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Role of *Pseudomonas aeruginosa* PhoP-PhoQ in resistance to antimicrobial cationic peptides and aminoglycosides

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Resistance to the polycationic antibiotic polymyxin B and expression of the outer-membrane protein OprH in the opportunistic pathogen *Pseudomonas aeruginosa* both involve the PhoP-PhoQ two-component regulatory system. The genes for this system form an operon with *oprH*, *oprH-phoP-phoQ*, that responds to Mg^{2+} starvation and PhoP levels. In this study, the Mg^{2+}-regulated promoter for this operon was mapped upstream of *oprH* by primer-extension experiments. An *oprH*::*xylE*-Gm^R mutant H855 was constructed and measurement of the catechol 2,3-dioxygenase activity expressed from this transcriptional fusion provided evidence for a second, weak promoter for *phoP-phoQ*. Wild-type *P. aeruginosa* PAO1 strain H103 was found to exhibit Mg^{2+}-regulated resistance to the α-helical antimicrobial cationic peptide CP28 in addition to its previously characterized resistance to polymyxin B. Resistance to this peptide was unchanged in the OprH-null mutant H855 and a PhoP-null mutant H851. In contrast, PhoQ-null mutant H854 demonstrated constitutive CP28 resistance. Northern blot analysis revealed constitutive expression of *phoP* in this strain, implicating PhoP-PhoQ in the resistance of *P. aeruginosa* to cationic peptides. Furthermore, all three null-mutant strains demonstrated increased resistance to the aminoglycoside antibiotics streptomycin, kanamycin and amikacin. Two additional mutant strains, H895 and H896, were constructed that carried unmarked deletions in *oprH* and were found to exhibit aminoglycoside susceptibility equivalent to that of the wild-type. This result provided definitive evidence that OprH is not involved in *P. aeruginosa* aminoglycoside resistance and that the changes in resistance in strain H855 and a previously reported *oprH* mutant were due to polar effects on *phoP-phoQ* rather than loss of OprH expression. A role for PhoP-PhoQ in resistance to aminoglycosides is envisaged that is distinct from that in resistance to cationic peptides and polymyxin B.

Keywords: PhoP-PhoQ, antimicrobial cationic peptides, aminoglycoside resistance, *Pseudomonas aeruginosa*

INTRODUCTION

Antibiotic resistance is becoming increasingly prevalent in pathogenic bacteria. As research into the mechanisms behind this resistance intensifies, the complex nature of the regulation of antibiotic resistance is becoming evident. Bacteria frequently employ two-component regulatory systems as a means of co-ordinating their often widely disparate responses to environmental stimuli. A growing number of such systems are being discovered that play a global regulatory role within the bacterial cell, and factors that influence antibiotic resistance and/or virulence frequently fall under their control. One notable example is PhoP-PhoQ in *Salmonella typhimurium*, which regulates the expression of at least forty genes in response to extracellular Mg^{2+} concentrations. The sensor protein PhoQ responds to extracellular concentrations of Mg^{2+} ions and, under conditions of Mg^{2+} starvation, first autophosphorylates at a conserved histidine residue, then activates the regulator protein PhoP by a phosphotransfer reaction (Garcia Vescovi *et al.*, 1996). The

phoP-phoQ locus is subject to auto-regulation, such that low extracellular Mg^{2+} concentrations upregulate transcription of this operon from an inducible promoter in a PhoP-dependent manner (Soncini *et al.*, 1995). Other regulators under the control of PhoP-PhoQ include PmrA-PmrB (Gunn & Miller, 1996; Soncini & Groisman, 1996), a two-component regulatory system responsible for structural changes in the outer-membrane lipopolysaccharide that lead to polymyxin B resistance (Gunn *et al.*, 1998; Helander *et al.*, 1994), and the transcriptional activator HilA, which is a key regulator of *Salmonella* pathogenicity island 1 and thus controls expression of several virulence factors (Bajaj *et al.*, 1996). In addition, PhoP-PhoQ also directly regulates *S. typhimurium* resistance to antimicrobial peptides (Fields *et al.*, 1989; Miller *et al.*, 1989).

The Gram-negative bacterium *Pseudomonas aeruginosa* is an important opportunistic pathogen. Chronic infections due to this organism are prevalent in cystic fibrosis patients and are frequently recalcitrant to treatment. In addition to displaying high levels of intrinsic antibiotic resistance, *P. aeruginosa* frequently converts to a mucoid state resulting in a rapid adaptive resistance that accounts for the high failure rate of antibiotic therapy in eradicating these infections.

Sequencing of the *P. aeruginosa* PAO1 genome has recently been completed (http://www.pseudomonas.com) and should provide some insight into the pathogenesis of this bacterium. One striking feature of the predicted protein complement of *P. aeruginosa* is the presence of a disproportionately large number of regulatory proteins. Recently we identified the PhoP-PhoQ homologues in *P. aeruginosa* (Macfarlane *et al.*, 1999). The genes for this regulatory system lie immediately downstream of the *oprH* gene, which encodes a small outer-membrane protein OprH that is highly expressed under Mg^{2+}-starvation conditions. The three genes *oprH-phoP-phoQ* form an operon that is under the joint control of PhoP and Mg^{2+} ion concentrations. Similar to its counterpart in *S. typhimurium*, *P. aeruginosa* PhoP-PhoQ is highly expressed under Mg^{2+}-starvation conditions and is involved in resistance to polymyxin B. *P. aeruginosa* PAK has been shown to exhibit LPS structural modifications when grown under Mg^{2+}-deficient conditions (Ernst *et al.*, 1999), and polymyxin resistance in *P. aeruginosa*, like that in *S. typhimurium*, may thus be related to altered outer-membrane permeability. In *S. typhimurium*, PhoP-PhoQ mediates resistance through activation of PmrA-PmrB, and a PhoP-null strain is therefore rendered supersusceptible to polymyxin B. However, our PhoP-null strain of *P. aeruginosa* PAO1 (strain H851) retained polymyxin B resistance under Mg^{2+}-deficient growth conditions, and a PhoQ-null strain (H854) exhibited constitutive polymyxin B resistance. In accord with the large number of regulators in *P. aeruginosa*, the involvement of another regulatory system(s) in the resistance of this organism appears likely. However, in contrast to the situation in *S. typhimurium*, PhoP is not required for activation of this system(s) under low-Mg^{2+} conditions.

In this paper we have further characterized the *oprH-phoP-phoQ* operon and demonstrated that the two-component regulatory system PhoP-PhoQ influences the resistance of *P. aeruginosa* to both cationic antimicrobial peptides and aminoglycoside antibiotics.

METHODS

Bacterial strains, growth conditions and general procedures. Bacterial strains and plasmids used in this study are listed in Table 1. All strains were maintained at 37 °C on LB agar, or grown in LB broth, supplemented with appropriate antibiotics unless otherwise stated. Both LB (Lennox) broth containing 5 g NaCl l^{-1}, and LB (Miller) broth containing 10 g NaCl l^{-1} were used. For conjugation experiments, *Escherichia coli* strain S17-1 (Simon *et al.*, 1983) was grown at 30 °C and *P. aeruginosa* strain H103 was grown at 42 °C. To study the effects of Mg^{2+} concentration, BM2-glucose minimal medium (Gilleland *et al.*, 1974) containing 20 μM (low) or 2 mM (high) $MgSO_4$ was used. Antibiotics were used at the following concentrations: for *E. coli*, 100 μg ampicillin ml^{-1}, 10 μg gentamicin ml^{-1}; for *P. aeruginosa*, 300–350 μg carbenicillin ml^{-1}, 15 μg gentamicin ml^{-1}. Unless otherwise specified, plasmids were transformed into bacterial strains by electroporation using a Gene Pulser (Bio-Rad) and 0·1 cm gap cuvettes following published protocols (Sambrook *et al.*, 1989; Dennis & Sokol, 1995). For DNA manipulations, standard protocols were followed (Sambrook *et al.*, 1989; Ausubel *et al.*, 1987). Total cellular RNA for Northern blots and primer-extension analysis was isolated from mid-exponential phase cultures using the RNeasy Mini Kit (Qiagen). Oligonucleotides were synthesized on an Applied Biosystems model 392 DNA–RNA Synthesizer. Catechol 2,3-dioxygenase activity assays were carried out as described previously (Macfarlane *et al.*, 1999).

Northern blots and primer-extension analysis. Northern blot experiments were carried out using methods and primers described previously for strain H103 (Macfarlane *et al.*, 1999). For the primer-extension experiments, primer OprH-rev2 (5′-TGGATGTTGTTGCTGGTCTC-3′) was designed that was complementary to nt 115–96 of the *oprH* gene. This primer was end-labelled with [γ-^{32}P]dATP and hybridized at 60 °C to RNA (5 μg) from strain H103 in 50 mM Tris/HCl, pH 8·3, containing 20 mM KCl (total volume 10 μl). Primer extension was initiated by the addition of 100 U Superscript reverse transcriptase (Gibco-BRL) in the presence of 50 mM Tris/HCl (pH 8), 40 mM KCl, 6 mM $MgCl_2$, 20 mM dNTPs and 10 U ribonuclease inhibitor (final volume 30 μl) and cDNA synthesis was allowed to proceed for 60 min at 45 °C. After treatment with DNase-free RNase for 15 min at 37 °C, the cDNA was isolated by ethanol precipitation followed by resuspension in 3–4 μl Promega *fmol* loading buffer. After denaturation, samples were loaded onto a 6% acrylamide sequencing gel together with dideoxy sequencing reactions covering the appropriate region of plasmid pGB22 (Bell *et al.*, 1991). Sequencing reactions used the OprH-rev2 primer and the Promega *fmol* DNA cycle sequencing system. Control primer-extension reactions using tRNA alone or RNA treated with RNase prior to cDNA synthesis were performed in parallel.

Construction of strains H855 (*oprH*::*xylE*-Gm^R), H895 (Δ*oprH*$_{73}$) and H896 (Δ*oprH*$_C$). To construct strain H855, a 1·2 kb *Sma*I–*Hin*cII fragment containing the *oprH* gene was excised from plasmid pAK9 (Macfarlane *et al.*, 1999) and cloned into the *Sma*I site of the gene-replacement vector

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype/description	Source or reference
***P. aeruginosa* strains**		
H103	Wild-type PAO1	Nicas & Hancock (1980)
H851	H103 *phoP*::*xylE*-Gm^R	Macfarlane *et al*. (1999)
H854	H103 *phoQ*::*xylE*-Gm^R	Macfarlane *et al*. (1999)
H855	H103 *oprH*::*xylE*-Gm^R	This work
H895	H103 Δ*oprH*$_{73}$ (deletion of bp 709–927 of *oprH*)	This work
H896	H103 Δ*oprH*$_C$ (deletion of bp 930–957 of *oprH*)	This work
Plasmids		
pUCP19, 20, 21	*Escherichia-Pseudomonas* shuttle vectors	Schweizer (1991), West *et al*. (1994)
pEMR3	Contains *phoP* as a 0·9 kb *Pst*I fragment cloned behind the *lac* promoter in pUCP19	Macfarlane *et al*. (1999)
pEMQ1a	Contains *phoQ* as a 1·55 kb fragment PCR amplified from H103 genomic DNA and cloned behind the *lac* promoter in pUCP20	Macfarlane *et al*. (1999)
pEMPQ2a	Contains *phoP-phoQ* as a 2·16 kb fragment PCR amplified from H103 genomic DNA and cloned in the opposite orientation to the *lac* promoter in pUCP20	Macfarlane *et al*. (1999)
pEMH4a	Contains *oprH* as a 0·7 kb fragment cloned behind the *lac* promoter in pUCP21	This work

pEX100T (Schweizer & Hoang, 1995) to give pEXH. The *xylE*-Gm^R cassette from plasmid pX1918GT (Schweizer & Hoang, 1995) was then cloned between the two *Pst*I sites that are situated 18 bp apart within the *oprH* gene. Gene replacement in strain H103 using this construct was carried out as described previously for strains H851 and H854 (Macfarlane *et al*., 1999). Southern blot analysis following standard protocols (Ausubel *et al*., 1987) and using probes complementary to either *oprH* or *xylE* confirmed the presence of the *xylE*-Gm^R cassette within *oprH* in strain H855. Plasmid pEMH4a was constructed by subcloning a 0·7 kb *Sst*I–*Xba*I fragment carrying the *oprH* gene from pBHR20 (Rehm & Hancock, 1996) into pUCP21 (West *et al*., 1994).

To construct the *oprH* deletion strains H895 and H896, the gene-replacement vectors pEXHΔ73 and pEXHΔC were constructed as follows. Primer ΔoprH2 was designed with the sequence 5′-CTGCTGGTGTCGGAGGCATTCTCGTAG-GTC-3′ that was complementary to nt 263–234 of *oprH*. The three underlined bases replaced CAT in the coding sequence and introduced a *Bsm*I site. A 410 bp fragment containing the 5′ end of *oprH* plus 144 bp of vector sequence was PCR amplified from pEMH4a using primer ΔoprH2 and a second primer, pUCPlac, that was complementary to the vector *lac* promoter. A fragment of 557 bp from the 5′ end of *oprH* was removed from pEXH by *Sma*I/*Bsm*I digestion and replaced with the *Hin*cII/*Bsm*I-digested PCR amplicon. The resulting plasmid, pEXHΔ73, contained the *oprH* gene with a 219 bp in-frame deletion (nt 709–927 inclusive). For plasmid pEXHΔC, primer ΔoprH1 (5′-CTGAGCAAGAATGCC-TC|ACCAACGCCAGCACCGAG-3′) was designed that contained a 28 bp deletion (nt 930–957 of *oprH*) indicated by the vertical line. A 337 bp fragment containing the 3′ end of *oprH* was PCR amplified from pGB22 (Bell *et al*., 1991) using this primer and RT-PCR3′ (Macfarlane *et al*., 1999). pGB22 was digested with *Sma*I and religated to remove the vector *Kpn*I site and give plasmid pEMH2. Replacement of a *Kpn*I–*Bsm*I fragment containing the 3′ end of *oprH* in pEMH2 with the *Kpn*I–*Bsm*I-digested PCR amplicon yielded plasmid pEMH2ΔC. The 28 bp deletion in the *oprH* gene resulted in a frameshift that would prematurely terminate translation 83 bp upstream of the regular *oprH* TAA stop codon. Finally, the modified *oprH* gene was excised from pEMH2ΔC by *Sma*I–*Hin*cII digestion and cloned into the *Sma*I site of pEX100T to give pEXHΔC.

Gene replacement in strain H103 using pEXHΔ73 and pEXHΔC was carried out as described previously (Macfarlane *et al*., 1999) with the exception that single crossover events were identified by selection on BM2-glucose minimal medium containing carbenicillin alone. Double crossover events were identified as sucrose-resistant, carbenicillin-sensitive colonies. Mid-exponential phase cultures of these strains were screened for OprH expression by Western immunoblot using anti-OprH specific antiserum (Macfarlane *et al*., 1999). Genomic DNA was subsequently isolated from OprH$^-$ strains and the appropriate deletion in *oprH* was confirmed by PCR.

MIC determinations and killing assays. Aminoglycoside MIC values were determined using the standard two-fold microtitre broth dilution protocol (Amsterdam, 1991) starting with mid-exponential phase cultures. Peptide MIC values were determined using a modified version of this protocol (Wu & Hancock, 1999) to prevent adhesion of the peptides to the walls of the microtitre plates that would artificially elevate the MIC values. All MIC values were read after a 48 h incubation

due to the slow growth rate of strain H854. Killing assays were carried out as described previously (Macfarlane *et al.*, 1999) with the following exceptions. For CP28, cultures were diluted 1:100 into 30 mM sodium phosphate buffer, pH 7, containing 150 mM NaCl and 8 μg CP28 ml^{-1}. For streptomycin, cultures were diluted 1:200 into pre-warmed LB (Miller) broth containing 16 μg streptomycin ml^{-1} and killing was carried out at 37 °C.

Streptomycin-assisted lysozyme lysis assays. Aliquots (1 ml each) of mid-exponential phase cultures (OD_{600} 0·4–0·8) grown in LB (Miller) broth were centrifuged and washed once with 30 mM sodium phosphate buffer, pH 7. The cells were then resuspended in 1 ml of the same buffer and placed in a cuvette. Streptomycin (450 μg ml^{-1}) and lysozyme (50 μg ml^{-1}) were added simultaneously to the cuvette and cell lysis was monitored by following the decrease in optical density at 600 nm.

RESULTS

A single Mg^{2+}-regulated promoter situated upstream of *oprH* transcribes the *oprH-phoP-phoQ* operon

In an earlier study we used Western blot analysis of OprH expression in *P. aeruginosa phoP* and *phoQ* mutants to demonstrate that Mg^{2+}-regulated transcription of the *oprH-phoP-phoQ* operon is dependent on the presence of PhoP. To confirm the effects of the *phoP* and *phoQ* mutations on transcription of this operon, we analysed total cellular RNA from strains H851 (*phoP*::*xylE*-Gm^R) and H854 (*phoQ*::*xylE*-Gm^R) (Table 1) by Northern blot hybridization. Primers complementary to regions internal to the *oprH* and *phoP* genes were used to probe RNA isolated from cultures of the two mutant strains grown to mid-exponential phase in high- and low-Mg^{2+} medium. Using this method, a high level of transcription was observed in the PhoQ-null mutant H854. Large quantities of transcripts corresponding to *oprH* and significant but diminishing amounts of *oprH-phoP* and *oprH-phoP-phoQ*::*xylE* transcripts were identified in RNA isolated from this strain under both high- and low-Mg^{2+} growth conditions (Fig. 1). We had previously observed by Western blot analysis that this mutant constitutively overexpressed OprH (Macfarlane *et al.*, 1999). The results of the Northern blot analysis confirmed our assumption that *phoP* is also constitutively overexpressed in strain H854. We were unable to detect any transcripts containing *phoP-phoQ* alone in this strain or in the wild-type strain H103 (Macfarlane *et al.*, 1999). In contrast to strain H854, no transcripts containing *oprH*, *phoP* or *phoQ* could be detected by Northern blot analysis of RNA isolated from the PhoP-null strain H851 under either growth condition (data not shown). These results were consistent with our previous observation that PhoP is an essential positive regulator of transcription for the *oprH-phoP-phoQ* operon.

To determine the transcriptional start site of this operon, we conducted primer-extension experiments using total cellular RNA isolated from the wild-type strain H103 and a primer complementary to an internal region of the *oprH* gene (see Methods). A single major product was

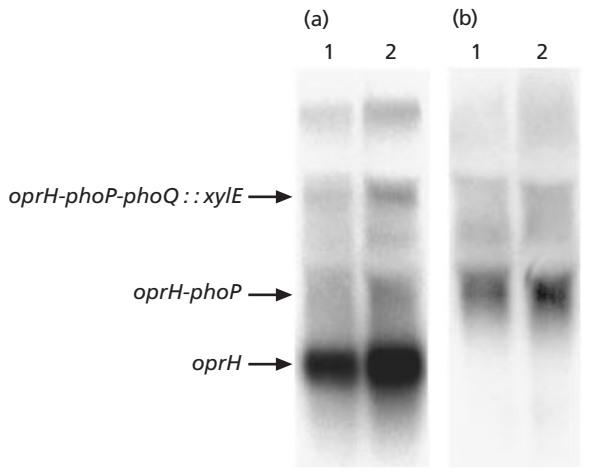

Fig. 1. Northern blot analysis of total cellular RNA from the PhoQ-null mutant H854 isolated under low-Mg^{2+} (lane 1) or high-Mg^{2+} (lane 2, both slightly overloaded) growth conditions. Blots were probed with primers complementary to (a) *oprH*, or (b) *phoP*.

observed under low-Mg^{2+} growth conditions (Fig. 2a, lane 2), which was only faintly visible under high-Mg^{2+} conditions (Fig. 2a, lane 1), and indicated the presence of a single, Mg^{2+}-regulated promoter upstream of *oprH*. Examination of the sequence upstream of the transcriptional start site revealed only a weak −10 consensus sequence beginning 12 bp downstream of the four hexanucleotide repeats found upstream of the operon (Fig. 2b). No −35 consensus sequence could be identified; however, this is a common finding for *P. aeruginosa* regulated promoters (Rothmel *et al.*, 1991). Analogous primer-extension experiments performed with a primer complementary to *phoP* failed to detect any transcriptional start sites immediately upstream of the *phoP* gene (data not shown).

Transcription of an *oprH*::*xylE*-Gm^R fusion is weakly Mg^{2+} regulated

Previous studies from this laboratory (Nicas & Hancock, 1980; Young *et al.*, 1992) ascribed a role for overexpressed OprH in polymyxin B and aminoglycoside resistance. However, our discovery that *oprH* formed an operon with *phoP* and *phoQ* cast some doubt on these conclusions. To further elucidate the role of each of the three genes in the *oprH-phoP-phoQ* operon, we constructed a chromosomal knockout of *oprH*. A *xylE*-Gm^R cassette was inserted into two *Pst*I sites within the *oprH* gene resulting in the removal of 18 bp of *oprH* coding sequence and the creation of a transcriptional fusion (see Methods). Gene replacement in the resulting *oprH*::*xylE*-Gm^R mutant strain H855 (Table 1) was confirmed by Southern blot analysis using probes complementary to *oprH* and to *xylE* (data not shown). As expected, Western blot analysis showed strain H855 to be deficient in expression of OprH protein under normal inducing conditions (data not shown).

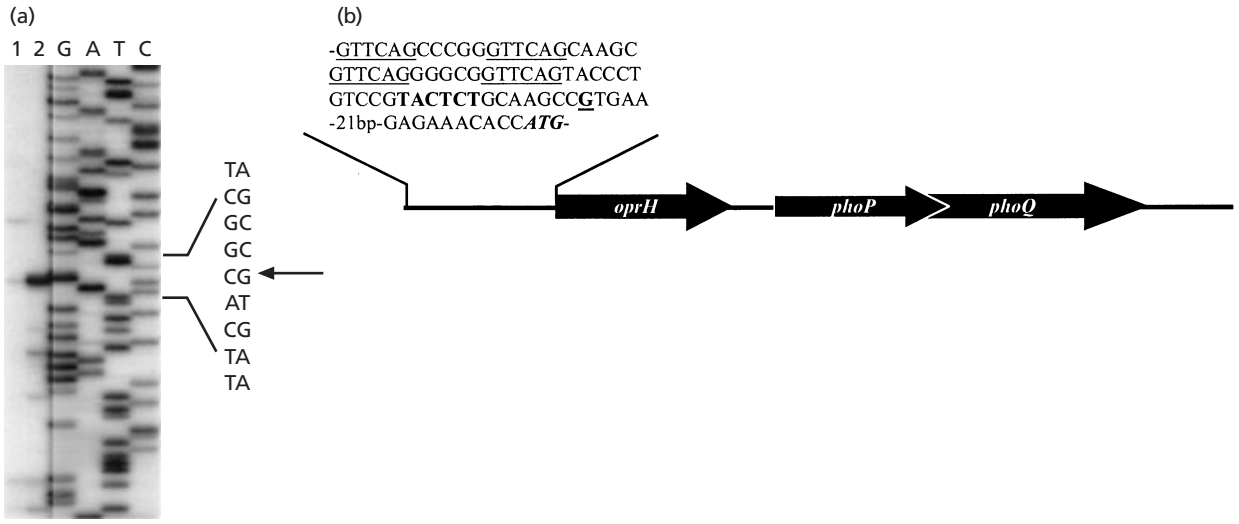

Fig. 2. (a) Primer-extension analysis using a primer complementary to *oprH* and total cellular RNA isolated from wild-type strain H103 grown under high-Mg^{2+} (lane 1) or low-Mg^{2+} (lane 2) growth conditions. Lanes G, A, T and C indicate the dideoxy sequencing reactions. The arrow indicates the transcriptional start. (b) Schematic representation of the *oprH-phoP-phoQ* operon showing the sequence immediately upstream of *oprH*. Hexanucleotide repeats are underlined, the putative −10 consensus sequence is shown in bold, the transcriptional start site determined by primer-extension experiments is shown underlined in bold and the *oprH* ATG start codon is in bold italics.

Table 2. Catechol 2,3-dioxygenase activity of the *oprH*::*xylE*-Gm^R transcriptional fusion in *P. aeruginosa* strain H855 harbouring PhoP, PhoQ and OprH expression plasmids

Plasmid	Catechol 2,3-dioxygenase activity*		Fold difference (low/high)
	Low Mg^{2+}	High Mg^{2+}	
None	4611 ± 622	204 ± 36	23
pUCP19 (vector control)	3597 ± 1401	230 ± 43	16
pEMR3 (*phoP*)	2889 ± 845	1800 ± 425	1·6
pEMQ1a (*phoQ*)	5272 ± 169	310 ± 5	17
pEMPQ2a (*phoP-phoQ*)	5314 ± 1308	7 ± 1	759
pEMH4a (*oprH*)	4736 ± 1227	234 ± 6	20

* Values shown are the mean ± SEM of three independent experiments, except those for pUCP19 and pEMQ1a, which were measured twice. Values are shown as pmol 2-hydroxymuconic semialdehyde produced min^{-1} (μg protein)$^{-1}$.

Strain H855 was transformed with plasmids carrying the *oprH*, *phoP*, *phoQ* and *phoP-phoQ* genes (pEMH4a, pEMR3, pEMQ1a and pEMPQ2a, respectively; Table 1). Cultures of H855 alone and harbouring each of these plasmids were grown to mid-exponential phase in both high- and low-Mg^{2+} media and the catechol 2,3-dioxygenase activity expressed from the *xylE* transcriptional fusion was measured. The results, shown in Table 2, indicated that transcription of *oprH* was still Mg^{2+} regulated in strain H855 with the level of catechol 2,3-dioxygenase activity measured under low-Mg^{2+} growth conditions being between 16- and 23-fold higher than that measured under high-Mg^{2+} conditions. The *xylE*-Gm^R cassette in strain H855 is flanked by omega fragments (Schweizer & Hoang, 1995); therefore the *oprH*::*xylE*-Gm^R construct was assumed to exert a polar effect on the downstream *phoP-phoQ* genes. Since we have shown that transcription of *oprH* is dependent on the presence of PhoP (Macfarlane *et al.*, 1999), the observed Mg^{2+} regulation of the transcriptional fusion in strain H855 strongly suggested that *phoP-phoQ* was being weakly transcribed from a second promoter. Presumably the level of this transcript was too low to be detected in the Northern blots and primer-extension

Table 3. Killing assays with polymyxin B (8 μg ml^{-1}) for strain H855 (*oprH*::*xylE*-Gm^{R}) harbouring PhoP, PhoQ and OprH expression plasmids

Values shown are the result of at least three independent determinations.

Plasmid	Survivors after 5 min (% ± SEM)	
	Low Mg^{2+}	High Mg^{2+}
None	85 ± 9	< 1·0
pUCP19 (vector control)	84 ± 8	< 1·0
pEMR3 (*phoP*)	100	59 ± 15
pEMQ1a (*phoQ*)	87 ± 13	< 1·0
pEMPQ2a (*phoP-phoQ*)	74 ± 15	< 1·0
pEMH4a (*oprH*)	95 ± 5	< 1·0
P. aeruginosa H103 (wild-type)	82 ± 12	< 1·0

experiments described above, even when the autoradiograms were overexposed (data not shown).

When introduced into strain H855, plasmids pEMR3 (*phoP*) and pEMPQ2a (*phoP-phoQ*) had opposing effects on the transcription of the *oprH*::*xylE* fusion. Overexpression of PhoP from pEMR3 led to a large increase in catechol 2,3-dioxygenase activity expressed in high-Mg^{2+} medium, and a slight decrease in low-Mg^{2+} medium, which essentially eliminated the Mg^{2+} regulation of *oprH*::*xylE* transcription. Conversely, the presence of both PhoP and PhoQ proteins expressed from pEMPQ2a led to a much higher degree of Mg^{2+} regulation than for strain H855 alone. A 759-fold difference in activity was measured between the two media, caused largely by the suppression of expression in high-Mg^{2+} medium. As would be expected, neither pEMQ1a (*phoQ*) nor pEMH4a (*oprH*) had any effect on transcription of the *oprH*::*xylE* fusion.

The OprH-null strain H855 retains Mg^{2+}-regulated polymyxin B resistance

The resistance of the OprH-null strain H855 to the polycationic antibiotic polymyxin B was determined by a killing assay. Cultures of this strain with and without the OprH, PhoP and PhoQ expression plasmids were grown to mid-exponential phase in BM2-glucose minimal medium and treated with 8 μg polymyxin B ml^{-1}. The number of survivors after 5 min was determined by a plate count (Table 3). Polymyxin B susceptibility in strain H855 remained similar to that of the wild-type strain H103 under both high- and low-Mg^{2+} growth conditions. Plasmids carrying the *phoQ* (pEMQ1a), *phoP-phoQ* (pEMPQ2a) or *oprH* (pEMH4a) genes had no effect on the polymyxin B resistance of strain H855. In contrast, constitutive overexpression of PhoP resulting from the presence of pEMR3 (*phoP*) led to constitutive polymyxin B resistance in this strain. This was a similar effect to that of pEMR3 on the polymyxin B resistance of the PhoP-null strain H851 (Macfarlane *et al*., 1999).

P. aeruginosa resistance to the α-helical cationic peptide CP28 is Mg^{2+} regulated and involves PhoP-PhoQ

The PhoP-PhoQ two-component regulatory system of *S. typhimurium* has been shown to regulate resistance to defensins and other antimicrobial peptides in response to extracellular Mg^{2+} ion concentrations (Garcia Vescovi *et al*., 1996; Guo *et al*., 1998; Miller *et al*., 1990). The Mg^{2+} regulation of polymyxin B resistance in *P. aeruginosa* is well documented (Brown & Melling, 1969; Nicas & Hancock, 1980), and we have shown that this resistance is also affected by PhoP and PhoQ (Macfarlane *et al*., 1999). To determine whether antimicrobial-peptide resistance in *P. aeruginosa* was similarly dependent on PhoP-PhoQ and the Mg^{2+} ion content of the growth medium, we conducted killing assays on the mutant strains H855 (*oprH*::*xylE*-Gm^{R}), H851 (*phoP*::*xylE*-Gm^{R}) and H854 (*phoQ*::*xylE*-Gm^{R}) (Table 1) using the antimicrobial peptide CP28 (Piers *et al*., 1994). CP28 is an α-helical cationic peptide, the sequence of which is based on the cecropin-mellitin hybrid peptide CEME (Piers *et al*., 1994). For the killing assays, mid-exponential phase cultures grown in BM2-glucose medium containing high or low concentrations of Mg^{2+} ions were diluted 100-fold into 30 mM sodium phosphate buffer, pH 7, containing 150 mM NaCl and 8 μg CP28 ml^{-1} and survivors were assessed by plate counts. The antimicrobial action of CP28 is unaffected by NaCl concentrations up to 300 mM (Friedrich *et al*., 1999), and the addition of NaCl to the assay buffer helped to stabilize the cells under the assay conditions. The results of these experiments, given in Table 4, clearly indicated that *P. aeruginosa* resistance to CP28 is Mg^{2+} regulated. After a 5 min exposure to 8 μg CP28 ml^{-1}, only a 3 % survival rate was observed for the wild-type strain H103 grown in high-Mg^{2+} medium compared to a 63 % survival rate for the same strain grown in low-Mg^{2+} medium. Results for the PhoP-null (H851) and OprH-null (H855) strains were comparable to the wild-type. However, the PhoQ-null mutant H854 demonstrated a higher level of resistance to CP28 under low-Mg^{2+} growth conditions (100 % survival) together with significant resistance in high-Mg^{2+} medium (86 % survival). These results were analogous to those previously reported for polymyxin B (Macfarlane *et al*., 1999).

In view of the effect of PhoP-PhoQ on CP28 resistance in *P. aeruginosa*, we screened the *oprH*, *phoP* and *phoQ* mutants for changes in resistance to a series of cationic peptides of varying structure by measurement of the MICs. Divalent cations exert an antagonistic effect on the action of many cationic peptides (Friedrich *et al*., 1999), making Mg^{2+} regulation of resistance to these compounds difficult to detect by measurement of the MIC values. However, the results of killing assays with these mutant strains and either polymyxin B

Table 4. Killing assays for *P. aeruginosa* wild-type and mutant strains with CP28 or streptomycin

Results shown are the mean ± SEM of at least three independent experiments.

Strain	Survivors after 5 min treatment with 8 μg CP28 ml^{-1} (%)*		Survivors after treatment with 16 μg streptomycin ml^{-1} in LB (%)†	
	Low Mg^{2+}	High Mg^{2+}	5 min	30 min
H103 (wild-type)	63 ± 8	3 ± 1	40 ± 16	< 1·0
H855 (*oprH*::*xylE*-Gm^R)	82 ± 12	7 ± 2	86 ± 19	70 ± 17
H851 (*phoP*::*xylE*-Gm^R)	46 ± 13	6 ± 3	100	100
H854 (*phoQ*::*xylE*-Gm^R)	100	86 ± 3	70 ± 6	15 ± 4

* Strains were grown to mid-exponential phase in BM2-glucose minimal medium supplemented with 20 μM (low) or 2 mM (high) $MgSO_4$. Killing was carried out in 30 mM sodium phosphate buffer, pH 7, containing 150 mM NaCl at ambient temperature.

† Strains were grown to mid-exponential phase in LB (Miller) broth. Killing was carried out in the same medium at 37 °C.

Table 5. MIC values determined for *P. aeruginosa* wild-type and mutant strains grown in BM2-glucose minimal medium supplemented with 2 mM $MgSO_4$

Values shown are the result of at least three independent determinations and are shown as μg ml^{-1}. PxB, polymyxin B; PMI, polyphemusin; Sm; streptomycin; Am, amikacin; Km; kanamycin; Er, erythromycin; LB, LB (Miller) broth. –, Not determined.

Strain	Peptide or antibiotic										
	PxB	IB-367	PMI	CP28	CP29	CP11CN	Sm (LB)	Sm (BM2)	Am	Km	Er
H103 (wild-type)	1·0*	1·0	0·5	4·0	4·0	4·0	1·0	8·0	1·0	64	256
H855 (*oprH*::*xylE*-Gm^R)	1·0*	1·0	0·5	4·0	4·0	8·0	8·0	16	4·0	256	512
H895 (Δ*oprH*$_{73}$)	1·0*	–	–	4·0	–	–	–	8·0	1·0	64	–
H896 (Δ*oprH*$_C$)	1·0*	–	–	4·0	–	–	–	8·0	1·0	64	–
H851 (*phoP*::*xylE*-Gm^R)	1·0*	1·0	0·5	2·0	4·0	8·0	4·0	16	2·0	256	256
H854 (*phoQ*::*xylE*-Gm^R)	4·0	2·0	0·5	8·0	8·0	4·0	4·0	16	2·0	128	256

* Values varied between 0·5 and 1·0 over 4–6 determinations for strains H103, H855, H895, H896 and H851.

(Macfarlane *et al.*, 1999) or CP28 indicated that PhoP-PhoQ related effects could be seen most clearly under high-Mg^{2+} conditions. Therefore, resistance to five peptides was determined for strains grown in BM2-glucose minimal medium supplemented with 2 mM $MgSO_4$ (Table 5). Although the differences in MICs for the mutant strains were modest (twofold), they were consistently observed in four to six independent experiments. It is noteworthy that under these conditions, the mutant strains demonstrated the same pattern of resistance to CP28 as that to polymyxin B, consistent with the killing-assay results. Resistance to the α-helical peptide CP29 [an analogue of CP28 (Friedrich *et al.*, 1999)] and to the protegrin-like peptide IB367 also followed this pattern, with increased resistance being seen in the PhoQ-null strain H854. Peptide CP11CN is a synthetic analogue of the bovine neutrophil peptide indolicidin with improved activity towards Gram-negative bacteria (Falla & Hancock, 1997). Resistance to this peptide was increased in the PhoP-null H851 and OprH-null H855 strains, but remained the same as wild-type for strain H854. Resistance to polyphemusin, a synthetic version of the natural peptide isolated from the haemocytes of horseshoe crabs (Iwanaga *et al.*, 1994), was unaffected in all three mutants.

P. aeruginosa resistance to aminoglycosides involves PhoP-PhoQ

In a manner analogous to polymyxin B resistance, *P. aeruginosa* resistance to the aminoglycoside antibiotics streptomycin and gentamicin has previously been shown to be dependent on the Mg^{2+} concentration of the growth medium (Hancock *et al.*, 1981). In view of the

influence of PhoP-PhoQ on resistance to both polymyxin B (Macfarlane *et al*., 1999) and cationic peptides, we investigated whether our mutant strains also displayed changes in aminoglycoside resistance.

MIC determinations revealed increased resistance (two- to fourfold) to a number of aminoglycoside antibiotics for the three mutant strains grown under high-Mg^{2+} conditions (Table 5). As was the case for cationic peptides, high divalent cation concentrations also interfered with the action of aminoglycosides (Zimelis & Jackson, 1973), which accounts for the increased MIC values for streptomycin measured under high-Mg^{2+} conditions compared to those in LB medium, which contains only moderate concentrations of Mg^{2+}. Although all three mutant strains carry the *aacC1* gene encoding the acetyltransferase-3-1 (AAC(3)I) on the *xylE*-Gm^R cassette, this enzyme has been shown to have very narrow substrate specificity (Phillips & Shannon, 1984) and, therefore, is unlikely to affect resistance to other aminoglycosides. Of the aminoglycosides used in these experiments, streptomycin lacks the requisite substituent for modification by AAC(3)I, and no modification of kanamycin A or amikacin by this enzyme has been observed (Phillips & Shannon, 1984).

We chose to study the resistance of the strains H851, H854 and H855 to streptomycin in more detail. Since the increased resistance in all of these strains (fourfold over wild-type) was observed in LB medium as well as high-Mg^{2+} BM2-glucose, we conducted killing assays in the former medium. After treatment with 16 μg streptomycin ml^{-1}, wild-type *P. aeruginosa* H103 was completely killed within 30 min (Table 4). All three mutant strains showed higher streptomycin resistance than the wild-type, but the extent of this resistance varied. The PhoP-null (H851) and OprH-null (H855) strains showed the highest levels of streptomycin resistance (100% and 70% survival, respectively, after 30 min), while the PhoQ-null strain H854 showed a level of resistance intermediate to these two strains and the wild-type (15% survival after 30 min).

To determine whether altered outer-membrane permeability could account for the observed increase in aminoglycoside resistance, we tested the ability of streptomycin to promote lysozyme lysis of the wild-type, and PhoP-, PhoQ- and OprH-null mutants. Lysozyme targets the peptidoglycan layer, but normally is unable to cross the outer-membrane barrier. Addition of aminoglycoside antibiotics disrupts the outer membrane sufficiently to allow the lysozyme molecules access to the cell. As can be seen from Fig. 3, all three mutant strains (H851, H854 and H855) were less susceptible to lysozyme lysis in the presence of 450 μg streptomycin ml^{-1} than wild-type H103. Of these mutants, the PhoQ-null strain H854 showed the highest level of resistance to streptomycin-assisted lysozyme lysis, and the OprH-null strain H855 the lowest. Treatment of cultures with 450 μg streptomycin ml^{-1} in the absence of lysozyme caused minimal cell lysis and no lysis was seen in cells treated with lysozyme alone (data not shown).

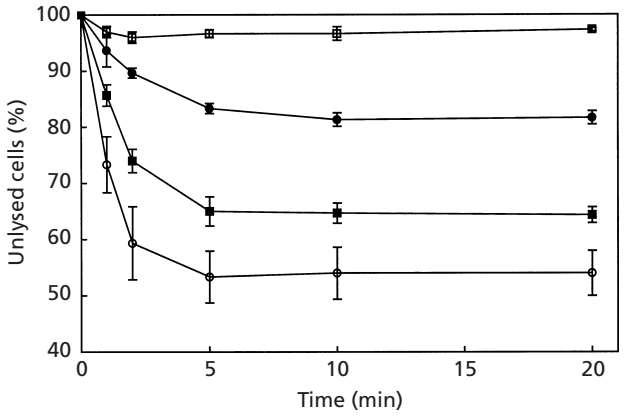

Fig. 3. Streptomycin-assisted lysozyme lysis of strains H103 (wild-type; ○), H851 (PhoP-null; ●), H854 (PhoQ null; □) and H855 (OprH null; ■). Results shown are the mean ± SEM of three independent experiments using 450 μg streptomycin ml^{-1} and 50 μg lysozyme ml^{-1}. Strains were grown in LB (Miller) broth and lysis was carried out in 30 mM sodium phosphate buffer, pH 7.

Aminoglycoside resistance of *P. aeruginosa* is unaffected by deletions in *oprH*

To confirm our hypothesis that the increased aminoglycoside resistance of strain H855 (*oprH*::*xylE*-Gm^R) was due to polar effects on the downstream *phoP-phoQ* genes rather than disruption of *oprH*, we constructed two strains, H895 and H896, with unmarked deletions in *oprH* (Table 1). In strain H895 ($\Delta oprH_{73}$), a 219 bp in-frame deletion within *oprH* was created that removed 73 amino acid residues, incorporating three of the eight proposed β-strands (Rehm & Hancock, 1996), from the central region of OprH. The 28 bp deletion in strain H896 ($\Delta oprH_C$) was placed towards the 3′ end of *oprH*, resulting in a frameshift mutation and creation of a premature stop codon 83 bp upstream of the regular TAA *oprH* stop codon.

Western blot analysis of mid-exponential phase cultures of both strains H895 and H896 grown under Mg^{2+}-limiting (inducing) conditions indicated that no proteins were expressed that reacted with anti-OprH specific antiserum (data not shown). This suggests that if a mutant OprH protein was produced by these strains, it was rapidly degraded within the cell.

The unmarked deletions in strains H895 and H896 should permit normal transcription of *phoP-phoQ* from the inducible promoter upstream of *oprH* and thus allow us to determine the effects of an OprH-null mutation without concomitant effects on PhoP-PhoQ expression. The MIC values for several antibiotic compounds were determined for the two *oprH* deletion strains grown in BM2-glucose minimal medium supplemented with 2 mM (high) $MgSO_4$. The values for polymyxin B, the aminoglycosides streptomycin, kanamycin and amikacin, and the α-helical cationic peptide CP28 are given in Table 5. No difference in the MIC

value for any of these five antibiotics was observed between strains H895, H896 and the wild-type *P. aeruginosa* strain H103. These results strongly suggest that the increased resistance of strain H855 (*oprH*::*xylE*-Gm^R) to aminoglycosides was due to polar effects of the *xylE*-Gm^R cassette on the transcription of *phoP-phoQ* and not to the loss of OprH expression.

DISCUSSION

In a previous paper we reported that resistance of *P. aeruginosa* PAO1 to polymyxin B involves the two-component regulatory system PhoP-PhoQ (Macfarlane *et al.*, 1999). In this study we have demonstrated that resistance to the α-helical cationic peptide CP28 is likewise regulated by both Mg^{2+} ion concentrations and by PhoP-PhoQ. Analogous to our findings with polymyxin, resistance to CP28 under Mg^{2+}-deficient conditions does not appear to depend on a functional PhoP protein. Under low-Mg^{2+} growth conditions, the PhoP-null mutant H851 exhibited a level of resistance equivalent to that of the wild-type strain H103 (Table 4). However, the PhoQ-null strain H854, in which PhoP is constitutively overexpressed, displayed constitutive resistance to CP28. Based on our prior observations of the transcriptional regulation of the *oprH-phoP-phoQ* operon (Macfarlane *et al.*, 1999), we have proposed that PhoQ acts primarily to dephosphorylate and thus downregulate the activity of PhoP under Mg^{2+}-sufficient conditions. In strain H854, constitutive high levels of PhoP expressed in the absence of a PhoQ protein may therefore result in the interaction of this response regulator with other resistance genes, most probably through cross-talk with another regulatory system. The observed resistance to CP28, like that to polymyxin B, is consistent with this proposal. As PhoP is presumably in an activated form in strain H854, phosphorylation of PhoP by a phospho-donor other than PhoQ remains a possibility.

OprH is not involved in polymyxin B resistance

In addition to the *P. aeruginosa phoP* and *phoQ* mutants, we also constructed an analogous chromosomal knockout of *oprH*, the first gene of the *oprH-phoP-phoQ* operon. Killing assays with polymyxin B revealed levels of resistance in this *oprH*::*xylE*-Gm^R strain (H855) similar to those of the wild-type strain H103 (Table 3), supporting our previous suggestion that OprH is not essential for polymyxin B resistance but may play an accessory role in stabilizing the outer membrane under Mg^{2+}-starvation conditions (Macfarlane *et al.*, 1999). The only plasmid-borne gene to affect the polymyxin B resistance of strain H855 was *phoP* (pEMR3) (Table 3), the presence of which resulted in significant resistance in high-Mg^{2+} medium. This pattern of polymyxin B resistance closely resembles that of the PhoP-null strain H851 (Macfarlane *et al.*, 1999), and thus supported our initial assumption that the polar nature of the *xylE*-Gm^R insertion in *oprH* rendered strain H855 PhoP- and PhoQ-null. This assumption was based on our inability to detect a second promoter for *phoP-phoQ* by Northern blotting (Macfarlane *et al.*, 1999) or primer-extension analysis (data not shown) of RNA transcripts in the wild-type strain H103, or by dot-blot analysis of RNA from strain H855 using *phoP* and *phoQ* primers (data not shown). Quantitation of the RNA dot blots indicated that if any *phoP-phoQ* transcript was present in strain H855, it occurred at a level at least twofold lower than that of any *phoP*-containing transcripts in strain H103 under high-Mg^{2+} (uninduced) conditions (data not shown).

The region between the *oprH* and *phoP* genes in the *P. aeruginosa* genome is 79 bp in length. A possible ribosome-binding site occurs 5 bp upstream of the *phoP* ATG start codon, but no promoter consensus sequences were identified. There are also no repeat sequences in this region resembling those found upstream of *oprH*. However, the Mg^{2+} regulation of catechol 2,3-dioxygenase expressed from the *oprH*::*xylE* transcriptional fusion in strain H855 (Table 2), together with our observation that transcription of *oprH* is wholly dependent on the presence of PhoP (Macfarlane *et al.*, 1999), provided indirect evidence for a very low level of *phoP-phoQ* transcription in this strain from an as yet unidentified second promoter. The presence of two promoters – one constitutive and one inducible – allowing a basal level of transcription is a common feature of two-component regulatory systems and has been reported for *phoP-phoQ* in *E. coli* (Kato *et al.*, 1999), and for both *phoP-phoQ* and *pmrA-pmrB* in *S. typhimurium* (Gunn & Miller, 1996; Soncini *et al.*, 1995). For the latter system, the second promoter lies within the 3′ region of *pmrC*, the first gene of the *pmrCAB* operon (Gunn & Miller, 1996). The *oprH-phoP* intergenic region was also inspected for possible secondary structures. Although no classic rho-independent terminator sequences were identified, an inverted repeat sequence predicted to be capable of forming a hairpin structure (predicted $\Delta G = -14{\cdot}3$ kcal mol^{-1}) was identified 36 bp downstream of the *oprH* stop codon. Northern blot analysis of strains H103 (Macfarlane *et al.*, 1999) and H854 (Fig. 1) revealed high levels of *oprH* transcripts and diminishing amounts of *oprH-phoP* and *oprH-phoP-phoQ* transcripts. Formation of a hairpin structure that functions as a transcriptional attenuator downstream of *oprH* and provides additional regulation of *oprH-phoP-phoQ* transcription would be consistent with these results. It is worth noting also that our results do not eliminate the possibility that *P. aeruginosa oprH-phoP-phoQ* may be subject to some form of global regulation, for example by another two-component regulatory system, that acts in conjunction with PhoP-mediated regulation.

PhoP-PhoQ selectively regulates cationic peptide resistance

The polymyxin B and CP28 resistance of the OprH-null strain H855 supported our conclusions that PhoP is not essential for resistance to these two antibiotics under

Mg^{2+}-starvation conditions. Although transcription of *phoP-phoQ* presumably occurs at a very low level in strain H855, *phoP* transcription cannot be induced through the promoter upstream of *oprH* and levels of PhoP protein, therefore, would be minimal in this strain.

Interestingly, a *phoP*::Gm mutant of *P. aeruginosa* PAK has recently been reported that is supersusceptible to polymyxin B, but resistant to the cationic peptide C18G, under Mg^{2+}-starvation conditions (Ernst *et al.*, 1999). The possibility that the Gm cassette inserted into the PAK *phoP* gene was non-polar and allowed transcription of *phoQ* could account for the discrepancy in polymyxin B resistance between the *phoP* mutants in the two strains (Macfarlane *et al.*, 1999).

The results of MIC determinations for cationic peptides other than CP28 (Table 5) indicated that *P. aeruginosa* resistance to a second α-helical peptide CP29 and the protegrin-like peptide IB-367 (currently undergoing clinical trials against *P. aeruginosa* lung infections in cystic fibrosis patients; see http://www.intrabiotics.com) probably involves a pathway similar to that for polymyxin B and CP28 resistance. However, resistance to the indolicidin analogue CP11CN, although still affected by mutations in PhoP-PhoQ, appears to be subject to different regulatory mechanisms. Increased resistance to this peptide was seen only in the two mutants, H851 and H855, that could not induce expression of PhoP. Resistance to polyphemusin, on the other hand, appeared to be independent of PhoP-PhoQ. While this was the only peptide we tested that was unaffected by the antagonistic effects of Mg^{2+} ions, resistance still appeared to be Mg^{2+} regulated (data not shown), indicating that other regulatory systems in *P. aeruginosa* must also respond to extracellular Mg^{2+} ion concentrations.

Although the mechanism of action of antimicrobial cationic peptides remains uncertain, the α-helical peptides CP28 and CP29, like polymyxin B, are believed to initially interact with and disrupt the outer membrane of Gram-negative bacteria (Hancock & Chapple, 1999). In *S. typhimurium*, increased resistance to polymyxin B and certain antimicrobial peptides is observed under Mg^{2+}-starvation conditions and is partly due to structural alterations of the outer membrane resulting from activation of genes in the PhoP-PhoQ regulon. Similarly, a *phoP* mutant of *P. aeruginosa* PAK was shown to lack certain modifications to core lipid A that were seen in the wild-type strain under Mg^{2+}-starvation conditions (Ernst *et al.*, 1999). Resistance of *P. aeruginosa* PAO1 to polymyxin B and CP28 likewise may be due to structural alterations to the outer membrane that are regulated in part by PhoP-PhoQ.

The small differences in MIC values measured for the *oprH*, *phoP* and *phoQ* mutants compared to wild-type indicate that PhoP-PhoQ is not a major factor in *P. aeruginosa* cationic peptide resistance. Our results are consistent with an indirect role for this regulatory system in the Mg^{2+} regulation of cationic peptide resistance. This conclusion contrasts peptide resistance in *P. aeruginosa* to that in *S. typhimurium*, where the PhoP-PhoQ two-component regulatory system has been proposed to play a key role. However, although killing assays comparing *S. typhimurium phoP* mutants with wild-type have showed substantial differences in peptide susceptibility (Fields *et al.*, 1989), examination of the susceptibility of *S. typhimurium* strain MS7953 (*phoP*::Tn*10*; Fields *et al.*, 1989) to the peptides CP28, CEME and CP11CN revealed decreases in MIC values of only two- to eightfold relative to the values for wild-type (Falla & Hancock, 1997; Piers *et al.*, 1994).

Aminoglycoside-resistance regulation

The differing effects of the *P. aeruginosa phoP* and *phoQ* mutations on cationic peptide resistance are indicative of the complex regulation of antibiotic resistance in this bacterium. The observed increase in resistance to aminoglycosides for all three mutants (PhoP-, PhoQ- and OprH-null) is a further indication of the intricacies of the PhoP-PhoQ regulatory system. The only feature common to strains H851, H854 and H855 is the inability to induce expression of *phoQ*. Strains H895 and H896, which carry deletions in *oprH* but which should allow fully inducible expression of *phoP-phoQ*, showed identical aminoglycoside resistance to the wild-type strain H103 (Table 5). The increased resistance observed in strain H855 (*oprH*::*xylE*-Gm^R), therefore, can be directly attributed to the polar effect of the *xylE*-Gm^R cassette on the downstream *phoP-phoQ* genes rather than to loss of OprH expression. Similarly, previous results that implicated OprH in *P. aeruginosa* resistance to aminoglycosides (Hancock *et al.*, 1981; Young *et al.*, 1992) can now be explained in terms of polar effects of the *tet* cassette inserted into *oprH* in strain H703 (Young *et al.*, 1992) on *phoP-phoQ* expression. Our results imply that PhoQ is responsible, either directly or through interaction with other regulatory systems, for the downregulation of resistance to the aminoglycosides streptomycin, kanamycin and amikacin. In this case, such a downregulation effect must be overridden by another system under Mg^{2+}-starvation conditions, as these conditions are known to induce both PhoP-PhoQ expression (Macfarlane *et al.*, 1999) and resistance to streptomycin and gentamicin (Hancock *et al.*, 1981). Interestingly, in killing assays with streptomycin, the overexpression of PhoP in the PhoQ-null mutant H854 was seen to partially counteract the positive effect of a PhoQ-null phenotype (Table 4). Hence it is feasible that high levels of PhoP expression in the absence of PhoQ affect the other system(s) involved in streptomycin resistance, most probably through cross-talk.

Decreased permeability of the outer membrane to the aminoglycoside streptomycin was observed for all three mutants (Fig. 3). However, the high concentrations of streptomycin (> 100-fold higher than the MIC) that were required for this experiment probably do not reflect the normal killing mechanism, and are consequently more indicative of a general increase in outer-membrane

stability in the mutants. A greater resistance to perturbation of the outer membrane was observed in strain H854, which overexpresses OprH, than in strains H851 and H855 (both unable to express OprH), lending support to our suggestion of an outer-membrane-stabilization role for OprH.

We noted earlier that an exceptionally large number of two-component regulatory systems have been revealed in *P. aeruginosa* by the *Pseudomonas* genome sequencing project. Our results support the concept of *P. aeruginosa* antibiotic resistance being the result of a complex interplay of several of these systems. Further studies aimed at unravelling such interactions, as well as defining genes that fall into the PhoP-PhoQ regulon, should greatly aid our understanding of antibiotic resistance in this important pathogen.

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