2 (Figure 4). Disruption of *adeN* by IS*Aba1* was identified within 22 isolates, whereas 1 isolate harboured IS*Aba12*. By cgMLST this isolate showed one allele difference to another isolate obtained from the same testing site and was therefore excluded from tables and figures in this study. However, these two isolates differed only by a T156M amino acid substitution in AdeS, associated with a shift in tigecycline MIC from 2 to 8 mg/L. Moreover, *adeN* was also truncated in a further 17 isolates by premature stop codons. These were caused by different events in the DNA sequence of *adeN*, such as an insertion, deletion or exchange of one or more nucleotides. The location of the premature stop codons varied, but was mainly within the beginning or middle of the gene. AdeN is 217 amino acids long and no premature stop codon was present after residue 138 of AdeN. The stop codons were found in isolates originating from Asia ($n = 5$), Europe ($n = 4$), North America ($n = 7$) and Latin America ($n = 1$), indicating that these mutations were also not limited to any given region. Remarkably, every investigated isolate belonging to USA-clone-1 revealed the same 2 bp deletion within *adeN*, causing a premature stop codon.

In addition, the amino acid substitutions P16T, A43P, G54S, G65D, N66Y, K141N and L173F in AdeN were detected each in a single isolate. Two isolates contained AdeN altered by a L35R substitution. Strains containing resistance-associated amino acid substitutions in AdeN were isolated in Asia ($n = 5$), Europe ($n = 3$) and Latin America ($n = 1$). One Asian isolate revealed a unique duplication of alanine after residue 32 of AdeN.

The sensor kinase AdeS and response regulator AdeR comprise the two-component regulatory system of *adeABC*. The sequence of *adeS* was interrupted by IS*Aba1* in two isolates that originated from Southern Europe (IC2). In both cases it was associated with amino acid substitutions in AdeR. In one of these isolates, the IS*Aba1* in *adeS* was combined with the amino acid substitution E204K in AdeR and the isolate had a tigecycline MIC of 4 mg/L, whereas the second isolate had a tigecycline MIC of 8 mg/L and this isolate combined the AdeR amino acid substitutions D21V and D26N.

Furthermore, a truncation of AdeS caused by a premature stop codon was found in a single Latin American isolate. AdeS amino acid substitutions were identified in 10 isolates. These isolates harboured a combination of I100N and T156M ($n = 4$, Latin America, IC1), T156M alone ($n = 2$, Asia and Europe, IC2), E121K ($n = 1$, Asia, IC1), D167N ($n = 1$, Europe, IC3), N268Y ($n = 1$, Africa, IC2) and Q339K ($n = 1$, Europe, IC1).

Six isolates harboured AdeR amino acid substitutions. The only substitutions in AdeR, which occurred in more than one isolate, were E19D ($n = 3$, North America, IC2) and L142I (Asia, IC8, and North America, IC3). In one IC2 isolate, which was obtained from North America, a nucleotide substitution in the AdeR binding site 85 bp upstream of *adeA* was detected.

Finally, analysis of AdeL, the LysR-like regulator of AdeFGH, revealed two different amino acid substitutions (P125L/C292G), which were each found in one Asian isolate (IC2). One European isolate (IC2) contained IS*Aba1* in *adeL*. However, in 48 tigecycline-resistant isolates, no RND-type efflux regulator alterations were observed. Of these isolates one revealed a unique amino acid substitution within the RND-type efflux pump AdeJ, which was not identified in any other isolate or reference strain. Tigecycline-resistant isolates with putative resistance mutations within RND-type efflux pump regulatory genes are summarized in Table 1.

Putative resistance mutations in *plsC* could not be identified in any included isolate, whereas frameshift mutations in *trm* were also present in tigecycline-susceptible isolates. Genes encoding 30S ribosomal proteins, as well as *rrf* and *msbA*, were found to be very conserved between resistant and susceptible isolates and did not reveal mutations present only in the resistant population. In contrast, the resistance determinant TviB revealed highly diverse amino acid sequences, including patterns and amino acid substitutions limited to resistant isolates (Figure S3, available as Supplementary data at *JAC* Online). In particular, a seven amino acid extended version of *tviB* was associated with tigecycline resistance, since it was limited to 17

Table 1. Overview of identified carbapenem-resistant *A. baumannii* isolates with putative tigecycline resistance mutations; cgMLST was used for determination of clonal lineages; tigecycline MICs were determined by broth microdilution

Isolate	IC	Year of collection	Country of origin	Tigecycline MIC (mg/L)	RND-type efflux regulatory gene mutations			
					adeL	*adeR*	*adeS*	*adeN*
ABC001	IC2	2012	Egypt	2				IS*Aba1*
ABC008	IC2	2012	Tunisia	2			N268Y	
ABC011	IC2	2013	Egypt	4				IS*Aba1*
ABC014	IC2	2013	Egypt	2				IS*Aba1*
ABC019	IC2	2014	Poland	2				PMSC
ABC020	IC2	2014	Poland	2				PMSC
ABC026	IC2	2014	Italy	2				G54S
ABC029	IC2	2014	Spain	1				IS*Aba1*
ABC032	IC2	2014	Romania	2				IS*Aba1*
ABC038	IC5	2014	Colombia	2			PMSC	
ABC040	IC2	2014	USA	2				IS*Aba1*
ABC041	USA-clone-1	2014	USA	2				PMSC
ABC047	IC2	2014	USA	2				IS*Aba1*
ABC051	IC2	2014	USA	4		E19D		IS*Aba1*
ABC052	IC2	2014	France	2				IS*Aba1*
ABC055	IC2	2014	USA	2				PMSC
ABC058	IC2	2014	USA	2		E19D		IS*Aba1*
ABC059	IC2	2014	USA	2				PMSC
ABC063	IC2	2014	USA	2		A101T		
ABC076	IC2	2015	Mexico	2				IS*Aba1*
ABC078	IC1	2015	Brazil	8			I100N; T156M	
ABC090	IC2	2015	Saudi Arabia	2				K141N
ABC091	IC2	2015	Saudi Arabia	4	P125L			IS*Aba1*
ABC093	IC2	2015	Philippines	4				amino acid insertion
ABC094	IC8	2015	Philippines	2		L142I		A43P
ABC095	IC2	2015	Philippines	1				PMSC
ABC103	IC2	2015	Pakistan	4				PMSC
ABC105	IC1	2015	Pakistan	2			E121K	
ABC110	IC2	2015	Pakistan	4				PMSC
ABC115	IC2	2015	Pakistan	4				PMSC
ABC124	unclassified	2015	Thailand	4				IS*Aba1*
ABC130	IC2	2015	France	2				P16T
ABC132	IC2	2015	Malaysia	2	C292G			
ABC137	IC7	2015	Argentina	4				N66Y
ABC140	IC2	2015	USA	8		E19D		IS*Aba1*
ABC144	IC2	2015	USA	1		nucleotide substitution in AdeR binding site		
ABC150	IC2	2015	Singapore	2				IS*Aba1*
ABC153	IC2	2016	Vietnam	8			T156M	IS*Aba12*
ABC156	IC2	2016	Vietnam	2				IS*Aba1*
ABC157	IC2	2016	Vietnam	2				L173F
ABC168	IC1	2015	Brazil	4			I100N; T156M	
ABC176	IC2	2015	France	4				IS*Aba1*
ABC180	IC1	2015	Germany	2			Q339K	
ABC181	USA-clone-1	2015	USA	2				PMSC
ABC185	USA-clone-1	2015	USA	2				PMSC
ABC189	IC3	2015	Spain	2			D167N	PMSC
ABC200	IC2	2016	Mexico	4				IS*Aba1*
ABC203	IC2	2016	Spain	1				PMSC
ABC204	IC2	2016	Hong Kong	4				PMSC

Continued

Table 1. *Continued*

Isolate	IC	Year of collection	Country of origin	Tigecycline MIC (mg/L)	RND-type efflux regulatory gene mutations			
					adeL	*adeR*	*adeS*	*adeN*
ABC210	IC2	2015	Italy	4		E204K	IS*Aba1*	
ABC226	IC2	2016	Argentina	1				PMSC
ABC250	IC1	2016	Brazil	1			I100N; T156M	
ABC251	IC1	2016	Brazil	8			I100N; T156M	
ABC262	IC2	2016	Spain	2				IS*Aba1*
ABC263	IC2	2015	Greece	8		D21V; D26N	IS*Aba1*	
ABC265	IC2	2016	Thailand	2				L35R
ABC266	IC2	2016	Thailand	2				IS*Aba1*
ABC271	IC2	2016	Austria	2				L35R
ABC278	USA-clone-1	2016	USA	1				PMSC
ABC282	IC3	2016	USA	4		L142I		
ABC300	USA-clone-1	2016	USA	2				PMSC
ABC303	IC2	2016	Netherlands	2				IS*Aba1*
ABC308	IC2	2016	Taiwan	2				G65D
ABC311	IC2	2016	France	2	IS*Aba1*			
ABC327	IC2	2014	USA	4			T156M	IS*Aba1*

PMSC, premature stop codon; IC, international clone.

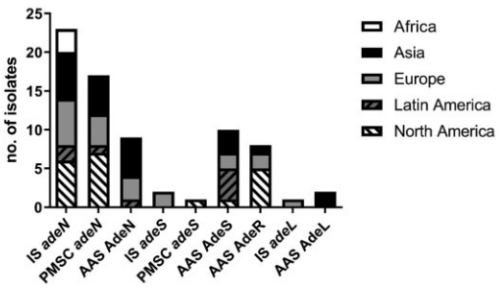

Figure 3. Geographical distribution of RND-type efflux regulatory gene alterations. AAS, amino acid substitution; PMSC, premature stop codon.

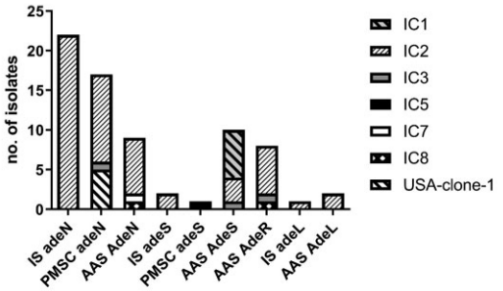

Figure 4. RND-type efflux regulatory gene alterations associated with clonal lineages. AAS, amino acid substitution; PMSC, premature stop codon.

tigecycline-resistant isolates that did not have any other putative resistance determinants or mutations.

Assessment of the impact of adeRS, adeN and adeL on tigecycline susceptibility

Analysis of the present cohort of isolates reveals that mutations of the various RND-type regulators differ in the degree of reduced tigecycline susceptibility that they confer. Among the isolates carrying a putative resistance mutation in *adeN*, 10% had a tigecycline MIC of 1 mg/L, while 60% had a tigecycline MIC of 2 mg/L and 30% had a tigecycline MIC of 4 mg/L or higher. In contrast to this, alterations in *adeRS* correlated with higher MICs, since 55% of these isolates had an MIC of $\geq$4 mg/L (Figure S1). Mutations within *adeL* have been excluded from Figure S1, since only three qualifying isolates were identified.

Discussion

Previously published studies analysing mutations in *A. baumannii* RND-type regulators were able to prove their impact on efflux pump overexpression and tigecycline resistance.[7,21,32,33] However, those studies have been predominantly based on few and locally limited isolates, due to lack of a comprehensive strain collection. The present study investigated the global prevalence of tigecycline resistance in *A. baumannii* in association with altered RND-type regulators. The isolates were carbapenem resistant and collected as part of a global multicentre study over five geographical regions: Africa, Asia, Europe, Latin America and North America. The fact that tigecycline resistance was found in almost 50% of the investigated isolates emphasizes the necessity to investigate the mechanisms of resistance, as the options for effective treatment decrease considerably.

The analysis was carried out by screening for known tigecycline resistance determinants and comparing the sequences of regulatory genes *adeN*, *adeRS* and *adeL* in resistant and susceptible isolates. Tigecycline resistance caused by regulator alterations of RND-type efflux pumps was the most common mechanism. Moreover, it was the disruption of the TetR-like repressor of the *adeIJK* gene cluster, *adeN*, with an IS element that was the most prevalent mechanism, which was identified in isolates from multiple countries located in Africa, Asia, Europe, Latin America and North America. Truncation of AdeN due to premature stop codons was also prevalent in tigecycline-resistant isolates investigated in this study. In accordance with our findings, it was described previously that dysfunction of *adeN* is associated with *adeIJK* overexpression and reduced antimicrobial susceptibility to macrolides, chloramphenicol, tetracyclines, β-lactams (including carbapenems), sulphonamides and quinolones.[34] Furthermore, Saranathan *et al*.[35] showed that disruption of *adeN* by IS*Aba1* causes increased virulence in *A. baumannii*. In the present study, we also identified AdeN amino acid substitutions that were limited to tigecycline-resistant isolates and we hypothesize that these will impair the repression of *adeIJK*.

Although tigecycline resistance was most frequently caused by a dysfunctional AdeN, our findings indicate that mutations within *adeRS* possess a greater potential, since these mutations correlate with higher tigecycline MICs. This hypothesis is supported by a study characterizing Southern European *A. baumannii* isolates, which also reported higher tigecycline MICs associated with *adeRS* mutations compared with changes in *adeN*.[7] Furthermore, the same study revealed the contribution of IS*Aba1* in *adeS*, the amino acid substitutions D21V and D26N in AdeR and the amino acid substitution D167N in AdeS to reduced tigecycline susceptibility. These mutations are presumably regionally limited and of low prevalence, since these *adeRS* alterations were limited to Southern European isolates in the present study as well. Since only a small number of isolates harbour mutations in AdeL, the LysR-like regulator of AdeFGH, we speculate that this efflux pump plays a minor role in tigecycline resistance. The amino acid substitution I37L in AdeL, which was thought to be associated with tigecycline resistance, was also identified in tigecycline-susceptible isolates in our study.[7] Therefore, I37L in AdeL might be a genetic polymorphism and does not contribute to reduced antimicrobial susceptibility.

Furthermore, our findings suggest that an accumulation of mutations within RND-type efflux regulatory genes contributes to a stepwise increase in tigecycline efflux, since isolates harbouring multiple regulator alterations correlate with higher tigecycline MICs (Figure S2). Consequently, the tigecycline MICs (Figure 2) creep upwards, in contrast to other resistance mechanisms, such as oxacillinase-mediated carbapenem resistance. For example, OXA-143 was shown to raise the meropenem MIC from 0.19 to 32 mg/L, whereas characterization of the AdeR amino acid substitution D20N revealed an increase in tigecycline MIC from 1 to 4 mg/L.[21,36]

Our study also identified tigecycline-resistant isolates that did not harbour mutations in the known RND-type regulatory genes and therefore other potential resistance mechanisms were investigated. The presence of *tet*(X)- and *tet*(A)-positive ESKAPE organisms contribute to tigecycline resistance.[5,8,37] However, no *tet*(X)-like or *tet*(A)-like resistance determinants were identified in our isolates. Mutations within the 30S ribosomal protein S10, encoded by *rpsJ*, were shown to emerge in tigecycline-resistant *A. baumannii*.[11,12] Our study did not reveal any mutations limited to tigecycline-resistant isolates and therefore the analysis was extended to 30S ribosomal proteins S1–S21. However, still no resistance mechanism was identified. These findings also apply for other tigecycline resistance-associated genes: *plsC*, *trm*, *msbA* and *rrf*.[9,10,12] On the other hand, *tviB*, which contributes to lipooligosaccharide and capsular polysaccharide synthesis and has been linked to tigecycline resistance previously, was found to be diverse among the presently investigated resistant and susceptible isolates.[12,13] An extension of this gene was limited to resistant isolates. However, the high variability of this gene indicates that care should be taken in interpretation of mutations and further characterization, e.g. using isolate pairs, is required. Taken together, these findings illustrate the complexity of tigecycline resistance mechanisms in *A. baumannii*.

In the revision of the EUCAST guidelines 2019, the Enterobacterales breakpoint for tigecycline was lowered from 4 to 1 mg/L, impairing comparison with previous publications.[7,21] Furthermore, the applied algorithm developed by Gerson *et al*.[31] had to be adapted, since the reference strain ACICU has to be considered as tigecycline resistant according to the current breakpoint value, and this strain was replaced by susceptible isolates from the TEST study. In the present study, most isolates with an unknown resistance mechanism had a tigecycline MIC of 1–2 mg/L and previously would have been classified as susceptible or intermediate. According to our findings, we hypothesize tigecycline MICs >4 mg/L to be predominantly caused by RND-type efflux overexpression, whereas lower MICs (EUCAST breakpoint) may be caused by currently unknown mechanism(s) in *A. baumannii*. This would then correlate with earlier published studies, based on the former EUCAST breakpoint for tigecycline.[7]

Since overexpression of RND-type efflux pumps may facilitate the persistence of Gram-negative bacteria in the hospital environment by reducing their susceptibility to antibiotics and biocides, it is important to elucidate the fundamental mechanism responsible for increased efflux.[38] This study contributes to the identification of regulatory alterations and assesses their worldwide prevalence. In addition, our results allow further elucidation of the impact of individual mutations on RND-type efflux pump expression and tigecycline susceptibility.

Funding

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Transparency declarations

None to declare.

Supplementary data

Figures S1 to S3 are available as Supplementary data at *JAC* Online.

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Supplementary data

Figure S1. Relative distribution of tigecycline MICs assigned to *adeN* and *adeRS* alterations.

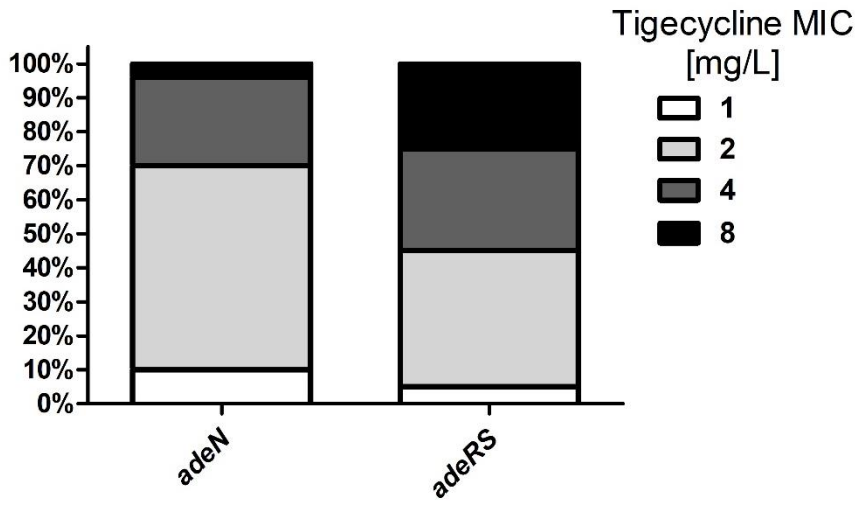


Figure S2. Distribution of tigecycline MICs assigned to RND-type efflux regulatory gene alterations.

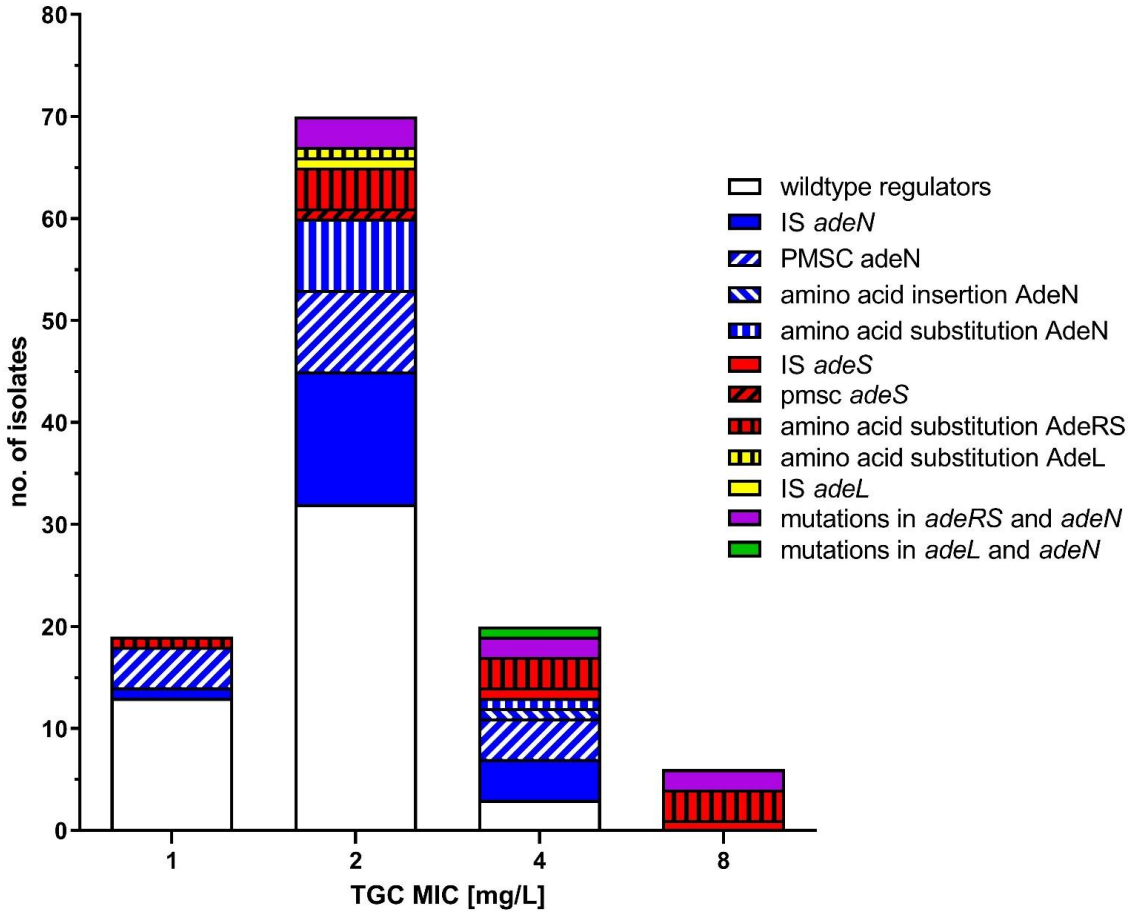
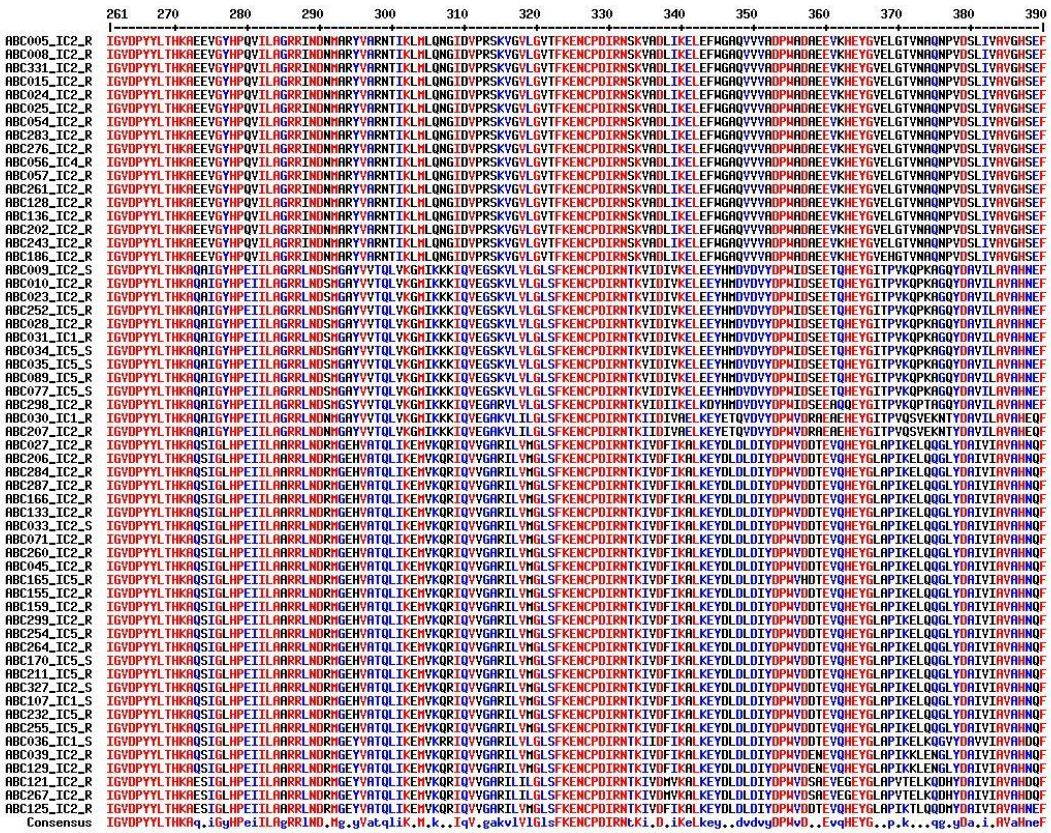


Figure S3. Analysis of *tviB* using MultAlin. Resistant (R) and susceptible isolates (S) of different international lineages (IC) have been aligned for detection of resistance patterns.

Figure S3. continued



3.2 Characterization of the two-component regulator system *adeRS* in the *A. baumannii* reference strains ATCC 17978 and ATCC 19606

Fundamental bacteriological research is mainly based on investigation of single or few reference strains of the corresponding species.[180, 181] These reference strains are intended to represent a norm, but some have considerable genetic differences from the isolates recovered from clinical specimens or the environment due to genetic plasticity and the necessity to adapt to external stimuli. For *A. baumannii* investigations, the reference strains *A. baumannii* ATCC 17978 and ATCC 19606 are mainly used.

In the following study we analyzed the regulation and expression of *A. baumannii* ATCC 17978 and ATCC 19606. We were able to show that *A. baumannii* ATCC 19606 exhibits increased AdeB efflux activity compared to ATCC 17978. Furthermore, an amino acid substitution at residue 172 of *adeS* was identified as a factor for deviating *adeB* expression. These results underscore the need to consider phenotypic variation within bacterial species based on genetic differences, to characterize fundamental bacterial mechanisms such as antimicrobial resistance.

In the preparation of the publication attended hereinafter, I was responsible for generation of gene knockout strains and introduction of amino acid substitution L172P in AdeS of ATCC 17978, as well for performing conduction and analysis of antimicrobial susceptibility testing, gene expression studies, accumulation studies, and manuscript composition.

Preliminary data of this study were presented as a poster at the 12th International Symposium on the Biology of Acinetobacter, Frankfurt, September 2019.

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MECHANISMS OF RESISTANCE

Comparison of the *Acinetobacter baumannii* Reference Strains ATCC 17978 and ATCC 19606 in Antimicrobial Resistance Mediated by the AdeABC Efflux Pump

K. Lucaßen,[a] S. Gerson,[a] K. Xanthopoulou,[a,b] J. Wille,[a,b] T. Wille,[a] H. Seifert,[a,b] P. G. Higgins[a,b]

[a]Institute for Medical Microbiology, Immunology and Hygiene, University of Cologne, Cologne, Germany
[b]German Center for Infection Research (DZIF), Partner Site Bonn-Cologne, Cologne, Germany

ABSTRACT The *Acinetobacter baumannii* RND efflux pump AdeABC is regulated by the 2-component regulator AdeRS. In this study, we compared the regulation and expression of AdeABC of the reference strains ATCC 17978 and ATCC 19606. A clearly stronger efflux activity was demonstrated for ATCC 19606. An amino acid substitution at residue 172 of *adeS* was identified as a potential cause for differential expression of the pump. Therefore, we recommend caution with exclusively using single reference strains for research.

KEYWORDS *Acinetobacter*, antibiotic resistance, cloning, efflux pumps

Infections with multidrug-resistant *Acinetobacter baumannii* in health care facilities are challenging due to limited treatment options (1). Antimicrobial resistance genes acquired by horizontal gene transfer are the main route toward multidrug resistance, and these genes are predominantly specific to single agents or classes (2). Contrary to this, intrinsic efflux mechanisms may contribute to reduced susceptibility to numerous antimicrobial classes simultaneously. The most common broad-spectrum efflux pumps in Gram-negative bacteria belong to the resistance-nodulation-division (RND) family. Besides their ability to pump out antimicrobials of different classes, they have been shown to affect susceptibility to diverse compounds used in medical practice, such as antiseptics, disinfectants, and detergents (3–6).

In *A. baumannii*, the RND efflux pump AdeABC, encoded on an operon and regulated by the two-component system AdeRS, has been shown to affect antimicrobial susceptibility and contribute to tigecycline resistance (7, 8). It was shown that single amino acid substitutions in AdeRS induce overexpression of AdeABC and are associated with antimicrobial resistance (9–11).

Bacteriological research into fundamental mechanisms of growth, metabolism, and antimicrobial resistance is mainly performed using a limited number of reference strains (12–14). For *A. baumannii* RND efflux investigations, the reference strains ATCC 17978 and ATCC 19606 are commonly used (7, 15). In this study, we compare and characterize AdeABC in terms of its regulation, expression, efflux activity, and role in antimicrobial susceptibility in these two *A. baumannii* reference strains.

We deleted *adeRS* in *A. baumannii* reference strains ATCC 17978 and ATCC 19606 according to the method of Stahl et al. (16). Briefly, the upstream and downstream regions of *adeRS* were integrated into pBIISK::*sacB_kanR* using the In-Fusion HD EcoDry cloning kit (TaKaRa Clontech, Saint-Germain-en-Laye, France) and primer pairs L49/L50 and L51/L52 or M66/M67 and M68/M69, respectively (Table S2 in the supplemental material). Transformed strains were selected overnight in 10 ml Luria-Bertani (LB) medium supplemented with 10% sucrose. Single colonies were obtained by plating appropriate dilutions onto LB agar with 10% sucrose. Colonies were tested for

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Address correspondence to P. G. Higgins, Paul.Higgins@uni-koeln.de.

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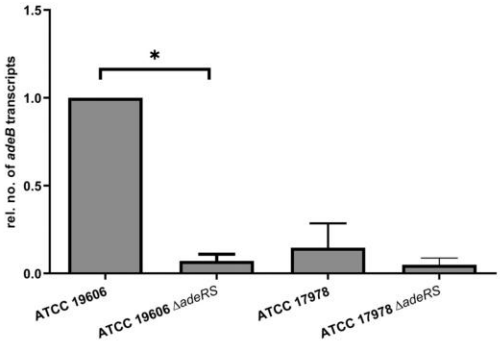

FIG 1 Relative *adeB* expression levels of *A. baumannii* ATCC 19606 and ATCC 17978 and the derived *adeRS* deletion strains determined by qRT-PCR. Results are represented as mean values ± standard errors of the means. Statistical analysis was done by using the unpaired *t* test on the absolute values. *, $P < 0.03$.

kanamycin sensitivity by replica plating and subjected to PCR to confirm the deletion of *adeRS*. To measure the expression of *adeB*, semiquantitative reverse transcription-PCR (qRT-PCR) was performed as described previously (17). Strains were grown in LB until mid-log phase. Samples were treated with RNAprotect (Qiagen, Hilden, Germany), followed by RNA isolation using the RNeasy minikit (Qiagen). cDNA was synthesized using the QuantiTect reverse transcription kit (Qiagen). Finally, RT-PCR was performed in a LightCycler (Roche, Mannheim, Germany) with QuantiFast SYBR green (Qiagen) in triplicates using freshly prepared cDNA. *rpoB* was used as the reference gene and was analyzed alongside *adeB*. Statistical analysis was performed using the unpaired *t* test. Strains and primers are listed in Tables S1 and S2.

We found that in ATCC 19606, *adeRS* deletion inhibited the expression of *adeB* ($P < 0.03$). In contrast, *adeB* expression in ATCC 17978 was already low in the wild-type strain, and there was no significant difference in expression levels ($P > 0.5$) compared to its *adeRS* knockout derivate (Fig. 1).

These findings were confirmed by accumulation studies using the AdeABC substrate ethidium bromide (18). Mid-log-phase cells were washed in potassium phosphate buffer (50 mM potassium phosphate buffer, 1 mM $MgSO_4$ [pH 7.4]), adjusted to an optical density of 20 at 600 nm, and finally supplemented with 0.2% (wt/vol) glucose (Sigma-Aldrich, Steinheim, Germany). Cells were kept on ice during washing. Ethidium bromide (Merck, Darmstadt, Germany) was applied at a final concentration of 10 μM. Fluorescence (excitation at 530 nm and emission at 600 nm) was measured in a 96-well Nunclon delta surface plate (Thermo Scientific) using an Infinite M1000 pro plate reader (Tecan, Crailsheim, Germany). Measurements were performed in triplicates, and mean values were analyzed for statistical significance using the unpaired *t* test. Accumulation of ethidium is facilitated by reduced expression of AdeABC, resulting in enhanced DNA binding of ethidium, which is revealed by increased fluorescence (9, 18, 19). Whereas ATCC 19606 Δ*adeRS* revealed higher fluorescence rates, indicating significantly reduced ($P < 0.0001$) efflux compared to its parental strain (Fig. 2), this effect of Δ*adeRS* was not detected for ATCC 17978 ($P < 0.05$) (Fig. 3).

Furthermore, deletion of *adeRS* in ATCC 19606 caused increased susceptibility to different antimicrobial classes, as determined by agar dilution or broth microdilution following the current CLSI guidelines (Table 1) (20). The biggest impact of *adeRS* deletion in ATCC 19606 was on azithromycin resistance, with a 16-fold-reduced MIC, emphasizing the affinity of azithromycin as a substrate of AdeABC. In accordance with our analysis of *adeABC* expression and efflux activity, the deletion of *adeRS* in ATCC 17978 had no detectable effect on antimicrobial susceptibility to the antimicrobials tested. All these findings illustrate the strain-dependent role of AdeABC expression,

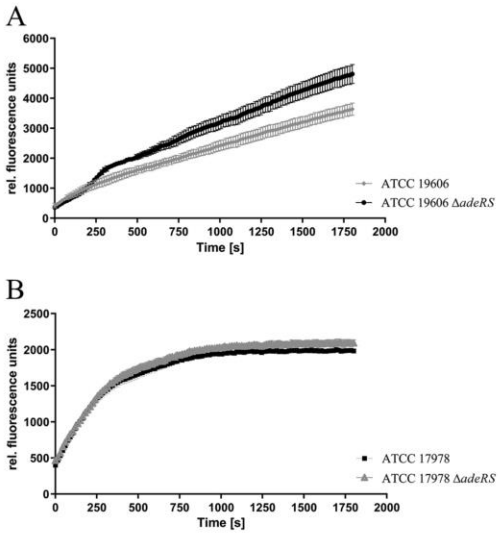

FIG 2 Ethidium bromide accumulation of ATCC 19606 and ATCC 19606 Δ*adeRS* (A) and ATCC 17978 and ATCC 17978 Δ*adeRS* (B). Fluorescence was measured every 10 s over 30 min. Data were collected from three independent experiments and are presented as mean values ± standard errors of the means.

which should be considered in future efflux studies based on findings in a single reference or laboratory strain.

Previous studies have shown that mutational hot spots in RND-type efflux regulatory genes identified in clinical isolates cause differential expression of the corresponding RND efflux pumps and thus contribute to antimicrobial resistance (9, 11, 21). To identify the cause of the different roles of AdeRS for ATCC 19606 and ATCC 17978, the amino acid sequences were aligned and compared (22). AdeR of ATCC 17978 revealed only one amino acid substitution, a change of L to P at position 241 (P241L), which was predicted by the PROVEAN online tool to be neutral (23). AdeS of ATCC 17978, however, revealed a P172L amino acid substitution, which was classified as deleterious. Furthermore, previous studies have shown that this region is a hot spot for resistance

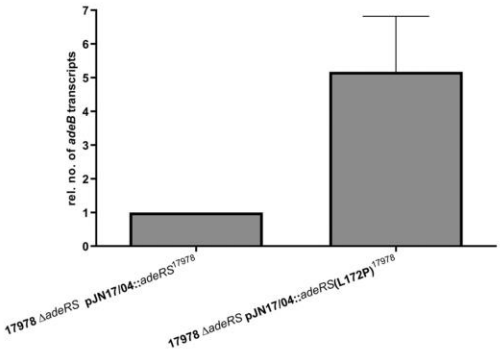

FIG 3 Relative *adeB* expression levels of *A. baumannii* ATCC 17978 Δ*adeRS* transformants determined by qRT-PCR. Results are represented as mean values ± standard errors of the means. Statistical analysis was done by using the unpaired *t* test on the absolute values ($P = 0.0699$).

TABLE 1 MICs of antimicrobials from different classes against ATCC 19606, ATCC 17978, and derived *adeRS* deletion strains

Strain	MIC (mg/liter) of[a]:											
	AMK	GEN	AZM	ERY	CIP	LVX	CHL	MEM	RIF	MIN	TET	TGC[b]
ATCC 19606	8	16	32	32	1	0.5	>128	0.5	4	0.25	8	1
ATCC 19606 Δ*adeRS*	2	8	2	16	0.5	0.25	128	0.25	4	≤0.125	4	≤0.125
ATCC 17978 wild type	2	1	2	16	0.25	≤0.125	64	0.5	4	≤0.125	1	0.25
ATCC 17978 Δ*adeRS*	2	1	2	16	0.25	≤0.125	64	0.5	4	≤0.125	1	≤0.125
ATCC 17978 Δ*adeRS*(pJN17/04)	2	0.5	2	8	≤0.125	≤0.125	64	0.5	4	≤0.125	1	≤0.125
ATCC 17978 Δ*adeRS*(pJN17/04::*adeRS*^{17978})	2	1	2	8	≤0.125	≤0.125	64	0.5	4	≤0.125	1	≤0.125
ATCC 17978 Δ*adeRS*[pJN17/04::*adeR*(L172P)*S*^{17978}]	2	2	4	16	0.25	≤0.125	64	0.5	4	≤0.125	2	≤0.125

[a]MICs were determined by agar dilution unless otherwise noted. AMK, amikacin; AZM, azithromycin; CHL, chloramphenicol; CIP, ciprofloxacin; ERY, erythromycin; GEN, gentamicin; MEM, meropenem; MIN, minocycline; LVX, levofloxacin; RIF, rifampicin; TET, tetracycline; TGC, tigecycline.
[b]TGC MICs were determined by broth microdilution.

mutations in *Acinetobacter baumannii* (10, 11, 24). Residue 172 is part of the dimerization and histidine-containing phosphotransfer domain, which includes the phosphorylation residue H149 and might therefore affect the activity of the sensor kinase. Moreover, analysis of available genome sequences revealed that clinical isolates predominantly harbor the P172 configuration, which is also present in other commonly used laboratory strains like ACICU and AYE (25).

Subsequently, ATCC 17978 Δ*adeRS* was either recomplemented with wild-type *adeRS* of ATCC 17978 or *adeRS* subjected to Q5 site-directed mutagenesis (New England Biolabs) to obtain the nucleotide triplet coding for proline at residue 172 cloned into shuttle vector pJN17/04. Sanger sequencing (LGC Genomics GmbH Berlin, Germany) was used to confirm the corresponding nucleotide exchange. This single amino acid substitution caused an approximately 5-fold increase in the expression of *adeB* as determined by qRT-PCR performed in triplicates (Fig. 4).

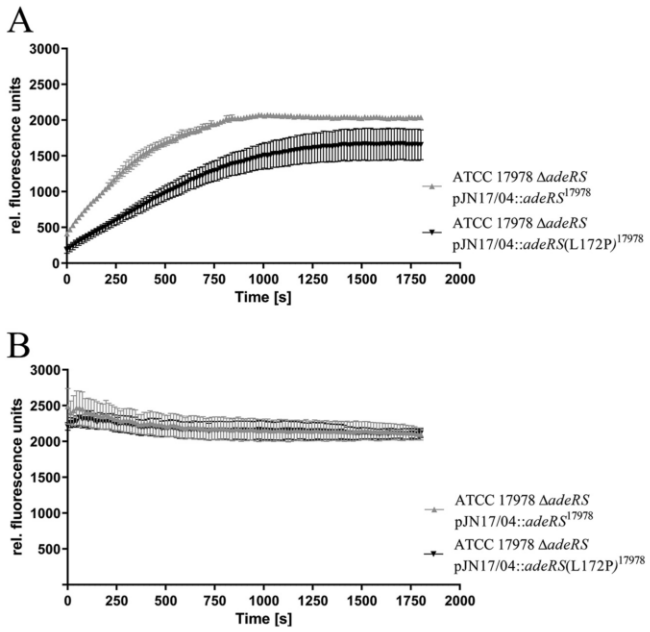

FIG 4 Ethidium bromide accumulation in untreated (A) and CCCP-treated (500 μm) (B) ATCC 17978 Δ*adeRS* transformants. Fluorescence was measured every 10 s over 30 min. Data were collected from three independent experiments and are presented as mean values ± standard errors of the means.

Analysis of ethidium accumulation revealed significantly lower accumulation levels ($P < 0.0001$) for ATCC 17978 Δ*adeRS*[pJN17/04::*adeRS*(L172P)^{17978}] than for the strain recomplemented with the ATCC 17978 wild-type *adeRS* (Fig. 4). To show that the difference in accumulation levels was induced by altered efflux activity, ethidium accumulation was measured while the strains were subjected to the proton motive force uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Sigma-Aldrich) at a final concentration of 500 μM. In this way, all proton-mediated efflux was inhibited, allowing the determination of the contribution of the amino acid substitution to efflux. The application of CCCP led to an alignment of the accumulation levels of both strains, indicating that the L172P amino acid substitution in AdeS caused increased efflux activity (Fig. 4).

Finally, the effect of the L172P amino acid substitution on antimicrobial susceptibility was determined, revealing a 2-fold increase in the MICs for gentamicin, azithromycin, erythromycin, ciprofloxacin, and tetracycline. These results confirm the increased efflux activity induced by the L172P amino acid substitution.

Overall, our results show that ATCC 17978 is comparatively weak in terms of AdeABC efflux because of its AdeS configuration, indicating that it is a rather inappropriate strain to study AdeABC efflux. Furthermore, it has to be considered that ATCC 17978 is derived from an isolate first described in 1951 (26) and therefore might not represent current *A. baumannii* isolates persisting in the hospital environment. This also applies for ATCC 19606, which was isolated in 1948.

Taking all these data into account, our results emphasize that focusing on single reference strains to characterize antibacterial resistance mechanisms can be problematic because of phenotypic variation within bacterial species based on genetic inconsistency.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

ACKNOWLEDGMENTS

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We declare no conflicts of interest.

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Supplementary Table 1. Strains and plasmids used in this study.

Strain or plasmid		**Reference or source**
E. coli	HST08 Stellar™	(TaKaRa Clontech)
	NEB 5 alpha competent cells	(New England BioLabs)
A. baumannii	ATCC 17978	(1)
	ATCC 19606	(2)
	ATCC 17978 Δ*adeRS*	(this study)
	ATCC 19606 Δ*adeRS*	(this study)
	ATCC 17978 Δ*adeRS* pJN17/04	(this study)
	ATCC 17978 Δ*adeRS* pJN17/04::*adeRS*^{17978}	(this study)
	ATCC 17978 Δ*adeRS* pJN17/04::*adeRS*(L172P)^{17978}	(this study)
Plasmids	pJN17/04::*adeRS*^{17978}	(3)
	pJN17/04::*adeRS*(L172P)^{17978}	(this study)
	pBIISK::*sacB_kanR*	(4)
	pBIISK::*sacB_kanR::adeRS*^{17978}	(this study)
	pBIISK::*sacB_kanR::adeRS*^{19606}	(this study)

Supplementary Table 2. Primers used in this study.

Target	Primer name	Sequence (5' - 3')	Size (bp)	Experiment
adeRS^{19606}	N66	gccactcatcgcagtGACATCGGCTAGAGCCTGA	2470	InFusion clonig into pJN17/04
	N67	atgaattacaacagtACTTAGGGTCAGGAGGTTATTTAGT		
adeRS^{19606} upstream	L49	ctggcagagcatcgaattccTAAACCTTGCTGACGTACAA	1716	InFusion clonig into pBIISK::*sacB_kanR*
	L50	acttagggtcaggaggttatAGATAATCTGGCTATAGAAAGTGC		
adeRS^{19606} downstream	L51	tttctatagccagattatctATAACCTCCTGACCCTAAGT	1712	
	L52	aactagtggatcccccgggcTATTCATGAACGGCCTGAAA		
adeRS^{17978} upstream	M66	ctggcagagcatcgaattccACTAGACAATTGCCCTTGAG	2062	InFusion clonig into pBIISK::*sacB_kanR*
	M67	ggggcactttaactctaagaAGAAAATCTGGCTATAGAAAG		
adeRS^{17978} downstream	M68	tttctatagccagattttctTCTTAGAGTTAAAGTGCCCC	1532	
	M69	aactagtggatcccccgggcCTCGCGCATCATTTTCATAT		
flanking *adeRS*	K25	AGTGTGGAGTAAGTGTGGAGA	2078/203	Control of *adeRS* deletion
	JE37	TGGATCGTTTTAAAGCTATTTTACTT	2600/700	
pJN17/04::*adeRS*	O37	GTTTTTAAAC**C**TGATGAAGTTCTATTTAAAAG	8581	Site-Directed Mutagenesis *adeS*(L172P)
	O38	GCCATCAATAATTCCCTG		
adeS	K28	AAACTTGCTCAATACGACGG	518	Sanger sequencing
	K42	AAAGCGTTTTATTGTGCCAA		
rpoB	C65	GAGTCTAATGGCGGTGGTTC	110	qRTPCR
	C66	ATTGCTTCATCTGCTGGTTG		
adeB	M24	AGGCATGAGTGTTATTCGGG	603	Standard for qRTPCR
	M25	CTATATCCCACGCCACGC		
	M26	CAAGGACGTATGCAACAAGT	103	qRTPCR
	M27	CTAATTGACCGCTTGAACCC		

Lowercase: tails for InFusion cloning
bold and in a box: site of nucleotide substitution in primer O37

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3.3 Characterization of amino acid substitutions in the two-component regulatory system AdeRS identified in multi-drug resistant *A. baumannii*

The previously designed *adeRS* knockout of *A. baumannii* ATCC 17978 (3.2) was used to investigate mutations identified as associated with RND efflux overexpression in clinical studies. To point out the effect of a specific amino acid substitution it was necessary to introduce it independently from any other alteration into a strain with normal levels of RND expression. Since the *A. baumannii* ATCC 17978 wildtype backbone of *adeRS* was shown to cause low level RND expression, we used the *adeRS* backbone of *A. baumannii* ACICU, which belongs to the most common clonal lineage IC2, and therefore shows higher similarity to recent clinical isolates identified within the clinical environment. The present study shows that the AdeR D26N and the AdeS T156M amino acid substitutions increase efflux expression and activity and consequently cause reduced antimicrobial susceptibility, whereas the D21V in AdeR does not account for the resistance phenotype.

I contributed to the following manuscript by generating the *adeRS* shuttle vector and introduce the *adeR* amino acid substitutions via site-directed mutagenesis. In addition, I performed antimicrobial susceptibility testing, gene expression studies, accumulation studies, data analysis, as well as manuscript composition. The following manuscript has been accepted for publication in the peer-reviewed journal mSphere.

Preliminary data of this study have been presented as a poster at the 31st ECCMID, Online-Conference, July 2021.

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RESEARCH ARTICLE

Characterization of Amino Acid Substitutions in the Two-Component Regulatory System AdeRS Identified in Multidrug-Resistant *Acinetobacter baumannii*

K. Lucaßen,[a] K. Xanthopoulou,[a,b] J. Wille,[a,b] T. Wille,[a] Y. Wen,[c] X. Hua,[d,e,f] H. Seifert,[a,b] P. G. Higgins[a,b]

[a]Institute for Medical Microbiology, Immunology and Hygiene, Faculty of Medicine and University Hospital Cologne, University of Cologne, Cologne, Germany
[b]German Center for Infection Research (DZIF), Partner Site Bonn-Cologne, Cologne, Germany
[c]Talent Highland and Center for Gut Microbiome Research of Med-X Institute, The First Affiliated Hospital, Xi'an Jiaotong University, Xi'an, China
[d]Department of Infectious Diseases, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou, China
[e]Key Laboratory of Microbial Technology and Bioinformatics of Zhejiang Province, Hangzhou, China
[f]Regional Medical Center for National Institute of Respiratory Diseases, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou, China

ABSTRACT In *Acinetobacter baumannii*, resistance-nodulation-cell division (RND)-type efflux is a resistance mechanism of great importance since it contributes to reduced susceptibility to multiple antimicrobial compounds. Some mutations within the genes encoding the two-component regulatory system AdeRS appear to play a major role in increased expression of the RND efflux pump AdeABC and, consequently, in reduced antimicrobial susceptibility, as they are commonly observed in multidrug-resistant (MDR) *A. baumannii*. In the present study, the impact of frequently identified amino acid substitutions, namely, D21V and D26N in AdeR and T156M in AdeS, on *adeB* expression, efflux activity, and antimicrobial susceptibility was investigated. Reverse transcription-quantitative PCR (qRT-PCR) studies revealed significantly increased *adeB* expression caused by D26N (AdeR) and T156M (AdeS). In addition, accumulation assays have shown that these mutations induce increased efflux activity. Subsequently, antimicrobial susceptibility testing via agar dilution and broth microdilution confirmed the importance of these substitutions for the MDR phenotype, as the MICs for various antimicrobials of different classes were increased. In contrast, the amino acid substitution D21V in AdeR did not lead to increased *adeB* expression and did not reduce antimicrobial susceptibility. This study demonstrates the impact of the D26N (AdeR) and T156M (AdeS) amino acid substitutions, highlighting that these regulators represent promising targets for interfering with efflux activity to restore antimicrobial susceptibility.

IMPORTANCE The active efflux of antimicrobials by bacteria can lead to antimicrobial resistance and persistence and can affect multiple different classes of antimicrobials. Efflux pumps are tightly regulated, and their overexpression can be mediated by changes in their regulators. Identifying these changes is one step in the direction of resistance prediction, but it also opens the possibility of targeting efflux pump regulation as a strategy to overcome antimicrobial resistance. Here, we have investigated commonly found changes in the regulators of the main efflux pumps in *Acinetobacter baumannii*.

KEYWORDS efflux pump, AdeABC, tigecycline

Acinetobacter baumannii has emerged as a serious nosocomial pathogen and has been implicated in various hospital outbreaks. It predominantly affects compromised and intensive care unit patients, causing ventilator-associated pneumonia, meningitis, and wound, urinary tract, and bloodstream infections (1).

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Address correspondence to P. G. Higgins, paul.higgins@uni-koeln.de.

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Multidrug resistance (MDR) is widespread among clinical *A. baumannii* isolates. In particular, the rise of carbapenem-resistant isolates is of major concern, indicating the need for novel treatment approaches (2). Resistance to the vast majority of antibiotic classes is primarily acquired through horizontal gene transfer, as can be observed from the dissemination of carbapenemase-encoding genes, or based on target-site mutations such as amino acid substitutions within GyrA and ParC, which are responsible for fluoroquinolone resistance (3–5). Both horizontal gene transfer and target site mutations are usually very specific, affecting a limited spectrum of antimicrobial compounds or antimicrobial classes.

In this respect, the above-described mechanisms differ from intrinsic efflux mechanisms, which are represented in particular by the resistance-nodulation-cell division (RND) family. The chromosomally encoded RND-type efflux pumps are tripartite and composed of an inner membrane-located pump and an outer membrane pore, which are connected via a linker protein. The broad range of RND substrates can reduce susceptibility of *A. baumannii* to multiple antimicrobials of different classes, as well as antiseptics, detergents, heavy metals, and disinfectants (6–11).

Characterized RND efflux pumps in *A. baumannii* are AdeABC, AdeFGH, and AdeIJK, whose expression is controlled by the two-component systems AdeRS (AdeABC), the LysR-like transcriptional regulator AdeL (AdeFGH), or the TetR-like repressor AdeN (AdeIJK) (12–14). Overexpression of AdeABC or AdeIJK has been shown to cause reduced antimicrobial susceptibility (15, 16). It was shown that overexpression of AdeABC is caused by amino acid substitutions within the dimerization and histidine-containing phosphotransfer domain (DHp) of its regulator sensor kinase AdeS, which includes the phosphorylation residue H149 (17–20). Moreover, amino acid substitutions within the receiver domain of the corresponding response regulator AdeR are also associated with AdeABC overexpression. In particular, changes of residues in spatial proximity of the phosphorylation site D63 have been shown to affect the function of AdeR (21, 22).

During a previous study of clinical southern European *A. baumannii* isolates, the AdeR double substitution D21V and D26N was observed in 17 of 65 isolates with high tigecycline MICs (18). The same double substitution was identified in a worldwide study; however, it was only found in a single European isolate (17). Within the same tigecycline surveillance study, the AdeS amino acid substitution T156M was identified in six different isolates with high tigecycline MICs from Asia and North and Latin America (17). The aim of this study was to investigate these hitherto-uncharacterized amino acid substitutions in terms of their contribution to increased efflux activity and reduced antimicrobial susceptibility (17, 18).

RESULTS

Antimicrobial susceptibility. The amino acid substitutions D21V and D26N have been identified in multiple clinical isolates in two different studies and were hypothesized to represent resistance mutations (17, 18). To characterize these substitutions, the *adeRS* knockout strain derived from ATCC 17978 was recomplemented with *adeRS* of the *A. baumannii* reference strain ACICU and cloned into the shuttle vector pJN17/04 with and without the corresponding nucleotide exchanges. To determine the impact of the amino acid substitutions, the mutant strains were subjected to antimicrobial susceptibility testing against eight different antimicrobial classes. MIC results are summarized in Table 1.

In comparison to 17978 *adeR*-wt, the amino acid substitution D26N in 17978 *adeR*-D26N caused a reduction in the susceptibility to aminoglycosides, macrolides, fluoroquinolones, carbapenems, and tetracyclines, including tigecycline. On the other hand, changing the amino acid from aspartic acid to valine at residue 21 of AdeR in 17978 *adeR*-D21V had no effect in antimicrobial susceptibility. However, combining D21V with D26N in 17978 *adeR*-D21V+D26N revealed the same antimicrobial resistance phenotype as the single D26N substitution in 17978 *adeR*-D26N, apart from a minor increase of the meropenem MIC. These data suggest that D21V has little or no impact on antimicrobial susceptibility.

Furthermore, the clinical isolate pair *A. baumannii* ABC153 and ABC154 was analyzed. These two isolates were genetically identical apart from a T156M amino acid substitution in

TABLE 1 MICs determined by agar dilution for amikacin, azithromycin, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, meropenem, minocycline, levofloxacin, rifampin, and tetracycline and by broth microdilution for tigecycline[a]

Strain	MIC (mg/liter) of:											
	AMK	GEN	AZI	ERY	CIP	LVX	CHL	MEM	RIF	MIN	TET	TGC
17978 *adeR*-wt	4	**8**	16	16	0.5	0.25	64	1	4	≤0.125	2	0.5
17978 *adeR*-D26N	8	**16**	32	32	1	0.5	64	2	4	0.25	4	2
17978 *adeR*-D21V	4	4	8	16	0.25	0.25	64	0.5	4	≤0.125	2	0.5
17978 *adeR*-D21V+D26N	8	**16**	32	32	1	0.5	64	1	4	0.25	4	2
ACICU	**>128**	**32**	32	32	**>128**	**32**	>128	1	8	2	32	2
ABC153	**>128**	**>128**	>128	>128	**>128**	**32**	>128	**64**	8	32	>128	8
ABC154	**>128**	**>128**	>128	>128	**64**	**16**	>128	**64**	8	16	>128	2

[a]AMK, amikacin; AZM, azithromycin; CHL, chloramphenicol; CIP, ciprofloxacin; ERY, erythromycin; GEN, gentamicin; MEM, meropenem; MIN, minocycline; LVX, levofloxacin; RIF, rifampin; TET, tetracycline (TET); TGC, tigecycline. Bold indicates resistance determined by EUCAST breakpoints (33).

AdeS of ABC153 (17). Within this isolate pair, the most striking change was revealed for tigecycline. The *adeS* wild-type strain ABC154 had a tigecycline MIC of 2 mg/liter, whereas ABC153 revealed a tigecycline MIC of 8 mg/liter. Furthermore, ABC153 revealed higher MICs for fluoroquinolones and minocycline.

AdeB expression studies. To determine whether the amino acid substitutions within AdeRS affect *adeB* expression, reverse transcription-quantitative PCR (qRT-PCR) was performed. Figure 1 shows that insertion of the D21V amino acid substitution into the WT *adeRS* backbone did not lead to a significant increase in expression of *adeB* ($P = 0.6876$). On the other hand, 17978 *adeR*-D26N exhibited a significant increase in *adeB* expression by a factor of 3.9-fold ($P = 0.0143$), compared to 17978 *adeR*-wt. Furthermore, comparing 17978 *adeR*-D21V and 17978 *adeR*-D26N revealed a 2.4-fold higher *adeB* expression for the strain carrying the D26N mutation ($P = 0.0106$). Additionally, combining D21V and D26N caused an *adeB* expression of a similar level to D26N alone. Analysis of *adeB* expression in the clinical isolate pair ABC153 and ABC154 exhibited a 15.8-fold higher expression in ABC153 ($P = 0.0094$), which is carrying the T156M amino acid substitution in AdeS, compared to ABC154 (Fig. 2).

Accumulation assay. To verify the direct correlation of increased *adeB* expression and efflux activity, the accumulation of ethidium was measured. Whereas insertion of the AdeR D21V mutation did not cause altered ethidium accumulation levels (Fig. S1 in the supplemental material), 17978 *adeR*-D26N revealed 25% lower ethidium accumulation in comparison to 17978 *adeR*-wt, indicating higher efflux activity induced by the D26N amino acid substitution in AdeR (Fig. 3A). Similar findings were revealed for the clinical isolate pair

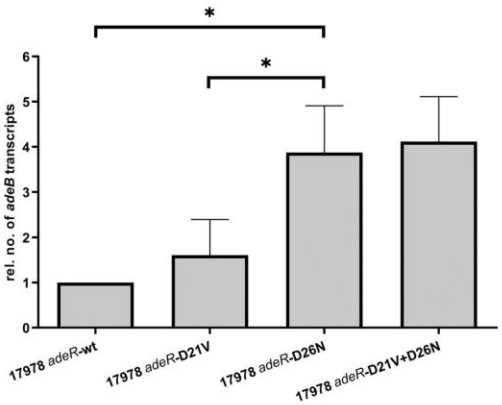

FIG 1 Relative *adeB* expression of *A. baumannii* ATCC 17978-derived *adeR* mutant strains determined by qRT-PCR. Results are represented as means ± standard errors of the means. Statistical analysis was done by using an unpaired *t* test of the absolute values. *, $P < 0.015$.

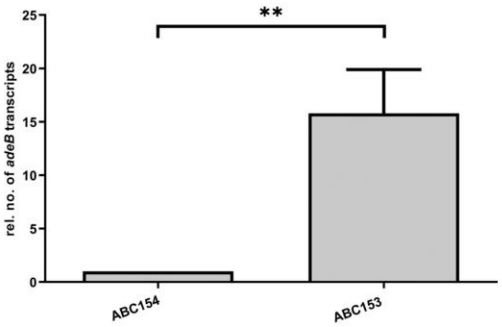

FIG 2 Relative *adeB* expression of *A. baumannii* isolates ABC153 and ABC154 determined by qRT-PCR. Results are represented as means ± standard errors of the means. Statistical analysis was done by using an unpaired *t* test of the absolute values. **, $P < 0.01$.

ABC153 and ABC154. Here, the *adeS* wild-type strain ABC154 was found to exhibit 12% higher accumulation than ABC153 (Fig. 4A). This effect was abolished within the compared strain couples by performing the ethidium accumulation assay in the presence of the proton motive force uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Inhibiting efflux with CCCP caused almost identical levels of ethidium accumulation for 17978 *adeR*-wt and 17978 *adeR*-D26N (Fig. 3B) and also for the isolates ABC153 and ABC154 (Fig. 4B). These results confirm that the described differences of ethidium accumulation are associated with increased efflux activity.

DISCUSSION

The contribution of mutations in the two-component regulatory system encoded by *adeRS* to increased expression of the RND-type efflux pump AdeABC and therefore

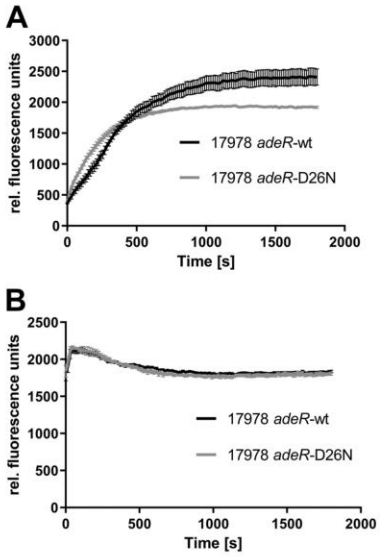

FIG 3 Ethidium bromide (EtBr)accumulation in ATCC 17978 Δ*adeRS* transformants that were untreated (A) and treated with CCCP (500 μm) (B). Fluorescence was measured every 15 s over 30 min. Data were collected from three independent experiments and are presented as means ± standard errors of the means.

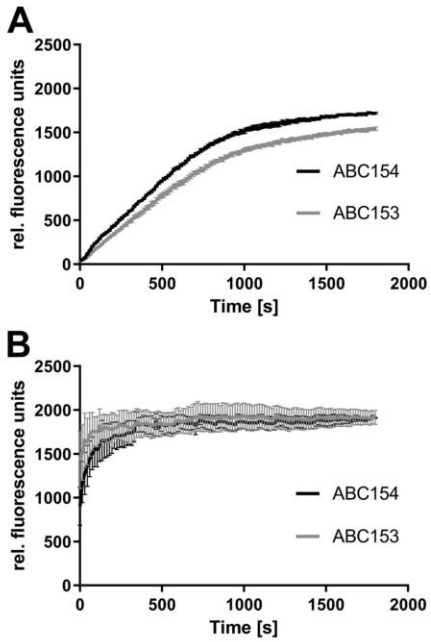

FIG 4 EtBR accumulation of clinical isolates ABC153 and ABC154 that were untreated (A) and treated with CCCP (500 μm) (B). Fluorescence was measured every 15 s over 30 min. Data were collected from three independent experiments and are presented as means ± standard errors of the means.

to reduced antimicrobial susceptibility has been described in various studies (19, 21). We have shown previously that *adeB* expression is abolished when *adeRS* is deleted, and we revealed a strain-dependent increased susceptibility to aminoglycosides, carbapenems, fluoroquinolones, glycylcyclines, macrolides, and tetracyclines (23). As the currently available efflux pump inhibitors have no therapeutic value due to their toxicity, more insight to the mechanisms of RND efflux pump regulation is required (24). Targeting the functionality of the two-component system AdeRS seems to be particular promising since the other MDR-associated RND efflux pump AdeIJK is regulated by the repressor AdeN, and affecting it is an inappropriate option to reduce efflux activity.

In the present study, we investigated three amino acid substitutions that were until now uncharacterized, D21V and D26N in AdeR, as well as T156M in AdeS, which were previously identified in clinical *A. baumannii* isolates which exhibited high tigecycline MICs (17, 18). Since tigecycline is known to be a substrate of RND-type efflux pump AdeABC (25), an increased activity of this pump caused by regulatory mutations was suspected and investigated in the present study.

Various mutations in *adeS* have already been associated with increased AdeABC efflux, including truncation by insertion sequence IS*Aba1*, and several amino acid substitutions (16–18, 26). These mutations were predominantly observed within the histidine kinase, adenylyl cyclase, methyl-accepting protein, and phosphatase (HAMP) and DHp domain (20). Of those, many substitutions were found adjacent or in close proximity to the autophosphorylation site H149, e.g., Q141R, R152K, T153A, T153M, and D167N, indicating a mutational hot spot (17–19, 27). Investigation of AdeS T156M was based on a clinical isolate pair described in a previous study, where the two isolates were identical except for the T156M amino acid substitution in AdeS and susceptibility to tigecycline (17).

Our study confirms that increased *adeB* expression, efflux activity, and, consequently, reduced antimicrobial susceptibility, is triggered by the AdeS T156M amino acid substitution. Since this mutation is located within the same hot spot region of the AdeS kinase domain, although the T156 is not directly involved in the dimerization or *cis*-autophosphorylation of AdeS as revealed by the structure (Fig. S2 in the supplemental material), our findings open up the possibility that these mutations increase the sensitivity of AdeS to environmental stimuli or facilitate activation of the response regulator AdeR by increasing its phosphorylation ratio, which results in increased AdeABC expression (28).

For detailed characterization, the AdeR with amino acid substitutions D21V and D26N was integrated into the wild-type (WT) *adeRS* backbone of *A. baumannii* international clone 2 (IC2) reference strain ACICU. Since ACICU is resistant to many commonly used selective markers, it could not be used for characterization of a distinct resistance mechanism. Therefore, *adeRS* of ACICU was cloned into the shuttle vector pJN17/04 and transferred to an *adeRS* deletion strain obtained from ATCC 17978, which is preferred for genetic manipulation because of its well-understood growth dynamics and efficient transformant rates. Although the wild-type ATCC 17978 was shown to be inappropriate for analysis of AdeABC efflux because of low *adeB* expression rates caused by an L172P amino acid substitution in AdeS, it was found to be suitable for characterizing *adeRS* mutations if the WT *adeRS* backbone was replaced with *adeRS* of other reference strains like ACICU (23). Previously, Wen et al. revealed the structure of the response regulator AdeR and showed that residues E19, D20, and K65 and the phosphorylation site D63 form a highly conserved magnesium binding pocket, and they consequently suggested that substitutions of contributing amino acids enhance phosphorylation by AdeS (22). A D20N substitution in AdeR was also shown to increase *adeB* expression and reduce antimicrobial susceptibility (21). Although residue 21 is in close proximity to the binding pocket and the amino acid substitution D21V has been detected in multiple isolates with high tigecycline MICs (17, 18), the contribution of this exchange to increased AdeABC efflux could not be verified in the present study. In contrast to these findings, the AdeR D26N mutation revealed a significant impact on AdeABC expression and subsequently on antimicrobial susceptibility. The residue is located in the α1 helix of AdeR and, consequently, is not directly involved in the dimerization interface (Fig. S3) (22). However, the substitution of aspartic acid to asparagine may alter the binding of AdeR to AdeS during phosphorylation since it is lacking the hydrogen bond acceptor carboxyl group. Nevertheless, the detailed mechanism caused by this mutation remains to be further investigated. Gaining mechanistic insight into the AdeS and AdeR interaction may shed light on the impact of the response regulator D26N substitution on the function of the AdeRS complex.

In summary, RND-type efflux pumps are an essential feature for *A. baumannii* to survive in harsh environments such as a modern intensive care unit by reducing its susceptibility to antimicrobials and biocides. The present study demonstrates the contribution of the AdeS T156M and AdeR D26N substitutions in causing overexpression of the AdeABC efflux pump, resulting in reduced antimicrobial susceptibility. Furthermore, the present data highlight that RND efflux regulators represent a promising target that should be considered in the development of novel antibacterial therapies.

MATERIALS AND METHODS

Strains and isolates used in the present study. The bacterial strains and plasmids used in this study are listed in Table 2. Generation of ATCC 17978 Δ*adeRS* was described previously (23). The clinical isolates ABC153 and ABC154 are an isolate pair, genetically identical apart from a point mutation in *adeS*, causing the amino acid substitution T156M (17).

Bacterial growth. Cells were grown at 37°C in Luria-Bertani (LB) broth or agar. Strains transformed with pJN17/04 were selected by media supplemented with 10 mg/liter kanamycin (21).

Plasmid purification. All plasmids used in this study were extracted using the QIAprep Spin miniprep kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Transformation of *A. baumannii* ATCC 17978 Δ*adeRS*. ATCC 17978 Δ*adeRS* was transformed with purified plasmids by electroporation as described previously (29), using the Gene Pulser II system (Bio-Rad, Munich, Germany). Selection of transformants was performed by growth on LB agar supplemented with 10 mg/liter kanamycin.

Generation of the shuttle vector pJN17/04::*adeRS*^{ACICU}. The *adeRS* backbone of the reference strain ACICU (30) was used since it represents the wild type of the globally spread *A. baumannii* international

TABLE 2 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic	Source or reference
E. coli strains		
HST08 Stellar	Chemically competent	TaKaRa Clontech
NEB 5-alpha competent cells	Chemically competent	New England BioLabs
A. baumannii strains		
ACICU	*A. baumannii* reference strain	30
ABC153	Genetically identical to ABC154 apart AdeS T156M substitution	17
ABC154	Genetically identical to ABC153 apart AdeS wild-type configuration	17
ATCC 17978 Δ*adeRS*	*A. baumannii* reference strain, *adeRS* deleted	23
17978 *adeR*-wt	ATCC 17978 Δ*adeRS* pJN17/04::*adeRS*^{ACICU}	This study
17978 *adeR*-D21V	ATCC 17978 Δ*adeRS* pJN17/04::*adeR*(D21V)*S*^{ACICU}	This study
17978 *adeR*-D26N	ATCC 17978 Δ*adeRS* pJN17/04::*adeR*(D26N)*S*^{ACICU}	This study
17978 *adeR*-D21V+D26N	ATCC 17978 Δ*adeRS* pJN17/04::*adeR*(D21V,D26N)*S*^{ACICU}	This study
Plasmids		
pJN17/04	Shuttle vector	21
pJN17/04::*adeRS*^{ACICU}	*adeRS* of ACICU fused to pJN17/04 backbone	This study
pJN17/04::*adeR*(D21V)*S*^{ACICU}	AdeR D21V substitution in pJN17/04::*adeRS*^{ACICU}	This study
pJN17/04::*adeR*(D26N)*S*^{ACICU}	AdeR D26N substitution in pJN17/04::*adeRS*^{ACICU}	This study
pJN17/04::*adeR*(D21V,D26N)*S*^{ACICU}	AdeR D21V and D26N double substitution in pJN17/04::*adeRS*^{ACICU}	This study

clone 2 (IC2) and was therefore more appropriate for characterization of amino acid substitutions identified in recent clinical isolates than the uncommon *adeRS* configuration of ATCC 17978 (23). Genomic DNA was extracted from a heat-induced crude cell lysate. Amplification of the target DNA was performed using Q5 high-fidelity DNA polymerase (New England BioLabs, Frankfurt, Germany). PCR settings were adjusted according to the PCR product size and the nucleotide sequence of the primer pair O47-O48 (Table S1 in the supplemental material). PCR products were purified using the QIAquick PCR purification kit (Qiagen). The shuttle vector pJN17/04 (21) was linearized by the restriction enzyme ScaI-HF (New England BioLabs). The In-Fusion HD cloning kit (TaKaRa Clontech, Saint-Germain-en-Laye, France) was used for directional cloning of *adeRS* into the shuttle vector. Cloned plasmids were transferred into chemically competent *Escherichia coli* HST08 cells via heat shock according to the manufacturer's instructions.

Introduction of amino acid substitutions into pJN17/04::*adeRS*^{ACICU}. The Q5 site-directed mutagenesis kit (New England BioLabs) was used to exchange single nucleotides within the sequence of *adeR* of pJN17/04::*adeRS*^{ACICU} to introduce the amino acid substitutions D21V and D26N. Primers for PCR amplification of the plasmid, including the corresponding nucleotide exchange, were designed using the online tool NEBaseChanger (New England BioLabs) (Table S1). Modified plasmids were transferred to competent NEB 5-alpha cells by heat shock. Sanger sequencing (LGC Genomics GmbH, Berlin, Germany) was used to confirm the correct nucleotide exchange.

Antimicrobial susceptibility testing. Susceptibility to tetracycline, gentamicin (Sigma-Aldrich, Steinheim, Germany), meropenem, amikacin, minocycline, rifampin (Molekula, Newcastle upon Tyne, UK), levofloxacin (Sanofi Aventis, Frankfurt, Germany), ciprofloxacin (Bayer Pharma AG, Berlin, Germany), azithromycin (Pfizer Pharma GmbH, Münster, Germany), chloramphenicol (Serva, Heidelberg, Germany), and erythromycin (AppliChem, Darmstadt, Germany) was tested by agar dilution following current CLSI guidelines (31).

The MIC for tigecycline (Molekula, Newcastle upon Tyne, UK) was determined by broth microdilution following CLSI guidelines (31).

qRT-PCR. Expression of *adeB* was evaluated by qRT-PCR as described previously (32). *rpoB* was used as a reference gene, and its expression was quantified concurrently with *adeB* expression. Primers used for the amplification of *adeB* and *rpoB* are listed in Table S1. Freshly prepared RNA (RNeasy; Qiagen) and cDNA (Quantiscript reverse transcriptase; Qiagen) were used to perform qRT-PCR with SYBR green master mix (Qiagen) in triplicates, and the experiment was repeated independently at least three times. The number of *adeB* transcripts in ATCC 17978 Δ*adeRS* pJN17/04::*adeRS*^{ACICU} (here referred to as 17978 *adeR*-wt), pJN17/04::*adeR*(D26N)*S*^{ACICU} (here referred to as 17978 *adeR*-D26N), pJN17/04::*adeR*(D21V)*S*^{ACICU} (here referred to as 17978 *adeR*-D21V), and pJN17/04::*adeR*(D21V, D26N)*S*^{ACICU} (here referred to as 17978 *adeR*-D21V+D26N) transformants were compared with each other. In a separate experiment, the number of *adeB* transcripts was compared between isolate ABC153 and ABC154.

Statistical analysis was performed via GraphPad Prism 9.2.0 (San Diego, California USA) with an unpaired *t* test using the recorded absolute values.

Accumulation studies. The AdeABC substrate ethidium bromide was used to investigate efflux activity. Cells grown in LB broth to log phase were washed twice in potassium phosphate buffer (50 mM potassium phosphate buffer, 1 mM $MgSO_4$ [pH 7.4]), and adjusted to an optical density at 600 nm of 20. Cells were kept on ice during washing. Pelleting was done in a centrifuge at 4°C and 4,000 × *g* for 5 min. Afterward, the suspension was pipetted to a 96-well Nunclon Delta surface plate (Thermo Fisher Scientific, Schwerte, Germany) and supplemented with 0.2% (wt/vol) glucose (Sigma-Aldrich) and a final ethidium bromide (Merck, Darmstadt, Germany) concentration of 10 μM. The fluorescence was measured in an Infinite M1000 Pro plate reader (Tecan, Crailsheim, Germany) every 15 s over a period of

30 min. The plate reader was set to an excitation wavelength of 530 nm and an emission wavelength of 600 nm.

Accumulation studies were carried out with and without the proton motive force uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Sigma-Aldrich), used at a final concentration of 500 μM.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 0.5 MB.

FIG S2, TIF file, 1.3 MB.

FIG S3, TIF file, 1.4 MB.

TABLE S1, DOCX file, 0.02 MB.

ACKNOWLEDGMENTS

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We have no conflict of interest to declare.

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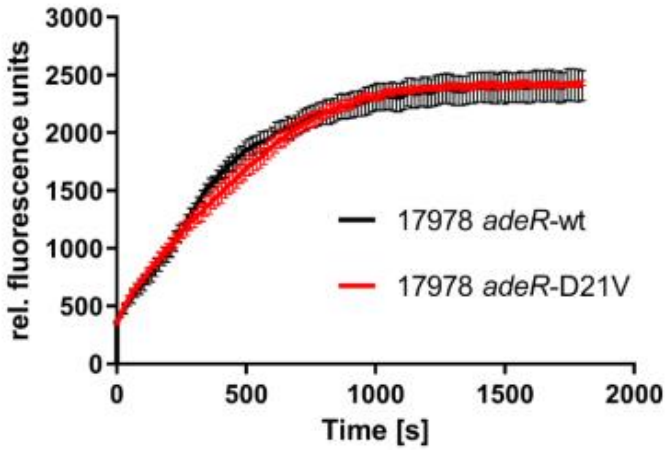
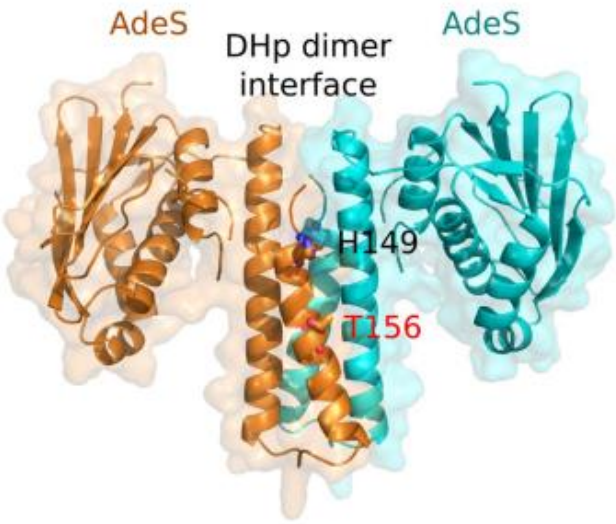
Supplementary table 1. Primers used in this study.

Target	Primer Name	Sequence (5′ - 3′)	Size (bp)	Experiment
adeRS^{ACICU}	O47	gccactcatcgcagtATTGCGGTTGAATGCTTAATACAC	2149	InFusion cloning into pJN17/04
	O48	atgaattacaacagtAGACAGCTTGGGATCAGGAAGTC		
pJN17/04::*adeRS*	N72	CGATATTGGC**A**ACATTATTGAAAATTATTTAAAAC	8473	Site-Directed Mutagenesis *adeR*(D26N)
	N73	TAGTCATCTTCTACCACAAG		
pJN17/04::*adeRS*	O27	GTAGAAGATG**T**CTACGATATTGG	8437	Site-Directed Mutagenesis *adeR*(D21V)
	O28	CACAAGAATAACTTTATCTTGG		
	O38	GCCATCAATAATTCCCTG		
adeR	D31	GGAGTAAGTGTGGAGAAATACGG	937	Sanger sequencing
	K57	ACCCAGTACTACAGAAAATAGCG		
adeS	K28	AAACTTGCTCAATACGACGG	518	Sanger sequencing
	K42	AAAGCGTTTTATTGTGCCAA		
rpoB	C65	GAGTCTAATGGCGGTGGTTC	110	qRT-PCR
	C66	ATTGCTTCATCTGCTGGTTG		
adeB	A48	GTATGAATTGATGCTGC	992	Standard for qRT-PCR
	A49	CACTCGTAGCCAATACC		
	M26	CAAGGACGTATGCAACAAGT	103	qRT-PCR
	M27	CTAATTGACCGCTTGAACCC		

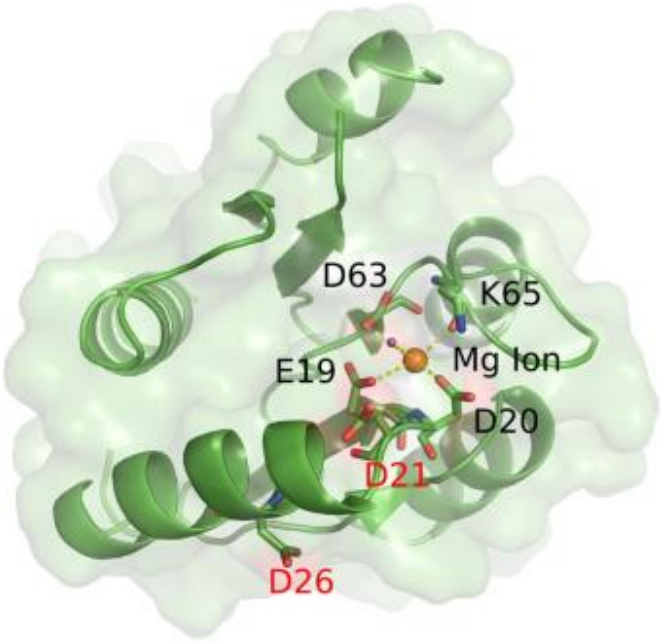
Lowercase: tails for InFusion cloning
Bold and in a box: site of nucleotide substitution



Supplementary figure 1. Comparison of EtBR accumulation between 17978 *adeR*-wt and 17978 *adeR*-D21V. Fluorescence was measured every 15 s over 30 min. Data were collected from three independent experiments and are presented as means ± standard errors of the means.



Supplementary figure 2. Crystal structure of AdeS. The residue T156 (red), which is changed to M156 in ABC 153, is located within the dimerization and histidine-containing phosphotransfer domain (DHp) on the same α helix as the phosphorylation residue H149. Crystal structure obtained from Ouyang et al. (33). Illustration designed with the PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.



Supplementary figure 3. Crystal structure of AdeR. The investigated residues D26 (red) and D21 (red) are adjacent to a magnesium binding pocket consisting of residues E19, D20, the phosphorylation site D63 and K65. Crystal structure obtained from Wen et al.(22). Illustration designed with the PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.

3.4 Characterization of *adeN* and *adeL* knockouts in *A. baumannii* ATCC 19606

The RND efflux pump AdeIJK, similar to AdeABC, has a wide substrate spectrum, which partly overlaps with AdeABC.[102] AdeIJK is reported to be constitutively expressed and to contribute to reduced susceptibility to antimicrobials like β-lactams, fluoroquinolones, tetracyclines, tigecycline, rifampin, chloramphenicol, and erythromycin.[87, 121] Expression of AdeIJK is regulated by the repressor AdeN and it was shown that disruption by IS or deletion of *adeN* causes reduced antimicrobial susceptibility in *A. baumannii*.[63, 128] The least characterized RND efflux pump in *A. baumannii* is AdeFGH. The expression of AdeFGH is controlled by the LTTR AdeL. It has been shown that mutations within *adeL* can cause overexpression of AdeFGH and consequently reduce susceptibility to chloramphenicol, fluoroquinolones, tetracyclines, and tigecycline.[129]

As deletion of *adeRS* in *A. baumannii* ATCC 19606 was shown previously to have a large impact on antimicrobial susceptibility, attention was turned to introducing gene knockouts for the other RND regulatory genes, *adeN* and *adeL*. *A. baumannii* ATCC 19606 Δ*adeN* was kindly provided by Stefanie Gerson, while the Δ*adeL* was generated using markerless mutagenesis. Strains used in this study are listed in Table 1, primers used for markerless mutagenesis are listed in Table 2. Antimicrobial susceptibility was tested by agar dilution or, in the case of tigecycline, by broth microdilution.

Table 1. Strains and plasmids used to investigate *adeN* and *adeL*.

Strain		**Relevant characteristics**	**Reference**
A. baumannii	ATCC 19606	reference strain	[182]
	ATCC 19606 Δ*adeN*		[183]
	ATCC 19606 Δ*adeL*		This study
E. coli	HST08 (Stellar™)	chemically competent	Clontech Takara
Plasmids	pBIISK::*sacB/kanR*	markerless mutagenesis	[172]
	pBIISK::*sacB/kanR::adeL*^{upstream} ::*adeL*^{downstream}	deletion of *adeL* by markerless mutagenesis	This study

Table 2. Primers used for *adeL* deletion in *A. baumannii* ATCC 19606.

Target	Primer Name	Sequence (5′ - 3′)	Size (bp)	Experiment
adeL^{19606} upstream	M54	ctggcagagcatcgaattcc CAGCAATCACTTTTGGGTTT	1623	Creation of the pBIISK::*sacB*::*kanR* ::*adeL*^{upstream}::*adeL*^{downstream} plasmid for *adeL* deletion in ATCC 19606
	M55	acggattaaatgtgaaaa GGCATGAAATAGATCCACAC		
adeL^{19606} down-stream	M58	gtgtggatctatttcatgcc TTTTCAACATTTAATCCGT	1987	
	M59	aactagtggatccccgggc CGTGGACATAATCAAAGCTA		

Lowercase: tails for In-Fusion cloning

In contrast to the deletion of *adeRS* in *A. baumannii* ATCC 19606, the loss of the regulators *adeN* and *adeL* revealed a much lesser effect on antimicrobial susceptibility (Figure 10). *A. baumannii* ATCC 19606 Δ*adeN* revealed increased MICs for chloramphenicol, minocycline, tetracycline, and tigecycline each by one dilution step. On the other hand, deletion of *adeL* only caused a reduction of the meropenem MIC by one dilution step.

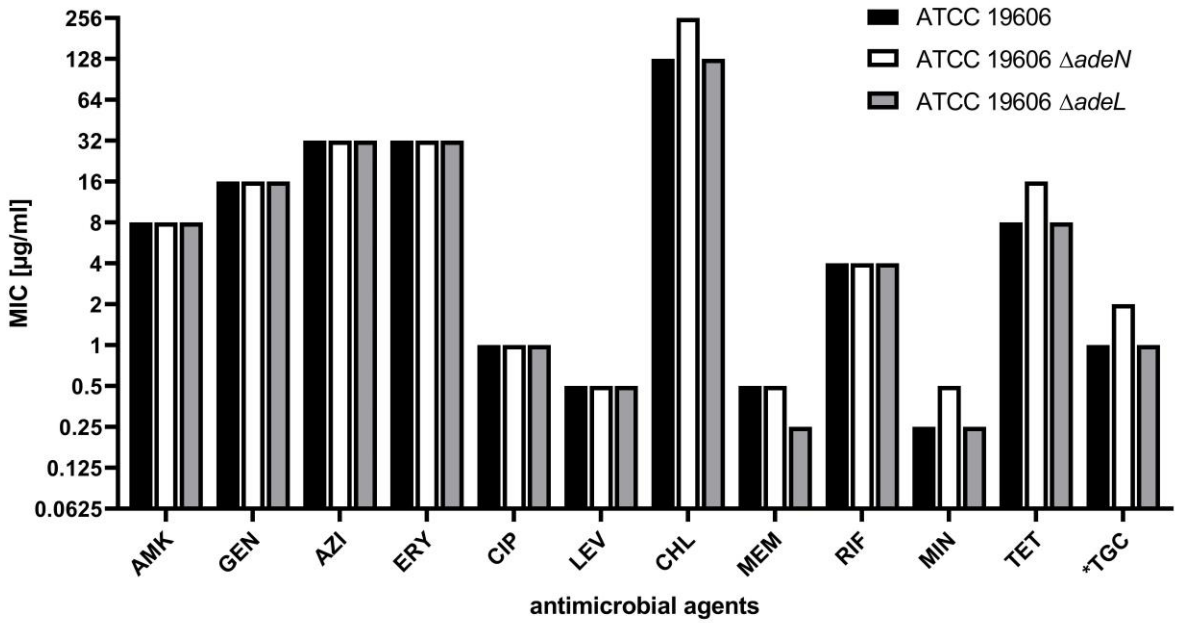


Figure 10. Minimal inhibitory concentrations determined by agar dilution for amikacin (AMK), azithromycin (AZM), chloramphenicol (CHL), ciprofloxacin (CIP), erythromycin (ERY), gentamicin (GEN), meropenem (MEM), minocycline (MIN), levofloxacin (LVX), rifampicin (RIF), tetracycline (TET), *and by broth micro-dilution for tigecycline (TGC).

It was described previously that overexpression of AdeFGH, which is regulated by AdeL, is associated with biofilm formation.[130] Therefore, the biofilm formation of *A. baumannii* ATCC 19606 Δ*adeL* and its parental strain were compared. For this purpose, biofilms were grown in clear flat bottomed 96-well plates containing 100 µl LB suspension of the respective strain adjusted to an OD_{600} of 0.03. After 24 h of static incubation at 37 °C the supernatants were aspirated, and adherent cells were washed gently with sterile phosphate buffered saline (PBS). Biofilm quantification was carried out as described previously using crystal violet staining and measuring the corresponding OD_{550} in the TECAN Infinite M1000 reader.[184] It was shown that the biofilm formation of the Δ*adeL* strain had significantly reduced (*P*=0.01) biofilm compared to wild-type (wt) *A. baumannii* ATCC 19606 (Figure 11).

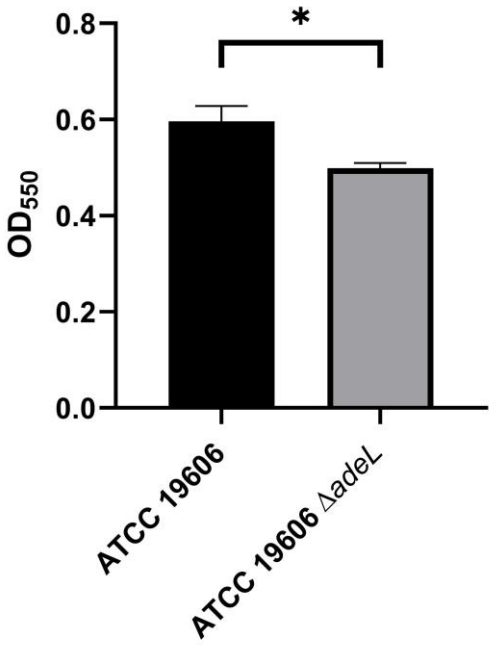


Figure 11. Biofilm quantification of *A. baumannii* ATCC 19606 wildtype and ATCC 19606 Δ*adeL* determined by crystal violet staining. Biofilm formation of *A. baumannii* ATCC 19606 Δ*adeL* was found significantly reduced compared to *A. baumannii* ATCC 19606. Biofilm quantification was performed in triplicates. Results are represented as means ± standard errors of the means. Statistical analysis was done by using an unpaired t-test. **P*<0.01.

3.5 Investigation of growth-dependent expression of RND efflux pumps and their corresponding regulatory genes

The preceding results highlighted the clinical importance of RND overexpression. Therefore, the project was extended for baseline RND efflux expression analysis. *A. baumannii* growth can take several different forms like sessile on an agar plate, motile on motility plates, planktonic in broth, and as a biofilm. Variation in the mode of growth is induced by differential transcriptome patterns.[185, 186] Little is known about how different growth conditions affect the expression of RND efflux pumps and their regulators. It was shown that *adeB*, *adeG*, and *adeJ* are constitutively expressed in early, mid and late log phases, as well as in the stationary phase.[187] The response to osmotic stress, temperature, and pH on expression of the pumps and their corresponding regulators remain to be investigated. Therefore, *lacZ*, *gfp*, and *luxCDABE* reporter systems were established to monitor the expression of RND efflux pump and regulator genes of *A. baumannii* (Table 3), by cloning the corresponding promoter regions into the reporter plasmids pIG14/09 (LacZ), pWH1266::*sfgfp* (sfGFP), and pLPV1Z (luciferase). Additionally, the promoter of the OMP gene *carO* was used to generate a positive control, because of its generally high expression levels, whereas strains transformed with promoterless reporter plasmids were used as negative control. The primers used for cloning insert generation are listed in Table 4.

Table 3. Strains and plasmids used to monitor RND pump and regulator expression.

Strain		Relevant characteristics	Reference
A. baumannii	ATCC 17978	reference strain	[182]
E. coli	HST08 (Stellar™)	chemically competent	Clontech Takara
Plasmids	pIG14/09	*lacZ* reporter plasmid	[188]
	pIG14/09::*adeR*	pIG14/09 backbone, *lacZ* fused to *adeRS* promoter	[176]
	pIG14/09::*adeA*	pIG14/09 backbone, *lacZ* fused to *adeABC* promoter	[188]
	pIG14/09::*adeN*	pIG14/09 backbone, *lacZ* fused to *adeN* promoter	This study
	pIG14/09::*adeI*	pIG14/09 backbone, *lacZ* fused to *adeIJK* promoter	This study
	pIG14/09::*adeL*	pIG14/09 backbone, *lacZ* fused to *adeN* promoter	This study
	pIG14/09::*adeF*	pIG14/09 backbone, *lacZ* fused to *adeFGH* promoter	This study
	pIG14/09::*carO*	pIG14/09 backbone, *lacZ* fused to *carO* promoter	[189]
	pLPV1Z	*luxCDABE* reporter plasmid	[178]
	pLPV1Z::*adeR*	pLPV1Z backbone, *luxCDABE* fused to *adeRS* promoter	This study
	pLPV1Z::*adeA*	pLPV1Z backbone, *luxCDABE* fused to *adeABC* promoter	This study
	pLPV1Z::*adeN*	pLPV1Z backbone, *luxCDABE* fused to *adeN* promoter	This study
	pLPV1Z::*adeI*	pLPV1Z backbone, *luxCDABE* fused to *adeIJK* promoter	This study
	pLPV1Z::*adeL*	pLPV1Z backbone, *luxCDABE* fused to *adeL* promoter	This study
	pLPV1Z::*adeF*	pLPV1Z backbone, *luxCDABE* fused to *adeFGH* promoter	This study
	pLPV1Z::*carO*	pLPV1Z backbone, *luxCDABE* fused to *carO* promoter	This study
	pWH1266::*sfgfp*	*sfgfp* reporter plasmid	[176]
	pWH1266::*sfgfp::adeR*	pWH1266::*sfgfp* backbone, *sfgfp* fused to *adeRS* promoter	[176]
	pWH1266::*sfgfp::adeA*	pWH1266::*sfgfp* backbone, *sfgfp* fused to *adeABC* promoter	[176]
	pWH1266::*sfgfp::adeN*	pWH1266::*sfgfp* backbone, *sfgfp* fused to *adeN* promoter	This study
	pWH1266::*sfgfp::adeI*	pWH1266::*sfgfp* backbone, *sfgfp* fused to *adeIJK* promoter	This study
	pWH1266::*sfgfp::adeL*	pWH1266::*sfgfp* backbone, *sfgfp* fused to *adeL* promoter	This study
	pWH1266::*sfgfp::adeF*	pWH1266::*sfgfp* backbone, *sfgfp* fused to *adeF* promoter	This study
	pWH1266::*sfgfp::carO*	pWH1266::*sfgfp* backbone, *sfgfp* fused to *carO* promoter	This study

Table 4. Primers used for reporter plasmid generation.

Target	Primer Name	Sequence (5′ - 3′)	Size (bp)	Experiment
adeR promoter region	K72	atccggggaattcccGCTGCTCATAACGTTCTAA	647	InFusion Clonig into pIG14/09
	K73	aacgacgggatccccCCGAATAACACTCATGCC		
adeN promoter region	M74	atccggggaattcccTAAGCCAATGGGCAATTCAA	604	InFusion Clonig into pIG14/09
	M75	aacgacgggatccccTGACTCAAGGACTGGATCA		
adeI promoter region	M76	atccggggaattcccTGTTATTTTCATTAGCTTGGG	469	InFusion Clonig into pIG14/09
	M77	aacgacgggatccccTGCCCAAAGCTTAGCCGA		
adeL promoter region	M72	atccggggaattcccTTGATCAATTGCTTCTAAACG	482	InFusion Clonig into pIG14/09
	M73	aacgacgggatccccAACAACTTTGTTGAATACTCTC		
adeF promoter region	M70	atccggggaattcccATACGGGCTGTCCGATCA	463	InFusion Clonig into pIG14/09
	M71	aacgacgggatccccCTGTTTGCGGGAAAATGAC		
adeR promoter region	P2	ttgatatcgaattccGCTGCTCATAACGTTCTAACTG	676	InFusion Clonig into pLPV1Z
	P3	gtggatcccccgggcCCGAATAACACTCATGCCTTCAC		
adeA promoter region	P4	ttgatatcgaattccAAGAATGATCAAACATAGAAAATCT	211	InFusion Clonig into pLPV1Z
	P5	gtggatcccccgggcAGTAAAAGATGCTTTTGCATACTGT		
adeN promoter region	P8	ttgatatcgaattccTAAGCCAATGGGCAATTCAAAAAC	574	InFusion Clonig into pLPV1Z
	P9	gtggatcccccgggcTGACTCAAGGACTGGATCATGCA		
adeI promoter region	P10	ttgatatcgaattccTGTTATTTTCATTAGCTTGGGGG	469	InFusion Clonig into pLPV1Z
	P11	gtggatcccccgggcTGCCCAAAGCTTAGCCGAC		
adeL promoter region	P12	ttgatatcgaattccTTGATCAATTGCTTCTAAACGACCG	482	InFusion Clonig into pLPV1Z
	P13	gtggatcccccgggcAACAACTTTGTTGAATACTCTCATG		
adeF promoter region	P14	ttgatatcgaattccATACGGGCTGTCCGATCA	463	InFusion Clonig into pLPV1Z
	P15	gtggatcccccgggcCTGTTTGCGGGAAAATGACA		

Lowercase: tails for In-Fusion cloning

Table 4 continued. Primers used for reporter plasmid generation.

Target	Primer Name	Sequence (5′ - 3′)	Size (bp)	Experiment
carO promoter region	P6	ttgatatcgaattcc**CAACAGCTTGGCGAATTTCG**	423	InFusion Clonig into pLPV1Z
	P7	gtggatcccccgggc**AATACTTTCATCGTTTTCTCCTTAA**		
sfgfp	L13	cgttgttgccattgctgcag**GAGAAAATGCGCAAAGGC**	800	InFusion Clonig into pWH1266
	L14	tgacaccacgatgcc**CAAATTTTATTACGCAACCAG**		
adeR promoter region	L30	cgttgttgccattgc**GCTGCTCATAACGTTCTAAC**	676	InFusion Clonig into pWH1266::*sfgfp*
	L32	ttgcgcattttctcc**ATAACACTCATGCCTTCACG**		
adeA promoter region	M14	cgttgttgccattgc**AAGAATGATCAAACATAGAAAAT**	211	InFusion Clonig into pWH1266::*sfgfp*
	M15	ttgcgcattttctcc**AGTAAAAGATGCTTTTGCATAC**		
adeN promoter region	N17	cgttgttgccattgc**TAAGCCAATGGGCAATTCAAAAAC**	574	InFusion Clonig into pWH1266::*sfgfp*
	N18	ttgcgcattttctcc**TGACTCAAGGACTGGATCATGCA**		
adeI promoter region	N19	cgttgttgccattgc**TGTTATTTTCATTAGCTTGGGGG**	469	InFusion Clonig into pWH1266::*sfgfp*
	N20	ttgcgcattttctcc**TGCCCAAAGCTTAGCCGAC**		
adeL promoter region	N13	cgttgttgccattgc**TTGATCAATTGCTTCTAAACGACCG**	482	InFusion Clonig into pWH1266::*sfgfp*
	N14	ttgcgcattttctcc**AACAACTTTGTTGAATACTCTCATG**		
adeF promoter region	N15	cgttgttgccattgc**ATACGGGCTGTCCGATCA**	463	InFusion Clonig into pWH1266::*sfgfp*
	N16	ttgcgcattttctcc**CTGTTTGCGGGAAAATGACA**		
carO promoter region	L60	cgttgttgccattgc**CAACAGCTTGGCGAATTTC**	423	InFusion Clonig into pWH1266::*sfgfp*
	L61	ttgcgcattttctcc**AATACTTTCATCGTTTTCTCCT**		

Lowercase: tails for In-Fusion cloning

3.5.1 Expression of RND efflux genes in the presence of antimicrobials and substrates altering osmotic pressure and pH

To evaluate the impact on RND pump and regulator expression in *A. baumannii* through different external substrates (Table 5) changing e.g. osmotic or pH conditions of the environment, *A. baumannii* ATCC 17978 *lacZ*-reporter transformants were grown on gradient plates.[190, 191] Gradient plates consisted of two different 20 ml MH-agar layers as shown in Figure 12; the bottom layer (orange), supplemented with a test substance and 40 mg/l X-gal was solidified obliquely so that the agar covered the whole plate, but not with an even thickness; the top layer (blue) supplemented with 40 mg/l X-gal only, was poured into the levelled plate. Thus, diffusion proportional to the thickness of the upper layer produced a steady substrate gradient across the agar surface. After solidification of the agar, a McF 0.5 suspension of the respective test strain was applied in a straight line along the gradient using a sterile swab. Additionally, MH-agar plates prepared with 40 mg/l X-gal were used to test the reporter transformants against a large set of antimicrobials applied by disc diffusion (Table 5). After incubation for 24 h at 37 °C, expression of all RND pumps and regulators was detected with every tested gradient setup, uncomplemented MH-agar, and antimicrobial disc until toxicity was reached, inhibiting bacterial growth. Accordingly, none of the tested substrates was found to impair RND pump or regulator expression in *A. baumannii*.

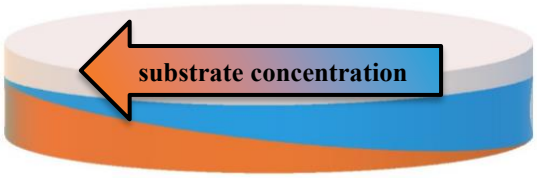


Figure 12. Schematic illustration of a gradient plate.

Preliminary data of this study have been presented as a poster at the 11th International Symposium on the Biology of Acinetobacter, September 20-22, 2017, Seville, Spain, and the 70th. DGHM-Conference, Bochum, February 2018, and have been part of my master thesis, University of Duisburg-Essen, 2017, as specified in Table 3.

Table 5. Substrates used for gradient assays.

Substance category	Substance class	Substance	Concentration
Acids		HCl	12.5 mM
		Ethylenediaminetetraacetic acid	1%
Azides		Sodium azide	40 mg/l
Alcohols		Ethanol	5% ,10%
		Glycerol	1%, 5%
		Methanol	0.5%
		Isopropanol	0.05%
Alkalis		NaOH	12.5 mM
Antimicrobials	Aminoglycosides	Kanamycin	2 mg/l
	ß-lactams	Ampicillin	64 mg/l
	Quinolones	Ciprofloxacin	0.25 mg/l
		Nalidixic acid	8 mg/l
	Bleomycins	Zeocin	10 mg/l
Bile salts		Sodium deoxycholate	20 mM
Detergents		Sarkosyl	1%
		SDS	1%
Dyes		Comassie blue	8 mg/l
		Safranin O	1 mg/l
Salts		NaCl	200 mM, 400 mM
		KCl	300 mM
		$ZnSO_4$	15 mM
Solvents		DMSO	5%
Sugars		Glucose	50 mM
		Sucrose	30 mM, 300mM

Table 6. Antimicrobial discs (Oxoid, Wesel, Germany).

Antimicrobial disc	Abbreviation	Concentration [µg]
Amikacin	AK	30
Ampicillin	AMP	10
Ampicillin/Sulbactam	SAM	20
Cefepime	FEP	30
Cefotaxime	CTX	5
Ceftazidime	CAZ	10
Cefuroxime	CXM	30
Ciprofloxacin	CIP	5
Cloxacillin	OB	5
Ertapenem	ETP	10
Erythromycin	E	15
Gentamicin	CN	10
Imipenem	IPM	10
Meropenem	MEM	10
Moxifloxacin	MXF	5
Oxacillin	OX	1
Penicillin G	P	10
Piperacillin	PRL	30
Piperacillin/tazobactam	TZP	30/6
Ticarcillin	TIC	75
Tobramycin	TOB	10
Trimethoprim/sulfamethoxazole	SXT	25

3.5.2 RND efflux gene expression in different modes of growth

Since *A. baumannii* exhibits various other growth phenotypes beyond sessile growth on agar plates, further reporter experiments were conducted to investigate gene expression in planktonic and motile cells, as well as biofilms. It is known that mechanisms of antimicrobial resistance in a biofilm differ from other phenotypes and that bacteria embedded in biofilms are less susceptible to antimicrobial agents, because of limited agent access. Moreover, it was shown that gene expression of biofilm-forming *A. baumannii* differs from that of other modes of growth, including gene overexpression as well as genes expressed only in biofilms. Therefore, *A. baumannii* biofilms were studied with respect to the expression of RND efflux pumps and regulatory genes, as in particular AdeFGH was described to contribute to biofilm formation.

Biofilms were grown in white 96-well nuclon delta surface plates containing 100 µl LB suspension of the respective *lux*-reporter transformant or the empty vector control (EV-ctrl) adjusted to an OD_{600} of 0.03 to rule out carryover of the planktonic phenotype. After 24 h of static incubation, the supernatants were aspirated, and adherent cells were washed gently with sterile phosphate buffered saline (PBS). Luciferase expression was measured in a TECAN Infinite M1000 reader. Biofilm quantification was carried out as described previously using crystal violet staining and measuring the corresponding OD_{550} in the TECAN Infinite M1000 reader.[184] Data of quantified biofilms was used for normalization of the luminescence signal. All tested regulators and their corresponding efflux pumps were expressed during biofilm. However, the expression of *adeA* was found to be at lower levels compared to other investigated genes. *adeN* revealed the highest expression levels, followed by *adeL* (Figure 13).

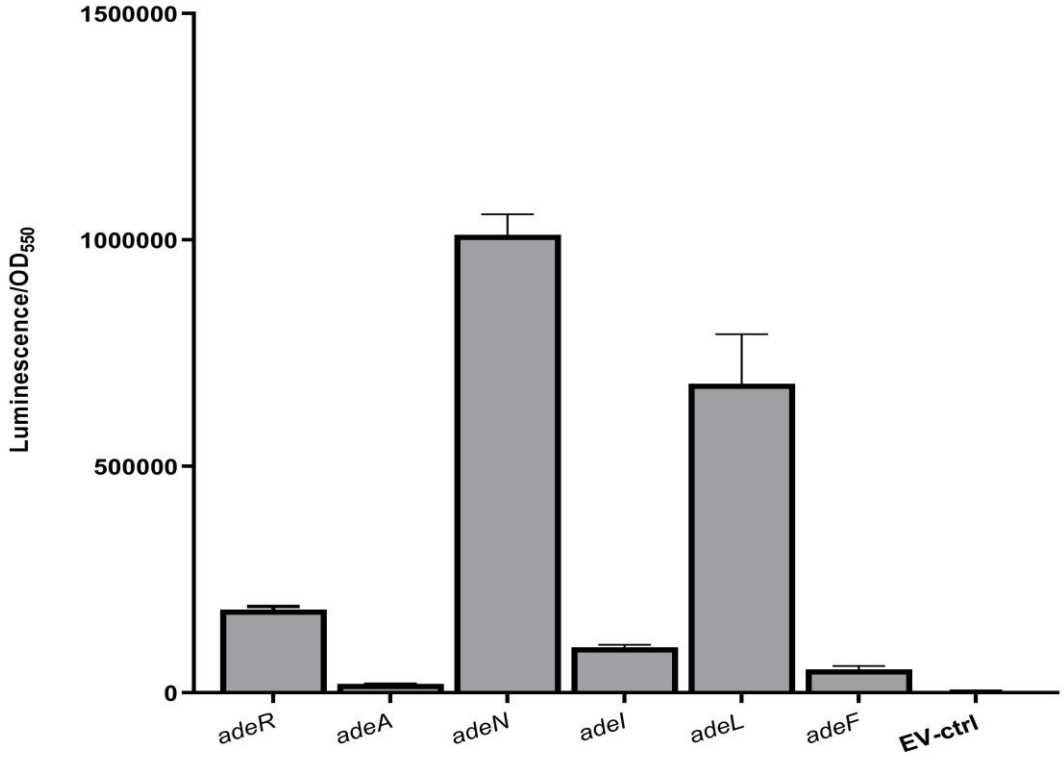


Figure 13. Luciferase reporter assay in *A. baumannii* growing as biofilms. Experiments were carried out in triplicates Results are represented as means ± standard errors of the means.

Analogous to this procedure, expression in planktonically growing *A. baumannii* cells was investigated using 10 ml LB suspensions adjusted to an OD_{600} of 0.03 supplemented with X-gal at a final concentration of 40 mg/l. Liquid cultures were incubated in an orbital incubator for 16 h at room temperature, 37 °C, and 40 °C in 30ml Falcon tubes and agitated at 220 rpm. Expression of every investigated RND pump and regulator was observed by means of blue coloration for all investigated reporter systems (Figure 14). Furthermore, no impact of incubation temperature was found since expression was also visible at room temperature and at 40 °C in planktonic and sessile cultures.

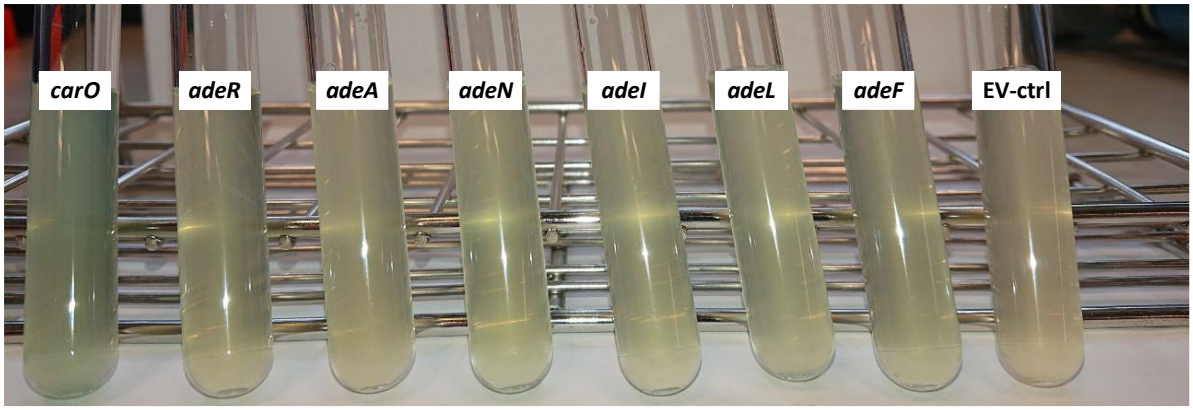


Figure 14. Visualization of RND efflux pump and regulator expression during planktonic growth using the *lacZ*-reporter. Gene expression is indicated by blue coloration of the LB medium due to X-gal hydrolysis.

To further investigate gene expression during planktonic growth and to additionally enable expression quantification, a sfGFP-reporter system was introduced into *A. baumannii* ATCC 17978. The *sfgfp*-reporter transformants were grown over night in LB and adjusted to fresh LB suspensions of an OD_{600} of 0.03. 200 µl of each suspension was loaded onto a black 96-well nuclon delta surface plate with clear bottom. The plate was incubated for 16 h at 37 °C with shaking in the TECAN Infinite M1000 reader. Emission at 535 nm after excitation at 483 nm was measured every 15 min and normalized by the corresponding OD600 value of each sample. Like the observation made with the *lacZ*-reporter, expression of RND type efflux pump and regulator genes was very low in comparison to the positive control represented by a the *carO-sfgfp* transformant, which revealed about 10 times higher fluorescence levels (not included in Figure 15).

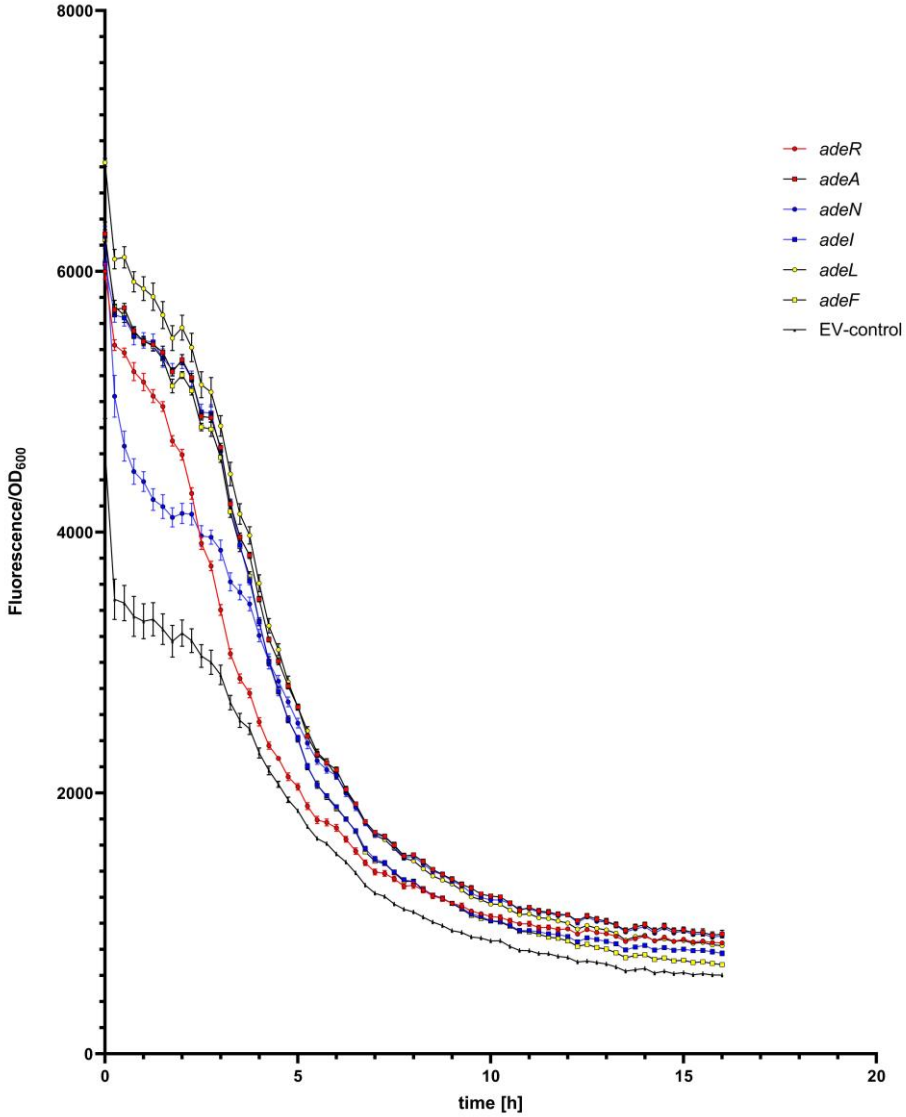


Figure 15. Real-time expression of RND-type efflux pumps and regulatory genes during planktonic growth monitored via a sfGFP-reporter system. Measured relative fluorescence was normalized for each time point using the corresponding OD_{600}.

Motility assays were carried out using agarose plates as described by Skiebe et al., which were supplemented with X-gal (40 mg/l).[37] Motility plates were inoculated on the surface with 2 µl of a McF 0.5 suspension. After inoculation, motility plates were sealed with parafilm to prevent desiccation and incubated for 48 h at 37 °C or RT. After 24 h incubation of the *A. baumannii* ATCC 17978 *adeRS-lacZ* transformant only the site of inoculation was blue while the migrating fringe remained white. The same observation was made for the *adeA-lacZ* transformant. This indicates, that the *adeRS* two-component system and the pump controlled by it, *adeABC*, are not expressed during motility. Moreover, in every other assay performed previously, both *adeN* and *adeIJK*, which is repressed by *adeN*, were expressed. However, during motility only *adeN* was expressed, whereas the *adeI-lacZ* transformant turned blue on site of inoculation,

but the migration zone remained white, suggesting inhibition of *adeIJK* expression (Figure 16). After 48 h incubation, the newly created migration fringes of the *adeR*-, *adeA*-, *adeI-lacZ* transformants were white, whereas the formerly white regions turned blue indicating induction of gene expression within the remaining cell layer.

Dysfunction of the *lacZ* reporter system by loss of genes or plasmids was excluded by subculturing of motile and white cells collected from the outer migration fringe on solid media supplemented with X-gal. After 24 h incubation at 37 °C, the colonies revealed the blue phenotype, demonstrating that the LacZ construct was not lost, only that it was not expressed under the motility growth condition.

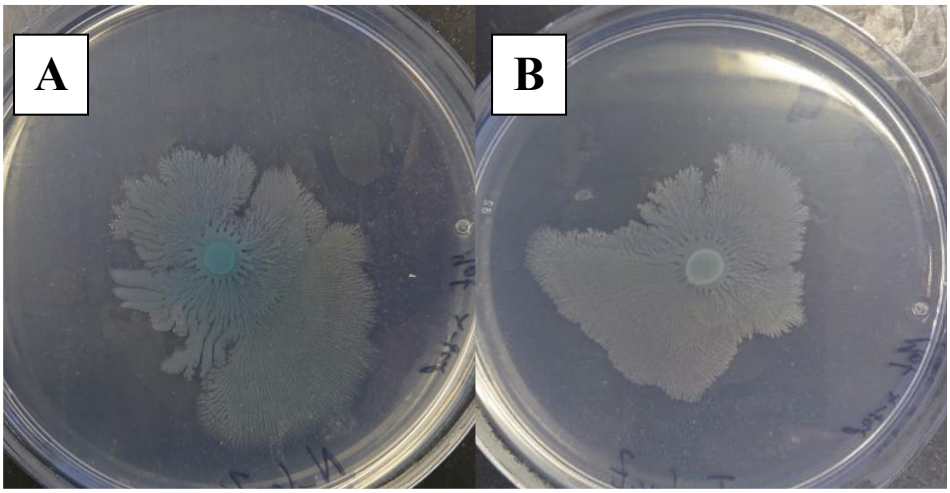


Figure 16. Expression of (A) *adeN* and (B) *adeI* after 24 h incubation on motility plates. Blue coloration of the entire growth zone indicates expression of *adeN* during motility. Expression of *adeI* is limited to the inoculation zone.

Preliminary data of this study have been presented as a poster at the 11th International Symposium on the Biology of Acinetobacter, September 20-22, 2017, Seville, Spain, and the 70th. DGHM-Conference, Bochum, February 2018, and have been part of my master thesis, University of Duisburg-Essen, 2017, as specified in Table 3.

3.6 Investigating the role of *adeRS* in the motile phenotype

The previous findings indicate that motility is the only condition of growth which leads to complete inhibition of *adeRSABC* expression in *A. baumannii* ATCC 17978. In *A. baumannii*, motility is usually mediated by type IV pili which are also associated with natural competence.[40, 192] Consequently, the motile phenotype enables *A. baumannii* to acquire novel resistance determinants which can lead to the development of multi-drug resistance. Therefore, further investigation of motility in *A. baumannii* was carried out using the motile *A. baumannii* reference strain ATCC 17978 and an *adeABC* overexpressing mutant named ATCC 17978 KI *adeRS* $^{\text{MB-R112}}$ that was created using markerless mutagenesis.[172] In detail, *A. baumannii* ATCC 17978 Δ*adeRS* was recomplemented with *adeRS* of the *adeABC* overexpressing clinical isolate MB-R112.[193] The insert *adeRS*$^{\text{MB-R112}}$, which is equivalent to *adeR*(D26N)*S*$^{\text{ACICU}}$ (3.3), was integrated into PCR-linearized pBIISK::*sacBkanR*::*adeRS*$^{\text{upstream}}$::*adeRS*$^{\text{downstream}}$. Subsequently, the plasmid was transformed into *A. baumannii* ATCC 17978 Δ*adeRS*. Insertion of *adeRS*$^{\text{MB-R112}}$ into the chromosome was confirmed by PCR. The environmental isolate *A. baumannii* 07-101 was used for natural competence studies, since its ability to pick up DNA during motility was described previously.[40] All strains and plasmids used for the present motility studies are listed in Table 7. Primers used for cloning experiments and gene expression analysis are listed in Table 8.

Table 7. Strains and plasmids used for investigation of motility.

Strain		Relevant characteristics	Reference
A. baumannii	ATCC 17978	Reference strain	[182]
	ATCC 17978 KI *adeRS* $^{MB\text{-}R112}$	ATCC 17978 knock-in *adeRS*$^{MB\ R112}$	This study
	07-101	Environmental isolate revealing natural competence during motility	[40]
E. coli	HST08 (Stellar^{TM})	Chemically competent	Clontech Takara
Plasmids	pBIISK::*sacB/kanR*	Markerless mutagenesis	[172]
	pBIISK_*sacBkanR*::*adeRS*^{upstream} ::*adeRS*^{downstream}	Markerless mutagenesis	[194]
	pBIISK_*sacBkanR*::*adeRS*^{upstream} ::*adeRS*$^{MB\text{-}R112}$::*adeRS*^{downstream}	Markerless mutagenesis	This study
	pBIISK::*tpm*	Tellurite resistance conferring shuttle plasmid	This study
	pBA03/05	IPTG inducible overexpression plasmid	[188]
	pBA03/05::*adeRS*	IPTG inducible *adeRS* overexpression plasmid	This study

Table 8. Primers used for motility studies.

Target	Primer Name	Sequence (5′ - 3′)	Size (bp)	Experiment
rpoB	C65	GAGTCTAATGGCGGTGGTTC	110	qRTPCR
	C66	ATTGCTTCATCTGCTGGTTG		
adeR	M24	AGGCATGAGTGTTATTCGGG	603	Standard for qRTPCR
	M25	CTATATCCCACGCCACGC		
adeR	D6	TGGGTTAAAAGGCTTCACCA	115	qRTPCR
	D7	ACGCCAAAAAGCTCAGACTC		
flanking *adeRS*	K25	AGTGTGGAGTAAGTGTGGAGA	2600 /700	Control of *adeRS* insertion
	JE37	AAGTAAAATAGCTTTAAAACGATCCA		
adeRS	N49	caccatcacggatccCTTTCTATAGCCAGATTTTCTATG	1882	InFusion Clonig into pBA03/05
	N50	ttggaattccgtggcTTACCCTATTTCTTCATGACCC		
pBIISK::*sacBkanR*::*adeRS*^{upstream} ::*adeRS*^{downstream}	O33	TCTTAGAGTTAAAGTGCCCCC	3594	PCR linearization
	O34	AGAAAATCTGGCTATAGAAAGTGC		
adeRS$^{MB-R112}$	O35	atagccagattttctATGTTTGATCATTCTTTTTC	1879	InFusion Clonig into pBIISK
	O36	actttaactctaagaTTAGTTATTCATAGAAATTTTTATG		
pBIISK::*sacB::kanR*	N21	acgtccatggtaagcAGAATTGGTTAATTGGTTG	4576	Deletion of *kanR*
	N22	gcttaccatggacgtAACACCCCTTGGTATTTATTTG		
tpm	N57	ggtgttacgtccatgTTTTGGCCTAGCAGCAAG	848	InFusion Clonig into pBIISK::*sacB*
	N58	attctgcttaccatgTGCTCAGAACTTGGCAGTG		
pBIISK::*sacB::tpm*	N51	acgtgatatctaagcAAGGGGTGTTACGTCCAT	3889	Deletion of *sacB*
	N52	gcttagatatcacgtTGTGCAATGTAACATCAGAGA		

Lowercase: tails for In-Fusion cloning

3.6.1 Induction of *adeRS* expression inhibits motility in *A. baumannii*

As written in 3.5.2, using the *lacZ*-reporter system revealed that *adeRS* was not expressed during motility. To test whether *adeRS* can be induced to expression during motility, *adeRS* of *A. baumannii* ATCC 17978 was cloned into BamHI-HF restricted overexpression vector pBA03/05. In this manner, the expression of *adeRS* was under the control of the isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible *lac*-promoter. Motility plates were prepared with 1nM IPTG and or ticarcillin (TIC) 150 mg/l.[188] Motility plates were inoculated on the surface using 2 µl of a McF 0.5 suspension. After inoculation, motility plates were sealed with parafilm to prevent desiccation and incubated for 48 h at 37 °C or room temperature. Analysis and documentation of the results followed after 24 h and 48 h of incubation. Whereas exposure to TIC 150 mg/l did not impair motility, *A. baumannii* ATCC 17978 pBA03/05::*adeRS* did not exhibit motility on plates supplemented with 1nM IPTG, indicating that *adeRS* expression hinders motility. However, sessile growth was not inhibited. Expression of *adeRS* induced by IPTG 1 nM was confirmed by qRT-PCR as described previously.[175]

3.6.2 Motility is abolished in *A. baumannii* at subinhibitory antimicrobial concentrations

The impact of the motile phenotype on antimicrobial susceptibility was investigated in the *A. baumannii* reference strain ATCC 17978, its derived transformants, and the environmental *A. baumannii* isolate 07-101 with a modified agar dilution method. Therefore, the corresponding antimicrobial dilutions were applied to motility agarose (2.4) instead of MH agar. Every tested strain exhibited a motile and a non-motile phenotype depending upon the concentration of azithromycin, ciprofloxacin, gentamicin, meropenem, tetracycline, or tigecycline on the plates. The concentration that inhibited motility only was defined as the minimum motility inhibitory concentration (MMIC). Thereafter the cells will grow in the inoculation zone, but no longer spread from this point (Figure 17 and Table 9). Due to low wt expression of *adeABC* (3.2), no difference in antimicrobial susceptibility using MH agar or motility agarose between *A. baumannii* ATCC 17978 and its *adeRS* knockout was found. Therefore, the *adeABC* overexpressing mutant ATCC 17978 KI *adeRS* $^{MB\text{-}R112}$ was created and investigated. Since this mutant strain recorded a higher MMIC than *A. baumannii* ATCC 17978 wt, it points to a mechanism that is decoupled from ade*RSABC*, and further that additional factors might affect susceptibility in motile growing *A. baumannii*.

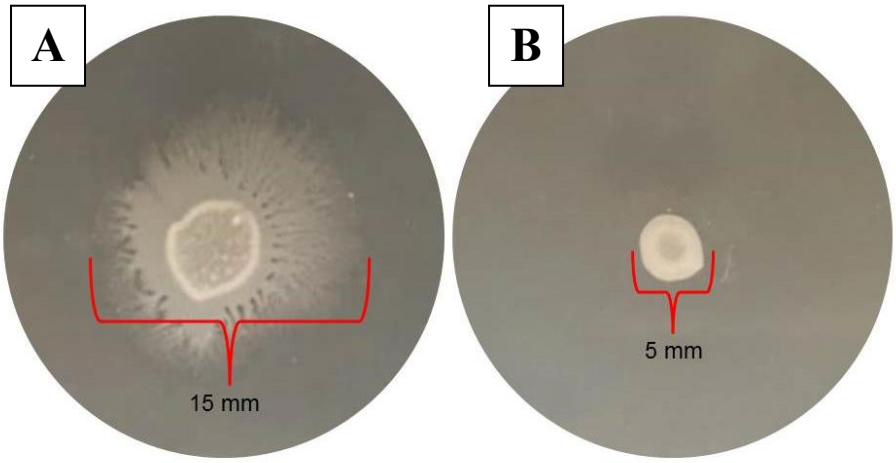


Figure 17. *A. baumannii* ATCC 17978 incubated for 24 h on motility plates (Agarose 0.5%) supplemented with 0.5 mg/l (A) and 1 mg/l (B) tetracycline. Both plates show a non-motile growth zone of 5 mm which corresponds to the drop size of the 2 µl McF 0.5 cell suspension used for inoculation. Increasing the tetracycline concentration (B) inhibited motility.

Table 9. MIC and MMIC [mg/l] of different *A. baumannii* strains as determined by a modified agar dilution method using motility agarose 0.5%.

Antimicrobial		ATCC 17978	ATCC 17978 Δ*adeRS*	ATCC 17978 KI *adeRS*$^{MB-R112}$	07-101
Azithromycin	MIC	2	1	64	4
	MMIC	0.125	0.125	2	0.25
Ciprofloxacin	MIC	0.25	0.25	1	0.5
	MMIC	0.0625	0.0625	0.25	0.125
Gentamicin	MIC	0.5	0.5	4	1
	MMIC	0.125	0.125	1	0.0625
Meropenem	MIC	0.25	0.25	0.25	0.125
	MMIC	0.0625	0.0625	0.125	0.0625
Tetracycline	MIC	2	2	8	2
	MMIC	1	1	2	0.25
Tigecycline	MIC	4	4	8	4
	MMIC	1	1	4	2

Preliminary data of this study have been presented at the 12th International Symposium on the Biology of Acinetobacter, Frankfurt, September 2019, the 71st DGHM-Conference, Göttingen, February 2019, and the 29th ECCMID, Amsterdam, April 2019.

3.6.3 Natural competence is hindered when motility is inhibited

Although loss of motility upon exposure to subinhibitory concentrations of antimicrobial was disproven to be solely linked to reduction of *adeABC* expression, the inhibition of motility was investigated further to determine its effect on natural competence.[40] Since natural competence was not observed for *A. baumannii* ATCC 17978, the environmental *A. baumannii* isolate 07-101 was used for characterizing the impact of motility inhibition on natural competence. The plasmid pBIISK::*tpm* was created by stepwise replacing the *sacB* and *kanR* genes of pBIISK::*sacB::kanR* with the tellurite resistance determinant *tpm,* which was obtained from *A. baylyi* ADP1. The advantage of tellurite as selective marker is the black color of positive colonies due to intracellular accumulation of crystals of metallic tellurium.[195] Liquid cultures of *A. baumannii* 07-101 were grown overnight and adjusted to an OD_{600} 0.1. 7 µl of the suspension were mixed with 14 µl plasmid DNA (200 ng/µl) and stab inoculated seven times per motility agarose plate as 2 µl fractions. As previously shown, *A. baumannii* 07-101 motility was inhibited with 0.5 mg/l azithromycin, whereas sessile growth on motility agarose was inhibited with azithromycin 2 mg/l (2.5.2). According to this natural transformation assays were carried out with and without azithromycin at a concentration of 0.5 mg/l.[40] After 24 h cells were flushed off the plate using sterile PBS, adjusted to an OD_{600} 1.0, and appropriate dilutions were plated on LB agar plates containing 20 mg/l tellurite. Incubation of selective plates was extended to 48 h due to slow growth of colonies. Expression of the motile phenotype was associated with an average transformation rate of 6.08E-07, whereas inhibition of motility using 0.5 mg/l azithromycin caused a reduction of transformation rates to 1.03E-07 on average which corresponds to a 5.9-fold decreased transformation rate in the presence of azithromycin.

4 Concluding discussion

This cumulative work complements the current knowledge on RND efflux pumps and their regulation in *A. baumannii*. It provides insight into the prevalence of alterations of RND efflux regulators affecting antimicrobial susceptibility in *A. baumannii* (see 3.1) and examines the immediate effects of *adeRS* alterations on *adeB* expression and the consequent changes of the resistance phenotype (see 3.2 and 3.3) as well as the role of the regulatory genes *adeN* and *adeL* in antimicrobial resistance (see 3.4). Subsequently, the growth and substrate dependent expression of RND efflux related genes was investigated (see 3.5). To a lesser extent, the ability to influence the motile phenotype of *A. baumannii* and the corresponding consequences on natural competence was assessed.

4.1 RND efflux pump regulator variants associated with antimicrobial resistance in *A. baumannii*

A. baumannii has emerged as a serious nosocomial pathogen implicated in various hospital outbreaks. Treatment of *A. baumannii* infections is challenging because of the organisms' frequent resistance to a wide range of antimicrobial agents, such as aminoglycosides, fluoroquinolones and β-lactams, including carbapenems.[11] Horizontal gene transfer builds the foundation of multi-drug resistance, and in this manner the spread of resistance determinants are predominantly specific to single agents or classes.[47] In contrast, overexpression of the chromosomally encoded RND-type efflux pumps reduces susceptibility of *A. baumannii* to multiple antimicrobials of different classes, as well as biocides used for disinfection in the healthcare setting. [81, 196-200] Characterized RND efflux pumps in *A. baumannii* are AdeABC, AdeFGH, and AdeIJK, whose expression is controlled by the two-component systems AdeRS (AdeABC), the LysR-like transcriptional regulator AdeL (AdeFGH), or the TetR-like repressor AdeN (AdeIJK).[201-203]

During the course of this thesis, the global prevalence of resistance-associated variants of the RND efflux pump regulatory genes *adeRS, adeN,* and *adeL* was examined based on decreased tigecycline susceptibility as an indicator for RND efflux pump overexpression. Reduced tigecycline susceptibility was found in almost 50% of the investigated isolates and was mainly caused by regulator alterations of RND-type efflux pumps. The disruption of *adeN,* which encodes the repressor of the *adeIJK* operon, caused by an IS element was the most prevalent mechanism, and was identified in isolates from Africa, Asia, Europe, North America and Latin

America. Additionally, truncation of AdeN due to premature stop codons was commonly detected. Disruption of *adeN* was also found to be associated with overexpression of *adeIJK* and reduced antimicrobial susceptibility to various antimicrobials in previous studies.[122] In accordance to this, AdeN amino acid substitutions, which could also be identified within isolates revealing reduced antimicrobial susceptibility, presumably harbor the potential to impair the repression of *adeIJK*. AdeN belongs to the family of TetR-type repressors. Characterization of TetR revealed that it is bound to the DNA over its N-terminal domains. Consequently, the identified AdeN amino acid substitutions A43P and G54S possibly interfere the effective binding of the repressor to DNA, resulting in increased expression of *adeIJK*. Other detected substitutions in AdeN like N66Y, K141N, and L173T might cause enhanced gene expression as these residues are similar to TetR substrate binding sites and might consequently affect repressor binding and conformational stability inducing dissociation of the repressor DNA complex.[123] Furthermore, follow up experiments showed that deletion of *adeN* in the *A. baumannii* reference strain ATCC 19606 Δ*adeN* caused increased MICs for chloramphenicol, minocycline, tetracycline, and tigecycline each by one dilution step. These findings align to the analyzed clinical isolates harboring *adeN* mutations associated with comparatively slightly elevated tigecycline MICs. Similar observations were made in a previous study that analyzed disruption of *adeN* by IS elements, and were associated with a one dilution increased tigecycline MIC too.[63] Additionally, the same study recorded reduced susceptibility to minocycline and fluoroquinolones in strains with disrupted *adeN*, which is consistent with the present results of *A. baumannii* ATCC 19606 Δ*adeN*.

While mutations within the two-component regulatory system AdeRS were found to be less prevalent than changes affecting AdeN, our findings indicate that variations of *adeRS* have greater influence on the antimicrobial resistance phenotype among RND-type regulators in *A. baumannii*. Moreover, the deletion of *adeRS* in *A. baumannii* ATCC 19606 revealed a bigger impact on antimicrobial susceptibility than deletion of *adeN* and *adeL* respectively. However, deletion of *adeRS* in *A. baumannii* ATCC 17978 had no significant effect. Therefore, *adeRS* of 19606 and 17978 were compared and further investigated and an AdeS L172P amino acid substitution was identified. Our studies revealed that the introduction of this amino acid substitution in *A. baumannii* ATCC 17978 increased expression rates of *adeB* and subsequently increased efflux activity and reduce antimicrobial susceptibility. Residue 172 of AdeS is part of the DHp domain, which includes the phosphorylation residue H149 and might therefore affect the activity of the sensor kinase.[111] Additional analysis of published *adeS* sequences

indicated that clinical isolates predominantly harbor the P172 configuration, which is also present in other commonly used *A. baumannii* laboratory strains like ACICU and AYE giving emphasis to genetic plasticity and strain dependent differences in studies analyzing fundamental bacterial mechanisms.[204]

Characterization of AdeRS was continued to investigate amino acid substitutions identified in the prevalence study (3.1). Since the AdeS amino acid substitution T156M was identified in 6 different isolates with high tigecycline MIC, it was subjected to further investigation. Analysis of AdeS T156M was initially based on a clinical isolate pair, as the two isolates were identical except for the T156M amino acid substitution in AdeS causing a shift in susceptibility to tigecycline.[205] Our study confirmed that increased *adeB* expression, efflux activity, and consequently reduced antimicrobial susceptibility is triggered by the AdeS T156M amino acid substitution. Since this mutation is located within the same hotspot region of the AdeS kinase domain, although the T156 is not directly involved in the dimerization of AdeS or its cis autophosphorylation as revealed by the structure, it is very likely that these mutations increase the sensitivity of AdeS sensing the environmental antimicrobial components stimuli, or facilitate activation of the response regulator AdeR, by increasing its phosphorylation ratio, which results in increased AdeABC expression.[111] Moreover, this amino acid substitution is also within the DHp domain in close proximity to the autophosphorylation site H149, which also applies to other resistance associated amino acid substitutions e.g., R152K, T153A, T153M, and D167N, indicating a mutational hotspot.

AdeR is activated by AdeS and acts as response regulator, initiating expression of AdeABC. Previously, Wen *et al.* revealed the structure of AdeR and showed that residues E19, D20, K65, and the phosphorylation site D63, form a highly conserved magnesium binding pocket, and consequently suggested that substitutions of contributing amino acids enhance phosphorylation by AdeS.[115] A D20N substitution in AdeR was also shown to increase *adeB* expression and reduce antimicrobial susceptibility.[64] During a previous study of clinical Southern European *A. baumannii* isolates, the AdeR double substitution D21V and D26N was observed in 17 of 65 isolates with high tigecycline MICs.[63] The same double substitution was identified again in the current worldwide study, once more in an European isolate. Therefore, this double mutation was further investigated. The AdeR amino acid substitution D21V, which has been detected in multiple isolates with high tigecycline MICs, is in close proximity to a conserved magnesium binding pocket formed by residues E19, D20, K65, and the phosphorylation site D63.[63, 205] However, the contribution of this exchange to increased AdeABC efflux was disproven in the

present study, since it did not induce a significantly increased efflux pump expression or reduced antimicrobial susceptibility. In contrast to these findings, the AdeR D26N mutation revealed a significant impact on AdeABC expression, efflux activity, and subsequently on antimicrobial susceptibility. Interestingly, the residue is located in the α1 helix of AdeR and consequently not directly involved in the dimerization interface.[115] However, the substitution of aspartic acid to asparagine may alter the binding of AdeR to AdeS during phosphorylation, since it is lacking the hydrogen bond acceptor carboxyl group. Nevertheless, the detailed mechanism caused by this mutation remains to be further investigated. Gaining mechanistic insight into the AdeS and AdeR interaction may shed light on the impact of the response regulator D26N substitution in the function of the AdeRS complex.

Although the double mutation D21V and D26N in AdeR did not reveal any synergistic effect on efflux activity and reduced antimicrobial susceptibility, our findings gained by the global RND regulatory study suggest that an accumulation of mutations within RND-type efflux regulatory genes contributes to a stepwise increase in tigecycline efflux, since isolates harboring multiple regulator alterations correlated with higher tigecycline MICs. A synergistic effect on multiple antimicrobials was shown previously using single and combined AdeABC and AdeIJK deleterious strains. Knockouts of either AdeABC or AdeIJK led to increased antimicrobial susceptibility. Subsequently, the inactivation of both efflux pumps simultaneously caused a further reduction of MICs for antimicrobials such as fluoroquinolones, tetracyclines, and tigecycline.[87, 121]

In the included prevalence study only two isolates harbored mutations in AdeL, the LysR-like regulator of AdeFGH. The isolates revealed the amino acid substitution P125L and C292G, respectively. Previously, substitutions at nearby residues have been shown to cause overexpression of *adeFGH* and to reduce antimicrobial susceptibility.[129] Furthermore, it was shown for the LysR-like regulator LeuO in *E. coli* that an amino acid substitution at residue 120 causes a structural change of the regulator similar to effector binding and consequently facilitates gene expression.[206] In concordance with this finding, it can be suggested that the P125L amino acid exchange presumably causes a signal-independent activation of AdeL. However, as the discovered mutations are associated with rather minimally increased tigecycline MIC and the deletion of *adeL* in *A. baumannii* ATCC 19606 did not affect a broad spectrum of antimicrobials, *adeL* might contribute less to the antimicrobial resistance phenotype than other RND type efflux regulators. However, it was shown to contribute to biofilm growth, indicated by reduced

biofilm formed by *A. baumannii* ATCC 19606 Δ*adeL*. These results are consistent with those of previous studies linking AdeFGH with biofilm formation.[130]

Persistence of *A. baumannii* in the healthcare environment does not only increase the likelihood to acquire antimicrobial resistance mediating determinants including IS-elements. In addition, the increased selective pressure by exposure to antimicrobials and biocides makes it more likely to develop point mutations that cause advantageous amino acid substitutions in RND efflux regulatory genes, as presented in this study. Moreover, *A. baumannii* survival on abiotic surfaces under desiccation conditions might contribute to acquisition of corresponding mutations, as desiccation and rehydration have been shown to cause a 50-fold increase in mutation frequency.[77] Consequently, overexpression of RND-type efflux pumps facilitates the survival of *A. baumannii* in the hospital environment by reducing the organisms' susceptibility to antimicrobials and biocides, and therefore again increase the likelihood of resistance gene acquisition.[81] Henceforth, the present data emphasize that RND efflux regulators represent a promising target that should be studied further for the development of novel antibacterial strategies.

4.2 Growth dependent expression of RND efflux pumps and their corresponding regulatory genes

To date, little is known about the influence of different growth conditions on the expression of RND efflux pumps and their regulators in *A. baumannii*. However, it has been shown that gene expression in Gram-negative organisms is altered by environmental conditions, as for example *Serratia marcescens* exhibited differential expression of *ompF* and *ompC* porin genes in response to osmotic stress, salicylate, temperature and pH.[207] Furthermore, these conditions affected antimicrobial susceptibility.[208] In *P. aeruginosa*, different stimuli and effector molecules, like reactive nitrogen species and chloramphenicol could be linked to the induced transcription of RND-type efflux pumps, enabling the cells to export toxic substances, including antimicrobials, disinfectants and detergents.[209, 210] In *A. baumannii*, it was shown that low pH can cause differences in fluoroquinolone susceptibility, as well as a strain-specific effect on carbapenem susceptibility.[211, 212] Furthermore, the transition between different growth phases displays a dynamic process which is associated with changes in gene expression, indicating the need to analyze the impact on RND efflux.[213, 214] Therefore, expression of RND efflux pumps and their regulators was investigated using different reporter system depending on the mode of growth, since *A. baumannii* showed efficient growth in various forms, such as sessile on agar plates, motile on motility agarose plates, planktonic in broth, and as biofilm.

Applying the *lacZ* reporters to investigate the impact of substrates like antimicrobials, detergents, salts, sugars, and dyes as well as pH altering acids and alkali via gradient plates, or by disc diffusion, revealed expression of all RND pumps and regulators with every tested gradient setup and antimicrobial disc, until toxicity was reached, inhibiting bacterial growth. This indicates that RND efflux pumps and their regulators are constitutively expressed during sessile growth, and expression is not altered by these substrates.

LacZ was also used to study gene expression of planktonic cells growing in liquid culture and revealed expression of every investigated RND pump and regulator. These results were verified by the application of an sfGFP assay. In this way, it could be shown that RND efflux associated genes are expressed at a low but steady level. These results fit with previous studies assuming consistent but low expression of RND efflux pumps, since strong overexpression may be associated with cellular toxicity.[122]

Combining biofilm quantification protocols with a luciferase assay enabled quantification of gene expression in biofilms. Biofilms are renowned for having a higher tolerance to harmful agents, including antimicrobials, UV light, acid exposure, dehydration, and phagocytosis, than other modes of growth.[33, 34] Furthermore, gene expression of *A. baumannii* biofilms was shown to exhibit an altered gene expression pattern.[35] However, all RND efflux pumps and regulatory genes were expressed, although we were able to detect clear differences within expression the investigated genes. AdeN revealed the highest expression, followed by AdeL, which also indicates its importance in biofilm formation, as described previously.[130]

The expression of RND efflux genes in planktonic and sessile growing *A. baumannii*, as well as exposure to environment altering substrates, indicates that RND efflux pumps are essential and that their function is not limited to extrusion of antimicrobial agents. Reviews of RND efflux pumps in other Gram-negative organisms suggested that the physiological role of this efflux mechanism might be the reduction of harmful naturally produced compounds.[80] In *Erwinia amylovora*, RND efflux pump AcrAB is associated with facilitated plant colonization by enabling extrusion of plant derived secondary metabolites that act upon antimicrobial toxins.[215] Furthermore, the role of the RND-family in clinically relevant pathogens such as *Salmonella* Typhimurium, *P. aeruginosa*, *Campylobacter jejuni*, and *Neisseria gonorrhoeae*, in colonization and host cell invasion, respectively, has been shown.[216-219]

Motility has turned out to be the most remarkable mode of growth in terms of expression of the studied genes. Motility enables *A. baumannii* to spread over surfaces and to take up

exogenous DNA, which is correlated with the presence of type IV pili.[40, 220] Furthermore, epidemiological studies of clinical *A. baumannii* isolates found blood isolates were more motile when compared to sputum isolates, and other studies found genes predicted to encode for proteins required for the biogenesis of type IV pili were shown to be upregulated during growth in human serum, indicate that motility may provide a fitness advantage in blood-stream infection.[22, 185] Additionally, it was previously shown that motile *P. aeruginosa* cells exhibited up-regulation of genes associated with antimicrobial resistance compared to planktonic cells.[221] In *A. baumannii* ATCC 17978, motility was the only investigated growth condition which revealed completely suppressed expression of regulatory (*adeRS)*, and RND efflux pump genes (*adeABC*, *adeIJK*) respectively, while the repressor *adeN* was still expressed. Interestingly, the corresponding reporter systems revealed expression within the point of inoculation after 24 h and within the older cell layers after 48 h, but never within the newly generated migration fringe. These findings indicate a heterogeneous population in terms of gene expression between motile cells at the migration zone and stationary cells within the central zone. Moreover, these observations resemble investigations in *P. aeruginosa*, which suggested differential gene expression patterns in motility assays. In particular, it was shown a deviating transcriptome between the cells in the center, which had a biofilm-like phenotype, whereas in migrating cells an up-regulation of genes involved in energy metabolism was discovered, which is suspected to facilitate migration into new niches.[222] Transferring this to *A. baumannii*, the present results may indicate expression of *adeRS*, *adeABC*, and *adeIJK* in the remaining biofilm, but not in motile cells. It still has to be determined, if the expression of other genes is increased to replace these RND pumps during motility.

Wannigama et al. pointed out the importance of considering growth conditions and phenotype and suggested an assay to determine antimicrobial effectiveness in *A. baumannii* biofilms.[223] This corresponds to the present study, which revealed that motile growing *A. baumannii* displays lower MICs for various antimicrobials compared to sessile growing cells. Furthermore, the present study shows that these results are not in dependency of inhibited *adeRSABC* expression, since *A. baumannii* ATCC 17978 exhibited low level *adeABC* expression, which does not contribute to reduced antimicrobial susceptibility. However, it was shown previously that extracellular stress affects motility in *A. baumannii* ATCC 17978 and other *A. baumannii* isolates.[224] Moreover, it has been shown that azithromycin can act as a quorum sensing inhibitor in *P. aeruginosa* and *A. baumannii*, and that the motile phenotype is in direct dependency of quorum sensing.[225-227] Therefore, the suppression of motility in the

presence of subinhibitory concentrations of azithromycin could be caused by loss of quorum sensing rather than increased antimicrobial susceptibility of the motile phenotype. Still, our results imply a correlation of growth phenotype and antimicrobial susceptibility as various other antimicrobials of different classes also were able to inhibit motility at subinhibitory concentrations. Hence, the role of AdeIJK-non-expression in increased antimicrobial susceptibility of the motile *A. baumannii* has to be investigated in future studies. As the impact of AdeABC is negligible in *A. baumannii* ATCC 17978, AdeIJK might be a more impactful RND efflux pump in this specific strain. Nevertheless, the finding of reduced natural competence at subinhibitory concentrations of azithromycin can provide the basis for further research in motility, natural competence, and the acquisition of resistance genes in the clinical setting. Additionally, control of the motile phenotype might be a possibility to control virulence, since *A. baumannii* hyper-motility has been associated with enhanced virulence in a *Caenorhabditis elegans* infection model.[36]

Technologically more advanced methods will be needed to gain further insight and analyze differential gene expression. Methods like RNA sequencing allow the comparison of environmental influences on an organism's global gene expression and furthermore allow a robust quantification of gene expression, in contrast to other methods limited to single targets, such as reporter systems (e.g. LacZ). Additionally, further elaborated analyses based on microscopy in combination with reporters like GFP or luciferase would allow single cell gene expression analysis and detailed determination of differential gene expression for example during the different phases of motility. Furthermore, a strain dependency in gene expression has to be considered during gene expression analysis as we have shown in a previous study; *A. baumannii* ATCC 17978 shows a significantly lower expression of *adeA* in comparison to *A. baumannii* reference strains like ATCC 19606 as well as clinical *A. baumannii* isolates. Nevertheless, it is the expression of *adeRS* which is of major interest as the expression of this regulatory system is crucial for expression of the *adeABC* operon, and therefore inhibition of *adeRS* displays a route to overcome broad range reduced antimicrobial susceptibility.

RND-type efflux pumps are crucial for Gram-negative bacteria to survive in inhospitable environments such as the hospital environment, by reducing their susceptibility to antimicrobials and biocides.[81] The present thesis gives is an overview of the significance in antimicrobial susceptibility of RND efflux in *A. baumannii* with particular emphasis on regulatory genes. It was shown that overexpression of AdeABC or AdeIJK associated mutations in *adeRS* and *adeN* respectively are of particular importance, as they cause reduced antimicrobial

susceptibility to various agents.[102, 120] Similar observations were made in the present thesis, as uncharacterized single point mutations within the two-component regulatory system *adeRS* were investigated. Surveillance of such putative resistance mutations has to be considered, as no efflux inhibition applicable in infection treatment is available up to now.[92] Additionally, consideration of the growth phenotype during infection may become more important, and has been highlighted in a previous study suggesting to routinely determine antimicrobial susceptibility of *A. baumannii* biofilms.[223] Interestingly, in the present thesis it was shown that the motile phenotype inhibits expression of RND genes and correlates with increased antimicrobial susceptibility. In conclusion, this thesis emphasizes the role of RND regulatory mutations and mode of growth in antimicrobial susceptibility of *A. baumannii*.

5 Summary

A. baumannii has been assigned as one of the most urgent threats among antimicrobial resistant bacterial organisms. The fundamental mechanism of action of antimicrobial agents is to bind to its bacterial target site and to interfere with cellular processes causing impaired bacterial cell growth or lead to bacterial cell death. *A. baumannii* is known for a broad spectrum of intrinsic and acquired resistance mechanisms, which are based on enzymatic inactivation of the drug, alterations in the structure of antimicrobial target sites preventing binding of antimicrobial agents. These mechanisms require specific adjustments of antimicrobial agents or targets. Thus, they are specific to single antimicrobials or antimicrobial classes. Contrary to this, mechanisms such as reduced permeability and increased efflux activity are able to diminish the intracellular concentration of antimicrobials and affect a large variety of agents.

Among efflux systems, the RND family embodies the highest clinical relevance. These efflux pumps are associated with a broad spectrum of substrates, including antimicrobials as well as antiseptics and disinfectants commonly used in healthcare. Characterized RND efflux pumps in *A. baumannii* are AdeABC, AdeFGH, and AdeIJK, whose expression are controlled by the two-component systems AdeRS (AdeABC), the LysR-like transcriptional regulator AdeL (AdeFGH), or the TetR-like repressor AdeN (AdeIJK). Overexpression of AdeABC or AdeIJK has been shown to cause reduced antimicrobial susceptibility. During the present thesis the prevalence of regulatory mutations putatively causing RND overexpression in 113 carbapenem-resistant *A. baumannii* isolates obtained from a global collection was investigated. The most commonly and worldwide detected mechanism was the disruption of the AdeIJK repressor *adeN*, either by IS elements or nucleotide deletions causing premature stop codons. Furthermore, amino acid substitutions and disruption by IS elements within the two-component regulatory system *adeRS*, which regulates expression of the AdeABC efflux pump, were found. These mutations were detected less often but correlated with higher tigecycline MICs than mutations in *adeN* and *adeL*, which were assigned a minor role in reduced antimicrobial susceptibility.

However, *adeRS* of *A. baumannii* ATCC 17978 was shown to have no impact on antimicrobial susceptibility in contrast to contemporary clinical isolates. Therefore, *adeRS* of *A. baumannii* ATCC 19606 and ATCC 17978 were compared and analyzed and an L172P amino acid substitution in AdeS was identified in *A. baumannii* ATCC 19606. Our studies revealed that the introduction of this amino acid substitution into AdeS of 17978 increased expression

of *adeB*, increased efflux activity and reduced antimicrobial susceptibility. Residue 172 of AdeS is part of the DHp domain, which includes the phosphorylation residue H149 and might therefore affect the activity of the sensor kinase. This substantial difference between two reference strains commonly used in *A. baumannii* research demands to consider genetic plasticity and strain-dependent differences in studies analyzing fundamental bacterial mechanisms.

Characterization of AdeRS was enhanced to investigate amino acid substitutions identified during the prevalence study. The AdeS amino acid substitution T156M was identified frequently, and the corresponding residue is positioned within the DHp domain in close proximity to the autophosphorylation site H149. An increased *adeB* expression, efflux activity, as well as reduced antimicrobial susceptibility was found to be induced by the AdeS T156M amino acid substitution.

Additionally, the AdeR D21V and D26N double substitution, which was identified in the present and previous studies, was investigated. Although the amino acid substitution D21V has been detected in multiple isolates with high tigecycline MICs and is in close proximity to a magnesium binding pocket, which affects the regulators' conformation, the contribution of this exchange to increased AdeABC efflux was disproven in the present study, since it did not cause increased efflux pump expression or reduced antimicrobial susceptibility. In contrast to these findings, the AdeR D26N mutation revealed a significant impact on AdeABC expression, efflux activity, and subsequently on antimicrobial susceptibility.

Moreover, expression of RND efflux pumps and regulatory genes in different growth phenotypes and under the impact of various substrates was monitored. In this scope, motility was the only investigated growth condition in *A. baumannii* ATCC 17978 which inhibited expression of regulatory (*adeRS*), and RND efflux pump genes (*adeABC, adeIJK*) respectively. In addition, a correlation between the motile phenotype and increased antimicrobial susceptibility was observed.

Summarizing, this thesis describes the worldwide distribution, the mechanism, and impact of RND regulatory mutations, as well as the effect of the mode of growth on gene expression and antimicrobial susceptibility in *A. baumannii*.

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Abbreviations

AbaR	*A. baumannii* resistance island
ABC	adenosine triphosphate-binding cassette
ABS	adjacent activation binding site
ACB	*A. calcoaceticus-A. baumannii*
Ade	*Acinetobacter* drug efflux
ATP	adenosine triphosphate
bp	base pair
CCCP	carbonyl cyanide m-chlorophenyl-hydrazone
cgMLST	core-genome multilocus sequence typing
DHp	dimerization histidine phosphotransfer
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EGCG	epigallocatechin 3-gallate
EPI	efflux pump inhibitor
EV-ctrl	empty vector control
GFP	green fluorescent protein
HAMP	histidine kinase, adenylyl cyclase, methyl-accepting chemotaxis protein and phosphatase
IC	international clone
ICU	intensive care unit
IPTG	isopropyl β-D-1-thiogalactopyranoside
IS	insertion sequence
kb	kilobase
KI	knock-in
LB	Luria Bertani
LPS	lipopolysaccharides
LTTR	LysR-type transcriptional regulator
MATE	multidrug and toxic compound extrusion
McF	McFarland
MDR	multi-drug resistant
MFP	membrane fusion protein
MFS	major facilitator superfamily

MH	Mueller Hinton
MIC	minimum inhibitory concentration
MLST	multilocus sequence typing
MMIC	minimum motility inhibitory concentration
mRNA	messenger ribonucleic acid
NMP	1-(1-napthylmethyl)-piperazine
OD	optical density
OMF	outer membrane factor
OMP	outer membrane porin
PACE	proteobacterial antimicrobial compound efflux
PAβN	phenylalanine-arginine-β-naphthylamide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDR	Pan-drug resistant
qRT-PCR	semi-quantitative real-time polymerase chain reaction
RBS	regulatory binding site
RNA	ribonucleic acid
RND	resistance nodulation-cell division
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulphate
sfGFP	super folder green fluorescent protein
SMR	small multi-drug resistance
tRNA	transfer ribonucleic acid
UV	ultra-violet
WGS	whole-genome sequencing
WHO	World Health Organization
wt	wild-type
XDR	extensive drug resistant
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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Erklärung

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne die Benutzung anderer als der angegebenen Hilfsmittel und Literatur angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen und eingebundenen Artikeln und Manuskripten - noch nicht veröffentlicht worden ist sowie, dass ich eine Veröffentlichung der Dissertation vor Abschluss der Promotion nicht ohne Genehmigung des Promotionsausschusses vornehmen werde. Die Bestimmungen dieser Ordnung sind mir bekannt. Darüber hinaus erkläre ich hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten der Universität zu Köln gelesen und sie bei der Durchführung der der Dissertation zugrundeliegenden Arbeiten und der schriftlich verfassten Dissertation beachtet habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen. Ich versichere, dass die eingereichte elektronische Fassung der eingereichten Druckfassung vollständig entspricht. Die in der vorliegenden Dissertation generierten Primärdaten und Materialien sind am Institut für Medizinische Mikrobiologie, Immunologie und Hygiene der Uniklinik Köln, Arbeitsgruppe Higgins, gesichert und zugänglich.

Eingebundene Publikationen

Lucaßen K., Xanthopoulou K., Wille J., Wille T., Wen, Y., Xua, X., Seifert H., Higgins P.G. Characterization of substitutions in the two-component regulatory system AdeRS identified in multi-drug resistant *Acinetobacter baumannii*. mSphere, Accepted for publication

Lucaßen K., Gerson S., Xanthopoulou K., Wille J., Wille T., Seifert H., Higgins P.G. Comparison of the A. baumannii reference strains ATCC 17978 and ATCC 19606 in antimicrobial resistance mediated by the AdeABC efflux pump. Antimicrobial Agents and Chemotherapy, 2021, DOI: 10.1128/AAC.00570-21

Lucaßen K., Müller C., Wille J., Xanthopoulou K., Hackel M., Seifert H., Higgins P.G. Prevalence of tigecycline resistance in dependency of resistance-nodulation-cell division efflux pump regulator alterations within carbapenem-resistant *Acinetobacter baumannii* observed in a worldwide survey. Journal of Antimicrobial Chemotherapy, 2021, DOI: 10.1093/jac/dkab079

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Lucaßen K., Müller C., Xanthopoulou K., Wille J., Seifert H., Higgins P.G. Characterization of the T156M amino acid substitution in the RND efflux pump regulator AdeS of *Acinetobacter baumannii.*
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Mutations of resistance-nodulation-cell division efflux pump regulators in tigecycline-resistant *Acinetobacter baumannii* observed in a worldwide surveillance study.
Vortrag: 71. DGHM-Jahrestagung, Leipzig, März 2020

Lucaßen K., Xanthopoulou K., Wille T., Seifert H., Higgins P.G. Inhibition of the RND-type efflux pump regulator AdeRS in *Acinetobacter baumannii.*
Posterpräsentation: 12th International Symposium on the Biology of Acinetobacter, Frankfurt, September 2019

Lucaßen K., Xanthopoulou K., Wille T., Seifert H., Higgins P.G. The role of *adeRS* in reduced antimicrobial susceptibility during motility in *Acinetobacter baumannii* ATCC 17978.
Posterpräsentation: 12th International Symposium on the Biology of Acinetobacter, Frankfurt, September 2019

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Posterpräsentation: 11th International Symposium on the Biology of Acinetobacter, Sevilla, September 2017 und 70. DGHM-Jahrestagung, Bochum, Februar 2018

Köln, 09. November 2021 Kai Lucaßen

Curriculum vitae

Name:	Kai Lucaßen
Anschrift:	Euskirchener Str. 42, 53947 Nettersheim
Geboren:	06.05.1987 in Hattingen
Tel.:	+49 176 70872516
Email:	kai.lucassen@outlook.de

Bildungsweg

Seit 05/2018 — **Promotionsstudium**

Institut für Medizinische Mikrobiologie, Immunologie und Hygiene, Universität zu Köln

Arbeitsgruppe: Dr. Paul Higgins und Prof. Dr. Harald Seifert

10/2015 – 11/2017 — **M.Sc. Medizinische Biologie**

Universität Duisburg-Essen

- Abschlussarbeit am Institut für Medizinische Mikrobiologie, Immunologie und Hygiene, Universität zu Köln: „Investigation of different physiological conditions inducing expression of the AdeRS two-component system and its association with antimicrobial resistance in *Acinetobacter baumannii*"

10/2012 – 09/2015 — **B.Sc. Medizinische Biologie**

Universität Duisburg-Essen

- Abschlussarbeit am Institut für Zellbiologie, Universitätsklinikum Essen: „Ansätze zur Wirkungssteigerung einer Platin-basierten Chemotherapie beim Ovarialkarzinom: Pharmakologische Modulation von Membrantransportern und DNA-Reparatur"

10/2007 – 09/2010 — **Ausbildung zum Gesundheits- und Krankenpfleger**

Marien Hospital Euskirchen

08/2000 – 05/2006 — **Allgemeine Hochschulreife**

Frankengymnasium Zülpich

Praxiserfahrung

10/2010 – 04/2019 — **Gesundheits- und Krankenpfleger**

Marien Hospital Euskirchen

08/2006 – 04/2007 — **Zivildienst**

Marien Hospital Euskirchen

- Bereich: Gesundheits- und Krankenpflege

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Gerson S., **Lucaßen K**., Wille J., Nodari C.S., Stefanik D., Nowak J., Wille T., Betts J.W., Roca I., Vila J., Cisneros J.M., Seifert H., Higgins P.G. Diversity of amino acid substitutions in PmrCAB associated with colistin resistance in clinical *Acinetobacter baumannii* isolates. International Journal of Antimicrobial Agents, 2020, DOI: 10.1016/j.ijantimicag.2019.105862

Betts J.W., Hornsey M., Higgins P.G., **Lucaßen K.**, Wille J., Salguero F.J., Seifert H., La Ragione R.M. Restoring the activity of the antibiotic aztreonam using polyphenol epigallocatechin gallate (EGCG) against multidrug-resistant clinical isolates of *Pseudomonas aeruginosa.* Journal of Medical Microbiology, 2019, DOI: 10.1099/jmm.0.001060

Gerson S., Betts J.W., **Lucaßen K**., Nodari C.S., Wille J., Josten M.., Göttig S., Nowak J., Stefanik D., Roca I., Vila J., Cisneros J.M., La Ragione R.M., Seifert H., Higgins P.G. Investigation of novel *pmrB* and *eptA* mutations in isogenic *Acinetobacter baumannii* Isolates associated with colistin resistance and increased virulence *in vivo*, Antimicrobial Agents and Chemotherapy, 2019, DOI: 10.1128/AAC.01586-18

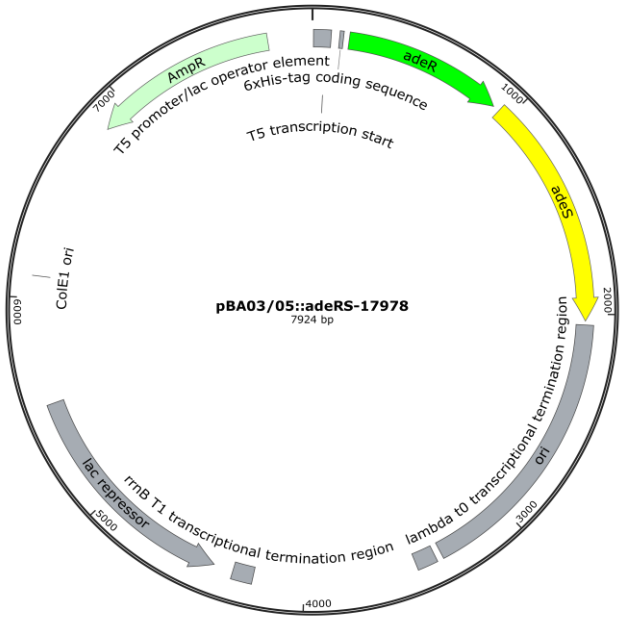
Appendix

Suppl. Table 1. Kits used in the present study.

Kit	Purpose	Manufacturer
Qiaprep Spin Miniprep Kit	Plasmid purification	Qiagen, Hilden, Germany
MagAttract HMW DNA Kit	DNA extraction	Qiagen, Hilden, Germany
Nextera XT library prep Kit	Library preparation for WGS	Illumina GmbH, Munich, Germany
Q5 High-Fidelity DNA Polymerase	PCR amplification of cloning inserts	New England BioLabs, Frankfurt, Germany
Qiaquick PCR Purification Kit	Purification of PCR products and linearized plasmids	Qiagen, Hilden, Germany
InFusio HD EcoDry cloning Kit	Cloning	Takara Clontech, Saint Germain-en-Laye, France
Site directed mutagenesis Kit	Induction of nucleotide exchanges	New England BioLabs, Frankfurt, Germany
RNeasy Mini Kit	RNA extraction	Qiagen, Hilden, Germany
Quantiscript Reverse Transcriptase Kit	cDNA synthesis	Qiagen, Hilden, Germany
SYBR Green Master Mix	RT-PCR	Qiagen, Hilden, Germany

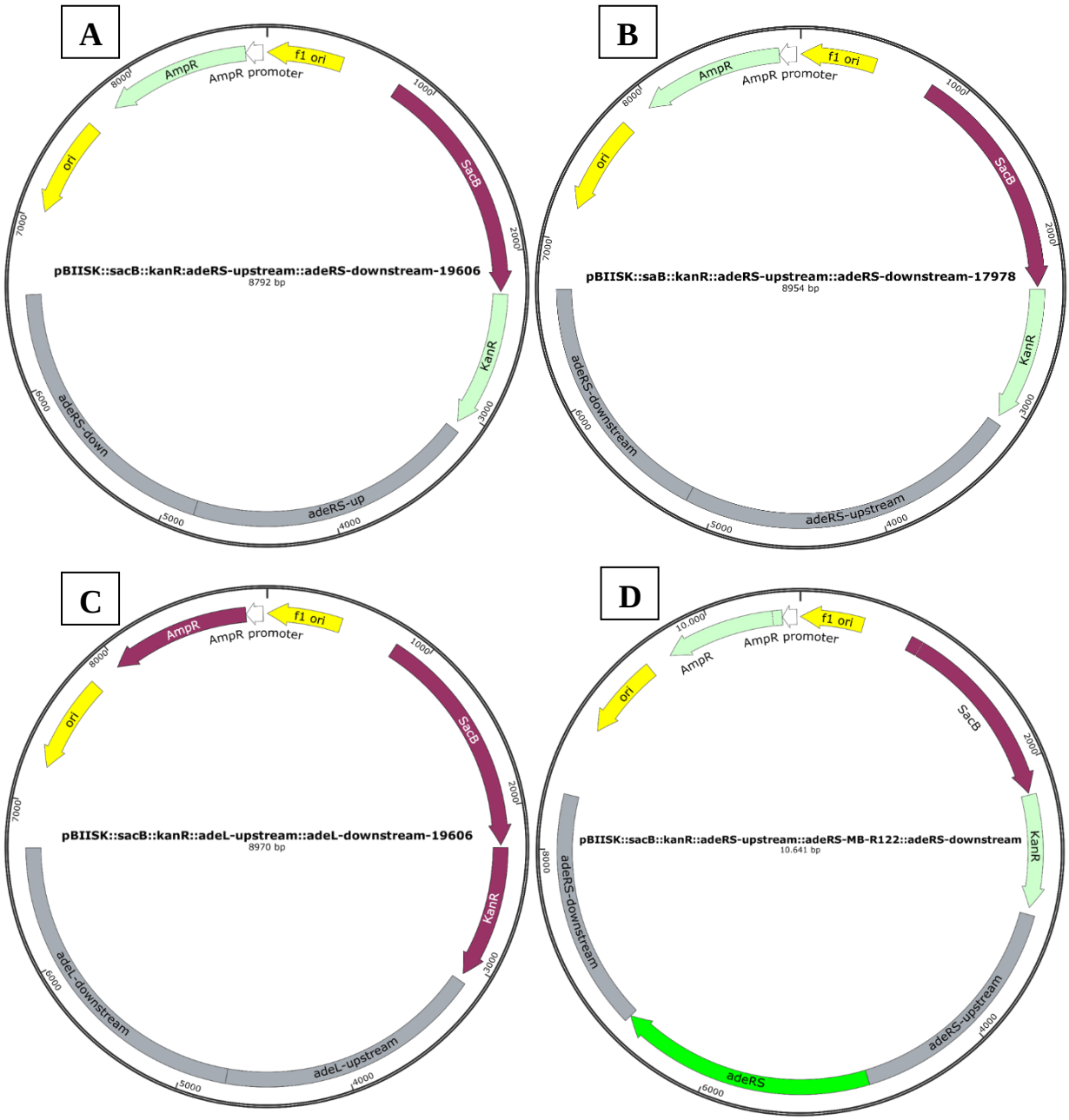
Plasmid cards

Suppl. Figure 1. Plasmid card pBA03/05::*adeRS*^{17978}.



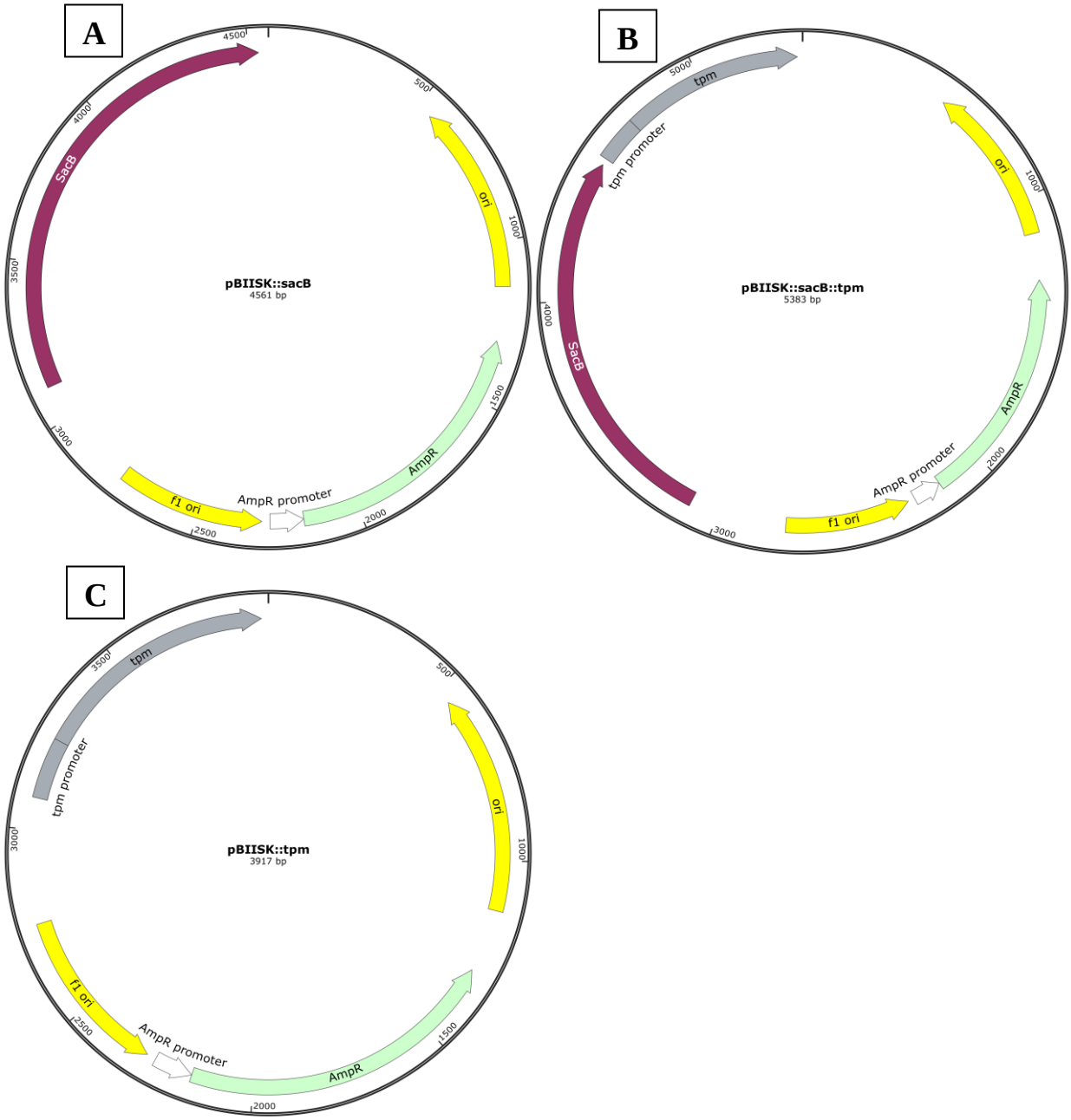
The *adeRS* gene of *A. baumannii* ATCC 17978 was cloned into the PstI restriction site in frame with the IPTG inducable T5 of promoter using the InFusion Cloning Kit.

Suppl. Figure 2. Plasmid cards of pBIISK plasmids generated for markerless mutagenesis.



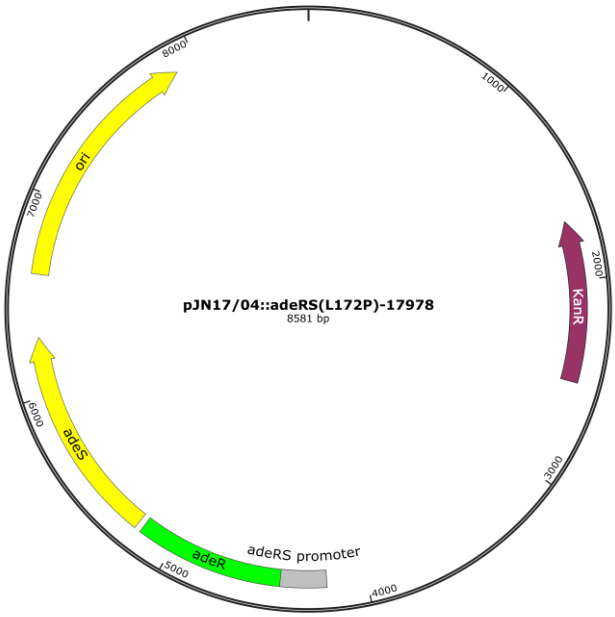
A-C: pBIISK was linearized using the restriction enzyme PstI. The ~1.5kb upstream and downstream regions of the target genes were fused together and cloned into the vector using the InFusion Cloning Kit. **D:** Plasmid B was linearized between the *adeRS* upstream and downstream inserts by PCR. *adeRS* of *A. baumannii* MB-R112 was fused into the plasmid using the InFusion Cloning Kit.

Suppl. Figure 3 Plasmid cards of vectors used for generation of pBIISK::*tpm.*



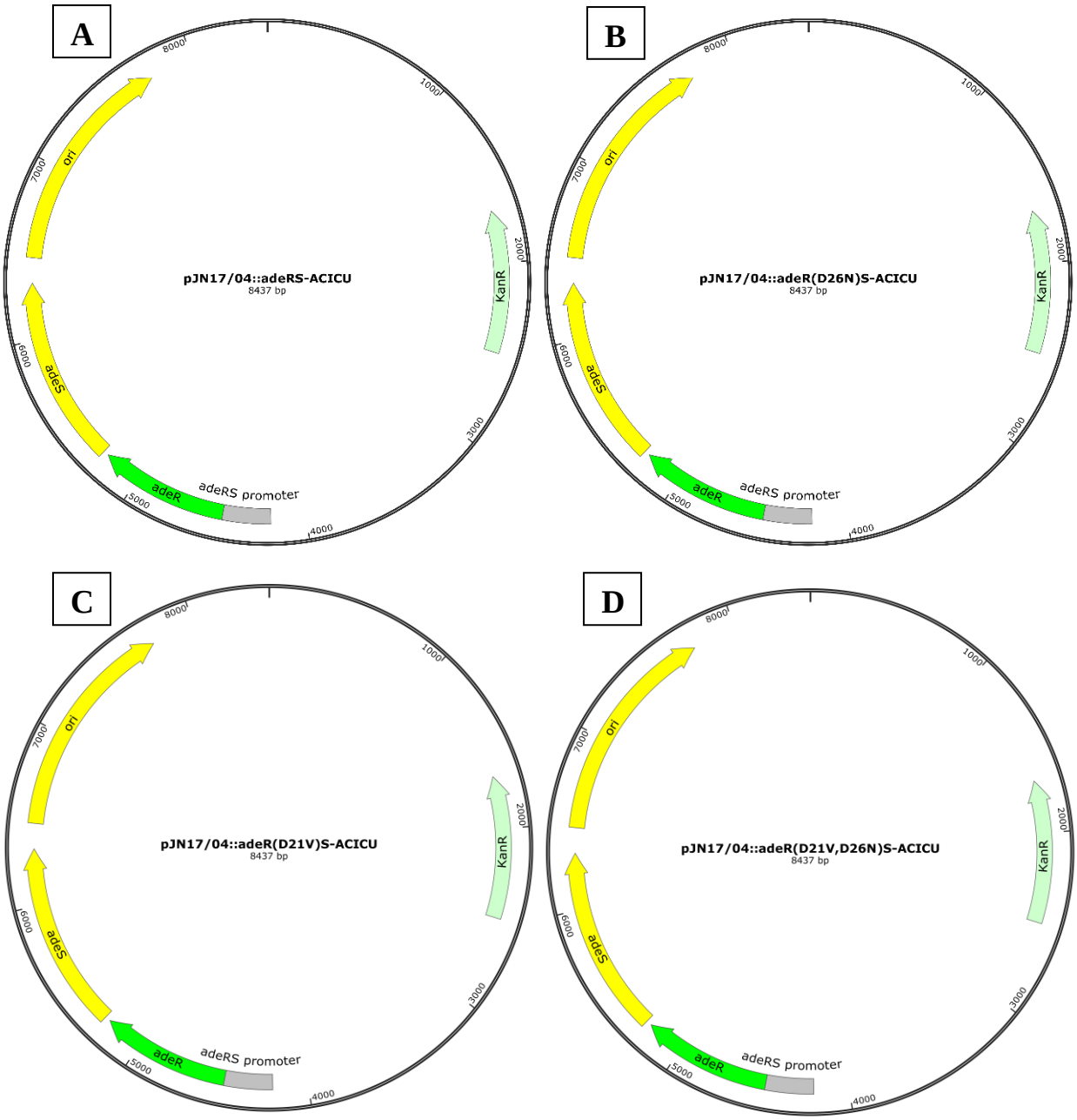
A: pBIISK::*sacB* was generated by PCR amplification of pBIISK::*sacBkanR* upstream and downstream of *kanR*. **B:** *tpm* obtained from *A. baylyi* ADP1 was cloned into NcoI linearized pBIISK::*sacB* using the InFusion Cloning Kit. **C:** pBIISK::*tpm* was generated by PCR amplification of pBIISK::*sacB*::*tpm* upstream and downstream of *sacB.*

Suppl. Figure 4. Plasmid card pJN17/04::*adeRS*(L172P)[17978].



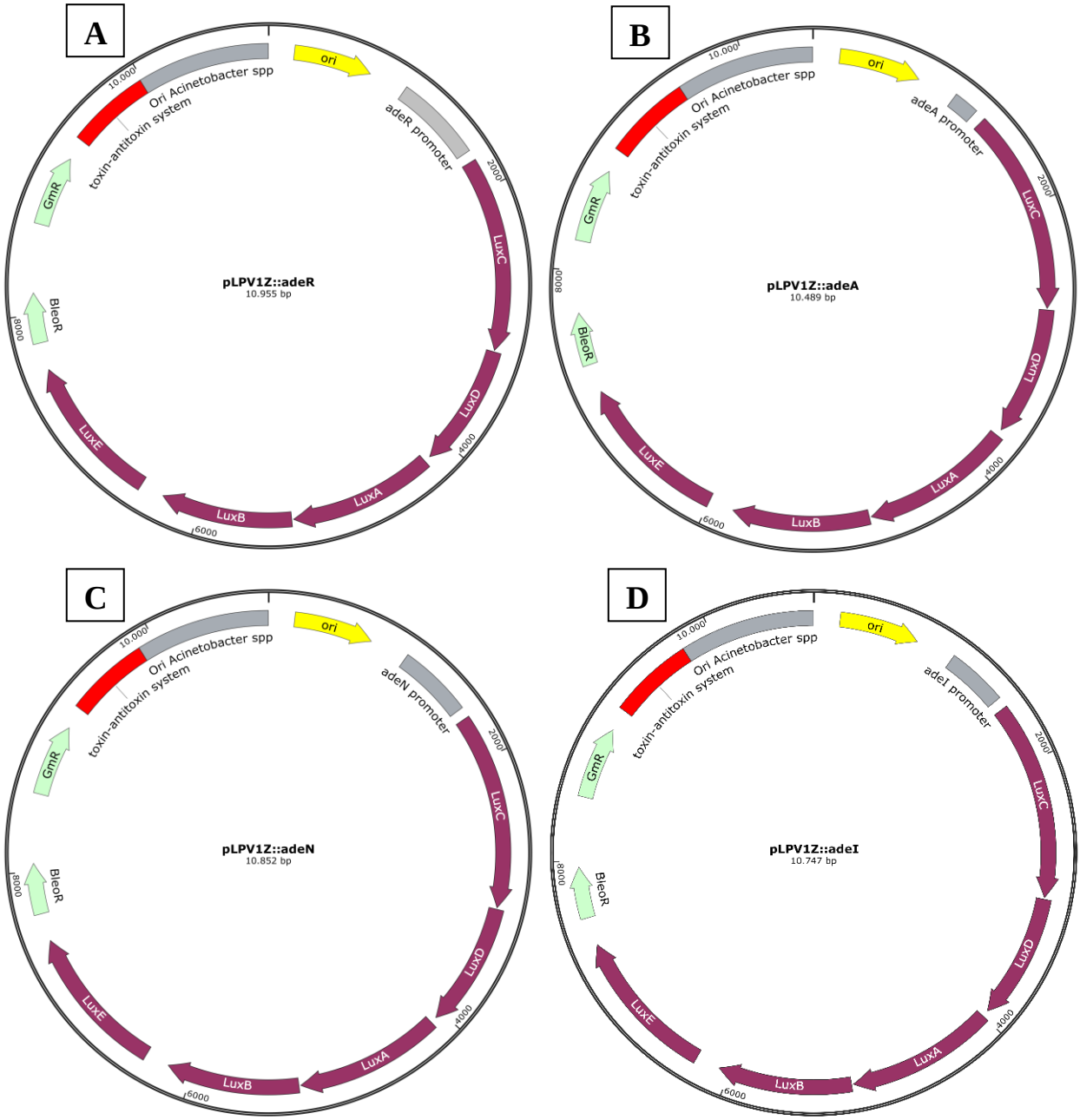
The shuttle vector pJN17/04::*adeRS*[17978] was subjected to Q5 site-directed mutagenesis to introduce the specific nucleotide exchange causing the L172P amino acid substitution.

Suppl. Figure 5. Plasmid cards of pJN17/04::*adeRS*^{ACICU} vectors.

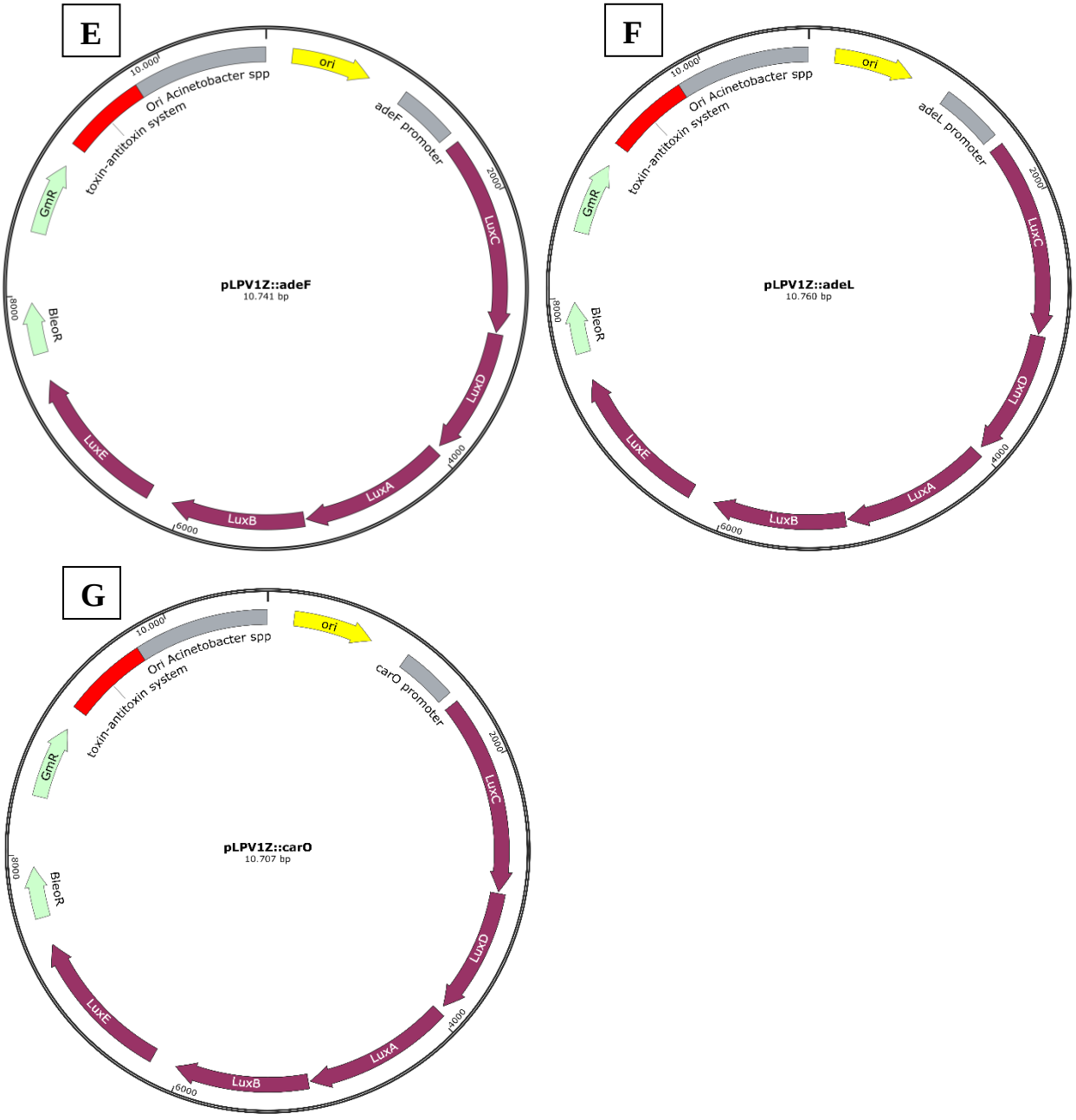


A: The shuttle vector pJN17/04::*adeRS*^{ACICU} was generated by fusing *adeRS* of *A. baumannii* ACICU into ScaI linearized pJN17/04 using the InFusion Cloning Kit. **B-D:** Introduction of the present amino acid substitutions was carried out using the Q5 site-directed mutagenesis kit.

Suppl. Figure 6. Plasmid cards of pLPV1Z luciferase reporter plasmids.

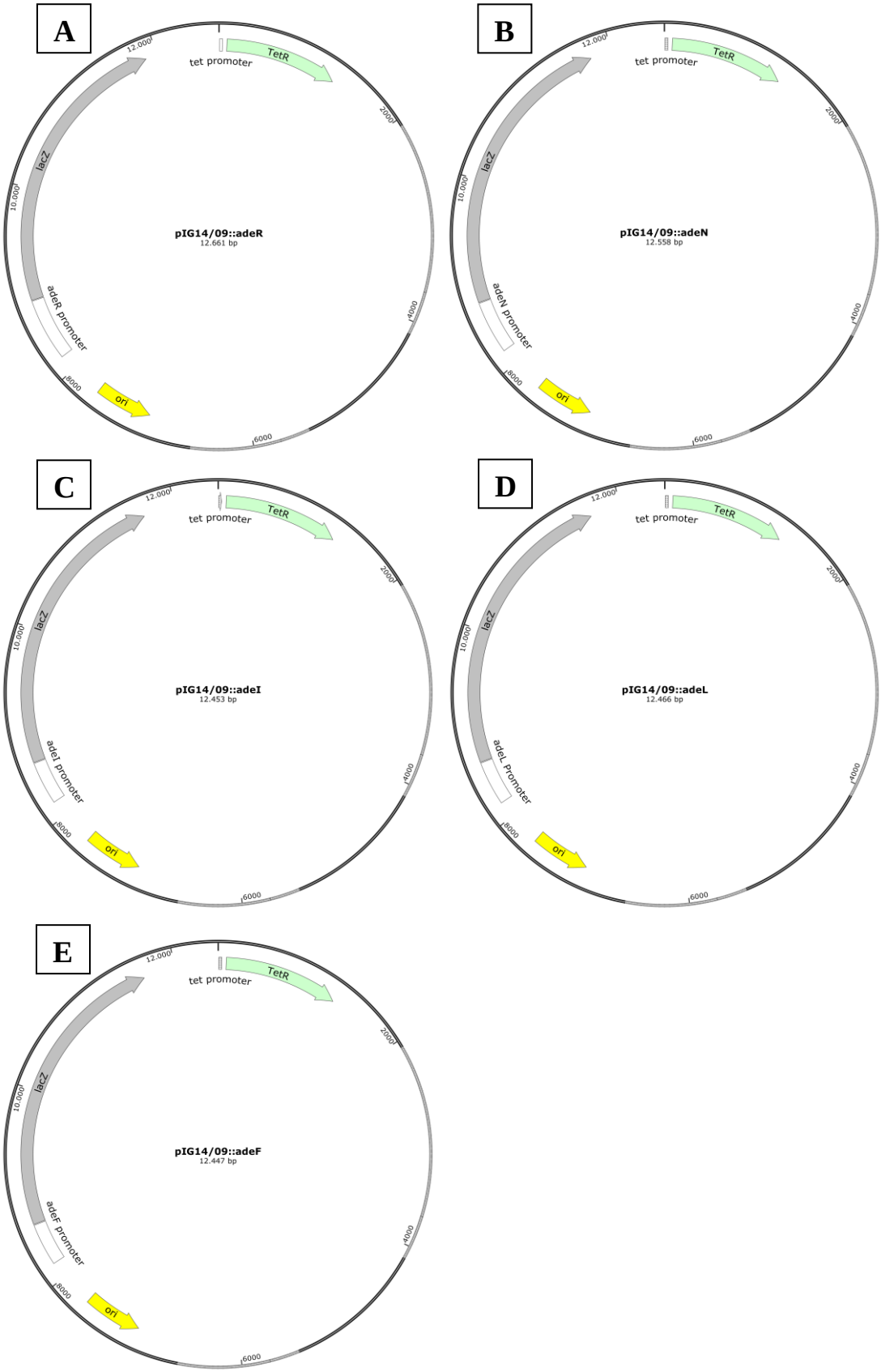


Suppl- Figure 6 continued.



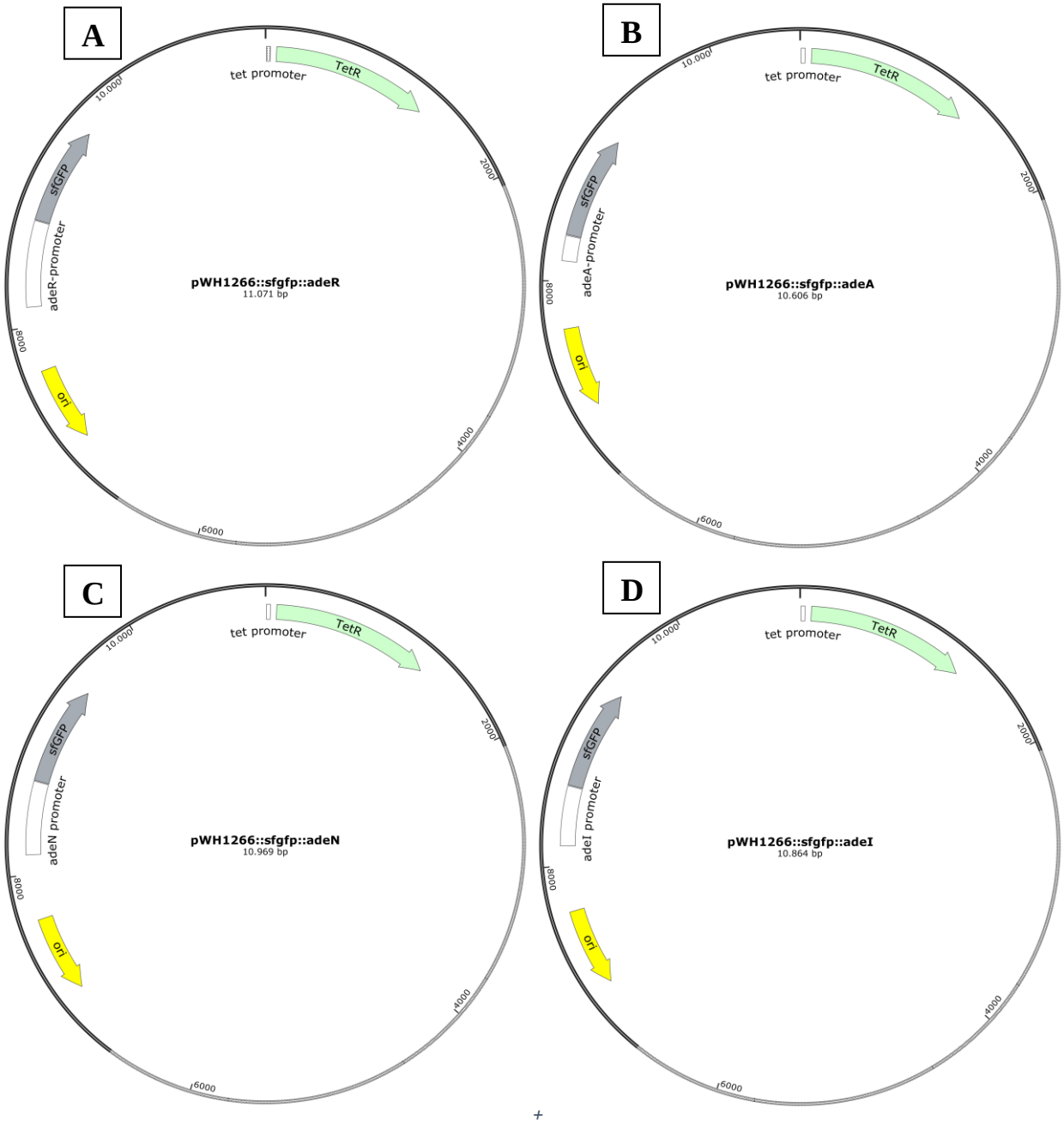
The respective predicted promoter was cloned via the InFusion Cloning Kit into the PstI linearized pLPV1Z vector in frame to the *luxCDABE* operon.

Suppl. Figure 7. Plasmid cards of pIG14/09 LacZ reporter plasmids.

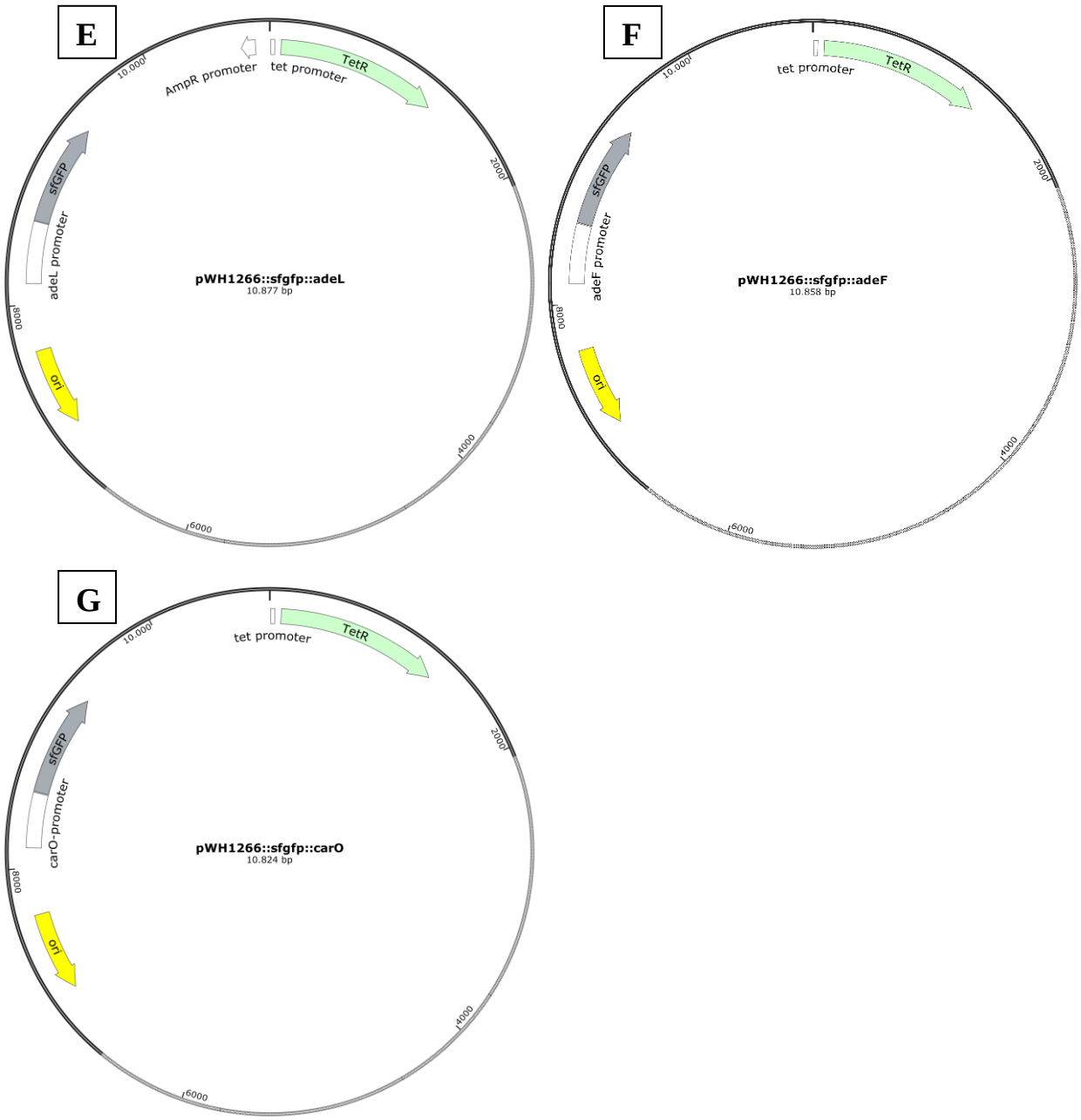


The respective predicted promoter was cloned via the InFusion Cloning Kit into the SmaI linearized pIG14/09 vector in frame to *lacZ*.

Suppl. Figure 8. Plasmid cards of pWH1266::*sfgfp* reporter plasmids.



Suppl. Figure 8 continued.



The respective predicted promoter was cloned via the InFusion Cloning Kit into the PstI linearized pWH1266::*sfgfp* in frame to *sfgfp*.

Other published contributions

In the following published contributions are mentioned, which have been established during the PhD period, but are not related to the thesis project.

Restoring the activity of the antibiotic aztreonam using the polyphenol epigallocatechin gallate (EGCG) against multidrug-resistant clinical isolates of *Pseudomonas aeruginosa*

Betts J.W., Hornsey M., Higgins P.G., **Lucaßen K.**, Wille J., Salguero F.J., Seifert H., La Ragione R.M. Journal of Medical Microbiology, 2019, 68(10):1552-1559.

This study investigated the ability of epigallocatechin (EGCG) to restore the activity of aztreonam against clinical MDR strains of *P. aeruginosa*. A synergy between aztreonam and EGCG was shown that restored susceptibility of MDR *P. aeruginosa* to azetreonam.[228]

I contributed to this manuscript by supporting and conducting experimental design of accumulation studies and their statistical analysis.

Investigation of novel *pmrB* and *eptA* mutations in isogenic *Acinetobacter baumannii* isolates associated with colistin resistance and increased virulence *in vivo*

Gerson S., Betts J.W., **Lucaßen K**., Nodari C.S., Wille J., Josten M.., Göttig S., Nowak J., Stefanik D., Roca I., Vila J., Cisneros J.M., La Ragione R.M. Antimicrobial Agents and Chemotherapy, 2019, 63(3):e01586-18.

In this study, we investigated the mechanisms of colistin resistance in four isogenic pairs of *A. baumannii* isolates displaying an increase in colistin MICs. Amino acid substitutions and deletions in *pmrB* with increased *pmrC* expression and resulted in reduced colistin susceptibility. Additionally, an IS-element upstream of *eptA* increased its expression and consequently lead to increased colistin MICs.[229]

To this publication I have contributed cloning and transformation experiments, gene expression analysis by qRT-PCR, and the determination of antimicrobial susceptibility.

Diversity of amino acid substitutions in PmrCAB associated with colistin resistance in clinical isolates of *Acinetobacter baumannii*

Gerson S., **Lucaßen K**., Wille J., Nodari C.S., Stefanik D., Nowak J., Wille T., Betts J.W., Roca I., Vila J., Cisneros J.M., Seifert H., Higgins P.G. International Journal of Antimicrobial Agents, 2020, 55(3):105862.

This study investigated the mechanisms of colistin resistance in clinical *A. baumannii* isolates. Most isolates exhibited amino acid substitutions in PmrCAB, predominantly in PmrB, indicating the importance of the histidine kinase for colistin resistance. Analysis of gene expression revealed increased *pmrC* expression in isolates harboring pmrCAB mutations. As complementation of *A. baumannii* reference strains with a *pmrAB* variants revealed increased *pmrC* expression but unchanged colistin MICs, additional unknown factors are suspected in colistin resistance, highlighting the diversity and complexity of colistin resistance *in A. baumannii*.[56]

I contributed to this manuscript by designing, preparing, and conducting gene expression analysis by qRT-PCR.

Characterization of a vancomycin-resistant and a vancomycin-susceptible *Enterococcus faecium* isolate from the same blood culture

Xanthopoulou K., Wille J., Zweigner J., **Lucaßen K**., Wille T., Seifert H., Higgins P.G. Journal of Antimicrobial Chemotherapy, 2021, 76(4):883-886.

In this manuscript, two *Enterococcus faecium* isolates with different resistance phenotypes obtained from the same bloodculture were analyzed. It was demonstrated that the isolates were identical but revealed heterogeneous resistance phenotypes due to acquisition or loss of plasmid segments.[230]

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