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The sensor kinase PhoQ mediates virulence in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is a ubiquitous environmental Gram-negative bacterium that is also a major opportunistic human pathogen in nosocomial infections and cystic fibrosis chronic lung infections. PhoP-PhoQ is a two-component regulatory system that has been identified as essential for virulence and cationic antimicrobial peptide resistance in several other Gram-negative bacteria. This study demonstrated that mutation of *phoQ* caused reduced twitching motility, biofilm formation and rapid attachment to surfaces, 2.2-fold reduced cytotoxicity to human lung epithelial cells, substantially reduced lettuce leaf virulence, and a major, 10 000-fold reduction in competitiveness in chronic rat lung infections. Microarray analysis revealed that PhoQ controlled the expression of many genes consistent with these phenotypes and with its known role in polymyxin B resistance. It was also demonstrated that PhoQ controls the expression of many genes outside the known PhoP regulon.

INTRODUCTION

The ubiquitous soil bacterium *Pseudomonas aeruginosa* is an important pathogen capable of infecting a wide range of hosts, including insects, nematodes, plants and mammals (Rahme *et al.*, 2000, 1995). In humans, *P. aeruginosa* rarely causes infections in healthy individuals, yet it is an efficient opportunist that causes serious infections in individuals with underlying medical conditions, such as severe burns, cystic fibrosis or HIV infection, or those undergoing mechanical ventilation (Pruitt *et al.*, 1998; Sadikot *et al.*, 2005). Indeed, *P. aeruginosa* is the most common cause of acute nosocomial pneumonia, and of chronic respiratory infections in cystic fibrosis patients. *P. aeruginosa* infections are characterized by their high incidence, severity, and increasing high-level resistance to antibiotics (Kipnis *et al.*, 2006). While the human lung inhales more than 10 000 litres of non-sterile air each day, the lower airway remains infection-free and maintains no commensal microbial population (Flato *et al.*, 1996). In light of this, the toxic effects of *P. aeruginosa* on the lung are of particular interest and importance.

Bacterial two-component regulatory systems are widely used by prokaryotes to sense and respond to environmental cues. They consist of a sensor histidine kinase and a transcriptional activator (Gooderham & Hancock, 2009; Rodrigue *et al.*, 2000). PhoP-PhoQ is one such two-component regulatory system, which has been identified as important for virulence in several *Enterobacteriaciae* but has yet to be linked to virulence in *P. aeruginosa*. This system has been best characterized in *Salmonella*, where it regulates a variety of bacterial functions, including invasion, motility, transport of small molecules, acid tolerance, antimicrobial peptide resistance and bacterial surface remodelling (Groisman, 2001; Prost & Miller, 2008). The *Salmonella* PhoQ sensor kinase responds to limiting divalent cations and to increasing acidity as well as conferring inducible resistance (via the PhoP response regulator) to host cationic antimicrobial peptides (Bader *et al.*, 2005; Groisman, 2001; Prost & Miller, 2008). Under Mg^{2+}- or Ca^{2+}-limiting (submicromolar) conditions, both *Salmonella* and *Pseudomonas* PhoP-regulated transcription is induced, while under high-Mg^{2+} or -Ca^{2+} conditions (i.e. millimolar), the PhoPQ system is strongly repressed.

Fundamental differences have been found between PhoQ in *Salmonella* and *P. aeruginosa*. Although *P. aeruginosa*

Abbreviations: CI, competitive index; LDH, lactate dehydrogenase; RT-qPCR, real-time quantitative PCR.

The ArrayExpress accession number for the microarray data associated with this paper is E-FPMI-18

Two supplementary tables are available with the online version of this paper, showing the oligonucleotides used and the microarray analysis of genes significantly dysregulated in the *phoQ* mutant relative to wild-type.

PhoQ responds normally to limiting concentrations of Mg^{2+} (causing resistance), unlike in *Salmonella*, *phoQ* mutants display increased resistance to peptides and polymyxin B when grown in high concentrations of Mg^{2+} (Macfarlane *et al.*, 2000; McPhee *et al.*, 2006). In contrast to *Salmonella* (Bader *et al.*, 2005; Prost *et al.*, 2008), *P. aeruginosa* PhoQ is not activated by cationic antimicrobial peptides and only very slightly activated under acidic conditions (McPhee *et al.*, 2006). Furthermore, while *Salmonella* PhoQ is able to both phosphorylate (activate) and dephosphorylate (repress) PhoP, the *Pseudomonas* PhoQ sensor appears to regulate PhoP only by dephosphorylation (Macfarlane *et al.*, 1999). These differences suggest that PhoQ in *P. aeruginosa* has different sensing capabilities from its *Salmonella* equivalent and that these differences might be due to the different ecological niches inhabited by these two bacterial genera. In particular *Salmonella* is a facultative intracellular pathogen for which PhoQ seems to play a role in intracellular survival and growth, while *Pseudomonas* is predominantly an opportunistic extracellular pathogen.

In this study we sought to determine whether the PhoQ sensor kinase of *P. aeruginosa* contributes to pathogenesis. We demonstrate that mutation of *phoQ* caused reduced cytotoxicity to human bronchial epithelial cells and reduced survival in chronic rat lung infections. Using microarray analysis, we show that PhoQ controls many genes that are consistent with these data and with the known role of PhoQ in polymyxin B resistance. We also show that PhoQ controls the expression of genes outside the known PhoP regulon.

METHODS

Tissue culture, bacterial strains, primers and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. Of note, a *phoQ* mutant was freshly constructed based on the originally described protocol (Macfarlane *et al.*, 2000) to avoid the phenotypic dilution that can occur with such mutants during storage. The sequences of the DNA primers used are supplied as Supplementary Table S1, available with the online version of this paper. All primers were synthesized by AlphaDNA (Montreal, QC, Canada). Cultures were routinely grown in Luria–Bertani (LB) broth or BM2-glucose minimal medium containing low (20 μM) or high (2 mM) $MgSO_4$ concentrations. Antibiotics for selection were used at the following concentrations: 50 μg tetracycline ml^{-1}, 300 μg carbenicillin ml^{-1}, and 30 μg gentamicin ml^{-1}. Routine genetic manipulations were carried out by standard molecular biology procedures (Ausubel, 1987).

The SV40-transformed, immortalized 16HBE14o- cell line (human bronchial epithelial cells) was a gift from Dr D. Gruenert (University of California, San Francisco, CA, USA). It was grown in cell culture flasks (Costar) at 37 °C in a 5 % CO_2 atmosphere in MEM with Earle's salts (Invitrogen) containing 10 % FBS and 2 mM L-glutamine. Cells were passaged by treating the monolayer with trypsin-EDTA (Invitrogen) at 37 °C for 5 min to detach the cells from the flask. The detached cells were transferred to a 50 ml centrifuge tube containing 20 ml complete MEM and then centrifuged for 5 min at 1000 ***g***. The supernatant was discarded and the cells were resuspended in complete MEM.

Competitive index determination. For *in vivo* assays, bacteria were enmeshed into agar beads and the rat model of chronic lung infection was used (Cash *et al.*, 1979). Bacterial cells embedded in agarose beads were prepared as described by van Heeckeren & Schluchter (2002). Briefly, cultures were grown in tryptic soy broth (TSB). A culture from the wild-type strain PAO1 containing the pUCP19 plasmid and a culture from each mutant strain were grown overnight at 37 °C. A 200 μl aliquot of an overnight culture was diluted 1 : 5 into fresh TSB to give a final concentration of approximately 1×10^{10} c.f.u. ml^{-1}. A 500 μl aliquot of a 1 : 1 mixture of wild-type and mutant bacteria was added to 4.5 ml TSB. This culture was mixed with 50 ml 2 % sterile agarose (Nusieve GTG; FMC, Rockland, ME, USA) in PBS, pH 7.2, at 48 °C. The agarose-broth mixture was added to a 250 ml Erlenmeyer flask containing 200 ml heavy mineral oil at 48 °C and rapidly stirred on a magnetic stirrer in a water bath (setting 500–600 r.p.m. on a hotplate stirrer, model M13; IKA, Germany). The mixture was cooled gradually with ice chips to 0 °C over a period of 5 min. The agarose beads were transferred to a sterile 500 ml Squibb separator funnel and washed once with 200 ml 0.5 % deoxycholic acid sodium salt (SDC)

Table 1. *P. aeruginosa* strains and plasmids used in this study

Strain or plasmid	Genotype or characteristics*	Source or reference
Strains		
WT	Wild-type *P. aeruginosa* PAO1; lab strain H103	Lab collection
phoQ	*phoQ*::*xylE-aacC1*; Gm^R, derivative of WT	Macfarlane *et al.* (1999)
phoQ (pUC-*phoQ*$^+$)	*phoQ* mutant with pUCP22-*phoQ*$^+$; Gm^R Cb^R	Macfarlane *et al.* (1999)
phoP	*phoP*::*xylE-aacC1*; Gm^R derivative of WT	Macfarlane *et al.* (1999)
pmrA	*pmrA*::*xylE-aacC1*; Gm^R derivative of WT	McPhee *et al.* (2003)
pmrB	*pmrB*::*xylE-aacC1*; Gm^R derivative of WT	McPhee *et al.* (2003)
putP	PA0783::*luxCDABE*-Tc^R; derivative of WT	Lewenza *et al.* (2005)
PA4773	PA4773::*luxCDABE*-Tc^R; inserted between PA4773 and PA4774; derivative of WT	Lewenza *et al.* (2005)
pmrE	PA3559::*luxCDABE*-Tc^R; derivative of WT	Lewenza *et al.* (2005)
feoA	PA4359::*luxCDABE*-Tc^R; derivative of WT	Lewenza *et al.* (2005)
Plasmid		
pUCP19	Polylinker *lacZ*, *lacI*^{q} selection; Amp^R	Schweizer (1991)

*Antibiotic resistance: Amp^R, ampicillin for *E. coli* and carbenicillin for *P. aeruginosa*; Gm^R, gentamicin; Tc^R, tetracycline.

in PBS, once with 200 ml of 0.25 % SDC in PBS, and three times with 200 ml PBS. Agarose beads were incubated on ice and the remaining PBS was removed. Prepared agarose beads were stored at 4 °C; bacterial counts were stable for up to a month.

To determine the competitive index (CI), 1 ml bead slurry was diluted in 9 ml PBS, homogenized, and serial dilutions were plated on *Pseudomonas* isolation agar (PIA) and on Mueller–Hinton agar (MHA; Difco) supplemented with appropriate antibiotics. C.f.u. were determined after 18 h at 37 °C and were used to calculate the input ratio of mutant to wild-type bacterial cells. Male Sprague–Dawley rats 500 g in weight were used according to the recommendations of the ethics committee for animal treatment. The animals were anaesthetized using isofluorane; inoculation into the lungs was done by intubation with ~120 µl of an agarose bead suspension containing a total of 10^5 bacterial cells. At 7 days post-infection, animals were sacrificed, lungs were removed and homogenized tissues were plated on Mueller–Hinton agar supplemented with appropriate antibiotics. After the *in vivo* passage, c.f.u. on plates represented the total number of bacteria present in the rat lungs. The CI is defined as the c.f.u. output ratio of mutant when compared to wild-type strain, divided by the c.f.u. input ratio of mutant to wild-type strain (Lehoux *et al.*, 2000). The final CI was calculated as the geometric mean for animals in the same group, and experiments were done at least in triplicate (Hava & Camilli, 2002). Each *in vivo* competition was tested for statistical significance by Student's two-tailed *t* test.

RNA extraction, cDNA synthesis and hybridization to DNA microarrays. *P. aeruginosa* PAO1 microarray slides were provided by the Institute for Genomic Research Pathogenic Functional Genomics Resource Center (http://pfgrc.tigr.org/). Microarrays were performed using five biologically independent experiments (for each strain, five independent cultures). Cultures were grown for 18 h in BM2-glucose medium supplemented with 2 mM $MgSO_4$. Cultures were then diluted 1/100 into fresh medium and cells were harvested at mid-exponential phase (high Mg^{2+} OD_{600} 0.5) after growth at 37 °C with shaking (250 r.p.m.). RNA was isolated using the Qiagen RNeasy Midi RNA isolation kit according to the manufacturer's protocols. Contaminating genomic DNA was removed by treatment with the DNA-free kit (Ambion). RNA was stored at −80 °C with 0.2 units μl^{-1} of SUPERase-In RNase Inhibitor (Ambion). RNA quality was assessed spectrophotometrically and by agarose gel electrophoresis. RNA was converted to cDNA and hybridized as previously described (McPhee *et al.*, 2006).

Analysis of DNA microarrays. Data analysis of DNA microarrays was carried out as previously described (McPhee *et al.*, 2006). Slide images from the five biologically independent experiments were quantified using ImaGene 6.0 Standard Edition software (BioDiscovery). Assessment of slide quality, normalization, detection of differential gene expression and statistical analysis were carried out with ArrayPipe (version 1.7), a web-based, semi-automated software specifically designed for processing of microarray data (http://koch.pathogenomics.ca/cgi-bin/pub/arraypipe.pl), using genome annotation from the *Pseudomonas* genome database (http://www.pseudomonas.com). The following processing steps were applied: (1) flagging of markers and control spots, (2) subgrid-wise background correction, using the median of the lower 10 % foreground intensity as foreground intensity as an estimate for the background noise, (3) data-shifting, to rescue most of the negative spots, (4) printTip LOESS normalization, (5) merging of replicate spots, (6) two-sided, one-sample Student's *t*-test on the $\log_2$-ratios within each experiment, and (7) averaging of biological replicates to yield overall fold-changes for each treatment group. Two-sided one-sample Student's *t* test was used for the $\log_2$ ratios within each experiment and averaging of the five biological replicates to obtain overall fold changes for *phoQ* mutant relative to wild-type. Changes $\geqslant$2-fold and a Student's *t* test *P*-value of $\leqslant$0.05 were used as the cut-offs for reporting expression changes.

Real-time quantitative PCR (RT-qPCR). Total RNA was isolated, using RNeasy mini columns (Qiagen), from mid-exponential-phase *P. aeruginosa* grown in BM2-glucose minimal medium with 2 mM Mg^{2+}. RNA samples were treated with DNase I (Invitrogen) to remove contaminating genomic DNA. Four micrograms of total RNA was combined with 0.5 µM dNTPs, 500 U SuperaseIN ml^{-1} (Ambion), 10 µM DTT in 1 × reaction buffer and reverse transcribed for 1 h at 37 °C and 2 h at 42 °C with 10 000 U Superscript II reverse transcriptase ml^{-1} (Invitrogen). The RNA was subsequently destroyed by the addition of 170 mM NaOH and incubation at 65 °C for 10 min. The reaction was then neutralized by the addition of HCl, and the cDNA was used as a template for qPCR. Analysis was carried out in the ABI Prism 7000 sequence detection system (Applied Biosystems) using the two-step RT-qPCR kit with SYBR Green detection (Invitrogen). Melting curve analysis was performed to ensure specificity. Fold-change was determined using the comparative C_t method by comparison to the *rpsL* gene, encoding the 30S ribosomal protein S12. Experiments were repeated with three independent cultures, each assayed in duplicate, with the mean ± SD reported.

Cytotoxicity assays. For the interaction assay, 16HBE14o- cells were seeded in 96-well plates (Corning Life Science) at a density of 2×10^4 cells per well and grown at 37 °C + 5 % CO_2 until 100 % confluent (~2 days). Bacteria (wild-type and *phoQ* mutant) were grown in LB medium until mid-exponential phase, washed with PBS, resuspended and diluted in MEM. The interaction assay was performed at a m.o.i. of 50 bacteria per cell in MEM without serum containing 2 mM L-glutamine, with incubation at 37 °C + 5 % CO_2. At the post-infection time point indicated in Results, medium was removed from the wells, placed in microfuge tubes and spun for 3 min at maximum speed to pellet the bacteria and host cell debris. The level of lactate dehydrogenase (LDH) in the supernatant was then assayed in triplicate using a colorimetric Cytotoxicity Detection kit (Roche). As a positive control for maximum LDH release, cells were treated with 1 % Triton X-100 (Sigma), resulting in complete cell lysis; untreated cells were used to assess background LDH release.

Minimal inhibitory concentration (MIC) determination. MICs were assessed using standard broth microdilution procedures in BM2-glucose minimal medium containing 2 mM Mg^{2+} (Macfarlane *et al.*, 2000). Growth was scored following 24 h incubation at 37 °C. For measuring MICs of cationic antimicrobial peptides, a modified assay was used to prevent artificially high MICs due to aggregation of peptides and binding to polystyrene (Wu & Hancock, 1999).

Twitching motility analysis. Twitch motility plates were made with LB medium containing 1 % agar. Bacteria were grown overnight in LB medium, diluted 1 : 100 into fresh LB medium and grown to mid-exponential phase, where 1 µl was used to inoculate twitch motility plates by stabbing down to the agar–plastic interface. The diameter of the twitch zone, visible at the interface between the agar and plastic bottom, was measured after 24 h incubation at 37 °C.

Biofilm experiments. Static microtitre biofilm assays were performed as previously described (Overhage *et al.*, 2007). After 20 h incubation at 37 °C without shaking in LB medium, medium and non-adherent cells were discarded, and the wells were gently washed with deionized H_2O. Surface-attached bacteria were then stained with 0.1 % (w/v) crystal violet for 20 min, followed by ethanol solubilization of crystal-violet-stained cells for quantification of absorbance at 600 nm using a microtitre plate reader (Bio-Tek Instruments).

Lettuce leaf model of infection. We followed a protocol previously described (Filiatrault *et al.*, 2006; Rahme *et al.*, 2000). Briefly, lettuce leaves from Romaine hearts were washed with distilled H_2O and 0.1 % bleach. Mid-exponential-phase cultures of *P. aeruginosa* were washed twice with 10 mM $MgSO_4$. Lettuce leaf midribs were then inoculated with 10 µl of *P. aeruginosa* at a density of 1×10^8 cells ml^{-1} ($\sim 1 \times 10^6$ cells). Leaves were then placed in humid plastic containers at 37 °C and symptoms were monitored for several days. Experiments were independently repeated at least five times.

Measurement of pyocyanin, pyoverdine and lipase. Strains were grown at 37 °C for 48 h in BM2 minimal medium with 2 mM $MgSO_4$, the same medium as used for our microarray studies. LB broth was used for detection of pyocyanin, as production of this pigment is negligible in BM2 for all strains. Cell-free supernatants were obtained by centrifugation of cultures at 3000 r.p.m. in a Beckman Coulter GH-3.8 rotor. Total protein secreted was measured by Pierce BCA assay (Thermo Fisher Scientific). Pyocyanin was extracted from cell-free supernatants with 1 vol. chloroform then re-extracted with 1 vol. 0.2 M HCl before measurement at 520 nm (Mavrodi *et al.*, 2001; Whooley & McLoughlin, 1982). For pyoverdine measurements, supernatants were diluted 1/70 to 1/100 in 10 mM Tris/HCl pH 7.5 and excited at 400 nm with a spectrofluorimeter (Baysse *et al.*, 2002; Mirleau *et al.*, 2000). Lipase secretion was determined by enzymic assay according to Furutani *et al.* (1995). Briefly, cell-free supernatants were diluted in 0.1 M NaH_2PO_4/K_2HPO_4 to give equal protein concentrations for loading. An equal volume of 0.4 M *p*-nitrophenyl palmitate in enzyme buffer was added as the substrate. The reactions were incubated at 37 °C overnight, then read at 410 nm compared to a standard curve of *p*-nitrophenol.

RESULTS

P. aeruginosa phoQ mutants are impaired in twitching motility

P. aeruginosa produces several surface-associated adhesins which promote attachment to epithelial cells and contribute to virulence. Among these appendages, the type IV pilus is the major virulence-associated adhesin, accounting for much of the adherence capability of this organism to human lung pneumocyte A549 cells, and it is responsible for more than 90 % of the virulence in AB.Y/SnJ mice (Hahn, 1997). Furthermore, swarming and twitching motility of *P. aeruginosa* are dependent on type IV pili, as is biofilm formation (O'Toole & Kolter, 1998). As a *phoQ* mutant had been previously shown to have reduced swarming motility (Brinkman *et al.*, 2001) and also a defect in biofilm formation (in strain PA14; Ramsey & Whiteley, 2004), we chose to analyse twitching motility in this mutant because of the interrelatedness of these processes. We found that the *phoQ*::*xylE*-Gm^R mutant displayed a significant ($P<0.001$) impairment in twitching motility (Fig. 1a). This impaired twitching phenotype could be successfully restored back to wild-type twitching levels by providing a wild-type $phoQ^+$ allele *in trans* on a plasmid (Fig. 1a), demonstrating that PhoQ was necessary for normal wild-type twitching motility. Similarly, the *phoQ* mutant showed a marked reduction in biofilm formation as assayed by microtitre plate assay, which could be successfully complemented *in trans* (Fig. 1b). Not surprisingly, considering the biofilm and twitching phenotypes, rapid attachment (partly based on functional type IV pili) to polystyrene was also impaired in the *phoQ* mutant (Fig. 1c). Of note, the *phoQ* mutant grew similarly to the wild-type strain in the LB broth medium used for these experiments (Fig. 1d).

Reduced lettuce virulence of *phoQ* mutants

PhoQ null mutants have shown reduced virulence in several Gram-negative pathogens but this has not been shown in opportunistic predominantly extracellular organisms such as *P. aeruginosa* (Groisman, 2001). As the virulence-associated processes of twitching (this study), swarming and biofilm formation (Brinkman *et al.*, 2001; Ramsey & Whiteley, 2004) were impaired in a *phoQ* mutant, we tested whether virulence was also impaired. Initially we chose the uncomplicated lettuce leaf model of virulence. This model was originally developed using whole *Arabidopsis* plants as a simple model geared towards analysing large differences in virulence phenotypes between different *P. aeruginosa* strains (Rahme *et al.*, 1997). Here, cells were inoculated into the Romaine lettuce leaf stem midrib and macroscopic symptoms elicited by infection were followed over several days. Relative to wild-type PAO1, the *phoQ* mutant was consistently impaired in its ability to cause spreading soft-rot destruction inside the leaf midrib outwards from the point of inoculation (Fig. 2). However, since AlgR was upregulated in our *phoQ* mutant it is unlikely that this gene contributed to attenuated virulence. Introduction of the cloned *phoQ* gene led to successful complementation of this impaired virulence towards wild-type levels of leaf tissue destruction (Fig. 2), demonstrating that PhoQ was necessary for full virulence expression in this model of plant infection.

phoQ mutants demonstrate reduced cytotoxicity toward human bronchial epithelial cells

A major concern regarding *P. aeruginosa* is its ability to cause opportunistic infections in humans, particularly in the lung, as it is a major cause of both acute and chronic lung infections. Therefore we examined the ability of the *phoQ* mutant to infect and destroy a monolayer of cultured 16HBE14o- epithelial cells. This cell line maintains many of the properties of primary airway epithelial cells, including the ability to form tight junctions and differentiation to produce microvilli and cilia (Cozens *et al.*, 1994). In control experiments, wild-type and *phoQ* mutant bacteria cultured on the epithelial cells displayed the same growth properties (data not shown). To measure the cytotoxic effects of both strains, the amount of lactate dehydrogenase (LDH) released from the 16HBE14o- cells was quantified using an enzyme assay. Both *phoQ* and wild-type strains induced LDH release from 16HBE14o- cells, showing that they both displayed some cytotoxicity toward this cell line. However, wild-type *P. aeruginosa* displayed a 2.2-fold ($P<0.05$) greater cytotoxicity than the *phoQ* mutant after

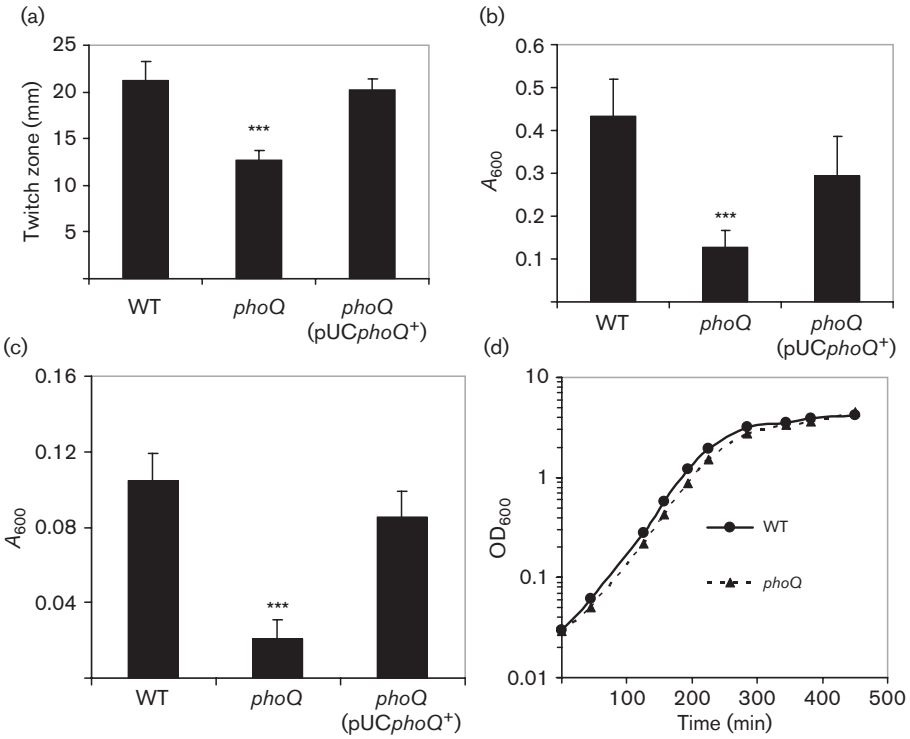

Fig. 1. Defects in twitching motility, biofilm formation and rapid attachment in *phoQ* mutants. (a) Twitching motility was assessed by inoculating cells from mid-exponential phase cultures into thin LB agar (1 % w/v) plates, down to the agar–plastic interface, and measuring colony diameter after 24 h incubation at 37 °C. Results shown are means of several independent biological replicates for each strain. (b) Biofilm formation was determined by crystal violet staining (quantified as A_{600}) of adherent cells after 20 h incubation at 37 °C. (c) Rapid attachment was assayed using mid-exponential-phase cells for 30 min, following the same basic protocol as used to assess biofilm development. Adherent cells were stained with crystal violet, followed by ethanol extraction of the crystal violet for quantification as A_{600}. Results shown are means with standard deviations for three biological experiments, each with six technical repeats. (d) Planktonic growth of the *phoQ* mutant was unaffected. Turbidity was periodically measured (OD_{600}). ***, Statistically significant difference ($P<0.001$) between *phoQ* mutant and wild-type as determined by Student's *t* test.

8.5 h of interaction (Fig. 3). This difference remained at 16 h, with the wild-type strain always showing greater cytotoxicity than the *phoQ* mutant. Of note, separate growth curves showed that, relative to the wild-type, the *phoQ* mutant was not impaired in aerobic growth in the tissue culture medium used for this assay (data not shown). Introducing the wild-type *phoQ*$^+$ allele into the *phoQ* mutant *in trans* restored cytotoxicity to wild-type levels at both time points. These results showed that *P. aeruginosa* PhoQ was necessary for wild-type levels of cytotoxicity towards 16HBE14o- cells *in vitro*.

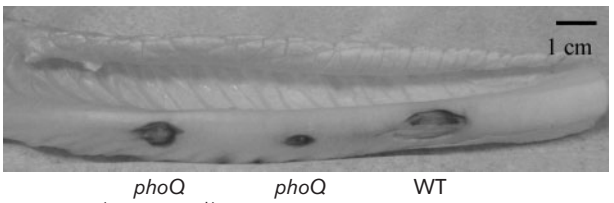

Fig. 2. *phoQ* mutants are attenuated for virulence in lettuce leaves. Day 3 symptoms of Romaine leaf infections after upper surface midribs were injected with 1×10^6 c.f.u. of *P. aeruginosa*. Leaves were incubated at 37 °C in small containers with moistened paper towels. Shown is the underside surface of the midrib from one representative leaf out of several independent experiments that produced similar trends.

phoQ mutants are highly attenuated for virulence in a model of chronic lung infection

As the *phoQ* mutant showed reduced virulence in lettuce leaves and reduced cytotoxicity toward cultured human lung epithelial cells, we analysed the *in vivo* competitive growth between the wild-type and the *phoQ* mutant in a rat model of chronic lung infection. First we confirmed that the two strains grew at the same rate by assessing their relative ability to grow in mixed culture, expressed as the *in vitro* competitive index (CI). To eliminate the possibility of

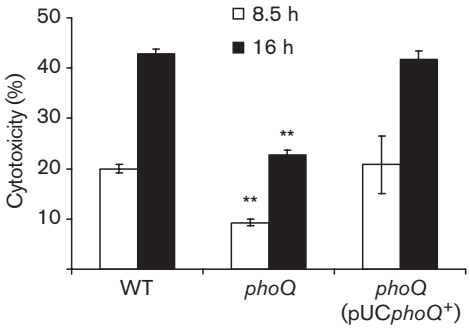

Fig. 3. *phoQ* mutants display reduced *in vitro* cytotoxicity towards human bronchial epithelial cells. The ability of the wild-type PAO1 and *phoQ* mutant strains to induce cell damage was determined by monitoring the release of intracellular LDH into the supernatant from human bronchial epithelial cells. Bacteria were co-cultured with the cells and LDH release was monitored at the time point indicated. Bars represent the mean of three independent biological repeats, each assayed in triplicate. **, Statistically significant difference ($P<0.05$) between *phoQ* mutant and wild-type as determined by Student's *t* test.

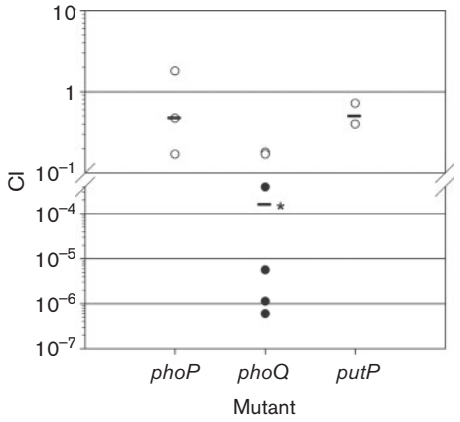

Fig. 4. *In vivo* competitive index (CI) analysis of *P. aeruginosa phoP*, *phoQ* and *putP* insertion mutants grown for 7 days in the rat lung model of chronic infection in competition with the wild-type PAO1 strain. Each circle represents the CI for a single animal in each group. A CI of <1 indicates a virulence defect. Each data point represents the results from an individual animal. Filled circles indicate animals from which no mutant bacteria were recovered; 1 was substituted in the numerator when calculating the CI. The geometric mean of the CIs for all rats is shown as a solid bar and a statistically significant value is indicated with an asterisk (*$P=0.02$ with the Mann–Whitney sum test). The *in vitro* CI values for each mutant were similar to the wild-type PAO1 strain. The CI values obtained 7 days post-infection *in vivo* with these and other mutants were *phoQ*, 0.00019; *phoP*, 0.52; *putP*, 0.54; *pmrA*, 1.27; *pmrB*, 0.7; PA4773, 1.06; and *pmrE*, 1.5.

auxotrophy, the *phoQ* strain was grown in liquid minimal medium; determination of colony forming units (c.f.u.) at 1 h intervals for a period of 24 h revealed similar growth curves compared to the wild-type PAO1 strain (data not shown). As shown in Fig. 4, a mutation in *phoQ* caused a severe defect in growth and maintenance *in vivo* as revealed 7 days after the initial infection. When compared to the wild-type strain, which by definition has a CI of 1.0, the *phoQ* mutant strain gave a CI value of <0.00019, indicating an unprecedented 10 000-fold relative decrease in c.f.u. in rat lung tissues ($P<0.02$, Fig. 4). Indeed four animals had no detectable bacteria 7 days post-infection. It therefore was apparent that the PhoQ sensor kinase was essential for virulence in this model of chronic lung infection. In contrast, no significant difference in competition was observed for the *phoP* response regulator mutant (CI 0.52; Fig. 4), in keeping with results indicating that PhoP and PhoQ demonstrate only partial interdependence (Macfarlane *et al.*, 2000). Of note, a control mutant strain *putP* (encoding a sodium/proline symporter that is not apparently involved in virulence) did not have a virulence defect.

PhoQ affects the expression of the cognate response regulators PhoP and PmrA

To investigate how *phoQ* contributed to the reduced virulence of *P. aeruginosa* in these three models, we performed microarray studies to compare the *phoQ* mutant to the wild-type. For the microarray, bacteria were grown in BM2-glucose containing 2 mM Mg^{2+}, a condition under which the wild-type response regulator PhoP is phosphorylated and constitutively active (Groisman, 2001; Macfarlane *et al.*, 2000; McPhee *et al.*, 2006). Using these conditions, we observed 474 genes that were differentially regulated by more than 1.5-fold ($P\leqslant0.05$) under high Mg^{2+} conditions, with 296 transcriptionally upregulated and 178 downregulated. A selection of these genes is presented in Table 2.

The *phoQ* mutation dysregulated many genes that we had previously shown to be regulated by the PhoP response regulator and/or the PmrA-PmrB two-component system in an Mg^{2+}-dependent manner (McPhee *et al.*, 2006). These genes are listed in Supplementary Table S2. Dysregulated PhoP-dependent genes included PA0921, *oprH-phoP*, *arnBCADTEF*, PA3885, PA4010–11, PA4453–5 and PA1343, while PmrA-dependent genes shown to be dysregulated included *feoAB*, PA1559–0, PA4357–9, PA4773–8 (including *pmrA* and *pmrB*), *arnBCADTEF* and PA4286 (McPhee *et al.*, 2006; Table S2). The upregulation of *pmrA* was confirmed using RT-qPCR, demonstrating that this gene was induced 4.1 ± 1.7-fold in the *phoQ* mutant (Table 3). Given the importance of the PhoPQ and PmrAB systems in cationic antimicrobial peptide resistance in both *P. aeruginosa* and *Salmonella* (Gunn *et al.*, 2000; Macfarlane *et al.*, 2000; Miller *et al.*, 1989), and their critical importance for virulence in *Salmonella* (Miller *et al.*, 1989), we determined the CI for *P. aeruginosa phoP* (CI 0.52), *pmrA* (CI 1.27) and *pmrB* (CI

Table 2. Microarray analysis of genes significantly dysregulated in the *phoQ* mutant relative to wild-type

Only selected genes showing ⩾2-fold change in the *phoQ* mutant are listed. Dysregulated hypothetical or conserved hypothetical genes (except those that were >10-fold upregulated) and/or ORFs are not included. The full set of dysregulated genes is given in Supplementary Table S2.

Gene ID*	Name	Fold change†	*P*-value	Description*
PA0059	*osmC*	2.1	0.003	Osmotically inducible protein OsmC
PA0198	*exbB1*	−2.1	0.01	Transport protein ExbB
PA0199	*exbD1*	−2.0	0.02	Transport protein ExbD
PA0355	*pfpI*	2.4	0.05	Intracellular protease PfpI
PA0459	*clpC*	−2.1	0.02	Probable ClpA/B protease ATP-binding subunit
PA0521	*nirO*	−2.2	0.02	Probable cytochrome *c* oxidase subunit
PA0523	*norC*	−13.2	0.002	Nitric-oxide reductase subunit C
PA0567	*yqaE*	2.0	0.01	Conserved hypothetical protein
PA0685	*hxcQ*	−3.0	0.03	Probable type II secretion system protein
PA0739		3.1	0.02	Probable transcriptional regulator
PA0762	*algU*	6.4	<0.0001	Sigma factor AlgU
PA0763	*mucA*	6.5	<0.0001	Anti-sigma factor MucA
PA0764	*mucB*	2.7	<0.0001	Negative regulator for alginate biosynthesis
PA0765	*mucC*	2.2	0.001	Positive regulator for alginate biosynthesis
PA0766	*mucD*	2.7	0.005	Serine protease MucD precursor
PA0854	*fumC2*	3.1	0.0001	Fumarate hydratase
PA0885	*dctQ*	−2.4	0.05	Probable C_4-dicarboxylate transporter
PA0929	*pirR*	2.3	0.0004	PirR two-component response regulator
PA0949	*wrbA*	4.5	0.003	Trp repressor-binding protein WrbA
PA0997	*pqsB*	2.5	0.0001	Quinolone signal (PQS) biosynthesis
PA0998	*pqsC*	2.4	0.0007	Quinolone signal (PQS) biosynthesis
PA0999	*pqsD*	2.3	0.0007	Quinolone signal (PQS) biosynthesis
PA1001	*phnA*	2.6	0.0002	Pyocyanin biosynthesis
PA1053	*slyB*	4.1	<0.0001	Outer-membrane lipoprotein
PA1178	*oprH*	33.1	<0.0001	Outer-membrane protein OprH
PA1179	*phoP*	82.6	<0.0001	Two-component response regulator PhoP
PA1317	*cyoA*	−2.8	0.005	Cytochrome *o* ubiquinol oxidase subunit II
PA1318	*cyoB*	−2.7	0.002	Cytochrome *o* ubiquinol oxidase subunit I
PA1319	*cyoC*	−2.4	0.01	Cytochrome *o* ubiquinol oxidase subunit III
PA1321	*cyoE*	−3.5	0.007	Cytochrome *o* ubiquinol oxidase protein CyoE
PA1344	*yvaG*	9.1	<0.0001	Probable short-chain dehydrogenase
PA1403		−2.6	0.03	Probable transcriptional regulator
PA1498	*pykF*	3.7	0.02	Pyruvate kinase I
PA1592		14.6	<0.0001	Hypothetical protein
PA1596	*htpG*	−3.2	0.03	Heat-shock protein HtpG
PA1715	*pscB*	−2.4	0.0004	Type III export apparatus protein
PA1868	*xqhA*	−3.9	0.02	Type II secretion protein XqhA
PA1920	*nrdD*	2.7	0.03	Ribonucleotide reductase
PA1978	*agmR*	−2.4	0.05	Transcriptional regulator AgmR
PA1979	*exaD*	−3.3	0.02	Two-component sensor kinase ExaD
PA1982	*exaA*	−10.9	0.003	Quinoprotein alcohol dehydrogenase
PA1983	*exaB*	−9.6	0.004	Cytochrome *c*550
PA1984	*exaC*	−9.6	0.02	Aldehyde dehydrogenase
PA2011	*gnyL*	2.6	0.04	Hydroxymethylglutaryl-CoA lyase
PA2019	*amrA*	2.7	0.02	RND multidrug efflux membrane fusion protein
PA2023	*galU*	2.2	0.001	UTP-glucose-1-phosphate uridylyltransferase
PA2121		2.0	0.02	Probable transcriptional regulator
PA2147	*katE*	−3.4	0.02	Catalase HPII
PA2194	*hcnB*	2.1	0.02	Hydrogen cyanide synthase HcnB
PA2254	*pvcA*	5.1	0.009	Pyoverdine biosynthesis protein PvcA
PA2258	*ptxR*	2.6	0.02	Transcriptional regulator PtxR
PA2280	*arsH*	−4.6	0.01	Conserved protein in arsenic resistance
PA2386	*pvdA*	4.3	0.01	Pyoverdine biosyntheis, ornithine oxygenase
PA2398	*fpvA*	4.0	0.0005	Ferripyoverdine receptor
PA2470	*gtdA*	2.4	0.05	Gentisate 1,2-dioxygenase

Table 2. cont.

Gene ID*	Name	Fold change†	*P*-value	Description*
PA2650	*ybaJ*	−2.5	0.04	Conserved methylase protein
PA2653	*yuiF*	−4.8	0.01	Probable transporter
PA2662		−10.1	<0.0001	Conserved hypothetical protein
PA2663		−14.9	0.008	Hypothetical protein
PA2664	*fhp*	−30.5	0.02	Flavohaemoprotein
PA2787	*cpg2*	2.7	0.03	Carboxypeptidase G2 precursor
PA2815	*yafH*	2.0	0.004	Probable acyl-CoA dehydrogenase
PA2987	*ycfV*	2.1	0.01	ATP-binding component of ABC transporter
PA3189	*gltF*	2.4	0.02	Probable permease of ABC sugar transporter
PA3391	*nosR*	−3.0	0.004	Regulatory protein NosR
PA3404	*opmM*	13.0	0.003	Outer-membrane protein
PA3405	*hasE*	−4.6	0.01	Metalloprotease secretion protein
PA3474	*yigM*	−2.1	0.02	Conserved membrane protein
PA3475	*pheC*	−2.4	0.003	Cyclohexadienyl dehydrogenase (Phe synthesis)
PA3530	*bfd*	3.4	0.02	Bacterioferritin-associated ferredoxin
PA3537	*argF*	2.5	0.04	Ornithine carbamoyltransferase, Arg biosynthesis
PA3552	*arnB*	44.9	<0.0001	Aminotransferase in L-Ara4N biosynthesis
PA3553	*arnC*	75.4	<0.0001	Glycosyltransferase in L-Ara4N biosynthesis
PA3554	*arnA*	77.8	<0.0001	Dual function enzyme in L-Ara4N biosynthesis
PA3556	*arnT*	45.4	<0.0001	Transferase in L-Ara4N biosynthesis
PA3557	*arnE*	45.6	<0.0001	Transport system for L-Ara4N
PA3558	*arnF*	69.0	<0.0001	Transport system for L-Ara4N
PA3559	*pmrE*	3.4	0.004	UDP-glucose dehydrogenase
PA3598	*ypqQ*	4.6	0.03	Conserved hypothetical protein
PA3607	*potA*	−2.5	0.04	Polyamine transport protein PotA
PA3610	*potD*	−3.2	0.02	Polyamine transport protein PotD
PA3819	*ycfJ*	12.0	<0.0001	Putative porin
PA4092	*hpaC*	−6.2	0.007	4-Hydroxyphenylacetate 3-monooxygenase
PA4154	*ygiM*	2.6	0.001	Conserved hypothetical protein
PA4229	*pchC*	10.7	0.003	Thioesterase PchC, pyochelin biosynthesis
PA4357	*yhgG*	3.2	0.009	Conserved hypothetical protein
PA4358	*feoB*	3.0	0.003	Ferrous iron transport protein
PA4359	*feoA*	3.7	<0.0001	Ferrous iron transport protein
PA4370	*icmP*	2.2	0.003	Outer-membrane insulin-cleaving protease
PA4454	*yrbD*	6.3	<0.0001	Conserved hypothetical protein
PA4455	*yrbE*	5.3	<0.0001	Probable permease of ABC transporter
PA4456	*yrbF*	4.1	<0.0001	ATP-binding component of ABC transporter
PA4479	*mreD*	3.2	0.05	Rod-shape-determining protein MreD
PA4514	*piuA*	2.9	0.03	Outer-membrane receptor for iron transport
PA4635	*mgtC*	3.1	<0.0001	Conserved membrane protein MgtC
PA4675	*iutA*	2.0	0.007	Probable TonB-dependent receptor
PA4762	*grpE*	−2.7	0.03	Heat-shock protein GrpE
PA4776	*pmrA*	4.0	0.0003	Two-component response regulator
PA4777	*pmrB*	3.2	0.005	Two-component sensor
PA4876	*osmE*	4.5	<0.0001	Osmotically inducible lipoprotein OsmE
PA5053	*hslV*	−2.0	0.002	Heat-shock protein HslV
PA5061	*phaI*	4.4	<0.0001	Polyhydroxyalkanoic acid biosynthesis
PA5107	*blc*	2.6	0.005	Outer-membrane lipoprotein Blc
PA5157	*marR*	−2.0	0.003	Transcriptional regulator MarR
PA5158	*opmG*	−2.8	0.001	Outer-membrane protein
PA5172	*arcB*	−2.7	0.0005	Ornithine carbamoyltransferase, catabolic
PA5231	*yhiH*	−3.4	0.03	ATP-binding/permease fusion ABC transporter
PA5302	*dadX*	−2.9	0.004	Catabolic alanine racemase
PA5493	*polA*	3.4	<0.0001	DNA polymerase I
PA5531	*tonB*	2.5	0.009	TonB protein

*Information according to the *P. aeruginosa* genome website (http://www.pseudomonas.com).
†Fold regulation of genes differentially expressed in the *phoQ* mutant relative to WT. A positive number indicates transcript upregulation in the *phoQ* mutant.

Table 3. RT-qPCR gene expression analysis of select genes in the *phoQ* mutant relative to wild-type

Gene ID	Name	Fold change	Description
PA0762	*algU*	5.1 ± 0.9	Sigma factor AlgU
PA2862	*lipA*	−3.2 ± 0.6	LipA lactonizing lipase precursor
PA3552	*arnB*	505 ± 21	Aminotransferase in L-Ara4N biosynthesis
PA4776	*pmrA*	4.1 ± 1.7	PmrA response regulator
PA5261	*algR*	4.6 ± 2.6	AlgR response regulator

0.7) mutants. However, no statistically significant differences from the wild-type were observed (data not shown).

The PmrA-regulated *feoAB* operon was upregulated in the *phoQ* microarray (Table 2). FeoAB is a well-conserved system that is involved in the transport of ferrous iron (Marlovits *et al.*, 2002). Previous studies have shown that FeoB mutants of *Escherichia coli* (Boyer *et al.*, 2002; Kammler *et al.*, 1993) and *Helicobacter pylori* (Velayudhan *et al.*, 2000) demonstrate reduced virulence and/or colonization activity, yet in our study the equivalent *P. aeruginosa* mutants demonstrated normal wild-type virulence in rat chronic lung infections (data not shown).

phoQ mutants upregulate an LPS modification system

The *arnBCADTEF-pmrE* operon involved in the modification of LPS was upregulated in the *phoQ* microarray. This system controls the addition of aminoarabinose to lipid A, a modification that has been shown to occur in *P. aeruginosa* grown in low-Mg^{2+} conditions and which has been associated with bacterial resistance to cationic peptides (Ernst *et al.*, 1999). We confirmed the upregulation of *arnB* (the first gene of the aminoarabinose lipid A LPS modification operon) by RT-qPCR as a remarkable 505 ± 21-fold change (Table 3). The massive upregulation of *arnB* together with the rest of the genes of this LPS modification operon directly correlated with the high-level resistance to antimicrobial peptides such as polymyxin B in the *phoQ* mutant. Indeed, the freshly made *phoQ* mutant was >64-fold more resistant to polymyxin B than wild-type cells as assayed by broth-dilution MIC in high-Mg^{2+} BM2-glucose medium. *In vivo* analysis of a *pmrE* mutant (the last gene in the operon), however, did not demonstrate any differences in virulence from the wild-type (CI 1.5, data not shown).

Alginate is dysregulated but not overexpressed in a *phoQ* mutant

The switch to the mucoid (alginate-producing) phenotype in most cystic fibrosis isolates of *P. aeruginosa* is caused by mutations in the *mucA* gene (encoding an anti-sigma factor), resulting in the activation of AlgU, an alternative sigma factor that induces alginate production. The *phoQ* microarray revealed transcriptional upregulation of the *algU-mucABCD* alginate regulatory gene cluster and corresponding AlgU-dependent (mucoidy) genes including *slyB*, encoding a lipoprotein, *osmC* and *osmE*, encoding osmotically induced lipoproteins, and the *pfpI* protease gene (Table 2). Although *slyB*, *osmC*, *osmE* and *pfpI* are among the strongest AlgU-dependent regulated genes (Firoved & Deretic, 2003), they were the only members of the AlgU stimulon that appeared on our microarray. This is not entirely unsurprising, as the *phoQ* mutant possessed a wild-type copy of *mucA* (which was similarly induced) and so the anti-AlgU sigma factor activity of MucA would likely therefore limit the degree to which less robust AlgU targets are induced. Of note, the alginate biosynthetic cluster (*algABCD*) did not appear to be significantly dysregulated in *phoQ* mutants, and *phoQ* mutants did not possess a mucoid phenotype on agar plates. Upon further analysis, an *algU* mutant showed no observable difference in cytotoxicity to 16HBE14o- cells when compared to the wild-type (data not shown). Thus it seems possible that PhoQ might contribute to mucoidy in particular mutants or environments but that it is not a primary regulator thereof.

Mutation of *phoQ* affects genes outside the PhoP regulon

Microarray analysis also indicated many interesting genes that were strongly affected by mutation of the *phoQ* gene, but that were not previously shown to be Mg^{2+} regulated or within the PhoP regulon (McPhee *et al.*, 2006). These included two type II secretion genes, *hxcQ* (PA0685; probable type II secretion system protein) and *xqhA* (PA1868; type II secretion protein), which were downregulated. Interestingly, the *P. aeruginosa* quinolone signal (PQS) biosynthetic genes *pqsBCD* were upregulated. Also induced were *pvcA* (PA2254; paerucumarin biosynthesis protein), and the iron-scavenging-related genes *pvdA (*PA2386; ornithine oxygenase in pyoverdine biosynthesis), *fpvA* (PA2398; ferripyoverdine receptor) and another siderophore gene *pchC* (PA4229; pyochelin biosynthesis thioesterase). Despite this, it was observed that *phoQ* mutants were a more yellow colour on plates and in broth culture compared to the wild-type. Furthermore, considerably less pyoverdine and pyocyanin were secreted from the *phoQ* mutant into the surrounding medium (Fig. 5). The increased transcription of genes related to iron scavenging may thus be a response to the *phoQ* mutant's inability to produce and/or secrete these same pigments

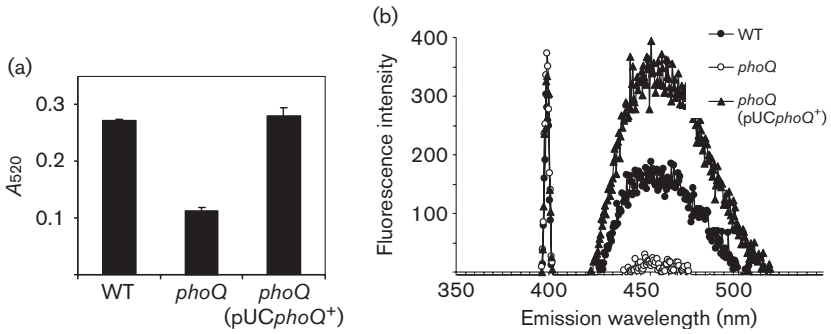

Fig. 5. Secretion of pigmented compounds into culture media. (a) The *phoQ* mutant was impaired in the secretion of pyocyanin into LB broth medium from 48 h cultures. Production of pyocyanin was negligible in BM2 medium for all strains. (b) Secretion of pyoverdine into high-Mg^{2+} BM2-glucose medium from 48 h cultures by the *phoQ* mutant was also impaired.

and siderophores. In turn, this may contribute to the mutant's reduced virulence.

Other genes that were strongly affected by mutation of the *phoQ* gene, but have not been shown to be Mg^{2+} regulated, included the energy-metabolism-related genes *nirO* (PA0521), *norC* (PA0523), the regulator *nosR* (PA3391), and the cytochrome *o* ubiquinol oxidase operon PA13170-21 (*cyoA–E*), each of which was downregulated in the *phoQ* mutant, implying positive regulation by this sensor kinase in wild-type *P. aeruginosa*. Mutants in the nitric oxide reductase operon containing PA0521–3 have previously demonstrated moderately attenuated virulence in the lettuce leaf model (Filiatrault *et al.*, 2006).

No fewer than 15 regulators were dysregulated in the *phoQ* mutant, namely PA0048, PA0739, *algR*, *algU*, *mucA*, *pirR*, *wrbA*, *phoP*, PA1403, *agmR*, PA2121, *ptxR*, *pmrA*, *pmrB* and *marR*, perhaps indicative of the position of PhoQ high in a regulatory hierarchy. Many of these genes have been linked to roles in virulence, including *algR*. The AlgR response regulator regulates a diverse assortment of virulence determinants, including twitching and swarming motility and biofilm formation (Belete *et al.*, 2008; Lizewski *et al.*, 2002). The transcriptional upregulation of *algR* was confirmed by RT-qPCR, which indicated that this gene was strongly induced (4.6 ± 2.6-fold) in the *phoQ* mutant (Table 3). An *algR* mutant in strain PA14 showed an attenuation of cytotoxicity (70 %) relative to the wild-type. However, since AlgR was upregulated in our *phoQ* mutant it is unlikely that this gene contributed to attenuated virulence.

Our transcriptome analysis also highlighted several other genes that play a role in virulence of *P. aeruginosa*, including the downregulation of the type II secreted lipase A (Table 3). Previously, using an enzymic assay, lipase secretion had been shown to be downregulated in a *phoQ* mutant (Brinkman *et al.*, 2001), a result confirmed here. Most interesting, however, were those virulence genes that did not appear on the array, indicating no change between the *phoQ* mutant and the wild-type. These genes included those that encoded exotoxin A (*toxA*), various phospholipases, and elastase (*lasB*). Interestingly, the master regulator of the type III secretion system, *exsA*, was only very slightly downregulated; however, the exotoxins co-regulated by this system did not appear on the array, indicating no difference in expression.

Previous work in this laboratory indicated some overlap in phenotypes between GacA and PhoPQ (Brinkman *et al.*, 2001); therefore we also analysed the level of regulation of the GacS/GacA/*rsmZ* pathway, which operates at the level of post-translational regulation, despite it not appearing on our array. RsmZ is a small non-coding RNA that post-translationally sequesters the RNA-binding protein RsmA and thus indirectly regulates a broad range of virulence properties including lipase production (Heurlier *et al.*, 2004). However, we were unable to demonstrate a significant level of regulation of *gacA* or *rsmZ*, thus indicating that this pathway may not play a role in the *phoQ* virulence defects.

DISCUSSION

In this study we have demonstrated that the two-component sensor kinase PhoQ is necessary for virulence in *P. aeruginosa* and controls the expression of a number of genes that are not part of the Mg^{2+}-dependent PhoP regulon. It is our proposal based on analysis of these data that PhoQ regulates virulence in a complex multi-faceted fashion, involving several discrete mechanisms, without strongly impacting on several other major virulence-related processes such as type III secretion, homoserine-lactone-based quorum sensing or most type II secretion processes, although both biofilm formation and cytotoxicity, which can be controlled by these systems, were altered.

The PhoP-PhoQ and PmrA-PmrB two-component systems of *P. aeruginosa* are responsible for regulating the adaptive response of this organism to limiting concentrations of cations (e.g. Mg^{2+}; McPhee *et al.*, 2006). Through regulation of the *arnBCADTEF-pmrE* LPS modification operon, they are critically important in controlling intrinsic and mutational resistance to cationic antimicrobial peptides (Ernst *et al.*, 1999; Lewenza *et al.*, 2005; Macfarlane *et al.*, 2000; McPhee *et al.*, 2006; Moskowitz *et al.*, 2004). However, mutants in the PhoP and PmrA response regulators, and the PmrB sensor kinase, were not found here to be attenuated for virulence in the rat model of chronic lung infection and were fully capable of competing

with the wild-type parental strain. Recent analysis of the *P. aeruginosa* PhoQ periplasmic sensing domain has demonstrated that while the sensing domain binds and is repressed by divalent cations, it is unable to respond to antimicrobial peptides (Prost *et al.*, 2008). This suggests that even though PhoQ may play a role in resistance to such peptides, it is not directly involved in sensing their presence. Importantly, here it was demonstrated that although the *phoQ* mutant was strongly resistant to the potential growth-antagonizing effects of cationic antimicrobial peptides, it was, perhaps counter-intuitively, actually attenuated for virulence. Thus this study confirmed that attenuated virulence and polymyxin B/antimicrobial peptide resistance in the PhoQ mutant are independent processes.

The PhoP-PhoQ system of *Salmonella* is also a master regulator of virulence (Miller *et al.*, 1989). We have previously compared the substantial differences in the architecture of these systems between *P. aeruginosa* and *Salmonella* (McPhee *et al.*, 2003), a theme that has been mirrored by studies of *Yersinia* (Marceau *et al.*, 2004; Winfield *et al.*, 2005). The *Yersinia* PhoP-PhoQ system is important for regulating virulence gene expression, since *phoP* mutants were less virulent during *Y. pseudotuberculosis* and *Y. pestis* intra-macrophage survival (Grabenstein *et al.*, 2004). Conversely, a *Salmonella* strain expressing PhoP constitutively was attenuated for virulence in mice, consistent with a requirement to be able to mediate positive autoregulation (Shin *et al.*, 2006). It seems possible that this constitutive PhoP phenotype in *Salmonella* is analogous to that occurring with the attenuated virulence phenotype displayed by the *P. aeruginosa phoQ* mutant, which also demonstrates a constitutive PhoP phenotype (Macfarlane *et al.*, 1999). One of the most fundamental differences between the *Salmonella* and *Pseudomonas* systems is that *Salmonella* PhoQ appears able to both phosphorylate and dephosphorylate PhoP whereas in *P. aeruginosa* the primary role of PhoQ appears to be in the dephosphorylation of PhoP, and we have proposed that another kinase activates PhoP (Macfarlane *et al.*, 1999, 2000). In accordance with this, our transcriptome analysis indicated that a number of genes outside the PhoP regulon were regulated by PhoQ, including several regulators, suggesting that PhoQ may mediate regulation through other transcription factors than PhoP.

In a previous study, a strain PA14 *phoQ* mutant was shown to be impaired in the formation of biofilms (Ramsey & Whiteley, 2004). While it was suggested that this reflected a potential role for LPS in this biofilm phenotype (due to upregulation of *arnBCADTEF*), our data are consistent with an influential role for the observed impaired twitching motility of the *phoQ* mutant (Fig. 1a), since type IV-pilus-dependent twitching motility has been implicitly linked to biofilm formation (O'Toole & Kolter, 1998). How the dysregulation of this form of motility occurs in the *phoQ* mutant is not immediately apparent, since the *phoQ* microarrays demonstrated only very modest downregulation of some type IV pili genes (changes were significant but less than twofold). Thus, it seems likely that the biofilm defect in this strain was multi-factorial, given the large number of genes differentially regulated in the *phoQ* mutant. As *P. aeruginosa* growth in the cystic fibrosis lung has been proposed to be biofilm-like (Garcia-Medina *et al.*, 2005; O'May *et al.*, 2006) this PhoQ-dependent adaptation may help protect the bacteria from the host. If true, this may explain why the *phoQ* mutant, deficient in biofilm formation, was deficient in survival in the rat lung.

In this study we have determined the transcriptional changes in a *phoQ* mutant to try to explain our findings regarding the attenuation of growth in an *in vivo* chronic rat lung model. We are aware of a number of problems with this type of comparison. The growth conditions (BM2-glucose with high Mg^{2+}) used for our *in vitro* microarray experiment are expected to be quite different from the growth conditions found in the rat lung, and as a result, the gene expression patterns might be quite different. Nevertheless, the *phoQ* mutation results in a constitutive expression of the PhoP response regulator that would be relatively insensitive to additional stimuli and might therefore be expected to have some of the same direct effects on gene expression regardless of growth conditions.

The goal of this study was to investigate the role of PhoQ in the virulence of *P. aeruginosa*. We have demonstrated that deletion of *phoQ* drastically reduces soft rot in lettuce leaves, impairs biofilm and siderophore production, reduces cytotoxicity towards cultured human lung epithelial cells and impairs the colonization of rat lungs. The role of PhoQ in virulence is probably multi-faceted, but most likely includes dysregulation of genes that control LPS modification, type IV pili, mucoidy and biofilm formation. As our study has suggested that PhoQ has the ability to regulate many other genes outside the PhoP regulon, other virulence-related targets of PhoQ are likely and will be sought. Further investigations into the mechanism of PhoQ virulence in this opportunistic pathogen are ongoing.

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