Antibiotic Inducibility of the mexXY Multidrug Efflux Operon of Pseudomonas aeruginosa: Involvement of the MexZ Anti-Repressor ArmZ

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Abstract

Expression of the mexXY multidrug efflux operon in wild type Pseudomonas aeruginosa is substantially enhanced by the ribosome-targeting antimicrobial spectinomycin (18-fold) and this is wholly dependent upon the product of the PA5471 gene. In a mutant strain lacking the mexZ gene encoding a repressor of mexXY gene expression, expression of the efflux operon increases modestly (5-fold) and is still responsive (18-fold) to spectinomycin. Spectinomycin induction of mexXY expression in the mexZ mutant is, however, independent of PA5471, suggesting that PA5471 functions as an anti-repressor (dubbed ArmZ for anti-repressor MexZ) that serves only to modulate MexZ’s repressor activity, with additional gene(s)/gene product(s) providing for the bulk of the antimicrobial-inducible mexXY expression. Consistent with PA5471/ArmZ functioning as a MexZ anti-repressor, an interaction between MexZ and ArmZ was confirmed using a bacterial 2-hybrid assay. Mutations compromising this interaction (P68S, G76S, R216C, R221Q, G231D and G252S) were identified and localized to one region of an ArmZ structural model that may represent a MexZ-interacting domain. Introduction of representative mutations into the chromosome of P. aeruginosa reduced (P68S, G76S) or obviated (R216C, R221Q) antimicrobial induction of mexXY gene expression, rendering the mutants pan-aminoglycoside-susceptible. These data confirm the importance of an ArmZ-MexZ interaction for antimicrobial-inducible mexXY expression and intrinsic aminoglycoside resistance in P. aeruginosa.

Introduction

Multidrug efflux systems of the 3-component Resistance-Nodulation-Division (RND) family are significant contributors to intrinsic and acquired antimicrobial resistance in a number of Gram-negative bacteria [1,2], including Pseudomonas aeruginosa [3]. P. aeruginosa expresses several RND type multidrug efflux systems of which four, MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM, are significant determinants of multidrug resistance in lab and clinical isolates [4,5]. MexXY-OprM is, however, somewhat unique in P. aeruginosa in providing resistance to the aminoglycoside class of antimicrobials [6-8] and in being inducible by many of its substrate antimicrobials [9].

The MexXY-OprM system is comprised of a cytoplasmic membrane antibiotic-proton antiporter (MexY), an outer membrane porin (OprM) and a periplasmic membrane fusion protein that joins the membrane-associated components together (MexX) [10]. The MexXY components are encoded by an operon under the control of an adjacent repressor gene, mexZ [10,11], while OprM, which functions as the outer membrane component of several multidrug efflux systems in P. aeruginosa [2], is encoded by the 3rd gene of an additional multidrug efflux operon, mexZAB-oprM [12]. Only the mexXY operon is antimicrobial-inducible, with only those agents known to target the ribosome promoting mexXY expression [9,13,14], and this is compromised by so-called ribosome protection mechanisms [13], suggesting that the MexXY efflux system is recruited in response to ribosome disruption or defects in translation. Consistent with this, mutations in fmt (encoding a methionyl-tRNA-formyltransferase) [15], folD (involved in folate biosynthesis and production of the formyl group added to initiator methionine) [15], and the ribosomal protein genes rplA [16], rplQ [17], the rplU-rpmA operon [18], all of which are expected to negatively impact protein synthesis, increase expression of mexXY. Upregulation of mexXY by antimicrobials [14] or mutations (fmt/folD [15], rplQ [17] and rplU-rpmA [18]) is dependent upon a gene, PA5471, encoding a conserved hypothetical protein. Expression of PA5471 is also promoted by ribosome-disrupting antimicrobials [14] and fmt/folD [15] or rplU-rpmA [18] mutations via a translational attenuation mechanism [19].

MexXY expression, while uncommon as a mechanism of aminoglycoside resistance in most clinical strains of P. aeruginosa, is the predominant mechanism of resistance to these agents in lung isolates of infected cystic fibrosis (CF) patients [6-8], with mutations in mexZ common in pan-aminoglycoside-resistant CF isolates expressing mexXY [6,20–23]. Indeed, mexZ has been identified as the most commonly mutated gene in P. aeruginosa CF isolates [24]. Recently, an interaction between MexZ and PA5471 has been reported, with PA5471 apparently modulating...
the repressor activity of MexZ [25]. We show here that the requirement for PA5471 for antimicrobial induction of mexXY expression is limited to wild type cells expressing the MexZ repressor, suggesting that PA5471 functions solely as an MexZ anti-repressor in the antimicrobial induction of mexXY. Further, we identify a putative MexZ-binding region in PA5471 and demonstrate that the interaction of this anti-repressor, dubbed ArmZ, with MexZ is key to the antimicrobial inducibility of this efflux system and its promotion of aminoglycoside resistance in P. aeruginosa.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. All bacterial strains were cultured at 37°C in Luria (L- broth or on L-agar, unless otherwise indicated, with antimicrobials added as necessary. Plasmid pEX18Tc and its derivatives were selected with 10 (in E. coli) or 50 (in P. aeruginosa) μg/ml tetracycline. Plasmid pMS604 and derivatives were maintained in E. coli with 10 μg/ml tetracycline. Plasmid pDP804 and derivatives were maintained in E. coli with 100 μg/ml ampicillin. Plasmid pSF004, a pMS604 derivative carrying the mexZ gene, was constructed by cloning a polymerase chain reaction (PCR)-generated product amplified from P. aeruginosa K767 chromosomal DNA using primers MSmexZ-F (5’-GACTCTGAGGGCGC- CAGGAAAACCAA-3’; PstI site underlined) and MSmexZ-R (5’- GACTCACTGTCAGGCTGCCGACGCAA-3’; PvuII site underlined). The 50-μl PCR reaction mixture contained 1 μg of chromosomal DNA, 0.2 μM of each primer, 0.2 mM each deoxynucleoside triphosphate (dNTP), 10% (vol/vol) dimethyl sulfoxide (DMSO), 1× Pfu polymerase buffer and 1.25 U Pfu DNA polymerase (Promega, Madison, WI). Following an initial denaturation step at 98°C for 3 min, the mixture was subjected to 30 cycles of 95°C for 45 sec, 60°C for 45 sec and 72°C for 2 min, before finishing with a 5-min incubation at 72°C. The mexZ-containing PCR product was gel-purified (see below), digested with PstI and PvuII and cloned into PstI-PvuII-restricted pMS604. PA5471 (renamed armZ) was cloned into pEX18Tc on a PCR product that was generated with primers DP5471-F (5’-GACTCTGAGGGCGC- CAGGAAAACCAA-3’; PstI site underlined) and DP5471-R (5’-GACTCACTGTCAGGCTGCCGACGCAA-3’; PvuII site underlined) in a reaction mixture formulated and processed as for mexZ. The armZ-containing PCR product was digested with Xhol and BglII and cloned into Xhol- BglII-restricted pDP804 to yield plasmid pSF005. armZ was also cloned into plasmid pEX18Tc on a PCR product generated with primers EX18armZ-F (5’-CGATATGGATCCGGCGAACGGAG- GATCTTCATGACCGTT-3’; KpnI site underlined) and EX18- armZ-R (5’-CGATATGGATCCGGCGAACGGAGTACCTCGGCT-3’; PstI site underlined). The reaction mixture was formulated as above except that primers and DMSO were included at 0.6 μM and 5% (vol/vol), respectively, and 1 U Phusion High Fidelity DNA polymerase (Promega, Madison, WI) was employed in 1× Phusion HF buffer. Following an initial denaturation step at 98°C for 30 sec, the mixture was subjected to 30 cycles of 98°C for 30 sec, 68°C for 30 sec and 72°C for 1 min, before finishing with a 7-min incubation at 72°C. The armZ-containing PCR product was digested with KpnI and PstI and cloned into KpnI-PstI-restricted pEX18Tc to yield plasmid pTH008.

DNA methods

Standard protocols were used for restriction endonuclease digestions, ligations, transformations, and agarose gel electrophoresis, as previously described [26]. Plasmid DNA was extracted from E. coli using the Fermentas GeneJet Plasmid Miniprep Kit or the Qiagen Plasmid Midi Kit according to protocols provided by the manufacturers. Chromosomal DNA was extracted from P. aeruginosa using the Qiagen DNeasy Blood & Tissue Kit according to a protocol provided by the manufacturer. PCR products and restriction endonuclease digestion products requiring purification were purified using the Promega Wizard SV Gel and PCR Clean-Up System (Promega Corp., Madison, WI) according to a protocol provided by the manufacturer. CaCl2-compent E. coli [26] and electrocompeint P. aeruginosa [27] cells were prepared as previously described. Oligonucleotide synthesis was performed by Integrated DNA Technologies (Coralville, Iowa), and nucleotide sequencing was performed by ACGT Corporation (Toronto, Canada).

Two-hybrid assay for ArmZ-MexZ interaction

To assess a potential ArmZ-MexZ interaction and the possible negative impact of armZ mutations on that interaction, E. coli SU202 harbouring the pMS604:mexZ plasmid pSF004 together with plasmid pDP804 derivatives carrying wild type or mutated armZ was cultivated overnight in L-broth containing tetracycline and ampicillin, diluted 1:49 into the same medium containing in addition 3 mM isopropyl-thio-β-D-galactopyranoside (IPTG), and grown to mid-log phase before being assayed for β-galactosidase activity as described previously [28]. E. coli strain SU202 harbours a chromosomal lacZ gene engineered to contain a hybrid lexA operator sequence in the promoter region to which a heterodimer only the LexAΔN-LexAΔC DNA-binding domains encoded by pDP804 and pMS604, respectively, can bind. pDP804- and pMS604-encoded LexA lack the natural dimerization domains of this protein, but fusion of the individual LexA DNA-binding domains encoded by these vectors to proteins that do interact can promote their dimerization and, ultimately, binding to the lexA hybrid operator upstream of lacZ in SU202, effectively repressing lacZ expression [29]. Thus, an interaction between ArmZ and MexZ, encoded by pDP804 and pMS604, respectively, should promote dimerization of the LexA DNA-binding domains of these vectors and repression of lacZ observable as lack of or reduction in β-galactosidase activity. As well, armZ mutations that compromise this interaction will interfere with LexA dimerization and lacZ repression, thereby increasing β-galactosidase activity. Production of ArmZ and MexZ proteins (as LexA fusions) was confirmed in all cases following immunoblotting of whole cell protein extracts with anti-LexA antibodies as described previously [30].

Screening hydroxylamine-mutagenized armZ for mutations abrogating ArmZ-MexZ interaction

The armZ-carrying pDP804 derivative pSF005 was mutagenised by a 50-min treatment with hydroxylamine at 70°C as described [31]. Twenty ml of the 225-μl mutagenesis mixture was treated with Tris-HCl (100 mM; to inactivate the hydroxylamine) and plasmid DNA was recovered (using the Promega Wizard SV Gel and PCR Clean-Up System) for transformation into E. coli SU202 harbouring the mexZ-carrying pMS604 derivative, pSF004. Transformants carrying both plasmids were selected on L-agar containing ampicillin and tetracycline, and supplemented with IPTG (5 mM) and 3-bromo-1-chloro-1-indolyl-β-D-galactopyranoside (X-Gal; 80 μg/ml). Colonies that appeared blue on these agar plates (i.e. β-galactosidase-positive), indicative of a lack of or reduced ArmZ-MexZ interaction, were recovered, increased β-galactosidase activity confirmed [28] and the pDP804-resident armZ genes sequenced.
Table 1. Bacterial strains and plasmids.

| Strain or plasmid | Relevant characteristics* | Reference |
|-------------------|---------------------------|-----------|
| **Strain**        |                           |           |
| E. coli           |                           |           |
| DH5s              | supE44 thi-1 recA gyrA96 relA1 F- Δ[lacZYA-argF] U169 | [41]      |
| S17-1             | thi pro hsdR recA Tra+    | [42]      |
| **P. aeruginosa** |                           |           |
| K767              | PAO1 wild-type            | [43]      |
| K2413             | K767 ΔPA5471              | [14]      |
| K2415             | K767 ΔMexZ                | [14]      |
| K2416             | K767 ΔArmZ ΔPA5471        | [14]      |
| K3240             | K767 armZ (P68S)          | This study|
| K3241             | K767 armZ (G76S)          | This study|
| K3242             | K767 armZ (R216C)         | This study|
| K3243             | K767 armZ (R221W)         |           |
| **Plasmid**       |                           |           |
| pMS604            | LexA1–87WT-Fos zipper fusion; Tc' | [29]      |
| pDP804            | LexA1–87A0B-Jun zipper fusion; Ap' | [29]      |
| pSF004            | pMS604::MexZ              | This study|
| pSF005            | pDP804::ArmZ (WT)         | This study|
| pTH001            | pDP804::ArmZ (P68S)       | This study|
| pTH002            | pDP804::ArmZ (G76S)       | This study|
| pTH003            | pDP804::ArmZ (R216C)      | This study|
| pTH004            | pDP804::ArmZ (R221D)      | This study|
| pTH005            | pDP804::ArmZ (R221W)      | This study|
| pTH006            | pDP804::ArmZ (G231D)      | This study|
| pTH007            | pDP804::ArmZ (G252S)      | This study|
| pEX18Tc           | Broad-host-range gene replacement vector; sacB, Tc' | [44]      |
| pTH008            | pEX18Tc::ArmZ (WT)        | This study|
| pTH009            | pEX18Tc::ArmZ (P68S)      | This study|
| pTH010            | pEX18Tc::ArmZ (G76S)      | This study|
| pTH011            | pEX18Tc::ArmZ (R216C)     | This study|
| pTH012            | pEX18Tc::ArmZ (R221W)     | This study|

*aTc', tetracycline resistance; Ap', ampicillin resistance.

*The amino acid substitutions in the ArmZ products produced by the indicated ArmZ mutant strains are highlighted in parentheses.

*The amino acid substitutions in the mutant ArmZ products encoded by the indicated plasmids are highlighted in parentheses. WT, wild type.

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Generation of armZ missense mutants

Selected armZ missense mutations were engineered into P. aeruginosa strain K767 following their creation in the armZ-carrying pEX18Tc plasmid, pTH008, and mobilization into strain K767. The mutations were introduced into pTH008 using the Quick-Change Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, La Jolla, CA) according to the manufacturer’s instructions. Mutations were confirmed by sequencing and the mutant pTH008 derivatives were mobilized into P. aeruginosa K767 from E. coli S17-1 as before [32]. P. aeruginosa harbouring chromosomal inserts of these pTH008 derivatives were selected on L-agar containing tetracycline (50 μg/ml) and chloramphenicol (5 μg/ml; to counter-select donor E. coli), and subsequently patched onto L-agar containing sucrose (10% [wt/vol]). Sucrose-resistant colonies were then screened on paromomycin (128 μg/ml; ½ MIC for K767) to identify colonies that were paromomycin-sensitive and, so, likely to harbour the armZ mutations. The armZ genes of these were sequenced following their amplification using primers armZ767-F (5’-CGATCTGGGCTTCCGTCATGCCG-3’) and armZ767-R (5’-CGATGGATCCACAGGCCTCGGCG-3’) and Phusion High Fidelity DNA polymerase. Reaction mixtures were formulated as above for construction of plasmid pTH008, with the exception that DMSO was included at 10% (vol/vol), and were subjected to an initial denaturation step at 98°C for 2.5 min, followed by 30 cycles of 98°C for 30 sec, 66.5°C for 30 sec and 72°C for 30 sec, before finishing with a 7-min incubation at 72°C.
Antimicrobial susceptibility testing

The susceptibility of bacterial strains to various antimicrobials was assessed using the two-fold serial dilution technique in 96-well microtiter plates as previously described [33].

Quantitative real-time PCR

RNA was prepared from log phase cells grown in L-broth without or with ¼ MIC of spectinomycin (128 µg/ml; added 90 min prior to harvesting) as described previously [18]. RNA conversion to cDNA and assessment of mexXY expression using quantitative real time PCR (qRT-PCR) was carried out as described [18].

Protein structure modelling

The ArmZ model was developed by threading the ArmZ sequence primarily onto the crystal structure of an RtcB homolog protein (PH1602-extein protein) from Pyrococcus horikoshii (PDB code: 1UC2, chain A) using the SWISS-MODEL program [34]. The model was visually inspected and subjected to energy minimization using GROMOS [35].

Results and Discussion

The requirement for PA5471 (ArmZ) for drug-inducible mexXY expression is MexZ-dependent

Previous studies have demonstrated that the induction of mexXY expression in response to ribosome-disrupting antibiotics is dependent upon the PA5471 gene product [14]. In agreement with this, induction of mexXY by a model ribosome-disrupting compound, spectinomycin, the agent observed to most strongly induce this efflux operon (C.H.F. Lau, unpublished), was wholly compromised in a ΔPA5471 mutant (Fig. 1; compare P. aeruginosa strains K767 and K2413). Given that PA5471 was itself inducible by the same ribosome-targeting agents as mexXY and that cloned PA5471 was able to promote mexXY expression in the absence of antimicrobials, it was reasoned that antimicrobial induction of mexXY resulted from antimicrobial induction of PA5471, which functions as a simple anti-repressor to block MexZ repression of mexXY and, so, promote expression of this efflux operon [14].

When examining the impact of a mexZ knockout on mexXY expression, however, it was noted that loss of this repressor enhanced mexXY expression only 5-fold (Fig. 1; see K1525), much less than the ca. 18-fold increase in mexXY expression that was afforded by spectinomycin exposure (Fig. 1). This result is in agreement with an earlier study which showed that a mutation causing hyperexpression of PA5471 only modestly enhanced mexXY expression, and less than was observed in antimicrobial-exposed cells [19]. Clearly, then, drug induction of mexXY involves more than drug inducible production of PA5471 which then operates as a MexZ anti-repressor. Indeed, exposure of the ΔmexZ mutant strain K1525 to spectinomycin markedly enhances mexXY expression, to levels seen for spectinomycin-exposed wild type strain K767 (Fig. 1), indicating that loss of MexZ repression contributes only modestly to spectinomycin induction of mexXY expression. Moreover, elimination of PA5471 in the ΔmexZ mutant had no effect on spectinomycin-inducible mexXY expression, in contrast to its negative impact in a MexZ- strain, K767 (Fig. 1), indicating that PA5471 was required for antimicrobial-inducible mexXY expression only when the MexZ repressor was present. This was consistent with PA5471 serving only as a MexZ anti-repressor, with additional gene(s)/gene product(s) responsible for maximal drug-inducible efflux gene expression. For this reason we have named PA5471 armZ (anti-repressor MexZ).

Figure 1. Influence of ArmZ on spectinomycin-inducible mexXY expression. mexXY expression was assessed in the indicated P. aeruginosa strains in the absence (–) or presence (+) of spectinomycin (SPC) using quantitative RT-PCR. The armZ and mexZ status (absent; +, present) of the strains is highlighted. Expression was normalized to rpoD and is reported relative (fold change) to the wild-type P. aeruginosa PA01 strain K767 not exposed to spectinomycin. Values represent the mean ± SEM from at least three independent determinations, each performed in triplicate.

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Identification and localization of mutations compromising the ArmZ-MexZ interaction

A previous study using a yeast two-hybrid approach demonstrated an interaction between MexZ and ArmZ [25]. In attempting to confirm this results we employed a bacterial 2-hybrid system [29] in which the armZ and mexZ genes were cloned in-frame to coding sequences for the DNA-binding domain of LexA on plasmids pDP804 and pMS604, respectively, and introduced into E. coli SU202 carrying a chromosomal lacZ gene under the control of a LexA operator. LexA binding to its operator and subsequent repression of lacZ in this strain requires prior dimerization of the LexA-binding domains encoded by pDP804 and pMS604, necessitating interaction of the ArmZ and MexZ sequences fused to the LexA DNA-binding domains of these vectors. As such, lack of or reduced β-galactosidase activity is a measure of the ArmZ-MexZ interaction. The two-hybrid vectors also contain sequences encoding Jun and Fos zipper motifs (which are known to interact) fused to lacZ, such that E. coli SU202 carrying these vectors demonstrates substantial repression of lacZ (Fig. 2A). The unaltered vectors thus provide a positive control for the system, although the Jun and Fos zipper-encoding sequences will be disrupted upon cloning of armZ and/or mexZ sequences, making lacZ repression dependent upon the ArmZ-MexZ interaction. Initially, no evidence for an interaction was obtained (no decrease in β-galactosidase activity was observed relative to SU202 carrying a chromosomal lacZ gene plasmid only (Fig. 2A). Apparently, the relatively lower levels of the armZ and mexZ products expressed from pMS604 and pDP804 in the absence of IPTG were insufficient for the assay to detect an interaction. This was in contrast with an earlier study of another anti-repressor in P. aeruginosa, ArmR, which targets the MexR repressor of the mexAB-oprM multidrug efflux operon and whose interaction with MexR was confirmed using the same bacterial 2-hybrid assay without IPTG induction [90]. This

MexZ Anti-Repessor ArmZ of P. aeruginosa
suggests that the relative affinity of ArmZ for MexZ is low, requiring higher protein levels to see it in the 2-hybrid assay, though it may be enhanced in vivo by the effects of ribosome perturbation. Consistent with a somewhat weaker ArmZ-MexZ interaction, only a 3-fold reduction β-galactosidase activity was observed when mexZ and armZ were both present in E. coli SU202, in contrast with the >100-fold reduction seen previously for armR and mexR [30]. Nonetheless, these data confirm an interaction between ArmZ and MexZ, consistent with ArmZ functioning as a MexZ anti-repressor.

To assess the details of the ArmZ-MexZ interaction, including identification of residues or regions of ArmZ important for or involved in this interaction, mutations in armZ compromising this interaction were selected using the two-hybrid system mentioned above. Thus, armZ-carrying pDP804 was mutagenized and introduced into pMS604::mexZ-carrying E. coli SU202, and LacZ+ colonies (i.e., blue colonies) were selected on L-agar containing X-Gal and IPTG since a defect in the ArmZ-MexZ interaction was expected to obviate lacZ repression in the SU202 reporter strain. Following confirmation that putative mutant ArmZ proteins (as fusions to LexA) defective in the MexZ interaction were expressed (to eliminate further study of mutations compromising ArmZ production or stability, which would also compromise the interaction with MexZ and obviate lacZ repression in SU202), armZ genes were sequenced and the genes carrying single point mutations were saved for further study. Seven mutant armZ genes producing stable ArmZ (-LexA) products (Fig. 2B) carried single mutations (P68S, G76S, R216C, R221W, R221Q, G231D and G252S) and were shown in β-galactosidase assays to be defective in MexZ interaction (i.e., the β-galactosidase activity increased relative to that of wild-type ArmZ) (Fig. 2A). Using a model for ArmZ constructed by threading the ArmZ sequence through the structure of the PH1602 intein from Pyrococcus horikoshii (PDB code: 1UC2), a homologue with 43% overall similarity (28% identity), these residues were mapped to a region of ArmZ within or in proximity to a large α-helix on one side of the protein, which may thus be or contribute to the MexZ interacting domain (Fig. 3). An ArmZ-interacting domain has also been proposed for MexZ, located within the C-terminal region of the protein [36], one of two regions where mexZ mutations tend to cluster in CF isolates of P. aeruginosa [24]. Unlike the mutations that occur in the second region, within the N-terminally-located DNA-binding helix-turn-helix where they compromise MexZ binding to target DNA [36], mutations in the C-terminal domain have a minimal impact on DNA binding [36]. In linking these latter mexZ mutations to mexXY derepression one possibility is that they enhance ArmZ binding to and modulation of the repressor activity of MexZ.

ArmZ mutations compromising its interaction with MexZ block drug-inducible mexXY expression

In order to assess the importance of ArmZ-MexZ interaction for drug-inducible mexXY expression in P. aeruginosa, representatives of the armZ mutations compromising its interaction with MexZ (P68S, G76S, R216C and R221W) were engineered into the chromosome of P. aeruginosa strain K767 and the impact on drug (i.e. spectinomycin) induction of mexXY was assessed. All mutations yielded a reduction in spectinomycin-induced mexXY expression, with the armZ (R216C) and armZ (R221W) mutations that occur within the aforementioned α-helix almost completely obviating spectinomycin induction of efflux gene expression (Fig. 4). Consistent with these results, the four mutants harbouring armZ mutations were pan-aminoglycoside susceptible, reminiscent of the armZ knockout strain K2413, with the armZ (R216C) and armZ (R221W) mutants the most susceptible overall (Table 2). The spectinomycin MICs in particular agree perfectly with the mexXY...
to additional signals/cofactors that impact the interactions with PpsR, respectively. Significantly, these anti-repressors respond/bind the PpsR repressor of photosynthesis genes in Proteobacteria [38] and Myxococcus xanthus is an 111-amino acid protein [37]. Indeed, ArmZ is more reminiscent of larger anti-repressors such as NifL (519 amino acids) and AppA (450 amino acids), which modulate the activity of a CarA repressor that controls expression of carotenoid biosynthetic genes in Myxococcus xanthus is an 111-amino acid protein [37]. Indeed, ArmZ is more reminiscent of larger anti-repressors such as NifL (519 amino acids) and AppA (450 amino acids), which modulate the activity of the NifR repressor of nitorgen fixation/nif genes in Proteobacteria [38] and the PpsR repressor of photosynthesis genes in Rhodobacter sphaeroides [39], respectively. Significantly, these anti-repressors respond/bind to additional signals/cofactors that impact the interactions with their cognate repressors [38,39], with the additional protein sequences responsible for cofactor binding/signal responding and ‘communicating’ with the repressor-binding domain. Thus, it is likely that ArmZ also responds to additional signals/s likely related to and downstream of ribosome perturbation as discussed above, and these promote ArmZ interaction with MexZ in vivo. What these co-factors/signals might be is, at present, unknown. Interestingly, this represents the 3rd example of multidrug efflux gene regulation in Pseudomonas involving an anti-repressor (the activity of the SrpS repressor of the srpABC solvent exporter operon in Pseudomonas putida is also modulated by an anti-repressor, SrpR [40]). The significance of this observation, if any, is unclear.

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Author Contributions

Conceived and designed the experiments: TH SF CHL CG.Performed the experiments: TH SF CHL CG. Analyzed the data: TH SF CHL KP. Wrote the paper: TH KP.

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Table 2. Influence of armZ mutations on aminoglycoside resistance in P. aeruginosa.

| Strain ArmZ | MIC (µg/ml) for:*b |
|---|---|
| TOB | AMI | GEN | KAN | PAR | SPC |
| K767 WT | 1 | 2 | 2 | 64 | 256 | 512 |
| K2413 --- | 0.5 | 1 | 1 | 32 | 16 | 64 |
| K3240 P68S | 0.5 | 1 | 1 | 32 | 32 | 128 |
| K3241 G76S | 0.5 | 1 | 1 | 32 | 32 | 128 |
| K3242 R216C | 0.5 | 1 | 1 | 32 | 32 | 64 |
| K3243 R221W | 0.5 | 1 | 1 | 32 | 32 | 64 |

*The amino acid change in ArmZ in the indicated armZ mutant strains is highlighted. WT, wild type ArmZ; ---, armZ deleted.

TOB, tobramycin; AMI, amikacin; GEN, gentamicin; KAN, kanamycin; PAR, paromomycin; SPC, spectinomycin.
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