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PhoQ Mutations Promote Lipid A Modification and Polymyxin Resistance of *Pseudomonas aeruginosa* Found in Colistin-Treated Cystic Fibrosis Patients▽†

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Pseudomonas aeruginosa **can develop resistance to polymyxin and other cationic antimicrobial peptides. Previous work has shown that mutations in the PmrAB and PhoPQ regulatory systems can confer low to moderate levels of polymyxin resistance (MICs of 8 to 64 mg/liter) in laboratory and clinical strains of this organism. To explore the role of PhoPQ in high-level clinical polymyxin resistance, *P. aeruginosa* strains with colistin MICs > 512 mg/liter that had been isolated from cystic fibrosis patients treated with inhaled colistin (polymyxin E) were analyzed. Probable loss-of-function *phoQ* alleles found in these cystic fibrosis strains conferred resistance to polymyxin. Partial and complete suppressor mutations in *phoP* were identified in some cystic fibrosis strains with resistance-conferring *phoQ* mutations, suggesting that additional loci can be involved in polymyxin resistance in *P. aeruginosa*. Disruption of chromosomal *phoQ* in the presence of an intact *phoP* allele stimulated 4-amino-L-arabinose addition to lipid A and induced transcription from the promoter of the *pmrH* (*arnB*) operon, consistent with the known role of this lipid A modification in polymyxin resistance. These results indicate that *phoQ* loss-of-function mutations can contribute to high-level polymyxin resistance in clinical strains of *P. aeruginosa*.**

Pseudomonas aeruginosa is capable of causing substantial morbidity and mortality in individuals with compromised host defense mechanisms. Those with cystic fibrosis (CF) are particularly susceptible to *P. aeruginosa* infections of the respiratory tract (41). In this disorder, unusually viscous secretions are retained within the airways, providing an ecological niche conducive to growth of such opportunistic pathogens (38).

In many cases, *P. aeruginosa* isolated from the respiratory tract of a patient with CF represents a unique episode of adaptation by a strain acquired from an environmental reservoir (16). However, in some cases, epidemic strains of *P. aeruginosa* have been observed to spread through local, regional, national, or even international CF patient populations (1, 5, 10, 52, 55). Such CF epidemic strains often display phenotypes such as specific antibiotic resistance patterns (10, 52), increased virulence (1, 3, 5, 44, 54), and enhanced transmissibility (51). These characteristics likely promote *P. aeruginosa* persistence in the lungs of CF patients despite aggressive use of antipseudomonal antibiotic combinations. In the face of such selection pressure, many CF strains of *P. aeruginosa* eventually become multidrug resistant or even pan-drug resistant, mediated by diverse mechanisms of resistance and tolerance (4, 21, 32).

As a consequence, clinicians increasingly rely on second-line antipseudomonal agents such as polymyxin (Pm) (48). Pm represents a family of antimicrobial cyclic oligopeptides synthesized by the Gram-positive organism *Bacillus polymyxa*. The clinically available forms, polymyxin B (PMB) sulfate and colistimethate, a prodrug form of colistin (CST; also known as polymyxin E), are administered to CF patients intravenously or by inhalation. Pm binds to lipopolysaccharide (LPS), the major constituent of the Gram-negative outer membrane, thus promoting membrane permeabilization and diffusion of peptide across the periplasm. Pm insertion at the inner membrane disrupts cellular respiration and results in cell lysis and death (57).

The Gram-negative pathogens for which Pm is most commonly used in clinical practice are *P. aeruginosa* and *Acinetobacter baumannii*. Unfortunately, Pm-resistant (Pm^r) clinical isolates of these organisms are increasingly being reported (2, 8, 15, 18, 30, 37, 40). Pm^r strains are generally resistant to both PMB and CST. In *P. aeruginosa*, Pm resistance is associated with covalent addition of 4-amino-L-arabinose (L-Ara4N) to phosphate groups within the lipid A and core oligosaccharide moieties of LPS (7, 47, 50). Genes in the *pmrHFIJKLME* operon (also known as *arnBCADTEFpmrE*) encode enzymes

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TABLE 1. Laboratory-adapted strains and CF patient isolates of *P. aeruginosa* used in this work

Strain no.	Designation (patient no.: yr of isolation)	Multilocus sequence type (ST)	CST MIC (mg/liter)	Strain or isolate origin or provider
Laboratory-adapted strains				
1026	PAK		0.5	S. Lory
1812	PAK Δ*pmrAB*		0.5	This work
1995	PAK Δ*pmrAB phoQ6*		8	This work
2248	1995 Δ*phoPQ*		0.5	This work
2244	PAK Δ*pmrAB* Δ*phoPQ*		2	This work
2307	PAK Δ*pmrAB* Δ*phoQ*		8	This work
2326	PAK Δ*phoQ*		32	This work
2243	PAK Δ*phoPQ*		0.5	This work
1555	PAO1		ND[a]	B. Iglewski
2898	PAO1 Δ*phoPQ* Ω[*attP*::Φ(P_{pmrH}-*lacZ*$^+$)]		ND	This work
Clinical isolates				
2047	CF isolate (patient 2: 1985)	ST387	1	N. Høiby
1016	CF isolate (patient 2: 1996)	ST387	>512	N. Høiby
1018	CF isolate (patient 4: 1998)	ST387	>512	N. Høiby
1019	CF isolate (patient 5: 1998)	ST387	>512	N. Høiby
1565	CF isolate (patient 7: 2002)	ST387	8	N. Høiby
1576	CF isolate (patient 8: 1999)	ST387	1	N. Høiby
1571	CF isolate (patient 8: 2002)	ST387	>512	N. Høiby
1579	CF isolate (patient 9: 1999)	(ST387-related)	>512	N. Høiby
1581	CF isolate (patient 9: 2001)	ST404 (ST387-related)	0.5	N. Høiby
1577	CF isolate (patient 9: 2002)	(ST387-related)	>512	N. Høiby
1585	CF isolate (patient 10: 1995)	ST387	1	N. Høiby
1582	CF isolate (patient 10: 2002)	ST387	>512	N. Høiby
1590	CF isolate (patient 11: 1992)	ST387	1	N. Høiby
1586	CF isolate (patient 11: 2002)	ST387	>512	N. Høiby
1597	CF isolate (patient 12: 1999)	ST387	>512	N. Høiby
1601	CF isolate (patient 13: 1997)	ST387	0.25	N. Høiby
1021	CF isolate (patient 13: 1998)	ST387	>512	N. Høiby
1598	CF isolate (patient 13: 2002)	ST387	>512	N. Høiby
1604	CF isolate (patient 14: 1991)	ST399	>512	N. Høiby
1605	CF isolate (patient 14: 1992)	ST400 (ST399 related)	16	N. Høiby
1603	CF isolate (patient 14: 2003)	ST398 (ST399 related)	>512	N. Høiby

[a] ND, not determined.

responsible for the synthesis and transfer of L-Ara4N to lipid A (26, 28). This amino-sugar modification is thought to hinder charge interactions between phosphate groups within LPS and amino groups within the cyclic Pm oligopeptide.

In *P. aeruginosa* and some other Gram-negative organisms, the PmrAB two-component regulatory system activates transcription of the *pmrHFIJKLME* operon in response to antimicrobial peptide exposure or divalent cation depletion (26, 28, 45) or as a consequence of mutation (8, 47, 53). However, many Pm^r CF isolates of *P. aeruginosa* have wild-type (WT) *pmrAB* alleles (8). We therefore hypothesized that mutations in additional regulatory systems stimulate the activation of the L-Ara4N operon in clinical Pm resistance. In this study, we used laboratory strains and CF isolates of *P. aeruginosa* to define mutation of the PhoPQ two-component regulatory system as an important mechanism of this resistance.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and genotyping. Laboratory-adapted strains and clinical isolates of *P. aeruginosa* used in this study are listed in Table 1. Clinical isolates of *P. aeruginosa* were collected from the sputum of CF patients in Denmark. The Institutional Review Board of Seattle Children's Hospital and the Institutional Review Board of Massachusetts General Hospital reviewed and approved the use of the clinical isolates for this study. *Escherichia coli* strain DH5α was used as the host strain for manipulation of recombinant plasmids. *P. aeruginosa* and *E. coli* were grown at 37°C on Luria-Bertani (LB) agar plates or in LB broth with aeration. Antibiotics were used for selection at the following concentrations: kanamycin (50 mg/liter) or gentamicin (GEN) (10 mg/liter) for *E. coli* DH5α and GEN (100 mg/liter) for *P. aeruginosa* strain PAK and its derivatives. Strains were stored in a 16% glycerol–LB broth solution at −80°C. Genotypes of clinical isolates were determined by multilocus sequence typing (13); novel alleles and sequence types have been added to a *P. aeruginosa* database available at http://pubmlst.org/paeruginosa.

Isolation of Pm^r mutants. A derivative of *P. aeruginosa* strain PAK from which the *pmrAB* locus had been deleted (strain 1812) was inoculated at a density of 10^9 CFU on 90-mm-diameter LB agar plates containing PMB (US Biochemical, Cleveland, OH) (50 to 100 mg/liter). Plates were incubated at 30°C overnight and then at ambient temperature (~22°C) for up to 2 weeks and inspected daily for the appearance of Pm^r colonies. Candidates were replated on PMB-containing LB agar to verify resistance. To assess phenotypic stability, putative PAK Δ*pmrAB* Pm^r mutants were inoculated into LB broth without Pm, grown overnight to stationary phase, and then subjected to passage using fresh Pm-free culture medium on each of five consecutive days; after the fifth passage, each putative mutant was retested on PMB-containing LB agar.

Molecular methods. Bacterial plasmids and chromosomal DNA were isolated using commercially available kits (QIAquick and DNeasy [Qiagen, Valencia, CA] and MasterPure [Epicentre Biotechnologies, Madison, WI]). Plasmids were introduced into *P. aeruginosa* by electroporation (11). Sequencing of plasmids and chromosomal DNA was performed on both strands by the use of oligonucleotide primers (see Table S1 in the supplemental material) spaced ~400 bp apart. PCR amplification was performed with the following final concentrations or amounts: 10% dimethyl sulfoxide, 0.2 mM (each) deoxynucleotide triphosphate, 0.4 μM (each) oligonucleotide primer (see Table S2 and Table S3 in the supplemental material), approximately 10 ng of template DNA, 5 U of *Pfu* Turbo polymerase, and 1× *Pfu* reaction buffer (Stratagene, La Jolla, CA).

To construct deletions, ~1-kb chromosomal DNA segments flanking the tar-

geted locus were PCR amplified using chromosomal DNA and specific oligonucleotide primers (see Table S2 in the supplemental material) and joined by splicing-by-overlap-extension PCR (33); deletions were marked by inclusion of a unique restriction site in the overlapping (internal) primers. For constructs made using SS-series primers, the resulting ~2-kb DNA fragment was inserted into the suicide plasmid pEX18Gm (34) by the use of XbaI and HinDIII restriction sites. For constructs made using SM-series primers, the DNA fragment was inserted into the Gateway entry vector pDONR201 (Gateway cloning system; Invitrogen, Carlsbad, CA) via a BP recombinase reaction and transferred into the Gateway destination vector pEXGmGW (60) via an LR recombinase reaction. Deletion construct plasmids introduced into *P. aeruginosa* were selected for chromosomal insertion on LB agar containing GEN and then counterselected for loss of the plasmid backbone on LB agar containing 5% sucrose (34). To confirm deletions, the region surrounding the target gene was PCR amplified from chromosomal DNA and digested with restriction endonucleases BamHI (for constructs made using SS-series primers) and HinDIII (for constructs made using SM-series primers) to detect the unique marker.

To construct expression plasmids, the target genes were PCR amplified using genomic DNA and specific oligonucleotide primers (see Table S3 in the supplemental material), and the Gateway cloning system was used to insert them into pJN105D, a version of plasmid pJN105 (49) that had been converted to a Gateway destination vector. Expression plasmids introduced into *P. aeruginosa* were selected on LB agar containing GEN.

Susceptibility testing. Pm agar dilution testing was performed as described previously (12); a Nunc 96-pin replicator with 1-mm pins and an OmniTray copier were used to inoculate the surface of OmniTray plates (Nunc International, Rochester, NY) containing 2-fold serial dilutions (0.125 to 512 mg/liter) of CST sulfate salt (Sigma-Aldrich, St. Louis, MO) in Difco Mueller-Hinton agar (Becton Dickinson Diagnostic Systems, Sparks, MD). Consistent with current CLSI recommendations, a CST MIC of ≤2 mg/liter was interpreted as indicating Pm susceptibility ("S"), a CST MIC of 4 mg/liter was interpreted as indicating intermediate Pm susceptibility ("I"), and a CST MIC of ≥8 mg/liter was interpreted as indicating Pm resistance ("R"). For Pm^r strains, a CST MIC of 8 to 32 mg/liter was interpreted as indicating low-level resistance, a CST MIC of 64 to 256 mg/liter was interpreted as indicating moderate-level resistance, and a CST MIC of ≥512 mg/liter was interpreted as indicating high-level resistance.

In an alternative PMB plate assay, bacterial strains were inoculated into LB broth containing 1 mM $MgCl_2$ and grown at 37°C with aeration for 16 to 20 h to an optical density at 600 nm (OD_{600}) of ~3.0 to 5.0. The culture was diluted 1:50 into fresh medium and grown for 2 to 3 h at 30°C with aeration to an OD_{600} of ~0.8. Dilutions containing ~50 to 200 CFU per 0.1 ml were spread on LB agar plates containing 1 mM $MgCl_2$ and PMB at 0 to 100 mg/liter. Susceptibility testing media used for strains with pJN105D-derived plasmids contained 0.1% L-arabinose and GEN or PMB. To estimate the PMB concentration representing a 50% lethal dose (LD_{50}), percent mean survival at each PMB concentration was calculated in relation to the mean CFU on the PMB-free control plates. A PMB LD_{50} of <4 mg/liter was interpreted as indicating susceptibility ("S"), a PMB LD_{50} of ≥4 mg/liter but <10 was interpreted as indicating intermediate susceptibility ("I"), and a PMB LD_{50} of ≥10 mg/liter was interpreted as indicating resistance ("R").

For quantitative bactericidal assays (47), strains were grown and subcultured as described above for the alternative PMB plate assay, diluted in Mueller-Hinton broth to a final density of 2×10^4 CFU per ml, exposed to 2-fold serial dilutions of PMB (0.5 to 512 mg/liter) as well as drug-free controls for 30 min at 37°C, spread on LB agar after 1:10 dilution, and incubated 16 to 20 h at 37°C for enumeration.

Lipid A isolation and analysis. LPS was isolated after growth in LB broth supplemented with 1 mM $MgCl_2$ (61). Lipid A was isolated from LPS by hydrolysis (9). Lipid A structure was analyzed using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry in negative-ion mode (17). All MALDI-TOF analyses were performed using a Bruker Autoflex II mass spectrometer (Bruker Daltonics). The matrix used for lipid A analysis was 5-chloro-2-mercaptobenzothiazole (20 mg/liter in 1:1 chloroform:methanol).

Transcriptional analysis. A *P. aeruginosa* reporter strain for transcriptional analysis was constructed in PAO1 (B. H. Iglewski). A *lacZ* fusion to the promoter of the *pmrHFIJKLME* operon (PA3552 to 3559 in the PAO1 genome) was constructed using a pMini-CTX::*lacZ* vector system (35). The region upstream of *pmrHFIJKLME* was amplified using specific oligonucleotide primers (see Table S4 in the supplemental material) and inserted into pMini-CTX::*lacZ* by the use of EcoRI and BamHI restriction sites. The fusion was integrated into the CTX site of the chromosome followed by removal of the plasmid backbone by the use of Flp recombinase. To confirm the reporter construct, the CTX region was PCR amplified from chromosomal DNA. Reporter strains carrying expression plasmids were grown for 16 to 20 h at 37°C with aeration in LB broth supplemented with 1 mM $MgCl_2$ and GEN. Cultures were diluted 1:100 into fresh medium supplemented with 0.1% arabinose, grown for 90 min, and assayed for β-galactosidase activity (39).

Nucleotide sequence accession numbers. The GenBank accession numbers for the new DNA sequences are JN868715 (*phoPQ6* allele, strain 1995), JN868716 (*phoPQ* WT allele, strain 2047), JN868717 (*phoPQ30* allele, strain 1016), JN868718 (*phoPQ21* allele, strain 1018), JN868719 (*phoP23Q23* allele, strain 1565), JN868720 (*phoPQ25* allele, strain 1571), JN868721 (*phoPQ22* allele, strain 1582), JN868722 (*phoP24Q24* allele, strain 1604), JN868723 (*pmrAB44* allele, strain 1018), and JN868724 (*pmrAB34* allele, strain 1603).

RESULTS

Isolation and genetic analysis of a Pm^r *phoQ* mutant in a Δ*pmrAB* strain of *P. aeruginosa*. We previously showed that specific point mutations in *pmrB*, which encodes the sensor kinase component of the PmrAB regulatory system, conferred Pm resistance in a laboratory-adapted strain of *P. aeruginosa* (47). However, a survey of Pm^r clinical isolates of *P. aeruginosa* from CF patients indicated that many do not have resistance-conferring mutations at the *pmrAB* locus (data not shown), suggesting that mutation of additional regulatory loci can confer clinical Pm resistance in this organism. To define such loci, we isolated spontaneous Pm^r mutants of strain PAK Δ*pmrAB* by plating on LB agar containing PMB at 50 to 100 mg/liter. Of nine putative mutants, strain 1995 displayed the strongest and most reproducible Pm resistance (Fig. 1A). Interestingly, in contrast to Pm^r *pmrB* mutants (47), strain 1995 had minimal cross-resistance to other cationic antimicrobial peptides (CAPs) such as protegrin-1 (56) and C18G (14) (Table 2).

Disruption of the gene encoding the PhoQ sensor kinase (designated PA1180 in the PAO1 genome) is known to confer CAP resistance in *P. aeruginosa* (42, 43); in this context, PhoQ appears to act as a repressor of PhoP transcriptional activity (43). Deletion of the *phoPQ* locus in strain 1995 (PAK Δ*pmrAB* Δ*phoPQ*) resulted in loss of Pm resistance (Fig. 1A). The *phoPQ* alleles from wild-type PAK (designated *phoPQ*$^+$) and strain 1995 (designated *phoPQ6*) were inserted into the broad-host-range expression plasmid pJN105D under the control of an arabinose-inducible promoter; expression of pJN105D::*phoPQ6* in PAK Δ*phoPQ* conferred Pm resistance, whereas expression of pJN105D::*phoPQ*$^+$ did not (Fig. 1B). Expression of pJN105D::*phoPQ6* in wild-type PAK failed to confer Pm resistance (data not shown), indicating a recessive effect of *phoPQ6* on Pm resistance.

Sequence analysis of the *phoPQ6* allele from strain 1995 revealed a single nonsynonymous nucleotide change relative to strain PAK, namely, deletion of thymidine 1262 in the *phoQ* gene. This resulted in a frameshift of codons 421 to 428 and a premature stop at codon 429, with truncation of the 28 carboxy-terminal amino acids of the PhoQ protein. This truncation removes a portion of the G-box, a Gly-rich motif in the cytosolic domain of PhoQ that is important for nucleotide binding (20). The nucleotide sequences of the *phoP* gene in strains 1995, PAK, and PAO1 were identical (58, 59).

Effect of PhoQ truncation or deletion on polymyxin resistance. To assess whether the frameshift associated with the *phoQ6* mutant allele was functionally equivalent to carboxy-terminal truncation of PhoQ, we constructed additional *phoQ* mutant alleles encoding in-frame truncations (Table 3). The

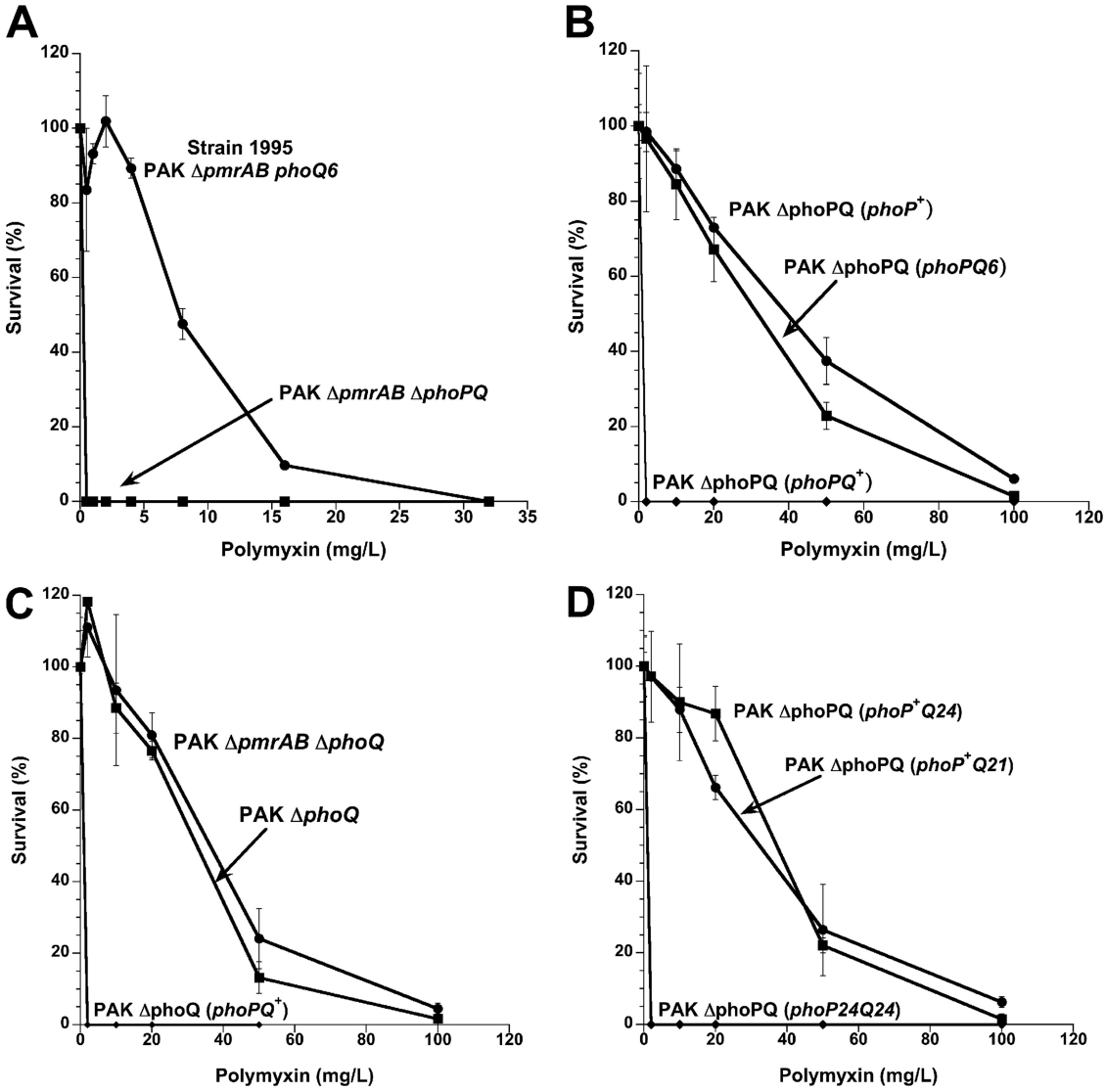

FIG. 1. Effect of *phoPQ* mutations on *P. aeruginosa* Pm resistance. (A) PMB quantitative bactericidal assay of strains 1995 (PAK Δ*pmrAB phoQ6*) and 2248 (PAK Δ*pmrAB* Δ*phoPQ*). (B) Alternative PMB plate assay of the PAK Δ*phoPQ* strain expressing the *phoPQ*$^+$, *phoPQ6*, or *phoP*$^+$ allele inserted in plasmid pJN105D. (C) Alternative PMB plate assay of chromosomal deletion mutants PAK Δ*phoQ* and PAK Δ*pmrAB* Δ*phoQ* as well as PAK Δ*phoQ* expressing the *phoPQ*$^+$ alleles inserted in plasmid pJN105D. (D) Alternative PMB plate assay of the PAK Δ*phoPQ* strain expressing mutant *phoPQ* alleles from CF clinical isolates in plasmid pJN105D. The *phoP*$^+$*Q21* and *phoP*$^+$*Q24* alleles encode deletions from PhoQ of 2 and 276 amino acids, respectively, while the *phoP24Q24* allele also encodes replacement in PhoP of Arg 118 with Cys.

phoQ11 mutant allele was designed to encode conversion of Ile 421 (the first altered codon of the *phoQ6* frameshift) to a stop codon (TGA), with loss of 28 carboxy-terminal residues. The *phoQ12* mutant allele was similarly designed to encode conversion of Asp 433 to a stop codon (TGA), with retention of the native G-box and loss of only 16 carboxy-terminal residues, including six residues highly conserved among *Salmonella enterica*, *E. coli*, and *P. aeruginosa*. Expression of pJN105D::*phoPQ11*, pJN105D::*phoPQ12*, or pJN105D::*phoP*$^+$ in strain PAK Δ*phoPQ* conferred Pm resistance at a level similar to expression of *phoPQ6* (Table 3 and Fig. 1B). Strains PAK Δ*pmrAB* Δ*phoQ* (2307) and PAK Δ*phoQ* (2326) were created to assess the effect of single-copy chromosomal expression of PhoP in the absence of PhoQ. These strains exhibited constitutive Pm resistance that expression of pJN105D::*phoPQ*$^+$ could repress (Fig. 1C). Interestingly, strain 1995 as well as the *phoQ* truncation mutants also displayed a small-colony morphology, reflecting a growth retardation phenotype; as with its effect in repressing Pm resistance, expression of WT *phoQ* from a plasmid restored these strains to the WT colony

TABLE 2. Antimicrobial peptide resistance of a *P. aeruginosa* PAK Δ*pmrAB phoQ6* mutant

CAP	PAK Δ*pmrAB phoQ*$^+$ LD_{50}[a] (mg/liter)	PAK Δ*pmrAB phoQ6*	
		LD_{50} (mg/liter)	Fold increase in Pm resistance
PMB	<0.5	8	>16
Protegrin-1	0.5	0.5	1.0
C18G	3	5	1.7

[a] LD_{50}, 50% lethal dose.

TABLE 3. *phoPQ* alleles and associated Pm susceptibility

Allele designation	Strain no.	Amino acid change(s)[a]		Pm susceptibility[b]
		PhoP	PhoQ	
phoPQ		WT	WT	S
phoPQ6		WT	frameshift at I421 (and 8 amino acids)	R
phoPQ11		WT	I421X	R
phoPQ12		WT	D433X	R
phoPQ21	1018 (and 9 other isolates)	WT	ΔL364-G365	R
phoPQ22	1582	WT	ΔL364-G365; R444C	R
phoP23Q23	1565	M175I	R6C; ΔL364-G365	I
phoP24Q24	1604 (and 2 other isolates)	R118C	ΔV57-Q332	S
phoPQ24		WT	ΔV57-Q332	R
phoPQ25	1571	WT	frameshift at V448 (and 30 amino acids)	S
phoPQ30	1016	WT	E72G	S

[a] C, Cys; D, Asp; E, Glu; G, Gly; I, Ile; L, Leu; M, Met; Q, Gln; R, Arg; V, Val; X, Ter.
[b] Alleles were inserted into pJN105D and expressed in PAK Δ*phoPQ*, with Pm susceptibility determined by alternative PMB plate assays and interpreted as defined in Materials and Methods. S, susceptible, I, intermediate, and R, resistant.

size (data not shown). However, several CF patient isolates of *P. aeruginosa* with small-colony morphology were found to be susceptible to Pm in the alternative PMB plate assay (data not shown), indicating that growth retardation *per se* does not account for the Pm^r phenotype of the *phoQ* mutants.

Analysis of *phoPQ* alleles in Pm^r isolates of *P. aeruginosa* from CF patients. To define the role of *phoPQ* mutations in clinical Pm resistance, we analyzed 15 Pm^r and 6 Pm-susceptible (Pm^s) isolates of *P. aeruginosa* from 11 Danish CF patients who had been continuously treated with inhaled CST for up to 15 years. The *phoPQ* and *pmrAB* alleles from these isolates, which represented two distinct genotypic backgrounds (ST387 and ST399; Table 1), were PCR amplified and inserted into the pJN105D broad-host-range expression plasmid. For six of the patients, the analysis included both resistant and susceptible isolates derived from the ST387 genotypic background (Table 1).

A mutant allele with a six-nucleotide deletion (*phoQ21*) confers Pm resistance in an epidemic CF strain of *P. aeruginosa*. The *phoPQ* allele from 10 Pm^r isolates and 2 Pm^s isolates conferred resistance when expressed in strain PAK Δ*phoPQ* (Fig. 1D and data not shown). These 12 isolates, cultured from eight different CF patients, were ST387 derivatives with wild-type (i.e., non-resistance-conferring) *pmrAB* alleles (M. K. Brannon and S. M. Moskowitz, unpublished results). All 12 isolates possessed the same resistance-conferring *phoQ* mutation, a six-nucleotide deletion at positions 1091 to 1096 corresponding to in-frame deletion of Leu 364 and Gly 365. For eight Pm-resistant isolates (1018, 1019, 1021, 1577, 1579, 1586, 1597, and 1598) and both Pm-susceptible isolates (1581 and 1601), this was the only mutation found in the *phoPQ* locus; the allele was designated *phoQ21* (Table 3). A plasmid bearing this allele, pJN105D::*phoPQ21*, conferred Pm resistance on PAK Δ*phoPQ* at a level similar to that conferred by pJN105D::*phoPQ6*. The two Pm-susceptible isolates carrying the *phoQ21* allele presumably harbor unidentified secondary suppressor mutations in one or more loci required for PhoPQ-dependent Pm resistance. A third Pm^s isolate (1590) cultured from patient 11 10 years earlier than Pm^r isolate 1586 had WT *phoPQ*. The *pmrB* gene in isolates with the *phoQ21* allele (or with related alleles as described below) contained a single nucleotide mutation (Ala 211 to Val) and was designated *pmrB44*; a plasmid bearing the *pmrAB44* allele failed to confer Pm resistance on wild-type strain PAK, indicating that this *pmrB* mutation represents a polymorphism.

Two resistance-conferring *phoQ* alleles are variants of *phoQ21*. In addition to the Leu 364 and Gly 365 deletions, the *phoQ* gene of one Pm^r isolate (1582) also contained a C-to-T transition at nucleotide 1330 of the WT sequence (designated *phoQ22*), corresponding to a change of Arg 444 to Cys (Table 3). A plasmid bearing this allele, pJN105D::*phoPQ22*, conferred Pm resistance on PAK Δ*phoPQ* at a level similar to that conferred by pJN105D::*phoPQ21*. The *phoQ* gene of another Pm^r isolate (1565) also contained an A-to-T transversion at nucleotide 16 (designated *phoQ23*), corresponding to a change of Arg 6 to Cys, while the *phoP* gene contained a G-to-A transition at nucleotide 545 (designated *phoP23*), corresponding to a change of Met 175 to Ile. A plasmid bearing this allele, pJN105D::*phoP23Q23*, conferred Pm resistance on PAK Δ*phoPQ* at a lower level than the those bearing the *phoQ6*, *phoQ21*, or *phoQ22* alleles (Table 3). Because pJN105D::*phoP23* conferred Pm resistance on PAK Δ*phoPQ* at the same diminished level as that conferred by pJN105D::*phoP23Q23*, whereas pJN105D::*phoP*$^+$*Q23* conferred Pm resistance at a level similar to that conferred by pJN105D::*phoPQ21* (data not shown), the mutation of Met 175 to Ile in *phoP23* represented a partial suppressor of *phoQ23*.

A *phoP* mutation completely suppresses the Pm resistance conferred by a *phoQ* mutant allele. The *phoQ* gene of a Pm^r isolate (1604) and two clonal Pm^r isolates from the same patient (1603 and 1605; all ST399 derivatives) contained an in-frame deletion of nucleotides 169 through 996 (designated *phoQ24*), corresponding to removal of Val 57 through Gln 332. This deletion encompasses part of the periplasmic domain, the entirety of the second transmembrane domain, and the H-box motif of PhoQ. The *phoP* gene of these isolates had a C-to-T transition at nucleotide 354 (designated *phoP24*), corresponding to a change of Arg 118 to Cys. A plasmid bearing the *phoQ24* allele in conjunction with wild-type *phoP*, pJN105D::*phoP*$^+$*phoQ24*, conferred Pm resistance on PAK Δ*phoPQ* (Fig. 1D). In contrast, both pJN105D::*phoP24Q24* and pJN105D::*phoP24* failed to confer Pm resistance on PAK Δ*phoPQ* (data not shown), confirming that *phoP24* is a complete suppressor of *phoQ24*. The *pmrB* gene in isolates with the

phoP24Q24 allele contained a single nucleotide mutation resulting in a change of Ala 248 to Thr (designated *pmrB34*); a plasmid bearing the *pmrAB34* allele failed to confer Pm resistance on wild-type strain PAK.

Some *phoQ* mutant alleles fail to confer Pm resistance on PAK Δ*phoPQ*. The *phoQ* allele of one Pm^r isolate (1571; an ST387 derivative) contained a frameshift mutation (deletion of T at nucleotide 1343; designated *phoQ25*) corresponding to a change of Val 448 (the carboxy-terminal residue) to Ala, loss of the normal opal termination codon, and addition of 30 amino acids to the C terminus of PhoQ before reaching an alternative termination codon; its *phoP* allele was WT. The *phoQ* allele of another Pm-resistant isolate (1016; also an ST387 derivative) contained an A-to-G transition at nucleotide 215 (designated *phoQ30*), corresponding to a change of Glu 72 to Gly; its *phoP* allele was WT. Pm^s isolates from the same patients (isolates 1576 and 2047, respectively) were both ST387 derivatives that contained wild-type *phoQ* alleles. Neither pJN105D::*phoPQ25* nor pJN105D::*phoPQ30* conferred Pm resistance on PAK Δ*phoPQ* (data not shown). Isolates 1571 and 1016 both contain resistance-conferring *pmrAB* alleles (S. M. Moskowitz et al., submitted for publication).

Lipid A modifications in PmrAB-independent Pm^r strains of *P. aeruginosa*. Addition of L-Ara4N to the 1 and 4′ phosphate groups of lipid A is associated with Pm resistance in many Gram-negative organisms (31). The *pmrHFIJKLME* operon encodes enzymes responsible for biosynthesis and attachment of L-Ara4N to lipid A (46). Other covalent modifications of *P. aeruginosa* lipid A (Fig. 2A) include an acyl-oxy-acyl addition of laurate (C12:0) to 3-hydroxylaurate at the 2 and 2′ positions by HtrB1 and HtrB2, 2-hydroxylation of the acyl-oxy-acyl laurates (2OH-C12:0) by LpxO1 and LpxO2, removal of 3-hydroxydecanoate (3OH-C10:0) from the 3 position by PagL, and acyl-oxy-acyl addition of palmitate (C16:0) to 3-hydroxydecanoate at the 3′ position by PagP (46). To assess the presence or absence of these modifications, lipid A was purified from Pm^r and Pm^s *P. aeruginosa* strains and isolates and analyzed qualitatively by MALDI-TOF mass spectrometry.

Lipid A from the PAK wild-type strain had major peaks at mass/charge ratios (*m/z*) 1,617 and 1,447 (data not shown), corresponding to hexa- and penta-acylated species that differ in the presence or absence of 3-hydroxydecanoate ($\Delta_{m/z}$ = 170). Lipid A from the PAK Δ*pmrAB* strain grown in LB broth with 1 mM Mg^{2+} had additional peaks at *m/z* 1,855 and 1,685 (Fig. 2B), corresponding to hepta- and hexa-acylated species of lipid A that contain palmitate ($\Delta_{m/z}$ = 238). Ernst et al. previously showed that Mg^{2+} depletion induces PhoPQ-dependent palmitoylation of lipid A (17); these results suggest that loss of PmrAB derepresses lipid A palmitoylation under non-inducing (Mg^{2+}-replete) conditions.

Lipid A from PAK Δ*pmrAB phoQ6* had peaks corresponding to all of the species found in PAK Δ*pmrAB* (Fig. 2C), as well as peaks corresponding to addition of one or two L-Ara4N moieties ($\Delta_{m/z}$ = 131) to the following species: *m/z* 1,748 (1617 + L-Ara4N), 1,578 (1447 + L-Ara4N), 1,709 (1447 + 2[L-Ara4N]), 1,986 (1855 + L-Ara4N), and 1,816 (1685 + L-Ara4N). Lipid A from PAK Δ*pmrAB* Δ*phoQ* (strain 2307) similarly had both palmitate and L-Ara4N (Table 4). Interestingly, lipid A from PAK Δ*phoQ* (strain 2326) also had peaks corresponding to addition of both palmitate and L-Ara4N

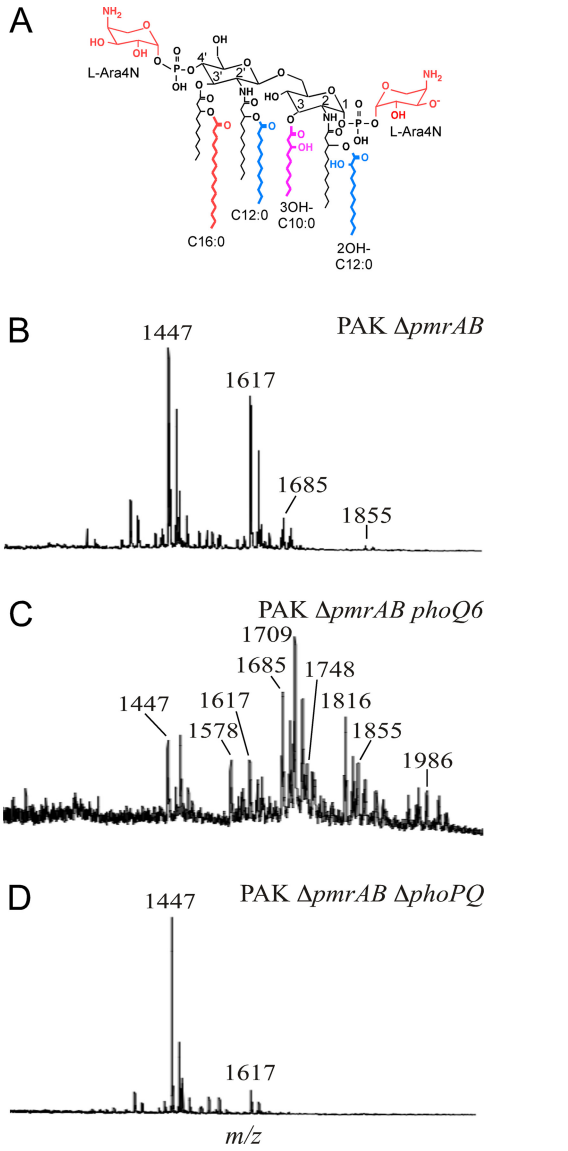

FIG. 2. Lipid A structures of Pm^s and Pm^r strains. (A) Diagram of lipid A structural modifications found in *P. aeruginosa*. (B to D) MALDI-TOF mass spectra for (B) PAK Δ*pmrAB* (strain 1812), (C) PAK Δ*pmrAB phoQ6* (strain 1995), and (D) PAK Δ*pmrAB* Δ*phoPQ* (strain 2244).

(data not shown), indicating that *phoQ* deletion promotes lipid A palmitoylation under noninducing conditions even in the presence of an intact PmrAB system. Conversely, lipid A from PAK Δ*pmrAB* Δ*phoPQ* (strain 2244) lacked both modifications (Fig. 2D), indicating that the effect of *pmrAB* deletion on lipid A palmitoylation is dependent on PhoP. In these laboratory strains, modification of lipid A with L-Ara4N consistently correlated with Pm resistance; in contrast, modification of lipid A with palmitate was seen in PAK Δ*pmrAB*, a strain that is Pm susceptible.

Among nine Pm^r CF isolates of *P. aeruginosa* that carry *phoQ21*, *phoQ22*, or *phoQ23* alleles, lipid A analysis demonstrated the addition of L-Ara4N to lipid A in all cases, and all but one of the isolates lacked 2-hydroxylaurate (Table 4). Six of

TABLE 4. Pm susceptibility and lipid A modifications of *P. aeruginosa phoPQ* mutants

Laboratory strain or clinical isolate(s)	*phoPQ* allele (*pmrAB* allele, laboratory strain)	Pm susceptibility[a]	Lipid A modification			
			Addition of L-Ara4N	Addition of C16:0	Presence of 2OH-C12:0	Removal of 3OH-C10:0
1026	*phoPQ*$^+$ (*pmrAB*$^+$)	S	−	−	+	+
1812	*phoPQ*$^+$ (Δ*pmrAB*)	S	−	+	+	+
1995	*phoQ6* (Δ*pmrAB*)	R	+	+	+	+
2307	Δ*phoQ* (Δ*pmrAB*)	R	+	+	+	+
2244	Δ*phoPQ* (Δ*pmrAB*)	S	−	−	+	+
2243	Δ*phoPQ* (*pmrAB*$^+$)	S	−	−	+	+
2326	Δ*phoQ* (*pmrAB*$^+$)	R	+	+	+	+
1018	*phoQ21*	R	+	+	+	+
1597, 1021	*phoQ21*	R	+	+	−	+
1019, 1586, 1598	*phoQ21*	R	+	−	−	+
1581, 1601	*phoQ21*	S	−	+	−	+
1582	*phoQ22*	R	+	+	−	+
1565	*phoP23Q23*	R	+	+	−	+
1603	*phoP24Q24*	R	+	+	−	+

[a] Data represent the results of measurements by colistin agar dilution. S, susceptible (CST MIC ≤ 2 mg/liter); R, resistant (CST MIC ≥ 8 mg/liter).

the nine isolates displayed palmitate addition; all exhibited 3-hydroxydecanoate deacylase activity. Two Pm^s isolates that harbor the *phoQ21* allele (1581 and 1601) displayed addition of palmitate but not L-Ara4N to lipid A; these isolates presumably have secondary suppressor mutations outside the *phoPQ* locus that interfere with this lipid A modification.

Effect of *phoPQ* alleles on transcriptional activation of the L-Ara4N operon. A transcriptional reporter was used to assess activation of the *pmrHFIJKLME* promoter by *phoPQ* mutant alleles. Plasmids encoding *phoPQ* alleles were expressed in a PAO1 Δ*phoPQ* strain in which a copy of the *pmrH* promoter region linked to a single-copy *lacZ* reporter had been inserted at the ΦCTX site {PAO1 Δ*phoPQ* Ω[*attP*::Φ(P_{pmrH}-*lacZ*$^+$)]}. The *phoPQ6*, *phoP*$^+$, and *phoPQ21* constructs conferred a high level of transcriptional activity, whereas the *phoPQ*$^+$ construct conferred minimal transcriptional activity (Fig. 3). The *phoP23Q23* and *phoP23* constructs conferred an intermediate level of transcriptional activity, consistent with their intermediate level of Pm resistance. The *phoP24Q24* and *phoP24* constructs conferred minimal transcriptional activity, whereas the *phoP*$^+$*Q24* construct conferred a high level of transcriptional activity, consistent with the Pm resistance phenotypes conferred by these allelic combinations.

DISCUSSION

This work establishes *phoQ* disruption as a mechanism of both epidemic and sporadic Pm resistance in CF isolates of *P. aeruginosa*, thus complementing and extending previous reports of Pm resistance induced by *phoQ* mutation in non-CF and laboratory-adapted strains (6, 42, 43). Mutations disrupting the *phoQ* gene (e.g., small and large in-frame deletions, frameshifts, or truncations that removed as few as 16 C-terminal residues) resulted in a multifaceted phenotype that encompassed growth retardation, Pm resistance, and addition of palmitate and L-Ara4N to lipid A; *phoQ* mutation stimulated the latter LPS modification via induction of the *pmrHFIJKLME* operon, as has been previously observed (23). Mutation of *phoQ* in *P. aeruginosa* is associated with a variety of phenotypes that would seem to decrease its fitness for *in vivo* infection, including impaired twitching motility and decreased cytotoxicity (23). The data presented here indicate that these phenotypes do not substantially mitigate the potential for Pm resistance and epidemic spread of clinical *phoQ* mutants.

Mutant *phoQ* alleles conferred Pm resistance that was dependent on *phoP*. Unlike its activity in other Gram-negative pathogens (25), the PhoQ sensor kinase of *P. aeruginosa* appears to act as a repressor of PhoP transcriptional activity (43). In the absence of functional PhoQ, native or episomal expression of a WT *phoP* allele promotes addition of palmitate and L-Ara4N to lipid A and induces Pm resistance. This contrasts with *S. enterica*, in which *phoP* alleles with specific activating mutations induce Pm resistance and associated lipid A modifications but a WT *phoP* allele does not (24). Whether the

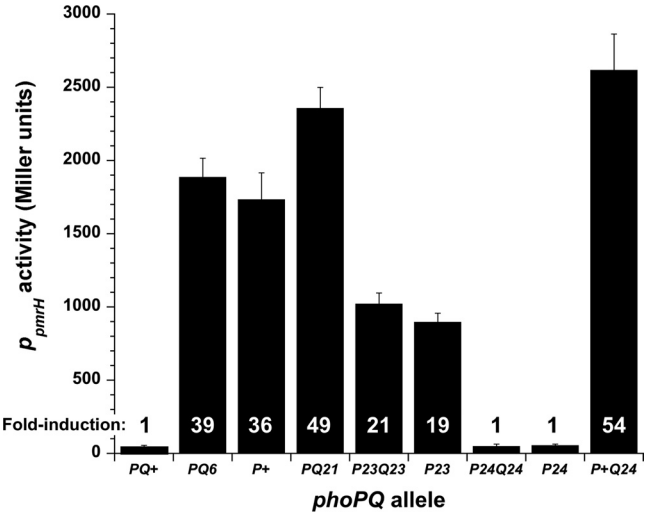

FIG. 3. Transcriptional activity of *phoPQ*$^+$, *phoPQ6*, *phoP*$^+$, *phoP*$^+$*Q21*, *phoP23Q23*, *phoP23*, *phoP24Q24*, *phoP24*, and *phoP*$^+$*Q24* alleles expressed from pJN105D in reporter strain PAO1 Δ*phoPQ* Ω[*attP*::Φ(P_{pmrH}-*lacZ*$^+$)]. Data shown represent the means of the results of triplicate biological experiments; error bars represent ± standard deviations. Fold induction values shown were calculated relative to the values determined for the WT *phoPQ*$^+$ allele.

transcriptionally active form of PhoP is phosphorylated in *P. aeruginosa* has not been determined; thus, it remains unclear whether PhoP repression by PhoQ is mediated by a phosphatase activity or a kinase activity.

Secondary suppressor mutations in the *phoP* gene were observed in some Pm^r clinical isolates with *phoQ* mutations; these isolates presumably have resistance-conferring mutations in other regulatory loci. Conversely, in the case of CST-exposed but Pm^s clinical isolates with resistance-conferring *phoPQ* mutant alleles, we inferred the occurrence of secondary suppressor mutations in other loci, presumably components of the *P. aeruginosa* PhoPQ regulon (23), that appear to be required for Pm resistance and addition of L-Ara4N to lipid A.

The PhoPQ and PmrAB systems of *P. aeruginosa* regulate L-Ara4N addition and Pm resistance in a convergent or redundant fashion, in contrast to the hierarchical relationship in which PhoPQ regulates PmrAB in *S. enterica* (26–28). In *P. aeruginosa*, mutation of *phoQ* can confer Pm resistance regardless of the presence or absence of a functional PmrAB system. Our data indicate a correlation between the level of Pm resistance that *phoPQ* alleles confer (Fig. 1D) and the level of transcriptional activity that they induce at the L-Ara4N operon promoter (Fig. 3).

In *P. aeruginosa*, the PhoPQ and PmrAB systems appear to regulate palmitoylation of lipid A in an antagonistic fashion; these systems also behave divergently with respect to their ability to induce resistance to CAPs other than Pm. Specifically, PhoP activation induces acyl-oxy-acyl palmitoylation of the 3′ 3-hydroxydecanoate without appearing to promote resistance to CAPs other than Pm, whereas PmrAB activation represses palmitoylation while promoting CAP resistance (47). In contrast, PhoPQ activation in *S. enterica* induces acyl-oxy-acyl palmitoylation at position 2 of lipid A, a modification that promotes resistance to CAPs other than Pm (29). This suggests that the specific position of the palmitate addition within lipid A determines the effect of this modification on CAP resistance.

Our results also indicate that PhoPQ and PmrAB are not the only two-component regulatory systems influencing Pm resistance in *P. aeruginosa*. This was confirmed recently when a third system (ParRS) was shown to control inducible Pm resistance (19). Whether the PhoPQ or PmrAB systems modulate CAP resistance through interactions with ParRS or other regulatory systems remains to be determined.

Analysis of Pm^r *P. aeruginosa* isolates collected from Danish CF patients revealed epidemic spread of a *phoQ* mutant strain as well as the occurrence of additional *phoQ* mutants in individual patients. A CF center in the United Kingdom has previously reported epidemic spread of a Pm^r *P. aeruginosa* strain among four patients; two others at that center had strains with unique genotypes (15). In Denmark, continuous treatment of many CF patients with inhaled CST over 10 to 15 years starting in the early to mid-1980s likely provided selection pressure for the emergence and spread of Pm^r *P. aeruginosa* strains (22, 36, 37).

The observation that some Danish CF patients were failing to respond clinically to continuous treatment with inhaled CST coincided with the detection of these *phoQ* mutant strains. CST inhalation was stopped for such patients, likely facilitating the emergence of Pm^s suppressor mutations in *phoP* and other loci. Such suppressor mutants readily outgrow *phoQ* strains that must divert a portion of their metabolic capacity to the modification of outer membrane components such as LPS. The subsequent resumption of CST inhalation in Danish CF patients with *phoP* suppressor mutants presumably provided renewed selection pressure that promoted reemergence of Pm^r strains with mutations in other loci that regulate L-Ara4N modification and other resistance mechanisms.

In summary, this work has shown that mutation of the PhoPQ system is one of several regulatory mechanisms underlying clinical Pm resistance. As with mutation of the PmrAB system, addition of L-Ara4N to lipid A is a consistent biochemical consequence of such mutations and appears to be required for Pm resistance. However, the PhoPQ system itself is not required for Pm resistance, as demonstrated by clinical strains that have lost PhoPQ functionality through secondary suppressor mutations in *phoP* but nonetheless display a high-level resistance phenotype. Most alarmingly, this work has shown that Pm^r *phoQ* mutant strains are capable of epidemic spread through CF clinical populations. This indicates the need for heightened infection control and clinical vigilance when CF patients are treated with inhaled CST or PMB for prolonged periods.

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