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

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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).
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
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

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**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

| Strain          | AMK (mg/liter) | 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  | >128| 32  | >128| 64  | 8   | 2   | 32   | 8   |
| ABC153         | >128          | >128| >128| >128| >128| >128| 64  | 8   | 32  | >128 | 8   |
| ABC154         | >128          | >128| >128| >128| 64  | 16  | 64  | 8   | 16  | >128 | 2   |

*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).

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**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.
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...
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, glycyclyclines, 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 ISAb1, and several amino acid substitutions (16–18, 26). These mutations were predominantly observed within the histidine kinase, adenyl 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).

**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.
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 ΔadeRS was described previously (23). The clinical isolates ABC153 and ABC154 are an isolate pair, genetically identical apart from a point mutation in adeD, 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 ΔadeRS. ATCC 17978 ΔadeRS was transformed with purified plasmids by electroporation as described previously (20), 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::adeRSΔaclicu. 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 AdeR T156M substitution | 17                   |
| ABC154            | Genetically identical to ABC153 apart AdeR wild-type configuration | 17                   |
| ATCC 17978 ΔadeRS | A. baumannii reference strain, adeR deleted | 23                   |
| 17978 adeR-wt     | ATCC 17978 ΔadeRS pJN17/04::adeR<sup>SCCU</sup> | This study           |
| 17978 adeR-D21V   | ATCC 17978 ΔadeRS pJN17/04::adeR<sup>(D21V)SCCU</sup> | This study           |
| 17978 adeR-D26N   | ATCC 17978 ΔadeRS pJN17/04::adeR<sup>(D26N)SCCU</sup> | This study           |
| 17978 adeR-D21V+D26N | ATCC 17978 ΔadeRS pJN17/04::adeR<sup>(D21V,D26N)SCCU</sup> | This study           |
| **Plasmids**      |                         |                     |
| pJN17/04          | Shuttle vector           | 21                   |
| pJN17/04::adeR<sup>SCCU</sup> | adeR of ACICU fused to pJN17/04 backbone | This study           |
| pJN17/04::adeR(D21V)<sup>SCCU</sup> | AdeR D21V substitution in pJN17/04::adeR<sup>SCCU</sup> | This study           |
| pJN17/04::adeR(D26N)<sup>SCCU</sup> | AdeR D26N substitution in pJN17/04::adeR<sup>SCCU</sup> | This study           |
| pJN17/04::adeR(D21V,D26N)<sup>SCCU</sup> | AdeR D21V and D26N double substitution in pJN17/04::adeR<sup>SCCU</sup> | This study           |

clone 2 (IC2) and was therefore more appropriate for characterization of amino acid substitutions identified in recent clinical isolates than the uncommon adeR 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 supplement material). PCR products were purified using the QIAquick PCR purification kit (Qiagen). The shuttle vector pJN17/04 (21) was linearized by the restriction enzyme Scal-HF (New England BioLabs). The In-Fusion HD cloning kit (TaKaRa Clontech, Saint-Germain-en-Laye, France) was used for directional cloning of adeR 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::adeR<sup>SCCU</sup>. The Q5 site-directed mutagenesis kit (New England BioLabs) was used to exchange single nucleotides within the sequence of adeR of pJN17/04::adeR<sup>SCCU</sup> 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 (Sano Fi, Frankfurt, Germany), chloramphenicol (Serva, Heidelberg, Germany), and erythromycin (Aventis, Frankfurt, Germany), ciprofloxacin (Bayer Pharma AG, Berlin, Germany), and azithromycin (P. A. Pharma GmbH, Münster, Germany), and supplemented with 0.2% (wt/vol) glucose (Sigma-Aldrich, Frankfurt, Germany) and ethidium bromide (Merck, Darmstadt, Germany) concentration of 10 μM. The MIC 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 (Quantscript 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 ΔadeRS pJN17/04::adeR<sup>SCCU</sup> (here referred to as 17978 adeR<sub>wt</sub>), pJN17/04::adeR<sup>(D26N)SCCU</sup> (here referred to as 17978 adeR<sub>D26N</sub>), pJN17/04::adeR<sup>(D21V)SCCU</sup> (here referred to as 17978 adeR<sub>D21V</sub>), and pJN17/04::adeR<sup>(D21V,D26N)SCCU</sup> (here referred to as 17978 adeR<sub>D21V+D26N</sub>) 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.
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.

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We have no conflict of interest to declare.

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