RESEARCH ARTICLE

Aminoglycoside-inducible expression of the mexAB-oprM multidrug efflux operon in Pseudomonas aeruginosa: Involvement of the envelope stress-responsive AmgRS two-component system

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Abstract

Exposure of P. aeruginosa to the aminoglycoside (AG) paromomycin (PAR) induced expression of the PA3720-armR locus and the mexAB-oprM multidrug efflux operon that AmgR controls, although PAR induction of mexAB-oprM was independent of armR. Multiple AGs promoted mexAB-oprM expression and this was lost in the absence of the amgRS locus encoding an aminoglycoside-activated envelope stress-responsive 2-component system (TCS). Purified AmgR bound to the mexAB-oprM promoter region consistent with this response regulator directly regulating expression of the efflux operon. The thiol-active reagent, diamide, which, like AGs, promotes protein aggregation and cytoplasmic membrane damage also promoted AmgRS-dependent mexAB-oprM expression, a clear indication that the MexAB-OprM efflux system is recruited in response to membrane perturbation and/or circumstances that lead to this. Despite the AG and diamide induction of mexAB-oprM, however, MexAB-OprM does not appear to contribute to resistance to these agents.

Introduction

Pseudomonas aeruginosa is a common nosocomial human pathogen [1, 2] often associated with pulmonary infections in patients with cystic fibrosis [3]. The organism has an impressive intrinsic antimicrobial resistance [4] and readily develops resistance during antimicrobial therapy via mutation and horizontal gene transfer [5–7]. Major contributors to intrinsic and acquired antimicrobial resistance in P. aeruginosa are a number of broadly-specific multidrug efflux systems of the RND family [8]. One of these, MexAB-OprM, which contributes to both intrinsic [9] and acquired (i.e., mutational) [6] resistance, exhibits one of the broadest substrate profiles of the RND pumps in P. aeruginosa, accommodating a wide range of clinically-relevant [10–12] and experimental [13] antimicrobials as well as biocides [14] and a variety of non-clinical agents (e.g., organic solvents [15], dyes [16, 17], detergents [17], herbicides [18] and acylhomoserine...
lactones (AHLs) associated with quorum-sensing (QS) [19]. Clinically, this efflux system is most noted for its contribution to acquired fluoroquinolone and β-lactam resistance [6].

Expression of mexAB-oprM is controlled by several regulators including two repressors, MexR [20] and NalD [21], that each act at one of two promoters occurring distal (PI, MexR) [22] and proximal (PII, NalD) [21] to the efflux locus. Mutations in each of these genes are associated with efflux gene hyperexpression and multidrug resistance in both lab [20] [23] [24, 25] and clinical [26] [25, 27, 28] isolates. MexR binding to its promoter is modulated by its redox status (in response to oxidative stress) [29, 30] and by the ArmR anti-repressor, whose binding to MexR abrogates promoter binding by the repressor [31]. armR occurs as part of the PA3720-armR operon that is regulated by the product of the divergently-transcribed nalC repressor gene [32], with nalC mutants showing elevated PA3720-armR expression and, so, elevated mexAB-oprM expression and multidrug resistance [32] as a result of ArmR modulation of MexR's repressor activity [31]. The function of PA3720, a protein with no homology to any characterised protein, remains unknown. nalC lab and clinical isolates expressing mexAB-oprM and showing a multidrug-resistant phenotype have been reported [32] [33, 34]. Recently, a homologue of the E. coli CpxR envelope stress response regulator has been identified in P. aeruginosa and shown to bind to the PI promoter of mexAB-oprM, where it positively influences efflux gene expression [35]. Interestingly, mexAB-oprM expression and the multidrug resistance of MexR nalB mutants were shown to be partially dependent on CpxR [35]. Finally, the BrlR regulator of antimicrobial tolerance of P. aeruginosa biofilms [36] has also been shown to bind to the mexAB-oprM promoter region where it positively influences expression of the efflux operon, which thus contributes to biofilm antimicrobial tolerance [37].

Despite the identification of a number of mexAB-oprM regulators, the naturally-occurring signals to which they respond and the intended substrates and, so, function of the efflux system remain largely unknown. Chlorinated phenols, including the pesticide pentachlorophenol (PCP) that is an uncoupler of oxidative phosphorylation [38], have been shown to induce expression of the PA3720-armR and mexAB-oprM operons, in part owing to their ability to bind and, so, modulate the repressor activity of NalC [39, 40]. Still, some PCP induction of mexAB-oprM is seen in mutants lacking the ArmR anti-repressor but dependent on MexR, an indication that this agent operates though multiple channels in driving expression of this efflux operon. In any case, it seems unlikely that PCP is the intended natural substrate/inducer but may mimic naturally-occurring plant-derived phenolic compounds that could be. NalD has been shown to bind novobiocin, a known MexAB-OprM substrate, with novobiocin binding abrogating NalD interaction with its DNA target, thereby enhancing PII promoter activity [41]. Still, it is far from clear that this gyrase-targeting [42, 43] antimicrobial is an intended inducer and substrate for this system. The identification of a CpxR homologue as a regulator of mexAB-oprM would suggest that the efflux system responds to envelope stress. Still, an envelope stress-response two-component system (TCS) that regulates genes reminiscent of E. coli CpxR targets, AmgRS, has already been described in P. aeruginosa, despite AmgRS being a homologue of the E. coli osmotic stress-responsive TCS, OmpR-EnvZ [44]. As such, P. aeruginosa CpxR may respond to signals other than those related to envelope perturbation. Finally, a recent report documents a transient, early log phase induction of mexAB-oprM in response to Ca^{2+} [45], although the regulator mediating this and the Ca^{2+}-generated signal(s) to which the system is responding have not been identified.

The current study was undertaken to follow up a preliminary observation that exposure of P. aeruginosa to the aminoglycoside (AG), PAR, induced the PA3720-armR operon, which suggested that this antimicrobial might also upregulate mexAB-oprM expression. We confirm that it and, in fact, several AGs, do indeed induce mexAB-oprM expression. Surprisingly, however, this was independent of the ArmR anti-repressor and instead dependent on the aforementioned
AmgRS envelope stress-responsive TCS. These results suggest that the generation of certain product(s) of envelope stress and/or cellular conditions that promote envelope perturbation require MexAB-OprM function and, so, promote its production.

**Materials and methods**

**Bacterial strains and plasmids**

Bacterial strains and plasmids used in this study are listed in Table 1. Plasmid pEX18Tc and its derivatives were maintained in *E. coli* with 5 (in L-broth) or 10 (on L-agar) μg/ml tetracycline. Plasmid pET23a and its derivatives were maintained in *E. coli* with ampicillin (100 μg/ml).

**DNA methods**

Standard protocols were used for restriction endonuclease digestions, ligations, transformations and agarose gel electrophoresis, as previously described [55]. 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 digest products requiring purification were purified using the Promega Wizard SV Gel and PCR Clean-Up System according to a protocol provided by the manufacturer. Plasmid DNA was introduced into CaCl2-competent *E. coli* cells, which were prepared as previously described [55].

**Table 1. Bacterial strains and plasmids.**

| Strain | Relevant genotype | Source |
|--------|------------------|--------|
| DH5α   | q80ΔlacZΔM15 endA1 recA1 hsdR17(tN mC ΔmB ) supE44 thi-1 gyrA96 relA1 F ΔlacZYA-argF U169 | [46] |
| S17-1  | thi pro hsdR recA Tra+ | [47] |
| E. coli Bl21 DE3 (pLysE) | F ompT (mB ) DE3 is a lambda derivative carrying lacI and a T7 RNA polymerase gene under placUV5 control | [48] |
| *P. aeruginosa* | | |
| K767   | PAO1 wild-type *P. aeruginosa* | [49] |
| K1491  | K767ΔmexR | [50] |
| K3415  | K767ΔarmR | [59] |
| K1523  | K767ΔmexB | [51] |
| K1525  | K767ΔmexXY | [52] |
| K1542  | K767ΔmexXYΔmexB | [53] |
| K3793  | K767ΔnalD | This study |
| K3794  | K767ΔmexR ΔnalD | This study |
| K3519  | K767ΔamgR | [54] |
| K3583  | K767ΔamgS | [54] |
| K3260  | K767 derivative carrying the *amgS*V121G mutation | [54] |
| K3249  | K767 derivative carrying the *amgS*B182C mutation | [54] |
| Plasmids | | |
| pET23a | Expression plasmid; Ap<sup>R</sup> | Novagen |
| pMJF34 | pET23a::amgR | This study |

<sup>Tc</sup><sup>R</sup>, tetracycline resistance;
<sup>Ap</sup><sup>R</sup>, ampicillin resistance.

https://doi.org/10.1371/journal.pone.0205036.t001
Oligonucleotide synthesis was performed by Integrated DNA Technologies (Coralville, Iowa), and nucleotide sequencing was performed by ACGT Corporation (Toronto, Canada).

Susceptibility testing
The antimicrobial susceptibility of various *P. aeruginosa* strains was assessed using the 2-fold microtiter broth dilution method as described previously [56]. The minimum inhibitory concentration (MIC) was recorded as the lowest concentration of antibiotic inhibiting visible growth after 18 hours of incubation at 37˚C.

Quantitative RT-PCR
*P. aeruginosa* cells grown overnight in L-broth at 37˚C were subcultured (1:49) in the same medium and incubated at 37˚C until cultures reached an optical density at 600 nm (OD<sub>600</sub>) of 0.6–0.8. Total RNA was isolated, purified and reverse transcribed into cDNA as described previously [57]. Where specified, the antimicrobials paromomycin (PAR; 256 μg/ml), neomycin (NEO; 64 μg/ml), gentamicin (GEN; 2 μg/ml), tobramycin (TOB; 1 μg/ml), kanamycin (KAN; 128 μg/ml), chloramphenicol (CAM; 32 μg/ml), tetracycline (TET; 16 μg/ml), erythromycin (ERY; 512 μg/ml), azithromycin (AZI; 512 μg/ml) and diamide (DIA; 4 mM) were added at their respective MICs 30 minutes prior to harvesting cells. In some experiments, *P. aeruginosa* was pretreated with CAM (128 μg/mL) for 15 min prior to the addition of neomycin at its MIC. The primers used in quantitative real-time PCR (qPCR) were designed to amplify gene fragments with lengths of 138 bp (*mexA*; Forward: CAGCAGCTCTACCAGATCG; Reverse: CGTACTGCTGCTTGCTCA), 123 bp (*armR*; Forward: CAACAAACCGTCCCGCAC; Reverse: GTAGAGGTCCCAGGCATTG) and 188 bp (PA3720; Forward: GATGCCTTTCCCTTGCTCCA; Reverse: TCCTTGAGCCACAAACACCAG). The *rpoD* reference gene was amplified as described previously [58]. The amplification efficiencies of the qRT-PCR primer sets were 102.8% (correlation co-efficient, r<sup>2</sup> = 0.997) for *mexA*, 100.3% for *armR* (r<sup>2</sup> = 0.997) and 101.2% for PA3720 (r<sup>2</sup> = 0.994). The qRT-PCR reaction mixtures, amplification parameters, and melt curve analyses were performed as previously described [39, 59]. The expression levels of *mexA*, PA3720 and *armR* were normalized to that of the reference gene *rpoD* using the ΔΔC<sub>t</sub> method provided by the CFX-manager software version 1.6 (Bio-Rad) and are reported as fold change relative to that in the wild-type *P. aeruginosa* PAO1 strain K767, unless otherwise specified. A minimum of three biological replicates each performed in triplicate were carried out for all samples. In all instances, no-template controls were carried out to ensure the absence of DNA contamination.

Expression and purification of AmgR
To facilitate the purification of AmgR, a C-terminal polyhistidine tag was engineered onto AmgR by cloning the *amgR* gene into plasmid pET23a. The *amgR* gene was amplified by PCR using primers AmgR-His-For (5’–GATCCATAATGCGAACCTTGCGCCCT–3’; NdeI site underlined) and AmgR-His-Rev (5’–GATCCTCGAGGCGCTTGGCCGTC–3’; XhoI site underlined) in a 50 μl PCR mixture that contained 1 μg *P. aeruginosa* PAO1 strain K767 chromosomal DNA, 0.6 μM of each primer, 0.2 mM of each dNTP, 1 x Phusion GC buffer and 1 U of Phusion polymerase (New England Biolabs). The mixture was heated for 30 sec at 98˚C, followed by 30 cycles of 30 sec at 98˚C, 30 sec at 65.0˚C, 30 sec at 72˚C, concluding with 7 min at 72˚C. The PCR product was gel-purified, digested with NdeI and XhoI and cloned into NdeI-XhoI-restricted pET23a to yield pET23a::*amgR* (pMJF34) encoding His-tagged AmgR. Following nucleotide sequencing of the cloned gene to confirm the absence of PCR-generated mutations, plasmid pMJF34 was introduced into *E. coli* BL21(DE3) harbouring the pLysE plasmid via transformation. AmgR-His was then expressed and purified from 50 ml
of culture as previously described [39], with the exception that IPTG-induced expression of the *amgR* gene on pMJF34 was carried out for 90 min.

**Electrophoretic mobility shift assay**

The binding of purified AmgR to a DNA fragment encompassing the intergenic region upstream of *mexAB-oprM* was assessed using the electrophoretic mobility shift assay (EMSA) as described previously [39]. The intergenic region was amplified on a 315-bp fragment using primers K9 and K10 [22] in a 50-μl reaction mixture containing 1 μg of purified *P. aeruginosa* PAO1 strain K767 chromosomal DNA, 0.6 μM of each primer, 0.2 mM of each dNTP, 1 x Phusion HF buffer, 5% (vol/vol) dimethylsulphoxide and 1 U of Phusion polymerase (New England Biolabs). The mixture was heated at 98˚C for 30 sec, followed by 30 cycles of 30 sec at 98˚C, 30 sec at 65.0˚C, 15 sec at 72˚C, concluding with 7 min at 72˚C. The DNA fragment was gel purified and quantified using a Nanodrop™ 2000 Spectrophotometer (Thermo Fisher Scientific). To assess the specificity of any binding observed, excess sheared salmon sperm DNA (100 μM) was added to the reaction mixtures prior to the addition of AmgR.

**Membrane depolarization assay**

A previously described fluorometric assay [60] involving the membrane potential-sensitive dye bis-(1,3-dibutylbarbituric acid) trimethine oxonol [DiBAC4(3)], was employed to measure the degree of cytoplasmic membrane (CM) depolarization promoted by diamide treatment of *P. aeruginosa*. Briefly, early log phase (OD$_{600}$ = 0.3–0.5) L-broth subcultures of *P. aeruginosa* were exposed (or not) to diamide (4 mM final concentration). Samples (5 mL) were taken immediately and then hourly over 3 h and incubated with DiBAC4(3) (Invitrogen, Burlington, Ontario, Canada; 10 μg/ml final concentration) in the dark for 5 min at 37˚C. Bacteria were then pelleted and resuspended in phosphate-buffered saline [61] to a final OD$_{600}$ of 0.1. The membrane depolarization-dependent fluorescence emitted by cells was measured using a Varian (now Agilent, Mississauga, Ontario, Canada) Cary Eclipse fluorescent spectrophotometer with excitation and emission wavelengths of 490 and 518, respectively.

**Results**

**Aminoglycoside-induced expression of *mexAB-oprM***

DNA microarray analysis of wild-type (WT) *P. aeruginosa* PAO1 strain K767 treated with the AG, PAR, revealed an increase in expression of the PA3720-*armR* two-gene operon (K. Poole, unpublished) whose products regulate expression of the *mexAB-oprM* multidrug efflux operon [31, 32]. To validate the results of the transcriptome analysis, log-phase K767 cells were similarly treated with the MIC of PAR, and expression of the PA3720 and *armR* genes were assessed using qRT-PCR. In agreement with the microarray data, treatment with PAR led to an increase (~4-fold) in expression of the PA3720 and *armR* genes (Fig 1). ArmR is an anti-repressor for the *mexAB-oprM* repressor [31], MexR, and its expression in so-called *nalC* mutants is responsible for the elevated *mexAB-oprM* expression and multidrug resistance of these mutants [32]. It was, therefore, hypothesized that the PAR-promoted increase in *armR* expression and, ultimately, ArmR production, would also drive derepression of the *mexAB-oprM* operon. Indeed, PAR provided for a ~2-fold increase in *mexA* gene expression (as a measure of *mexAB-oprM* expression) relative to the untreated K767 strain (Fig 1). Surprisingly, however, *mexAB-oprM* expression was still PAR-inducible in a mutant lacking *armR* (Fig 1), an indication that PAR induction of *mexAB-oprM* expression was not mediated by ArmR.
To assess whether PAR induction of this efflux system was specific to this AG or a more general property of this antimicrobial class, additional AGs were assessed for an ability to induce mexAB-oprM expression. Like PAR, the AGs KAN and NEO induced mexAB-oprM expression at their MICs, with NEO showing the best induction amongst all of the AGs tested (Fig 2). In contrast, GEN provided for a minimal increase in mexAB-oprM expression and TOB failed to induce this efflux operon, also at their MICs (Fig 2). Despite the induction of mexAB-oprM, however, this efflux system does not appear to contribute to AG resistance in P. aeruginosa – loss of MexAB-OprM in both WT strain K767 and a mutant derivative lacking the MexXY efflux system linked to intrinsic AG resistance did not impact AG susceptibility (Table 2).

AG induction of the mexAB-oprM operon is independent of the MexR and NalD repressors

The mexAB-oprM operon is regulated by 2 direct repressors, MexR [22] and NalD [21], one or both of which might mediate the observed AG inducibility of this efflux operon. To assess this, the impact of a mexR or nalD deletion on AG induction of mexAB-oprM expression was

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Fig 1. PAR induction of PA3720-armR and mexAB-oprM expression. Expression of PA3720, armR and mexA was assessed in log-phase WT P. aeruginosa strain K767 following a 30-minute exposure to the MIC of the AG, paromomycin (PAR; 256 μg/ml) using qRT-PCR. mexA expression was also assessed in a ΔarmR derivative of K767, K3415, following exposure (30 min) to the MIC of PAR (256 μg/ml) using qRT-PCR. In all cases, expression was normalized to rpoD and is reported relative to the untreated WT P. aeruginosa strain K767 (fold-change). Values shown are means ± standard errors of the means (SEMs) from at least three independent determinations, each performed in triplicate. t-test: *, P < 0.05; ***, P < 0.001.

https://doi.org/10.1371/journal.pone.0205036.g001
assessed. If AGs somehow obviate MexR or NalD repressor activity in promoting increased \textit{mexAB-oprM} expression, AG induction of \textit{mexAB-oprM} expression should be lost in either a \textit{mexR} or \textit{nalD} knockout strain. Individual deletions of the \textit{mexR} and \textit{nalD} genes in the WT \textit{K767} strain led to the expected increase in \textit{mexAB-oprM} expression (Fig 3). Still, exposure of these mutants to the AG, NEO, still produced an increase in \textit{mexAB-oprM} expression (Fig 3). Similar results were obtained with a mutant lacking both repressor genes, where loss of both \textit{mexR} and \textit{nalD} provided for an increase in \textit{mexAB-oprM} expression that was, nonetheless,

![Fig 2. AG induction of \textit{mexAB-oprM} expression. \textit{mexA} expression was assessed in log-phase WT \textit{P. aeruginosa} strain K767 following a 30-minute exposure to the MIC of the AGs, paromomycin (PAR; 256 \text{ g/ml}), neomycin (NEO; 64 \text{ g/ml}), gentamicin (GEN; 2 \text{ g/ml}), tobramycin (TOB; 1 \text{ g/ml}), and kanamycin (KAN; 128 \text{ g/ml}) using qRT-PCR. Expression was normalized to \textit{rpoD} and is reported relative to the untreated K767 (fold change). Values are means ± SEMs from at least three independent determinations, each performed in triplicate. t-test: *, P < 0.05; **, P < 0.001.](https://doi.org/10.1371/journal.pone.0205036.g002)

Table 2. Impact of \textit{mexAB-oprM} loss on AG susceptibility.

| Strain | Genotype | GEN (\text{\mu g/ml}) | PAR | NEO | KAN |
|--------|----------|------------------------|-----|-----|-----|
| K767   | WT\textsuperscript{b} | 4                      | 256 | 64  | 128 |
| K1523  | \textit{\Delta}mexB | 4                      | 256 | 64  | 128 |
| K1525  | \textit{\Delta}mexXY | 2                      | 32  | 32  | 64  |
| K1542  | \textit{\Delta}mexXY \textit{\Delta}mexB | 2                      | 32  | 32  | 64  |

\textsuperscript{a}GEN, gentamicin; PAR, paromomycin; NEO, neomycin; KAN, kanamycin.

\textsuperscript{b}WT, wild type

[https://doi.org/10.1371/journal.pone.0205036.t002](https://doi.org/10.1371/journal.pone.0205036.t002)
elevated further upon exposure to NEO (Fig 3). Thus, AG induction of this efflux operon is independent of the MexR and NalD repressors.

AmgRS mediates AG induction of *mexAB-oprM* expression

AmgRS, an AG-responsive TCS in *P. aeruginosa* that functions as part of an envelope stress response to AG-induced CM-damaging aberrant polypeptides, was recently shown to play a role in AG-induced expression of the AG resistance-promoting *mexXY* multidrug efflux operon [44, 54, 62]. This TCS might, thus, mediate the AG induction of *mexAB-oprM*. To assess this, the impact of AmgRS loss on PAR induction of *mexAB-oprM* expression was determined. As seen in Fig 4A, mutants lacking *amgR* or *amgS* were deficient for PAR-inducible expression of *mexAB-oprM*, an indication that AG induction of this efflux system was dependent on the AmgRS TCS. Consistent with this, *mexAB-oprM* expression promoted by the other AGs was similarly lost in the *amgR* deletion strain (Fig 4B). As expected, activation of AmgRS as a result of *amgS* gain-of-function mutations (V121G and R182C) also promoted an increase in *mexAB-oprM* expression, independent of AG exposure (Fig 4C). This did not, however, parallel an increase in resistance to representative MexAB-OprM substrate antimicrobials (e.g. carbenicillin, chloramphenicol, nalidixic acid; Table 3). As well, despite its link to *mexAB-oprM* and a common inducibility by PAR, the PA3720-ARMR operon retained its PAR
**Fig 4. AmgRS-dependent AG-inducible mexAB-oprM expression.** (A) mexA expression was assessed in log-phase cultures of WT *P. aeruginosa* strain K767 and its ΔamgR (strain K3159) and ΔamgS (strain K3583) derivatives following exposure to the MIC of PAR (K767, 256 μg/ml; K3159, 16 μg/ml; K3583, 16 μg/ml) for 30 min using qRT-PCR. (B) mexA expression was assessed in log-phase cultures of *P. aeruginosa* strains K767 (WT; AmgR⁺) and K3159 (ΔamgR; AmgR⁻) exposed to the MIC of the indicated antimicrobials (−, no antimicrobial; NEO, neomycin; GEN, gentamicin; KAN, kanamycin) using qRT-PCR. Antimicrobials were used at: NEO, 64 (K767) and 4 (K3159) μg/ml; GEN, 2 (K767) and 0.5 (K3159) μg/ml; KAN, 128 (K767) and 16 (K3159) μg/ml. (C) mexA expression was assessed in strains harbouring WT (K767) and mutant (K3288, AmgSV121G, K3249, AmgSR182C) amgS genes using qRT-PCR. (D) armR and PA3720 expression was assessed in log-phase cultures of *P. aeruginosa* strains K767 (WT), K3159 (ΔamgR), and K3583 (ΔamgS) exposed to the MIC of PAR for 30 min using qRT-PCR. Expression in all cases was normalized to rpoD and is reported relative to the untreated K767 strain (fold change). Values are means ± SEMs from at least three independent determinations, each performed in triplicate. t-test: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

https://doi.org/10.1371/journal.pone.0205036.g004

**Table 3. Impact of amgS gain-of-function mutations on antibiotic susceptibility.**

| Strain | AmgS | CAR | CAM | NAL | NOV |
|--------|------|-----|-----|-----|-----|
| K767   | WT   | 64  | 64  | 128 | 1024|
| K3260  | V121G| 64  | 32  | 128 | 2048|
| K3249  | R182C| 64  | 64  | 128 | 1024|

*aThe amino acid change in AmgS in the indicated mutants is highlighted. WT, wild type.

*bCAR, carbenicillin; CAM, chloramphenicol; NAL, nalidixic acid; NOV, novobiocin.

https://doi.org/10.1371/journal.pone.0205036.t003
Inducibility in the amgR deletion strain (Fig 4D) an indication that these operons can be independently regulated.

**AmgR binds to the mexAB-oprM promoter region**

The regulation of mexAB-oprM by AmgRS is most simply explained by AmgR directly controlling expression of the efflux operon from a promoter upstream of the efflux genes. To assess this, binding of AmgR to the mexAB-oprM promoter region was assessed using an EMSA. As seen in Fig 5A, purified AmgR bound to a DNA fragment that encompassed the intergenic region upstream of mexAB-oprM. Moreover, binding was retained in the presence of excess competitor DNA (Fig 5B), an indication that binding was specific.

**Role of cytoplasmic membrane perturbation in AG-induced mexAB-oprM expression**

It has been demonstrated that AmgRS responds to and protects cells from envelope stress caused by AG-generated mistranslated/misfolded proteins that damage the cytoplasmic membrane (CM) [44, 54, 62]. Previous reports demonstrated that treatment of WT *P. aeruginosa* with CAM, a protein translation inhibitor, prior to the addition of an AG, blocked AG-promoted CM damage, consistent with is blockage of mistranslated protein synthesis [62]. To assess if AG-promoted CM damage is responsible for AmgRS-dependent induction of mexAB-oprM expression by AGs, WT *P. aeruginosa* K767 cells were pre-treated with CAM prior to the addition of the AG, NEO, and mexA expression was measured. CAM pre-treatment of cells prevented NEO induction of mexAB-oprM expression (Fig 6), consistent with it blocking AG-generated mistranslated/misfolded proteins and subsequent CM damage. Still, it was also observed that CAM alone negatively influenced mexAB-oprM expression (Fig 6). As such, it is unclear whether AG-induced mexAB-oprM expression follows from AG generation of membrane-damaging mistranslation products.

**mexAB-oprM is inducible by additional ribosome-perturbing agents**

The observation that AG induction of mexAB-oprM is AmgRS-dependent and that CAM, another ribosome-targeting agent, fails to induce the efflux system, is consistent with AG-generated membrane-damaging mistranslation products being key to AG-promoted mexAB-oprM expression. As such, only mistranslation-promoting AGs are likely to induce this efflux system. To assess this, a number of additional agents that target the ribosome but do not...
promote mistranslation (TET, ERY, AZI) [63] were assessed for an ability to induce expression of the mexAB-oprM operon. As with CAM, treatment of WT P. aeruginosa K767 with the MIC of TET yielded a decrease in mexAB-oprM expression (2-fold; Fig 7A). In contrast, treatment of K767 with ERY or AZI increased mexAB-oprM gene expression (2.5-fold; Fig 7A). Still, this was independent of AmgR–induction of mexAB-oprM expression by ERY and AZI was.
retained in an amgR null mutant (Fig 7B; see K3519). Thus, ribosome perturbation alone is insufficient for AmgRS-promoted mexAB-oprM expression. While macrolide-induced mexAB-oprM expression is clearly mediated by a different regulatory pathway, it is unclear whether it follows from ribosome perturbation or some other impact of these agents on P. aeruginosa.

**Diamide-promoted mexAB-oprM expression**

In addition to their contribution to CM damage, AGs [e.g. streptomycin (STR)] have also been shown to cause protein aggregation in E. coli, also as a result of AG-induced mistranslation [64]. Like AGs, the thiol-specific oxidant, diamide (DIA) has also been shown to promote protein misfolding and aggregation [65–67] although an impact on membranes has never been assessed. To assess whether protein aggregation might be playing a role in AmgRS-driven mexAB-oprM expression the impact of diamide on expression of the efflux operon was assessed. Treatment of WT P. aeruginosa strain K767 with the MIC of diamide induced mexAB-oprM expression (~3.5-fold; Fig 8) and this was dependent on AmgR (Fig 8). Interestingly diamide was also shown to be modestly CM perturbing, promoting a transient increase in membrane depolarization (at 1 h post-diamide addition; Fig 9) and, as with AGs [60, 62], this was enhanced in a mutant lacking amgR (Fig 9). Thus, AmgRS appears to protect P. aeruginosa from CM perturbation promoted by diamide.

**Discussion**

AmgR represents yet another direct regulator of the mexAB-oprM multidrug efflux operon, highlighting the complexity of the regulation of this locus and the apparent diversity of signals and growth conditions to which it responds. Given the apparent responsiveness of the AmgRS TCS to envelope stress resultant from cytoplasmic membrane perturbation by AG-generated mistranslation products, it is likely that the observed AmgRS-dependent induction of mexAB-
oprM by AGs reflects an envelope stress-inducibility of this efflux locus and, so, a need for MexAB-OprM under certain conditions of envelope stress. Consistent with this, another agent shown here to be membrane perturbing, diamide, also showed AmgRS-dependent induction of \textit{mexAB-oprM} expression and this TCS appeared to protect \textit{P. aeruginosa} somewhat from diamide-mediated membrane damage. While CM perturbation is not a heretofore reported property of diamide, it has been shown that a mutant of \textit{Xanthomonas campestris} pv. \textit{campestris} lacking the RpoE envelope stress response sigma is more sensitive to diamide than WT \textit{X. campestris} [68], further support for this agent being membrane-damaging. It is interesting to note, too, that the AG induction of \textit{mexAB-oprM} was variable and reflected the AG responsiveness of AmgRS [54] with PAR and NEO being the better inducers/activators while, for

![Graph](https://doi.org/10.1371/journal.pone.0205036.g008)
example, TOB failed to induce/activate (at 1X MIC) and, indeed, perturb membranes [62]. Still, despite the induction of this multidrug efflux locus by AGs and diamide, MexAB-OprM does not appear to play any role in resistance to these agents. It is also possible that AG induction of mexAB-oprM serves primarily to increase OprM levels, this outer membrane protein functioning with the proteins products of the similarly AmgRS-regulated and AG-inducible mexXY efflux operon, with MexXY-OprM a known contributor to AG resistance. Still, oprM appears to possess its own promoter [69], such that its expression can be driven independently of mexAB, to ensure, for example, adequate levels of OprM to partner with MexXY without the need for unnecessary and wasteful induction of the mexAB genes.

While attempts were made to show that AG-promoted mexAB-oprM expression was dependent on protein translation (as evidence for potentially membrane-damaging mistranslation products being key to AG induction of the efflux locus) the results were inconclusive since classical translation inhibitors (CAM and TET) themselves had a negative impact on mexAB-oprM expression, rendering the observed CAM prevention of AG-promoted mexAB-oprM expression not readily interpretable. Possibly, TET and CAM inhibition of translation interferes with endogenous mistranslation that might provide basal levels of membrane-damaging aberrant polypeptides that activate AmgRS and provide for some mexAB-oprM expression. Still, loss of amgR did not adversely affect endogenous mexAB-oprM expression, arguing against this. Alternatively, since CAM is known to induce expression of a 2nd RND type multidrug efflux operon,
mexEF-oprN [70], and mutational upregulation of this efflux operon has been shown to parallel a possibly compensatory decrease in mexAB-oprM expression [71, 72], the CAM-driven decrease in mexAB-oprM expression may be secondary to its upregulation of mexEF-oprN. TET (and CAM) also induces the mexXY RND efflux operon [73] although there is as yet no evidence that this parallels a decrease in mexAB-oprM expression, and other mexXY inducers (AGs and macrolides) [73, 74] were shown here to promote mexAB-oprM expression. This may, however, simply reflect these ribosome-targeting agents impacting the P. aeruginosa in ways that require MexAB-OprM and, so, induce mexAB-oprM expression, masking the negative impact their possible induction of a 2nd efflux operon might otherwise have on it.

Adding to the complexity of mexAB-oprM regulation is the observed induction of this efflux locus by the macrolide antibiotics ERY and AZI. That such induction is independent of AmgRS, consistent with this TCS responding to membrane perturbation and macrolides not known to provide for membrane damage, speaks to a possibly additional mexAB-oprM regulator mediating this, though at this point one cannot rule out the involvement of one of the other regulators identified to date. Interestingly, and in contrast to results presented here, it has been reported that AZI suppresses mexAB-oprM expression in P. aeruginosa [75], which we can only attribute to differences in growth medium and/or the WT strain used in the two studies. The observed AG induction of the PA3720-armR operon that encodes the MexR anti-repressor is also interesting, given that it does not provide for AG induction of mexAB-oprM expression and, so, suggests that this locus may also influence expression of additional loci.

It is becoming clear that the mexAB-oprM multidrug efflux locus is inducible by a number of antimicrobial agents although, with few exceptions, the actual inducing signals and intended efflux substrates are unknown. Thus, while clearly stress-responsive, the actual function of MexAB-OprM remains to be elucidated.

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