Molecular Microbiology (1999) **34**(2), 305–316

PhoP–PhoQ homologues in *Pseudomonas aeruginosa* regulate expression of the outer-membrane protein OprH and polymyxin B resistance

Emma L. A. Macfarlane, Agnieszka Kwasnicka, Martina M. Ochs and Robert E. W. Hancock*
Department of Microbiology, # 300, 6174 University Boulevard, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3.

Summary

Rapid adaptation to environmental challenge is essential for the survival of many bacterial species, and is often effectively mediated by two-component regulatory systems. Part of the adaptive response of *Pseudomonas aeruginosa* to Mg^{2+} starvation is overexpression of the outer-membrane protein OprH and increased resistance to the polycationic antibiotic polymyxin B. Two overlapping open reading frames that encoded proteins with high similarities to the PhoP–PhoQ two-component regulatory system of *Salmonella typhimurium* were identified downstream of the *oprH* gene. A *P. aeruginosa* PhoP-null mutant, H851, was constructed by means of a *phoP::xylE*-Gm^R transcriptional fusion, and shown to be deficient in OprH expression. In contrast, an analogous PhoQ-null mutant, H854 (*phoQ::xylE*-Gm^R), exhibited constitutive overexpression of OprH. Normal Mg^{2+}-regulated OprH expression could be restored in both mutants by complementation with a plasmid carrying the *phoP* and *phoQ* genes. Measurement of the catechol-2,3-dioxygenase activity, expressed from the *xylE* transcriptional fusion in strains H851 and H854, indicated that PhoP–PhoQ is involved in the regulation of *phoP–phoQ* as well as *oprH*. Reverse transcription polymerase chain reaction experiments and Northern blot analysis revealed linkage of *oprH*, *phoP* and *phoQ* into an operon that was demonstrated to be under the joint control of PhoP–PhoQ and Mg^{2+} ion concentration. In addition, studies of the polymyxin B resistance of the two mutant strains, H851 and H854, indicated that PhoP–PhoQ is involved in regulating *P. aeruginosa* polymyxin resistance in response to external Mg^{2+} concentrations.

Introduction

Two-component regulatory systems are a ubiquitous family of proteins that allow bacteria to modulate the expression of genes in response to various environmental cues. The pleiotropic nature of these systems makes them exceptionally efficient at mediating adaptive changes to environmental stress, and as a result many are involved in the virulence and antibiotic resistance pathways of pathogenic bacteria. Classic two-component regulatory systems comprise a membrane-associated sensor kinase and a DNA-binding response regulator. The sensor kinase responds to specific environmental stimuli by autophosphorylation at a conserved histidine residue at the expense of ATP. A transphosphorylation reaction follows, by which the kinase activates its cognate response-regulator protein; this phosphoprotein in turn activates or represses its target genes by binding to specific upstream sequences. In some instances these target genes encode a second two-component regulatory system, creating a signal transduction cascade that not only allows a single environmental signal to have far-reaching effects in the cell, but also provides bacteria with a means of expressing important genes by integrating responses to a variety of signals (Soncini and Groisman, 1996).

The ability of the pathogen *Salmonella typhimurium* to grow under Mg^{2+} starvation conditions is mediated by the two-component regulatory system PhoP–PhoQ (Soncini *et al*., 1996). This major regulon controls over 40 genes, including those responsible for Mg^{2+} transport (Soncini *et al*., 1996), virulence (Miller *et al*., 1989), lipopolysaccharide (LPS) structure (Helander *et al*., 1994; Guo *et al*., 1997) and resistance to both defensins (Fields *et al*., 1989) and polycationic antibiotics, such as polymyxin B (Groisman *et al*., 1997; Gunn *et al*., 1998). LPS modifications and polymyxin resistance are controlled by a second two-component regulatory system, PmrA–PmrB, which is itself regulated by PhoP–PhoQ (Gunn and Miller, 1996; Soncini and Groisman, 1996). The sensor kinase protein, PhoQ, has been shown to be a Mg^{2+} sensor that responds to low external concentrations

Received 15 February, 1999; revised 5 July, 1999; accepted 23 July, 1999. E.L.A.M. and A.K. contributed equally to this work. *For correspondence. E-mail bob@cmdr.ubc.ca; Tel. (+1) 604 822 2682; Fax (+1) 604 822 6041.

© 1999 Blackwell Science Ltd

of this cation by activating PhoP (Véscovi *et al*., 1996). Thus, transcription of PhoP-activated genes is increased under Mg^{2+} starvation conditions, whereas that of PhoP-repressed genes is decreased.

Homologues of the *S. typhimurium* PhoP–PhoQ two-component regulatory system have been isolated from *Escherichia coli* (Groisman *et al*., 1992; Kasahara *et al*., 1992) and *Shigella flexneri* (Groisman *et al*., 1989), and identified in a number of other Gram-negative bacterial species, both pathogenic and non-pathogenic (Groisman *et al*., 1989). The widespread occurrence of this regulon suggests that its primary function may be in the routine control of physiological adaptations common to most bacteria.

When grown under Mg^{2+} starvation conditions, the opportunistic pathogen *Pseudomonas aeruginosa* exhibits resistance to EDTA and the polycationic antibiotics gentamicin and polymyxin B (Brown and Melling, 1969; Nicas and Hancock, 1980), together with structural alterations in the outer-membrane LPS (Moore *et al*., 1984) and overexpression of the outer-membrane protein, OprH (Nicas and Hancock, 1980). In this study, we have shown that a homologue of PhoP–PhoQ is present in *Pseudomonas aeruginosa* PAO1 and that the genes for this two-component regulatory system are located immediately downstream of the *oprH* gene.

OprH is a small (21 kDa), slightly basic protein, with a proposed eight-stranded β-barrel structure, (Rehm and Hancock, 1996) that has been shown to be structurally related to the porin family (Bell *et al*., 1991). Under low-Mg^{2+} growth conditions, OprH becomes the major protein in the outer membrane. Although the exact function of OprH remains unknown, it has been proposed that it occupies Mg^{2+} ion binding sites in the outer membrane when these cations are limited, and thus contributes to membrane stability, resistance to EDTA and possibly resistance to polymyxin B (Bell *et al*., 1991).

In the results reported here, we have established the role of *P. aeruginosa* PhoP–PhoQ in the regulation of OprH expression, PhoP–PhoQ expression and polymyxin B resistance, through the construction of PhoP- and PhoQ-null mutants and complementation experiments.

Results

Identification of PhoP–PhoQ homologues downstream of oprH *in* P. aeruginosa PAO1

The release of sequences from the *Pseudomonas aeruginosa* genome project provided us with the opportunity to search for homologues of the two-component regulatory system PhoP–PhoQ in this organism. We identified two open reading frames (ORFs) that, using BLASTX analysis (Altschul *et al*., 1997), showed high similarities to PhoP–PhoQ from *Salmonella typhimurium* (Miller *et al*., 1989) and *Escherichia coli* (Kasahara *et al*., 1992). These ORFs were found to start 79 bases downstream of the gene for the outer-membrane protein *oprH*, and were transcribed in the same direction as this gene (Fig. 1). The two ORFs overlapped by four nucleotides suggesting that they form a single transcriptional unit with the start codon for the second ORF (*phoQ*) being the less common GTG. Identities with the *S. typhimurium* and *E. coli* proteins were 53–54% for PhoP and 33% for PhoQ (Fig. 2). The *P. aeruginosa phoP* and *phoQ* genes possessed high GC contents (>65%), typical of *Pseudomonas* genes.

An interesting feature noted in the region upstream of the *oprH* gene was the presence of four hexanucleotide GTTCAG repeats, each separated by five base pairs, situated 60 bp upstream of the ATG codon (Fig. 1). Direct repeats have been found a similar distance upstream of *phoP–phoQ* in *S. typhimurium* (Groisman *et al*., 1989) and *E. coli* (Groisman *et al*. 1992).

Co-transcription of oprH *and* phoP

The observed proximity of the *oprH* and *phoP–phoQ* coding regions in the genome, together with the presence of direct repeats upstream of *oprH,* presented the possibility that the three genes might be transcriptionally linked and form an operon. In which case, the genes would share a common promoter located upstream of *oprH*.

We obtained preliminary evidence that *oprH* and *phoP* could be found on a single RNA transcript by conducting reverse transcription polymerase chain reaction (RT-PCR) experiments. Primers were designed to amplify a 346 bp fragment from bases 729–749 within *oprH* (5′ primer) to bases 1055–1075 within *phoP* (3′ primer) (Fig. 1). Total cellular RNA was isolated from wild-type *P. aeruginosa* (strain H103) grown in low-Mg^{2+} medium, growth conditions known to induce OprH expression (Nicas and Hancock, 1980). Reverse transcription followed by PCR yielded a product of the expected size (≈ 350 bp; Fig. 3, lane 1). The same size of fragment was amplified from the control genomic DNA (lane 4).

Additional evidence for the cotranscription of *oprH–phoP* and of *oprH–phoP–phoQ* was subsequently obtained from Northern blot analysis of total cellular RNA isolated from strain H103 grown in either high- or low-Mg^{2+} media (Fig. 4). DNA probes complimentary to *oprH, phoP* and *phoQ* were used for hybridization. Three separate mRNAs were observed to hybridize to the *oprH* probe in RNA isolated from cells grown under low-Mg^{2+} conditions (panel A, lane 2), but only a very low level of the smallest transcript hybridized under high-Mg^{2+} growth conditions (panel A, lane 1). The major transcript produced in low-Mg^{2+} medium, which corresponded

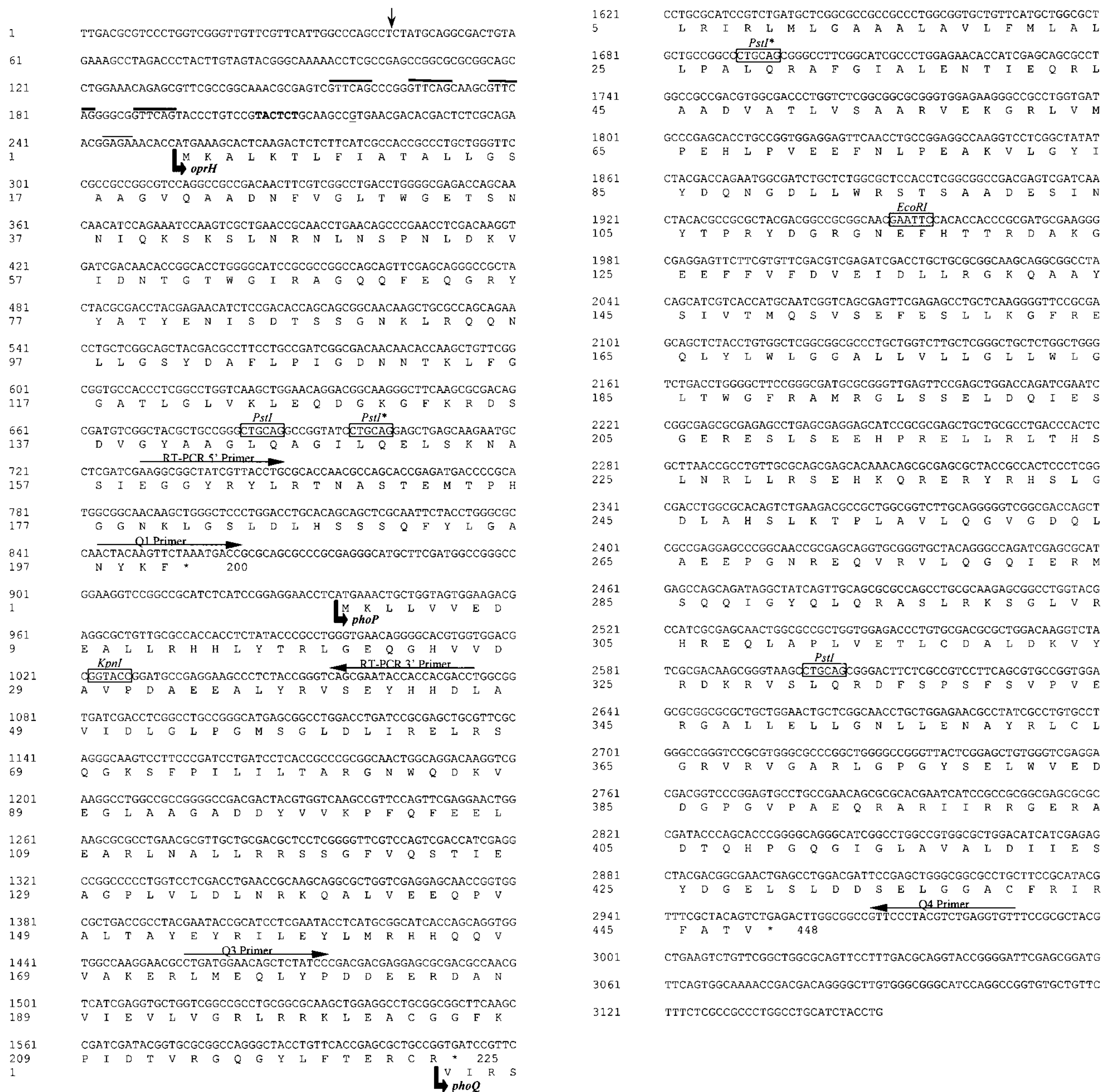

Fig. 1. Nucleotide sequence and translation for the *oprH, phoP* and *phoQ* genes in *Pseudomonas aeruginosa PAO1.* The start of each gene is labelled. The hexanucleotide direct repeats upstream of *oprH* are indicated by bold lines. A putative −10 sequence is indicated in bold and the proposed start of transcription is underlined, both are based on preliminary data from primer extension experiments. Primers used for RT-PCR and for amplification of the *phoQ* and *phoP–phoQ* coding regions from genomic DNA are indicated by arrows. Important restriction sites are boxed. The *Pst*I sites used to construct pEMR2 and pEMR3 are distinguished with an asterisk. The *EcoR*I site within *phoQ* marks the end of the insert in pGB22. The start of the insert in pAK9, the plasmid used to construct the *phoP::xylE*-Gm^R fusion, is indicated by a downwards arrow.

to the single transcript seen in high-Mg^{2+} medium, was equivalent in size to the *oprH* coding region (0.7 kB signal). The other two transcripts were of an appropriate size to represent *oprH–phoP* (1.3 kB) and *oprH–phoP–phoQ* (2.7 kB). The former transcript also hybridized to a probe complimentary to *phoP* (panel B, lane 2), whereas the latter hybridized to probes complimentary to both *phoP* and *phoQ* (panels B and C, lane 2), but only in RNA isolated from cells grown in low-Mg^{2+} medium. No transcripts containing *phoP* or *phoQ* could be detected in RNA isolated from cells grown in high-Mg^{2+} medium (panels B and C, lane 1).

A

```
PaPhoP  :  -MKLLVVEDEALLRHHLYTRLGEQGHVVDAVPDAEEALYRVSEYHHDLAVIDLGLPGMSGLDLIRELRSQGKSFPILILT  :  79
StPhoP  :  MMRVLVVEDNALLRHHLKVQLQDSGHQVDAAEDAREADYYLNEHLPDIAIVDLGLEDEDGLSLIRRWRSSDVSLPVLVLT  :  80
EcPhoP  :  -MRVLVVEDNALLRHHLKVQIQDAGHQVDDAEDAKEADYYLNEHIPDIAIVDLGLEDEDGLSLIRRWRSNDVSLPILVLT  :  79

PaPhoP  :  ARGNWQDKVEGLAAGADDYVVKPFQFEELEARLNALLRRSSGFVQSTIEAGPLVLDLNRKQALVEEQPVALTAYEYRILE  :  159
StPhoP  :  AREGWQDKVEVLSSGADDYVTKPFHIEEVMARMQALMRRNSGLASQVINIPPFQVDLSRRELSVNEEVIKLTAFEYTIME  :  160
EcPhoP  :  ARESWQDKVEVLSAGADDYVTKPFHIEEVMARMQALMRRNSGLASQVISLPPFQVDLSRRELSINDEVIKLTAFEYTIME  :  159

PaPhoP  :  YLMRHHQQVVAKERLMEQLYPDDEERDANVIEVLVGRLRRKLEACGGFKPIDTVRGQGYLFTERCR  :  225
StPhoP  :  TLIRNNGKVVSKDSLMLQLYPDAELRESHTIDVLMGRLRKKIQAQYPHDVITTVRGQGYLFELR--  :  224
EcPhoP  :  TLIRNNGKVVSKDSLMLQLYPDAELRESHTIDVLMGRLRKKIQAQYPQEVITTVRGQGYLFELR--  :  223
```

B

```
PaPhoQ  :  --------VIRSLRIRLMLGAAALAVLFMLALLPALQRAFGIALENTIEQRLAADVATLVSAARVEKGRLVMPEHLPV  :  70
StPhoQ  :  MNKFARHFLPLSLRVRFLLATAGVVLVLSLAYGIVALVGYSVSFDKTTFRLLRGESNLFYTLAKWENN----KISVEL  :  74
EcPhoQ  :  MKKLLRLFFPLSLRVRFLLATAAVVLVLSLAYGMVALIGYSVSFDKTTFRLLRGESNLFYTLAKWENN----KLHVEL  :  74

PaPhoQ  :  EEFNLPEAKVLGYIYDQNGDLLWRSTSAADESINYTPRYDGRG--NEFHTTRDAKGEEFFVFDVEIDLIRGKQ-----  :  141
StPhoQ  :  PENLRMQSPTMTLIYDETGKLLWTQRNIPWLIKSIQPEWLKTNGFHEIETNVDATSTLLSEDHSAQEKLKEVREDDDD  :  152
EcPhoQ  :  PENIDKQSPTMTLIYDENGQLLWAQRDVPWLMKMIQPDWLKSNGFHEIEADVNDTSLLLSGDHSIQQQLQEVREDDDD  :  152

PaPhoQ  :  ------------AAYSIVTMQSVSEFESLLKGFREQLYLW------LGGALLVLLGLLWLGLTWGFRAMRGLSSELDQ  :  201
StPhoQ  :  AEMHSVAVNNIYPATARMPQLTIVVVDTIPIELKRSYMVWSWFVYVLAANLLLVIPLLWIAAWWSLRPIEALAREVRE  :  230
EcPhoQ  :  AEMTHSVAVNVYPATSRMPKLTIVVVDTIPVELKSSYMVWSWFIYVLSANLLLVIPLLWVAAWWSLRPIEALAKEVRE  :  230

PaPhoQ  :  IESGERESLSEEHPRELLRLTHSLNRLLRSEHKQRERYRHSLGDLAHSLKTPLAVLQGVGDQLAEE-PGNREQVRVLQ  :  278
StPhoQ  :  LEDHHREMLNPETTRELTSLVRNLNQLLKSERERYNKYRTTLTDLTHSLKTALAVLQSTLRSLRNEKMSVSKAEPVML  :  308
EcPhoQ  :  LEEHNRELLNPATTRELTSLVRNLNRLLKSERERYDKYRTTLTDLTHSLKTPLAVLQSTLRSLRSEKMSVSDAEPVML  :  308

PaPhoQ  :  GQIERMSQQIGYQLQRASLRK--SGLVRHREQLAPLVETLCDALDKVYRDKRVSLQRDFSPSFSVPVERGALLELLGN  :  354
StPhoQ  :  EQISRISQQIGYYLHRASMRGSGVLLSRELHPVAPLLDNLISALNKVYQRKGVNISMDISPEISFVGEQNDFVEVMGN  :  386
EcPhoQ  :  EQISRISQQIGYYLHRASMRG-GTLLSRELHPVAPLLDNLTSALNKVYQRKGVNISLDISPEISFVGEQNDFVEVMGN  :  385

PaPhoQ  :  LLENAYRLCLGRVRVGARLGPGYSELWVEDDGPGVPAEQRARIIRRGERADTQHPGQGIGLAVALDIIESYDGELSLD  :  432
StPhoQ  :  VLDNACKYCLEFVEISARQTDDHLHIFVEDDGPGIPHSKRSLVFDRGQRADTLRPGQGVGLAVAREITEQYAGQIIAS  :  464
EcPhoQ  :  VLDNACKYCLEFVEISARQTDEHLYIVVEDDGPGIPLSKREVIFDRGQRVDTLRPGQGVGLAVAREITEQYEGKIVAG  :  463

PaPhoQ  :  DSELGGACFRIRFATV-------  :  448
StPhoQ  :  DSLLGGARMEVVFGRQHPTQKEE  :  487
EcPhoQ  :  ESMLGGARMEVIFGRQHSAPKDE  :  486
```

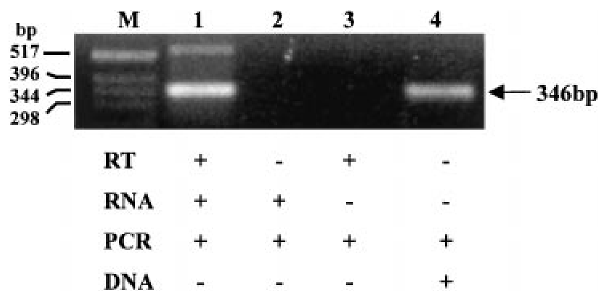

Fig. 3. Co-transcription of *oprH* and *phoP* demonstrated by RT-PCR. Lane 1, whole cell RNA from H103 grown in low-Mg^{2+} medium after reverse transcription and PCR using the RT-PCR 5′ and 3′ primers shown in Fig. 1; lane 2, whole cell RNA submitted to PCR without prior reverse transcription; lane 3, control reaction containing no RNA or DNA template; lane 4, control PCR using H103 genomic DNA and RT-PCR 5′ and 3′ primers; M, molecular weight standard. The results shown are representative of three independent experiments.

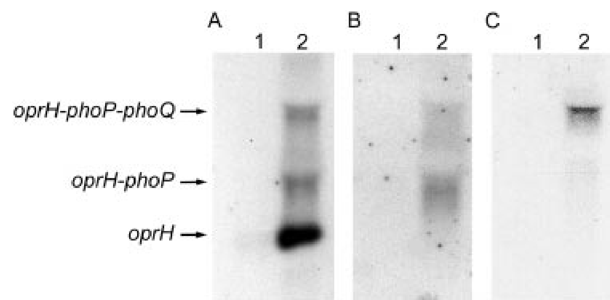

Fig. 4. Northern blot analysis of RNA isolated from strain H103 and probed with DNA complimentary to A, *oprH;* B *phoP*; and C *phoQ*. Total cellular RNA was isolated from mid-log phase cells grown BM2-glucose minimal medium containing 2 mM (high Mg^{2+}, lane 1) or 20 μM (low Mg^{2+}, lane 2) $MgSO_4$. Approximate transcript sizes are: *oprH* 0.7 kB, *oprH–phoP* 1.3 kB, *oprH–phoP–phoQ* 2.7 kB.

Regulation of OprH expression in P. aeruginosa *by PhoP–PhoQ*

The transcriptional linkage of the *oprH–phoP–phoQ* genes, and the known regulation of *oprH* by Mg^{2+} and Ca^{2+} deficiency (Nicas and Hancock, 1980), indicated a potential involvement of PhoP–PhoQ in the regulation of *oprH* transcription. To investigate this possibility, we constructed mutants of *P. aeruginosa* that were either PhoP-null (strain H851, *phoP::xylE*-Gm^R) or PhoQ-null (strain H854, *phoQ::xylE*-Gm^R) (Table 1). Gene replacement in these mutants was confirmed by Southern blot analysis using probes complimentary to either *phoP* or *phoQ,* and to *xylE*. The gentamicin resistance gene, *aacC1,* carried by the *xylE*-Gm^R cassette used to construct these mutants, is flanked by omega fragments that prevent read-through from the *aacC1* promoter affecting expression of downstream genes (Schweizer and Hoang, 1995). Strain H851 was therefore assumed to be phenotypically both $PhoP^-$ and $PhoQ^-$, as the overlap of the PhoP–PhoQ genes implied that a separate promoter for *phoQ* was unlikely. Preliminary data from Northern blot analysis of RNA from strain H851 has confirmed the absence of any transcripts containing *phoQ* (A. Kwasnicka and R. Hancock, unpublished).

SDS–PAGE and Western blot analysis of whole cell lysates from wild-type H103 and the mutant H851 and H854 strains grown to mid-log phase in low-Mg^{2+} (20 μM $MgSO_4$) and high Mg^{2+} (2 mM $MgSO_4$) media are shown in Fig. 5. In wild-type *P. aeruginosa,* OprH expression is known to be induced in low-Mg^{2+} medium (lane 3) and repressed in high-Mg^{2+} medium (lane 2) (Nicas and Hancock, 1980). OprH expression was seen to be suppressed in the PhoP-null strain H851 ($PhoP^-$ $PhoQ^-$) under both growth conditions (lanes 4 and 5). In contrast, elevated levels of OprH expression were observed in the PhoQ-null strain H854 ($PhoP^+$ $PhoQ^-$) in both low- and high-Mg^{2+} media (lanes 10 and 11).

Introduction of *phoP*, carried on the multicopy pUC-based plasmid pUCP19 (pEMR3, Table 1), into strain H851 resulted in unregulated expression of OprH (lanes 6 and 7) similar to that seen in the PhoQ-null strain, H854. Introduction of the *phoP*$^+$*phoQ*$^+$ plasmid, pEMPQ2a (Table 1), into strain H851, however, resulted in full complementation of Mg^{2+}-regulated OprH expression (lanes 8 and 9).

Normal Mg^{2+}-regulated OprH expression could be restored to the PhoQ-null mutant H854, which still carried a functional *phoP* gene, by either *phoQ* alone (plasmid pEMQ1a, lanes 12 and 13), or by *phoP–phoQ* (plasmid pEMPQ2a, lanes 14 and 15). As expected, the levels of OprH expression in strain H854 were unaffected by the *phoP*$^+$ plasmid, pEMR3. Similarly, OprH expression in strain H851 ($PhoP^-$ $PhoQ^-$) could not be restored by the *phoQ*$^+$ plasmid, pEMQ1a.

Fig. 2. Alignment of the PhoP response-regulator proteins (A) and the PhoQ sensor-kinase proteins (B) from *P. aeruginosa* (Pa), *Salmonella typhimurium* (St) and *Escherichia coli* (Ec). Alignments were performed using the CLUSTALW program (Thompson *et al*., 1994) and shaded using the GENEDOC program Version 1.1.004 (Nicholas and Hughes, 1996). Residues indicated by arrows in A are highly conserved in response regulators and form the phosphorylation site (Volz, 1993). Predicted transmembrane domains are indicated in B by double underlines for *S. typhimurium* PhoQ (Miller *et al*., 1989) and by dotted overhead lines for *P. aeruginosa* PhoQ. Predictions for Pa PhoQ were made with the SAPS program (Brendel *et al*., 1992). The histidine residue conserved amongst sensor kinases, and believed to be the site of autophosphorylation, is indicated by an asterisk. Arrows indicate other conserved residues; the series of glycine residues at the C-terminus is thought to be part of the ATP-binding domain (Stock *et al*., 1989).

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotype or relevant phenotype	Source or reference
Pseudomonas aeruginosa		
H103	Wild-type PAO1	Nicas and Hancock (1980)
H851	H103 *phoP::xylE*-Gm^R	This work
H854	H103 *phoQ:: xylE*-Gm^R	This work
Plasmids		
pUCP19, 20, 21	*Escherichia–Pseudomonas* shuttle vectors	Schweizer (1991), West *et al.*(1994)
pX1918GT	pUC-based plasmid containing *xylE*-Gm^R cassette flanked by multiple cloning site from pUC19	Schweizer and Hoang (1995)
pEX100T	Gene replacement vector with *sacB* marker, *oriT* for conjugation-mediated transfer and unique *Sma*I and I-*Sce*I cloning sites	Schweizer and Hoang (1995)
pGB22	2.8 kB *Eco*RI fragment from H103 containing *oprH* *phoP* and part of *phoQ* cloned into pUC18	Bell and Hancock (1989),
pEMR3	*phoP,* as a 0.9-kB *Pst*I fragment from pGB22, cloned behind *lac* promoter in pUCP19	This work
pEMQ1a	*phoQ*, as a 1.55 kB fragment PCR-amplified from H103 genomic DNA, cloned behind *lac* promoter in pUCP20	This work
pEMPQ2a	*phoP–phoQ*, as a 2.16 kB fragment PCR-amplified from H103 genomic DNA, cloned in the opposite orientation to *lac* promoter in pUCP20	This work
pAK9	pGB22 deletion (see Fig. 1)	This work

Autoregulation of the phoP–phoQ *locus*

The effects of the levels of PhoP and PhoQ, and of Mg^{2+} concentration, on the *phoP* and *phoQ* loci were investigated by measurement of catechol-2,3-dioxygenase activity expressed from the *xylE* transcriptional fusion in strains H851 and H854 (Table 2). Mid-log phase cells of these strains, with and without plasmids, grown in both high- (2 mM) and low- (20 μM) Mg^{2+} media were harvested, and assayed by standard methods for catechol dioxygenase activity.

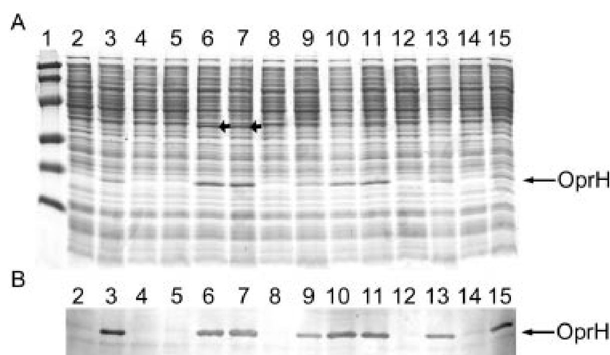

Fig. 5. SDS–PAGE (A) and Western blot (B) of whole-cell lysates from cultures grown in BM2-glucose minimal medium containing 20 μM (low) or 2 mM (high) $MgSO_4$. Cells were harvested at mid-log phase. The arrowheads in lanes 6 and 7 indicate the band corresponding to the putative PhoP–catechol-2,3-dioxygenase fusion protein. Lane 1, molecular weight marker; lane 2, H103 control, high Mg^{2+}; lane 3, H103 control, low Mg^{2+}; lane 4, H851 (*phoP::xylE*-Gm^R), high Mg^{2+}; lane 5, H851, low Mg^{2+}; lane 6, H851/pEMR3 (*phoP*$^+$), high Mg^{2+}; lane 7, H851/pEMR3 (*phoP*$^+$), low Mg^{2+}; lane 8, H851/pEMPQ2a (*phoP*$^+$*, phoQ*$^+$), high Mg^{2+}; lane 9, H851/pEMPQ2a, low Mg^{2+}; lane 10, H854 (*phoQ::xylE*-Gm^R), high Mg^{2+}; lane 11, H854, low Mg^{2+}; lane 12, H854/pEMQ1a (*phoQ*$^+$), high Mg^{2+}; lane 13, H854/pEMQ1a, low Mg^{2+}; lane 14, H854/pEMPQ2a (*phoP*$^+$*, phoQ*$^+$), high Mg^{2+}; lane 15, H854/pEMPQ2a, low Mg^{2+}.

A low level of enzyme activity was observed in the PhoP-null strain, H851, under both growth conditions. When the *phoP*$^+$ plasmid, pEMR3, was introduced into this strain, a dramatic increase in the catechol dioxygenase activity measured in both media was observed, up to 350-fold over the level observed in strain H851, with or without vector control.

Consistent with these observations, strain H851, harbouring the *phoP*$^+$ plasmid, pEMR3, showed high expression, not only of OprH, but also of a second protein with an apparent mass of 45 kDa (Fig. 5, lanes 6 and 7). The size of this protein was exactly that expected from an in-frame fusion of the 5′ end of *phoP* with intervening plasmid sequences and *xylE* creating a PhoP–catechol dioxygenase fusion protein. This protein was unique to H851/pEMR3, confirming that it resulted solely from the action of PhoP on the *phoP::xylE*-Gm^R fusion.

Complementation with the *phoP*$^+$ *phoQ*$^+$ plasmid, pEMPQ2a, conferred Mg^{2+} regulation on the catechol dioxygenase expression in strain H851, with a 62-fold difference in activity observed between cells grown in low- and high-Mg^{2+} media. It was interesting to note that the levels of catechol dioxygenase activity in H851/pEMPQ2a were substantially lower than those measured in H851/pEMR3. In addition to plasmid pEMPQ2a, we constructed an analogous plasmid, pEMPQ1b, that carried *phoP–phoQ* behind the *lac* promoter. In direct contrast to

Table 2. Catechol-2,3-dioxygenase activity in strains H851 and H854 harbouring PhoP and PhoQ expression plasmids.

Strain	Plasmid[a]	Catechol-2,3-dioxygenase activity, pmol min^{-1} Low $MgSO_4$ Mean activity[b] ± S. E. M	High $MgSO_4$ Mean activity[b] ± S. E. M	Activity increase in low $MgSO_4$
H851	None	60 ± 5	43 ± 4	1.4
(*phoP::xylE*-Gm^R)	pEMR3 (*phoP*^{F})	17 427 ± 4554	15 545 ± 4675	1.1
	pEMQ1a (*phoQ*^{F})	66 ± 9	44 ± 9	1.5
	pEMPQ2a (*phoPQ*^{R})	3428 ± 194[c]	55 ± 6[c]	62
H854	None	2039 ± 206	1165 ± 10	1.7
(*phoQ::xylE*-Gm^R)	pEMR3 (*phoP*^{F})	1771 ± 166	1178 ± 128	1.5
	pEMQ1a (*phoQ*^{F})	326 ± 51	24 ± 3	14
	pEMPQ2a (*phoPQ*^{R})	207 ± 33	4.9 ± 0.2	41

a. Genes cloned behind the *lac* promoter are labelled with a superscript F; genes cloned in the opposite orientation to the *lac* promoter are labelled with a superscript R.
b. Values are the mean for three independent experiments.
c Measurements for each strain/plasmid combination were performed in parallel with strain H851 alone as a control. The values for the H851 control performed in parallel with H851/pEMPQ2a were lower than those shown in the above table therefore the values shown for H851/pEMPQ2a were scaled up by an appropriate factor to allow direct comparison with the other catechol dioxygenase activity measurements in strain H851.

pEMPQ2a, this plasmid led to the repression of OprH expression, and substantial decreases in the catechol dioxygenase activity in both strains H851 and H854, regardless of the growth conditions (data not shown).

In contrast to strain H851, the PhoQ-null strain, H854, showed a significant level of catechol dioxygenase activity in the absence of any plasmid. A small (twofold) difference in activity was observed between the two media, with the higher activity seen consistently under low-Mg^{2+} conditions. Complementation of H854 with the *phoQ*$^+$ plasmid, pEMQ1a, established a much greater difference (14-fold) in activity between high- and low-Mg^{2+} media, and also resulted in a substantial decrease in the overall level of catechol dioxygenase activity. An even greater decrease in overall catechol dioxygenase activity resulted from complementation with the *phoP*$^+$ *phoQ*$^+$ plasmid, pEMPQ2a, together with an enhanced Mg^{2+} regulation effect (41-fold).

In agreement with the results from OprH expression studies, the *phoP*$^+$ plasmid, pEMR3, in strain H854 and the *phoQ*$^+$ plasmid, pEMQ1a, in strain H851 had little or no effect on catechol dioxygenase activity in the respective strains.

Polymyxin B resistance in P. aeruginosa *is influenced by PhoP–PhoQ*

It has been known for some time that *P. aeruginosa* grown under the low-Mg^{2+} (< 0.5 mM) conditions that induce overexpression of OprH (Nicas and Hancock, 1980) is resistant to polymyxin B (Brown and Melling, 1969). For this reason, it has been proposed that OprH may play a role in *P. aeruginosa* polymyxin B resistance by helping to stabilize the outer membrane when it is cation depleted (Nicas and Hancock, 1980, 1983; Bell *et al.*, 1991). As we had demonstrated that PhoP–PhoQ regulated OprH expression, we investigated whether this two-component regulatory system was also involved in polymyxin B resistance in *P. aeruginosa*.

Polymyxin B resistance was determined by killing assays on mid-log phase cultures that had been grown in high- (2 mM) or low- (20 μM) Mg^{2+} media. The concentration of polymyxin B used in these assays (8 μg ml^{-1}) was between eight and 16 times greater than the minimum inhibitory concentration determined for the wild-type strain, H103. The results of the killing assays are summarized in Table 3. The PhoP-null mutant, H851, demonstrated wild-type resistance to polymyxin B in low-Mg^{2+} medium and remained susceptible when grown in high-Mg^{2+} medium. In contrast, the PhoQ-null strain, H854, displayed high levels of resistance to polymyxin B in both high- and low-Mg^{2+} media.

Introduction of the *phoP*$^+$ plasmid, pEMR3, into strain H851 resulted in polymyxin B resistance in high-Mg^{2+} medium, a similar resistance phenotype to that seen in strain H854. Both these strains would be phenotypically $PhoP^+$ $PhoQ^-$, and these results confirmed that PhoP was involved in *P. aeruginosa* polymyxin B resistance. Complementation of strain H854 with the *phoQ*$^+$ plasmid, pEMQ1a, restored polymyxin sensitivity to this strain when grown in high-Mg^{2+} medium, and demonstrated that PhoP activation is normally modified by PhoQ in response to external Mg^{2+} levels. Introduction of the *phoP*$^+$ *phoQ*$^+$ plasmid, pEMPQ2a, into either of the mutant strains, H851 or H854, fully restored the wild-type pattern of polymyxin B resistance.

A somewhat surprising result was that introduction of the *phoQ*$^+$ plasmid, pEMQ1a, into the PhoP-null strain,

Table 3. Effect of PhoP and PhoQ proteins on OprH expression and polymyxin B resistance in *P. aeruginosa*.

Strain	Plasmid[c]	Relative OprH expression[a]		Polymyxin B resistance[b] (mean percentage survival ± SEM)	
		Low $MgSO_4$	High $MgSO_4$	Low $MgSO_4$	High $MgSO_4$
H103 ($PhoP^+$,$PhoQ^+$)	None	+++	+/−	82 ± 12	0
H851 (*phoP::xylE*-Gm^R)	None	−	−	95 ± 3	0
	pUCP19(control)	−	−	65 ± 6	0
	pEMR3(*phoP*^{F})	+++	+++	100	81 ± 17
	pEMQ1a(*phoQ*^{F})	−	−	13 ± 6	0
	pEMPQ2a(*phoPQ*^{R})	+++	−	100	0.2 ± 0.1
H854 (*phoQ::xylE*-Gm^R)	None	+++	+++	100	69 ± 17
	pUCP19(control)	+++	+++	100	87 ± 13
	pEMR3(*phoP*^{F})	+++	+++	100	42 ± 12
	pEMQ1a(*phoQ*^{F})	+++	−	100	0
	pEMPQ2a(*phoPQ*^{R})	+++	−	100	0

a. Determined by SDS–PAGE experiments, such as those shown in Fig. 5, and based on a +++ rating for wild-type H103 grown in low-Mg^{2+} medium.
b. After treatment with 8 $\mu g\ ml^{-1}$ of polymyxin B for 5 min, as described in *Experimental procedures*. Results are the average of three independent experiments.
c. Genes cloned behind the *lac* promoter are labelled with a superscript F; genes cloned in the opposite orientation to the *lac* promoter are labelled with a superscript R.

H851, lowered the polymyxin B resistance of this strain in low-Mg^{2+} medium. It is most likely that this result is attributable to an increased amount of PhoQ protein in the cell in the absence of PhoP protein, and implies a possible interaction with other regulatory systems.

Discussion

In this study, we have identified a homologue of the *Salmonella typhimurium* PhoP–PhoQ two-component regulatory system in *Pseudomonas aeruginosa* PAO1. The *phoP–phoQ* genes are located immediately downstream of the gene for the outer-membrane protein OprH. We have demonstrated, using RT-PCR and Northern blot analysis, that the three genes *oprH–phoP–phoQ* are cotranscribed, and therefore form a small operon. This gene arrangement is distinct from that found in *S. typhimurium* in which no transcriptionally linked genes are found upstream of *phoP–phoQ* (Soncini *et al*., 1995).

Transcription of the *oprH–phoP–phoQ* operon displayed distinct Mg^{2+} regulation. Only very low amounts of the *oprH* transcript were observed when cells were grown in high-Mg^{2+} medium, which is in agreement with the level of OprH expression in wild-type H103 under these conditions. No transcripts containing *phoP* or *phoQ* could be detected under high-Mg^{2+} growth conditions. As the major transcript observed by Northern blot analysis in strain H103 corresponded to the *oprH* coding region alone (Fig. 4), post-transcriptional processing of the full-length *oprH–phoP–phoQ* mRNA may occur that accounts for the appearance of the shorter transcripts. We propose that the promoter for the *oprH–phoP–phoQ* operon is located upstream of *oprH*, between the hexanucleotide repeats and the ATG start codon. Preliminary results from primer extension experiments conducted in our laboratory have identified a single promoter in this region (A. Kwasnicka and R. Hancock, unpublished). A possible −10 sequence and transcriptional start site based on this data are shown in Fig. 1.

The results we have presented here for OprH expression and catechol-2,3-dioxygenase activity measured in the *P. aeruginosa* PhoP-null (H851, *phoP::xylE*-Gm^R) and PhoQ-null (H854, *phoQ::xylE*-Gm^R) mutants have demonstrated that *oprH–phoP–phoQ* transcription is dependent upon PhoP–PhoQ as well as Mg^{2+} ion concentration. The presence of the response regulator, PhoP, is an absolute requirement for *oprH* transcription, as the PhoP-null strain, H851, was unable to express OprH under either high- or low-Mg^{2+} growth conditions (Fig. 5). The high levels of OprH expression observed in both media when this strain was complemented by the *phoP*$^+$ plasmid, pEMR3, demonstrate that PhoP is an activator of transcription. The same pattern of OprH overexpression was seen in the PhoQ-null strain, H854, which is phenotypically $PhoP^+$ $PhoQ^-$. Measurement of the catechol dioxygenase activity expressed from the transcriptional fusions in strains H851 (*phoP::xylE*-Gm^R) and H854 (*phoQ::xylE*-Gm^R) was in agreement with the observed OprH expression (Table 2). Transcription of *phoP–phoQ* is therefore also activated by PhoP; an observation that is consistent with the arrangement of *oprH–phoP–phoQ* in a single transcriptional unit.

Although our results indicate that PhoP is a positive regulator of *oprH–phoP–phoQ* transcription, they also demonstrate that the role of PhoQ is to modulate and impose Mg^{2+} regulation on this activation. Complementation of

the PhoQ-null strain, H854, with either the *phoQ*$^+$ plasmid, pEMQ1a, or the *phoP*$^+$ *phoQ*$^+$ plasmid, pEMPQ2a, led to a substantial decrease in the catechol dioxygenase activity expressed from the *phoQ::xylE*-Gm^R transcriptional fusion. Both plasmids established strong Mg^{2+} regulation on this activity – a 14-fold difference between high- and low-Mg^{2+} media for pEMQ1a and a 41-fold difference for pEMPQ2a. The latter plasmid also restored Mg^{2+}-regulated OprH expression and catechol dioxygenase activity to the PhoP-null strain, H851. The difference in activity between high- and low-Mg^{2+} media in this case was 62-fold.

This delineation of the roles of *P. aeruginosa* PhoP and PhoQ in *oprH–phoP–phoQ* activation raises the possibility that a second phosphorylation agent exists for PhoP. We have shown that PhoP by itself was capable of activating a very high level of *oprH–phoP–phoQ* transcription under both high- and low-Mg^{2+} conditions, whereas the presence of PhoQ restored regulation, and generally reduced this level of activation. Thus, in the absence of PhoQ, PhoP becomes an unregulated activator of the *oprH–phoP–phoQ* operon. In view of the fact that the active form of response regulators is generally accepted to be the phospho-form, a second kinase for PhoP may exist in *P. aeruginosa* capable of phosphorylating this response regulator irrespective of Mg^{2+} levels in the growth medium. The main role of PhoQ would then be to act as a phosphatase that dephosphorylates – and therefore deactivates – PhoP selectively, in response to Mg^{2+} concentrations. In addition, we observed that PhoP activated the *phoQ* locus to a greater extent in the absence of PhoQ than in its presence (H854 alone compared with H854/pEMQ1a or H851/pEMPQ2a, Table 2), even under low-Mg^{2+}-inducing conditions. Therefore, it is possible that an alternative signal to Mg^{2+} starvation suppresses the dephosphorylation of PhoP by PhoQ, and allows this high level of transcription to occur in wild-type *P. aeruginosa*.

In addition to the regulation of OprH expression, our results have demonstrated that PhoP–PhoQ is involved in polymyxin B resistance in *P. aeruginosa.* However, the patterns of resistance displayed by strains H851 (PhoP$^-$ PhoQ$^-$) and H854 (PhoP$^+$ PhoQ$^-$), with and without complementing plasmids (Table 3), clearly indicated that the regulation of polymyxin resistance is much more complex than that of OprH expression. The fact that both mutant strains remained resistant to polymyxin under Mg^{2+}-deficient conditions implies that one or more other regulatory systems are involved in the activation of resistance genes. The polymyxin resistance of strain H851, which is OprH deficient, also confirms that OprH itself is not essential for a polymyxin-resistant phenotype. However, the fact that the gene is found in the same operon as a two-component regulatory system that responds to Mg^{2+} levels supports the proposed accessory role of this protein in membrane stabilization under Mg^{2+} starvation conditions (Bell *et al*., 1991).

A role for the PhoP–PhoQ regulatory system in *P. aeruginosa* polymyxin B resistance was defined by the resistance of strains H854 and H851/pEMR3 (both PhoP$^+$ PhoQ$^-$) in high Mg^{2+} and the susceptibility exhibited by H851/pEMQ1a (PhoP$^-$ PhoQ$^+$) in low Mg^{2+} (Table 3). Normal wild-type susceptibility to polymyxin could be restored to strain H854 by complemention with the *phoQ*$^+$ plasmid, pEMQ1a, or the *phoP*$^+$ *phoQ*$^+$ plasmid, pEMPQ2a. These results indicate that, under high-Mg^{2+} growth conditions in the absence of PhoQ, PhoP is capable of activating one or more genes that are involved in the polymyxin resistance pathway; behaviour that parallels its activation of OprH expression. Polymyxin B resistance in *S. typhimurium* is dependent upon the PmrA–PmrB two-component regulatory system (Roland *et al*., 1993), which in turn is both regulated by, and interacts with, PhoP–PhoQ (Gunn and Miller, 1996; Soncini and Groisman, 1996). An alternative explanation for our results therefore could be that, rather than directly activating resistance genes, PhoP ‘cross-talks’ to a second two-component regulatory system involved in polymyxin resistance. Likewise, in the absence of PhoP, PhoQ may engage in cross-talk that leads to a decrease in polymyxin resistance under low-Mg^{2+} growth conditions.

In *S. typhimurium*, one of the major roles of PhoP–PhoQ is in virulence (Fields *et al*., 1986; Miller *et al*., 1989). Preliminary results of LD_{50} tests conducted in our laboratory indicate that PhoP–PhoQ in *P. aeruginosa* may also be involved in virulence determination. In tests using neutropenic mice, the PhoQ-null mutant, H854, displayed significantly lower virulence than the wild-type strain, H103 (H. Yan and R. Hancock, unpublished).

In conclusion, we have shown that the PhoP–PhoQ two-component regulatory system in *P. aeruginosa* regulates expression of the OprH protein through the activation of transcription of *oprH* under low-Mg^{2+} growth conditions. We have also demonstrated the regulation of PhoP–PhoQ transcription by PhoP and PhoQ in response to external Mg^{2+} concentration, and the transcriptional linkage of the *oprH–phoP–phoQ* genes. In addition, PhoP–PhoQ was shown to play a role in both the polymyxin B resistance and virulence of *P. aeruginosa.* The observed effects of the cloned PhoP and PhoQ proteins in PhoP-null and PhoQ-null mutants suggest both a primary role as a phosphatase for PhoQ and interaction with other regulatory systems. In view of the large number of putative two-component regulators revealed in *P. aeruginosa* by the genome project, we believe that PhoP–PhoQ will be found to be part of an intricate regulatory network worthy of further investigation.

Experimental procedures

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. All strains were maintained on Luria–Bertani (LB) agar or grown in LB broth, supplemented with appropriate antibiotics at 37°C as rich media. For conjugation experiments, *E. 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 different Mg^{2+} concentrations, 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*, ampicillin 100 μg ml^{-1}, gentamicin 10 μg ml^{-1}; for *P. aeruginosa*, carbenicillin 300–350 μg ml^{-1}, gentamicin 15 μg ml^{-1}. Unless otherwise specified, plasmids were transformed into bacterial strains by electroporation using a Gene Pulser™ (Bio-Rad Laboratories) and 0.1-cm-gap cuvettes using published protocols (Sambrook *et al.*, 1989; Dennis and Sokol, 1995).

DNA/RNA techniques

Restriction and modification enzymes were purchased from New England Biolabs (NEB) or Gibco-BRL and used according to the manufacturer's recommendations. Other procedures were taken from Sambrook *et al.* (1989) or Current Protocols in Molecular Biology (Ausubel *et al.*, 1987, and updates). Total cellular RNA for RT-PCR and Northern blot analysis was isolated from mid-logarithmic phase cultures using the RNeasy™ Mini Kit (Qiagen Inc.). Oligonucleotides for PCR and for DNA sequencing were synthesized on an Applied Biosystems model 392 DNA/RNA Synthesizer. The 5′ and 3′ primers for RT-PCR are shown in Fig. 1. Cloned genes that had been amplified from genomic DNA by PCR were sequenced by the dideoxy-chain termination method using the ABI Prism Dye Terminator Cycle Sequencing Kit with AmpliTaq DNA polymerase, FS (PE-Applied Biosystems) and an Applied Biosystems model 373 DNA Sequencer.

Cloning phoP *and* phoQ

A 0.9 kB *Pst*I fragment containing the entire *phoP* gene together with the *oprH–phoP* intergenic region, 156 bp of *oprH* and 82 bp of *phoQ* (Fig. 1), was excised from plasmid pGB22 and cloned into pUCP19 (Schweizer, 1991). The orientation of the *phoP* gene with respect to the *lac* promoter was determined by restriction analysis, and single clones with the gene in forward (pEMR3) and reverse (pEMR2) orientation were selected for further study. Primers Q1, Q3 and Q4 (see Fig. 1) were used to amplify *phoQ* and *phoP–phoQ* from *P. aeruginosa* genomic DNA using the polymerase chain reaction (PCR). Both Q1 and Q3 contained a 9 bp 5′ extension that introduced a unique *Sst*I restriction site, and Q4 carried a 10 bp 5′ extension that introduced a unique *Xba*I site. All PCRs were carried out using Vent DNA polymerase (NEB) with the addition of 8% dimethyl sulphoxide. The Q3+Q4 (*phoQ*) PCR product was digested with *Sst*I and *Xba*I, and cloned into the corresponding sites of plasmid pUCP20 (West *et al.*, 1994) to give pEMQ1a. The Q1+Q4 (*phoP–phoQ*) product was treated with bacteriophage T4 polynucleotide kinase (Gibco-BRL) and blunt-end ligated into the *Sma*I site of pUCP20. The orientation of *phoP–phoQ* relative to the *lac* promoter was determined using restriction analysis. Single clones with the *phoP–phoQ* genes in the forward (pEMPQ1b) and reverse (pEMPQ2a) orientation were isolated. Sequences of all cloned genes were confirmed by dideoxy DNA sequencing. It should be noted that the *phoP* and *phoQ* genes proved difficult to clone together. Very low ligation efficiencies were observed and frequent base changes were found in the clones obtained. Consequently, the sequence of plasmid pEMPQ2a revealed a G to A base change at position 1197 that resulted in a conservative valine to isoleucine substitution at residue 88 in PhoP. However, as this plasmid successfully complemented strains H851 and H854, we considered it reasonable to use pEMPQ2a in the reported studies.

Northern blot analysis

Purified RNA (5 μg) was denatured at 65°C for 15 min in the presence of 2.2 M formaldehyde and 50% formamide and resolved on a 1.2% agarose gel containing 0.6 M formaldehyde in MOPS buffer. The RNA was transferred from the gel onto positively charged nylon membrane (Boehringer Mannheim) by downward alkaline blotting (Ingelbrecht *et al.*, 1998). Double-stranded DNA probes for the Northern blots were synthesized by PCR using the following primers and templates: *oprH* probe (316 bp), 5′ primer: 5′-CAACTTCGTC GGCCTGACCT-3′, 3′ primer: 5′-GCCGTCCTGTTCCAGC TTGA-3′, template: plasmid pBHR20 (Rehm and Hancock, 1996); *phoP* probe (260 bp), 5′ primer: 5′-CTGCTGGTAGTG GAAGACGA-3′, 3′ primer: 5′-TCGACCTTGTCCTGCCAG TT-3′, template: plasmid pEMR3 (Table 1); *phoQ* probe (287 bp), 5′ primer: 5′-AGGAGTTCTTCGTGTTCGAC-3′, 3′ primer: 5′-CAACAGGCGGTTAAGCAGTG-3′, template: plasmid pEMQ1a (Table 1). All probes were labelled with [α-P^{32}]-dCTP using the Rediprime™ DNA-labelling system (Amersham Life Science). Blots were hybridized overnight at 60°C (*oprH* probe) or 45°C (*phoP* and *phoQ* probes), washed for 2 × 5 min at room temperature in 2 × SSC, 0.1% SDS, 2 × 5 min at room temperature in 0.2 × SSC, 0.1% SDS and 2 × 15 min at 65°C in 0.1 × SSC, 0.1% SDS and then exposed to Kodak Biomax AR film (Eastman Kodak Company).

Construction of strains H851 and H854

For strain H851, the *xylE*-Gm^R cassette from plasmid pX1918GT (Schweizer and Hoang, 1995) was cloned into the unique *Kpn*I site within the *phoP* coding region in plasmid pAK9. The *phoP::xylE*-Gm^R fusion was isolated by *Sma*I/*Psh*AI digestion and subcloned into the *Sma*I site of the gene replacement vector pEX100T (Schweizer and Hoang, 1995). For strain H854, a 1.7 kB *Hinc*II fragment, containing *phoQ* from plasmid pEMPQ1b, was first subcloned into the *Sma*I site of pEX100T, and the *xylE*-Gm^R cassette was then inserted into a unique *Eco*RI site within the *phoQ* coding region (Fig. 1). Both constructs were independently transformed by electroporation into the mobilizing *E. coli* strain S17-1, then transferred by conjugation into *P. aeruginosa*

H103. Single cross-over events were screened for by plating onto BM2-glucose minimal media containing carbenicillin and gentamicin. The sucrose-sensitive phenotype, encoded by the pEX100T *sacB* gene, allowed double cross-over events to be identified by plating cointegrates isolated from the initial screen onto LB containing 5% sucrose. Colonies capable of growing on this medium were screened for carbenicillin sensitivity to confirm the excision of plasmid sequences. The presence of the *xylE*-Gm^R cassette within the appropriate genes was confirmed by Southern blot analysis following standard protocols (Ausubel *et al.*, 1987,) using probes complimentary to either *phoP, phoQ* or *xylE* labelled with alkaline phosphatase (GeneImages AlkPhos Direct Labelling and Detection System, Amersham Life Science).

Western immunoblot analysis

Cells from mid-logarithmic phase cultures (OD_{600} 0.4–0.6) of *P. aeruginosa* were collected by centrifugation and resuspended in 50 mM Tris HCl pH 8.0 buffer. Samples were resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 15% acrylamide gel according to previously published protocols (Bell *et al.*, 1991), and transferred onto Immobilon^{TM}-P polyvinylidene difluoride (PVDF) membrane using a Mini-PROTEAN II system (Bio-Rad Laboratories) following the manufacturer's protocols. Membranes were blocked in a solution of 3% bovine serum albumin BSA, (Boehringer Mannheim) in phosphate-buffered saline (PBS) at ambient temperature for 3–16 h prior to treatment with anti-OprH specific antiserum (1:6000 dilution in 1% BSA, 0.05% Tween-20 in PBS). Alkaline phosphatase-conjugated secondary antibody [goat anti-rabbit IgG(H+L), Bio-Rad Laboratories] was used at 1:3000 dilution, and blots were visualized with 5-bromo-4-chloro-3-indoyl phosphate–nitroblue tetrazolium (BCIP/NBT) according to published protocols (Ausubel *et al.*, 1987).

Killing curves

Killing was carried out at room temperature by diluting mid-log phase cultures of *P. aeruginosa* (OD_{600} 0.4–0.6) 1:100 into 30 mM sodium phosphate buffer, pH 7.0, containing 8 $\mu g\ ml^{-1}$ of polymyxin B sulphate (Sigma Chemical Company). Samples were shaken gently, and aliquots removed at specified time intervals were assayed for survivors by plating appropriate dilutions onto proteose peptone #2 agar.

Enzymatic assays

Catechol-2,3-dioxygenase assays were performed on mid-log phase cultures ($OD_{600} \approx 0.7$) of *P. aeruginosa.* Cells from 50 ml of culture were collected by centrifugation, resuspended in 50 mM potassium phosphate buffer, pH 7.5, containing 10% acetone, and broken by sonication. After removal of cell debris and unbroken cells using centrifugation, the protein content of the supernatant was determined using published procedures (Sandermann and Strominger, 1972), and catechol-2,3-dioxygenase activity was measured following the protocol of Konyecsni and Deretic (1988).

Acknowledgements

This work was supported by a grant from the Canadian Cystic Fibrosis Foundation. R.E.W.H. has an MRC Distinguished Scientist Award. We thank Hong Yan for performing the LD_{50} experiments.

References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25:** 3389–3402.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1987 and updates) *Current Protocols in Molecular Biology*. New York: John Wiley and Sons.

Bell, A., and Hancock, R.E.W. (1989) Outer membrane protein H1 of *Pseudomonas aeruginosa*: Purification of the protein and cloning and nucleotide sequence of the gene. *J Bacteriol* **171:** 3211–3217.

Bell, A., Bains, M., and Hancock, R.E.W. (1991) *Pseudomonas aeruginosa* outer membrane protein OprH: expression from the cloned gene and function in EDTA and gentamicin resistance. *J Bacteriol* **173:** 6657–6664.

Brendel, V., Bucher, P., Nourbakhsh, I., Blaisdell, B.E., and Karlin, S. (1992) Methods and algorithms for statistical analysis of protein sequences. *Proc Natl Acad Sci USA* **89:** 2002–2006.

Brown, M.R.W., and Melling, J. (1969) Role of divalent cations in the action of polymyxin B and EDTA on *Pseudomonas aeruginosa*. *J Gen Microbiol* **59:** 263–274.

Dennis, J.J., and Sokol, P.A. (1995) Electrotransformation of *Pseudomonas*. *Methods Mol Biol* **47:** 125–133.

Fields, P.I., Swanson, R.V., Haidaris, C.G., and Heffron, F. (1986) Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. *Proc Natl Acad Sci USA* **83:** 5189–5193.

Fields, P.I., Groisman, E.A., and Heffron, F. (1989) A *Salmonella* locus that controls resistance to microbicidal proteins from phagocytic cells. *Science* **243:** 1059–1062.

Gilleland Jr, H.E., Stinnett, J.D., and Eagon, R.G. (1974) Ultrastructural and chemical alteration of the cell envelope of *Pseudomonas aeruginosa* associated with resistance to ethylenediaminete-traacetic acid resulting from growth in Mg^{2+}-deficient medium. *J Bacteriol* **117:** 302–311.

Groisman, E.A., Chiao, E., Lipps, C.J., and Heffron, F. (1989) *Salmonella typhimurium phoP* virulence gene is a transcriptional regulator. *Proc Natl Acad Sci USA* **86:** 7077–7081.

Groisman, E.A., Heffron, F., and Soloman, F. (1992) Molecular genetic analysis of the *Escherichia coli phoP* locus. *J Bacteriol* **174:** 486–491.

Groisman, E.A., Kayser, J., and Soncini, F.C. (1997) Regulation of polymyxin resistance and adaptation to low-Mg^{2+} environments. *J Bacteriol* **179:** 7040–7045.

Gunn, J.S., and Miller, S.I. (1996) PhoP–PhoQ activates transcription of *pmrAB*, encoding a two-component regulatory system involved in *Salmonella typhimurium* antimicrobial peptide resistance. *J Bacteriol* **178:** 6857–6864.

Gunn, J.S., Lim, K.B., Krueger, J., Kim, K., Guo, L., Hackett, M., and Miller, S.I. (1998) PmrA–PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. *Mol Microbiol* **27:** 1171–1182.

Guo, L., Lim, K.B., Gunn, J.S., Bainbridge, B., Darveau, R.P., Hackett, M., and Miller, S.I. (1997) Regulation of lipid A modifications by *Salmonella typhimurium* virulence genes PhoP–PhoQ. *Science* **276:** 250–253.

Helander, I.M., Kilpeläinen, I., and Vaara, M. (1994) Increased substitution of phosphate groups in lipopolysaccharides and lipid A of the polymyxin-resistant *pmrA* mutants of *Salmonella typhimurium*: a ^{31}P-NMR study. *Mol Microbiol* **11:** 481–487.

Ingelbrecht, I.L., Mandelbaum, C.I., and Mirkov, T.E. (1998) Highly sensitive Northern hybridization using a rapid protocol for downward alkaline blotting of RNA. *Biotechniques* **25:** 420–425.

Kasahara, M., Nakata, A., and Shinagawa, H. (1992) Molecular analysis of the *Escherichia coli* PhoP–PhoQ operon. *J Bacteriol* **174:** 492–498.

Konyecsni, W.M., and Deretic, V. (1988) Broad-host range plasmid and M13 bacteriophage-derived vectors for promoter analysis in *Escherichia coli*. *Gene* **74:** 375–386.

Miller, S.I., Kukral, A.M., and Mekalanos, J.J. (1989) A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. *Proc Natl Acad Sci USA* **86:** 5054–5058.

Moore, R.A., Chan, L., and Hancock, R.E.W. (1984) Evidence for two distinct mechanisms of resistance to polymyxin B in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **26:** 539–545.

Nicas, T.I., and Hancock, R.E.W. (1980) Outer membrane protein H1 of *Pseudomonas aeruginosa:* involvement in adaptive and mutational resistance to ethylenediaminetetraacetic acid, polymyxin B and gentamicin. *J Bacteriol* **143:** 872–878.

Nicas, T.I., and Hancock, R.E.W. (1983) Alteration of susceptibility to EDTA, polymyxin B and gentamicin in *Pseudomonas aeruginosa* by divalent cation regulation of outer membrane protein H1. *J Gen Microbiol* **129:** 509–517.

Nicholas, K.B., and Hughes Jr, H.B. (1996) GeneDoc: a tool for editing and annotating multiple sequence alignments. Distributed by author.

Rehm, B.H.A., and Hancock, R.E.W. (1996) Membrane topology of the outer membrane protein OprH from *Pseudomonas aeruginosa*: PCR-mediated site-directed insertion and deletion mutagenesis. *J Bacteriol* **178:** 3346–3349.

Roland, K.L., Martin, L.E., Esther, C.R., and Spitznagel, J.K. (1993) *pmrA* mutants of *Salmonella typhimurium* LT2 define a new two-component regulatory system with a possible role in virulence. *J Bacteriol* **175:** 4154–4164.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual,* 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sandermann Jr, H., and Strominger, J.L. (1972) Purification and properties of C55-isoprenoid alcohol phosphokinase from *Staphylococcus aureus*. *J Biol Chem* **247:** 5123–5131.

Schweizer, H.P. (1991) *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19. *Gene* **97:** 109–112.

Schweizer, H.P., and Hoang, T.T. (1995) An improved system for gene replacement and *xylE* fusion analysis in *Pseudomonas aeruginosa*. *Gene* **158:** 15–21.

Simon, R., Preifer, U., and Puhler, A. (1983) A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. *Bio/Technology* **1:** 784–790.

Soncini, F.C., and Groisman, E.A. (1996) Two-component regulatory systems can interact to process multiple environmental signals. *J Bacteriol* **178:** 6796–6801.

Soncini, F.C., Véscovi, E.G., and Groisman, E.A. (1995) Transcriptional autoregulation of the *Salmonella typhimurium phoPQ* operon. *J Bacteriol* **177:** 4364–4371.

Soncini, F.C., Véscovi, E.G., Soloman, F., and Groisman, E.A. (1996) Molecular basis of the magnesium deprivation response in *Salmonella typhimurium*: identification of PhoP-regulated genes. *J Bacteriol* **178:** 5092–5099.

Stock, J.B., Ninfa, A.J., and Stock, A.M. (1989) Protein phosphorylation and regulation of adaptive responses in bacteria. *Microbiol Rev* **53:** 450–490.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignments through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22:** 4673–4680.

Véscovi, E.G., Soncini, F.C., and Groisman, E.G. (1996) Mg^{2+} as an extracellular signal: environmental regulation of *Salmonella* virulence. *Cell* **84:** 165–174.

Volz, K. (1993) Structural conservation in the CheY superfamily. *Biochemistry* **32:** 11741–11752.

West, S.E.H., Schweizer, H.P., Dall, C., Sample, A.K., and Runyen-Janecky, L.J. (1994) Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. *Gene* **128:** 81–86.