Acinetobacter nosocomialis: Defining the Role of Efflux Pumps in Resistance to Antimicrobial Therapy, Surface Motility, and Biofilm Formation

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Acinetobacter nosocomialis is a member of the Acinetobacter calcoaceticus-Acinetobacter baumannii (ACB) complex. Increasingly, reports are emerging of the pathogenic profile and multidrug resistance (MDR) phenotype of this species. To define novel therapies to overcome resistance, we queried the role of the major efflux pumps in A. nosocomialis strain M2 on antimicrobial susceptibility profiles. A. nosocomialis strains with the following mutations were engineered by allelic replacement; ∆adeB, ∆adeJ, and ∆adeB/adeJ. In these isogenic strains, we show that the ∆adeJ mutation increased susceptibility to beta-lactams, beta-lactam/beta-lactamase inhibitors, chloramphenicol, monobactam, tigecycline, and trimethoprim. The ∆adeB mutation had a minor effect on resistance to certain beta-lactams, rifampicin and tigecycline. In addition, the ∆adeJ mutation resulted in a significant decrease in surface motility and a minor decrease in biofilm formation. Our results indicate that the efflux pump, AdeIJK, has additional roles outside of antibiotic resistance in A. nosocomialis.

Keywords: Acinetobacter, RND-efflux, motility, biofilm, antimicrobial resistance

INTRODUCTION

Acinetobacter nosocomialis is a Gram-negative opportunistic pathogen that is grouped into the Acinetobacter calcoaceticus-Acinetobacter baumannii (ACB) complex (Nemec et al., 2011; Visca et al., 2011). The ability of A. nosocomialis to cause disease in humans is well-recognized (Wisplinghoff et al., 2012; Chusri et al., 2014; Huang et al., 2014), although studies suggest the virulence of this bacterium may be lower than the closely related bacterium Acinetobacter baumannii (Peleg et al., 2012; Lee et al., 2013; Yang et al., 2013; Fitzpatrick et al., 2015). Many potential virulence factors have been identified in A. nosocomialis and include a CTFR inhibitory factor (Cif), a protein O-glycosylation system, a type-I secretion system, a type-II secretion system, secretion of outer membrane vesicles, the OmpA protein, the CpaA protease, and quorum sensing (Niu et al., 2008; Bahl et al., 2014; Harding et al., 2015, 2016, 2017; Nho et al., 2015; Weber et al., 2015; Kim et al., 2016; Kinsella et al., 2017).
A. nosocomialis strain M2 was isolated in 1996 from a hip infection and has been extensively studied, particularly with respect to the virulence factors described above. M2 was formerly classified as A. baumannii, but whole genome sequencing resulted in its reclassification (Carruthers et al., 2013). While A. nosocomialis can be highly resistant to antibiotics, the role of RND-type efflux pumps in this process has not been investigated in this bacterium. Two primary efflux systems in the closely related A. baumannii are the AdeABC and AdeIJK efflux systems (Magnet et al., 2001; Damier-Piolle et al., 2008). Each efflux system is composed of an outer membrane channel (AdeC, AdeK), a membrane fusion protein (AdeA, AdeI) and an inner membrane transporter (AdeB, AdeJ). In addition to the efflux of antimicrobials, these systems can impact additional phenotypes in the cell, such as surface motility, biofilm formation, and virulence (Yoon et al., 2015; Richmond et al., 2016).

In this study, we investigated the role of AdeABC and AdeIJK orthologs in A. nosocomialis. Similar to what is observed in A. baumannii, loss of AdeIJK had a major impact on antibiotic susceptibility profiles. In contrast, the loss of AdeABC had a minimal impact on susceptibility. Interestingly, the loss of AdeIJK reduced surface motility, indicating additional roles for this RND-type efflux system in A. nosocomialis.

MATERIALS AND METHODS

Bacterial Growth Conditions, Strains, and Plasmids

A. nosocomialis strain M2 was used for all studies and has been described previously (Carruthers et al., 2013). E. coli strains EC100D and CC118 were used for general cloning. E. coli strain SM10 was used for bacterial conjugations. Growth media consisted of 10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter. Agar was added at 15 g per liter. For sucrose counter-selections, media was prepared as described above, but without NaCl and containing 10% sucrose. Cloning vectors were pBC.SK- (Agilent) and pKNG101 (Kaniga et al., 1991).

Construction of adeB and adeJ Mutations

Internal fragments of the adeB and adeJ genes were obtained by PCR amplification of M2 genomic DNA using the following primers. peg93for 5'- TTGCTAAGTATTCCTAAATTAC-3' and peg93.rev 5'- TTAGGAAGAGATTTTTTTC-3' for adeB, and peg1681for 5'- ATGGCACAATTTTTTATTCATC-3' and peg1681.rev 5'- TCAGATTATGCTCCTGAG-3' for adeJ. The resulting PCR generated fragments were cloned into the pBC.SK digested plasmid with SmaI, creating padeB and padeJ. The padeB plasmid was then digested with NarI, which digests once in the middle of the adeB gene and treated with T4 DNA polymerase to create blunt ends. This was then re-ligated to create a frameshift mutation in adeB. The plasmid padeJ was digested with SphI, which cuts once in the middle of adeJ, treated with T4 DNA polymerase to create blunt ends and re-ligated to create an adeJ frameshift mutation. The mutated adeB and adeJ genes were then excised as an XbaI-Sall fragment and cloned into the suicide vector pKNG101 digested with XbaI and Sall. Each plasmid was transformed into E. coli SM10 and then introduced into the A. nosocomialis M2 chromosome by conjugation. Exconjugants were grown for 10 generations in LB broth without antibiotic and dilutions were plated on lysogeny broth (LB) plates without sodium chloride and containing 10% sucrose. Colonies containing the adeB or adeJ frameshift mutations were identified by PCR amplifying each gene and the digesting the resulting PCR products with either NarI for adeB or SphI for adeJ. The presence of each chromosomal mutation was indicated by the failure of each enzyme to digest the fragment and each mutation was verified by DNA sequence analysis. To create an adeB, adeJ double mutant, the adeB mutant was used as the parent and the adeJ mutation was crossed into the chromosome as described above. To create an adeB::Km mutation, an EZ-Tn5<Kan-2-> insertion centrally located in the adeB gene present in pKNG101 was recombined into the chromosomal copy of adeB as described above.

Antimicrobial Susceptibility Testing

A. nosocomialis strain M2 and its isogenic derivatives were subject to antimicrobial susceptibility testing using E-Test Strips, Trek, and MicroScan platforms. Additionally, disk diffusion assays were performed using Mueller Hinton agar for several antibiotics alone and in combination with boronic acid transition state inhibitor (BATSI) compounds SM23 and S02030 (Powers et al., 2014; Nguyen et al., 2016). For TREQ, strains were tested once. For the disc diffusion and Etest assays, strains were tested in duplicate.

Motility Assays

The base media for motility assays consisted of 10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter. Media was solidified with 1.5% agar and grown up to early log phase, adjusted to the same optical density of A600 = 0.15 by the addition of sterile LB broth and a 1 µl drop was placed on the center of the plate. Plates were incubated at 30°C and motility was measured after 14 h. Statistical analysis was done using the Student's T-test.

Biofilm Analysis

Cells for biofilm analysis were taken directly from freezer stocks and grown in 2 ml 0.5X LB without shaking at room temperature to an optical density A600 of 0.1. Each tube was then used to inoculate wells of a 96 well microtiter plate with 150 µl of culture. Plates were incubated stationary at 30 or 37°C for 24 h. The optical density of each well was read at A600 for cell growth and the planktonic cells were removed. To stain biofilms, 250 µl of 10% crystal violet was added to each well for 30 min. The crystal violet was gently decanted and each well was gently washed three times with distilled water. Three hundred microliters of 33% acetic acid was added to each well to solubilize the crystal violet and the absorbance of a 1/10 dilution was read at A585. Statistical analysis was done using the Student's T-test.
TABLE 1 | Antimicrobial susceptibility profiles.

| Antibiotic (mg/L) | M2 wild-type | M2 ΔadeB | M2 ΔadeJ | M2 ΔadeB, ΔadeJ |
|-------------------|-------------|---------|---------|----------------|
| **E-TEST (mg/L)** |             |         |         |                |
| Ampicillin        | 24          | 16      | 8       | 8              |
| Cefotaxime        | >32         | 24      | 1.5     | 1.5            |
| Ceftriaxone       | >32         | >32     | 3       | 3              |
| Chloramphenicol   | 64          | 64      | 6       | 6              |
| Amikacin          | 4           | 3       | 3       | 3              |
| Rifampin          | 12          | 8       | 3       | 3              |
| Tigecycline       | 0.25        | 0.19    | 0.032   | 0.023          |
| Trimethoprim      | >32         | >32     | 1.5     | 1.5            |
| **TREK (mg/L)**   |             |         |         |                |
| Piperacillin/tazobactam | 16/4 | ≤8/4 | ≤8/4 | ≤8/4 |
| Cefazidime        | 4           | 4       | ≤1      | ≤1             |
| Cefuroxime        | 16          | 16      | ≤4      | ≤4             |
| Amikacin          | ≤4          | ≤4      | ≤4      | ≤4             |
| Aztreonam         | >16         | >16     | 4       | 4              |
| Meropenem         | <1          | <1      | <1      | <1             |

RESULTS

Analysis of AdeABC and AdeIJK
RND-Efflux Systems in A. nosocomialis

A. nosocomialis strain M2 contains orthologs of AdeA, AdeB and AdeC that share 94, 98, and 92 percent amino acid identity, respectively, to the corresponding proteins in A. baumannii strain AB5075.UW. In addition, orthologs of the AdeIJK proteins were found with 97, 99, and 98 percent identity to the corresponding proteins in A. baumannii AB5075.UW. To investigate the function of each RND-type efflux system, null alleles in the adeB and adeJ genes, encoding the inner membrane transporter for each system were constructed by introducing frameshift mutations in each gene into the chromosome by allelic replacement (section Materials and Methods).

The antibiotic susceptibility profile of each mutant was then determined for a panel of antibiotics representing different classes (Table 1). The loss of adeB had a minimal effect on the overall levels of resistance and a slight increase in susceptibility was observed for ampicillin, cefotaxime, amikacin, rifampin, and tigecycline (Table 1). This result was surprising as the AdeABC system has a prominent role in antibiotic resistance in A. baumannii (Magnet et al., 2001). To determine if this adeB frameshift mutation was somehow being suppressed or was not a null allele, we constructed an adeB::Km mutation, where the adeB gene was disrupted in the middle of the coding region. However, this adeB::Km mutant displayed the same level of resistance to ampicillin (128 µg/ml), tetracycline (2 µg/ml), and ciprofloxacin (0.38 µg/ml) as wild-type, indicating that the previously isolated frameshift mutation in adeB was non-functional.

The effect of a mutation in adeJ on antibiotic susceptibility was far more pronounced, where cells became more susceptible to the following antibiotics; ampicillin (3-fold), cefotaxime (>15-fold), ceftriaxone (>10-fold), chloramphenicol (>10-fold), rifampin (4-fold), tigecycline (8-fold), and trimethoprim (≤20-fold) (Table 1). The antibiotic susceptibility profiles were also examined for an adeB/adeJ double mutant to determine if the loss of both efflux systems had additional effects. However, the adeB/adeJ double mutant essentially phenocopied the adeJ single mutant (Table 1).

We next assayed the role of AdeB and AdeJ efflux pumps in the handling of the boronic acid transition state inhibitors (BATSIs) SM23 and S02030 (Powers et al., 2014; Nguyen et al., 2016). These BATSIs either mimic the acylation or deacylation transition state. Paired with a penicillin (ampicillin), carbenapenem (meropenem), or cephaporphin (ceftazidimne or cefepime) as performed herein, the BATSI can act to inhibit serine based beta-lactamases in-vitro. As a result of this mechanism of action, the double mutant to determine if the adeB/adeJ double mutant essentially phenocopied the adeJ single mutant (Table 1).

Role of AdeABC and AdeIJK in Motility

A. nosocomialis strain M2 is capable of rapidly translocating across soft agar surfaces (Clemmer et al., 2011). Although the mechanism responsible for this motility is unclear, a number of genes have been identified that reduce motility including mutations in the abai autoinducer synthase responsible for quorum sensing signal production (Clemmer et al., 2011). We tested the wild-type M2 parent and the isogenic adeB mutant for their motility phenotypes at 30 degrees. The adeB mutation did not significantly alter surface motility (Figures 1A,B). In contrast, the adeJ mutation had a pronounced effect on surface motility, with a greater than 50% reduction relative to the wild-type M2 parent (Figure 1). Interestingly, this motility defect was temperature dependent, at 37 degrees...
adeJ mutant exhibited a similar level of motility as wild-type (Figure 1C).

In a previous study, the motility of A. nosocomialis M2 was shown to be dependent on production of the quorum sensing signal 3-OH C₁₂-HSL (Clemmer et al., 2011). To investigate the possibility that the motility defect in the adeJ mutant was due to the failure to export 3-OH-C₁₂-HSL, an Agrobacterium tumefaciens traG-lacZ biosensor strain was used to assay signal production in the adeJ mutant and wild-type M2 (Niu et al., 2008). However, no significant differences in signal production were observed between these strains (Supplementary Figure 1).

**FIGURE 1 |** Surface motility of wild-type M2 and efflux mutants. Wild-type M2 and the isogenic adeB and adeJ mutants were assayed for surface motility as described in the Materials and Methods. In (A), motility of the indicated strains is shown after 14 h at 30°C. Motility was quantitated from 4 separate experiments at 30°C (B) and 37°C (C). Error bars represent standard deviation of the mean. N.S. indicates a p-value > 0.05.

**FIGURE 2 |** Biofilm formation. Wild-type M2 and the isogenic adeB and adeJ mutants were assayed for biofilm formation in microtiter wells grown at 30 or 37°C for 24 h. Values represent crystal violet staining/cell density (A₅₈₅ / A₆₀₀) ratio and error bars represent standard deviation of the mean. N.S. indicates a p-value > 0.05.

**Role of AdeABC and AdeIJK in Biofilm Formation**

The role of AdeABC and AdeIJK in biofilm formation was also examined. When biofilms were formed on the surface of polystyrene microtiter wells, biofilm formation by the adeB and adeJ mutants were similar to wild-type M2 after 24 h of growth at 30°C (Figure 2). At 37°C, only the adeJ mutant showed a statistically significant reduction in biofilm formation, with a 24% decrease relative to wild-type (Figure 2).

**DISCUSSION**

In this study, the roles of AdeABC and AdeIJK orthologs in A. nosocomialis were addressed. Both a frameshift allele in the adeB gene and an adeB::Km disruption did not result in a major change in antibiotic resistance profiles, which is in contrast to that observed in A. baumannii (Magnet et al., 2001). Several possibilities can account for these differences. First, the adeABC genes may be expressed at very low levels in A. nosocomialis M2, therefore, the loss of this efflux system would have a minimal impact. In A. baumannii, the AdeABC system is typically expressed at low levels and inactivation of these genes in some strains does not produce a phenotype (Yoon et al., 2015; Leus et al., 2018). Increased expression can
result from mutations in the AdeRS two-component system. In *A. nosocomialis* M2, the AdeR and AdeS proteins did not contain amino acid substitutions previously associated with increased AdeABC expression (Marchand et al., 2004; Yoon et al., 2013; Gerson et al., 2018). Based on this information, we propose that the AdeABC genes are tightly regulated by AdeRS and the levels of expression in the M2 strain do not contribute to intrinsic resistance. We also tested the role of AdeABC in both surface motility and biofilm formation and no significant changes were observed in the adeB mutant relative to wild-type (Figures 1, 2).

In contrast, the AdeIJK efflux system was shown to play a significant role in antibiotic efflux, where a mutation inactivating this system had a pronounced effect on antibiotic susceptibility (Table 1). This observation is consistent with previous studies in *A. baumannii* demonstrating that efflux mediated by AdeIJK contributes substantially to antibiotic resistance. In addition, the loss of AdeIJK strongly reduced surface motility with a greater than 50% reduction compared to wild-type (Figure 1). The loss of AdeIJK resulted in a modest (24%) reduction in biofilm formation, which is also consistent with previous studies in *A. baumannii*, where the loss of AdeIJK resulted in a 20% reduction in biofilm formation (Yoon et al., 2015). The decreased surface motility and biofilm formation in the adeI mutant were not the result of decreased production of the quorum sensing signal 3-OH C12–HSL, which has been shown to be important for both surface motility and biofilm formation in *A. nosocomialis* (Niu et al., 2008; Clemmer et al., 2011).

The mechanism that results in loss of motility when the AdeIJK system in inactivated is unknown, but may indicate a role for AdeIJK in secretion of a lipopeptide surfactant that is required for motility (Clemmer et al., 2011; Rumbo-Feal et al., 2017) or in the secretion of 1,3-diaminopropane, also required for motility (Skiebe et al., 2012). This also indicates that in addition to antibiotic efflux, there are cellular functions mediated by AdeIJK, indicating a general role for this RND-type efflux system in general physiology of *A. nosocomialis*.

**AUTHOR CONTRIBUTIONS**

DK, SR, and PR conducted experiments. PR and RB wrote the manuscript. SR, RB, and PR edited the manuscript.

**FUNDING**

This work was supported by grant R01 AI072219 from the National Institutes of Health to PR and RB. PR is also supported by grants from the Merit Review program I01BX001725 and a Research Career Scientist Award IK6BX004470, both from Department of Veterans Affairs.

**ACKNOWLEDGMENTS**

We thank Katy Clemmer for assistance with strain constructions.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: [https://www.frontiersin.org/articles/10.3389/fmicb.2018.01902/full#supplementary-material](https://www.frontiersin.org/articles/10.3389/fmicb.2018.01902/full#supplementary-material)
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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