Molecular cloning and functional characterization of two novel membrane fusion proteins in conferring antimicrobial resistance in *Acinetobacter baumannii*

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Objectives: The aim of this study was to elucidate the role of two novel membrane fusion proteins (MFPs) in the susceptibility of *Acinetobacter baumannii* to antimicrobial agents.

Methods: The genome sequence of *A. baumannii* ATCC 17978 contains two open reading frames (ORFs) annotated as AdeT in the NCBI genome database. Both the putative efflux genes display >30% similarity to known MFPs. The antimicrobial susceptibility profiles of *Escherichia coli* KAM32 cells carrying the genes were monitored by the broth dilution method. Different efflux pump inhibitors were used for fluorimetric efflux assays. The functions of putative ORFs were confirmed in *A. baumannii* by insertional inactivation and complementation.

Results: *E. coli* cells carrying the ORFs had decreased susceptibility to antibiotics, disinfectants, dyes and detergents, with enhanced efflux activity. Inactivation of the ORFs and further characterization in *A. baumannii* confirmed its role in antimicrobial resistance by active efflux.

Conclusions: This report describes the functions of novel resistance determinants, members of the MFP family, for the first time in *A. baumannii*.

Keywords: periplasmic accessory proteins, nosocomial pathogens, active efflux, *A. baumannii*

Introduction

*Acinetobacter baumannii* is a rapidly emerging nosocomial pathogen that causes severe infections, including bacteraemia, pneumonia, meningitis, urinary tract and wound infections. It has now become a major cause of hospital-acquired infections worldwide due to its remarkable propensity to rapidly acquire resistance determinants to a wide range of antibacterial agents. In bacteria, resistance to antibiotics is often associated with multidrug efflux pumps functioning to decrease cellular drug accumulation. In Gram-negative bacteria, including *A. baumannii*, the pumps belonging to the resistance nodulation division (RND) family are significantly effective in generating resistance, as they form a tripartite complex together with the periplasmic proteins belonging to the membrane fusion protein (MFP) family and the outer membrane protein (OMP) channels, so that drugs are pumped out directly to the external medium. The RND pumps often have wide substrate specificity. The best-studied members of the RND group are the MexAB-Opm system of *Pseudomonas aeruginosa* and the AcrAB-TolC system of *Escherichia coli*, where AcrA and MexA are the MFPs in the system.

The RND efflux complex in *A. baumannii*—AdeI (the MFP), AdeJ (transporter), together with AdeK (OMP)—was found to confer resistance to β-lactams, aminoglycosides, fluoroquinolones and structurally unrelated compounds. The first member of the RND family of exporters discovered in *A. bauman- nii* BM4454 was the AdeABC system, and it is known to pump out mostly aminoglycosides, tetracycline, erythromycin and fluoroquinolones. Availability of bacterial genome sequences enable us to trace the presence of putative drug resistance genes in the *A. baumannii* ATCC 17978 genome. We focused our research efforts on a pair of open reading frames (ORFs) that displayed homologies to known members of the MFP family. Therefore, the aim of the present study was to delineate the functions of...
two previously uncharacterized ORFs from a multidrug-resistant (MDR) clinical isolate A. baumannii AC0037.

**Materials and methods**

**Bacterial strains, growth medium and chemicals**

A. baumannii AC0037, an MDR strain used in this study, was isolated from a patient with a respiratory tract infection in the Ohio State University Medical Center, Columbus, Ohio. E. coli KAM32 (ΔaadA ΔydhE) was used as the host for genetic studies. Bacteria were grown at 37°C in Luria-Bertani (LB) and agar (Difco Laboratories, Sparks, NV, USA). Antibiotics, dyes, detergents and inhibitors were purchased from Sigma-Aldrich.

**Cloning of the putative efflux genes**

The genome of A. baumannii ATCC 17978 (accession number CP000521.1) has a pair of ORFs, both annotated as AdeT. The ORF1 (A1S_1755) and ORF2 (A1S_3092) are two separate, distinct and distinctly placed putative efflux genes. Genomic DNA from A. baumannii AC0037 was extracted using the DNeasy Tissue Kit (Qiagen, Valencia, CA, USA). These ORFs were amplified from A. baumannii AC0037 using gene-specific primers adeT1F/R and adeT2F/R, as listed in Table 1, by PCR as described previously with minor modifications. The digested PCR fragments were ligated with PstI-kanamycin resistance cassette (K) from the pUC-4K vector. The resulting AKB fragment was inserted into the XbaI site of pWH-Spc to yield a recombinant plasmid padeT1-Kan. The recombinant plasmids denoted pAdet1 and pAdet2 were transformed into E. coli KAM32 to generate E. coli KAM32/pAdet1 and E. coli KAM32/pAdet2.

**MICs**

MICs were determined according to the guidelines of the CLSI by the broth dilution method in Mueller–Hinton broth (MHB; Difco Laboratories, Sparks, NV, USA). Antibiotics, used as the host for genetic studies. Bacteria were grown at 37°C in Luria-Bertani (LB) and agar (Difco Laboratories, Sparks, NV, USA). Antibiotics, dyes, detergents and inhibitors were purchased from Sigma-Aldrich.

**Accumulation studies**

Accumulation of ciprofloxacin was monitored as described previously. Briefly, bacteria were grown to log phase, harvested and suspended in PBS to an OD600 of 0.2. Ciprofloxacin was added to a final concentration of 10 mg/L. Fluorimetric measurements were performed using an LS 55 Fluorescence Spectrometer, 120V (Perkin-Elmer). The assay was also performed using 4',6-diamidino-2-phenylindole (DAPI). DAPI binds to specific cellular components and undergoes a change in fluorescence that can be used to indicate how much of the substrate is retained by the cell and therefore its accumulation can be used to assess the relative level of active efflux in bacterial cells. The excitation and emission wavelengths used for the fluorescent compounds were as follows: ciprofloxacin, 275/440 nm; DAPI, 345/455 nm, respectively.

**Construction of deletion mutants in A. baumannii AC0037**

To construct an adeT1 defective mutant, 400 bp upstream (amplicon A) with primers UpP1/ParP2 (Table 1) and 350 bp downstream with primer ParP3, DwnP4 (amplicon B) was amplified by PCR from A. baumannii AC0037. The products A and B were digested with respective enzymes present in their primer sequences. The digested PCR fragments were ligated with PstI-kanamycin resistance cassette (K) from the pUC-4K vector. The resulting AKB fragment was inserted into the XbaI and BamHI double-digested plasmid pUC18 (unable to replicate in A. baumannii), generating the padeT1-Kan plasmid. The recombinant plasmid padeT1-Kan was introduced into the kanamycin-susceptible, ticarcillin-resistant A. baumannii AC0037 by electroporation (Eppendorf, Hamburg, Germany). Selection of the adeT1 null mutant was made on plates containing 50 mg/L kanamycin and 80 mg/L ticarcillin and confirmed by DNA sequencing; the mutant was designated AC0037adeT1. A similar strategy was used to inactivate adeT2 using primers UpP5 and ParP6 (300 bp) and ParP7 and DwnP8 (250 bp). The adeT2 null mutant was designated AC0037adeT2.

**Complementation studies with AdeT deletion mutants**

Briefly, a DNA fragment containing a functional aadA1 gene was amplified from the AC0019 clinical isolate (accession number EU977568.1) by PCR using primers aadA1F/R and was ligated into the E. coli–Acinetobacter shuttle vector pWH1266. The resulting plasmid, pWH-Spc, was modified by cloning a PCR-amplified parental gene adeT1 from A. baumannii strain AC0037 into the PstI site of pWH-Spc to yield a recombinant plasmid pWH-adeT1. Electroporation of the recombinant plasmid pWH-adeT1 into deletion mutant AC0037adeT1 resulted in an AC0037adeT1adeT2 mutant. Selection of the complemented mutant was made on plates containing 200 mg/L streptomycin and 50 mg/L kanamycin. A similar approach was followed to generate AC0037adeT2adeT2.

**Data analysis**

The assays were performed in triplicate on independent occasions to confirm reproducibility. All data are presented as means ± SEM.

**Accession numbers for the gene sequences**

The nucleotide sequences have been deposited in the GenBank nucleotide sequence database under the accession numbers GQ338837 and GQ338838.
Table 2. Susceptibility testing of various compounds in heterologous and native hosts

<table>
<thead>
<tr>
<th>Compounds</th>
<th>MIC (mg/L) for strains</th>
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|                 | control pUC18/KAM32    | pAdeT1/KAM32 | pAdeT2/KAM32 | AC0037 AdeT1 | AC0037 AdeT2 | AC0037 AdeT1
ded | AC0037 AdeT2
ded | AC0037 AdeT1 AdeT2 | AC0037 AdeT2 AdeT2 |
| Chloramphenicol | 0.2 1.2 1.6            | 256 | 64 | 4 | 32 | 8 | 128 |
| Ciprofloxacin   | 0.002 0.002 0.01       | 120 | 120 | 1 | 24 | 5 | 120 |
| Erythromycin    | 4 24 20                | 64 | 32 | 2 | 16 | 4 | 64 |
| Minocycline     | 0.5 1 1                | 2 1 | 2 | 1 | 2 | 2 |
| Nalidixic acid  | 1 2 1                 | 256 | 128 | 2 | 256 | 1 | 256 |
| Norfloxacin     | 0.6 1.2 2.4           | 512 | 512 | 1 | 128 | 4 | 512 |
| Novobiocin      | 4 20 24               | 120 | 60 | 2 | 20 | 6 | 120 |
| Rifampicin      | 2 2 2                | 2 | 2 | 1 | 2 | 2 |
| Tetracycline    | 0.5 1 1               | 64 | 32 | 2 | 32 | 2 | 64 |
| Trimethoprim    | 0.125 0.2 0.2         | 64 | 32 | 2 | 32 | 2 | 64 |
| Acridine Orange | 16 64 96              | 200 | 50 | 4 | 40 | 5 | 128 |
| Acriflavine     | 2 8 16               | 128 | 64 | 2 | 32 | 4 | 128 |
| Benzalkonium chloride | 1 3 4 | 48 | 32 | 1.5 | 16 | 3 | 48 |
| Chlorhexidine   | 2 4 4                | 32 | 32 | 1 | 32 | 1 | 32 |
| Crystal Violet  | 0.01 0.01 0.03        | 0.05 | 0.05 | 1 | 0.01 | 5 | 0.05 |
| DAPI            | 0.5 2 2              | 2 | 1 | 2 | 0.5 | 4 | 2 |
| Deoxycholate    | 125 500 750          | >2048 | 1024 | 2 | 1024 | 2 | 2048 |
| Ethidium bromide| 4 4 24              | 1024 | 1024 | 1 | 256 | 4 | 1024 |
| Methyl viologen | 64 128 128           | 800 | 400 | 2 | 400 | 2 | 800 |
| Pyronin Y       | 4 8 8               | 4 | 4 | 1 | 4 | 1 | 4 |
| Rhodamine 123  | 8 16 16             | 128 | 64 | 2 | 64 | 2 | 128 |
| SDS             | 50 200 400         | >256 | 64 | 4 | 42 | 6 | >128 |
| Synergize (%)   | <0.005 0.01 0.01    | 0.01 | 0.002 | 5 | 0.002 | 5 | 0.01 |
| TPPCl           | 8 16 32             | 1500 | 1024 | 1.5 | 512 | 3 | 1500 |
| Virkon-S (%)    | 0.02 0.02 0.02      | 0.3 | 0.3 | 1 | 0.3 | 1 | 0.3 |
| Wex-cide-128 (%)| 0.01 0.01 0.01     | 0.1 | 0.05 | 2 | 0.005 | 2 | 0.1 |

Different classes of disinfectant formulations used were: Virkon-S (an oxidizing agent: peroxygen blend and organic acid) (Antec International, UK); Wex-cide-128 (a phenolic agent) (Wexford Laboratories, USA); Synergize [a glutaraldehyde disinfectant containing quaternary ammonium compound (QAC)] (Preserve International, USA); chlorhexidine gluconate (MP Biomedicals, LLC); benzalkonium chloride (a QAC) (MP Biomedicals, LLC); and TPPCl, tetraphenylphosphonium chloride.

*aSusceptibility profiles of E. coli expressing AdeT1, AdeT2 and control having the pUC18 plasmid alone.
*bMIC data for A. baumannii AC0037 and adeT null mutants and complemented strains used in this study.
*cFold change is the ratio of MICs for strain AC0037 to \( \Delta \text{adeT} \).
Results and discussion

Cloning of two putative ORFs from A. baumannii AC0037

The two ORFs annotated as AdeT were amplified by PCR from A. baumannii AC0037 and cloned in pUC18 to yield recombinant plasmids pAdeT1 and pAdeT2. Sequencing revealed that pAdeT1 harboured one complete ORF (100% identical to A1S_1755) and plasmid pAdeT2 harboured the other ORF (100% identical to A1S_3092). Further sequence analysis revealed that adeT1 is a 915 bp gene with a start and stop codon and specifies a 304 amino acid protein with a predicted mass of 33.46 kDa. The adeT2 gene is a 717 bp gene with a start and stop codon and specifies a 238 amino acid protein with a predicted mass of 27.28 kDa.

Using the Basic Local Alignment Search Tool (BLAST) at www.ncbi.nlm.nih.gov, it was observed that the AdeTs exhibit >30% similarity to CmeA of Campylobacter jejuni (accession number ACQ85275),17 MexA of P. aeruginosa (accession number AAG03814)5 and AdeA of A. baumannii (accession number YP_002319438).8 Thus, initial assessment indicated

Figure 1. Fluorimetric efflux assay. The accumulation profiles of ciprofloxacin (a) or DAPI (b) by AC0037, AC0037ΔadeT1, AC0037ΔadeT2 and E. coli KAM32. The accumulation study was done using 10 mg/L of the antimicrobial agent as described previously.10,13 Arrows indicate the timepoints when the inhibitor carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was added to the assay mixture. Relative fluorescence intensity (y-axis) represents the levels of accumulated ciprofloxacin or DAPI. The graph shows the fluorescence value of the supernatant collected in the absence (5–20 min) and the presence (25–40 min) of the inhibitor CCCP. Each data point represents the mean ± SD of three independent experiments.
that both AdeT1 and AdeT2 were members of the MFP family of proteins.

**Contributions of the accessory proteins AdeT1 and AdeT2 in mediating antimicrobial resistance**

The MICs of antimicrobial agents for E. coli KAM32 carrying AdeT1 (E. coli KAM32/pAdeT1) and AdeT2 (E. coli KAM32/pAdeT2) were monitored and compared with the control strain (E. coli KAM32/pUC18) to obtain the fold differences in susceptibilities. Expression of AdeT1 or AdeT2 in E. coli KAM32 from the vector exhibited a similar fold higher resistance to erythromycin (6- and 5-fold, respectively), Acridine Orange (4- and 5-fold, respectively) and DAPI (4- and 4-fold, respectively).

AdeT1-carrying E. coli cells displayed elevated MICs of chloramphenicol (5-fold), novobiocin (5-fold), gentamicin (4-fold), kanamycin (3-fold), acriflavine (4-fold), deoxycholate (4-fold), SDS (4-fold) and benzalkonium chloride (3-fold) (Table 2). AdeT2 also conferred a similar profile of increased MICs (Table 2).

**Characterization of efflux genes in MDR clinical strain A. baumannii AC0037**

To decipher the role of adeT in A. baumannii, AC0037ΔadeT1 and AC0037ΔadeT2 mutants were constructed as described in the ‘Materials and methods’ section. Susceptibility testing was performed with these mutants along with their parental strain AC0037 as control. Data showed that deletion of adeT resulted in increased susceptibility to different antibiotics, dyes, detergents and disinfectants (Table 2).

**Complementation studies**

The role of A. baumannii adeTs was confirmed by performing complementation experiments as described in the methodology. The expression of AdeT1 and AdeT2 in complemented strains was confirmed at the RNA level (data not shown).Susceptibility testing indicated that complementation with wild-type gene restored the ability of the deletion mutants to confer resistance to antimicrobial agents similar to that displayed by AC0037 (Table 2).

**Fluorimetric efflux assay using A. baumannii null mutants**

Fluorimetric experimentation was performed to elucidate the participation of adeT in active efflux. The ciprofloxacin accumulation data indicated that efflux was decreased in AC0037ΔadeT1 compared with the wild-type AC0037, whereas it was increased (>3-fold increased accumulation) in the AC0037ΔadeT2 mutant. Expression of AdeT2 increased the MIC of ciprofloxacin; upon deleting adeT2 in the native host (AC0037ΔadeT2 mutant), ciprofloxacin efflux was decreased in the AC0037ΔadeT2 mutant, which was obvious, as the mutant lacked the functional AdeT2 protein. The addition of CCCP increased the ciprofloxacin accumulation levels, which eventually reached a plateau in the tested strains (Figure 1a). However, upon inactivating a single gene we did not observe a complete loss in resistance profile. It is worthy to state here that other efflux pumps in the genome of the bacteria have a role in conferring decreased susceptibility to these agents.

An increase in the MIC of DAPI was observed upon expressing either gene in E. coli, thus their absence led to an increased accumulation of DAPI in AC0037ΔadeT1 (>1.5-fold) and AC0037ΔadeT2 (>2.3-fold) mutants (Figure 1b). Data presented here indicate that AdeTs possibly cooperate with other A. baumannii transporters such as AdeB or AdeJ and OMPs such as AdeC or AdeK to mediate the antimicrobial resistance phenomenon.

**Conclusions**

Overall, using genetic tools, we demonstrated for the first time the importance of two MFPs in mediating antimicrobial resistance in a MDR clinical isolate, A. baumannii AC0037.

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**Transparency declarations**

None to declare.

**References**