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# Polymyxin Resistance of *Pseudomonas aeruginosa phoQ* Mutants Is Dependent on Additional Two-Component Regulatory Systems

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***Pseudomonas aeruginosa*** **can develop resistance to polymyxin as a consequence of mutations in the PhoPQ regulatory system, mediated by covalent lipid A modification. Transposon mutagenesis of a polymyxin-resistant *phoQ* mutant defined 41 novel loci required for resistance, including two regulatory systems, ColRS and CprRS. Deletion of the *colRS* genes, individually or in tandem, abrogated the polymyxin resistance of a Δ*phoQ* mutant, as did individual or tandem deletion of *cprRS*. Individual deletion of *colR* or *colS* in a Δ*phoQ* mutant also suppressed 4-amino-L-arabinose addition to lipid A, consistent with the known role of this modification in polymyxin resistance. Surprisingly, tandem deletion of *colRS* or *cprRS* in the Δ*phoQ* mutant or individual deletion of *cprR* or *cprS* failed to suppress 4-amino-L-arabinose addition to lipid A, indicating that this modification alone is not sufficient for PhoPQ-mediated polymyxin resistance in *P. aeruginosa*. Episomal expression of *colRS* or *cprRS* in tandem or of *cprR* individually complemented the Pm resistance phenotype in the Δ*phoQ* mutant, while episomal expression of *colR*, *colS*, or *cprS* individually did not. Highly polymyxin-resistant *phoQ* mutants of *P. aeruginosa* isolated from polymyxin-treated cystic fibrosis patients harbored mutant alleles of *colRS* and *cprS*; when expressed in a Δ*phoQ* background, these mutant alleles enhanced polymyxin resistance. These results define ColRS and CprRS as two-component systems regulating polymyxin resistance in *P. aeruginosa*, indicate that addition of 4-amino-L-arabinose to lipid A is not the only PhoPQ-regulated biochemical mechanism required for resistance, and demonstrate that *colRS* and *cprS* mutations can contribute to high-level clinical resistance.**

The polymyxins (Pm), a family of cyclic oligopeptides with activity against *Pseudomonas aeruginosa* and other Gram-negative pathogens, are increasingly important in the treatment of invasive infections in critically ill patients and airway infections in those with cystic fibrosis (CF) (1, 2). First-line treatment of these infections often involves intravenous or inhaled combinations of antipseudomonal beta-lactams, aminoglycosides, fluoroquinolones, and other agents. Repeated use of these first-line agents imposes selection pressure leading to multidrug-resistant strains of *P. aeruginosa* (3–5). When this occurs, the clinically available forms of Pm, namely, Pm B sulfate (PMB) and colistimethate, the prodrug form of colistin (CST) (also known as Pm E), become key components of second-line regimens.

Pm binds to lipopolysaccharide (LPS), the major constituent of the Gram-negative outer membrane, promoting membrane permeabilization and diffusion of peptide through the periplasm to the inner membrane, where Pm insertion disrupts cellular respiration and results in cell lysis (6). Unfortunately, the prevalence of Pm-resistant (Pm$^r$) clinical strains of *P. aeruginosa* and other Gram-negative pathogens is increasing (7–13); such strains are generally resistant to both PMB and CST.

At a biochemical level, Pm resistance of *P. aeruginosa* and other Gram-negative pathogens is strongly associated with covalent modification of LPS, most specifically with the addition of 4-amino-L-arabinose (L-Ara4N) to the phosphate groups of its lipid A and core oligosaccharide components (14–16). Genes in the *arnBCADTEF-pmrE* operon encode enzymes responsible for synthesis and transfer of L-Ara4N to LPS (17, 18). This aminosugar modification is thought to hinder charge interactions between phosphate groups within LPS and amino groups within the cyclic Pm oligopeptide.

In contrast to their hierarchical regulation of Pm resistance in *Salmonella enterica*, the PhoPQ and PmrAB two-component systems of *P. aeruginosa* regulate Pm resistance convergently, at least in part by activating transcription of the *arnBCADTEF-pmrE* operon in response to antimicrobial peptide exposure or divalent cation depletion (17–20) or as a consequence of mutation (13, 16, 21–25). Recently, the ParRS two-component system has also been found to play a role in Pm resistance in *P. aeruginosa* (26, 27). We hypothesized that additional regulatory systems interact with these known two-component systems to modulate Pm resistance and that mutations in such systems might contribute to high-level clinical resistance. The primary objective of this study was to identify additional regulatory systems contributing to PhoPQ-mediated Pm resistance in highly resistant clinical strains; a secondary objective was to define loci encoding additional structural elements required for PhoPQ-mediated Pm resistance.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Laboratory strains and clinical isolates of *P. aeruginosa* used in this study are listed in Table 1. Clinical

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

| Strain | Description | Strain origin or reference |
|---|---|---|
| 1026 | PAK WT | S. Lory |
| 1995 | PAK Δ*pmrAB phoQ6* | 23 |
| 3882 | PAK Δ*pmrAB phoQ6 colR*::mTn*5-aacC1* | This work |
| 3897 | PAK Δ*pmrAB phoQ6 cprS*::mTn*5-aacC1* | This work |
| 2326 | PAK Δ*phoQ* | 23 |
| 3932 | PAK Δ*phoQ* Δ*colR* | This work |
| 3934 | PAK Δ*phoQ* Δ*colS* | This work |
| 3936 | PAK Δ*phoQ* Δ*colRS* | This work |
| 3749 | PAK Δ*colR* | This work |
| 3751 | PAK Δ*colS* | This work |
| 3752 | PAK Δ*colRS* | This work |
| 3969 | PAK Δ*phoQ* Δ*cprR* | This work |
| 3970 | PAK Δ*phoQ* Δ*cprS* | This work |
| 3975 | PAK Δ*phoQ* Δ*cprRS* | This work |
| 3806 | PAK Δ*cprR* | This work |
| 3802 | PAK Δ*cprS* | This work |
| 3804 | PAK Δ*cprRS* | This work |
| 1579 | PA clinical isolate (Copenhagen CF patient 9, 1999) | 23 |
| 1581 | PA clinical isolate (Copenhagen CF patient 9, 2001) | 23 |
| 1577 | PA clinical isolate (Copenhagen CF patient 9, 2002) | 23 |
| 1601 | PA clinical isolate (Copenhagen CF patient 13, 1997) | 23 |
| 1021 | PA clinical isolate (Copenhagen CF patient 13, 1998) | 23 |
| 1598 | PA clinical isolate (Copenhagen CF patient 13, 2002) | 23 |
| 4177 | PAK Δ*phoQ* pJN105 ($GEN^r$) | This work |
| 4041 | PAK Δ*phoQ* Δ*colR* pJN105D::*colR*$^+$ ($GEN^r$) | This work |
| 4039 | PAK Δ*phoQ* Δ*colR* pJN105D::*colR21* ($GEN^r$) | This work |
| 4046 | PAK Δ*phoQ* Δ*colR* pJN105 ($GEN^r$) | This work |
| 4037 | PAK Δ*phoQ* Δ*colS* pJN105D::*colS*$^+$ ($GEN^r$) | This work |
| 4035 | PAK Δ*phoQ* Δ*colS* pJN105D::*colS21* ($GEN^r$) | This work |
| 4048 | PAK Δ*phoQ* Δ*colS* pJN105 ($GEN^r$) | This work |
| 4044 | PAK Δ*phoQ* Δ*colRS* pJN105D::*colRS*$^+$ ($GEN^r$) | This work |
| 4043 | PAK Δ*phoQ* Δ*colRS* pJN105D::*colR21S21* ($GEN^r$) | This work |
| 4050 | PAK Δ*phoQ* Δ*colRS* pJN105 ($GEN^r$) | This work |
| 4115 | PAK Δ*colRS* pJN105D::*colRS*$^+$ ($GEN^r$) | This work |
| 4113 | PAK Δ*colRS* pJN105D::*colR21S21* ($GEN^r$) | This work |
| 4117 | PAK Δ*colRS* pJN105 ($GEN^r$) | This work |
| 4099 | PAK Δ*phoQ* Δ*cprR* pJN105D::*cprR*$^+$ ($GEN^r$) | This work |
| 4101 | PAK Δ*phoQ* Δ*cprR* pJN105 ($GEN^r$) | This work |
| 4065 | PAK Δ*phoQ* Δ*cprS* pJN105D::*cprS*$^+$ ($GEN^r$) | This work |
| 4063 | PAK Δ*phoQ* Δ*cprS* pJN105D::*cprS21* ($GEN^r$) | This work |
| 4069 | PAK Δ*phoQ* Δ*cprS* pJN105 ($GEN^r$) | This work |
| 4103 | PAK Δ*phoQ* Δ*cprRS* pJN105D::*cprRS*$^+$ ($GEN^r$) | This work |
| 4200 | PAK Δ*phoQ* Δ*cprRS* pJN105D::*cprR*$^+$*S21* ($GEN^r$) | This work |
| 4105 | PAK Δ*phoQ* Δ*cprRS* pJN105 ($GEN^r$) | This work |
| 4128 | PAK Δ*cprR* pJN105D::*cprR*$^+$ ($GEN^r$) | This work |
| 4130 | PAK Δ*cprR* pJN105 ($GEN^r$) | This work |
| 4091 | PAK Δ*cprS* pJN105D::*cprS*$^+$ ($GEN^r$) | This work |
| 4093 | PAK Δ*cprS* pJN105D::*cprS21* ($GEN^r$) | This work |
| 4097 | PAK Δ*cprS* pJN105 ($GEN^r$) | This work |
| 4131 | PAK Δ*cprRS* pJN105D::*cprRS*$^+$ ($GEN^r$) | This work |
| 4240 | PAK Δ*cprRS* pJN105D::*cprR*$^+$*S21* ($GEN^r$) | This work |
| 4133 | PAK Δ*cprRS* pJN105 ($GEN^r$) | This work |
| 4175 | PAK Δ*phoQ* pJN105D::*colRS*$^+$ ($GEN^r$) | This work |
| 4173 | PAK Δ*phoQ* pJN105D::*colR21S21* ($GEN^r$) | This work |
| 4236 | PAK Δ*phoQ* pJN105D::*cprRS*$^+$ ($GEN^r$) | This work |
| 4238 | PAK Δ*phoQ* pJN105D:: *cprR*$^+$*S21*($GEN^r$) | This work |

isolates were from the sputum of patients followed in the CF clinic at Rigshospitalet, Copenhagen, Denmark; the Institutional Review Board of Massachusetts General Hospital reviewed and approved their use in this study. *Escherichia coli* DH5α was used as a host for manipulation of recombinant plasmids. *P. aeruginosa* and *E. coli* were grown at 30°C or 37°C on lysogeny agar (LA) plates or in lysogeny broth (LB) with aeration. Antibiotics were used at the following concentrations for selection and maintenance of plasmids: 50 mg/liter kanamycin or 10 mg/liter gentamicin (GEN) for *E. coli* DH5α and 50 to 100 mg/liter GEN for *P. aeruginosa* PAK and its derivatives. Strains were stored at −80°C in LB supplemented with 16% glycerol.

**Molecular methods.** Bacterial plasmids were isolated using the QIAprep spin kit (Qiagen, Valencia, CA), and bacterial chromosomal DNA was isolated using the MasterPure kit (Epicentre Biotechnologies, Madison, WI). Plasmids were introduced into *P. aeruginosa* by electroporation (28). PCR amplification was performed as described previously (23) using oligonucleotide primers (see Table S1 in the supplemental material) that were designed according to data from the Pseudomonas.com database (29). Sanger sequencing of bacterial plasmids and chromosomal DNA was performed using oligonucleotide primers listed in Table S1. Whole-genome sequencing of bacterial chromosomal DNA was performed as described previously (30). For each genome, a random-fragment library was constructed using a custom protocol. Briefly, genomic DNA (gDNA) samples were sheared by sonication and end repaired using the End-It DNA end repair kit (Epicentre). Repaired fragments were subjected to A tailing using *Taq* DNA polymerase (Roche Inc. USA, Chicago, IL); custom "Y" adaptors were then ligated to A-tailed fragments using T4 DNA ligase (New England BioLabs, Beverly, MA). Libraries were size selected and quantified using standard agarose gel electrophoresis. Sequencing (single-end, 36-bp reads) was performed on an Illumina GA IIx genome analyzer according to the manufacturer's instructions (Illumina, San Diego, CA). Alignments of short reads were performed using the software program MAQ (31). *De novo* assembly of unmappable and poorly mapped reads was performed using the program EDENA (32).

**Transposon mutagenesis.** To create a set of random transposon mutants, the $Pm^r$ strain PAK Δ*pmrAB phoQ6* (23) was conjugated with the donor strain SM10 λpir pUT::mTn*5-aacC1* (kindly provided by E. P. Greenberg, University of Washington, Seattle, WA), using the filter method (33). The conjugation mix was plated on LA supplemented with 100 mg/liter GEN and 10 mg/liter chloramphenicol to select for transconjugants and counterselect against the donor strain. Individual transconjugants were manually arrayed in 96-well plates containing LB with 20% glycerol (0.2 ml per well) and stored at −80°C.

To screen the set of PAK Δ*pmrAB phoQ6* mTn*5-aacC1* transconjugants ($n = 8{,}836$) for loss of Pm resistance, a 96-pin replicator was used to inoculate 96-well plates containing LB with 20 mg/liter GEN (0.2 ml per well), which were then incubated overnight at 30°C. The 96-pin replicator was used to transfer the regrown transconjugant set to OmniTray plates containing LA with 1 mM $MgCl_2$ and 0 or 100 mg/liter PMB, the highest concentration that consistently permitted growth of pin-inoculated PAK Δ*pmrAB phoQ6* in pilot experiments. The inoculated plates were incubated at 30°C overnight and then at ambient temperature for six additional days. Growth was examined and recorded on days 1, 2, 3, and 7. Transconjugants with loss of Pm resistance ($n = 127$) were retested using the PMB plate assay (0, 5, and 20 mg/liter).

Transposon insertion sites were identified using a two-step semidegenerate PCR method (34). A mixture of semidegenerate primers (SM1368 and SM1369) was used in combination with the mini-Tn*5* (mTn*5*)-specific primer (SM1373) in the first PCR step. A primer designed to hybridize with the 5′ (nondegenerate) portion of the semidegenerate primers (SM1370) was used in combination with a nested mTn*5*-specific primer (SM1371) in the second PCR step. The product of the second PCR step was sequenced using primer SM1371. The insertion site for some transconjugants was reconfirmed using locus-specific primers. Primer sequences are listed in Table S1 in the supplemental material.

**Strain construction.** Chromosomal deletions were constructed as described previously (23) using the Gateway cloning system (Invitrogen, Carlsbad, CA), with specific oligonucleotide primers listed in Table S1 in the supplemental material. Each deletion was marked with a unique HindIII restriction site; to confirm deletions, the region surrounding the target gene was PCR amplified from chromosomal DNA and digested with the restriction endonuclease HindIII to detect the unique marker. 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 (23). Expression plasmids were constructed using Gateway technology as described previously (23), with specific oligonucleotide primers listed in Table S1. The Quick-Change site-directed mutagenesis method (Stratagene) was used to introduce the CprS R241C mutation into expression plasmids containing a wild-type (WT) *cprS* allele, according to the manufacturer's instructions. The PCR amplification step was performed using the oligonucleotide primers SM1620 and SM1621, with plasmid pDONR221::*colR*$^{+}$*S*$^{+}$ as the template. To eliminate the parental DNA template, this PCR mixture was incubated with DpnI (New England BioLabs) at 37°C for 1.5 h. To confirm the mutagenesis product, the oligonucleotide primers SM1562, SM1563, SM1516, SM1517, and SM1624 were used.

**Susceptibility testing.** CST agar dilution testing and PMB plate assay were carried out and interpreted as described previously (23, 35) with the following modifications. LA plates and LB were supplemented with 1 mM $MgCl_2$. For testing of strains that contained expression plasmids, inocula were prepared using prolonged induction (24-h exposure to inducer) or brief induction (4-h exposure to inducer). For prolonged induction, strains with pJN105D-derived plasmids were streaked to LA containing GEN and L-arabinose (L-Ara) at 0.1% (strong induction) or 0.025% (weak induction) and incubated at 30°C for 20 h. Several colonies from this plate were inoculated into LB containing GEN and 0.1% or 0.025% L-Ara and incubated at 30°C with aeration for 4 h. For brief induction, strains with pJN105D-derived plasmids were streaked to LA containing GEN and incubated at 30°C for 20 h. Several colonies from this plate were inoculated into LB containing GEN and incubated at 30°C with aeration for 20 h. An aliquot from this culture was diluted 1:50 into LB containing GEN and 0.1% or 0.025% L-Ara and incubated at 30°C with aeration for 4 h. To test the plasmid-containing strains, 0.1% or 0.025% L-Ara was added to LA for the PMB plate assay. For the PMB plate assay, the MIC was defined as the lowest concentration with <10% survival. For CST agar dilution and PMB plate assays, an MIC of ≥8 but <64 mg/liter was interpreted as low-level resistance, ≥64 but <512 mg/liter was interpreted as moderate resistance, and ≥512 mg/liter was interpreted as high-level resistance.

**Lipid A isolation and analysis.** LPS was isolated after growth in LB with 1 mM $MgCl_2$ (36). Lipid A was isolated from LPS by hydrolysis (37). The lipid A structure was analyzed using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry in negative-ion, linear mode (38). All MALDI-TOF analyses were performed on a Waters MALDI micro MX mass spectrometer. The matrix used for lipid A analysis was 5-chloro-2-mercaptobenzothiazole (20 mg/liter in 1:1 chloroform-methanol).

## RESULTS

**Transposon insertion in PA4381 (*colR*), PA3078 (*cprS*), or 39 other loci abrogates PhoPQ-mediated Pm resistance.** To define loci necessary for PhoPQ-mediated Pm resistance, we analyzed a bank of transposon mutants of the Pm$^{r}$ *P. aeruginosa* strain PAK Δ*pmrAB phoQ6* (CST MIC, 8 mg/liter) (23) that had been created with a mini-Tn*5* transposon carrying a selectable GEN resistance marker (mTn*5*-*aacC1*). A primary screen of transconjugants ($n$ = 8,836) on medium with or without PMB yielded 127 candidates. PMB plate assays repeated on at least two occasions confirmed 71 transconjugants with partial or complete loss of Pm resistance. PCR amplification and sequence analysis of 63 transconjugants revealed transposon insertion in 41 distinct loci (Table 2); for the other eight transconjugants, the transposon insertion site was not identified.

Given the *phoQ* strain background and the known role of the L-Ara4N biosynthetic operon in Pm resistance, identification of transposon insertions within *phoP* (PA1179), *arnC* (PA3553), and *arnT* (PA3556) provided an important validation of the method. The transposon analysis implicated cell wall biogenesis genes, such as *oprF* (PA1777), *galU* (PA2023), *oprI* (PA2853), *prc* (PA3257), and *mpl* (PA4020); the *pagL* gene (PA4661) encoding a lipid A deacylase (39, 40); genes within the LPS core biosynthesis cluster (PA5002-5004), including *wapH* and *mig-14* (41, 42); and *algC* (PA5322), a gene required for biosynthesis of full-length LPS (41). The analysis also implicated a locus (PA4476) encoding a large hypothetical protein with C-terminal homology to the AsmA proteins of *E. coli* and *Yersinia pestis*; null mutations in *asmA* are associated with decreased LPS levels in *E. coli* (43), while induction of *asmA* transcription is associated with increased antimicrobial peptide resistance in *Y. pestis* (44). In addition to these loci, which are important for outer membrane stability, the analysis implicated an operon (PA1768-1766) encoding a probable zinc protease, a membrane-tethered transglutaminase, and an α-L-glutamate ligase homologue; each of these could plausibly contribute to antimicrobial peptide resistance through modification of proteins and peptides at the cell wall.

Some *P. aeruginosa* clinical isolates with stable Pm resistance do not have mutations in *phoPQ*, *pmrAB*, or *parRS* (data not shown), which encode three regulatory systems known to be involved in such resistance. This suggests that additional regulatory systems are involved in Pm resistance and might be mutated in such isolates. Transposon-disrupted loci encoding novel regulatory systems were therefore deemed to be of special interest for further analysis. Two such loci, PA4381 (disrupted in transconjugant strain 3882) and PA3078 (disrupted in transconjugant strain 3897), encode homologues of the ColRS two-component regulatory system of *Pseudomonas fluorescens* (45) and *Pseudomonas putida* (46). PA4380, downstream of PA4381 on the minus strand of the PAO1 chromosome, encodes a sensor kinase previously designated *colS* (47); PA4381 encodes a response regulator that we designated *colR*. *P. aeruginosa* ColS shares 76% similarity with that of *P. fluorescens*, while the ColR homologues are 91% similar (29). PA3078 encodes a sensor kinase, and the upstream locus PA3077 encodes the cognate response regulator; these were recently designated *cprS* and *cprR*, respectively (48). *P. aeruginosa* CprS is 45% similar to ColS of *P. fluorescens*, while CprR is 68% similar to ColR of *P. fluorescens*. In addition, the transposon analysis identified PA1559, encoding a hypothetical protein; the CprRS system was previously shown to regulate this locus (48).

**Deletion of *colRS* or *cprRS* abrogates the Pm resistance of a *P. aeruginosa* Δ*phoQ* strain.** Similar to the phenotypic effect of the *phoQ6* allele, which contains a frameshift mutation, *phoQ* deletion or disruption also confers Pm resistance in a *phoP*-dependent fashion (20, 22, 23); the CST MIC is 32 mg/liter for the PAK Δ*phoQ* mutant, compared to 0.5 mg/liter for the PAK wild-type (WT) strain. To confirm the dependence of PhoPQ-mediated Pm resistance on the ColRS and CprRS two-component systems, strains with clean deletion of genes in the *colRS* and *cprRS* loci were constructed in the PAK WT and Δ*phoQ* backgrounds. Deletion of *colRS* largely abrogated the Pm resistance that *phoQ* deletion confers in strain PAK (Fig. 1A). Deletion of *colR* or *colS* in the Δ*phoQ* background was similar in phenotypic effect to deletion of *colRS* (Fig. 1A). The *colRS* deletions (Δ*colR*, Δ*colS*, and Δ*colRS*; strains 3749, 3751, and 3752, respectively) did not have a detectible effect on Pm susceptibility in the WT background (see Table S2 in the supplemental material, which lists PMB plate assay MICs for strains without expression plasmids).

Deletion of *cprRS* in the Δ*phoQ* background resulted in substantial loss of Pm resistance (Fig. 1B). Deletion of *cprR* or *cprS*

**TABLE 2** Transposon insertions in PAK Δ*pmrAB phoQ6* that abrogate Pm resistance[a]

| Strain and gene ortholog | Gene name and/or function | Functional category | No. of hits |
|---|---|---|---|
| PAO1 | | | |
| PA0020 | Hypothetical; LysM-like domain | Cell wall degradation | 1 |
| PA0666 | Hypothetical; chaperone/anhydro-*N*-acetylmuramate kinase | Posttranslation modification/cell wall | 1 |
| PA0667 | Hypothetical; LysM-like domain | Cell wall biogenesis | 1 |
| PA0766 | *mucD*; trypsin-like serine protease | Posttranslation modification/alginate | 1 |
| PA0809 | Transporter (probable) | Inorganic ion transport | 1 |
| PA0846 | Sulfate transporter (probable) | Inorganic ion transport | 1 |
| PA1049 | *pdxH*; pyridoxine 5′-phosphate oxidase | Coenzyme metabolism | 1 |
| PA1064 | Hypothetical lipoprotein | LPS biogenesis | 2 |
| PA1179 | *phoP*; response regulator | Polymyxin resistance | 1 |
| PA1559 | Hypothetical; nucleoside-sugar epimerase domain | Cell wall biogenesis/$CH_2O$ metabolism | 1 |
| PA1766 | Hypothetical; probable alpha-L-glutamate ligase | Posttranslation modification/cell wall | 3 |
| PA1767 | Hypothetical; probable transglutaminase | Posttranslation modification/cell wall | 6 |
| PA1768 | Hypothetical; probable zinc protease | Posttranslation modification/cell wall | 1 |
| PA1777 | *oprF*; major outer membrane porin | Cell wall biogenesis | 1 |
| PA1802 | *clpX*; ATP-dependent protease | Posttranslation modification/cell wall | 1 |
| PA2023 | *galU*; Glc-1-P uridylyl transferase | Cell wall biogenesis | 1 |
| PA2397 | *pvdE*; pyoverdine biosynthesis | Secondary metabolite biosynthesis | 1 |
| PA2653 | Permease (probable) | Small molecule transport | 1 |
| PA2853 | *oprI*; outer membrane lipoprotein | Cell wall biogenesis | 1 |
| PA2854 | Hypothetical; LD transpeptidase domain | Cell wall biogenesis | 1 |
| **PA3078** | ***cprS*; two-component sensor kinase** | **Signal transduction** | **1** |
| PA3182 | 6-Phosphogluconolactonase | $CH_2O$ metabolism | 2 |
| PA3257 | *prc*; periplasmic protease | Cell wall biogenesis | 1 |
| PA3553 | *arnC*; 4-amino-L-arabinose transferase | Lipid A modification | 3 |
| PA3556 | *arnT*; 4-amino-L-arabinose transferase | Lipid A modification | 1 |
| PA3737 | *dsbC*; thiol-disulfide interchange | Posttranslation modification/chaperone | 4 |
| PA4020 | *mpl*; UDP-*N*-acetylmuramate:L-alanyl-gamma-D-glutamyl-meso-diaminopimealate ligase | Cell wall biogenesis | 1 |
| PA4265/PA4277 | *tufA*/*tufB*; elongation factors (paralogs) | Translation (ribosome structure/biogenesis) | 1 |
| **PA4381** | ***colR*; two-component response regulator** | **Signal transduction** | **1** |
| PA4476 | Hypothetical; AsmA homology | LPS biogenesis | 4 |
| PA4661 | *pagL*; lipid A 3-*O*-deacylase | Lipid A modification | 1 |
| PA4689 | Hypothetical; paraquat-inducible protein B | Pathogenesis | 1 |
| PA4732 | *pgi*; Glc-6-P isomerase | $CH_2O$ metabolism | 2 |
| PA5002 | Hypothetical; LmbE homology | Cell wall biogenesis | 1 |
| PA5003 | *mig14*; antimicrobial resistance protein | Antimicrobial resistance | 3 |
| PA5004 | *wapH*; glycosyltransferase ($Glc^{II}$, LPS outer core) | LPS biogenesis | 3 |
| PA5296 | *rep*; ATP-dependent DNA helicase | DNA replication and repair | 1 |
| PA5322 | *algC*; phosphomannomutase | $CH_2O$ metabolism/LPS and alginate | 1 |
| PA5332 | *crc*; catabolite repression control protein | DNA replication and repair/carbon metabolism | 1 |
| PALES (Liverpool epidemic strain) | | | |
| 19121 | Upstream of *wbpR*; GT1 glycosyltransferase | Cell wall biogenesis | 1 |
| 19131 | *asnB*; asparagine synthase | Amino acid metabolism/cell wall | 1 |

[a] Loci chosen for further analysis are indicated in bold.

alone in the Δ*phoQ* background was similar in phenotypic effect to Δ*cprRS*. None of these *cprRS* deletions had a detectible effect on Pm susceptibility in the WT background (strains 3806, 3802, and 3804, respectively) when the mutants were grown under $Mg^{2+}$-replete conditions (see Table S2 in the supplemental material).

**Effect of *colRS* or *cprRS* deletion on lipid A 4-amino-L-arabinose modification in the PAK Δ*phoQ* strain.** Addition of L-Ara4N to the 1 and 4′ phosphate groups of lipid A is associated with Pm resistance in *P. aeruginosa* and other Gram-negative organisms (49). The *arnBCADTEF-pmrE* operon encodes enzymes responsible for biosynthesis and attachment of L-Ara4N to lipid A (50). To examine the hypothesis that deletions in the *colRS* and *cprRS* loci abrogate Pm resistance through their effect on this modification, lipid A from the WT strain, Δ*phoQ* mutant, and *colRS* or *cprRS* deletion mutants in the Δ*phoQ* background was purified and analyzed by MALDI-TOF mass spectrometry.

Lipid A from the WT had major peaks at mass/charge ratio (*m*/*z*) 1,617 and 1,447 (Table 3), corresponding to hexa- and penta-acylated species that differ in the presence or absence of 3-hydroxydecanoate ($\Delta m/z = -170$) (Fig. 2A). It also had a minor peak at *m*/*z* 1,685, corresponding to addition of palmitate (C16:0) to the penta-acylated species. Lipid A from the Δ*phoQ* mutant had the major peaks at *m*/*z* 1,617 and 1,447, with addi-

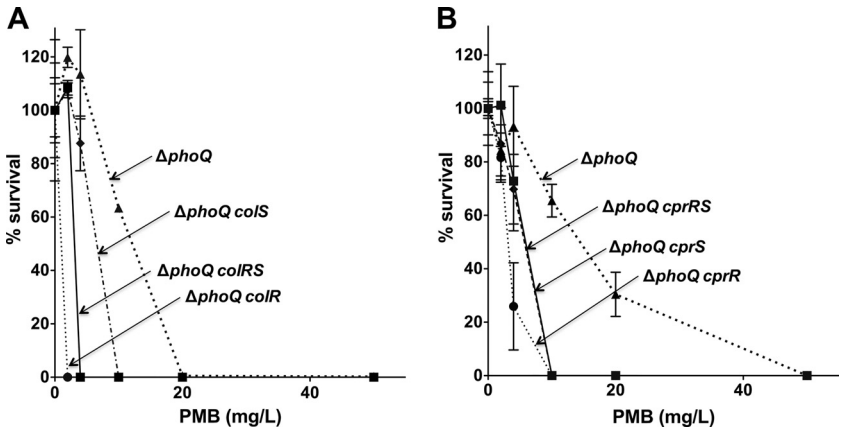


**FIG 1** Effect of *colRS* or *cprRS* deletion on Pm resistance in a Δ*phoQ* background. (A) PMB plate assay of *P. aeruginosa* PAK-derived strains 3932 (Δ*phoQ colR*, circles), 3934 (Δ*phoQ colS*, diamonds), and 3936 (Δ*phoQ colRS*, squares), with 2326 (Δ*phoQ*, triangles) as a positive control. (B) PMB plate assay of strains 3969 (Δ*phoQ cprR*, circles), 3970 (Δ*phoQ cprS*, diamonds), and 3975 (Δ*phoQ cprRS*, squares), with 2326 as a positive control.

tional major peaks at *m*/*z* 1,578, 1,748, and 1,879 (Fig. 2B), corresponding to addition of one or two L-Ara4N residues (Δ*m*/*z* = +131) to the hexa- and penta-acylated species (Fig. 2A). It also had minor peaks at *m*/*z* 1,709, corresponding to addition of two L-Ara4N residues to the penta-acylated species, and at *m*/*z* 1,685, 1,947, and 1,986, corresponding to addition of C16:0 to various species.

Lipid A from the Δ*phoQ* Δ*colR* strain had major peaks at *m*/*z* 1,617 and 1,447 but none of the additional major peaks present in lipid A from the Δ*phoQ* strain (Fig. 2C) and thus was largely indistinguishable from WT lipid A. Lipid A from the Δ*phoQ* Δ*colS* strain had major peaks at *m*/*z* 1,617 and 1,447 but only minor peaks at *m*/*z* 1,578 and 1,748 (Table 3), indicating decreased L-Ara4N abundance compared to that for lipid A from the Δ*phoQ* strain. Lipid A from these mutants had one or more minor peaks indicating the presence of C16:0.

Surprisingly, lipid A from the Δ*phoQ* Δ*colRS* strain had major peaks at *m*/*z* 1,617, 1,447, 1,578, and 1,748 (Fig. 2D), indicating L-Ara4N abundance comparable to that of lipid A from Δ*phoQ*, despite the diminished Pm resistance of this *colRS* mutant. It also had minor peaks indicating the presence of C16:0. Similarly, lipid A from the Δ*phoQ* Δ*cprR*, Δ*phoQ* Δ*cprS*, and Δ*phoQ* Δ*cprRS* strains had the same major peaks as lipid A from the Δ*phoQ* strain, indicating abundant L-Ara4N modification, despite the diminished Pm resistance of these mutants (Table 3).

**Prolonged strong induction of episomal *colRS* WT allele in PAK Δ*phoQ* Δ*colRS* restores Pm resistance.** Episomal expression of the *colRS* WT allele (derived from strain PAK) in the Δ*phoQ* Δ*colRS* strain restored Pm resistance (Fig. 3A), in response to prolonged strong induction (0.1% L-Ara for 24 h), compared to that of the Δ*phoQ* strain as a positive control. In contrast, brief strong induction (0.1% L-Ara for 4 h) of this allele was insufficient to complement the Pm susceptibility phenotype of the Δ*phoQ* Δ*colRS* strain (data not shown). Prolonged strong induction of $colR^+$ in the Δ*phoQ* Δ*colR* strain or of $colS^+$ in the Δ*phoQ* Δ*colS* strain also failed to complement the Pm susceptibility phenotype of these strains (see Table S3 in the supplemental material, which lists PMB plate assay MICs for strains with expression plasmids).

**Prolonged weak induction of episomal *colR21S21* mutant allele in PAK Δ*phoQ* Δ*colRS* restores Pm resistance.** Previous work had shown that a *P. aeruginosa* clinical isolate (strain 1577) with high-level Pm resistance (CST MIC > 512 mg/liter) carries the *phoQ21* mutant allele (23). This strain was isolated from a Copenhagen CF patient continuously treated with inhaled CST.

Whole-genome sequencing of this strain revealed a point mutation in *colR*, C4912731T, resulting in a missense mutation (Asp32Asn) in the ColR receiver domain near the Asp51 active site. This mutation was not found in the whole-genome sequences of earlier *phoQ21* mutants from the same patient,

**TABLE 3** Lipid A modifications of *colRS* and *cprRS* mutants in PAK Δ*phoQ* background

| Strain | Genotype | PMB plate assay MIC[a] (mg/liter) | Major peaks in MALDI-TOF spectrum (*m*/*z*) | Lipid A modifications[b] | |
|---|---|---|---|---|---|
| | | | | L-Ara4N | C16:0 |
| 1026 | WT | 2 | 1447, 1617 | − | +/− |
| 2326 | Δ*phoQ* | 50 | 1447, 1578, 1617, 1748, 1879 | ++ | + |
| 3932 | Δ*phoQ colR* | 2 | 1447, 1617 | − | + |
| 3934 | Δ*phoQ colS* | 10 | 1447, 1617 | + | +/− |
| 3936 | Δ*phoQ colRS* | 4 | 1447, 1578, 1617, 1748, 1879 | ++ | + |
| 3969 | Δ*phoQ cprR* | 7 | 1447, 1578, 1617, 1748, 1879 | ++ | + |
| 3970 | Δ*phoQ cprS* | 10 | 1447, 1578, 1617, 1748, 1879 | ++ | + |
| 3975 | Δ*phoQ cprRS* | 4 | 1447, 1578, 1617, 1748, 1879 | ++ | + |

[a] Median MICs are shown with rounding to nearest whole number.
[b] ++, present in ≥2 major peaks; +, present in ≥2 minor peaks; +/−, single minor peak; −, absent.

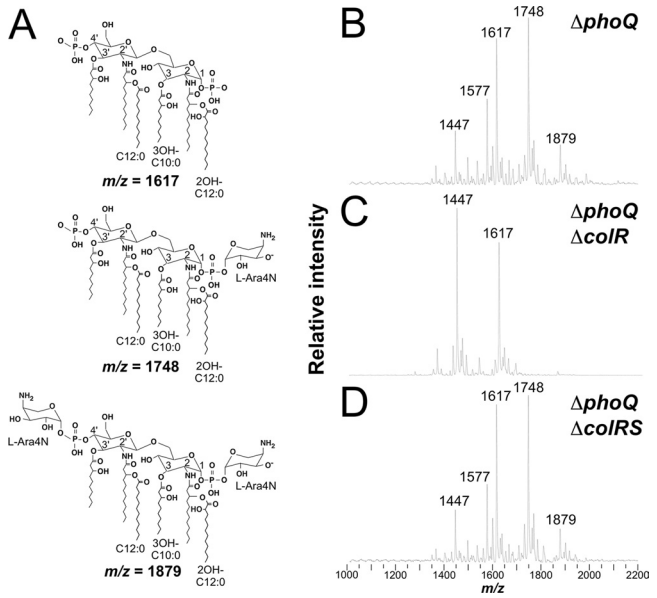


**FIG 2** Lipid A structures of *colRS* deletion strains. (A) Diagram of lipid A modifications found in strain 2326 (Δ*phoQ*). Position of acyl-oxy-acyl 2-hydroxy-laurate groups at the 2 position of the structures is based on the work of Karunaratne et al. (73). MALDI-TOF mass spectra for strain 2326 (Δ*phoQ*) (B), strain 3932 (Δ*phoQ* Δ*colR*) (C), and strain 3936 (Δ*phoQ* Δ*colRS*) (D) are shown.

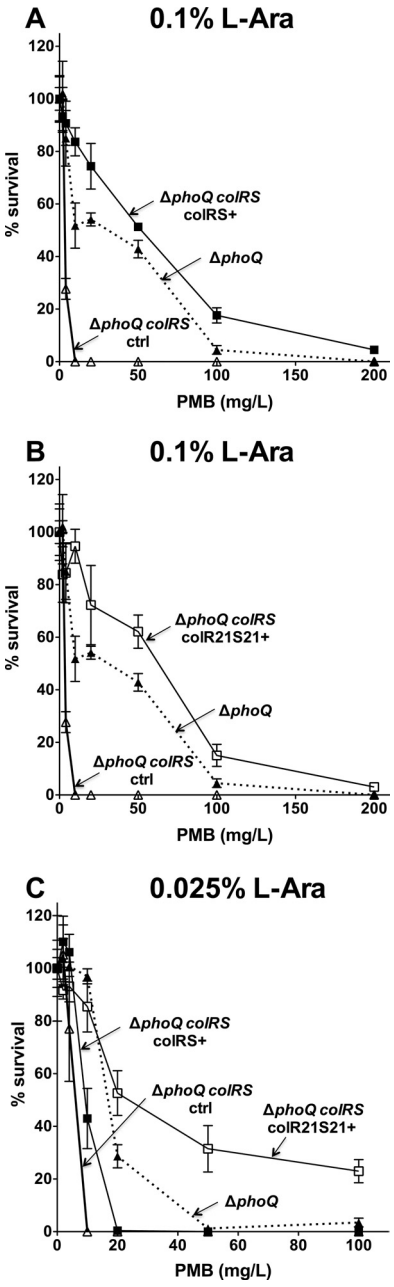


**FIG 3** Effect of episomal *colRS* WT or mutant alleles on Pm resistance in a Δ*phoQ* Δ*colRS* background. (A) PMB plate assay of strain 4044 (Δ*phoQ colRS* colRS+, squares) induced with 0.1% L-Ara for 24 h, with strain 2326 (Δ*phoQ*, triangles) as a positive control and strain 4050 (Δ*phoQ colRS* ctrl, open triangles) as a negative control. (B) PMB plate assay of strain 4043 (Δ*phoQ colRS* colR21S21+, open squares) induced with 0.1% L-Ara for 24 h, with the same controls as in panel A. (C) PMB plate assay of strains 4044 and 4043 induced with 0.025% L-Ara for 24 h, with the same controls as in panel A.

namely, Pm-susceptible (Pm$^s$) strain 1581 (CST MIC 0.5 mg/liter) and highly Pm$^r$ strain 1579 (CST MIC > 512 mg/liter). Whole-genome sequencing of strain 1577 also revealed a point mutation in *colS*, G4911835A, resulting in a missense mutation (Ala106Val) in the ColS extracytosolic loop; this mutation was found in highly Pm$^r$ strain 1579 but not Pm$^s$ strain 1581. Neither of these *colRS* mutations was found in *phoQ21* mutants from a different Copenhagen CF patient, Pm$^s$ strain 1601 (CST MIC 0.25 mg/liter) and highly Pm$^r$ strains 1021 and 1598 (CST MIC > 512 mg/liter).

To define their role in clinical Pm resistance, these *colRS* mutant alleles, designated *colR21S21*, were PCR amplified and inserted into pJN105D. Prolonged strong induction of the bicistronic *colR21S21* mutant allele in the Δ*phoQ* Δ*colRS* strain restored a level of Pm resistance similar to that conferred by the WT allele (Fig. 3B). As seen with the WT allele, brief strong induction was insufficient to complement the Pm susceptibility phenotype, and prolonged strong induction of monocistronic *colR21* in the Δ*phoQ* Δ*colR* strain (strain 4039) or of monocistronic *colS21* in the Δ*phoQ* Δ*colS* strain (strain 4035) failed to complement Pm susceptibility (see Table S3 in the supplemental material). Interestingly, prolonged weak induction (0.025% L-Ara for 24 h) of *colR21S21* in the Δ*phoQ* Δ*colRS* strain was sufficient to restore Pm resistance, whereas prolonged weak induction of the *colRS* WT allele was not (Fig. 3C).

To determine the effect of the *colRS* WT and *colR21S21* mutant alleles on Pm susceptibility independent of *phoQ* deletion, each was expressed in the Δ*colRS* background (strains 4115 and 4113, respectively) with prolonged strong induction. These alleles did not decrease the Pm susceptibility of this strain, compared to results for an empty vector (see Table S3).

More surprisingly, prolonged strong induction of these alleles in the Δ*phoQ* background (strains 4175 and 4173) failed to augment Pm resistance (see Table S3). This indicates that the effect of *colR21S21* on Pm susceptibility is genetically recessive to the chromosomal *colRS* WT allele present in this strain background.

**Duration of *cprR* WT allele induction in Δ*phoQ* Δ*cprRS* strains determines Pm resistance level.** The *cprRS*, *cprR*, and *cprS* WT alleles from PAK were inserted into pJN105D. In response to

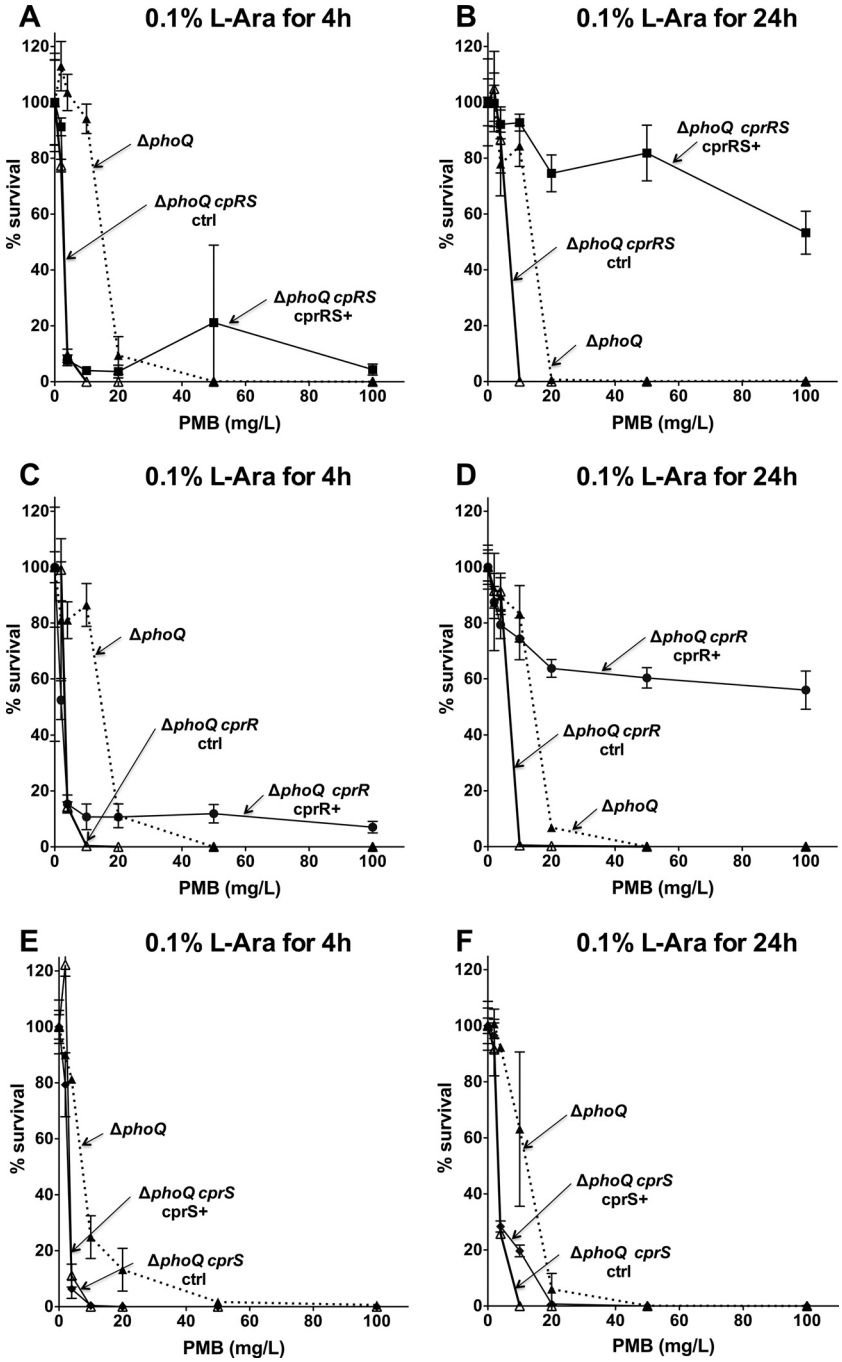


**FIG 4** Effect of episomal *cprRS* WT alleles on Pm resistance in corresponding Δ*phoQ* Δ*cprRS* backgrounds. (A and B) PMB plate assay of strain 4103 (Δ*phoQ cprRS* cprRS+, squares) induced with 0.1% L-Ara for 4 h (A) or 24 h (B), with strain 2326 (Δ*phoQ*, filled triangles) as a positive control and strain 4105 (Δ*phoQ cprRS* ctrl, open triangles) as a negative control. (C and D) PMB plate assay of strain 4099 (Δ*phoQ cprR* cprR+, circles) induced with 0.1% L-Ara for 4 h (C) or 24 h (D), with strain 2326 as a positive control and strain 4101 (Δ*phoQ cprR* ctrl, open triangles) as a negative control. (E and F) PMB plate assay of strain 4065 (Δ*phoQ cprS* cprS+, diamonds) induced with 0.1% L-Ara for 4 h (E) or 24 h (F), with strain 2326 as a positive control and strain 4069 (Δ*phoQ cprS* ctrl, open triangles) as a negative control.

brief strong induction (0.1% L-Ara for 4 h), expression of the bicistronic *cprRS* WT allele in the Δ*phoQ* Δ*cprRS* strain restored moderate Pm resistance to a subpopulation of ~10 to 20% (Fig. 4A), compared to results for the Δ*phoQ* strain as a positive control. With prolonged strong induction (0.1% L-Ara for 24 h), this strain displayed moderate resistance for ~60 to 80% of the population, substantially greater than that of the Δ*phoQ* positive control (Fig. 4B).

Similarly, brief strong induction of the monocistronic *cprR* WT allele in the Δ*phoQ* Δ*cprR* strain restored moderate resistance to a subpopulation of ~10% (Fig. 4C), while prolonged strong induction conferred moderate resistance on ~60% of

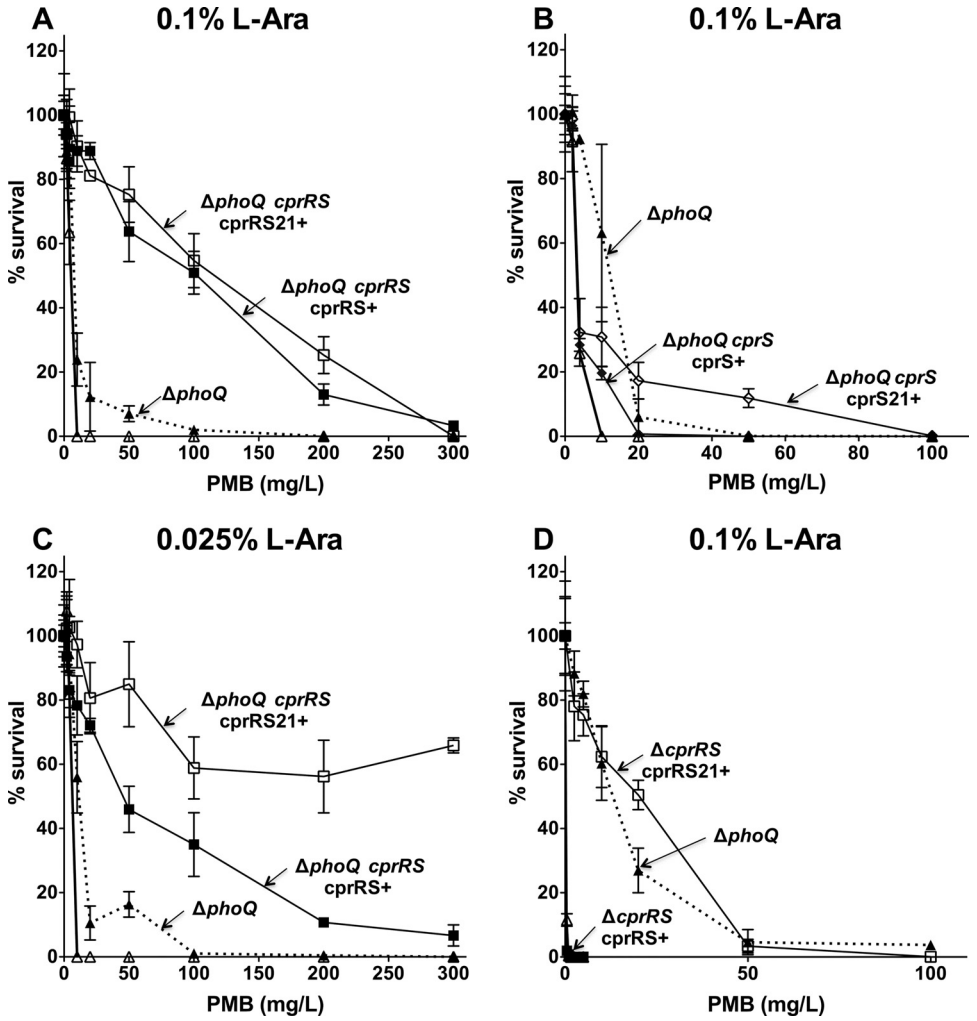


**FIG 5** Effect of episomal *cprS21* mutant allele, with or without *cprR* WT allele, on PhoPQ-mediated Pm resistance. (A) PMB plate assay of strain 4200 (Δ*phoQ cprRS* cprRS21+, open squares) and strain 4103 (Δ*phoQ cprRS* cprRS+, squares) induced with 0.1% L-Ara for 24 h, with strain 2326 (Δ*phoQ*, triangles) as a positive control. The unlabeled line (open triangles) is a negative control (strain 4105, Δ*phoQ cprRS* with empty vector). (B) PMB plate assay of strain 4063 (Δ*phoQ cprS* cprS21+, open diamonds) and strain 4065 (Δ*phoQ cprS* cprS+, filled diamonds) induced with 0.1% L-Ara for 24 h, with strain 2326 as a positive control. The unlabeled line (open triangles) is a negative control (strain 4069, Δ*phoQ cprS* with empty vector). (C) PMB plate assay of the same strains as in panel A, induced with 0.025% L-Ara for 24 h. (D) PMB plate assay of strains 4240 (Δ*cprRS* cprRS21+, open squares) and 4131 (Δ*cprRS* cprRS+, filled squares) induced with 0.1% L-Ara for 24 h, with strain 2326 as a positive control. The unlabeled line (open triangles) is a negative control (strain 4133, Δ*cprRS* with empty vector).

the population (Fig. 4D). In contrast, both brief and prolonged strong induction of the monocistronic *cprS* WT allele in the Δ*phoQ* Δ*cprS* strain conferred negligible resistance (Fig. 4E and F).

**Prolonged weak induction of *cprR*$^+$*S21* mutant allele in the Δ*phoQ* Δ*cprRS* strain maximally enhances Pm resistance.** Whole-genome sequencing of highly Pm$^r$ strain 1577 revealed a single point mutation in *cprS*, C3452226T, resulting in a missense mutation (Arg241Cys) in the CprS histidine kinase domain, close to the predicted His245 active site. This mutant allele, which we have designated *cprS21*, was also found in the whole-genome sequences of earlier *phoQ21* mutants from the same Copenhagen CF patient (Pm$^s$ strain 1581 and Pm$^r$ strain 1579) and of *phoQ21* mutants from a different patient (Pm$^s$ strain 1601 and highly Pm$^r$ strains 1021 and 1598). To define the role of *cprS21* in clinical Pm resistance, it was PCR amplified and inserted into pJN105D.

Prolonged strong induction of the bicistronic *cprR*$^+$*S21* mutant allele in the Δ*phoQ* Δ*cprRS* strain conferred moderate Pm resistance on ~50% of the population, with an MIC of >100 mg/liter, comparable to the effect of the bicistronic *cprRS* WT allele (Fig. 5A). In contrast, prolonged strong induction of the monocistronic *cprS21* mutant allele in the Δ*phoQ* Δ*cprS* strain conferred moderate resistance on only ~10% of the population, with an MIC of 100 mg/liter; however, this was substantially greater than the effect of the monocistronic *cprS* WT allele, which conferred 20% low-level resistance with an MIC of only 20 mg/liter (Fig. 5B). Unexpectedly, prolonged weak induction (0.025% L-Ara for 24 h) of the bicistronic *cprR*$^+$*S21* mutant allele conferred high-level Pm resistance (MIC > 300 mg/liter) on ~65% of the population (Fig. 5C), thus enhancing PhoPQ-mediated Pm resistance to a greater degree than did prolonged strong induction. In contrast, for the bicistronic WT allele, the effect of prolonged weak induction was similar to that of prolonged strong induction, con-

ferring ~20% moderate resistance and only ~5 to 10% high-level resistance.

To determine the effect of *cprRS* alleles on Pm susceptibility independent of *phoQ* deletion, bicistronic *cprRS* WT and *cprR*$^+$*S21* mutant alleles were each expressed in the Δ*cprRS* strain with prolonged strong induction. In this strain background, the *cprR*$^+$*S21* mutant allele conferred Pm resistance comparable to that of Δ*phoQ*, whereas the *cprRS* WT allele had no effect (Fig. 5D). Prolonged strong induction of the monocistronic *cprR* WT allele in the Δ*cprR* strain (strain 4128) conferred low-level resistance on ~5 to 10% of the population, while such induction of the monocistronic *cprS* WT or *cprS21* mutant alleles in a Δ*cprS* background (strains 4091 and 4093, respectively) had no effect on Pm susceptibility (see Table S3 in the supplemental material).

Prolonged strong induction of the bicistronic *cprRS* WT or *cprR*$^+$*S21* mutant alleles in Δ*phoQ* (strains 4236 and 4238, respectively) failed to augment Pm resistance (see Table S3). This indicates that the effect of *cprR*$^+$*S21* is genetically recessive to the chromosomal *cprRS* WT allele present in this strain background.

## DISCUSSION

This work has defined multiple loci necessary for PhoPQ-mediated Pm resistance in *P. aeruginosa*, including four that encode novel two-component regulatory systems, ColRS and CprRS. Deletion of *colR*, *colS*, *cprR*, or *cprS* resulted in diminished Pm resistance in a Δ*phoQ* background but had no significant effect on susceptibility in a WT background. Prolonged strong induction of bicistronic ColRS in a Δ*phoQ* Δ*colRS* background restored Pm resistance, whereas brief strong induction of ColRS, or prolonged strong induction of monocistronic ColR or ColS, did not. In contrast, brief strong induction of bicistronic CprRS in a Δ*phoQ* Δ*cprRS* background restored Pm resistance, as did brief strong induction of monocistronic CprR in a Δ*phoQ* Δ*cprR* background; prolonged strong induction markedly enhanced the resistance of these strains.

Two-component systems function via phosphorylation of histidine on one protein (the histidine sensor kinase), followed by transfer of phosphate to aspartic acid, usually on a second protein (the response regulator). The histidine sensor kinase is generally bifunctional, with a kinase activity that phosphorylates the response regulator and a phosphatase activity that dephosphorylates it. Overexpressed sensor kinases may exhibit excessive phosphatase activity relative to kinase activity, leading to inactivation of the cognate response regulator (51); this may explain the failure of monocistronic ColS and CprS overexpression to complement the corresponding deletion mutants. The failure of monocistronic ColR overexpression to complement Δ*phoQ* Δ*colR* is more difficult to explain but may be attributable to the timing and/or intensity of its expression under the control of a constitutive non-native promoter. Nonetheless, the deletion and complementation results presented here indicate that the ColRS and CprRS two-component regulatory systems interact with the PhoPQ system to regulate Pm resistance.

The identification of mutant *colRS* and *cprS* alleles in a clinical *phoQ* mutant strain provides additional evidence of interaction among these systems. After transfer to an expression plasmid, prolonged weak induction of the *colR21S21* allele in a Δ*phoQ* Δ*colRS* background or of the *cprR*$^+$*S21* allele in a Δ*phoQ* Δ*cprRS* background enhanced Pm resistance more than the respective WT alleles did under the same conditions. This suggests that the mutations in the *colR21S21* and *cprS21* alleles heighten the capacity of each system to activate its regulon. Though episomal expression of these alleles resulted in complementation, the inducing conditions used to accomplish this were chosen through trial and error, without knowledge of the timing and strength of induction of these loci from their native promoters. These empirically determined inducing conditions could have led to a substantial underestimation of resistance enhancement.

In terms of clinical relevance, these results indicate that in Pm-exposed clinical strains, second-step mutations in the CprRS and ColRS systems enhance the Pm resistance that first-step mutations in the PhoPQ system confer. Continuous treatment of Copenhagen CF patients with inhaled CST since the early 1980s (11, 52, 53) provided selection pressure for the emergence of highly Pm$^r$ CF isolates of *P. aeruginosa* (23). The observation that some of these patients were failing to respond to continuous inhaled CST coincided with clinical detection of the *phoQ21* mutant and other *phoQ* mutant strains in the late 1990s. CST inhalation was stopped for such patients, likely facilitating the emergence of Pm$^s$ mutants with suppressor mutations in *phoP* and other loci.

The *cprS21* mutant allele was found in six *phoQ21* mutants isolated from two Copenhagen CF patients between 1997 and 2002, including two Pm$^s$ isolates with presumed secondary suppressor mutations. This indicates that the *cprS21* allele arose no later than 1997, presumably in response to the same initial selection pressure that selected for the *phoQ21* allele. First-step mutations in *phoQ* alone result in only low to moderate Pm resistance; data presented here support the hypothesis that second-step mutations, such as that found in the *cprS21* allele, contribute to high-level resistance. Here we have shown that the *cprS21* allele remains active in enhancement of PhoPQ-mediated Pm resistance even at low levels of induction, compared to the *cprS* WT allele. We speculate that this enhanced activity is attributable to the effects of the Arg241Cys H-box mutation in the cytosolic histidine kinase domain, which likely alters phosphatase activity relative to kinase activity of the CprS sensor protein. Such mutations typically activate the cognate response regulator (in this case, CprR), as shown for other two-component systems, including PmrAB (16, 54–56).

The subsequent resumption of CST inhalation in Copenhagen CF patients with suppressor mutants provided renewed selection pressure, which presumably promoted reemergence of Pm$^r$ strains with mutations in additional loci, such as *colRS*, that regulate L-Ara4N modification and other resistance mechanisms. The *colS21* allele was detected in Pm$^r$ *phoQ21* mutants isolated in 1999 and 2002, whereas the *colR21* allele was detected only in the *phoQ21* mutant isolated in 2002. This suggests that the *colS21* periplasmic domain mutation represents a discrete step resulting in increased Pm resistance in the *phoQ21 cprS21* background, but specific evidence to support this hypothesis is presently lacking. Similarly, we speculate that the activity-enhancing effect of the *colR21* allele, which has an Asp32Asn receiver domain mutation, is partial interference with the ColR-ColS interaction. This would diminish the capacity of the ColS histidine sensor kinase to act as a phosphatase, as shown for similar receiver domain mutations in other response regulators (57, 58). Taken together, these findings support the notion that mutations in the ColRS and CprRS systems represent regulatory mechanisms contributing to high-level clinical Pm resistance.

Other work in *P. aeruginosa* has shown that the *colRS* promoter is differentially expressed in the presence of Gram-positive oral

flora (*Streptococcus* and *Staphylococcus* spp.) from CF patients (59) and that the ColS sensor kinase is necessary for virulence in a *Caenorhabditis elegans* infection model (47), implicating the ColRS system in polymicrobial and host-pathogen interactions. ColRS-CprRS homologues were defined initially for *P. fluorescens*, in which the ColRS system is required for Pm tolerance and efficient root colonization of host plants (45, 60) and subsequently for *P. putida*, in which this system is required for root colonization (46), specific transpositional activity (61), phenol tolerance (62, 63), heavy metal tolerance (64), and protection against cell lysis in the context of metabolic responses to glucose limitation (63, 65). Interestingly, *oprF* (PA1777) and *crc* (PA5332) mediate this metabolic response in *P. putida* (65), and ColRS also regulates the gene product of *pagL* (PA4661), a lipid A deacylase, in this organism (66). The present work has implicated all three of these loci in *P. aeruginosa* Pm resistance. This suggests that the pseudomonads express these genes as part of a coordinated metabolic response protecting against membrane stress.

Single-gene deletion of *colS* or *colR* in a Δ*phoQ* background decreased or completely eliminated L-Ara4N modification of lipid A, presumably by interfering, either directly or indirectly, with expression of the *arnBCADTEF-pmrE* operon. Although the mechanism of this interference remains unknown, a variety of noncognate interactions have been observed among two-component regulatory systems of *P. aeruginosa* and other microbes (67–69). Thus, it is plausible that ColR or ColS could, in the absence of its cognate partner, interact aberrantly with PhoP, CprR, or CprS to inhibit aminoarabinosylation of lipid A.

In contrast, tandem deletion of *colRS* or *cprRS* in the Δ*phoQ* background or single-gene deletion of *cprRS* in this background abrogated Pm resistance without changing the mass spectral pattern of L-Ara4N modification of lipid A seen for the Pm$^r$ Δ*phoQ* mutant. These findings provide the first conclusive evidence that L-Ara4N modification alone is not sufficient for Pm resistance in *P. aeruginosa* and suggest that the ColRS and CprRS systems regulate additional factors necessary for resistance. This finding does not diminish the potential of lipid A aminoarabinosylation as a drug target, as confirmed through transposon (Tn) mutants with *arnC* or *arnT* disruption (Table 2), but it indicates that other factors could represent additional targets for the development of resistance inhibitors.

Insight into the potential identities of such factors comes from a recent screen of the comprehensive PA14 Tn mutant library, which contains ~5800 Tn mutants representing coverage of ~4,600 distinct nonessential open reading frames (ORFs), or ~90% of the nonessential *P. aeruginosa* genome. This screen defined only 17 genes as necessary for $Mg^{2+}$ depletion-induced PMB resistance (70). The present study analyzed a set of ~8,800 Tn mutants estimated to provide coverage of ~3,000 distinct nonessential ORFs, or ~60% of the nonessential genome (34), implicating 41 genes as necessary for PhoPQ-mediated PMB resistance. Aside from *phoPQ* itself (PA1179-1180), only two individual genes were implicated in both studies: *galU* (PA2023) and *mpl* (PA4020). Both of these studies also implicated adjacent genes (*wapR-ssg*-hypothetical-*mig14-wapH*, PA5000-5004) within the LPS core biosynthesis gene cluster (PA4996-5012) (41, 42). Among the other 12 genes implicated in $Mg^{2+}$ depletion-induced PMB resistance (70), *pmrA* (PA4776) and *parR* (PA1798) were described previously (16, 19, 26, 27). The present study has implicated 35 additional genes, of which only *arnC* (PA3553), *arnT* (PA3556), and *cprS* have been previously linked to *P. aeruginosa* Pm resistance (13, 48, 71). In addition, our study identified PA1559, encoding a hypothetical protein, as required for PhoPQ-mediated Pm resistance; the CprRS system has been shown to regulate this locus (48).

Our transposon screen may have included false positives as a consequence of sporadic secondary suppressor mutations of the *phoQ6* allele, which complicated a previous analysis of clinical *phoQ* mutants (23). On the other hand, this transposon screen provided only ~60% coverage of the nonessential genome and thus may significantly underestimate the total number of genes necessary for PhoPQ-mediated PMB resistance. In addition, both studies fail to account for resistome components that are necessary for PmrAB- or ParRS-mediated PMB resistance but that are not required for PhoPQ-mediated and $Mg^{2+}$ depletion-induced resistance. These limitations notwithstanding, ≥50 novel genes are presently implicated in the overall PMB resistome of *P. aeruginosa*, and we strongly suspect that others remain to be discovered.

This work has increased our understanding of *P. aeruginosa* Pm resistance, revealing unexpected complexity in key molecular mechanisms. At present, five two-component regulatory systems of *P. aeruginosa* are known to influence resistance to Pm and other cationic antimicrobial peptides: PmrAB (13, 16, 19, 24, 25, 72), PhoPQ (20, 22, 23, 25, 38, 72), ParRS (26, 27), CprRS (48), and ColRS. Of these, PmrAB, PhoPQ, and ParRS are known targets of first-step mutations resulting in low- to moderate-level Pm resistance. The ColR response regulator represents a novel, nonredundant intermediary that interacts with the PhoPQ system to regulate aminoarabinosylation of lipid A but also other undefined cellular factors necessary for Pm resistance. The CprR response regulator is similarly required for PhoPQ-mediated Pm resistance and has previously been shown to regulate the L-Ara4N biosynthetic locus (48) but is apparently redundant with the PhoP response regulator in this function. Nonetheless, evidence presented here indicates that both the ColRS and CprRS systems regulate required cellular factors besides L-Ara4N. Such cellular factors likely include the hypothetical protein encoded by PA1559 and others of the >50 novel gene products that transposon mutagenesis has defined as required for PhoPQ-mediated and $Mg^{2+}$ depletion-induced Pm resistance. Further delineation of these cellular factors is expected to broaden the range of potential targets for development of Pm resistance inhibitors beyond the current focus on L-Ara4N biosynthesis.

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## REFERENCES

1. **Michalopoulos A, Falagas ME.** 2008. Colistin and polymyxin B in critical care. Crit. Care Clin. **24**:377–391.
2. **Moskowitz SM, Silva SJ, Mayer-Hamblett N, Pasta DJ, Mink DR, Mabie**

**JA, Konstan MW, Wagener JS.** 2008. Shifting patterns of inhaled antibiotic use in cystic fibrosis. Pediatr. Pulmonol. **43**:874–881.
3. **Alonso A, Campanario E, Martinez JL.** 1999. Emergence of multidrug-resistant mutants is increased under antibiotic selective pressure in *Pseudomonas aeruginosa*. Microbiology **145**(Part 10):2857–2862.
4. **Henrichfreise B, Wiegand I, Pfister W, Wiedemann B.** 2007. Resistance mechanisms of multiresistant *Pseudomonas aeruginosa* strains from Germany and correlation with hypermutation. Antimicrob. Agents Chemother. **51**:4062–4070.
5. **Foweraker JE, Laughton CR, Brown DF, Bilton D.** 2009. Comparison of methods to test antibiotic combinations against heterogeneous populations of multiresistant *Pseudomonas aeruginosa* from patients with acute infective exacerbations in cystic fibrosis. Antimicrob. Agents Chemother. **53**:4809–4815.
6. **Storm DR, Rosenthal KS, Swanson PE.** 1977. Polymyxin and related peptide antibiotics. Annu. Rev. Biochem. **46**:723–763.
7. **Adams MD, Nickel GC, Bajaksouzian S, Lavender H, Murthy AR, Jacobs MR, Bonomo RA.** 2009. Resistance to colistin in *Acinetobacter baumannii* associated with mutations in the PmrAB two-component system. Antimicrob. Agents Chemother. **53**:3628–3634.
8. **Denton M, Kerr K, Mooney L, Keer V, Rajgopal A, Brownlee K, Arundel P, Conway S.** 2002. Transmission of colistin-resistant *Pseudomonas aeruginosa* between patients attending a pediatric cystic fibrosis center. Pediatr. Pulmonol. **34**:257–261.
9. **Falagas ME, Bliziotis IA.** 2007. Pandrug-resistant Gram-negative bacteria: the dawn of the post-antibiotic era? Int. J. Antimicrob. Agents **29**:630–636.
10. **Hawley JS, Murray CK, Jorgensen JH.** 2008. Colistin heteroresistance in *Acinetobacter* and its association with previous colistin therapy. Antimicrob. Agents Chemother. **52**:351–352.
11. **Johansen HK, Moskowitz SM, Ciofu O, Pressler T, Høiby N.** 2008. Spread of colistin resistant non-mucoid *Pseudomonas aeruginosa* among chronically infected Danish cystic fibrosis patients. J. Cyst. Fibros. **7**:391–397.
12. **Li J, Rayner CR, Nation RL, Owen RJ, Spelman D, Tan KE, Liolios L.** 2006. Heteroresistance to colistin in multidrug-resistant *Acinetobacter baumannii*. Antimicrob. Agents Chemother. **50**:2946–2950.
13. **Moskowitz SM, Brannon MK, Dasgupta N, Pier M, Sgambati N, Miller AK, Selgrade SE, Miller SI, Denton M, Conway SP, Johansen HK, Høiby N.** 2012. PmrB mutations promote polymyxin resistance of *Pseudomonas aeruginosa* isolated from colistin-treated cystic fibrosis patients. Antimicrob. Agents Chemother. **56**:1019–1030.
14. **Boll M, Radziejewska-Lebrecht J, Warth C, Krajewska-Pietrasik D, Mayer H.** 1994. 4-Amino-4-deoxy-L-arabinose in LPS of enterobacterial R-mutants and its possible role for their polymyxin reactivity. FEMS Immunol. Med. Microbiol. **8**:329–341.
15. **Nummila K, Kilpelainen I, Zahringer U, Vaara M, Helander IM.** 1995. Lipopolysaccharides of polymyxin B-resistant mutants of *Escherichia coli* are extensively substituted by 2-aminoethyl pyrophosphate and contain aminoarabinose in lipid A. Mol. Microbiol. **16**:271–278.
16. **Moskowitz SM, Ernst RK, Miller SI.** 2004. PmrAB, a two-component regulatory system of *Pseudomonas aeruginosa* that modulates resistance to cationic antimicrobial peptides and addition of aminoarabinose to lipid A. J. Bacteriol. **186**:575–579.
17. **Gunn JS, Lim KB, Krueger J, Kim K, Guo L, Hackett M, Miller SI.** 1998. PmrA-PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. Mol. Microbiol. **27**:1171–1182.
18. **Gunn JS, Ryan SS, Van Velkinburgh JC, Ernst RK, Miller SI.** 2000. Genetic and functional analysis of a PmrA-PmrB-regulated locus necessary for lipopolysaccharide modification, antimicrobial peptide resistance, and oral virulence of *Salmonella enterica* serovar Typhimurium. Infect. Immun. **68**:6139–6146.
19. **McPhee JB, Lewenza S, Hancock RE.** 2003. Cationic antimicrobial peptides activate a two-component regulatory system, PmrA-PmrB, that regulates resistance to polymyxin B and cationic antimicrobial peptides in *Pseudomonas aeruginosa*. Mol. Microbiol. **50**:205–217.
20. **Macfarlane EL, Kwasnicka A, Ochs MM, Hancock RE.** 1999. PhoP-PhoQ homologues in *Pseudomonas aeruginosa* regulate expression of the outer-membrane protein OprH and polymyxin B resistance. Mol. Microbiol. **34**:305–316.
21. **Roland KL, Martin LE, Esther CR, Spitznagel JK.** 1993. Spontaneous *pmrA* mutants of *Salmonella typhimurium* LT2 define a new two-component regulatory system with a possible role in virulence. J. Bacteriol. **175**:4154–4164.
22. **Macfarlane EL, Kwasnicka A, Hancock RE.** 2000. Role of *Pseudomonas aeruginosa* PhoP-PhoQ in resistance to antimicrobial cationic peptides and aminoglycosides. Microbiology **146**:2543–2554.
23. **Miller AK, Brannon MK, Stevens L, Johansen HK, Selgrade SE, Miller SI, Høiby N, Moskowitz SM.** 2011. PhoQ mutations promote lipid A modification and polymyxin resistance of *Pseudomonas aeruginosa* found in colistin-treated cystic fibrosis patients. Antimicrob. Agents Chemother. **55**:5761–5769.
24. **Abraham N, Kwon DH.** 2009. A single amino acid substitution in PmrB is associated with polymyxin B resistance in clinical isolate of *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. **298**:249–254.
25. **Barrow K, Kwon DH.** 2009. Alterations in two-component regulatory systems of *phoPQ* and *pmrAB* are associated with polymyxin B resistance in clinical isolates of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **53**:5150–5154.
26. **Fernandez L, Gooderham WJ, Bains M, McPhee JB, Wiegand I, Hancock RE.** 2010. Adaptive resistance to the "last hope" antibiotics polymyxin B and colistin in *Pseudomonas aeruginosa* is mediated by the novel two-component regulatory system ParR-ParS. Antimicrob. Agents Chemother. **54**:3372–3382.
27. **Muller C, Plesiat P, Jeannot K.** 2011. A two-component regulatory system interconnects resistance to polymyxins, aminoglycosides, fluoroquinolones, and beta-lactams in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **55**:1211–1221.
28. **Choi KH, Kumar A, Schweizer HP.** 2006. A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: application for DNA fragment transfer between chromosomes and plasmid transformation. J. Microbiol. Methods **64**:391–397.
29. **Winsor GL, Lam DK, Fleming L, Lo R, Whiteside MD, Yu NY, Hancock RE, Brinkman FS.** 2011. *Pseudomonas* Genome Database: improved comparative analysis and population genomics capability for *Pseudomonas* genomes. Nucleic Acids Res. **39**:D596–D600.
30. **Hayden HS, Lim R, Brittnacher MJ, Sims EH, Ramage ER, Fong C, Wu Z, Crist E, Chang J, Zhou Y, Radey M, Rohmer L, Haugen E, Gillett W, Wuthiekanun V, Peacock SJ, Kaul R, Miller SI, Manoil C, Jacobs MA.** 2012. Evolution of *Burkholderia pseudomallei* in recurrent melioidosis. PLoS One **7**:e36507. doi:10.1371/journal.pone.0036507.
31. **Li H, Ruan J, Durbin R.** 2008. Mapping short DNA sequencing reads and calling variants using mapping quality scores. Genome Res. **18**:1851–1858.
32. **Hernandez D, Francois P, Farinelli L, Osteras M, Schrenzel J.** 2008. De novo bacterial genome sequencing: millions of very short reads assembled on a desktop computer. Genome Res. **18**:802–809.
33. **de Lorenzo V, Herrero M, Jakubzik U, Timmis KN.** 1990. Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J. Bacteriol. **172**:6568–6572.
34. **Jacobs MA, Alwood A, Thaipisuttikul I, Spencer D, Haugen E, Ernst S, Will O, Kaul R, Raymond C, Levy R, Chun-Rong L, Guenthner D, Bovee D, Olson MV, Manoil C.** 2003. Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. U. S. A. **100**:14339–14344.
35. **Clinical and Laboratory Standards Institute.** 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard M7-A7, 7th ed. Clinical and Laboratory Standards Institute, Wayne, PA.
36. **Yi EC, Hackett M.** 2000. Rapid isolation method for lipopolysaccharide and lipid A from gram-negative bacteria. Analyst **125**:651–656.
37. **Caroff M, Tacken A, Szabo L.** 1988. Detergent-accelerated hydrolysis of bacterial endotoxins and determination of the anomeric configuration of the glycosyl phosphate present in the "isolated lipid A" fragment of the *Bordetella pertussis* endotoxin. Carbohydr. Res. **175**:273–282.
38. **Ernst RK, Yi EC, Guo L, Lim KB, Burns JL, Hackett M, Miller SI.** 1999. Specific lipopolysaccharide found in cystic fibrosis airway *Pseudomonas aeruginosa*. Science **286**:1561–1565.
39. **Geurtsen J, Steeghs L, Hove JT, van der Ley P, Tommassen J.** 2005. Dissemination of lipid A deacylases (pagL) among gram-negative bacteria: identification of active-site histidine and serine residues. J. Biol. Chem. **280**:8248–8259.
40. **Ernst RK, Adams KN, Moskowitz SM, Kraig GM, Kawasaki K, Stead CM, Trent MS, Miller SI.** 2006. The *Pseudomonas aeruginosa* lipid A

deacylase: selection for expression and loss within the cystic fibrosis airway. J. Bacteriol. **188**:191–201.
41. **Lam JS, Taylor VL, Islam ST, Hao Y, Kocincova D.** 2011. Genetic and functional diversity of *Pseudomonas aeruginosa* lipopolysaccharide. Front. Microbiol. **2**:118. doi:10.3389/fmicb.2011.00118.
42. **Jochumsen N, Liu Y, Molin S, Folkesson A.** 2011. A Mig-14-like protein (PA5003) affects antimicrobial peptide recognition in *Pseudomonas aeruginosa*. Microbiology **157**:2647–2657.
43. **Deng M, Misra R.** 1996. Examination of AsmA and its effect on the assembly of *Escherichia coli* outer membrane proteins. Mol. Microbiol. **21**:605–612.
44. **Guo J, Nair MK, Galvan EM, Liu SL, Schifferli DM.** 2011. Tn5AraOut mutagenesis for the identification of *Yersinia pestis* genes involved in resistance towards cationic antimicrobial peptides. Microb. Pathog. **51**:121–132.
45. **Dekkers LC, Bloemendaal CJ, de Weger LA, Wijffelman CA, Spaink HP, Lugtenberg BJ.** 1998. A two-component system plays an important role in the root-colonizing ability of *Pseudomonas fluorescens* strain WCS365. Mol. Plant Microbe Interact. **11**:45–56.
46. **Ramos-González MI, Campos MJ, Ramos JL.** 2005. Analysis of *Pseudomonas putida* KT2440 gene expression in the maize rhizosphere: in vivo expression technology capture and identification of root-activated promoters. J. Bacteriol. **187**:4033–4041. (Erratum, **187**:5504.)
47. **Garvis S, Munder A, Ball G, de Bentzmann S, Wiehlmann L, Ewbank JJ, Tummler B, Filloux A.** 2009. *Caenorhabditis elegans* semi-automated liquid screen reveals a specialized role for the chemotaxis gene cheB2 in *Pseudomonas aeruginosa* virulence. PLoS Pathog. **5**:e1000540. doi:10.1371/journal.ppat.1000540.
48. **Fernandez L, Jenssen H, Bains M, Wiegand I, Gooderham WJ, Hancock RE.** 2012. The two-component system CprRS senses cationic peptides and triggers adaptive resistance in *Pseudomonas aeruginosa* independently of ParRS. Antimicrob. Agents Chemother. **56**:6212–6222.
49. **Helander IM, Nummila K, Kilpelainen I, Vaara M.** 1995. Increased substitution of phosphate groups in lipopolysaccharides and lipid A of polymyxin-resistant mutants of *Salmonella typhimurium* and *Escherichia coli*. Prog. Clin. Biol. Res. **392**:15–23.
50. **Moskowitz SM, Ernst RK.** 2010. The role of *Pseudomonas* lipopolysaccharide in cystic fibrosis airway infection. Subcell. Biochem. **53**:241–253.
51. **Batchelor E, Goulian M.** 2003. Robustness and the cycle of phosphorylation and dephosphorylation in a two-component regulatory system. Proc. Natl. Acad. Sci. U. S. A. **100**:691–696.
52. **Jensen T, Pedersen SS, Garne S, Heilmann C, Høiby N, Koch C.** 1987. Colistin inhalation therapy in cystic fibrosis patients with chronic *Pseudomonas aeruginosa* lung infection. J. Antimicrob. Chemother. **19**:831–838.
53. **Frederiksen B, Koch C, Høiby N.** 1997. Antibiotic treatment of initial colonization with *Pseudomonas aeruginosa* postpones chronic infection and prevents deterioration of pulmonary function in cystic fibrosis. Pediatr. Pulmonol. **23**:330–335.
54. **Atkinson MR, Ninfa AJ.** 1992. Characterization of *Escherichia coli glnL* mutations affecting nitrogen regulation. J. Bacteriol. **174**:4538–4548.
55. **Hsing W, Silhavy TJ.** 1997. Function of conserved histidine-243 in phosphatase activity of EnvZ, the sensor for porin osmoregulation in *Escherichia coli*. J. Bacteriol. **179**:3729–3735.
56. **Hsing W, Russo FD, Bernd KK, Silhavy TJ.** 1998. Mutations that alter the kinase and phosphatase activities of the two-component sensor EnvZ. J. Bacteriol. **180**:4538–4546.
57. **Pioszak AA, Ninfa AJ.** 2004. Mutations altering the N-terminal receiver domain of NRI (NtrC) that prevent dephosphorylation by the NRII-PII complex in *Escherichia coli*. J. Bacteriol. **186**:5730–5740.
58. **Gao R, Stock AM.** 2013. Probing kinase and phosphatase activities of two-component systems in vivo with concentration-dependent phosphorylation profiling. Proc. Natl. Acad. Sci. U. S. A. **110**:672–677.
59. **Duan K, Dammel C, Stein J, Rabin H, Surette MG.** 2003. Modulation of *Pseudomonas aeruginosa* gene expression by host microflora through interspecies communication. Mol. Microbiol. **50**:1477–1491.
60. **de Weert S, Dekkers LC, Bitter W, Tuinman S, Wijfjes AH, van Boxtel R, Lugtenberg BJ.** 2006. The two-component *colR/S* system of *Pseudomonas fluorescens* WCS365 plays a role in rhizosphere competence through maintaining the structure and function of the outer membrane. FEMS Microbiol. Ecol. **58**:205–213.
61. **Hõrak R, Ilves H, Pruunsild P, Kuljus M, Kivisaar M.** 2004. The ColR-ColS two-component signal transduction system is involved in regulation of Tn4652 transposition in *Pseudomonas putida* under starvation conditions. Mol. Microbiol. **54**:795–807.
62. **Kivistik PA, Putrins M, Puvi K, Ilves H, Kivisaar M, Horak R.** 2006. The ColRS two-component system regulates membrane functions and protects *Pseudomonas putida* against phenol. J. Bacteriol. **188**:8109–8117.
63. **Putrins M, Ilves H, Kivisaar M, Horak R.** 2008. ColRS two-component system prevents lysis of subpopulation of glucose-grown *Pseudomonas putida*. Environ. Microbiol. **10**:2886–2893.
64. **Hu N, Zhao B.** 2007. Key genes involved in heavy-metal resistance in *Pseudomonas putida* CD2. FEMS Microbiol. Lett. **267**:17–22.
65. **Putrins M, Ainelo A, Ilves H, Horak R.** 2011. The ColRS system is essential for the hunger response of glucose-growing *Pseudomonas putida*. BMC Microbiol. **11**:170. doi:10.1186/1471-2180-11-170.
66. **Kivistik PA, Kivi R, Kivisaar M, Horak R.** 2009. Identification of ColR binding consensus and prediction of regulon of ColRS two-component system. BMC Mol. Biol. **10**:46. doi:10.1186/1471-2199-10-46.
67. **Sivaneson M, Mikkelsen H, Ventre I, Bordi C, Filloux A.** 2011. Two-component regulatory systems in *Pseudomonas aeruginosa*: an intricate network mediating fimbrial and efflux pump gene expression. Mol. Microbiol. **79**:1353–1366.
68. **Goodman AL, Merighi M, Hyodo M, Ventre I, Filloux A, Lory S.** 2009. Direct interaction between sensor kinase proteins mediates acute and chronic disease phenotypes in a bacterial pathogen. Genes Dev. **23**:249–259.
69. **Venkatesh GR, Kembou Koungni FC, Paukner A, Stratmann T, Blissenbach B, Schnetz K.** 2010. BglJ-RcsB heterodimers relieve repression of the *Escherichia coli bgl* operon by H-NS. J. Bacteriol. **192**:6456–6464.
70. **Fernandez L, Alvarez-Ortega C, Wiegand I, Olivares J, Kocincova D, Lam JS, Martinez JL, Hancock RE.** 2013. Characterization of the polymyxin B resistome of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **57**:110–119.
71. **McPhee JB, Bains M, Winsor G, Lewenza S, Kwasnicka A, Brazas MD, Brinkman FS, Hancock RE.** 2006. Contribution of the PhoP-PhoQ and PmrA-PmrB two-component regulatory systems to Mg2+-induced gene regulation in *Pseudomonas aeruginosa*. J. Bacteriol. **188**:3995–4006.
72. **Schurek KN, Sampaio JL, Kiffer CR, Sinto S, Mendes CM, Hancock RE.** 2009. Involvement of *pmrAB* and *phoPQ* in polymyxin B adaptation and inducible resistance in non-cystic fibrosis clinical isolates of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **53**:4345–4351.
73. **Karunaratne DN, Richards JC, Hancock RE.** 1992. Characterization of lipid A from *Pseudomonas aeruginosa* O-antigenic B band lipopolysaccharide by 1D and 2D NMR and mass spectral analysis. Arch. Biochem. Biophys. **299**:368–376.