Multiple roles for a novel RND-type efflux system in *Acinetobacter baumannii* AB5075

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**INTRODUCTION**

*Acinetobacter baumannii* is a Gram-negative pathogen typically associated with infections in hospital settings (Bergogne-Berezin & Towner, 1996; Gootz & Marra, 2008; Joly-Guillou, 2005; Peleg, Seifert, & Paterson, 2008). Although most *A. baumannii* infections are seen in immunocompromised patients or those with severe injuries, community acquired infections and infections in otherwise healthy patients have increased in recent years (Antunes, Visca, & Towner, 2014; Charnot-Katsikas et al., 2009; Guerrero et al., 2010; Lowman, Kalk, Menezes, John, & Grobusch, 2008). This development, combined with the increasing frequency of multidrug resistance, has made *A. baumannii* an extremely problematic pathogen for clinicians to treat and mortality rates for these infections has approached 70% (Lee, Chen, Wu, Huang, & Chiu, 2014). It has become widely recognized that new therapies are needed to help combat these infections (Gootz & Marra, 2008; Hujer et al., 2006; Joly-Guillou, 2005; Scott et al., 2007).

Members of the resistance–nodulation–cell division (RND) class of efflux systems in Gram-negative bacteria are composed of three proteins: an inner membrane transporter, an outer membrane protein that serves as a pore, and a periplasmic adapter protein that interacts with both the inner and outer membrane proteins to form a conduit for the extrusion of small molecules. RND-type systems typically capture toxic compounds or metabolites and remove them from the cell, and because...
of this function they can be involved in resistance to antibiotics, disinfectants, and heavy metals (Alvarez-Ortega, Olives, & Martinez, 2013; Anes, McCusker, Fanning, & Martins, 2015; Delmar, Su, & Yu, 2014; Magnet, Courvalin, & Lambert, 2001; Routh et al., 2011; Venter, Mowla, Ohene-Agyei, & Ma, 2015). They have also been shown to have roles in virulence, resistance to host antimicrobial peptides, and in cellular homeostasis by removing excess metabolites (Helling et al., 2002; Warner, Folster, Shafer, & Jerse, 2007). Acinetobacter baumannii possesses a number of RND-type efflux systems that have roles in antibiotic resistance, virulence, and biofilm formation (Damier-Piolle, Magnet, Bremont, Lambert, & Courvalin, 2008; Magnet et al., 2001; Yoon et al., 2015), and these systems are reviewed in Coyne, Courvalin, and Perichon (2011). The expression of these genes in A. baumannii is often regulated at the level of transcription by activator and/or repressor proteins (Lin, Lin, & Lan, 2015; Marchand, Damier-Piolle, Courvalin, & Perichon, 2011). To begin understanding the mechanism underlying this high-frequency colony opacity switch, transposon mutagenesis was used to generate mutations in strain AB5075 that greatly reduced the frequency of phase variation from opaque to translucent. One mutant revealed a role for a previously uncharacterized RND-type system in this process. Mutations in the genes encoding this RND system significantly decreased phase variation in the opaque to translucent direction, but had little to no effect on phase variation in the translucent to opaque direction. Moreover, mutations inactivating this RND system were pleiotropic and resulted in altered surface motility, aminoglycoside resistance, and virulence in a Galleria mellonella waxworm model.

2 EXPERIMENTAL PROCEDURES

2.1 Bacterial strains, plasmids, and growth conditions

Both A. baumannii and Escherichia coli were grown in modified Luria Broth containing 10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter. For screening opaque and translucent colonies of AB5075, LB was prepared at 0.5× of the normal concentration with 8 g agar per liter. For screening opaque and translucent colonies of AB5075, LB was prepared at 0.5× of the normal concentration with 8 g agar per liter. Escherichia coli transformants were selected with chloramphenicol (25 μg/ml), ampicillin (200 μg/ml), or kanamycin (20 μg/ml) when appropriate. Acinetobacter baumannii AB5075 transformants were selected with tetracycline (3 μg/ml). Plasmid pEX18Tc was used for allelic replacement in AB5075 (Hoang, Karkhoff-Schweizer, Kutchma, & Schweizer, 1998). For all experiments involving opaque and translucent variants, cells were obtained from freezer stocks that were grown to low density and contained <0.5% of the opposite cell type. Strain AB00075 arpB::Tc was obtained from the A. baumannii transposon insertion library maintained by Dr. Colin Manoil's laboratory at the University of Washington (Gallagher et al., 2015).

2.2 Phase variation assays

Freezer stocks of each strain that were verified as 99.99% of a single colony type were grown in LB broth to an OD of 1.8–1.9. Colonies were resuspended in LB broth and serial dilutions were plated on 0.5× LB, 0.8% agar plates. After overnight growth, the total number of viable cells per ml was determined and the number of opaque or translucent colonies present was determined on plates with at least 200 colonies/plate by using oblique light to illuminate the colonies.

2.3 Transposon mutagenesis

A culture of the A. baumannii AB5075 opaque variant was grown in 25 ml of LB broth at 37°C with vigorous shaking. Cells were harvested from cultures at an OD ~0.5 by centrifugation at 4°C for 10 min. Pellets were washed twice with 10% glycerol to prepare electrocompetent cells. Transposon (0.1 pmole) and transposase (1 unit) components of an EZ-Tn5 <TET-1> kit (Epicentre Biotechnologies) were combined with 10% glycerol and incubated at 37°C for approximately 45 min. Transpososome complex mixture was then cooled on ice prior to electroproporation. Aliquots of 1.25 μl transpososome mixture and 1 μl of TypeOne Restriction Inhibitor (Epicentre Biotechnologies) were electroporated into 60 μl of competent AB5075 O cells at 2.5 kV. Following electroporation, 1 ml of room temperature LB broth was added following electroporation. This cell suspension was transferred to tubes and incubated at 37°C with shaking for 1 hr. Mutagenized cells were plated in 100 μl aliquots on 0.5× LB, 0.8% agar plates supplemented with 3 μg/ml tetracycline to select for insertion mutants and plates were incubated at 37°C. Colonies were evaluated at 24 and 48 hr postplating for altered colony morphology or reduced colony sectoring when viewed under a dissecting scope with oblique illumination from below. Putative mutants were restreaked on 0.5× LB, 0.8% agar with Tet³ to ensure colony morphology was stable and that picked colonies were pure. Identification of insertion sites was accomplished by rescue cloning of the tetracycline resistance gene and DNA sequencing of plasmids generated from rescue cloning.

2.4 Construction of in-frame deletions in arpA and arpR

An in-frame deletion in arpA was generated as described previously (Hoang et al., 1998) from AB5075 genomic DNA by PCR amplification of two approximately 1 kb fragments containing small portions of the arpA coding region using the primers arpA Up-1 (5′-AAAAAGGATCCATACTACGGTACGCTAC-3′), arpA
Up-2 (5′-GTGAAAAATTCAGGGAGCCA-3′), arpA Down-1 (5′-TTGTGTGTAATGAGCATTTACCACCTAATAATTGCGCAATAGC-3′). Oligonucleotide primers arpA Up-1 and arpA Down-2 were engineered to contain BamHI restriction sites at the 5′ end. The up- and downstream fragments were ligated together to produce an approximately 2-kbp fragment containing the ΔarpA allele which was subsequently gel purified. The ΔarpA allele contains an in-frame deletion in the arpA coding sequence corresponding to amino acids 48–359 of the 366 (85% of coding region). Purified fragment was reamplified with outer primers (arpA Up-1 and arpA Down-2) and gel purified. The ΔarpA fragment and pEX18Tc were digested with BamHI and gel purified. Digested fragments were then ligated and transformed into competent E. coli DH5α cells. To transfer the mutant alleles to the chromosome of A. baumannii AB5075, the suicide vector containing the in-frame arpA deletion was electroporated into competent AB5075 cells which had been grown overnight in LB and washed with 300 mM sucrose as described previously (Choi & Schweizer, 2006). Integrants were selected on LB + tetracycline at 5 μg/ml. For switching frequency assays, overnight starter cultures of LB + tetracycline (3 μg/ml) were inoculated with AB5075 or ΔarpA harboring pWH1266 or pArpA and incubated at room temperature overnight without shaking. Strains were subcultured into LB without antibiotics and incubated at 37°C with vigorous shaking. CFU/ml and number of translucent colonies (switching frequency) were quantified at OD₆₀₀ 0.7 and 1.7.

2.6 Preparation of conditioned media

To prepare conditioned media, cells were grown in 25 ml LB cultures at an optical density of 1.7. At this time, aliquots were restreaked to verify that the cultures remained at least 95% opaque or translucent. Cells were removed by centrifugation and the resulting media was adjusted to pH 7.0 with HCl and filter sterilized by passing through a 0.22-μm filter. Aliquots were frozen at ~80°C and used within 2 weeks. To grow cells in conditioned media, a 2-ml aliquot was thawed and a 10× concentrate of tryptone and yeast extract (TY) was added back to a final concentration of 0.25×. Cells were grown by shaking at 270 rpm at 37°C to an optical density of 1.1 and dilutions were then plated to determine phase variation frequencies. Growth of cells in the LB control was done at 0.25× LB, a concentration that gave a similar growth rate as the TY-supplemented conditioned media.

2.7 Galleria mellonella infections

Galleria mellonella larvae between 200 and 250 mg were utilized for infection studies. Acinetobacter baumannii strains were grown in LB broth at 37°C with shaking to an OD₆₀₀ ~0.5. Cultures were serially diluted in LB broth and plated to determine CFU/ml for each bacterial strain. Strains diluted to 10⁻² were chilled on ice prior to injection into larvae. Four μl aliquots of each strain were injected into the hemolymph of G. mellonella larvae (10 larvae per strain in three replicates, ~30 total larvae per strain). The average CFU for the infected wild-type cells in the three experiments was 8.2 × 10³ and 9.5 × 10³ for ΔarpA mutant. Infected larvae were incubated at 37°C for up to 5 days in a humidified incubator and mortality was assessed at daily intervals.

2.8 Construction of an arpR expression plasmid

The full-length arpR gene including 146 bp of sequence upstream from the GTG start codon and 71 bp downstream from
the stop codon were amplified from ABS075 genomic DNA by PCR (Phusion Hot-Start Polymerase, Thermo Scientific). Primers arpR Exp.1 (5′-CATTTTAATGCGCTTATAAC-3′) and arpR Exp.2 (5′-TTATCGCTTATTTCAACT-3′) were phosphorylated prior to PCR amplification to add 5′ phosphates with T4 polynucleotide kinase by DNA sequencing prior to introduction into replicates.

2.12 | Antimicrobial resistance assays

Strains to be tested were grown to an OD<sub>600</sub> of 0.05 with sterile LB. The minimum inhibitory concentration for various antibiotics was determined using E-test strips on LB agar plates according to the manufacturer's instructions (Biomerieux). The MICs for each antibiotic were determined after 12 hr of growth at 37°C. All susceptibility tests were done in duplicate.

3 | RESULTS

3.1 | An RND-type efflux system is required for the opaque to translucent colony opacity phase variation

Previously, our laboratory reported on a phase variable mechanism in A. baumannii that results in the interconversion between opaque and translucent colonies (Tipton et al., 2015). This phase variation was stimulated at high density and when opaque colonies are grown for 36–48 hr, they become highly mottled in appearance due to translucent variants arising at high frequency within the colony (Figure 1a). This mottled appearance formed the basis for a genetic screen to identify mutants with a reduced frequency of phase variation in the opaque to translucent direction, as these mutants would have a reduction or absence in the mottled appearance of the colony. A library of EZ-Tn5 <Tet-1> insertions in ABS075 was screened for colonies that did not exhibit the mottled appearance after 36 hr of growth and mutants with this phenotype were isolated and confirmed by subsequent replating. One mutant 5075.8B (Figure 1a) was characterized further and the transposon insertion was mapped to nucleotide 64 of a 1,101 nucleotide open reading frame (ABUW_0034) encoding a putative periplasmic membrane fusion component of RND-type efflux systems (Figure 1b). This protein exhibited the highest degree of identity to annotated RND-type transporters from Pseudomonas mendocina (47% identity/69% similarity) and Pseudomonas pseudaerocaligenes (47% identity/68% similarity). A second open reading frame (ABUW_0035) that encoded a putative inner membrane transporter component of RND-type systems. This protein exhibited the highest identity to an annotated RND-type transporter from Bacillus mycoides (85% identity/93% similarity) and to Alkanindiges illinoisensis (75% identity/86% similarity). Given their close proximity, these two genes likely formed an operon and were previously identified in A. baumannii as a locus upregulated in the presence of farnesol and were
designated as acrAB (Kostoulias et al., 2016). However, the overall similarity of these proteins to the E. coli AcrA (23% identity/39 similarity) and AcrB (25% identity/43% similarity) was low and there are other RND-type systems in E. coli that exhibit greater identity to the A. baumannii proteins. In addition, as outlined below, this RND system confers aminoglycoside resistance, therefore, these genes were designated arpA and arpB (aminoglycoside resistance pump). The frequency of phase variation from opaque to translucent was quantitated in individual colonies of the 5075.8B mutant and wild-type cells after 48 hr of growth, which revealed an average 55-fold decrease in the opaque to translucent switch in the arpA:: EZ-Tn5 <Tet-1> mutant 5075.8B. While this analysis was being conducted, it was observed that there was significant colony to colony variation within the same strain with respect to phase variation frequencies, leading to large standard deviations. As a result of this, broth grown cells were used for subsequent experiments, which did reduce variability, although significant variability still existed. In broth cultures, the phase variation rate of the 5075.B mutant was 77-fold lower than wild type (Table 1).

To verify the role of the arpA gene in phase variation and to avoid polar effects on the downstream arpB gene that likely occurred from the arpA:: EZ-Tn5 <Tet-1> insertion, an in-frame deletion in arpA (ΔarpA2) was constructed. This mutation had a similar effect on phase variation as the original arpA::EZTN<Tc> mutant, where the frequency of switching from opaque to translucent was reduced 71-fold in cultures at an OD<sub>600</sub> = 1.9 (Table 1). This ΔarpA2 mutant strain was designated KT2.

Complementation analysis was performed to determine if the loss of arpA was responsible for the phase variation defect. When a plasmid containing the arpA gene was introduced into the arpA deletion mutant (KT2), colonies regained the ability to form the mottled phenotype due to translucent variants arising at high frequency (data not shown). In addition, the frequency of phase variation in 24 hr colonies was increased by 3,600-fold in the arpA deletion mutant containing the cloned arpA gene (parpA), relative to the arpA deletion mutant containing the pWH1266 vector alone (Figure S1, panel A).

**FIGURE 1** Decreased phase variation in an arpA mutant. (a) A typical wild-type opaque colony variant of AB5075 is shown compared to 5075.8B arpA:: EZ-Tn5 <Tet-1> after 36 hr of growth on a 0.5× LB, 0.8% agar plate. The mottled appearance at the outside edge of the wild type is due to translucent variants arising within the opaque colony. (b) The organization of the arpAB region and the site of the arpA:: EZ-Tn5 <Tet-1> insertion that blocks phase variation is depicted by an arrow. Proteins exhibiting the closest match to ArpA and ArpB are shown below each gene.

**TABLE 1** Phase variation frequencies

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relative O to T phase variation frequency&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB5075 wild type</td>
<td>1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5075.8B arpA::EZTn-5Tc</td>
<td>0.013 ± 0.02 (77-fold decrease)</td>
</tr>
<tr>
<td>KT2 ΔarpA2</td>
<td>0.014 ± 0.03 (71-fold decrease)</td>
</tr>
<tr>
<td>AB00075 arpB::Tc</td>
<td>0.0013 ± 0.001 (769-fold decrease)</td>
</tr>
<tr>
<td>KT3 ΔarpR</td>
<td>0.0009 ± 0.0002 (1,916-fold decrease)</td>
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</table>

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relative T to O phase variation frequency&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB5075 wild type</td>
<td>1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>KT2 ΔarpA2</td>
<td>1.5 ± 0.6</td>
</tr>
<tr>
<td>AB00075 arpB::Tc</td>
<td>1.2 ± 0.5</td>
</tr>
<tr>
<td>KT3 ΔarpR</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

<sup>a</sup>Determined in cultures at an OD<sub>600</sub> of 1.85–1.9.  
<sup>b</sup>Phase variation frequency from O to T was typically 25%–35%.  
<sup>c</sup>Phase variation frequency from T to O was typically 40%–50%.
3.2 | An arpB mutation also decreases the opaque to translucent phase variation

The gene located immediately downstream from arpA is predicted to encode a putative inner membrane transporter of RND efflux systems and likely functions with ArpA in this process. This gene was designated arpB. To determine if arpB also regulates phase variation, two separate transposon insertions in arpB (AB00075, AB00076) were obtained from the A. baumannii AB5075 transposon insertion library at the University of Washington (Gallagher et al., 2015). Like the arpA mutant, opaque colonies of the arpB mutants did not produce motiled colonies at 48 hr. The frequency of phase variation for AB00075 arpB::Tc in broth grown cells at an OD_{600} of 1.9 was reduced an average of 769-fold compared to wild-type cells (Table 1).

3.3 | arpA and arpB mutations do not alter the translucent to opaque switch

The effect of the ΔarpA mutation on the reciprocal opacity switch, from translucent to opaque, was measured in KT2 grown to an OD of 1.9, where the rate of phase variation was increased 1.5-fold over wild type (Table 1). The arpB::Tc mutant AB00075 exhibited a phase variation rate for translucent to opaque that was 1.2-fold greater than wild type.

3.4 | An arpB mutant still produces an extracellular signal that stimulates phase variation

Previous work demonstrated that the increase in phase variation at high cell density is mediated, in part, by the accumulation of a secreted signal (Tipton et al., 2015). If this signal is dependent on ArpAB for secretion and sensed at the cell surface or the cytoplasmic membrane, then this could explain the reduction in phase variation in the arpA mutant. To test this possibility, conditioned media were prepared from opaque variants of wild-type and the ΔarpB mutant at an optical density of 1.7 and tested for the ability to stimulate phase variation in the opaque to translucent direction. Conditioned media from wild-type cells stimulated the opaque to translucent conversion 11-fold and the conditioned media from the arpB mutant stimulated phase variation ninefold (Table 2). There was not a statistically significant difference between these values (p = .39) indicating that the arpB mutant produced a similar level of extracellular signal activity as the wild-type parent.

3.5 | Role of ArpAB in antimicrobial resistance

It is well established that RND-type systems can export antimicrobials and confer higher levels of resistance (Alvarez-Ortega et al., 2013; Anes et al., 2015; Coyne et al., 2011; Magnet et al., 2001; Rosenfeld et al., 2012; Routh et al., 2011; Venter et al., 2015). Therefore, both the arpA and arpB mutants were tested for levels of resistance to various antibiotics. For this analysis, both the opaque and translucent variants of each mutant and wild-type cells were tested (Table 3). Due to the multiple resistances that already exist in AB5075, the number of antibiotics that could be tested was limited. There were no significant differences between wild-type and the arpA mutant for any of the antibiotics tested (Table 3), although there were subtle differences between opaque and translucent variants of wild type as reported previously (Tipton et al., 2015). However, the arpB mutant AB00075 was more sensitive to the two aminoglycosides that were tested, amikacin and tobramycin (Table 3). This sensitivity was seen in both the opaque and translucent variants of AB00075 arpB::Tc.

3.6 | arpA and arpB mutations selectively increase surface motility in the opaque variants

Both the arpA and arpB mutants formed irregular, slightly spreading colonies on 0.8% agar plates that were distinct from wild-type colonies that contained smooth rounded edges. This observation suggested that motility may be enhanced by the arpA and arpB mutations. To test this hypothesis, the motility of both opaque and translucent arpA and arpB mutants were compared to opaque and translucent wild-type AB5075 on 0.3% Eiken agar plates. In opaque colonies, both the arpA and arpB mutants exhibited increased motility, 53 mm and 75 mm, respectively, over wild-type value of 33 mm (Figure 2). However, in the translucent form, there was no significant difference in motility between wild-type and either the arpA or arpB mutants (Figure 2). The increased motility observed in the arpA mutant was restored back to wild-type levels by parpA containing the cloned arpA gene relative to cells containing the pWH1266 vector alone (Figure S1, panel B).

### TABLE 2 | Effect of conditioned media on relative phase variation frequency

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>Opaque to translucent switching frequency</th>
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<tr>
<td>LB broth</td>
<td>0.35% ± 0.29</td>
</tr>
<tr>
<td>Wild-type opaque conditioned media</td>
<td>3.9% ± 2.8 (11-fold increase)</td>
</tr>
<tr>
<td>ΔarpB opaque conditioned media</td>
<td>3.2% ± 2.7 (9-fold increase)</td>
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*Determined in cells grown to an optical density A_{600} of 1.0.

### TABLE 3 | Minimum inhibitory concentrations for antibiotics

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (μg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>COL</td>
</tr>
<tr>
<td>WT opaque</td>
<td>0.38</td>
</tr>
<tr>
<td>ΔarpA opaque</td>
<td>0.38</td>
</tr>
<tr>
<td>arpB::Tc opaque</td>
<td>0.38</td>
</tr>
<tr>
<td>WT translucent</td>
<td>0.38</td>
</tr>
<tr>
<td>ΔarpA translucent</td>
<td>0.38</td>
</tr>
<tr>
<td>arpB::Tc transluent</td>
<td>0.38</td>
</tr>
</tbody>
</table>

COL, colistin; TET, tetracycline; AK, amikacin; RIF, rifampicin; TIG, tigecycline; TOB, tobramycin.
3.7 | ArpAB is required for virulence in *Galleria mellonella*

RND-type efflux systems have been shown to have roles in virulence in a number of bacterial pathogens (Alvarez-Ortega et al., 2013; Delmar et al., 2014; Routh et al., 2011; Taylor, Bina, & Bina, 2012; Venter et al., 2015; Warner et al., 2007; Yoon et al., 2015). Therefore, the impact of *arpA* and *arpB* mutations on virulence were examined in a *Galleria mellonella* waxworm model that has previously been shown to be a useful model of virulence in *A. baumannii* (Esterly et al., 2014; Gaddy et al., 2012; Heindorf, Kadari, Heider, Skiebe, & Wilharm, 2014; Iwashkiw et al., 2012; Jacobs et al., 2014; Nwugo et al., 2012; Peleg et al., 2009; Repizo et al., 2015; Stahl, Bergmann, Gottig, Ebersberger, & Averhoff, 2015; Tipton et al., 2015). Since previous work indicated that the opaque form was more virulent, these studies were done with the opaque forms of wild-type AB5075, KT2 Δ*arpA* and the *arpB::Tc* mutant (Tipton et al., 2015). Relative to wild-type AB5075, both the Δ*arpA* and *arpB* mutations substantially reduced the ability of *A. baumannii* to kill *G. mellonella* waxworms (Figure 3a). The decreased virulence exhibited by the *arpA* mutant was restored by parpA containing the cloned *arpA* gene relative to cells containing the pWH1266 vector alone (Figure S1, panel C).

3.8 | A divergently transcribed gene encoding a TetR-type regulator represses *arpAB* and is required for the opaque to translucent switch

Adjacent to the *arpAB* genes in *A. baumannii* was a divergently transcribed gene encoding a predicted TetR-type repressor of the AcrR family (Figure 1). In *E. coli*, the AcrR protein acts to repress the *acrAB* operon, which is organized in a similar manner as *arpAB*. To determine if a similar function was present in *A. baumannii*, an in-frame deletion was constructed in this gene, designated *arpR*. The Δ*arpR* deletion did not have a significant impact on *arpAB* expression, with the levels of expression 1.1-fold higher in the mutant (Figure 4). However, the *arpR* mutation reduced the frequency of the opaque to translucent conversion by 1,916-fold (Table 1).

As a second method to determine if ArpR could function as a repressor, the *arpR* gene was overexpressed by cloning the gene into pWH1266, where expression was driven from the promoter for the β-lactamase gene. When this plasmid was introduced into wild-type AB5075, the colony morphology was altered with irregular slightly spreading colonies that were similar to *arpA* and *arpB* mutants,
FIGURE 4 Effect of ArpR on arpA expression. The levels of arpA expression were determined by quantitative RT-PCR and the values shown are relative to the control gene clpX. The values for the left side represent the levels of arpA expression in the arpR mutant relative to wild-type. The values on the right side represent the levels of arpA expression in cells overexpressing arpR from a plasmid relative to cells containing the vector alone.

suggesting that expression of both arpA and arpB were reduced when ArpR was overexpressed. To investigate this possibility, expression of the first gene arpA was examined by qRT-PCR and was found to be 7.3-fold lower in cells with parpR versus the pWH1266 vector alone (Figure 4). Next, we investigated if the reduced ArpAB expression resulting from ArpR overexpression impacted the opaque to translucent switch. In cells overexpressing the arpR gene, the frequency of phase variation was reduced threefold, relative to cells containing the vector only (data not shown).

4 | DISCUSSION

In this study, a previously undescribed RND-type efflux system in A. baumannii was found to regulate phase variation in the opaque to translucent direction. The original mutation defining this phenotype was a transposon insertion in arpA, a gene encoding a membrane fusion component of a putative RND-type efflux system. This arpA mutation resulted in a 77-fold decrease in the frequency of phase variation from the opaque to translucent colony opacity phenotype (Table 1). The role of arpA in this process was confirmed by the construction of a nonpolar, in-frame deletion within arpA, which decreased the conversion to translucent colonies by 71-fold, and by complementation analysis with the wild-type arpA gene. In addition, a transposon insertion in the downstream arpB gene obtained from the A. baumannii AB5075 transposon library at the University of Washington (Gallagher et al., 2015) exhibited a stronger effect with a 769-fold reduction in phase variation (Table 1). Interestingly, neither the arpA or arpB mutations had a significant effect on phase variation in the translucent to opaque direction. This provides strong evidence that a distinct mechanism regulates phase variation in each direction.

Two additional phenotypes were altered by the loss of arpA and arpB. First, surface motility of the opaque variants on 0.3% agar plates was increased, with the arpB mutant exhibiting a more substantial increase than the arpA mutant (Figure 2). Interestingly, in translucent variants, the motility of both the arpA and arpB mutants was similar to wild-type. The basis for the selective motility increase in opaque variants is unknown, but previously it was noted that in wild-type cells, the opaque variants were more motile (Tipton et al., 2015). Although the actual mechanism responsible for motility in AB5075 has not been identified, if this mechanism was only expressed in opaque cells and also repressed by ArpAB, it could explain the selective increase in motility seen in arpA or arpB mutants in the opaque background. Second, the arpA mutation decreased the ability of A. baumannii AB5075 to kill G. mellonella waxworms. Additional examples exist where the loss of an RND-type system decreases virulence (Alvarez-Ortega et al., 2013; Delmar et al., 2014; Routh et al., 2011; Taylor et al., 2012; Venter et al., 2015; Warner et al., 2007; Yoon et al., 2015). The decreased virulence may be due to a role for ArpAB in efflux of host antimicrobial peptides or other antibacterial compounds present inside G. mellonella.

The arpB mutant consistently exhibited stronger effects than the arpA mutant for phase variation (Table 1), surface motility (Figure 2), and antibiotic sensitivity (Table 3). It is unlikely that this results from the arpA mutation being leaky, as the in-frame deletion removed most of the coding region. The weaker phenotype of the arpA mutation may indicate that a periplasmic fusion protein from another RND-type system can partially substitute for ArpA. The stronger effect of a arpB mutation suggests that ArpB is unable to be substituted for by another RND component and has a more critical role than ArpA. However, this hypothesis is speculative at the current time. In addition, the outer membrane channel that works with ArpAB is unknown, but AbuO, a TolC-like protein is a possible candidate (Srinivasan, Vaidyanathan, & Rajamohan, 2015).

A TetR-type transcriptional regulator was encoded adjacent to the arpAB genes and divergently transcribed, a genetic organization similar to that of acrR-acrAB in E. coli and mexR-mexAB in P. aeruginosa. A ΔarpR mutation did not alter arpAB expression, however, overexpressing the arpR gene decreased arpAB expression by 7.3-fold. In addition, cells overexpressing arpR exhibited phenotypes that at least partially mimicked the arpAB mutations, as the colonies formed the irregular spreading appearance and the frequency of the opaque to translucent conversion was reduced threefold. It is unclear why the effects of ArpR on arpAB expression are only seen when it is overexpressed, but under the laboratory growth conditions we tested, it does not have a role in arpAB regulation, possibly because it is not expressed at high enough levels to mediate repression. If certain environmental conditions increased arpR expression, this condition would be predicted to repress arpAB and reduce the opaque to translucent conversion.

To our knowledge, this represents the first report of an RND-type efflux system regulating bacterial phase variation. A direct role for ArpAB in this process is unlikely and loss of ArpAB may indirectly modulate a regulatory pathway controlling phase variation. At the present time, the regulatory mechanism regulating the interconversion between opaque and translucent colonies is unknown. However, whole genome sequencing suggests that nucleotide changes are not responsible. This information, together with the observation that phase variation between colony opacity phenotypes is essentially undetectable in cells at low density, but is sharply activated at high density and reaches frequencies above 10% (Table 1) suggests a nonmutational mechanism is involved.
A possible mechanism to control phase variation is via a bistable switch involving one or more regulatory proteins (Casadesus & Low, 2013; Chang et al., 2010; Dubnau & Losick, 2006; Ferrell, 2002; Maamar & Dubnau, 2005; Mitrophanov & Groisman, 2008; Piggot, 2010; Turner, Vallet-Gely, & Dove, 2009; Veening, Smits, & Kuipers, 2008). Bistability has previously been shown to regulate colony opacity in *Pseudomonas fluorescens* (Gallie et al., 2015). Based on this information, there are several possibilities for how ArpAB might regulate phase variation. First, the extracellular signal that stimulates the opaque to translucent phase variation might require ArpAB for secretion. This would imply that the signal works via a sensing mechanism that operates at the cell surface or at the cytoplasmic membrane and then regulates the bistable switch controlling the phase variation. However, spent culture supernatants from an arpB mutant and wild-type cells appear to activate the opaque to translucent switch at equal frequencies (Table 2). A second possibility is that a metabolite normally secreted by the ArpAB system accumulates in arpAB mutants and this alters a regulatory pathway that controls phase variation. The isolation of both extragenic and high-copy suppressors that restore phase variation to an *arpA* or *arpB* mutant should help uncover the role of this efflux system in regulating the process of colony opacity phase variation in *A. baumannii* and these studies are in progress.

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**CONFLICT OF INTEREST**

None declared.

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