Pentachlorophenol Induction of the *Pseudomonas aeruginosa mexAB-oprM* Efflux Operon: Involvement of Repressors NalC and MexR and the Antirepressor ArmR

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**Abstract**

Pentachlorophenol (PCP) induced expression of the NalC repressor-regulated PA3720-<em>armR</em> operon and the MexR repressor-controlled <em>mexAB-oprM</em> multidrug efflux operon of *Pseudomonas aeruginosa*. PCP’s induction of PA3720-<em>armR</em> resulted from its direct modulation of NalC, the repressor’s binding to PA3720-<em>armR</em> promoter-containing DNA as seen in electromobility shift assays (EMSAs) being obviated in the presence of this agent. The NalC binding site was localized to an inverted repeat (IR) sequence upstream of PA3720-<em>armR</em> and overlapping a promoter region whose transcription start site was mapped. While modulation of MexR by the ArmR anti-repressor explains the upregulation of <em>mexAB-oprM</em> in <em>nalC</em> mutants hyperexpressing PA3720-<em>armR</em>, the induction of <em>mexAB-oprM</em> expression by PCP is not wholly explainable by PCP induction of PA3720-<em>armR</em> and subsequent ArmR modulation of MexR, inasmuch as <em>armR</em> deletion mutants still showed PCP-inducible <em>mexAB-oprM</em> expression. PCP failed, however, to induce <em>mexAB-oprM</em> in an <em>armR</em> deletion strain, indicating that MexR was required for this, although PCP did not modulate MexR binding to <em>mexAB-oprM</em> promoter-containing DNA as seen in electromobility shift assays. One possibility is that MexR responds to PCP-generated in vitro effector molecules in controlling <em>mexAB-oprM</em> expression in response to PCP. PCP is an unlikely effector and substrate for NalC and MexAB-OprM – its impact on NalC binding to the PA3720-<em>armR</em> promoter DNA occurred only at high μM levels, suggesting that it mimics an endogenous phenolic effector/substrate(s). In this regard, plants are an abundant source of phenolic antimicrobial compounds and, so, MexAB-OprM may function to protect *P. aeruginosa* from plant antimicrobials that it encounters in nature.

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**Introduction**

*Pseudomonas aeruginosa* is an opportunistic pathogen associated with debilitating infections of immunocompromised individuals and individuals with cystic fibrosis [1] and is characterized by an innate resistance to many antimicrobials and the ability to develop/acquire resistance [2]. Significant contributors to this intrinsic and/or acquired multidrug resistance are several members of the RND family of multidrug efflux systems [2–4], particularly that encoded by the <em>mexA-mexB-oprM</em> efflux operon. MexAB-OprM exhibits one of the broadest substrate profiles of the RND pumps in *P. aeruginosa*, accommodating a wide range of clinically-relevant antimicrobials [5] and biocides [6] as well as a variety of non-clinical agents (e.g., organic solvents [7,8], dyes [9–11], detergents [10], metabolic inhibitors [12], acylhomoserine lactones (AHLs) associated with quorum-sensing (QS) [13,14]). An important determinant of intrinsic multidrug resistance [9] this efflux system contributes to acquired fluoroquinolone [15–17] and β-lactam [18–23] resistance in clinical isolates of *P. aeruginosa*.

Expression of the <em>mexA-mexB-oprM</em> operon is regulated by the product of a local repressor gene, <em>mexR</em> [24], occurring upstream of the efflux genes and the target of mutation in MexAB-OprM-overexpressing multidrug-resistant <em>nalB</em> lab [24,25] and clinical [26–28] isolates. MexR acts to control <em>mexA-mexB-oprM</em> expression from one of two promoters (PI; most distal from the efflux genes) for this efflux operon [29,30], binding as a dimer [31] to a site that also overlaps the <em>armR</em> promoter [29,32] and, so, effects negative autoregulation. Recent studies show that its ability to bind and so repress <em>mexA-mexB-oprM</em> is governed by the redox status of the protein, MexR serving to regulate <em>mexA-mexB-oprM</em> expression in response to oxidative stress [33,34]. MexR repressor activity is also modulated by the product of the <em>armR</em> anti-MexR repressor gene, which binds to MexR and negatively impacts its ability to bind to the <em>mexA-mexB-oprM</em> PI promoter region [35,36]. <em>armR</em> occurs as part of the 2-gene operon PA3720-<em>armR</em> that is regulated by the product of the divergently-transcribed <em>nalC</em> repressor gene [37], with <em>nalC</em> mutants showing elevated PA3720-<em>armR</em> expression and, so, elevated <em>mexA-mexB-oprM</em> expression and multidrug resistance [37] as a result of ArmR modulation of MexR’s repressor activity [35]. <em>nalC</em> lab and clinical isolates expressing <em>mexA-mexB-oprM</em> and showing a multidrug-resistant phenotype have been reported [24,37,38]. A third repressor involved in regulating <em>mexA-mexB-oprM</em> expression, NalD, is encoded by a gene unlinked to <em>mexA-mexB-oprM</em> and <em>nalC</em>/PA3720-<em>armR</em> and regulates <em>mexA-mexB-oprM</em> from a second, efflux
operon-proximal promoter (P11) [30]. Mutations in nalD have been described in lab and clinical multidrug-resistant mutants [39].

Despite their contribution to antimicrobial resistance, RND family multidrug efflux systems in P. aeruginosa are increasingly appreciated as having other than drug efflux as an intended function, most of these systems being regulated independently of antimicrobials and instead induced in response to environmental stress (e.g., MexCD-OpE), envelope stress [40]; MexEF-OprN, nitrosative stress [41]; MexXY-OprM, oxidative [42] and ribosome [43,44] stress.

Recently, the mexAB-oprM efflux system and its regulatory locus nacL/PA3720-amrR have been shown to be inducible by the uncoupler of oxidative phosphorylation and environmental contaminant, pentachlorophenol (PCP) [45], the implication being that this compound is being ‘sensed’ by NalC and mexAB-oprM recruited as a result of ArmR-mediated MexR modulation. While MexAB-OprM appears able to accommodate this toxin (mutants lacking the pump are more sensitive to PCP) [45], it is unclear whether PCP is the stimulant of oxidative phosphorylation are responsible for nacL/PA3720-amrR and armR/mexR/mexAB-oprM upregulation. The current study was undertaken to address this and to ascertain the mechanism by which PCP ultimately induces mexAB-oprM. We report here that NalC is a PCP-responsive repressor that mediates PCP induction of PA3720-amrR and, possibly, mexAB-oprM but that PCP is also able to induce mexAB-oprM expression independently of PA3720-amrR, possibly as a result of PCP-generated oxidative stress and, so, redox regulation of MexR. During the course of this work another study demonstrating PCP control of NalC repressor activity was published [46] although the mechanism(s) by which this toxin induced mexAB-oprM were not assessed, and the assumption that it was via ArmR are not supported by our study.

Materials and Methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. Bacterial cells were cultured in Luria broth (L broth) and on Luria agar with antibiotics, as necessary, at 37°C. Plasmid pET23a and its derivatives were maintained in E. coli with ampicillin (100 μg/ml). Plasmid pEX18Tc and its derivatives were maintained in E. coli with tetracycline (10 μg/ml). Plasmid pLysS was maintained in E. coli with chloramphenicol (30 μg/ml). ΔPA3720 derivatives of P. aeruginosa strains K767 and K3145 (ΔarmR) were generated by constructing a PA3720 deletion in K767. These were subsequently streaked onto L agar containing suroce (10% [wt/vol]), as before [48], with sucrose-resistant colonies screened for the appropriate deletion using colony PCR [49] with primers 3720 UF and 3720 DR and parameters detailed above (except for an annealing temperature of 55°C). A ΔarmR derivative of P. aeruginosa K767 was constructed using pEX18Tc::ΔarmR plasmid pLC8 as described previously [37] except that transconjugants carrying chromosomal inserts of the deletion vector were selected on L-agar plates containing tetracycline and chloramphenicol as above. ΔarmR mutants were verified by colony PCR [49] using primers 3719UF (5'-CGGTAGCGTCGCGGCGGC-3') and 3720DR (5'-TGGGCTCTTACCTGAAGG-3'). Reaction mixtures were heated to 95°C for 5 min followed by 30 cycles of 30 sec at 94°C, 30 sec at 60°C, 1 min at 72°C and a final 5-min elongation at 72°C.

DNA methods

Standard protocols were used for restriction endonuclease digestion, ligation, transformation, plasmid isolation, agarose gel electrophoresis, and preparation of chemically competent (CaCl₂) E. coli cells as described by Sambrook and Russell [50]. Electrocompetent P. aeruginosa cells were prepared as described by Choi and Shiweierz (51). Chromosomal DNA was extracted from P. aeruginosa using the DNeasy Tissue Kit (Qiagen, Inc., Mississauga, Ontario). Plasmid DNAs were also prepared from E. coli or P. aeruginosa using a GenJet Plasmid Mini Prep kit (Fermentas Canada Inc., Burlington, Ontario, Canada) according to a protocol provided by the manufacturer. The Wizard® SV Gel and PCR Clean-Up System kit (Promega Corp., Nepean, Ontario) was used to purify PCR products and to gel-purify DNA fragments generated by restriction endonuclease digestion. Oligonucleotides were synthesized by Integrated DNA Technologies (IDT, Coralville, Iowa, USA) and nucleotide sequencing was performed by AGCT Corp. (Toronto, Ontario).

Susceptibility testing

The antimicrobial susceptibilities of the various P. aeruginosa strains were assayed in 96-well microtitrator plates using twofold serial dilutions as described [52]. In assessing the impact of PCP exposure on antimicrobial susceptibility, 0.75 mM PCP (one quarter of the MIC) was included in the growth medium used to prepare the bacterial inoculum and to generate the serial dilutions.

Quantitative RT-PCR

Overnight cultures of P. aeruginosa strains were subcultured (1:49) in fresh L-broth and incubated at 37°C with shaking for 2.5 hours, at which time cells were harvested by centrifugation. In some experiments, PCP (0.75 mM final concentration; ¼ MIC) was added to 1.5 hr before harvesting. Total bacterial RNA was isolated from 1 ml of late-log phase culture using the RiboPure™ Bacteria kit (Ambion, Inc., Streeterville, Ontario) or the High Pure RNA Isolation Kit (Roche Diagnostics) using the manufacturers’ protocols, and resuspended in 50 μl elution buffer. Samples were treated with Turbo DNA-Free (Ambion, Inc; 2 U enzyme per 10 μg of RNA for 60 min at 37°C). DNA-free RNA (confirmed using PCR; 1 μg) was used to synthesize cDNA using an iScript cDNA Synthesis kit (Bio-Rad, Mississauga, Ontario) in a reaction mixture formulated as described by the kit manufacturer and incubated for 5 min at 25°C, 30 min at 42°C and 5 min at 85°C. cDNA was then stored until needed at −20°C. Quantification of the cDNA

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Table 1. Bacterial strains and plasmids.

| Strain | Relevant characteristic | Reference or source |
|--------|-------------------------|---------------------|
| E. coli | DH5α ΔlacZΔM15 endA1 recA1 hsdR17 (rK- mK+) supE44 thi-1 gyrA96 relA1 F− Δ(lacZYA-argF)U169 | [59] |
| K113 | BL21 (DE3) (pLysS) | [60] |
| K114 | BL21 (DE3) (pLysS) | [60] |
| SI7-1 | thi-1 pro hsdR reca Tra+ | [61] |
| Sm10(pir) | thi-1 tet rpsL lacI supE recA::RP4-2-Tc::Mu; Km’ pir | [62] |
| NovaBlue | recA’ endA’ lacI’ Novagen | |
| P. aeruginosa | K767 PA01 prototroph | [63] |
| K1454 | Spontaneous nalC mutant of K767 | [24] |
| K2276 | K1454 ΔarmR | [37] |
| K2568 | K767 ΔmexR | [30] |
| K3145 | K767 ΔarmR | This study |
| K3146 | K767 ΔPA3720-ΔarmR | This study |
| K3130 | K767 ΔPA3720-ΔarmR | This study |
| K3151 | K1445 ΔPA3720 | This study |

Plasmids

| Plasmids | pET23a | His-tag expression vector: Ap’ | Novagen |
|----------|--------|--------------------------|--------|
| pKLE1 | pET23αΔmexR | | |
| pLM53 | pET23α-ΔnalC | This study |
| pEX18Tc | Broad-host-range gene replacement vector; sacB; Tc’ | [64] |
| pLC8 | pEX18Tc-ΔarmR | [37] |
| pLM52 | pEX18Tc-ΔPA3720 | This study |
| pMF1 | pEX18Tc-ΔPA3720-ΔarmR | This study |

Ap’, ampicillin resistant; Km’, kanamycin resistant; Tc’, tetracycline resistant.  
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was carried out using a Bio-Rad, CFX96™ Real-Time PCR Detection System (Bio-Rad, Mississauga, Ontario). PCR amplification reactions were performed in 20 μl reaction volumes each containing 10 μl of SsoFast EvaGreen Supermix (Bio-Rad, Mississauga, Ontario), 0.6 μM each of 2 primers per gene being amplified (mexA: mexAQ-F, 5’-GATCCATATGAGATGCTTCTCCCGTCTG-3’ and mexAR-R, 5’-CGTACTGCTGCTTGCTCA-3’; PA3720: 3720Q-F, 5’-GATGCTTTCCTCGTTGTC-CA-3’ and 3720Q-R, 5’-CGTTTGGACCCACACACACAG-3’; rpsL: rpsLQ-F, 5’-GGT-TCCTCTGTAACATGGTG-3’ and rpsQ-R, 5’-TGCTTACGGTCTTTGACACC-3’) and 5 μl of 1:49 diluted cDNA. Following an initial 3-min denaturation at 95°C, the mixture was subjected to 40 cycles of 10 sec at 95°C and 30 sec at 60°C. A melt curve, obtained following an initial 10-sec treatment at 95°C and involving 5-sec incubations of 0.5°C increments beginning at 65°C, was run at the end of 40 cycles to test for the presence of a unique PCR product. The expression levels of mexA and PA3720 were normalized to that of the reference gene rpsL. Biological duplicates and technical triplicates were performed for all samples. To confirm the absence of genomic DNA contamination, ‘no-template’ controls were performed in technical triplicates for all primer sets employed.

Expression and purification of polyhistidine (His)-tagged NalC protein

The nalC gene was amplified by PCR using primers: nalC-Fwde-NdeI (5’-GATCCATATGAGATGCTTCTCCCGTCTG-3’; NdeI site underlined) and nalC-Rev-Sall (5’-GATCCAT- GACCGCCTGCGGGGCGCTG-3’; Sall site underlined) in a 50 μl PCR reaction that contained 1 μg P. aeruginosa strain K767 chromosomal DNA, 0.2 mM each dNTP, 3% (vol/vol) DMSO, 1× Phusion® High Fidelity (HF) buffer and 1 U Phusion® polymerase (Finnzymes, New England Biolabs, Pickering, Ontario). The reaction mixture was heated for 30 sec at 94°C followed by 30 cycles of 30 sec at 94°C, 30 sec at 54°C and 30 sec at 72°C, before finishing with 10 min at 72°C. The nalC-containing PCR product was purified, digested with Sall and NdeI and cloned into NdeI-XhoI-restricted pET23a (Novagen, Madison, Wisconsin, USA) to yield pLMS3 encoding His-tagged NalC (i.e, NalC-His). Following nucleotide sequencing of the cloned gene to confirm the absence of PCR-generated mutations, plasmid pLMS3 was introduced into E. coli BL21 (DE3) carrying the pLysS plasmid and NalC-His production induced with IPTG (1 mM) for 2 hr. Cells were harvested by centrifugation (10 min at 10000 x g) at 4°C and pellets were resuspended in 6 ml buffer A (0.3 M NaCl, 50 mM Na2HPO4) containing 5 mM imidazole and sonicated (three sonic bursts of 30 sec, power 40 with a VibraCell Sonicator [Sonic & Material Inc., Danbury, Connecticut, USA]).
Following centrifugation for 60 min at 16 000×g, the NaCl-His-containing supernatant was mixed with 500 μl of Ni-NTA Agarose resin (Qiagen, Inc.) equilibrated with 10 ml buffer A containing 5 mM imidazole and incubated, with shaking, for 10 min. The resin was subsequently pelleted by centrifugation (3 min at 3000×g) and washed twice with 10 ml buffer A containing 5 mM imidazole and once with 2 ml buffer A containing 5 mM imidazole, the resin again being centrifuged after each wash. Bound protein was eluted stepwise with 500 μl buffer A containing increasing amounts of imidazole (50, 100, 150, 250 mM); at each step the resin was incubated with shaking at room temperature and centrifuged as above. The NaCl-His protein was recovered in the supernatant following elution with buffer A containing 250 mM imidazole. Protein concentration was determined using the BCA Protein Assay Kit (Pierce, Illinois, USA), and purified protein (verified using SDS-polyacrylamide gel electrophoresis [10]) was stored at −20°C in 20% (vol/vol) glycerol.

Electromobility shift assay

The binding of purified NaLC (see above) and MexR (prepared as above but using pET23a:mexR vector pKLE1 in place of pLMS3) to PCR-amplified target DNAs was assessed using the electromobility shift assay (EMSA) as described previously [30]. Briefly, 50 ng target DNA was incubated with purified NaLC or MexR for 20 min at room temperature in a 15 μl reaction mixture containing 1× binding buffer (750 mM KCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, 50 mM Tris-HCl, pH 7.4). Following the addition of EMSA gel-loading solution, mixtures were separated by electrophoresis on a non-denaturing 8% (wt/vol) polyacrylamide gel in 0.5× TBE buffer and stained with 1× SYBR Green DNA gel-loading solution. DNA was then visualized using digital photography with a S6656 SYPRO photographic filter (S6656).

NaLC target DNAs included the entire nalC-PA3720 intergenic region (205 bp), ca. 100 bp fragments corresponding to the putative NalC binding site. Amplification of the 205 bp fragment was achieved using shorter oligonucleotides corresponding to the putative NalC region (205 bp), ca. 100 bp fragments corresponding to the proximal fragment was amplified using primers nalC-3720 Fwd 1 and nalC-3720 Rev 2 (5′-GAGCGGTAT-CTGGTCA-3′) in a reaction mixture containing 1× binding buffer (750 mM KCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, pH 8.0) and gels were stained with 1× SYBR Green EMSA nucleic acid stain. DNA was then visualized using digital photography with a S6656 SYPRO photographic filter (S6656).

NaLC target DNAs included the entire nalC-PA3720 intergenic region (205 bp), ca. 100 bp fragments corresponding to the putative NaLC binding site. Amplification of the 205 bp fragment was achieved using primers nalC-3720 Fwd 1 (5′-GGGGGCGTATCGCGGG-3′) and nalC-3720 Rev 2 (5′-GAGCGGTATCGGGGCTCG-3′) in a reaction mixture containing 1× binding buffer (750 mM KCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, pH 8.0) and gels were stained with 1× SYBR Green EMSA nucleic acid stain. DNA was then visualized using digital photography with a S6656 SYPRO photographic filter (S6656).

NalC target DNAs included the entire nalC-PA3720 intergenic region (205 bp), ca. 100 bp fragments corresponding to the putative NaLC binding site. Amplification of the 205 bp fragment was achieved using shorter oligonucleotides corresponding to the putative NalC region (205 bp), ca. 100 bp fragments corresponding to the proximal fragment was amplified using primers nalC-3720 Fwd 1 and nalC-3720 Rev 2 (5′-GAGCGGTAT-CTGGTCA-3′) in a reaction mixture containing 1× binding buffer (750 mM KCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, pH 8.0) and gels were stained with 1× SYBR Green EMSA nucleic acid stain. DNA was then visualized using digital photography with a S6656 SYPRO photographic filter (S6656).

Mapping the PA3720-armR transcription start site

To identify the transcription start site for the PA3720-armR operon, the 5′ rapid amplification of cDNA ends (RACE) protocol [53] and a 5′/3′ RACE Kit, 2nd Generation (Roche Diagnostics) were used as described previously with modifications [29]. Total RNA was prepared from PA3720-armR-expressing P. aeruginosa nalC mutant K1454 using the Roche High Pure RNA Isolation Kit according to the manufacturer’s instructions, and contaminating DNA was removed using the Turbo DNAse Kit (Ambion). cDNA was synthesized from total RNA (1 μg) with a RACE kit-provided reverse transcriptase and a PA3720-specific primer, 3720 RACE Sp1 (5′-GGCGGATAGACAGGGCGGAATG-3′; anneals 131 bp downstream of the PA3720 ATG start site), using a protocol provided by the manufacturer. A homopolymeric A-tail was added to the 3′ end of the total cDNA using terminal transferase and dATP, and the dA-tailed cDNA subsequently PCR amplified using a kit-provided oligo (dT)-anchored primer and a second PA3720-specific primer, 3720 RACE Sp2 (5′-CGGCCAGTCGCCGGGGCATCT-3′; anneals 57 bp downstream of the PA3720 ATG start site). The resulting PCR product was purified and cloned into the pETBlue-1 AccpTor™ Vector (EMD Chemicals, Gibbstown, New Jersey, USA) as per instructions provided by the manufacturer. Following transformation of NovaBlue Giga Slices Competent Cells (Novagen), plasmid-carrying E. coli cells were selected on L-agar kanamycin (50 μg/ml) and tetracycline (15 μg/ml). Plasmids carrying inserts of the amplified 5′ end of PA3720 mRNA were identified and the inserts sequenced and aligned with the PAO1 genome sequence to determine the PA3720-armR transcription start site.

Results

NaLC mediates PCP inducibility of PA3720-armR

Microarray data has shown that PCP induces expression of the NaLC-regulated PA3720-armR operon [45], results that have been confirmed here using quantitative RT-PCR (qRT-PCR) (Fig. 1).
PCP induction of PA3720-armR was lost in a nalC mutant (K1454) – although the mutant showed the expected increase in PA3720-armR expression compared with its wild type parent K767, PCP treatment did not enhance expression of this operon in K1454 (Fig. 1). This indicated that NalC was mediating the PCP induction of PA3720-armR, either via a direct response to PCP or via recognition of PCP-generated cellular products (PCP is an energy inhibitor). To assess this NalC was purified and the impact of PCP on its binding to the ca. 200 bp nalC-PA3720 intergenic region was examined using EMSA. As seen in Fig. 2, NalC bound the nalC-PA3720 intergenic region (Fig. 2A) with binding compromised by the addition of PCP (some loss of binding was detected at 10 μM PCP with almost complete loss of binding seen at 750 μM) (Fig. 2B). Thus, NalC responds directly to PCP in mediating its induction of PA3720-armR.

Mapping the NalC binding site

In an attempt to localize the NalC binding site NalC binding to ca. 100 bp PA3720-proximal and ca. 100 bp PA3720 distal fragments was assessed, with binding confirmed only for the PA3720-proximal fragment (Fig. 3A, cf Fig. 3B). This fragment (with endpoints II) included putative promoters for nalC and PA3720-armR as well as an inverted repeat sequence that might serve as the NalC-binding site (Fig. 4). To assess the latter, a 45 bp fragment encompassing the inverted repeat (IR; endpoints III in Fig. 4) was used in an EMSA and NalC binding was observed (Fig. 3C). An identically-sized fragment lacking the IR and encompassing a region more PA3720-proximal (endpoints IV in Fig. 4) failed to bind NalC (Fig. 3D) and little if any binding was observed to a fragment encompassing the repeat region (endpoints III) in which the repeat sequence was mutated, and then only at the highest concentration of NalC (Fig. 3E). This confirmed the repeat region as the NalC-binding site. Interestingly, a 21-bp fragment carrying only the IR failed to bind NalC (data not shown) indicating that the proper disposition of the repeat region for NalC binding required flanking sequence. The IR-containing region to which NalC bound contains the putative promoter regions for nalC and PA3720-armR [predicted using neural network promoter prediction (http://www.fruitfly.org/seq_tools/promoter.html)] (Fig. 4) as would be expected. Using 5′-RACE the PA3720-armR transcription start site was mapped to an adjacent CT pair of bases (exact base could not be determined unequivocally owing to limitations of the RACE), which occurred downstream of the putative PA3720-armR-10 region and immediately downstream of the predicted (using neural network) start site (Fig. 4). This is consistent with NalC regulating expression of the PA3720-armR operon.

Figure 1. PCP induction of PA3720-armR. qRT-PCR results showing impact of PCP and/or a nalC mutation on PA3720 expression (as a measure of PA3720-armR expression). Expression of PA3720 is reported relative to the rpsL internal control for wild type P. aeruginosa K767 and its nalC derivative, K1454 exposed or not to PCP (0.75 mM; 1.5 hr). Results shown are the mean +/- standard error of one cDNA sample for each, processed in triplicate, and are representative of 2 independent experiments. doi:10.1371/journal.pone.0032684.g001

Figure 2. PCP modulation of NalC repressor binding to the PA3720-armR upstream region. A) Mobility shift assay in which 50 ng of a 209-bp DNA fragment encompassing the nalC-PA3720 intergenic region was incubated without (lane 1) or with 200 (lane 2), 300 (lane 3), 400 (lane 4), 500 (lane 5), 800 (lane 6), 1000 (lane 7), 2000 (lane 8) or 3000 (lane 9) ng of purified NalC-His. B) Mobility shift assay in which purified NalC-His (800 ng) was incubated with 50 ng of a 209-bp DNA fragment encompassing the nalC-PA3720 intergenic region and increasing amounts of PCP as indicated. Lane 1, DNA only control. doi:10.1371/journal.pone.0032684.g002
PCP induction of *mexAB-oprM* is NalC and MexR-dependent but PA3720-ArmR-independent

In addition to its induction of PA320-armR, PCP was previously shown to induce the MexR-regulated *mexAB-oprM* multidrug efflux operon [45], results that have been confirmed here (modest ca. 3-fold induction; Fig. 5). Mutations in *nalC* and *mexR* yielded elevated expression of *mexAB-oprM* (*nalC*, 3-fold; *mexR*, 5-fold; Fig. 5), as seen previously [45], and this was no longer responsive to PCP (Fig. 5). Thus, PCP operates through these regulators in promoting *mexAB-oprM* expression. Given that NalC controls expression of the ArmR anti-repressor that modulates MexR repression of *mexAB-oprM*, the obvious interpretation of these results is that PCP as a NalC effector affords PA3720-armR expression, with ArmR modulating MexR activity to effect *mexAB-oprM* expression. To assess this, PCP induction of the efflux operon was examined in *armR* mutant strain K3145. As seen in Fig. 6, *mexAB-oprM* was still induced in response to PCP treatment, albeit to a lesser extent, indicating that PCP induction of this efflux operon can occur independently of its promotion of ArmR expression. PA3720 encodes a protein of unknown function that, like *armR*, is NalC regulated [37] and PCP inducible [45] (Fig. 1) and which might, therefore, play a role in PCP induction of *mexAB-oprM*. Thus, PCP induction of *mexAB-oprM* was assessed in a mutant, K3146, lacking PA3720. Again, however, PCP induction of *mexAB-oprM* was retained in the mutant (Fig. 6). Similarly, PCP induction of *mexAB-oprM* was seen in a mutant strain, K3130, ruling out both genes independently mediating PCP induction of the efflux operon. Similarly, while loss of ArmR compromised the *mexAB-oprM* overexpression seen in a *nalC* mutant (Fig. 5) in agreement with previous results [37], PCP induction of *mexAB-oprM* was retained in the *nalC ΔarmR* mutant K2276 (Fig. 5) again indicating that PCP induction of the efflux operon can occur independently of ArmR. Interestingly, however, and in contrast to what was observed when ArmR was eliminated in wild type strain K767, where there was little or no impact on basal levels of *mexAB-oprM* expression (in the absence of PCP) (Fig. 6), loss of ArmR in a *nalC* background reduced expression of *mexAB-oprM* to below the basal levels seen in K767 (Fig. 5).

PCP is not a MexR effector

The observation that PCP induction of *mexAB-oprM* is dependent on MexR but independent of PA3720-armR suggested that PCP may be capable of directly modulating MexR activity to effect efflux gene expression. To assess this, the impact of PCP on MexR binding to a DNA fragment containing the *mexAB-oprM* promoter region was examined using EMSA. As seen in Fig. 7 MexR binding was observed, revealing a complex pattern of DNA

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**Figure 3. Identifying the NalC binding site upstream of PA3720-armR.** A–B) Mobility shift assay in which 50 ng of A) the 107-bp PA3720-distal DNA fragment (sequence delineated by endpoints I in Fig. 4A) or B) the 102-bp PA3720-proximal DNA fragment (sequence delineated by endpoints II in Fig. 4A) were incubated without NalC (lane 1) or with 300 (lane 2), 500 (lane 3), 800 (lane 4) and 1000 (lane 5) ng of purified NalC-His. C–E) Mobility shift assay in which 0 (lane 1), 300 (lane 2), 500 (lane 3), 800 (lane 4) and 1000 (lanes 5) and 2000 (lanes 6) ng of purified NalC-His was incubated with 40 ng of C) the annealed oligonucleotide NalC-1 and its reverse complement (corresponds to sequence delineated by endpoints III in Fig. 4A), D) the annealed oligonucleotide NalC-2 and its reverse complement (corresponds to sequence delineated by endpoints IV in Fig. 4A), and E) annealed oligonucleotide NalC-3 and its reverse complement, in which the AGAAGCTGT sequence of NalC-1 corresponding to the first half of the inverted repeat highlighted in Fig. 4(A) (shaded sequence overlapping the putative −10 regions for *nalC* and PA3720-armR) is changed to TCTTGACA.

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binding suggestive of variable occupancy of multiple MexR binding sites and/or cooperative binding of MexR to its target DNA. In any case, PCP had no impact on MexR binding at concentrations up to 100 μM, and while there were qualitative differences in the pattern of shifted DNA at the highest concentrations (500 and 750 μM) virtually all of the \textit{mexAB-oprM} promoter-containing DNA remained bound to MexR at these concentrations of PCP. Thus, in contrast to NalC, PCP is not an effector for MexR.

**Discussion**

ArmR is an anti-repressor that modulates MexR repressor activity in \textit{nalC} mutants that hyperexpress the \textit{PA3720-armR} operon, accounting for the elevated expression of the \textit{mexAB-oprM} multidrug efflux operon in such mutants [37]. In light of the impact of PCP on NalC repression of the \textit{PA3720-armR} operon and the NalC dependence of PCP induction of \textit{mexAB-oprM} it seemed reasonable to suppose that PCP induction of the efflux operon followed from elevated \textit{armR} expression and subsequent ArmR-dependent modulation of MexR repression of \textit{mexAB-oprM}. Indeed, this was the conclusion of a recent study, published while this work was in progress, demonstrating that PCP induction of \textit{PA3720-armR} expression results from PCP directly modulating NalC's repression of this operon [46]. Still, the requirement for ArmR in PCP-inducible \textit{mexAB-oprM} expression was not assessed in that study and results presented here clearly demonstrate that PCP induction of the efflux operon occurs in the absence of the anti-repressor. While there seemed to be some reduction in the

**Figure 4. Mapping the \textit{PA3720-armR} transcriptional start site.** The \textit{nalC/PA3720-armR} intergenic region highlighting the RACE-determined transcription start site for \textit{PA3720-armR} (bolded and italicized), the predicted transcriptional initiation site (marked with an asterisk; assessed using neural network promoter prediction software provided by M. G. Reese at http://www.fruitfly.org/seq_tools/promoter.html), the \textit{nalC} and \textit{PA3720} start codons (arrows), and putative −10/−35 sites for \textit{nalC} (overlined) and \textit{PA3720-armR} (underlined) promoters. The end-points of PCR-generated \textit{PA3720}-distal (I) and -proximal (II) fragments and oligonucleotides (III and IV) used in EMSAs with purified NalC (see Fig. 2B) are identified by arrowheads above the sequence. The shaded sequence corresponds to an inverted repeat and possible NalC-binding site. doi:10.1371/journal.pone.0032684.g004

**Figure 5. NalC and MexR dependence of PCP induction of \textit{mexAB-oprM}.** Expression of \textit{mexA} (as a measure of \textit{mexAB-oprM}) was assessed in \textit{P. aeruginosa} strains K767 (wild type; WT), K1454 (\textit{nalC}), K2276 (\textit{nalC ΔarmR}) and K2568 (\textit{ΔmexR}) exposed or not to PCP (0.75 mM; 1.5 hr) using qRT-PCR. Expression was normalized to \textit{rpsL} controls and is reported relative (fold change) to untreated \textit{P. aeruginosa} K767. Results shown are the mean +/− standard error of one cDNA sample for each, processed in triplicate, and are representative of 2 independent experiments. doi:10.1371/journal.pone.0032684.g005

**Figure 6. ArmR-/PA3720-independence of PCP induction of \textit{mexAB-oprM}.** Expression of \textit{mexA} (as a measure of \textit{mexAB-oprM}) was assessed in \textit{P. aeruginosa} strains K767 (wild type; WT), K3145 (\textit{ΔarmR}), K3146 (\textit{ΔPA3720}) and K3130 (\textit{ΔPA3720-armR}) exposed or not to PCP (0.75 mM; 1.5 hr) using qRT-PCR. Expression was normalized to \textit{rpsL} controls and is reported relative (fold change) to untreated \textit{P. aeruginosa} K767. Results shown are the mean +/− standard error of one cDNA sample for each, processed in triplicate, and are representative of 2 independent experiments. doi:10.1371/journal.pone.0032684.g006
absolute levels of PCP-inducible mexAB-oprM expression in the absence of armR, all mutants lacking armR (i.e., K767ΔarmR, \(nalC\)ΔarmR, K767ΔPA3720ΔarmR) showed fold increases in expression of the efflux genes in the presence of PCP (2- to 3-fold increase) that were similar to wild type strain K767 (ca. 3-fold).

While it is unlikely that elevated armR expression in PCP-treated \(P. \) aeruginosa would not contribute to mexAB-oprM expression as a result of ArmR modulation of MexR repressor activity, these results do indicate that there are other mechanisms by which PCP can promote mexAB-oprM expression, probably in parallel with the ArmR-dependent mechanism. Such a mechanism appears not to involve PA3720, whose function still remains to be elucidated, but does require MexR, which may respond to PCP. Given the observation that PCP does not impact MexR binding to the mexAB-oprM promoter containing DNA in vitro, one possibility is that \(P. \) aeruginosa exposure to PCP promotes in vivo production of MexR effector molecule(s) that facilitate mexAB-oprM derepression. In this vein, it has been shown that MexR binding to the mexAB-oprM promoter region and its control of efflux gene expression is impacted by oxidative stress, MexR being a redox responsive regulator whose ability to bind and repress mexAB-oprM is compromised when the protein is oxidized [33,34]. Thus, exposure to hydroperoxide oxidizes MexR and alleviates promoter binding in vitro and induces mexAB-oprM expression in vivo [34]. One possibility, then, is that PCP exposure generates some form of oxidative stress in \(P. \) aeruginosa. Intriguingly, PCP has been shown to dramatically increase \(O_2\) flux in \(P. \) aeruginosa, generating an oxidative stress that could impact MexR activity [34]. Puzzlingly, PCP induction of mexAB-oprM is absent in \(nalC\) mutant K1454, despite the fact that efflux gene expression is less than what is seen in a mexR null strain (K2568) — while much of MexR is inactive as a repressor in K1454, being bound by ArmR, clearly some MexR repression of mexAB-oprM is still occurring and would, presumably, be susceptible to modulation by PCP (or PCP-generated effectors).

Still, there is some unexplained complexity to mexAB-oprM expression in a \(nalC\) strain given the greater impact of armR loss on efflux gene expression in a \(nalC\) background as compared to an otherwise wild type background where in absolute terms mexAB-oprM expression is less (2-fold; Fig. 5) in a \(nalC\) ΔarmR mutant (K2276) than a ΔarmR mutant (K3145). Whether this explains the anomaly, there is likely to be differences in the expression of at least some genes in K2276 vs. K3145, with PA3720 presumably up-regulated in the former but not the latter and any other genes impacted by loss of \(nalC\) and/or upregulation of PA3720 differentially expressed in the two strains. How these might influence mexAB-oprM expression is uncertain.

The modulation of NaCl repressor activity by PCP (and other chlorinated phenols [46]) occurs at high levels (high \(\mu M\) to low mM) for an effector molecule suggesting that it only mimics an intended NaCl effector and ultimate inducer of mexAB-oprM, likely a phenolic of some kind. Plants are common sources of phenolic compounds, many of which have antimicrobial activity [55–58], and these may be the intended inducers/substrates for MexAB-OprM. While attempts were made to assess the ability of plant extracts to ‘interact’ with NaCl and to induce mexAB-oprM expression technical issues (extract insolubility in aqueous solutions) compromised these efforts.

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Author Contributions

Conceived and designed the experiments: LMS MF KP. Performed the experiments: LMS MF. Analyzed the data: LMS MF KP. Contributed reagents/materials/analysis tools: KP. Wrote the paper: LMS KP.

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