

Pseudomonas aeruginosa High-Level Resistance to Polymyxins and Other Antimicrobial Peptides Requires *cprA*, a Gene That Is Disrupted in the PAO1 Strain

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The *arn* locus, found in many Gram-negative bacterial pathogens, mediates resistance to polymyxins and other cationic antimicrobial peptides through 4-amino-L-arabinose modification of the lipid A moiety of lipopolysaccharide. In *Pseudomonas aeruginosa*, several two-component regulatory systems (TCSs) control the *arn* locus, which is necessary but not sufficient for these resistance phenotypes. A previous transposon mutagenesis screen to identify additional polymyxin resistance genes that these systems regulate implicated an open reading frame designated PA1559 in the genome of the *P. aeruginosa* PAO1 strain. Resequencing of this chromosomal region and bioinformatics analysis for a variety of *P. aeruginosa* strains revealed that in the sequenced PAO1 strain, a guanine deletion at the end of PA1559 results in a frameshift and truncation of a full-length open reading frame that also encompasses PA1560 in non-PAO1 strains, such as *P. aeruginosa* PAK. Deletion analysis in the PAK strain showed that this full-length open reading frame, designated *cprA*, is necessary for polymyxin resistance conferred by activating mutations in the PhoPQ, PmrAB, and CprRS TCSs. The *cprA* gene was also required for PmrAB-mediated resistance to other cationic antimicrobial peptides in the PAK strain. Repair of the mutated *cprA* allele in the PAO1 strain restored polymyxin resistance conferred by an activating TCS mutation. The deletion of *cprA* did not affect the *arn*-mediated lipid A modification, indicating that the CprA protein is necessary for a different aspect of polymyxin resistance. This protein has a domain structure with a strong similarity to the extended short-chain dehydrogenase/reductase family that comprises isomerases, lyases, and oxidoreductases. These results suggest a new avenue through which to pursue targeted inhibition of polymyxin resistance.

Polymyxins (Pm) are acylated cationic peptides active against *Pseudomonas aeruginosa* and other Gram-negative pathogens. As pharmaceuticals, Pm B sulfate (PMB) and colistimethate, the prodrug form of colistin (also known as Pm E), are increasingly used to treat multidrug-resistant strains of *P. aeruginosa* that cause serious infections in critically ill patients and in those with cystic fibrosis (CF) (1, 2). Multidrug-resistant strains emerge when first-line agents, such as antipseudomonal β -lactams, aminoglycosides, and fluoroquinolones, are used repeatedly, imposing major selective pressure (3–5). Unfortunately, the prevalence of clinical isolates of *P. aeruginosa* and other Gram-negative pathogens resistant to PMB and colistin is increasing (6–13).

Pm binds to lipopolysaccharide (LPS), the major constituent of the Gram-negative outer membrane, promoting its own uptake and diffusion through the periplasm to the inner membrane, where it disrupts cellular respiration and leads to cell lysis (14). The resistance of Gram-negative pathogens to Pm and other cationic antimicrobial peptides (CAPs) is associated with covalent modification of LPS, namely, the addition of 4-amino-L-arabinose (L-Ara4N) to phosphate groups within its lipid A and core oligosaccharide components (13, 15–17). This amino-sugar modification likely hinders charge interactions between phosphate groups within LPS and amino groups within CAPs. Genes of the *arnBCADTEFpmrE* operon encode enzymes responsible for synthesis and transfer of L-Ara4N to LPS (18, 19).

Several two-component systems (TCSs) of *P. aeruginosa*, including PhoPQ, PmrAB, ParRS, CprRS, and ColRS, convergently regulate Pm resistance. These systems generally induce *arn* operon transcription in response to CAP exposure or divalent cation depletion (18–21), or as a consequence of mutation (12, 17, 22–29). However, disruption of the *cprRS* locus (deletion or transposon

insertion) results in a partial loss of PhoPQ-mediated Pm resistance without a loss of L-Ara4N from lipid A (29). Moreover, some clinical strains of *P. aeruginosa* lose Pm resistance after drug-free passage without the concomitant loss of L-Ara4N from lipid A (12). These observations indicate that in *P. aeruginosa*, Pm resistance requires not only L-Ara4N modification of lipid A, but also the expression of one or more CprRS-regulated gene products.

Prior studies in the *P. aeruginosa* PAO1 reference strain have shown that the annotated PA1559 and PA1560 open reading frames (ORFs) (30) are positively regulated through the CprRS (31), ParRS (27), and PmrAB TCSs (32) and are induced by CAPs, such as indolicidin (27) and LL-37 (33). We previously observed, in a Pm-resistant (Pm^r) *phoQ* mutant of the *P. aeruginosa* PAK reference strain, that transposon insertion within the PA1559 ORF is associated with a loss of Pm resistance (29). We hypothesized that PhoPQ-, PmrAB-, and CprRS-mediated Pm resistance

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TABLE 1 Strains of *P. aeruginosa* used in this work

Strain	Strain description (source/resistance profile)	Reference
1026	PAK laboratory-adapted WT (S. Lory)	65
1027	PAO1 laboratory-adapted WT (S. Lory)	46
1555	PAO1 laboratory-adapted WT (M. Franklin)	66
2114	PAO1 laboratory-adapted WT (C. Manoil)	67
2178	PAO1 laboratory-adapted WT (M. Vasil)	68
1016	Clinical isolate (Aarhus CF patient 2, 1996)	12
1020	Clinical isolate (Copenhagen CF patient 6, 1998)	12
1603	Clinical isolate (Copenhagen CF patient 14, 2003)	12
1611	Clinical isolate (Copenhagen CF patient 15, 2001)	12
4240	PAK $\Delta cprRS$ pJN105D::cprR ⁺ S21 (GEN ^r)	29
4537	PAK $\Delta phoPQ \Delta cprRS$ pJN105D::cprR ⁺ S21 (GEN ^r)	This study
4541	PAK $\Delta phoPQ \Delta cprRS$ pJN105 (GEN ^r)	This study
4472	PAK $\Delta pmrAB \Delta cprRS$ pJN105D::cprR ⁺ S21 (GEN ^r)	This study
4470	PAK $\Delta pmrAB \Delta cprRS$ pJN105 (GEN ^r)	This study
4543	PAK $\Delta phoPQ \Delta pmrAB \Delta cprRS$ pJN105D::cprR ⁺ S21 (GEN ^r)	This study
4547	PAK $\Delta phoPQ \Delta pmrAB \Delta cprRS$ pJN105 (GEN ^r)	This study
4232	PAK $\Delta pmrAB \Delta cprRS$ pJN105D::pmrAB12 (GEN ^r)	This study
4234	PAK $\Delta pmrAB \Delta cprRS$ pJN105 (GEN ^r)	This study
4343	PAK $\Delta cprA2$	This study
4458	PAK $\Delta cprA2$ pJN105 (GEN ^r)	This study
4460	PAK $\Delta cprA2$ pJN105D::cprA2 (GEN ^r)	This study
2326	PAK $\Delta phoQ$	24
4374	PAK $\Delta cprA2 \Delta phoQ$	This study
4614	PAK $\Delta cprA2 \Delta phoQ$ pJN105D::cprA2 (GEN ^r)	This study
4497	PAK $\Delta cprA2 \Delta phoQ$ pJN105 (GEN ^r)	This study
3420	PAK $\Delta pmrAB$ pJN105 (GEN ^r)	This study
2735	PAK $\Delta pmrAB$ pJN105D::pmrAB12 (GEN ^r)	12
4519	PAK $\Delta cprA2 \Delta pmrAB$ pJN105D::pmrAB12 (GEN ^r)	This study
4521	PAK $\Delta cprA2 \Delta pmrAB$ pJN105 (GEN ^r)	This study
4425	PAK $\Delta cprA2 \Delta cprRS$ pJN105D::cprR ⁺ S21 (GEN ^r)	This study
4447	PAK $\Delta cprA2 \Delta cprRS$ pJN105 (GEN ^r)	This study
4200	PAK $\Delta cprRS \Delta phoQ$ pJN105D::cprR ⁺ S21 (GEN ^r)	12
4608	PAK $\Delta cprA2 \Delta cprRS \Delta phoQ$ pJN105D::cprR ⁺ S21 (GEN ^r)	This study
4612	PAK $\Delta cprA2 \Delta cprRS \Delta phoQ$ pJN105 (GEN ^r)	This study
4581	PAK $\Delta arnC \Delta cprRS$ pJN105D::cprR ⁺ S21 (GEN ^r)	This study
4586	PAK $\Delta arnC \Delta cprRS$ pJN105 (GEN ^r)	This study
4734	PAO1 $\Delta pmrAB$ pJN105D::pmrAB12 (GEN ^r)	This study
4763	PAO1 $\Delta pmrAB \Delta cprA1::cprA^+$ pJN105D::pmrAB12 (GEN ^r)	This study
4765	PAO1 $\Delta pmrAB \Delta cprA1::cprA^+$ pJN105 (GEN ^r)	This study

in *P. aeruginosa* is dependent on the PA1559/PA1560 ORFs, in addition to the known dependence of this resistance phenotype on the *arn* locus. The objectives of this study were to assess the degree of interdependence among the PhoPQ, PmrAB, and CprRS systems in mediating Pm resistance and to define the role of the PA1559/PA1560 ORFs in TCS-induced resistance to Pm and other CAPs.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The laboratory-adapted wild-type (WT) strains and clinical isolates of *P. aeruginosa* used in this study are listed in Table 1. The institutional review board of Massachusetts General Hospital reviewed and approved the use of CF clinical isolates of *P. aeruginosa* for this study. *Escherichia coli* strain DH5 α was used as host for plasmid manipulation, and *E. coli* strain SM10 was used for conjugative transfer of plasmids into *P. aeruginosa*. The strains were stored at -80°C in lysogeny broth (LB) supplemented with 16% glycerol and grown at 30°C or 37°C on lysogeny agar (LA) or in LB with aeration.

Antibiotics were used at the following concentrations for plasmid selection and maintenance: 50 mg/liter kanamycin or 10 mg/liter gentamicin (GEN) for *E. coli* and 50 to 100 mg/liter GEN for *P. aeruginosa*. For conjugative transfer of plasmids, *Pseudomonas* isolation agar (PIA) containing appropriate antibiotics and low-salt LA (10 g/liter tryptone, 5 g/liter yeast extract, and 15 g/liter agar) containing 5% sucrose were used. For induction, 0.1% L-arabinose (L-Ara) was included in LA or LB.

Bacterial DNA sequencing and bioinformatics. For Sanger sequencing of the *cprA* gene, the primers used are listed in Table S1 in the supplemental material. For whole-genome sequencing, a random-fragment library was constructed from each genome using a custom protocol, as described previously (34). Briefly, genomic DNA was purified using the MCD 85201 kit (Epicentre Biotechnologies, Madison, WI), and 2 μg was sheared using a Covaris S2 sonicator (5% intensity; 10% duty cycle; 200 cycles/burst, processing time, 4 min). The ends were repaired using T4 DNA polymerase, T4 polynucleotide kinase, DNA polymerase large Klenow fragment, and deoxynucleoside triphosphate (dNTP) mix (New England Biolabs, Beverly, MA). The reaction was purified on MinElute columns (Qiagen, Valencia, CA). The repaired fragments were subjected to A-tailing using Klenow fragment 3'-5' exonuclease and dATP (New England Biolabs), followed by treatment with RNase (Roche Diagnostics, Indianapolis, IN) and purification using SPRI beads (Beckman Coulter, Indianapolis, IN). Custom "Y" adaptors (Illumina, San Diego, CA) were ligated to A-tailed fragments using T4 DNA ligase, indexed using a universal primer combined with one of 24 index primers (Illumina) and the Phusion high-fidelity PCR master mix (New England Biolabs), followed by purification using SPRI beads. The libraries were analyzed and quantified using the HiSeq 2100 bioanalyzer (Illumina) and high-sensitivity DNA kit (Agilent Technologies, Santa Clara, CA). After pooling and sequencing of the libraries, alignment of demultiplexed short reads was performed using the Burrows-Wheeler Aligner (BWA) (35), SAMtools (36), and Picard (<http://broadinstitute.github.io/picard>). Calling of single-nucleotide polymorphisms and short insertions/deletions was performed using the GATK UnifiedGenotyper (37) and SnpEff (38).

The nucleotide sequences of PAO1 strains from various laboratories and the single PAK strain were aligned with *cprA* reference sequences from the PAO1 genome (PA1559 and PA1560 ORFs, in the genomic region between positions 1697188 and 1698344) and from the PA14 genome (PA14_44311 ORF) via Clustal W2 (39). The *cprA* reference nucleotide sequences were obtained from the *Pseudomonas* Genome database. Bio-PHP (www.biophp.org) was used to translate the nucleotide sequences to corresponding protein sequences in six different reading frames. Protein BLAST and Nucleotide BLAST were performed using the NCBI website (40).

Bacterial strain construction. Expression plasmids and chromosomal deletions were constructed as described previously (24, 29) using the Gateway cloning system (Invitrogen, Carlsbad, CA), with the specific oligonucleotide primers listed in Table S1 in the supplemental material. A unique HindIII restriction endonuclease site was used to mark and confirm the deletions. For strains in which the *phoQ* gene was deleted, this was performed as the final step of strain construction to minimize the occurrence of secondary suppressor mutations (24).

To perform allelic replacement of *cprA* on the bacterial chromosome, the native *cprA* allele was first deleted as described above. The allelic replacement construct was produced through PCR amplification of the replacement *cprA* allele and flanking chromosomal sequences (~ 1 kb on each side) from genomic DNA using specific oligonucleotide primers (see Table S1 in the supplemental material). This DNA fragment was inserted into the Gateway entry vector pDONR221 (Invitrogen) via a BP recombination reaction and then transferred into the Gateway destination vector pEXGmGW (41) via an LR recombination reaction. Allelic replacement plasmids introduced into *P. aeruginosa* were selected for chromosomal insertion on LA containing GEN and then counterselected for loss of the plasmid backbone on LA containing 5% sucrose (42). To confirm allelic

replacement, the *cprA* coding sequence was PCR amplified from chromosomal DNA and subjected to Sanger sequencing.

For the PAO1 *cprA1* allele, the QuikChange site-directed mutagenesis method (Agilent Technologies) was used, according to the manufacturer's instructions, to reinsert a deleted guanine nucleotide at codon 224, thereby restoring the WT translational reading frame. The PCR amplification step was performed using specific oligonucleotide primers (see Table S1 in the supplemental material), with plasmid pDONR221::*cprA1* as the template. To eliminate the parental DNA template, the amplification product was incubated with DpnI (New England BioLabs) at 37°C for 1.5 h. To confirm the site-directed mutagenesis product, Sanger sequencing was performed using specific oligonucleotide primers (see Table S1).

Antibiotic susceptibility testing. For all susceptibility assays, LA and LB were supplemented with 1 mM MgCl₂ to avoid inducing resistance through Mg²⁺ depletion. When preparing inocula of *P. aeruginosa* strains containing expression plasmids, the media were also supplemented with 0.1% L-Ara and 50 mg/liter GEN.

Pm agar dilution testing and the PMB plate assay were performed and interpreted as described previously (24, 29). For the PMB plate assay of strains containing expression plasmids, prolonged (24-h) strong induction with 0.1% L-Ara was performed as described previously (29).

For the quantitative bactericidal assay (17), strains containing expression plasmids were streaked onto LA containing 0.1% L-Ara and GEN and then incubated at 30°C for 20 h. Colonies of each strain were suspended in LB containing 0.1% L-Ara and GEN to achieve an optical density at 600 nm (OD₆₀₀) of 0.5, back-diluted 1:10 (strains with empty vectors) or 1:5 (strains with episomal TCS mutant alleles), and incubated at 30°C for 3 h to achieve an OD₆₀₀ of 0.8 to 1.2. Each inoculum was then diluted in Mueller-Hinton broth to a final density of 1 × 10⁴ to 2 × 10⁴ CFU per ml and incubated at 37°C for 30 min in 2-fold serial dilutions of PMB (0.5 to 500 mg/liter) and a Pm-free control, or for 120 min in 2-fold serial dilutions of C18G (0.5 to 500 mg/liter) or protegrin-1 (0.0625 to 64 mg/liter) and a CAP-free control. To enumerate the surviving CFU, the cells were then diluted 1:10, spread on LA, and incubated at 37°C for 16 to 20 h.

Lipid A isolation and analysis. LPS was isolated from cells grown in LB, supplemented with 1 mM MgCl₂, 0.1% L-Ara, and GEN when required (43). Lipid A was prepared as described previously (29) from hydrolyzed LPS (44). The lipid A structure was analyzed using matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF MS) in negative-ion linear mode (45). All MALDI-TOF MS analyses were performed on an autoflex speed LRF mass spectrometer (Bruker Daltonics, Billerica, MA).

Reverse transcription-quantitative PCR. Cultures of WT and mutant strains were grown in LA and LB media supplemented with 1 mM MgCl₂ and 0.1% L-Ara. The media for the expression strains also included 50 mg/liter GEN; such strains were induced for 24 h, as described previously (29). The strains were grown in 25 ml of medium with aeration at 30°C to an OD₆₀₀ of 0.2 to 0.5. Total RNA was extracted using TRI reagent (Life Technologies, Grand Island, NY), bromo-chloropropane (Fisher), and the RNeasy kit (Qiagen), with DNase I (Qiagen) treatment performed as part of the RNeasy column purification step. A reverse transcription kit (Qiagen) was used to synthesize cDNA from 100 ng of total RNA; to analyze 50 ng of cDNA, Phusion master mix (New England BioLabs), 5× SYBR green (Invitrogen), ROX passive reference dye (U.S. Biochemical Corporation, Cleveland, OH), and 400 nM oligonucleotide primers (see Table S1 in the supplemental material) designed with IDT PrimerQuest were used in an Applied Biosystems 7500 Fast quantitative PCR (qPCR) system (Life Technologies), as recommended by the manufacturer. cDNA derived from the PA4268 ORF, which encodes the 30S ribosomal protein S12, was used as an internal control to normalize the results (32), and the comparative cycle threshold was used for quantification. For each qPCR, technical duplicates were performed; for each strain analyzed, biological triplicates were performed. Two-sided *P* values were calculated using the one-sample *t* test.

Nucleotide sequence accession numbers. New genomic DNA sequences for the *cprA* region of *P. aeruginosa* strain 1026 (PAK) and four clinical strains (1020, 1603, 1016, and 1611) have been deposited in GenBank under accession numbers KR131719 to KR131723, respectively.

RESULTS

Pm resistance mediated through the *P. aeruginosa* CprRS two-component system is partially dependent on the PhoPQ and PmrAB systems. Previous work demonstrated that the Pm resistance conferred by *phoQ* mutation in the *P. aeruginosa* PAK strain is partially dependent on an intact CprRS TCS (29). That work also defined a mutant allele, *cprRS21*, which confers Pm resistance when overexpressed in a PAK Δ*cprRS* strain. To assess the extent to which Pm resistance conferred by *cprRS21* is dependent on the PhoPQ TCS, this mutant allele was overexpressed in a PAK strain in which the WT *phoPQ* and *cprRS* genes had been deleted from the chromosome. The resulting strain exhibited a partial loss of Pm resistance in comparison to the positive control (Fig. 1A).

To broaden these observations to the PmrAB TCS, which also regulates Pm resistance, the extent to which resistance conferred by the mutant allele *pmrAB12* (17) is dependent on the CprRS TCS was assessed. This allele was overexpressed in a PAK strain in which the WT *pmrAB* and *cprRS* genes had been deleted. The resulting strain exhibited a partial loss of Pm resistance (see Fig. S1 in the supplemental material), the extent of which was dependent on the conditions used to induce expression of the *pmrAB12* allele; prolonged induction partially overcame the effect of *cprRS* deletion. To determine the reciprocal relationship, i.e., the degree to which resistance conferred by the *cprRS21* mutant allele is dependent on the PmrAB system, the *cprRS21* allele was overexpressed in the same *pmrAB cprRS* deletion strain. The resulting strain exhibited a partial loss of Pm resistance in comparison to the positive control (Fig. 1B).

To assess the extent to which Pm resistance conferred by *cprRS21* is dependent on having at least one of the other TCSs intact, this mutant allele was overexpressed in a PAK strain in which the *phoPQ*, *pmrAB*, and *cprRS* genes had all been deleted. The resulting triple-deletion overexpression strain exhibited substantial but incomplete loss of Pm resistance in comparison to the positive-control and the double-deletion overexpression strains (Fig. 1C). Thus, even though the deletion of both the PhoPQ and PmrAB TCSs has a combined effect on CprRS-mediated Pm resistance, in their absence, the *cprRS21* mutant allele still conferred some residual Pm resistance. These findings are consistent with the possibility of cross talk among these TCSs but also indicate that their regulatory activities are not completely interdependent.

CprRS, PhoPQ, and PmrAB systems jointly regulate PA1559 and PA1560, distinct ORFs in the PAO1 strain that together constitute a single ORF in PAK and other *P. aeruginosa* strains. Having established the partial interdependence of the CprRS, PhoPQ, and PmrAB TCSs with respect to Pm resistance, we sought to define loci, in addition to the *arn* operon, jointly regulated by these TCSs; the availability of transcriptional array data for each TCS facilitated this task (31, 32). One such jointly regulated locus in those analyses is the PA1559 ORF, which we previously defined as being required for PhoPQ-mediated Pm resistance in the PAK strain (29). Bioinformatics analysis of completely assembled *P. aeruginosa* genomes available in the *Pseudomonas* Genome Database (30) as of April 2015 revealed that in all non-PAO1 genomes (*n* = 12), PA1559 corresponds to the 5'

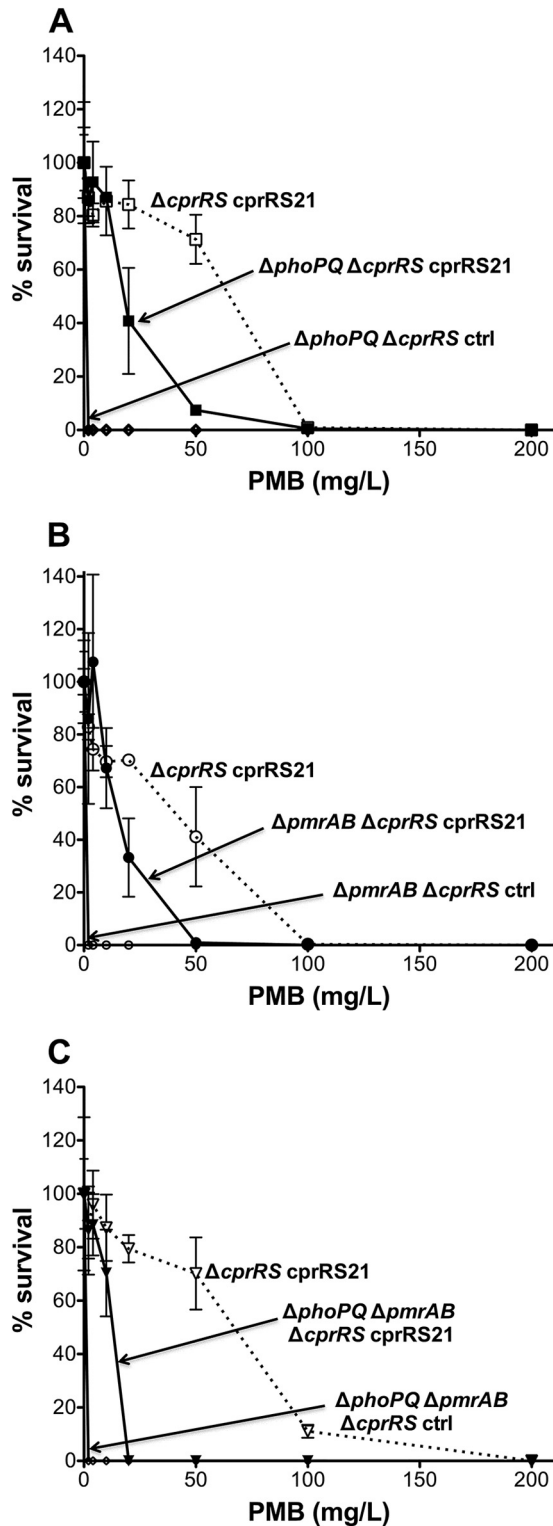


FIG 1 Effect of *phoPQ*, *pmrAB*, or *phoPQ* and *pmrAB* deletion on Pm resistance in a PAK $\Delta cprRS$ cprRS21 background. For this and subsequent figures, Pm resistance experiments were performed twice; if discrepancies were seen, the experiment was performed a third time. Each panel shows a representative experiment, with the results expressed as means from three technical replicates. The error bars represent the standard deviations (SD). The nonitalicized allele name indicates the presence of an episomal version, i.e., an inducible expression strain (ctrl, empty vector control). Results from a PMB plate assay

end of a conserved ORF. However, only PAO1 genomes were found to have a distinct adjacent ORF corresponding to the annotated PA1560. In the non-PAO1 genomes, PA1560 instead corresponds to the 3' end of the conserved ORF that contains PA1559-encoded residues at its 5' end (see Fig. S2 in the supplemental material).

To confirm the unitary relationship of the PA1559 and PA1560 ORFs in a non-PAO1 strain, Sanger sequencing of the genomic region corresponding to these ORFs was performed for the PAK strain of *P. aeruginosa* (1026). This analysis revealed a single ORF of 1,158 bp encoding a hypothetical protein comprised of 385 amino acids; the completely assembled genome of the PA14 strain and those of 11 other *P. aeruginosa* strains in the *Pseudomonas* Genome database (30) also contain this single ORF. Considering the role that the CprRS TCS plays in regulating PA1559 and PA1560 of the PAO1 strain (31) and the role that this locus plays in cationic peptide resistance (this work), we designated it the *cprA* gene.

Allelic forms of the *cprA* gene. Analysis of assembled whole-genome sequences from the *cprA* genomic region for four Pm^r CF clinical isolates of *P. aeruginosa* (1020, 1603, 1016, and 1611) with colistin agar dilution MICs of >512 mg/liter (12) revealed amino acid sequences identical to those of 13 completely assembled genomes of non-PAO1 strains in publicly available databases (see Table S2 in the supplemental material). We designated the *cprA* allele of these clinical strains and the other non-PAO1 strains *cprA*⁺ (WT).

Sanger sequencing of *cprA* for four Pm-susceptible (Pm^s) PAO1 substrains (1027, 1555, 2114, and 2178) revealed a single guanine deletion in codon 224 that results in a translational frameshift and premature termination at codon 244 (see Fig. S2 and Table S2 in the supplemental material). This mutation, which is responsible for the annotation of *cprA* as adjacent ORFs (PA1559 and PA1560) within the published PAO1 genome (30, 46), is also found in three completely assembled PAO1 genomes in the public NCBI database (see Table S2). We have designated the *cprA* allele of the PAO1 strain *cprA1*.

Sanger sequencing of *cprA* for the Pm^s PAK parental strain revealed nonsynonymous changes at codons 192 (Glu→Gln) and 196 (Gly→Asp) relative to *cprA*⁺; one non-PAO1 genome in the NCBI database has these same amino acid changes (see Table S2 in the supplemental material). We designated the *cprA* allele of the PAK strain *cprA2*.

Predicted structure of the *P. aeruginosa* *cprA* gene product. The predicted CprA structure is typical of an extended short-chain dehydrogenase/reductase (SDR) family member that (i) is >250 amino acids, (ii) contains a Rossmann fold having an N-terminal dinucleotide cofactor binding motif with consensus [S/T]GXX GXXG (CprA, TGATGFLG, codons 22 to 29), and (iii) possesses a catalytic residue motif with consensus YXXXK (CprA, YTASK, codons 179 to 183) (47). In addition to these conserved motifs, the

of strains induced with 0.1% L-Ara for 24 h are shown for strain 4537 ($\Delta phoPQ \Delta cprRS$ cprRS21) with strain 4240 ($\Delta cprRS$ cprRS21) as a positive control and strain 4541 ($\Delta phoPQ \Delta cprRS$ ctrl) as a negative control (A), strain 4472 ($\Delta pmrAB \Delta cprRS$ cprRS21) with strain 4240 ($\Delta cprRS$ cprRS21) as a positive control and strain 4470 ($\Delta pmrAB \Delta cprRS$ ctrl) as a negative control (B), and strain 4543 ($\Delta phoPQ \Delta pmrAB \Delta cprRS$ cprRS21) with strain 4240 ($\Delta cprRS$ cprRS21) as a positive control and strain 4547 ($\Delta phoPQ \Delta pmrAB \Delta cprRS$ ctrl) as a negative control (C).

SDR enzymes typically have a low pairwise sequence identity of 20 to 30% (48) and are functionally diverse (47). Protein BLAST searches performed versus other Gram-negative bacteria indicate that CprA has ~50% sequence identity with a *Klebsiella pneumoniae* protein, hemolysin F (HlyF). An alphanumeric search of the *Pseudomonas* Genome database revealed 69 proteins annotated as SDRs in the PAO1 genome (30). The enzymatic function is known for 15 of these proteins; eight are involved in LPS biosynthesis.

Deletion of *cprA* abrogates Pm resistance conferred by *phoQ*, *pmrB*, and *cprS* mutant alleles. To define the role of *cprA* in Pm resistance, clean deletions of this gene were constructed in the Pm^s PAK parental strain and in various Pm^r derivatives. The *cprA* gene does not appear to be part of an operon; thus, deleting or otherwise disrupting it has minimal potential for polar effects on adjacent loci.

In the PAK parental background, neither deletion of the *cprA2* allele nor *in trans* expression of this allele under prolonged strong induction (0.1% L-Ara) had a detectible effect on Pm susceptibility (data not shown). In contrast, deletion of *cprA2* abrogated the Pm resistance of a PAK *phoQ* deletion mutant, as shown in a PMB plate assay (Fig. 2A). The Pm resistance of the $\Delta cprA2 \Delta phoQ$ strain was similar to that of the PAK parental strain, indicating that the loss of Pm resistance was more complete than that previously seen for strains in which the CprRS TCS or the ColRS TCS had been deleted in a *phoQ* mutant background (29). Moreover, *in trans* expression of *cprA2* under prolonged strong induction in the $\Delta cprA2 \Delta phoQ$ strain partially restored Pm resistance (Fig. 2A). Decreasing the L-Ara concentration used for induction to 0.025%, which enhanced the resistance phenotype that an episomal *cprRS21* allele conferred in a *phoQ* mutant (29), did not enhance this partial complementation (data not shown).

The deletion of *cprA2* led to a loss of Pm resistance in a PAK $\Delta pmrAB$ strain that carries a resistance-conferring *pmrAB12* allele *in trans* (12) (Fig. 2B). The deletion of *cprA2* also caused a substantial loss of Pm resistance (~10-fold) in a PAK $\Delta cprRS$ strain that carries a resistance-conferring *cprRS21* allele *in trans* (29) (Fig. 2C). The deletion of *cprA2* caused a similar loss of resistance in a PAK $\Delta phoQ \Delta cprRS$ strain expressing *cprRS21* *in trans* (see Fig. S3 in the supplemental material).

Deletion of *cprA* abrogates CAP resistance conferred by a *pmrB* mutant allele. Pm resistance-conferring mutations in the *P. aeruginosa pmrB* gene, such as that found in the *pmrAB12* allele, also confer cross-resistance to CAPs, such as protegrin-1 and C18G (12), as measured through a standard CAP killing assay (17). To determine whether resistance to CAPs other than Pm is dependent on *cprA*, we used the killing assay to test resistance to protegrin-1 and C18G of PAK $\Delta pmrAB$ and PAK $\Delta cprA2 \Delta pmrAB$ mutants harboring an episomal L-Ara-inducible version of the *pmrAB12* allele. The expression of *pmrAB12* in the $\Delta pmrAB$ strain (positive control) resulted in a >256-fold increase in resistance (FIR) to Pm, relative to that of the negative-control strains, and FIRs of 96 and 128 to protegrin-1 and C18G, respectively (Table 2). Expression of this allele in the $\Delta cprA2 \Delta pmrAB$ strain resulted in a 16-fold decrease in Pm resistance relative to the positive control, similar to the effect seen in the PMB plate assay (Fig. 2B), and in 6- and 4-fold decreases in resistance to protegrin-1 and C18G, respectively (Table 2). These results indicate that the CAP resistance of *P. aeruginosa pmrB* mutants is partially dependent on the functionality of the *cprA* gene.

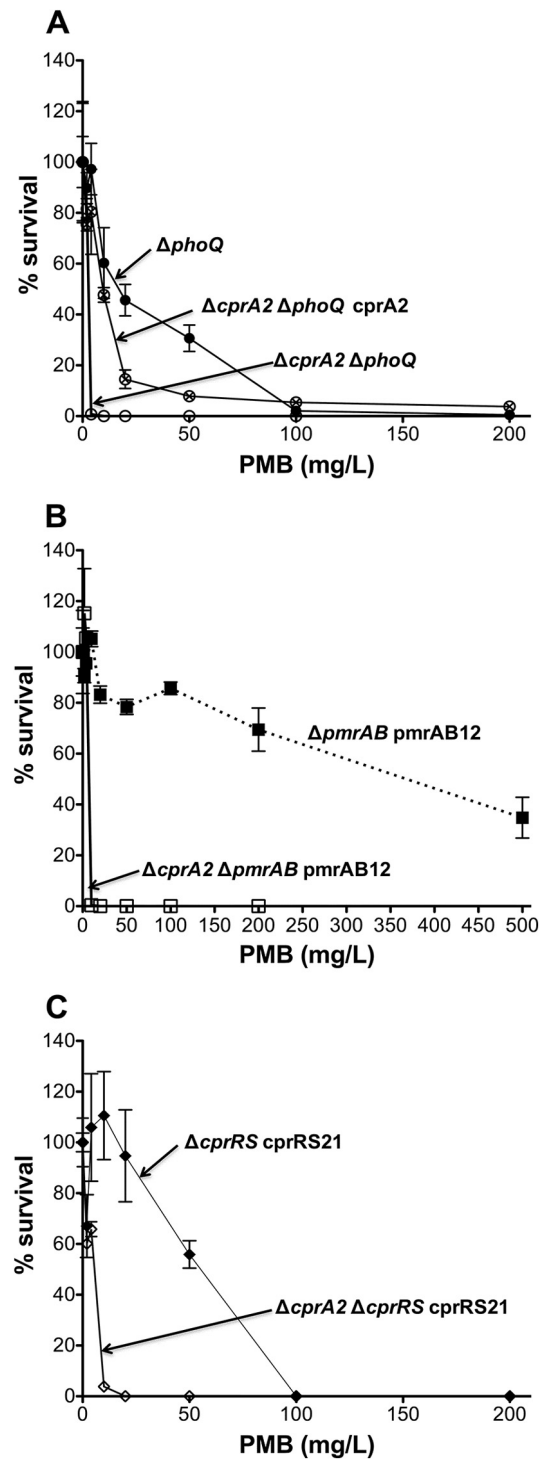


FIG 2 Effect of *cprA2* deletion on Pm resistance in various PAK strain backgrounds. Shown are the results of a PMB plate assay of strain 4374 ($\Delta cprA2 \Delta phoQ$) and strain 4614 ($\Delta cprA2 \Delta phoQ cprA2$) induced with 0.1% L-Ara for 24 h, with strain 2326 ($\Delta phoQ$) as a positive control (A), strain 4519 ($\Delta cprA2 \Delta pmrAB pmrAB12$) induced with 0.1% L-Ara for 24 h, with strain 2735 ($\Delta pmrAB pmrAB12$) as a positive control (B), and strain 4425 ($\Delta cprA2 \Delta cprRS cprRS21$) induced with 0.1% L-Ara for 24 h, with strain 4240 ($\Delta cprRS cprRS21$) as a positive control (C). Empty vector control strains 4497 ($\Delta cprA2 \Delta phoQ pJN105$), 4521 ($\Delta cprA2 \Delta pmrAB pJN105$), and 4447 ($\Delta cprA2 \Delta cprRS pJN105$) had Pm susceptibility values similar to those of the corresponding parental strains (not shown).

TABLE 2 Effect of *cprA2* deletion in the PAK strain on *pmrAB12*-induced cationic antimicrobial peptide resistance

Strain/genotype	PMB ^a		Protegrin-1		C18G	
	EC ₅₀ (mg/liter)	FIR	EC ₅₀ (mg/liter)	FIR	EC ₅₀ (mg/liter)	FIR
PAK WT	<0.125	1	0.125	1	2	1
PAK $\Delta pmrAB$ pJN105	<0.125	1	0.125	1	2	1
PAK $\Delta pmrAB$ pJN105D:: <i>pmrAB12</i>	32	>256	12	96	256	128
PAK $\Delta cprA2$ $\Delta pmrAB$ pJN105D:: <i>pmrAB12</i>	2	>16	2	16	64	32

^a EC₅₀, effective concentration that kills 50% of CFU; FIR, fold increase in resistance to the indicated antimicrobial peptide of each strain with respect to PAK WT (ratio of EC₅₀s).

Repair of *cprA1* mutant allele in the PAO1 strain markedly increases PmrAB-mediated Pm resistance. In the PAO1 strain, increased Pm resistance is conferred by mutational activation of the PhoPQ system or is induced by specific environmental conditions (e.g., Mg²⁺ depletion or subinhibitory CAP exposure) that act through the PmrAB, ParRS, and CprRS systems (20, 21, 23, 27, 31, 32). However, the degree of Pm resistance seen with the PAO1 strain is much more modest than that with the PAK strain or clinical strains (12, 17, 24, 29, 45). To test the hypothesis that the difference in Pm resistance levels seen with these strains is attributable to the frameshift mutation in the *cprA1* allele, its guanine deletion was repaired in a PAO1 $\Delta pmrAB$ strain through plasmid-based site-directed mutagenesis and chromosomal allelic replacement. The pJN105D::*pmrAB12* plasmid was introduced into the *cprA*-repaired PAO1 $\Delta cprA1::cprA^+$ $\Delta pmrAB$ strain, and as a control, into a PAO1 $\Delta pmrAB$ strain in which the *cprA1* allele had not been repaired. A PAO1 $\Delta cprA1::cprA^+$ $\Delta pmrAB$ strain carrying an empty plasmid served as an additional control. Restoration of the *cprA^+* allele in the *pmrAB12*-expressing PAO1 was associated with a marked increase in Pm resistance: an effective concentration yielding 50% lethality (EC₅₀) of ≥ 500 mg/liter PMB for the repaired strain versus ~ 6 mg/liter PMB for the control strain (Fig. 3). The level of Pm resistance that episomal *pmrAB12* expression conferred on the *cprA*-repaired PAO1 $\Delta pmrAB$ strain was comparable to that conferred on the PAK $\Delta pmrAB$ strain (Fig. 2B). Thus, the difference in Pm resistance

between the PAO1 and PAK strains is largely attributable to the frameshift mutation in the *cprA1* allele of the PAO1 strain.

Deletion of *cprA* does not abolish lipid A modifications conferred by *phoQ*, *pmrB*, and *cprS* mutant alleles. Covalent modification of lipid A is a biochemical hallmark of resistance to Pm and other CAPs in Gram-negative bacilli (13, 45, 49, 50) and is commonly analyzed using mass spectral methods, such as MALDI-TOF MS (18). Analysis of lipid A from the Pm^s PAK parental strain revealed unmodified penta- and hexa-acylated species (Fig. 4A, top) corresponding to major mass spectral peaks at mass/charge ratio (*m/z*) 1,447 and 1,617 (Fig. 4B). In contrast, analysis of lipid A from the Pm^r PAK $\Delta phoQ$ strain had modified species with the addition of one or two L-Ara4N residues ($\Delta m/z$, +131 or +262) to the 1- and 4'-phosphates of the penta- and hexa-acylated species (Fig. 4A, middle and bottom), corresponding to peaks at *m/z* 1,578, 1,748, and 1,879 (Fig. 4C). The deletion of *cprA2* in the PAK $\Delta phoQ$ strain did not qualitatively alter the complex pattern of modified lipid A species resulting from the addition of L-Ara4N in this strain background (Fig. 4D), indicating that the *cprA* dependence of Pm resistance in the $\Delta phoQ$ background is not attributable to a loss of this lipid A modification. This is not surprising in light of our previous observation that deletion of the CprRS TCS in a $\Delta phoQ$ background did not abolish the robust addition of L-Ara4N to lipid A, despite a substantial loss of Pm resistance associated with the *cprRS* deletion (29).

Analysis of lipid A from the Pm^r PAK $\Delta pmrAB$ pJN105D::*pmrAB12* expression strain revealed unmodified penta-acylated species with one or two 2-hydroxy lauroyl residues, corresponding to minor peaks at *m/z* 1,447 and 1,463, and unmodified hexa-acylated species with one or two 2-hydroxy lauroyl residues, corresponding to major peaks at *m/z* 1,617 and 1,633 (see Fig. S4A in the supplemental material). This lipid A also had modified species with one L-Ara4N residue added, corresponding to minor peaks at *m/z* 1,578 and 1,594 and major peaks at *m/z* 1,748 and 1,764, with adjacent Na⁺ adduct peaks at *m/z* 1,771 and 1,787 ($\Delta m/z$, +23). Peaks corresponding to Na⁺ adducts at *m/z* 1,902 and 1,918 dwarfed the species from which they derived that had two L-Ara4N residues, corresponding to minor peaks at *m/z* 1,879 and 1,895. The deletion of *cprA2* in the $\Delta pmrAB$ pJN105D::*pmrAB12* expression background did not alter the pattern of modified lipid A species, except that the peaks corresponding to the Na⁺ adducts were minimal (see Fig. S4B in the supplemental material).

Similarly, analysis of lipid A from the Pm^r PAK $\Delta cprRS$ pJN105D::*cprRS21* expression strain revealed unmodified penta-acylated species with one or two 2-hydroxy lauroyl residues, corresponding to minor peaks at *m/z* 1,447 and 1,463, and unmodified hexa-acylated species with one or two 2-hydroxy lauroyl residues, corresponding to major peaks at *m/z* 1,617 and 1,633

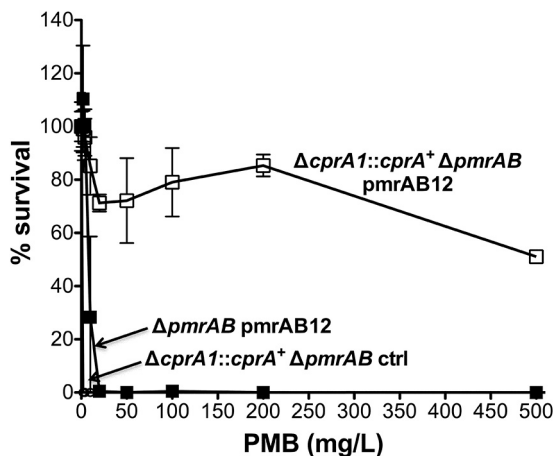


FIG 3 Repair of the *cprA1* mutant allele promotes high-level Pm resistance in the PAO1 $\Delta pmrAB$ strain expressing *pmrAB12* in trans. Shown are the results of a PMB plate assay for strain 4763 ($\Delta cprA1::cprA^+ \Delta pmrAB pmrAB12$) induced with 0.1% L-Ara for 24 h, with strains 4734 ($\Delta pmrAB pmrAB12$) and 4765 ($\Delta cprA1::cprA^+ \Delta pmrAB ctrl$) as negative controls.

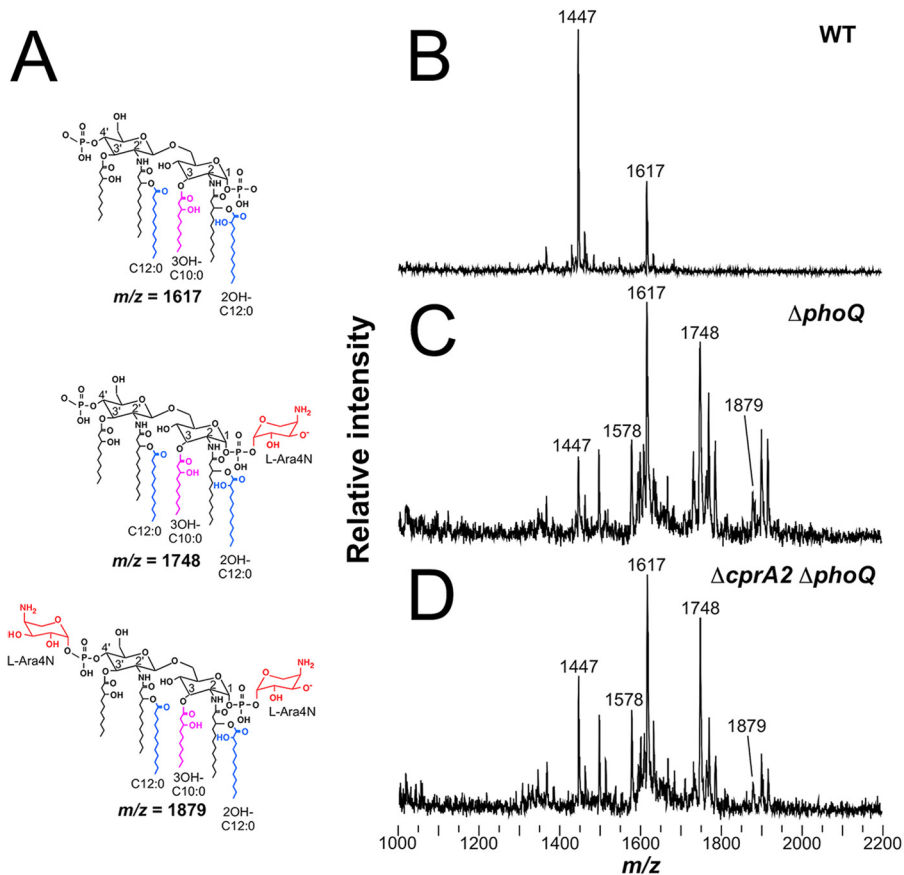


FIG 4 Deletion of *cprA2* does not affect L-Ara4N modification of lipid A from the PAK strain. (A) Structure of lipid A with baseline hexa-acylation (m/z , 1,617), single L-Ara4N addition (m/z , 1,748), and double L-Ara4N addition (m/z , 1,879). Also shown are the MALDI-TOF spectra of lipid A isolated from strain 1026 (WT) (B), strain 2326 ($\Delta phoQ$) (C), and strain 4374 ($\Delta cprA2 \Delta phoQ$) (D).

(see Fig. S4C in the supplemental material). This lipid A also had modified species with one or two L-Ara4N residues, corresponding to minor peaks at m/z 1,578 and 1,594, major peaks at m/z 1,748 and 1,764, and minor peaks at m/z 1,879 and 1,895. The deletion of *cprA2* in the $\Delta cprRS$ pJN105D::*cprRS21* expression background did not alter this modification pattern (see Fig. S4D in the supplemental material).

Taken together, these results indicate that in *pmrB* and *cprS* mutant backgrounds, as in the *phoQ* mutant background, the dependence of Pm resistance on *cprA* is not attributable to a loss of L-Ara4N addition or other modifications detectible through MALDI-TOF MS analysis of lipid A derived from hydrolyzed LPS.

Both *cprA* and *arn* are required for Pm resistance. We previously showed that the deletion of the *arnC* gene, which encodes an enzyme that transfers L-Ara4N from UDP to undecaprenyl phosphate at the inner membrane (51), causes a loss of lipid A L-Ara4N modification and a loss of Pm resistance in a *P. aeruginosa* clinical strain with a resistance-conferring *pmrB* mutation (12). Thus, PmrAB-mediated Pm resistance requires both *cprA* (Fig. 2B) and *arnC*. Pm resistance conferred through *phoQ* deletion requires *cprA* (Fig. 2A) and presumably also *arnC*, although to our knowledge, the dependence of PhoPQ-mediated resistance on *arnC* has not been directly documented in the published literature. Given that CprRS-mediated Pm resistance requires *cprA* (Fig. 2C), we examined the extent to which Pm resistance also depends on the

arnC locus in the *cprRS21* expression background. The deletion of *arnC* resulted in a marked decrease in Pm resistance (Fig. 5), indicating that similarly to PmrAB-mediated Pm resistance, CprRS-mediated resistance requires both *cprA* and *arnC*. However, the

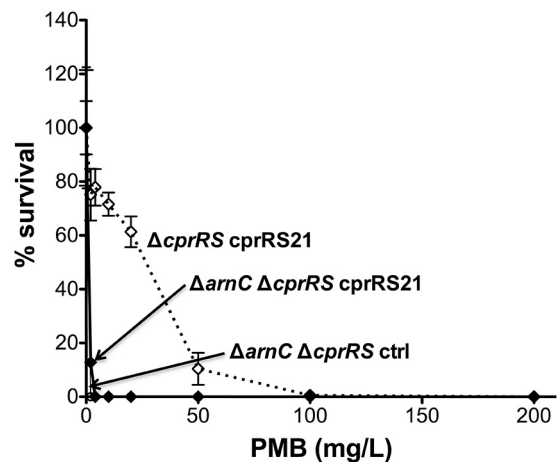


FIG 5 Effect of *arnC* deletion in the PAK $\Delta cprRS$ *cprRS21* strain. Shown are the results of a PMB plate assay of strain 4581 ($\Delta arnC$ $\Delta cprRS$ *cprRS21*) induced with 0.1% L-Ara for 24 h, with strain 4240 ($\Delta cprRS$ *cprRS21*) as a positive control and strain 4586 ($\Delta arnC$ $\Delta cprRS$ ctrl) as a negative control.

TABLE 3 Relative abundance of *cprA* and *arnC* mRNA transcripts in *phoQ* deletion and *pmrAB12* or *cprRS21* expression backgrounds, determined by RT-qPCR analysis ($n = 3$ biological replicates)

Genotype	Relative mRNA abundance \pm SD ^a	
	<i>cprA</i>	<i>arnC</i>
PAK Δ <i>phoQ</i>	1 \pm 0.4	34 \pm 17
PAK Δ <i>pmrAB</i> pJN105D:: <i>pmrAB12</i>	94 \pm 26	21 \pm 7
PAK Δ <i>cprRS</i> pJN105D:: <i>cprRS21</i>	165 \pm 37	34 \pm 18
PAK Δ <i>cprA2</i> Δ <i>cprRS</i> pJN105D:: <i>cprRS21</i>	NA	30 \pm 9
PAK Δ <i>arnC</i> Δ <i>cprRS</i> pJN105D:: <i>cprRS21</i>	172 \pm 70	NA
PAK Δ <i>phoQ</i> Δ <i>cprRS</i> pJN105D:: <i>cprRS21</i>	236 \pm 162	51 \pm 40

^a Normalized to mRNA abundance in the PAK WT strain. NA, not applicable.

cprRS21 expression strain still had a minor degree of residual Pm resistance (~10 to 15% survival at 2 mg/liter Pm) compared to that of the negative control (0% survival at 2 mg/liter Pm), indicating that *arnC* deletion is not epistatic to the resistance-confering effect of the *cprRS21* allele.

To quantify the correlation of *cprA* and *arnC* expression, reverse transcription-quantitative PCR (RT-qPCR) was used to measure the abundance of their mRNA transcripts in a *phoQ* deletion strain, a *pmrAB12* expression strain, a *cprRS21* expression strain, a combined *phoQ* deletion/*cprRS21* expression strain, and *cprRS21* expression strains in which either *cprA2* or *arnC* had been deleted. The *cprA* and *arnC* transcripts were both increased in the *pmrAB12* and *cprRS21* expression strains (Table 3). In contrast, only the *arnC* transcript was increased in the *phoQ* deletion strain. Considering that CprA is required for Pm resistance conferred through *phoQ* mutation (Fig. 2A), this indicates that a basal level of *cprA* expression is permissive for resistance in the Δ *phoQ* strain. The deletion of either *arnC* or *cprA* in the *cprRS21* expression background did not significantly alter the mRNA expression level of the other locus (Table 3). Both transcripts were increased when *cprRS21* was expressed in the *phoQ* *cprRS* double-deletion background, indicating that *phoQ* deletion does not have a suppressive effect on CprRS-mediated *cprA* expression.

DISCUSSION

Previous studies have established that the Pm resistance of *P. aeruginosa* is dependent on *arn*-mediated addition of L-Ara4N to lipid A (12). However, recent work has shown that to achieve substantial Pm resistance, additional loci are required. For example, loss of the CprRS TCS was found to diminish PhoPQ-mediated resistance without a loss of L-Ara4N addition (29). That study used transposon mutagenesis of a Pm-resistant PAK mutant (*phoQ6* Δ *pmrAB*) to define such additional loci, including PA1559, an ORF in the PAO1 genome which transcriptional analysis had previously defined as being CprRS regulated (31). Prior PAO1 transcriptional analyses indicated that the ParRS and PmrAB systems also regulate the expression of PA1559 (27, 32). Nonetheless, experiments performed in the PAO1 strain background failed to demonstrate a role for PA1559 in inducible Pm resistance (27), even though this prior work with the PAO1 strain clearly established that specific TCSs mediate differential CAP sensing and thus trigger transcription from the *arn* promoter (27, 31). Bioinformatics analysis and resequencing of the chromosomal region containing the PA1559 and PA1560 ORFs has revealed that in *P. aeruginosa* strains other than PAO1, this region

contains only a single ORF (annotated PA14_44311 in the PA14 genome) that we named *cprA*. A comparison of *cprA* alleles *in silico* reveals that this discrepancy is attributable to a frameshift mutation and associated premature termination codon in the *cprA1* allele of the PAO1 strain, resulting in annotation as adjacent ORFs.

The relatively low levels of mutational and adaptive resistance to Pm and other CAPs that have been reported for the PAO1 strain (20, 21, 23, 27, 31) are likely attributable to its nonfunctional *cprA1* allele and may thus need to be revisited. This work has shown that in *P. aeruginosa*, *cprA* is required not only for robust Pm resistance mediated through *phoQ* mutation but also for resistance mediated through activating mutations in *cprS* and *pmrB*. Moreover, *cprA* is likely critical for the induction of adaptive Pm resistance via TCS sensing of subinhibitory CAP concentrations and other physiological conditions, responses that appear to be impaired in the PAO1 strain. Because PAO1 has undergone genotypic and phenotypic microevolution over decades under laboratory conditions (52), it may not accurately represent the adaptive capacity of typical pathogenic strains *in vivo*, in which *P. aeruginosa* encounters CAPs as a front-line component of host innate immunity.

Several TCSs mediate resistance to Pm and other CAPs (Fig. 6), controlling *P. aeruginosa* loci that encode LPS-modifying enzymes, such as those necessary for the synthesis and transfer of L-Ara4N to lipid A. Key insights into this regulatory complexity have gradually emerged over the past 15 years. Initial work focused on the role of the *P. aeruginosa* PhoPQ TCS in Pm resistance (21, 45). Subsequent work defined a stronger effect on this resistance phenotype by the PmrAB TCS, which appears to be independent of PhoPQ in *P. aeruginosa* (17, 20). More recently, the ParRS, ColRS, and CprRS TCSs have been recognized as making

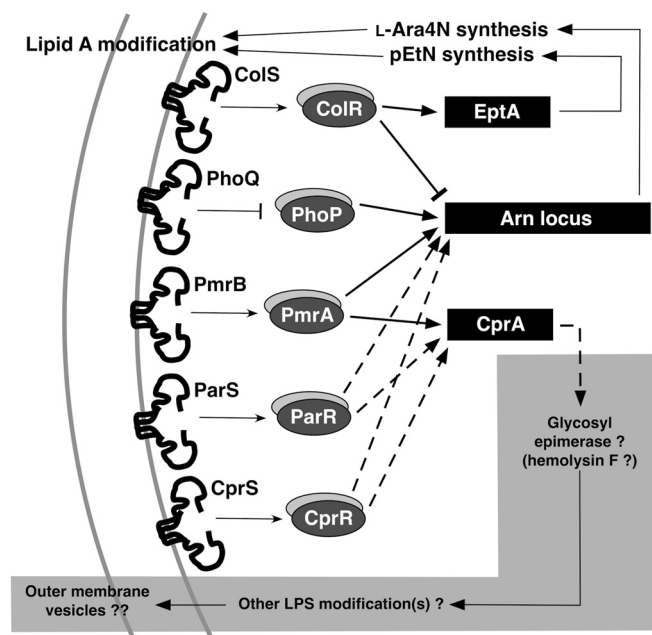


FIG 6 Two-component regulatory systems mediating resistance to Pm and other CAPs modulate expression of *arn* and *eptA*, loci that enable specific lipid A modifications, and *cprA*, a locus potentially implicated in other LPS modification(s) and vesiculation from the outer membrane.

additional contributions to Pm resistance, although the ParRS effect is modest, and the ColRS effect appears to be dependent on the PhoPQ TCS (27–29, 31). The ColRS TCS was also recently shown to promote phosphoethanolamine addition at the 4' position of lipid A under specific environmental conditions, through positive regulation of the newly defined *eptA* gene (corresponding to the PA1972 ORF) and negative regulation of *arn*, but without a detected effect on Pm resistance (53). These prior studies indicate that most of these TCSs regulate both the *arn* operon and the *cprA* gene, although some of the regulatory effects may be indirect. However, the extent to which Pm resistance induced via a given TCS is dependent on the functionality of the other TCSs and their regulons has not been comprehensively defined.

The present work increases our understanding of these regulatory relationships, showing that the ability of an activating mutation in *cprS* to confer Pm resistance is partially dependent on the presence of functional PmrAB and PhoPQ TCSs. Conversely, the ability of an activating mutation in *pmrB* to confer Pm resistance is partially dependent on the presence of a functional CprRS TCS. However, the induction of L-Ara4N addition by such *pmrB* mutations is not strictly CprRS dependent. Collectively, these findings corroborate the observation that in addition to the *arn* locus, other key components of the CprRS regulon, such as *cprA*, are required for maximal Pm resistance. The deletion or disruption of the *cprA* coding sequence causes diminished Pm resistance in several different Pm^r strain backgrounds and results in diminished resistance to other CAPs.

Activating the PmrAB or CprRS TCS through a resistance-conferring mutation of *pmrB* or *cprS* increases the transcription of both *arn* and *cprA* in a PAK strain background (Table 3). In contrast, activating the PhoPQ TCS through the deletion of *phoQ* increases *arn* transcription and Pm resistance but does not increase *cprA* transcription relative to the WT background (Table 3). Nonetheless, experiments with the Pm^r *phoQ* deletion strain demonstrate that its Pm resistance is dependent on *cprA* (Fig. 2A). This suggests that a basal level of *cprA* transcription prevails in the Δ *phoQ* strain background, enough to permit an L-Ara4N-mediated increase in Pm resistance, albeit at a lower level than that seen with the *pmrAB12* or *cprRS21* alleles that actively promote *cprA* transcription.

Understanding the biochemical role of the CprA protein in Pm resistance is of paramount importance, given the genetic and transcriptional evidence implicating *cprA* in this phenotype. Bioinformatics analysis of *P. aeruginosa* CprA indicates strong similarity to the extended SDR enzyme family, which comprises isomerases (e.g., glycosyl epimerases), lyases (e.g., glycosyl dehydratases), and some oxidoreductases not classified as classical SDRs (e.g., certain multifunctional enzymes with both dehydrogenase and isomerase activities) (54). Several functionally defined SDRs participate in LPS biosynthesis in *P. aeruginosa*, such as WbpM and WbpK (55); CprA may similarly function as an LPS-modifying enzyme. However, the deletion of *cprA* in Pm^r strain backgrounds had no effect on *arn* transcription or on mass spectrometric detection of the L-Ara4N lipid A modification, indicating that the CprA protein does not affect this specific LPS modification.

A comparison of CprA to the genomes of other sequenced bacterial species also indicates strong similarity to hemolysin F (*hlyF*), a *K. pneumoniae* gene sometimes carried on large plasmids found in pathogenic clinical isolates of *E. coli* (56, 57). Hemolysin F was recently shown to contribute to *E. coli* virulence in an avian

model of colibacillosis (58). Moreover, hemolysin F appears to enhance the production of outer membrane vesicles (OMVs) and release of the cytolysin ClyA, potentially explaining its hemolytic phenotype. Among Gram-negative pathogens, outer membrane turnover and OMV production provide a general mechanism of protection against environmental sources of envelope stress, such as antimicrobials and agents of host innate immunity (59–61). Of potential relevance to the work reported here, exposure of the *P. aeruginosa* PA14 strain to subinhibitory concentrations of PMB was found to increase OMV production via unknown mechanisms (62). Thus, CprA may participate directly in *P. aeruginosa* OMV production. Alternatively, if CprA has enzyme activity typical of extended SDRs, it may function as a nucleoside diphosphate-sugar epimerase and thus play a role in LPS synthesis (as suggested above). This might have an indirect effect on *P. aeruginosa* OMV production, which was shown to be dependent on the synthesis of B-band LPS (62).

Our discovery that *P. aeruginosa* resistance to Pm and other CAPs requires both the *arn* locus and an active CprA protein moves the field beyond a narrow focus on enzymes involved in L-Ara4N synthesis (63, 64), opening the way to novel resistance inhibition strategies. The observation that laboratory-adapted strains with mutated *cprA* alleles, such as PAO1, have diminished resistance suggests the need to examine how Pm resistance correlates with CprA expression and function in clinical isolates of *P. aeruginosa*. Moreover, we found that several of the TCSs regulating *cprA* transcription influence Pm resistance in a convergent and partially interdependent fashion, suggesting that an analysis of clinical isolates may reveal additional correlations between patterns of TCS mutations and levels of CprA protein expression. CprA is predicted to be an extended SDR that appears not to affect lipid A modification but might participate in the modification of other LPS moieties (O antigen or core oligosaccharide), thus influencing OMV production or another aspect of outer membrane remodeling. We are currently pursuing functional characterization of CprA to delineate its enzymatic activity and assess its viability as a potential drug target.

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