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The *Pseudomonas aeruginosa* PhoP-PhoQ Two-Component Regulatory System Is Induced upon Interaction with Epithelial Cells and Controls Cytotoxicity and Inflammation

Shaan L. Gellatly,[a] Brittany Needham,[c] Laurence Madera,[a] M. Stephen Trent,[b,c] and Robert E. W. Hancock[a]

Department of Microbiology and Immunology and Centre for Microbial Diseases and Immunity Research, University of British Columbia, Vancouver, British Columbia, Canada,[a] and Section of Molecular Genetics and Microbiology[b] and The Institute of Cellular and Molecular Biology,[c] The University of Texas at Austin, Austin, Texas, USA

The adaptation of *Pseudomonas aeruginosa* to its environment, including the host, is tightly controlled by its network of regulatory systems. The two-component regulatory system PhoPQ has been shown to play a role in the virulence and polymyxin resistance of *P. aeruginosa* as well as several other Gram-negative species. Dysregulation of this system has been demonstrated in clinical isolates, yet how it affects virulence of *P. aeruginosa* is unknown. To investigate this, an assay was used whereby bacteria were cocultured with human bronchial epithelial cells. The interaction of wild-type (WT) bacteria that had adhered to epithelial cells led to a large upregulation of the expression of the *oprH-phoP-phoQ* operon and its target, the *arn* lipopolysaccharide (LPS) modification operon, in a PhoQ-dependent manner, compared to cells in the supernatant that had failed to adhere. Relative to the wild type, a *phoQ* mutant cocultured on epithelial cells produced less secreted protease and lipase and, like the *phoQ* mutant, *piv*, *lipH*, and *lasB* mutants demonstrated reduced cytotoxicity toward epithelial cells. Mutation in *phoQ* also resulted in alterations to lipid A and to increased inflammatory LPS. These data indicate that mutation of *phoQ* results in a phenotype that is similar to the less virulent but more inflammatory phenotype of clinical strains isolated from chronic-stage cystic fibrosis lung infections.

Pseudomonas aeruginosa is a Gram-negative soil bacterium that causes opportunistic infections of plants and animals. While rarely causing infections in healthy humans, *P. aeruginosa* is the third most common cause of nosocomial infections in the United States and is the most common bacterial pathogen associated with eventually fatal chronic lung infections in cystic fibrosis (CF) patients (34). Infections can be severe, with high mortality, and are usually complicated by the increasing prevalence of antibiotic-resistant isolates (9). In light of this, improved understanding the mechanisms by which *P. aeruginosa* contributes to pathogenesis can lead to improved prevention and treatment regimens.

One of the most clinically important adaptations is that of *P. aeruginosa* to the lungs of cystic fibrosis patients. Several virulence factors have been characterized for *P. aeruginosa*; however, their expression and contributions to CF lung disease are still for the most part incompletely defined. It was previously observed in CF that many of the virulence factors proposed to be essential for establishing a lung infection are actually downregulated or lost during the progression to a chronic infection (13, 16, 51, 59, 61). Notable bacterial factors that are required for the initial colonization of the lungs but that are downregulated or lost in chronic CF isolates include flagella, pili, proteases, and smooth lipopolysaccharide (LPS). Furthermore, isolates from chronic infections typically overproduce the exopolysaccharide alginate to become mucoid, can have an altered LPS which lacks the B-band O-antigen (33), and add aminoarabinose and palmitic acid to lipid A (17). Despite these observations, the adaptive response of *P. aeruginosa* to the lung environment, whereby gene expression is immediately altered to adjust to the new environment, is not well understood.

Bacterial adaptations to environmental changes are mediated by two-component regulatory systems. PhoP-PhoQ is one such two-component system that has been identified in many Gram-negative bacteria, including *Salmonella*, *Yersinia*, and *P. aeruginosa* (21, 32, 50), as playing roles in the detection of Mg^{2+} levels, cationic peptide resistance, and pathogenesis. *P. aeruginosa* polymyxin-resistant clinical isolates and early-stage CF isolates have shown dysregulation of this system (4, 43, 57, 61), highlighting its role in resistance and suggesting a possible role in colonization.

The PhoP-PhoQ system of *P. aeruginosa* is distinctly different from those of the intracellular pathogens *Salmonella* and *Yersinia*, possibly reflecting the distinct infection strategies and ecological niches of these organisms. With *Salmonella* and *Yersinia*, both *phoP* and *phoQ* mutants demonstrate reduced virulence and have been shown to be required for invasion of macrophages and intracellular survival. Furthermore, both mutants are supersusceptible to cationic antimicrobial peptides, such as polymyxin B (7, 54). In contrast, *P. aeruginosa* is an extracellular pathogen, and only *phoQ* mutants demonstrate a distinct phenotype compared to wild type, including reduced virulence and increased resistance rather than increased susceptibility to polymyxins (21, 39). In both *Salmonella* and *P. aeruginosa*, resistance to cationic antimicrobial peptides (CAMPs) is believed to be mediated in part by the addition of aminoarabinose (L-Ara4N) to lipid A, which reduces the overall negative charge of the membrane and thereby reduces the attraction of CAMPs (24, 33). In *Salmonella*, PhoQ senses the presence of CAMPs, leading to upregulation of *phoP* and *phoQ*

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Address correspondence to Robert E. W. Hancock, bob@hancocklab.com.

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TABLE 1 Strains and plasmids used in this study

Strain	Genotype[a]	Reference(s) or source
WT	Wild-type *P. aeruginosa* PAO1	Lab strain
phoP	*phoP*::*xylE-aacC1*; Gm^r derivative of WT	39
phoQ	*phoQ*::*xylE-aacC1*; Gm^r derivative of WT	21, 39
phoQ pUCP(*phoQ*$^+$)	*phoQ* mutant with pUCP22-*phoQ*$^+$; Gm^r Cb^r	39
exsA	Ω insertion at bp 2358 within *exsA*	19
lipH	PAO1 transposon mutant ID51115	31
piv	PAO1 transposon mutant ID31339, ID31393	31
lasB	PAO1 transposon mutant ID32737	31
62	Environmental strain from soil	65

[a] Antibiotic resistance: Cb^r, carbenicillin; Gm^r, gentamicin.

and the induction of PhoP-regulated genes (3), while in *P. aeruginosa* PhoQ has no CAMP-sensing ability and this role is instead performed by the ParR-ParS system. The observation that *phoQ* disruption in *P. aeruginosa* results in both reduced pathogenesis and increased CAMP resistance indicates that its effects on virulence and persistence *in vivo* are not mediated through such resistance to host peptides (which might logically lead to increased competitiveness *in vivo*). Furthermore, mammals are not deficient in divalent cations, having millimolar levels of Mg^{2+} and Ca^{2+} in most tissues and body fluids, and although cationic peptides serve as the major potential host-induced mechanism for induction of this system in the cases of *Salmonella* and *Yersinia*, the mechanism by which *P. aeruginosa* PhoPQ might be upregulated in the host has not yet been elucidated. In this study, we demonstrate that the PhoPQ system is substantially induced during adherence to epithelial cells compared to planktonic cells that have failed to adhere. Moreover, we confirm that PhoQ controls the ability of *P. aeruginosa* to destroy epithelial cells and demonstrate that this is mediated primarily via lipases and proteases rather than toxins from the type 3 secretion system (T3SS). We also found that PhoQ mutation led to altered structure and inflammatory properties of the lipid A portion of LPS.

MATERIALS AND METHODS

Bacterial and mammalian cell cultures. *P. aeruginosa* strains and plasmids used in this study are listed in Table 1. Strains were grown in Lauria-Bertani (LB) broth or BM2-glucose minimal medium with a high (2 mM) or low (20 μM) $MgSO_4$ concentation at 37°C with aeration. The antibiotics gentamicin (50 μg/ml) and carbenicillin (300 μg/ml) were added to overnight cultures where necessary to maintain plasmids or mutant phenotypes. The simian virus 40-transformed, immortalized human bronchial epithelial cell line 16HBE14o$^-$ (HBE) was a gift from D. Gruenert (University of California, San Francisco, CA). It was grown in minimal essential medium (MEM) with Earle's salts (Gibco) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine and incubated at 37°C and 5% CO_2. Cells were passaged (for no more than 16 passages from the primary culture) by treating the monolayer with trypsin-EDTA (Invitrogen) at 37°C for 5 min to detach the cells from the flask. Detached cells were diluted with complete MEM.

RNA extraction, cDNA synthesis, and quantitative PCR (qPCR). HBE cells (HBEs) were seeded into tissue culture-treated culture dishes and grown to 100% confluence (2 to 3 days) at 37°C and 5% CO_2 in complete MEM. On the day of the experiment, HBEs were washed with MEM containing reduced FBS (1%) and rested for a minimum of 30 min. *P. aeruginosa* wild type (WT) and its *phoQ* mutant were grown in LB to mid-log phase, washed with phosphate-buffered saline (PBS), and resuspended in the MEM with reduced serum. Bacterial cells were added to the HBE monolayer at a multiplicity of infection (MOI) of 100. HBEs and bacteria were cocultured at 37°C and 5% CO_2 for 5 h. This MOI maximized the amount of adhered bacteria that could be obtained for RNA extraction, while the 5-h time point gave ample time for transcriptional changes to occur while minimizing cytotoxic effects to the HBEs. Medium containing planktonic bacteria was removed after coculture, the cells were precipitated by centrifugation, and the pellet was treated with RNAprotect (Qiagen) and stored at −80°C. Monolayers containing adhered bacteria were washed twice with PBS and incubated at 37°C for 5 min with 0.48 mM EDTA in PBS, followed by scraping to detach the cells, resuspension, and washing of the culture dish with PBS. The combined detached adhered fraction was centrifuged, treated with RNAprotect, and stored at −80°C.

RNA was extracted with an RNEasy minikit (Qiagen) using the company protocol adapted for RNAprotect. Contaminating DNA was removed by using a DNA-free kit (Ambion). RNA quality was checked by spectrophotometry and by agarose gel electrophoresis and stored at −80°C with 20 U of SUPERase-In RNase inhibitor (Ambion). Six micrograms of random primers (Invitrogen) was annealed to 3 μg total RNA at 70°C for 10 min, followed by 25°C for 10 min. RNA was then reverse transcribed with 600 U SuperScript II reverse transcriptase (Invitrogen) in solution containing 1× first-strand buffer, 10 mM dithiothreitol, 500 μM deoxynucleoside triphosphates, and 30 U SUPERase-In at 37°C for 1 h, 42°C for 3 h, and 72°C for 10 min. Analysis was carried out in an ABI Prism 7300 sequence detection system (Applied Biosystems) using the two-step qPCR kit with SYBR green detection (Invitrogen). Melting curve analysis was performed to ensure specificity. The fold change was determined using the comparative threshold cycle method with comparison to results with the *rpsL* gene, which encodes the 30S ribosomal protein S12. Experiments were repeated with three independent cultures.

Adherence assay. HBE cells were seeded into 6-well tissue culture-treated plates and grown to confluence as described above. The monolayer was infected with bacteria at a final optical density at 600 nm (OD_{600}) of 0.15 to 0.2 in MEM containing reduced serum (1%). Medium was removed after 5 h of coculturing at 37°C and 5% CO_2, and the turbidity was measured to estimate the number of CFU in the planktonic fraction. The HBE monolayer was washed three times with PBS to remove noninteracting bacteria, and then HBEs were lysed in 0.5 ml of 1% Triton X-100 in PBS on a gentle rotary shaker for 5 min at room temperature. This fraction (adhered) was removed to a microcentrifuge tube, the wells were washed with 0.5 ml PBS, and the washings were added to the microcentrifuge tube. Serial dilutions of both the planktonic and adhered fractions were plated onto LB agar plates and incubated overnight at 37°C. Adherence was calculated as the total number of adhered cells divided by the total number of cells in the coculture (adhered plus planktonic), multiplied by 100 to give a percentage.

Lipase and protease enzyme assays and cytotoxicity measurements. HBE cells were seeded into 24-well tissue culture-treated plates and grown to confluence in MEM containing 10% FBS and 2 mM L-glutamine without phenol red (Gibco). The monolayer was infected at an MOI of 100 for 5 h for lipase and protease assays and an MOI of 50 for 16 to 18 h for

TABLE 2 Gene expression of *P. aeruginosa* strain PAO1 WT and the *phoQ* mutant when adhered to HBE cells or unadhered in the supernatant

PA no.	Gene	Description	Mean fold change (±SEM) in ratio of adhered vs unadhered[a]	
			Wild type	*phoQ*[b]
PA1178	*oprH*	Outer membrane protein in operon with *phoP-phoQ*	5,200 ± 950	2.8 ± 0.8
PA1179	*phoP*	Two-component response regulator	116 ± 43	2.8 ± 0.5
PA4777	*pmrB*	Two-component response regulator	3.7 ± 1.6	6.0 ± 1.7
PA3552	*arnB*	Aminoarabinose synthesis; lipid A modification	995 ± 223	2.2 ± 0.7
PA4661	*pagL*	Lipid A 3-O-deacylase	3.9 ± 1.0	3.5 ± 0.5
PA1092	*fliC*	Flagellin subunit	5 ± 2.1	14.9 ± 3.6
PA2862	*lipA*	Lipase	4.3 ± 1.8	13.1 ± 2.5
PA1249	*aprA*	Alkaline protease	2.6 ± 0.8	2.2 ± 0.7
PA3841	*exoS*	Type 3 secreted effector, exoenzyme S	1.36 ± 0.3	1.31 ± 0.2
PA4626	*pilB*	Type IV pilin biogenesis protein	1.5 ± 0.3	1.1 ± 0.3
PA0026	*plcB*	Phospholipase C	1.2 ± 0.2	1.06 ± 0.4
PA1148	*toxA*	Exotoxin A	1.1 ± 0.7	0.9 ± 0.3
PA1430	*lasR*	Transcriptional regulator	1.9 ± 0.3	2.5 ± 0.6

[a] Results of interaction assays; the data are from at least three biological replicates.
[b] Note that both *phoQ* adherent and unadhered cells showed similar high expression levels of the *oprH* and *phoP* gene products compared to the wild type.

cytotoxicity assessments in MEM containing reduced serum (1%). Medium was removed after coculturing, centrifuged to remove bacteria, and stored at 4°C. Supernatants for enzyme assays were further purified by filtration through a 0.22-μm-pore-size membrane Lipase assays were performed as described as adapted elsewhere, from the method of Furutani (20). Briefly, 180 μl of cell-free supernatant was combined with 20 μl of 4 mM *p*-nitrophenyl palmitate (Sigma) in enzyme buffer (0.1 M NaH_2PO_4/K_2HPO_4, pH 7.6) in a 96-well plate. The reaction mixtures were incubated at 37°C overnight and then read at 410 nm and compared to a standard curve for *p*-nitrophenol (Sigma). For the protease assay, equal volumes of cell-free supernatant and 0.8 mM *N*-succinyl-(alanine)$_3$-*p*-nitroaniline (Sigma) were combined in a 96-well plate. The reaction mixtures were incubated at 37°C overnight, read at 410 nm, and compared to a standard curve for *p*-nitroaniline (Sigma). Cytotoxicity was measured by the release of lactate dehydrogenase (LDH) by using a kit (Roche Applied Sciences).

LPS and lipid A extraction and analysis. Bacteria were grown in LB broth at 37°C with aeration for LPS extraction. LPS was isolated using the Darveau-Hancock method (12), then extracted twice with chloroform-methanol (2:1) to remove contaminating phospholipids (19), and dialyzed 5 times against 0.5 mM HEPES (pH 7.4)–5 mM Na_2EDTA (pH 8), twice against 5 mM HEPES (pH 7.4)–50 mM NaCl, and twice against distilled water for conversion to sodium salts (52). The concentration of LPS was estimated from the amount of 2-keto-3-deoxyoctonate (49). Bacteria were grown in BM2-glucose high (2 mM) or low (20 μM) $MgSO_4$ for lipid A isolation until cultures reached an OD_{600} of ~1. Lipid A was isolated according to the method of Zhou et al. (67) from bacteria grown in LB or in BM2-glucose with 2 mM or 20 μM $MgSO_4$. Briefly, cells were harvested in a clinical centrifuge and washed with PBS. Cell pellets were resuspended and lysed in a single-phase Bligh/Dyer mixture of chloroform-methanol-water (1:2:0.8 [vol/vol]). After 20 min, the insoluble material was released by hydrolysis for 30 min at 100°C in the presence of 12.5 mM sodium acetate, pH 4.5, and 1% SDS. Lipid A was recovered by two-phase Bligh/Dyer extraction and then dried under a stream of air, resuspended in 2 ml distilled water and 10 ml of acidified ethanol (100 μl HCl to 20 ml ethanol), and centrifuged. The pellet was washed twice with 95% ethanol and dried under a stream of air. Lipid A species were analyzed using a matrix-assisted laser desorption ionization–time of flight mass spectrometer (MALDI-TOF/TOF ABI 4700 proteomics analyzer) in the negative ion mode with the linear detector as previously described (26).

LPS stimulation of PBMCs and cytokine analysis by ELISA. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy human donors in accordance with UBC ethics guidelines as described previously (45). PBMCs were seeded at 5×10^5 cells/well in a 24-well tissue culture-treated plate in RPMI medium (Invitrogen) supplemented with 10% FBS and 2 mM L-glutamine. The cells were rested at 37°C and 5% CO_2 for a minimum of 30 min prior to addition of LPS at 50 ng/ml. Medium was collected at 4 and 24 h after stimulation and centrifuged, and supernatants were stored at −20°C. Cytokine levels were measured by enzyme-linked immunosorbent assay (ELISA) with anti-human interleukin-6 (IL-6) antibody clones MQ2-1345 and Mq2-39C3 (eBioscience), anti-human CCL2 (MCP1) antibody clones 5D3-F7 and 2H5 (eBioscience), anti-human tumor necrosis factor alpha (TNF-α) antibody clones MAb1 and MAb11 (eBioscience), and anti-human IL-10 antibody clones JES3-9D7 and JES3-12G8 (eBioscience), all followed by avidin-horseradish peroxidase (eBioscience) conjugation as per the manufacturer protocols. ELISA reactions were developed using a tetramethylbenzidine liquid substrate system (Sigma) and imaged with a PowerWave ×340 plate reader (Bio-Tek Instruments). Cytokine quantification was performed against standard curves of recombinant cytokines IL-6 (catalog number 14-8069;

TABLE 3 Gene expression of *P. aeruginosa* environmental strain 62 when adhered to HBE cells versus unadhered in the supernatant

PA no.	Gene	Description	Mean fold change (±SEM) in ratio for adhered vs unadhered[a]
PA1178	*oprH*	Outer membrane protein in operon with *phoP-phoQ*	159,000 ± 44,000
PA1179	*phoP*	Two-component response regulator	640 ± 90
PA4777	*pmrB*	Two-component response regulator	20 ± 2
PA3552	*arnB*	Aminoarabinose synthesis; lipid A modification	14,000 ± 3,400

[a] Data are from at least three biological replicates.

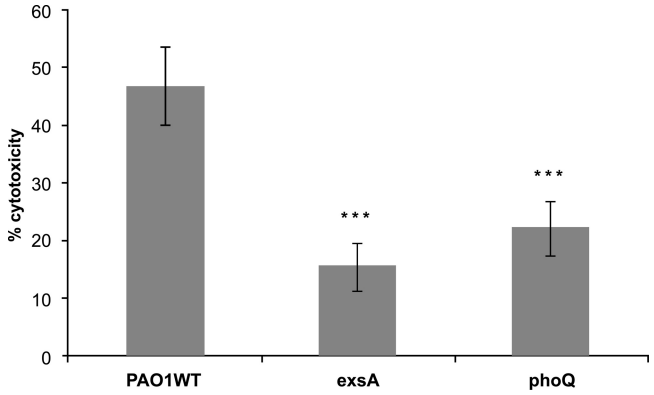

FIG 1 Cytotoxicity was reduced in a type 3 secretion system *exsA* mutant and in a *phoQ* mutant. The mutants and the WT were added to HBE cells at an MOI of 50 and cocultured for 20 to 24 h. Cytotoxicity was measured as the amount of lactate dehydrogenase released from the HBE cells compared to a Triton X-100 lytic control. Data shown are means and standard errors of means from more than three independent experiments. ***, $P < 0.001$ (Student's *t* test).

eBioscience), TNF-α (catalog number 14-8329; eBioscience), and IL-10 (catalog number 14-8109; eBioscience).

RESULTS

Gene expression was altered during interaction with human epithelial cells *in vitro*. Adherence to epithelial surfaces is considered a key factor in the initial stages of infection in most bacterial infections. It is well documented that strains of *P. aeruginosa* isolated from chronic infections are genetically distinct from commonly used lab strains, from environmental isolates, and from strains isolated from acute infections (8, 17, 30, 42, 59). It is believed that over time unknown selective pressures during a chronic infection give rise to strains that have lost the ability to express numerous virulence factors, such as the type 3 secretion apparatus or lipopolysaccharide containing O-antigen, yet little is known about the transcriptional and physiological changes that occur during or immediately following colonization. To provide insights into how *P. aeruginosa* initially adapts itself to the host lung environment, we analyzed by qPCR the transcriptional profiles of several bacterial genes pertaining to virulence when the well-studied *P. aeruginosa* strain PAO1 was coincubated with HBE cells for 5 h.

The two-component regulatory system sensor kinase PhoQ has demonstrated roles in virulence, the response to limiting Mg^{2+} levels, and cationic antimicrobial peptide resistance in several Gram-negative bacteria, including *P. aeruginosa* (21). *phoQ* is transcribed as two transcripts, *oprH-phoP-phoQ* and *phoP-phoQ*, where *oprH* is an outer membrane protein and *phoP* is a cognate response regulator (38). In bacteria that had adhered to HBE cells, *phoP* was upregulated 116-fold and *oprH* by an astonishing >5,000-fold compared to bacterial cells that did not adhere (Table 2). This was despite the fact that the cell culture medium contained millimolar concentrations of Mg^{2+} that would normally suppress transcription from the *oprH-phoP-phoQ* operon (38). That *oprH* would be upregulated much more so than *phoP* possibly reflects its demonstrated ability to be transcribed as a single gene without *phoP* or *phoQ* (38). Genes in the known PhoQ regulon (21, 64) were also upregulated in adhered bacteria, including *arnB* (1,000-fold), encoding an enzyme involved in the addition of aminoarabinose to lipid A, and *pmrB* (3.7-fold), a two-component regulator that also regulates the expression of *arnB* in response to limiting Mg^{2+} levels. Another lipid A modification gene, *pagL*, which encodes a 3-O-deacylase that removes a C_{10} acyl chain from lipid A, was also upregulated by 3.7-fold, although this gene has not been shown to be part of the PhoQ regulon. Strains of *P. aeruginosa* isolated from early CF lung disease (i.e., prior to development of a chronic infection) have been shown to differ substantially from strains isolated during the chronic state, and instead

TABLE 4 Effects of the *phoQ* mutation during adherence to HBEs on the expression of known cytotoxicity-associated virulence factors compared to WT

PA no.	Gene	Description	Mean fold change (±SEM) in ratio for *phoQ* vs WT[a]
PA3724	*lasB*	Elastase B	−(26 ± 10.5)
PA4175	*piv*	Protease IV	−(3.0 ± 1.3)
PA1249	*aprA*	Alkaline metalloproteinase	−(1.2 ± 0.2)
PA2862	*lipA*	Lipase A	−(4.4 ± 2.2)
PA2863	*lipH*	Lipase modulator protein	−(5.7 ± 2.9)
PA4813	*lipC*	Lipase C	−(3.0 ± 0.4)
PA0026	*plcB*	Phospholipase C	1.1 ± 0.4
PA1092	*fliC*	Flagellin subunit	−(13.1 ± 4.3)
PA4526	*pilB*	Type IV pilin subunit	−(1.1 ± 0.1)
PA1178	*oprH*	Outer membrane protein, OprH, in operon with *phoP-phoQ*	5,500 ± 1,000
PA1179	*phoP*	Response regulator	10.5 ± 1.8
PA3552	*arnB*	Lipid A aminoarabinosylation	143 ± 59
PA4661	*pagL*	Lipid A 3-O-deacylase	−(7.4 ± 2.1)
PA4209	*phzM*	Pyocyanin biosynthesis	−(4.1 ± 1.4)
PA2399	*pvdD*	Pyoverdine synthetase	−(1.5 ± 0.1)
PA3841	*exoS*	Exoenzyme S, type 3 secretion effector	0.9 ± 0.3
PA1148	*toxA*	Exotoxin A	−(1.5 ± 0.5)
PA3477	*rhlR*	Quorum-sensing regulator, RhlR	−(2.8 ± 0.8)
PA1430	*lasR*	Quorum-sensing regulator, LasR	−(2.6 ± 0.9)

[a] Gene expression data from qPCRs are based on at least three biological replicates.

the isolates from early CF disease resemble environmental isolates. Therefore, we repeated the transcriptional analysis in an environmental strain, strain 62. Similar to PAO1, strain 62 demonstrated substantial upregulation of *oprH*, *phoP*, *arnB*, and *pmrB* during interaction with HBE cells (Table 3), indicating that strain PAO1 adequately reflected an early colonizing strain of *P. aeruginosa* in CF lung disease.

Other virulence-related genes were analyzed by qPCR to see if there were changes in expression between bacteria that had adhered to HBE cells and bacteria that had not adhered. Previous studies by Chugani and Greenberg (10) demonstrated that bacteria cocultured with epithelial cells upregulated quorum-sensing systems and secreted increased proteases and lipases compared to the same bacteria grown in normal lab medium. In this study, the comparison was between adhered and nonadhered bacteria within the same coculture. We found that three genes were upregulated: *fliC* (5-fold), encoding the flagellin subunit, *aprA* (2.6-fold), encoding the secreted alkaline protease, and *lipA* (4.3-fold), encoding a secreted lipase. These genes were also upregulated in a *phoQ* mutant when it adhered to HBE cells compared to a nonadhered *phoQ* mutant (Table 2). Interestingly, several known virulence factors displayed no altered transcription between adhered and nonadhered bacterial cells in our system, including the type 3 secreted exoenzyme S (*exoS*), a type IV pilus biogenesis protein (*pilB*), a phospholipase (*plcB*), exotoxin A (*toxA*), and the quorum-sensing regulator LasR. Many of these virulence factors have been shown to play roles in virulence in acute infections. In particular, the type 3 secretion system is correlated with increased negative outcomes in acute clinical infections (27, 36, 62). To check that the laboratory strain PAO1 used here could effectively induce HBE cell cytotoxicity via the type 3 secretion system, we utilized a mutant of ExsA, the master regulator for this system, and compared its cytotoxic effect to that of the WT (Fig. 1). The *exsA* mutant demonstrated 3-fold-reduced cytotoxicity to HBEs compared to the WT at 24 h, indicating that the type 3 secretion system was effective in causing cytotoxicity in strain PAO1. A 2-fold reduction in cytotoxicity was observed for the *phoQ* mutant, confirming its contribution to this aspect of virulence (21).

Virulence factors were downregulated in a *phoQ* mutant during interaction with human epithelial cells *in vitro*. We previously demonstrated in a microarray that mutation of *phoQ* from *P. aeruginosa* resulted in the dysregulation of >450 genes (21). Although some of these genes were involved in virulence, we were unable to satisfactorily explain how a *phoQ* mutant showed such a marked attenuation, possibly because these data were obtained from bacteria grown in defined laboratory medium. To resolve this, we utilized qPCR to compare the gene expression of known virulence genes in a *phoQ* mutant to that in the WT when cocultured on a monolayer of HBEs (Table 4).

Several proteases and lipases are secreted by *P. aeruginosa*, and many have well-established roles in virulence (18, 29, 34, 55). Many of these were downregulated in the *phoQ* mutant based on qPCR analysis (Table 4), including several that were not dysregulated compared to these strains grown in normal lab broth medium (21). Proteases contribute significantly to tissue damage in respiratory infections, influencing the degradation of host surfactant proteins, immunoglobulin, and fibrin and the disruption of epithelial tight junctions (6, 34). Of the proteases examined, elastase B (*lasB*) revealed the biggest difference from the WT, demonstrating 26-fold-reduced expression in the *phoQ* mutant (Table 4).

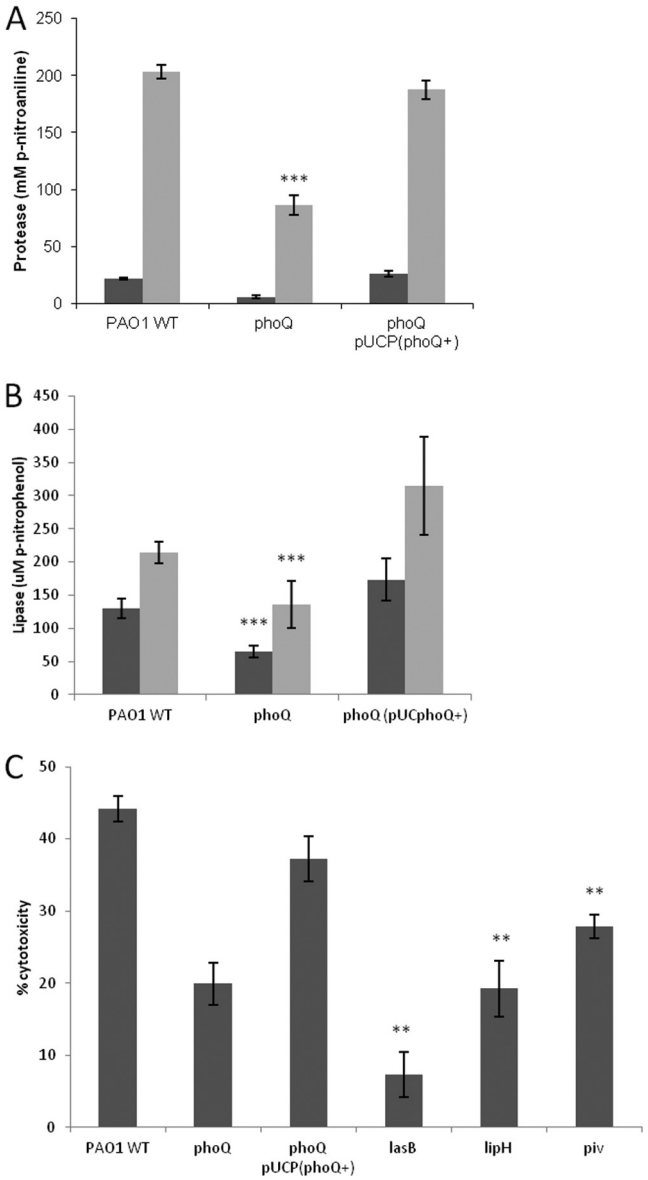

FIG 2 Mutation of *phoQ* led to decreased protease and lipase secretion during interaction with epithelial cells. (A and B) Protease (A) and lipase (B) activities in the media of cocultured HBEs and *P. aeruginosa* strain PAO1 and a *phoQ* mutant at 5 h (dark gray) and 20 h (light gray). (C) Cytotoxicities of protease (*piv*) and lipase (*lipH*) mutants compared with the wild type, *phoQ* mutant, and complemented *phoQ*$^+$ strain. **, $P < 0.01$; ***, $P < 0.001$ (Student's *t* test).

The *P. aeruginosa* elastase B is regulated by the *lasI* quorum-sensing system (14, 56), which was downregulated to a much lesser degree (−2.6-fold). We also found a 3-fold downregulation of protease IV (*piv*), a protease which has an established role in bacterial keratitis but is less frequently recognized in lung infections (29, 40, 41). No difference was found for alkaline protease (*aprA*; −1.2-fold). Lipases *lipA* and *lipC*, as well as the lipase modulator *lipH*, which controls both LipA and LipC, were downregulated in the *phoQ* mutant by 3- to 5.7-fold. Both lipases and phospholipases have been shown to break down lung surfactant, which is composed of 90% lipid, and to directly target host cell membranes

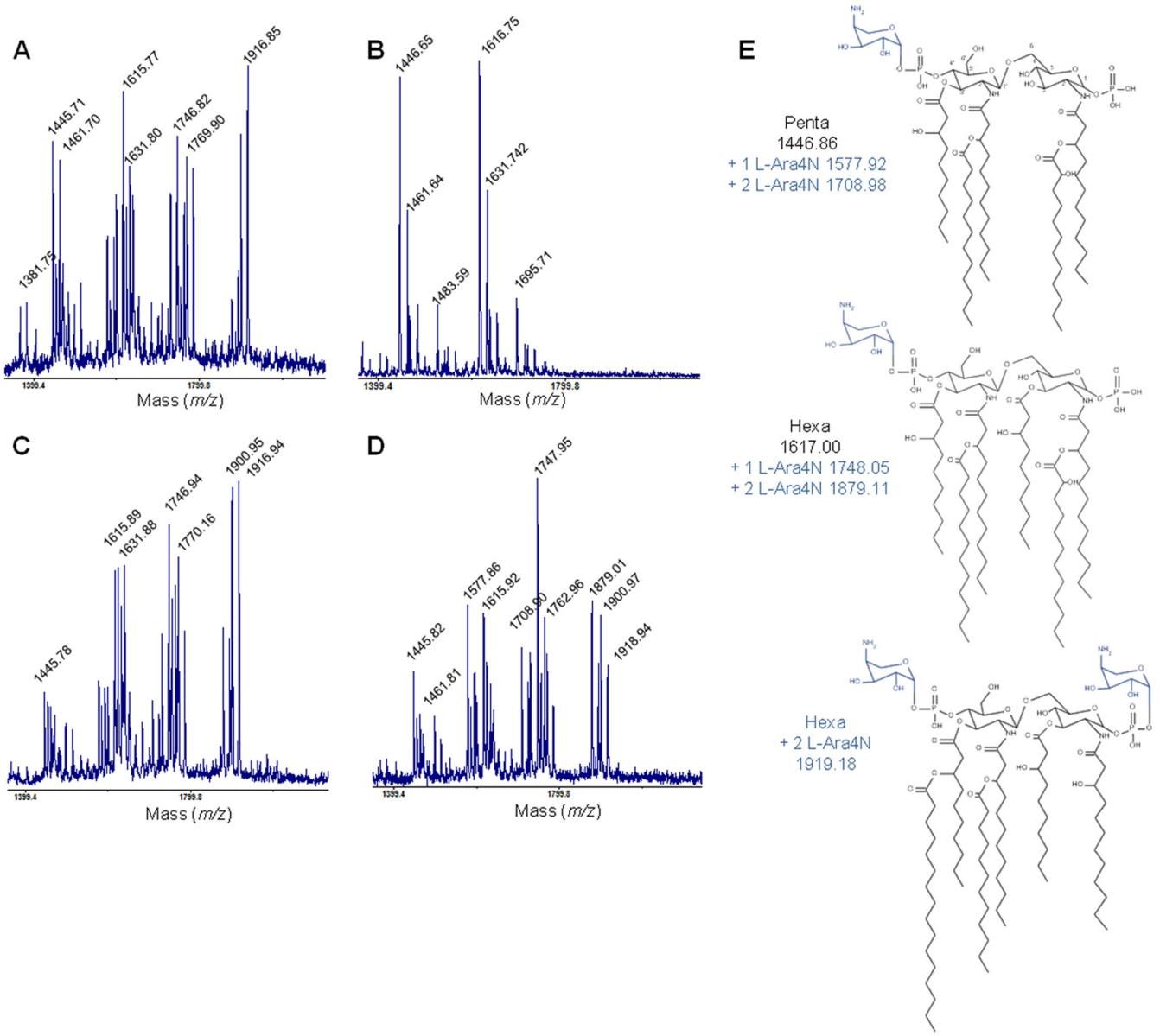

FIG 3 Lipid A was altered in a *phoQ* mutant. MALDI-TOF mass spectrometry results show the relative levels of intensities of different lipid A species isolated from *P. aeruginosa* PAO1. (A and B) The wild type when grown in BM2-glucose containing 20 μM $MgSO_4$ demonstrated addition of L-Ara4N to lipid A (A), but not when grown with 2 mM $MgSO_4$ (B). (C and D) L-Ara4N was added to the lipid A of the *phoQ* mutant grown in either 20 μM $MgSO_4$ (C) or 2 mM $MgSO_4$ (D). Adjacent peaks that differed by 16 *m/z* units represented the addition of a hydroxyl group (OH). (E) Lipid A structures of common mass peaks, showing exact masses. L-Ara4N can be added to either or both of the 1- and 4′-phosphate groups (shown in blue).

(28, 60). No dysregulation was found for a phospholipase (*plcB*; 1.1-fold). Similarly, neither the T3SS (*exoS*; 0.9-fold) nor the type 2 system-secreted exotoxin A (*toxA*; −1.5-fold) was dysregulated in a *phoQ* mutant (Table 4). From the results, we were able to infer that PhoQ controls cytotoxicity through lipases and proteases, but not through T3SS or exotoxin A. Both the WT and the *phoQ* mutant have demonstrated similar growth rates in tissue culture medium (21) and during coculture with HBE cells.

Lipases and proteases were reduced in the *phoQ* mutant. As the transcription of proteases and lipases was downregulated in the *phoQ* mutant, enzyme assays were used to see if the quantities of proteases and lipases were also reduced (Fig. 2A and B). These assays showed statistically significant reductions in both classes of enzymes in the *phoQ* mutant, particularly for proteases, which showed a 2.5-fold reduction for the *phoQ* mutant. However, these assays did not distinguish the specific proteases and lipases produced by *P. aeruginosa*. The *phoQ* mutant was previously shown to have a deficiency in rapid attachment to polystyrene (21); therefore, the lipase and protease transcriptional differences seen here for the *phoQ* mutant could have been influenced by differing abilities of the *phoQ* mutant and the WT to adhere to HBE cells. It was determined that 10.4 ± 1.4% of WT and 9.5 ± 3.5% of the *phoQ* mutant adhered to epithelial cells, and this difference was not statistically significantly different ($P = 0.84$).

TABLE 5 Peak assignments from MALDI-TOF[a]

Exact mass or *m/z* peak	Proposed lipid A structure
Exact mass (kDa) forms	
1,367.72	Tetra-acylated, includes a single L-Ara4N
1,498.78	Tetra-acylated, includes two L-Ara4N
1,446.86	Penta-acylated, missing C_{10} acyl chain at position 3
1,462.85	Hydroxylation of 1,446.86 form
1,577.92	1,446.86 structure including a single L-Ara4N
1,708.98	1,446.86 structure including two L-Ara4N
1,617.00	Hexa-acylated, includes C_{10} acyl chain at position 3
1,632.99	Hydroxylation of 1,617.00 form
1,748.05	1,617.00 structure including a single L-Ara4N
1,764.04	Hydroxylation of 1,748.05 form
1,879.11	1,617.00 structure including two L-Ara4N
1,919.18	Hexa-acylated, includes secondary acylation (C_{16}) at 3′ position, missing secondary acylation (C_{12}) at position 2
m/z peaks	
1,484	Sodium adduct of 1,462.85 form
1,770	Sodium adduct of 1,748.05 form
1,901	Sodium adduct of 1,879.11 form

[a] Based on MALDI-TOF results shown in Fig. 3.

To confirm that the dysregulated lipases and proteases might be involved in the altered virulence of the *phoQ* mutant, lipase- and protease-deficient strains were tested, and these strains demonstrated reduced cytotoxicity toward HBEs (Fig. 2C). In particular, a mutation in *lasB* almost totally abrogated epithelial cell cytotoxicity. This was consistent with the known ability of *P. aeruginosa* elastase to degrade a variety of host proteins and cause tissue damage, as well as the attenuation of virulence of a *lasB* mutant in a murine acute lung infection model (35).

The LPS of the *phoQ* mutant was more inflammatory. Both the presence of the C_{10} acyl chain at the 3-position of lipid A and an L-Ara4N sugar at the 1- and 4′-phosphate groups have been demonstrated in laboratory strain *phoQ* mutants and in cystic fibrosis clinical isolates that contain a *phoQ* mutation (17, 43). The results here indicated >100-fold upregulation of the *arn* operon in the *phoQ* mutant when incubated with HBEs and a 7.4-fold downregulation of the lipid A deacylase, *pagL*, responsible for the removal of the C_{10} acyl chain. Consistent with this, our *phoQ* mutant grown under both Mg^{2+}-rich and depleted conditions demonstrated the presence of a C_{10} acyl chain (to give a hexa-acylated species) and the addition of L-Ara4N (Fig. 3). Interestingly, the *phoQ* mutant revealed a variety of modified lipid As, including penta- (*m/z* 1,447) and hexa-acylated species (*m/z* 1,617) containing one (*m/z* 1,578, 1,747) and two (*m/z* 1,709, 1,879) L-Ara4N sugars and another hexa-acylated species that differed in acyl chain and length and contained L-Ara4N (*m/z* 1,919). A similar trend was observed for the WT and *phoQ* mutant grown in LB (data not shown). Additional peak assignments are presented in Table 5. While such changes have been proposed to impact the resistance to polymyxins and antimicrobial peptides, we examined here whether they could also affect the inflammatory response.

The LPS of *P. aeruginosa* plays a key role in virulence and in host innate and acquired responses during infection. The lipid A portion of LPS triggers an inflammatory response by sequentially binding host coreceptors CD14 and MD2, leading to activation of Toll-like receptor 4 (TLR4) in the NF-κB pathway and triggering the production of proinflammatory cytokines, inflammation, and eventually endotoxic shock (1, 63). Hyperacylated forms of lipid A (i.e., hexa- and hepta-acylated) have been shown to be more inflammatory (2, 63), and isolates of *P. aeruginosa* from chronic infections of the CF lung demonstrate increased lipid A acylation and increased NF-κB-mediated responses in the host (16, 46). As the lipid A of the *phoQ* mutant was modified, we hypothesized that its inflammatory properties may also be altered. To ascertain whether the lipid A from the *phoQ* mutant was more or less inflammatory, whole LPS was isolated from this mutant and the wild type grown in LB and used to stimulate human PBMCs (Fig. 4), as the HBE cell line has been shown to be comparatively weakly responsive to TLR4 agonists (48, 53, 58). After 4 and 24 h, the proinflammatory cytokine TNF-α was produced at over-5-fold and -9-fold higher levels, respectively, from PBMCs stimulated with LPS from the *phoQ* mutant than WT. Similarly, the secretion of proinflammatory IL-6 was approximately 5-fold and 2-fold greater with *phoQ* LPS at 4 and 24 h. The anti-inflammatory cytokine IL-10 was also secreted to a greater extent (3-fold), although this effect was seen only at 24 h, consistent with its role as a homeostatic regulator of inflammation (5).

DISCUSSION

Isolates of *P. aeruginosa* from chronically infected CF patients have revealed genotypic changes that result in marked phenotypic differences from isolates of newly acquired or acute infections (30, 42, 59, 66). Noticeably, these changes include conversion to a mucoid, nonmotile state due to the overproduction of alginate and the loss of pili and flagella (59), changes to lipid A, including aminoarabinosylation and hyperacylation (15), LPS that displays shortened or a complete lack of O-antigen (25), and a reduction in secreted virulence factors, including elastase (30). These changes have been proposed to reflect adaptations to a chronic infection state aided by changes in mutators (42) or stepwise mutations as an adaptation to specific selective pressures. However, the early-term adaptive responses of *P. aeruginosa* to the lung environment are not well understood. Here we have demonstrated that the PhoPQ regulon is substantially upregulated in response to interaction with epithelial surfaces.

The PhoPQ two-component system has a profound role in the pathogenesis of several Gram-negative organisms (21–23, 32, 37, 44). We have revealed that the *P. aeruginosa* PhoPQ system is upregulated during adherence to epithelial cells, as are a number of genes known to be PhoQ or Mg^{2+} regulated, suggesting that PhoPQ may partially control the adaptation of *P. aeruginosa* to epithelial surfaces. Several studies have identified *phoQ* mutations in polymyxin-resistant clinical isolates from both acute and chronic infections (4, 43, 57, 61). Since the PhoPQ system is transcriptionally upregulated upon adherence of the WT to epithelial cells, it is possible this contributes to early adaptations to the lung environment that are later stabilized by a mutation. Since polymyxins are increasingly utilized as a drug of last resort in patients with recalcitrant multiresistant infections and aerosolized polymyxin is routinely used in European CF patients (11, 31, 47), the combined selective pressures of adherence and polymyxins may

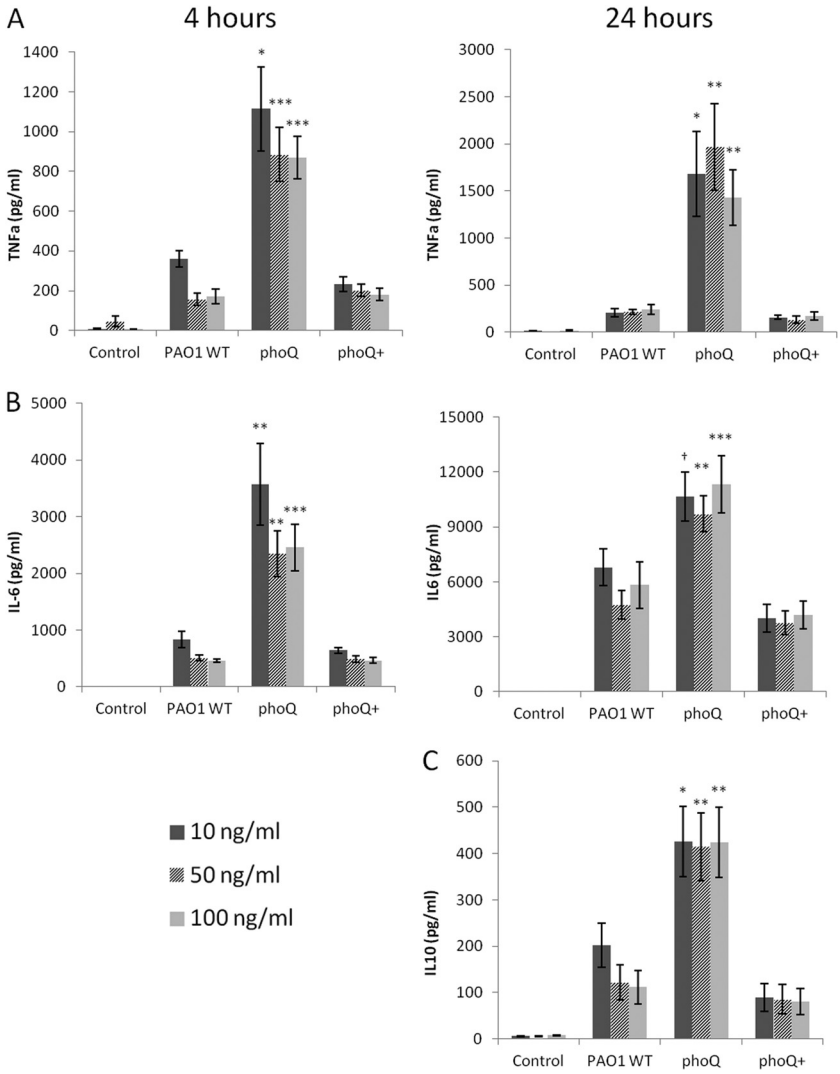

FIG 4 LPS from a *phoQ* mutant elicits a greater inflammatory response. LPS was isolated from log-phase bacteria grown in LB and used to stimulate human PBMCs from healthy donors at 10, 50, and 100 ng/ml. Secretion of TNF-α (A), IL-6 (B), and IL-10 (C) from PBMCs was analyzed at 4 h and 24 h. Shown are means (and standard errors of the means) for at least three donors. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; †, $P = 0.052$ (Student's *t* test).

expedite the emergence of resistant strains due to mutations in *phoQ*.

The acylation state of lipid A is known to affect the extent to which it can initiate an inflammatory response. CF is already described as a hyperinflammatory disease, and part of this may be due to the more inflammatory lipid A produced by infecting strains of *P. aeruginosa* (46). In this study, a *phoQ* mutant similarly produced a more inflammatory LPS, which in turn was likely due to its altered lipid A. It is interesting that a *phoQ* mutant demonstrated reduced virulence and cytotoxicity while possessing a more inflammatory LPS. Together, these attributes could contribute to the lowered competitiveness previously shown in a chronic lung infection model (21) in which less cytotoxic damage is caused to the host epithelia while a strong inflammatory response is initiated by the host in response to a more inflammatory LPS. While further work is required to understand this phenotype in the context of the CF lung, it is well known that both antibiotic resistance development and uncontrolled inflammation are hallmarks of chronic disease in CF. Possibly, the suppression of specific virulence factors and decreased toxicity toward epithelial cells favor the chronic lifestyle established in this disease.

In this study, it was shown that the PhoPQ system is induced upon adherence to epithelial cells and controls virulence through reduced secreted cytotoxic enzymes and altered inflammatory properties of LPS. We suggest that a PhoQ-mediated control of virulence may occur as an inducible adaptation to the lung environment which involves adherence to the epithelia and which would contribute to and be exacerbated by the selective pressure of inhaled polymyxins. Since evidence suggests that PhoQ acts primarily as a phosphatase rather than a kinase for PhoP (38, 39), adherence may be a primary signal that promotes PhoP phosphorylation *in vivo*. Consistent with this was the fact that the epithelial cell medium employed contained sufficient divalent cations to prevent induction of the *oprH-phoP-phoQ* operon through divalent cation deficiency. Understanding how *P. aeruginosa* adapts to favor a chronic infection in the CF lung may provide

avenues for preventing the advancement of this debilitating disease as well as suggest antibiotic treatment regimens that are less likely to result in the emergence of resistant strains.

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