Identification of PhoP-PhoQ homologues in *Pseudomonas aeruginosa* responsible for regulation of the outer membrane protein OprH

by
Agnieszka Kwasnicka

B.Sc.(Honours) in Genetics, 1997.
University of Manitoba, Winnipeg, Manitoba

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

In

THE FACULTY OF GRADUATE STUDIES
(MICROBIOLOGY PROGRAM)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

September 1999

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Microbiology

The University of British Columbia
Vancouver, Canada

Date Sept. 27, 1999

Abstract

Expression of the *Pseudomonas aeruginosa* outer membrane protein OprH is induced in low magnesium growth conditions (Nicas and Hancock, 1980; 1983). This protein has been proposed to play a role in stabilizing the outer membrane in the absence of Mg^{2+} by interacting with LPS at sites where these cations would bind. Adaptation to magnesium limitation in *Salmonella typhimurium* has been shown to occur through activation of the two-component regulatory system, PhoP-PhoQ (Soncini *et al.*, 1996). Putative PhoP and PhoQ proteins were identified in the *P. aeruginosa* genome through homology searches using the corresponding *S. typhimurium* protein sequences. The genes encoding these proteins were located directly downstream of the gene encoding OprH. Transcriptional linkage of *oprH*, *phoP* and *phoQ* was demonstrated and the hypothesis that this system regulates expression of OprH in *P. aeruginosa* was tested in the following study.

Through construction of a *phoP* null mutants and transformation of this mutant with PhoP encoding plasmids, it was shown that PhoP is required for expression of OprH. Furthermore, PhoP was demonstrated to be an activator of *oprH*, *phoP* and *phoQ* transcription from a promoter upstream of *oprH*. In contrast, a *phoQ* null mutant showed high-level, unregulated activation of *oprH* and *phoP* transcription and OprH expression. Complementation of this mutant demonstrated a requirement for PhoQ in down regulation of transcription and response to magnesium. Analysis of the *oprH* promoter enabled identification of the start of transcription and delineation of the sequences required for regulated OprH expression to within 90 basepairs of the ATG.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

aa	amino acids
Asp	aspartate
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
CF	Cystic Fibrosis
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
DMSO	dimythelsulfoxide
dNTPs	deoxynucleoside triphosphates
EDTA	ethylenediaminotetraacetate
ExoIII	exonuclease III
ΔG	free energy change
Gm^R	gentamicin resistance
IgG	immunoglobulin G
Ile	isoleucine
kb	kilobase pairs
kDa	kiloDaltons
LB	Luria Bertani
LPS	lipopolysaccharide
Lys	lysine
MCS	multiple cloning site
min	minute
OD	optical density
Pag(s)	PhoP-activated gene(s)
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol

Prg(s)	PhoP-repressed gene(s)
S.E.M.	standard error of the mean
SSC	sodium acetate and sodium chloride
TAE	Tris-HCl, acetic acid and EDTA
TBE	Tris-HCl, boric acid and EDTA
TE	Tris-HCl and EDTA
Thr	threonine
UV	ultra-violet

Acknowledgement

I would like to extend my sincere thanks to my supervisor, Dr. Robert E. W. Hancock, for his guidance, insight and support during the course of this project. I would also like to thank Dr. Emma Macfarlane, without whom this project and thesis would not have been possible. I would also like to acknowledge Dr. Martina Ochs, for her invaluable technical and intellectual advice and for proofreading of this thesis. Special thanks goes out to my committee members, Drs. Fernandez and Spiegelman for their help and encouragement, the rest of the Hancock lab for keeping me sane, and Dr. Meg Pope for her guidance. Last, but not least, I would like to thank my dearest Greg Wasney for all the emotional support he has provided me over the past two years.

1 Introduction

1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is ubiquitous in the environment, residing in water and soil, and associating with both plants and animals (Clarke and Slater, 1986). This Gram negative, motile rod is harmless to a healthy host, though infection is problematic for the immunocompromised individual. In recent years, *P. aeruginosa* has become one of the most common nosocomial pathogens, second only to the enterococci (Botzenhurt and Rüden, 1987). It most commonly causes respiratory infections that are particularly severe for Cystic Fibrosis (CF) patients. Infection caused by mucoid stains of *P. aeruginosa* is the most common cause of death in CF patients (Høiby *et al.*, 1987). *P. aeruginosa* has also been implicated in burn, eye and post-surgical infections. Colonization by this organism is aided by its many virulence factors. Exotoxin A, proteases and the exopolysaccharide alginate are just a few of the factors contributing to virulence (Döring *et al.*, 1987; Liu, 1974).

A unique feature of *P. aeruginosa* is its intrinsic resistance to many antimicrobial agents, specifically those used in the treatment of infection. Aminoglycosides, some β-lactams, quinolones (Hancock and Speert, 1996) and polymyxins are becoming the few effective antibiotics able to treat *P. aeruginosa* infection. Antibiotic inactivating enzymes like aminoglycoside acetyltransferase and β-lactamases contribute to this resistance (Bryan, 1979), but the intrinsic low outer membrane permeability of *P. aeruginosa* likely contributes to resistance to many unrelated antimicrobials.

1.2 The Outer Membrane of *P. aeruginosa*

Like all Gram negative bacteria, *P. aeruginosa* has both an inner, cytoplasmic membrane and an outer membrane. Located between these are the periplasmic space and the peptidoglycan (Nikaido and Vaara, 1985). The outer membrane consists of a lipid bilayer interspersed with proteins. The lipid content of the outer membrane differs from the cytoplasmic membrane in that its outer leaflet is predominantly made up of lipopolysaccharide (LPS). LPS is an amphipathic molecule with a hydrophobic lipid A anchor and hydrophilic core oligosaccharide and O-side chain oligosaccharide. Although the study of *P. aeruginosa* LPS has lagged behind that of other Gram negative organisms, it has been shown that there are an exceptionally high number of phosphate groups in the core oligosaccharide (Kropinski *et al.*, 1979).

Nine major proteins occupy the outer membrane (for review see Hancock *et al.*, 1990). The most abundant outer membrane protein, OprF, acts as a non-specific, water filled channel. Nutrient inducible proteins include; OprP, induced by phosphate limitation, OprB, induced by glucose, OprD, induced by certain carbon sources, OprG, induced in high magnesium and iron, and OprH, induced in low magnesium but repressed in high magnesium, calcium, strontium and manganese. In addition to that observed in OprF, channel forming ability has also been observed for OprP (specific for small anions), OprB (glucose channel), OprD2 (selective for the β-lactam, imipenem) and possibly OprC and E. The lipoproteins OprL and OprI are peptidoglycan associated and play structural rules. OprF is also associated with peptidoglycan and has a role in structure and cell shape. OprH, the focus of this study, is described in detail below.

In order to exert their bacteriocidal or bacteriostatic effects, antibiotics must first penetrate the outer membrane. In *P. aeruginosa* three methods of antibiotic uptake have been proposed (Moore *et al.*, 1987). Entry of hydrophilic compounds occurs mainly through the water filled channel of OprF. Hydrophobic molecules may cross the lipid bilayer but the close association of LPS molecules described below, largely restricts this type of entry. A third method has been proposed through the study of OprH and polymyxin B. Hancock (1997) described a method for entry by cationic antimicrobial like polymyxin B termed self-promoted uptake. Binding of these molecules to the negatively charged outer membrane is thought to displace LPS stabilizing divalent cations. These molecules, larger than the ions they displace, disrupt and permeabilize the membrane and facilitate their own uptake. This mechanism was proposed through the study of OprH overexpressing mutants and under inducing conditions. Under conditions that induce overexpression of this slightly basic outer membrane protein, *P. aeruginosa* also becomes resistant to polymyxin B, gentamicin and EDTA (Brown and Melling, 1969; Nicas and Hancock, 1980, 1983). OprH is thought to inhibit self-promoted uptake by replacing divalent cations in the outer membrane and interacting with LPS. The role of OprH in resistance is described further in the following section.

The low permeability of the *P. aeruginosa* outer membrane is conferred by restriction of entry of hydrophilic molecules due to the small channel size of porins like OprF and the blockage of entry of hydrophobic molecules by the tight packing of the LPS. The negative charge imparted by phosphate ions on LPS requires it to associate with cations such as magnesium and calcium for stability (Schindler and Osborn, 1979). This interaction imparts stability to the outer membrane as is evident in the

permeabilizing effect of the chelator EDTA and the ability of magnesium ions to antagonize permeabilization (Nikaido and Hancock, 1986).

1.3 The Outer Membrane Protein OprH

The outer membrane protein, OprH, is a 21 kDa, slightly basic (theoretical pI 8.6) protein. Its structure has been proposed to be an eight-stranded β-barrel (Rehm and Hancock, 1996). Although β-barrel structures are characteristic of porins, no channel forming ability has been detected in OprH (Bell *et al.*, 1991). Expression of this outer membrane protein is induced when *P. aeruginosa* is grown in limiting concentrations of the divalent cation magnesium (Nicas and Hancock, 1980; 1983). OprH has been shown to associate with LPS and may stabilize the outer membrane in the absence of these stabilizing cations (Bell and Hancock, 1989; Hancock and Carey, 1997). OprH has been proposed to interact with LPS at magnesium binding sites and it has been shown that the amount of OprH is inversely proportional to the level of magnesium ions in the outer membrane (Nicas and Hancock, 1980; 1983).

This interaction may contribute to polymyxin B, gentamicin and EDTA resistance. Resistance to these antimicrobials was observed to be associated with overexpression of OprH, whether due to mutation causing overexpression of OprH or adaptation to low magnesium (Hancock *et al.*, 1981; Bell *et al.*, 1991; Young *et al.*, 1992). In contrast, no resistance to β-lactams or tetracycline was observed. Nicas and Hancock (1983) proposed that OprH interferes with self-promoted uptake of cationic antimicrobials such as polymyxin B. Interaction of this integral membrane protein with LPS at sites where magnesium ions are usually found would prevent displacement by

polymyxin B and thus prevent the initial interaction with the outer membrane. In support of this hypothesis, rough mutations of *P. aeruginosa* LPS were shown to abolish resistance to polymyxin B in low magnesium (Bell *et al.*, 1991).

Overexpression of plasmid encoded OprH in an OprH deficient mutant demonstrated that this protein is largely responsible for EDTA resistance but may only contribute in a minor way to gentamicin and polymyxin B resistance (Bell *et al.*, 1991). Preliminary data suggested that LPS alteration may be the primary resistance mechanism. In *E. coli*, expression of OprH encoded on a multicopy plasmid did not confer resistance to gentamicin or polymyxin B on this organism. Thus the OprH overexpressing mutant (*P. aeruginosa* strain H181) which is also polymyxin B resistant is likely a regulatory mutant with effects elsewhere in the cell.

1.4 PhoP-PhoQ: A Two-Component Regulatory System

Adaptation by *Salmonella typhimurium* to depletion of extracellular magnesium and calcium ions occurs via the two-component regulatory system PhoP-PhoQ (Soncini *et al.*, 1996). PhoP-PhoQ was first identified, and named, for its regulation of expression of a nonspecific acid phosphatase, PhoN (Kiers *et al.*, 1979). Although best characterized in *S. typhimurium*, PhoP-PhoQ systems have been identified in *Salmonella typhi* (Baker *et al.*, 1997), *E. coli* (Groisman *et al.*, 1992; Kasahara *et al.*, 1992) and *Shigella flexneri* (Groisman *et al.*, 1989). These two proteins show sequence similarity to the classical two-component systems OmpR-EnvZ and PhoB-PhoR (Groisman *et al.*, 1989). PhoP is the response regulator and PhoQ the sensor-kinase.

Response via two-component signal transduction occurs first through sensing of the environmental condition by the cytoplasmic membrane bound sensor-kinase (Figure 1). This protein usually autophosphorylates at a conserved histidine residue. This is hypothesized to occur by cross phosphorylation of two interacting sensor-kinases molecules. Transfer of the phosphate to an aspartate residue in the response-regulator propagates the signal. Phosphorylation of these DNA binding proteins may alleviate constraints between the C- and N-termini or may promote dimerization, resulting in enhancement of its DNA binding ability. This regulator can then activate or repress a subset of genes whose transcription is controlled by the signal (see Stock *et al.*, 1989 for review).

When magnesium or calcium ion concentrations in the extracellular environment are low, *S. typhimurium* PhoQ autophosphorylates and in turn phosphorylates PhoP (Figure 1), a regulator of transcription of over 40 genes. PhoP activated genes (Pags) include genes for virulence (Miller *et al.*, 1989), intramacrophage survival (Fields *et al.*, 1989), resistance to cationic antimicrobials (Miller *et al.*, 1990; Guo *et al.*, 1998) and defensins (Fields *et al.*, 1989), magnesium uptake systems (García Véscovi *et al.*, 1996) and LPS modifications (Groisman *et al.*, 1997; Gou *et al.*, 1997; Gunn *et al.*, 1998). A number of PhoP repressed genes (Prgs) have been identified including a possible type III secretion system (Pegues *et al.*, 1995).

PhoP regulated genes fall into two distinct categories; those regulated by PhoP-PhoQ directly and those regulated by PmrA-PmrB, a PhoP-PhoQ activated two-component regulatory system (Soncini and Groisman, 1996). Genes for polymyxin B resistance and LPS modifications are activated in low magnesium by PmrA-PmrB

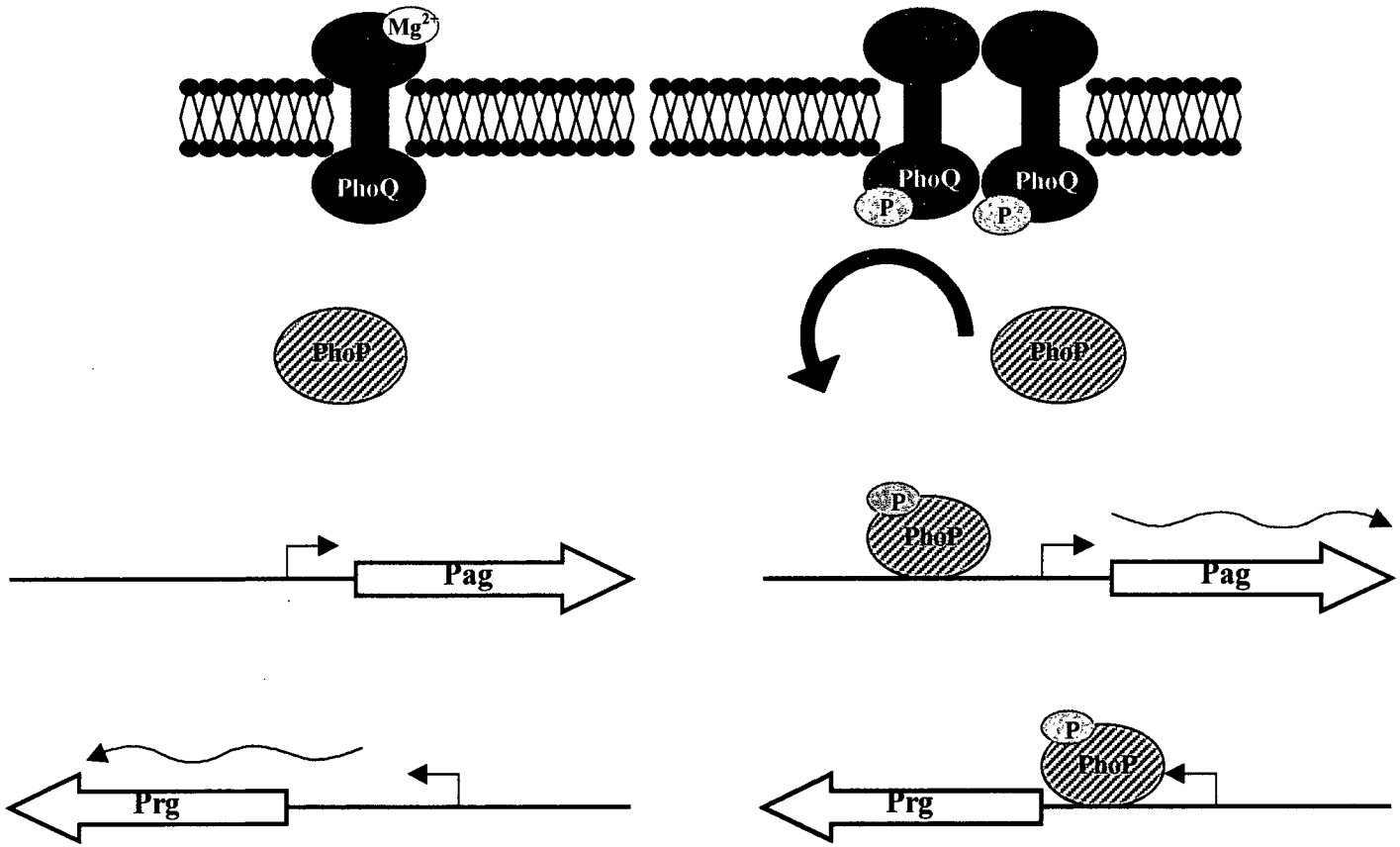


Figure 1. Signal transduction and transcriptional regulation by the PhoP-PhoQ two-component regulatory system in *Salmonella typhimurium*.

PhoQ binds Mg^{2+} when this ion is available. In absence of sufficient Mg^{2+} PhoQ autophosphorylates. After phosphotransfer from PhoQ to PhoP, phospho-PhoP turns on expression of PhoP activated genes (Pag) and turns off expression of PhoP repressed genes (Prg).

through its activation by PhoP (Roland *et al.*, 1994; Groisman *et al.*, 1997; Gunn *et al.*, 1998). The PmrA-PmrB system also responds to pH as an environmental signal independent of PhoP-PhoQ (Soncini and Groisman, 1996). LPS modification is postulated to occur, in part, through expression of the products of the following PhoP-activated genes; *pmrE* (also called *pagA* or *ugd*, Gunn *et al.*, 1998) encoding a UDP-glucose dehydrogenase, *pmrF* (Gunn *et al.*, 1998), part of an operon responsible for complex carbohydrate biosynthesis, *pmrD* (Roland *et al.*, 1994), encoding a peptide responsible for polymyxin B resistance, and *pagP* (Gou *et al.*, 1998), which increases lipid A modification. These changes may enable the organism to respond to depletion of the LPS stabilizing divalent cation and indirectly enable resistance to cationic antimicrobials by preventing their interaction and binding to the outer membrane.

Though many of the PhoP-regulated genes identified thus far have been shown to be involved in virulence, possible *phoP* and *phoQ* genes have been identified in a number of non-pathogenic species. This system is also necessary for response to the environmental stress of divalent cation depletion. Magnesium ions are essential not only for membrane stabilization but also cellular reactions that require ATP. In *S. typhimurium*, PhoP-PhoQ activates transcription of genes encoding two magnesium uptake systems, *mgtA* and *mgtBC* (García Véscovi *et al.*, 1996). PhoP mutants have been shown to be deficient for growth in low magnesium liquid media, though some growth is observed, presumably due to the *corA* magnesium uptake system (Soncini *et al.*, 1996).

The genes encoding PhoP and PhoQ are themselves PhoP-activated genes and thus autoregulated in response to magnesium (Soncini *et al.*, 1996). Transcription of this operon in *S. typhimurium* occurs from two promoters upstream of *phoP*; one

constitutively expressing PhoP and PhoQ at low levels and a second promoter for magnesium regulated expression (Gunn and Miller, 1996). Though no consensus PhoP binding sequence has been identified in the promoter regions of genes activated by this regulator, hexanucleotide repeats are observed upstream of *phoP* in both *S. typhimurium* and *E. coli* (Groisman *et al.*, 1989 and 1992; Kasahara *et al.*, 1992). Two sets of GTTTAT sequences, spaced five nucleotides apart, are observed 11 bases upstream of the –10 region in *S. typhimurium*. These sequences overlap the –35 region and have been proposed to be involved in PhoP mediated regulation.

PhoQ has been shown to bind both magnesium and calcium, but at different sites in the periplasmic region of this protein (García Véscovi *et al.*, 1997). A mutation, *pho-24*, which results in constitutive activation of Pags and repression of Prgs, has been isolated in *S. typhimurium* (Gunn *et al.*, 1996). The mutation occured in *phoQ* and resulted in a substitution of isoleucine for threonine at position 48 in the periplasmic domain of the protein. This mutation resulted in increased phosphorylation of PhoP without an increase in the overall amount of PhoQ protein in the membrane. Explanations for the increase in phospho-PhoP proposed included enhanced phosphotransfer by the mutant PhoQ, the inability of this mutant PhoQ to act as a phosphatase towards PhoP, or an increase in interaction and cross phosphorylation between these proteins. Although the first explanation is favoured, no definitive evidence to discount the latter two hypotheses has been presented.

1.5 Aims of This Study

P. aeruginosa from the sputum of CF patients was shown to possess elevated levels of OprH (Brown *et al.*, 1984). Thus, study of the regulation of expression of this protein may be relevant to the determination of a) other genes induced during infection and b) which antimicrobials will be effective in treatment. As OprH in *P. aeruginosa* is induced in low magnesium and PhoP-PhoQ in *S. typhimurium* responds to this signal, it was hypothesized that homologues of this two-component system regulate expression of OprH in *P. aeruginosa*.

In this study, a PhoP-PhoQ two-component regulatory system in *P. aeruginosa* responsible for adaptation to growth in low magnesium was identified. Furthermore, I have shown that PhoP-PhoQ regulates expression of OprH and itself in response to extracellular magnesium ion concentration. The study of the transcriptional behavior of the *oprH*, *phoP* and *phoQ* genes and determination of sequences necessary for their transcription and regulation was also achieved.

2 Materials and Methods

2.1 Sequence Analysis

Homology searches against the database of contig sequences at the *Pseudomonas* Genome Project (http://www.pseudomonas.com, released Sept 17, 1997) for identification of putative *phoP* and *phoQ* genes were performed using the TBLASTN algorithm (Altschul *et al.*, 1990). BLASTX analysis against the National Center for Biotechnology Investigation (NCBI) non-redundant set of databases (http://www.ncbi.nlm.nih.gov/BLAST/) was performed to determine the genomic context of *oprH*. Amino acid sequence alignment of *S. typhimurium*, *E. coli* and *P. aeruginosa* PhoP and PhoQ was performed using the CLUSTAL W (Thompson *et al.*, 1994) application and shaded using the GeneDoc program version 1.1.004 (Nicholas and Hughes, 1996). Protein structure predictions were performed using the TMBASE application (Hofmann and Stoffel, 1993). Sequence alignment of promoter deletion construct sequences and identification of direct and inverted repeats was accomplished using the PCGene program (Korn and Queen, 1984).

2.2 Strains, Plasmids and Growth Conditions.

Bacterial strains and plasmids used in this study are listed in Tables 1 and 2, respectively. For rich media, strains were grown in Luria Bertani (LB) broth (1.0% Tryptone, 0.5% yeast extract, 0.5% NaCl) and maintained on LB solid media (agar added to 1.5% (w/v)) at 37°C. Media components were purchased from Difco Laboratories (Detroit, MI). Antibiotic concentrations used for *E. coli* were 100μg/mL ampicillin and

Table 1. Bacterial strains used in this study

Strain	Relevant genotype	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	Macfarlane *et al.* (1999)
H855	H103 *oprH*::*xylE*-Gm^R	Macfarlane (unpublished data)
***Escherichia coli*:**		
DH5α	[*supE*44, *lacU*169(ϕ 80*lacZ* M15), *hsdR*17, *recA*1, *endA*1, *gyrA*96, thi-1, *relA*1]	Gibco BRL
S17-1	[*pro endA*::*RP4(*Tc::Mu-Km::Tn*7*)]	Simon *et al.* (1983)

Table 2. Plasmids used in this study

Plasmid	Relevant characteristic	Source or reference
pUCP19	*Escherichia-Pseudomonas* shuttle vector	Schweizer (1991)
pX1918GT	pUC-based plasmid containing *xylE*-Gm^R cassette flanked by restriction sites from pUC19	Schweizer and Hoang (1995)
pEX100T	Gene replacement vector with *sacB* marker, *lacZ* allele, *oriT* for conjugation-mediated transfer and unique *Sma*I and I-*Sce*I cloning sites	Schweizer and Hoang (1995)
pEXP	*phoP*::*xylE*-Gm^R cloned into the *Sma*I site of plasmid pEX100T	This work
pGB22	2.8kB *Eco*RI chromosomal fragment from H103 containing *oprH*, *phoP* and part of *phoQ* cloned into pUC18	Bell and Hancock (1989)
pEMR2	0.9kB *Pst*I fragment from pGB22 containing *phoP* cloned into pUCP19 in reverse orientation to the *lac* promoter	Macfarlane *et al.* (1999)
pEMR3	As pEMR2 but insert cloned behind the *lac* promoter	Macfarlane *et al.* (1999)
pEMQ1a	1.55kB fragment containing *phoQ* and 156 bases of *phoP* cloned behind the *lac* promoter in pUCP20	Macfarlane *et al.* (1999)
pEMQ3c	As pEMQ1a but insert cloned in reverse orientation to the *lac* promoter	Macfarlane *et al.* (1999)
pEMPQ1b	2.16kB fragment containing *phoP* and *phoQ* and 93 bases of upstream sequence cloned behind the *lac* promoter in pUCP20	Macfarlane *et al.* (1999)
pEMPQ2a	As pEMPQ1b but insert in reverse orientation to the *lac* promoter [a]	Macfarlane *et al.* (1999)
pAKP8	*Ssp*I- *Hin*cII fragment of an exonuclease III treated pGB22 cloned into the *Sma*I site of pUCP19 (see Figure 12A)	This work
pAKP9	*Ssp*I- *Hin*cII fragment of an exonuclease III treated pGB22 cloned into the *Sma*I site of pUCP19 (see Figure 12A)	This work
pAKP9.5	*SmaI*- *Hin*cII fragment of pAK9 cloned into the *Sma*I site of pUCP19 (see Figure 11, A)	This work
pAKP12	*Ssp*I- *Hin*cII fragment of an exonuclease III treated pGB22 cloned into the *Sma*I site of pUCP19 (see Figure 12A)	This work
pAK17	a exonuclease III treated pGB22 which resulted in deletion of the *oprH* region (see Figure 4)	This work
pBHR20	PCR amplified *oprH* gene cloned into pUCP19	Rehm and Hancock (1996)

[a] the *phoP* gene on this plasmid has a G→A base change resulting in a Val88→Ile change in the protein

for *P. aeruginosa* 300-350 μg/mL carbenicillin and 15 μg/mL gentamicin. To study the effects of external magnesium concentration, *P. aeruginosa* strains were grown in BM2 minimal media (Gilleland *et al.*, 1974) with glucose as the sole carbon source and supplemented with 0.02mM (low magnesium) or 2mM (high magnesium) $MgSO_4$ (Macfarlane *et al.*, 1999). To study expression of OprH in *E. coli*, cells were grown in M9 minimal media (Sambrook *et al.*, 1989) supplemented with magnesium at the high and low concentrations stated above. Plasmids were transformed into *P. aeruginosa* and *E. coli* by electroporation using a Gene Pulser™ (BioRad Laboratories, Hercules, CA) and 0.1cm gap cuvettes (BioRad Laboratories) according to published protocols (Sambrook *et al.*, 1989; Dennis and Sokol, 1995).

2.3 Reagents

Commonly used buffers including PBS, SSC, TAE, TBE, TE (pH 8.0), and 50mM potassium phosphate buffer were made as described in Sambrook *et al.* (1989). All DNA restriction and modification enzymes were purchased from Gibco BRL (Burlington, ON) or New England Biolabs (Mississauga, ON) and used according to the manufacturer's instructions.

2.4 DNA Techniques

General molecular biology methods were performed according to Sambrook *et al.* (1989) or Ausubel *et al.* (1987 and updates). Small-scale plasmid preparations were performed using the QIAprep Spin Miniprep System (Qiagen Inc., Chatworth, CA). DNA

fragments were purified from agarose gels using the GeneClean kit (Bio101 Inc., Vista, CA). Large-scale isolation of plasmid pGB22 was performed as previously described (Sambrook *et al.*, 1989) and the plasmid DNA was purified by PEG precipitation (Sambrook *et al.*, 1989). Deletions of plasmid pGB22 (Table 2) were introduced by controlled exonuclease III (ExoIII) digestion (Henihoff, 1984) with the aid of the Erase-a-Base® System according to the manufacturer's directions (Promega, Madison, WI). Briefly, to ensure targeted deletion, nicked and linear DNA was removed by acid-phenol extraction. Digestion of plasmid pGB22 with appropriate restriction enzymes followed by controlled ExoIII digestion and re-ligation resulted in nested deletions of the *oprH* promoter region. Digestion with the selected enzymes (*Xba*I and *Aat*II) resulted in the loss of 0.5kb of the plasmid containing part of the MCS and the reverse primer binding site. After initiation of ExoIII mediated deletion, samples were taken every 30 seconds for the first 3 minutes and every 1 minute for the following 6 minutes.

2.5 DNA Sequencing

Sequencing primer 1 (Table 3) was chosen using the Primer Designer 2.01 program and synthesized on a 392 DNA/RNA Synthesizer (PE-Applied Biosystems, Foster City, CA). Oligonucleotides were purified after deprotection at 55°C by precipitation with butanol and quantitated using a Perkin-Elmer (Lambda3) dual-beam spectrophotometer. Plasmid DNA for sequencing was quantitated using a Hoefer spectroflourometer (model TKO100, Hoefer Scientific Instruments, San Francisco, CA). DNA was sequenced using the ABI Model 373 automated DNA sequencer and the ABI Prism Dye Terminator Cycle Sequencing Kit with AmpliTaq DNA polymerase, FS (PE-

Table 3. Primers used in this study

Primer Name	Primer Sequence	Complementary to Nucleotides
Sequencing primer 1	5'-GGCGACACGGAAATGTT-3'	895-911 of pUC18/19
Rt-forward	5'-GAAGGCGGCTATCGTTACCT-3'	475-494 of *oprH*
Rt-reverse	5'-GGTCGTGGTGGTATTCGCTG-3'	120-139 of *phoP* (R)[a]
HpF	5'-CAACTTCGTCGGCCTGACCT-3'	72-91 of *oprH*
HpR	5'-GCCGTCCTGTTCCAGCTTGA-3'	367-386 of *oprH* (R)
Q1	5'-AACTACAAGTTCTAAATGACC-3'	589-603 of *oprH* and 1-6 of the *oprH* and *phoP* intergenic region
Pout1	5'-TAGAGCTGTTCCATCAGG-3'	519-536 of *phoP* (R)
PpF	5'-CTGCTGGTAGTGGAAGACGA-3'	7-26 of *phoP*
PpR2	5'-TCGACCTTGTCCTGCCAGTT-3'	247-266 of *phoP* (R)
QpF	5'-GGAGAACACCATCGAGCAGC-3'	108-127 of *phoQ*
Qseq3	5'-CGATAGACCTTGTCCAGC-3'	955-982 of *phoQ* (R)
Qseq1	5'-AGGAGTTCTTCGTGTTCGAC-3'	388-407 of *phoQ*
Qin1	5'-CAACAGGCGGTTAAGCAGTG-3'	666-685 of *phoQ* (R)
OprH-rev2	5'-TGGATGTTGTTGCTGGTCTC-3'	96-115 of *oprH* (R)

[a](R) indicates primer complementary to the non-coding strand

Applied Biosystems). The method of DNA sequencing used for primer extension experiments is described below.

2.6 OprH Expression Analysis

Whole cell lysates of logarithmic phase cells ($OD_{600} \cong 0.6$) were prepared by harvesting 1mL of *E. coli* or *P. aeruginosa* cells and resuspending in 10mM Tris-HCl, pH 7.4 buffer. Protein quantitation was carried out according to published protocols (Sandermann and Strominger, 1972). Samples were prepared for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) by addition of loading buffer containing 2% (w/v) SDS and 2% (v/v) 2-mercaptoethanol and heating to 100°C for 5 minutes. Proteins were resolved on a 15% SDS-polyacrylamide gel, with 70mM NaCl included in the gel for resolution of OprH and OprL (Hancock and Carey, 1979). Western immunoblotting onto Imobilon-P membranes (Millipore, Bedford, Ma) was performed using BioRad Trans-Blot electrophoretic transfer cell with cooling pack at constant voltage, 100V for 1 hour according to standard methodology (Sambrook *et al.*, 1989). Blots were probed with polyclonal anti-OprH serum (Bell and Hancock, 1989) at 1:10,000 dilution in PBS containing 1% (w/v) BSA for 1 hour followed by goat anti-rabbit alkaline phosphatase conjugated IgG (BioRad Laboratories, Hercules, CA) at 1:3,000 dilution. Immunoblots were developed using 50μg/mL 5-bromo-4-chloro-3-indolyl-phosphate, 10μg/mL nitroblue tetrazolium and 10μg/mL $MgCl_2$ in 0.1M Tris-HCl, pH 9.6 (Sambrook *et al.*, 1989).

2.7 Construction of the *phoP* Mutant H851

The *xylE*-Gm^R cassette from pX1918GT was cloned into the unique *Kpn*I site of pAK9 (Table 2 and Figure 4). A plasmid containing a *phoP::xylE*-Gm^R fusion (pEXP, Table 2) was constructed by subsequent digestion with *Sma*I and *Psh*AI and subcloning into the unique *Sma*I site of pEX100T. This construct was transferred into the mobilizing *E. coli* strain S17-1 for conjugative transfer into *P. aeruginosa* PAO1. For biparental mating experiments *P. aeruginosa* was grown at 42°C and *E. coli* at 30°C, both in rich media. Mating was performed by a modified method of the triparental mating experiments previously described by Goldberg and Ohman (1984). Single crossover events were selected for on BM2 minimal media containing carbenicillin and gentamicin. To select for the double crossover events, colonies were subsequently streaked on LB containing 5% sucrose. Sucrose resistant strains were checked for carbenicillin sensitivity and four sensitive clones were verified by Southern blot analysis. One of these (H851) was chosen for further study.

2.8 Southern Blot Analysis

Genomic DNA from wild-type *P. aeruginosa* and the *phoP* mutant H851 was prepared according to standard protocols (Ausubel *et al.*, 1987 and updates). Approximately 20μg of each DNA was digested with *Pst*I or *Bbs*I overnight and the fragments were resolved on a 1% agarose gel. The digested DNA was transferred in alkaline buffer onto a positively charged nylon membrane (Boeringer Mannheim, Laval,

Quebec). The membrane was prehybridized for 30 minutes at 55°C prior to addition of probe.

A *xylE* probe was generated by digesting plasmid pX1918GT with *Aat*II and a second probe complementary to bases 92-629 of the *phoP* gene, was generated by digestion of plasmid pAK17 with *Kpn*I and *Pvu*I. Probe labeling with alkaline phosphatase, prehybridization, hybridization and blot development was performed as described in the AlkPhos Direct Manual (Amersham Life Science, Oakville, Ontario). Signal detection was carried out using CDP-Star™ detection reagent (Amersham Life Science). Blots were exposed to Kodak Biomax MR Film (Eastman Kodak Company, Rochester, NY).

2.9 Determination of Catechol-2,3-dioxygenase Activity

Assays were performed on mid-logarithmic phase cells ($OD_{600} \cong 0.7$) according to published methods (Dereic and Konyecsni, 1988). Cells from 50mL of culture were harvested and resuspended in 750μL 50mM potassium phosphate buffer, pH 7.5 containing 10% acetone. Cells were broken by sonication on ice for 30 seconds. Unbroken cells and debris were removed by centrifugation for 20 minutes at 5000rpm at 4°C. Protein concentration of the supernatant was determined as described above. Two samples of the supernatant, each diluted in a total volume of 1mL containing 50mM potassium phosphate buffer, pH7.5 and 0.3mM catechol, were assayed for catechol-2,3-dioxygenase activity. The conversion of catechol to 2-hydroxymuconic semialdehyde was recorded on a Perkin-Elmer (Lambda3) dual-beam spectrophotometer coupled to a Perkin-Elmer 561 chart recorder by following the change in absorbance at 375nm. The

molar extinction coefficient of the product, 2-hydroxymuconic semialdehyde, at this wavelength is 4.4 x 10^4 M^{-1} cm^{-1}. All experiments were performed in triplicate.

2.10 RNA Isolation and RT-PCR

Total cellular RNA was isolated from logarithmic phase cultures of *P. aeruginosa* grown in BM2 containing 0.02mM $MgSO_4$ using the RNeasy™ Mini Kit (Qiagen Inc.) according to the manufacturer's instructions. Samples were treated for 30 minutes with 50U DNase (RNase-free; Gibco BRL) in the presence of 15U RNA Guard (Amersham Pharmacia Biotech, Buckingamshire, England) in a total volume of 100μL, followed by ethanol precipitation of the RNA. The quality was assessed by running approximately 5μg on a 0.7% agarose-formaldehyde gel using standard procedures (Sambrook *et al.*, 1989). Reverse transcription was performed using 10μg RNA template, 15pmoles of the 5' primer (RT-rev, Table 4), 13.5U AMV reverse transcriptase in 1x RT Buffer (Gibco BRL), 50mM $MgCl_2$ and 2.5mM each of dATP, dTTP, dGTP and dCTP. Reactions were performed in a Minicycler (Fisher Scientific, Nepean, ON) at 42°C for 15 minutes followed by a denaturation step (99°C for 5 minutes) to inactivate the reverse transcriptase. After reverse transcription, PCR was performed in a 100μL final volume after addition of the following components: 2U of Taq DNA polymerase, 1x PCR Buffer (Gibco BRL), an additional 50pmoles each of 5' primer and of 3' primer, $MgCl_2$ to a final concentration of 50mM. Amplification was achieved by 31 cycles of 95°C for 1 minute, 50°C for 1 minute and 72°C for 2 minutes. As a positive control, PCR was also performed using 10μL of DNA isolated from whole cells by chloroform extraction as a

template. PCR products were resolved on a 1% agarose gel according to standard protocols (Sambrook *et al.*, 1989).

2.11 Generation of Probes for RNA Dot Blots and Northern Blots

A probe complementary to *oprH* was generated by PCR amplification of the appropriate genetic regions using the HpF and HpR primers (Table 3) and pBHR20 (Table 2) as a template. A probe complementary to *phoP* was obtained by amplification of 630 bp region of pEMR3 using the primers Q1 and Pout1 (Table 3). To minimize the presence of non*phoP*-coding regions, the product of this PCR was subsequently used as a template in a second round of PCR using the primers PpF and PpR (Table 3) to amplify a 260 bp fragment internal to *phoP*. Similarly, a probe complementary to *phoQ* was generated by two round of PCR; the first using primers QpF and Qseq3 (Table 3) and pEMQ1a as a template (Table 2) to amplify a 866 bp fragment, the second using primers Qseq1 and Qin1 amplify an internal a 287 bp fragment suitable for use as a probe. A probe complementary to *rpoB* (generated as described in Och *et al.*, 1999) for use in RNA dot blots was a kind gift from Dr. Och. PCR was performed in a 50µL volume with 1X Vent® polymerase buffer, 1U Vent polymerase (New England Biolabs), 4mM dNTPs and 50pmoles of each primer. For amplification of the *oprH* probe, 5%(v/v) DMSO was included in the PCR and for the *phoP* large fragment and both *phoQ* fragments 10%(v/v) DMSO was included. The PCR program used for amplification of all fragments was: 94°C for 5 minutes, 65°C or 67°C for the *phoP* probe for 1 minute; 72°C for 1 minute, 25-31 cycles of 94°C for 1 minute, 65°C or 67°C for 1 minute and 72°C for 1 minute; then one cycle of 94°C for 1 minute, 65°C or 67°C for 1 minute and 72°C for 10 minutes.

Approximately 25ng of each DNA probe was labeled with [αP^{32}]-dCTP (Amersham Pharmacia Biotech) using the *redi*prime™ II random primer labeling system (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

2.12 Northern Blot and RNA Dot Blot Analysis

RNA was isolated from *P. aeruginosa* cells grown in BM2 media containing 2mM or 0.02mM $MgSO_4$ as described above. Approximately 5μg of each sample were denatured at 65°C for 5 minutes in the presence of 2.2M formaldehyde and 50% formamide and resolved on a 1.2% agarose gel containing 0.6M formaldehyde in MOPS buffer. RNA was transferred onto a positively charged nylon membrane (Boehringer Mannheim) by downward alkaline blotting in 50mM NaOH (Ingelbrecht *et al.*, 1998). Blots were hybridized overnight at 60°C (*oprH* probe) or 45°C (*phoP* and *phoQ* probes) in formamide prehybridization/hybridization buffer as described by Ausubel *et al.* (1987 and updates). Blots were washed twice for 5 minutes at room temperature in 2XSSC, 0.1% SDS, twice for 5 minutes at room temperature in 0.2XSSC, 0.1% SDS then twice for 15 minutes at hybridization temperature in 0.1XSSC, 0.1% SDS. Autoradiography was performed as described by Ausubel *et al.* (1987 and updates) and blots were exposed to Kodak Biomax AR Film (Eastman Kodak Company).

For RNA dot blots, 3μg of RNA isolated from wildtype *P. aeruginosa* and the *phoP*, *phoQ* and *oprH* mutants were denatured at 65°C for 5 minutes and spotted onto positively charged nylon membranes (Boehringer Mannheim). Crosslinking was carried out by exposure to a UV source. Hybridization and washing was performed as described above for the Northern blots. Dot blots were quantitated by exposure to the

PhosphoImager SI system using the ImageQuant v.1.1 software (Molecular Dynamics Inc., Sunnyvale, CA). The amount of *rpoB* transcript was used as a standard to quantitatively compare the amount of *oprH*, *phoP* and *phoQ* transcripts. All dot blots were performed in triplicate.

2.13 Primer Extension

Total cellular RNA was isolated as described above. The OprH-rev2 primer (Table 3), which hybridized within the *oprH* gene was end-labeled with [γ^{32}-P]dATP (Amersham Canada Ltd.) then added to 5μg of RNA in 50mM Tris-HCl, pH 8.3 containing 20mM KCl in a final volume of 10μL for hybridization at 60°C. The primer was extended by addition of 100U Superscript™ reverse transcriptase (Gibco BRL) in the presence of 50mM Tris-HCl, pH 8, 40mM KCl, 6mM $MgCl_2$, 20mM dNTPs and 10U Ribonuclease Inhibitor (Gibco BRL) in a final volume of 30μL. Reaction mixtures were incubated at 45°C for 60 minutes then stopped by addition of 1μL 0.5M EDTA, pH 8. Following treatment of the samples with DNase-free RNase (Boehringer Mannheim) for 15 minutes at 37°C the, cDNA was isolated by ethanol precipitation . The cDNA pellets were resuspended in 3-4μL *fmol* loading buffer (Promega), denatured and loaded on a 6% acrylamide sequencing gel. Dideoxy-sequencing of the appropriate region of plasmid pGB22 was carried out using the *fmol* DNA cycle sequencing system (Promega) with the OprH-rev2 and the reactions were loaded alongside the primer extension reactions. As controls, reactions were performed with tRNA only or RNA treated with RNase prior to cDNA synthesis.

3 Results

3.1 Identification of *phoP* and *phoQ* Genes in *P. aeruginosa*

Identification of PhoP and PhoQ homologues in *P. aeruginosa* was facilitated by the September 17, 1997 release of a database of contig sequences by the *Pseudomonas* Genome Project. Using the TBLASTN algorithm (Altschul *et al.*, 1997), two open-reading frames (ORFs) were identified in the same direction as the *oprH* gene encoding proteins with high sequence similarities to both *S. typhimurium* (Miller *et al.*, 1989) and *E. coli* (Kasahara *et al.*, 1992) PhoP and PhoQ proteins. The putative *P. aeruginosa* PhoP and PhoQ proteins showed 53-54 % and 33% sequence identity, respectively, to the corresponding proteins from *S. typhimurium* and *E. coli* (Figures 2 and 3). The *phoP* and *phoQ* genes have GC contents typical of *P. aeruginosa* genes (>65%). The start of the putative *phoP* gene is located 79 bp downstream of *oprH*, but no obvious σ^{70} type consensus sequence was evident in the intergenic region. The *phoQ* ORF was proposed to begin with the less common GTG start codon and overlap *phoP* by four nucleotides (Figure 4), suggesting the genes are transcribed as a single unit.

Despite extensive analysis, no –10 or –35 consensus sequences could be identified in the region upstream of *oprH*. However, four direct hexanucleotide repeats (GTTCAG) were identified 60 bp upstream of *oprH*, each separated by five nucleotides (Figure 4). Four similar repeats were identified on the opposite strand within the *oprH* gene; three in the reverse direction to and one in the same direction as *oprH* transcription (Figure 12A). One of these repeats contains an adenine to guanine change and the spacing between the repeats was not conserved. It should be noted that direct hexanucleotide repeats

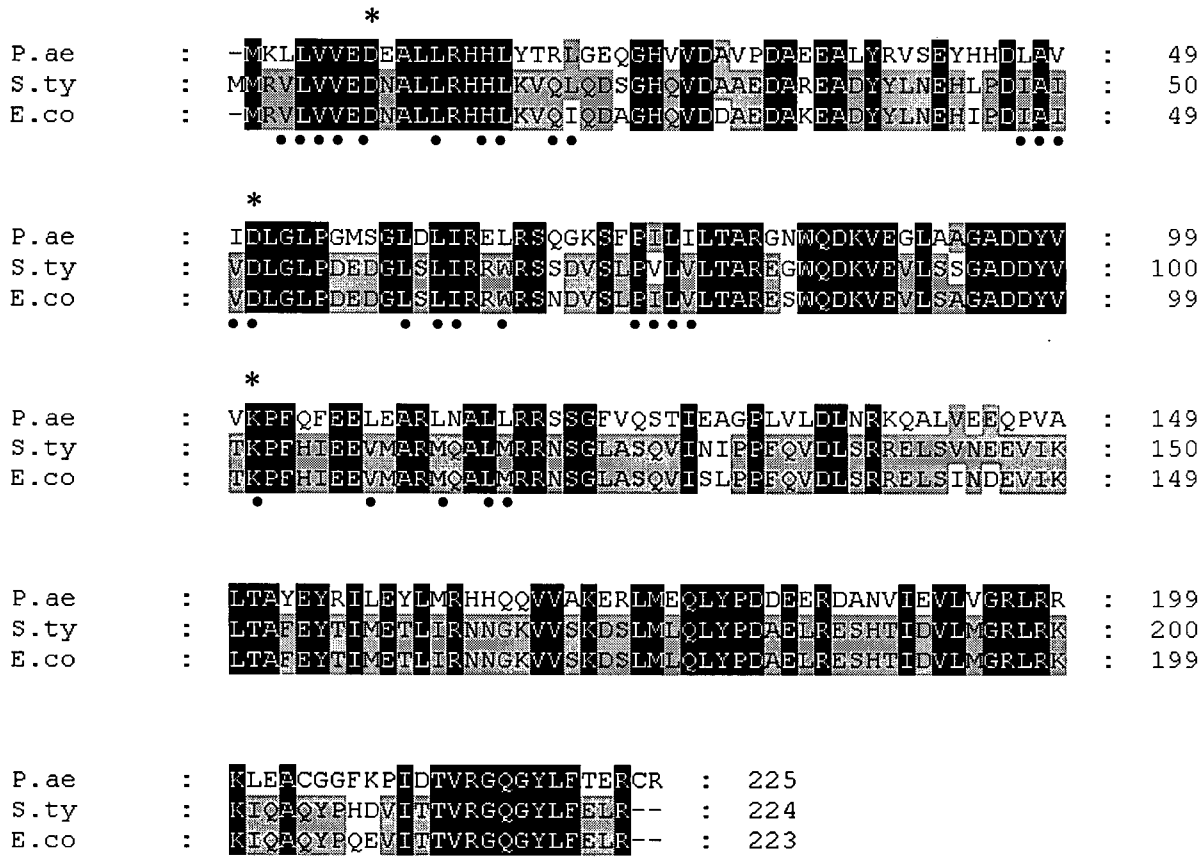


Figure 2. Amino acid sequence alignment of the PhoP proteins.
PhoP of *P. aeruginosa* (P.ae) was aligned with the protein sequence from *E. coli* (E.co) and *S. typhimurium* (S.ty) using the CLUSTALW application and shaded with GeneDoc. *P. aeruginosa* PhoP shows 53 and 54% sequence identity to the PhoP proteins of *S. typhimurium* and *E. coli* respectively. Residues marked by dots below the sequence are conserved among response regulators while those marked by asterisks above are highly conserved. One of the aspartate residues may constitute the site of phosphorylation.

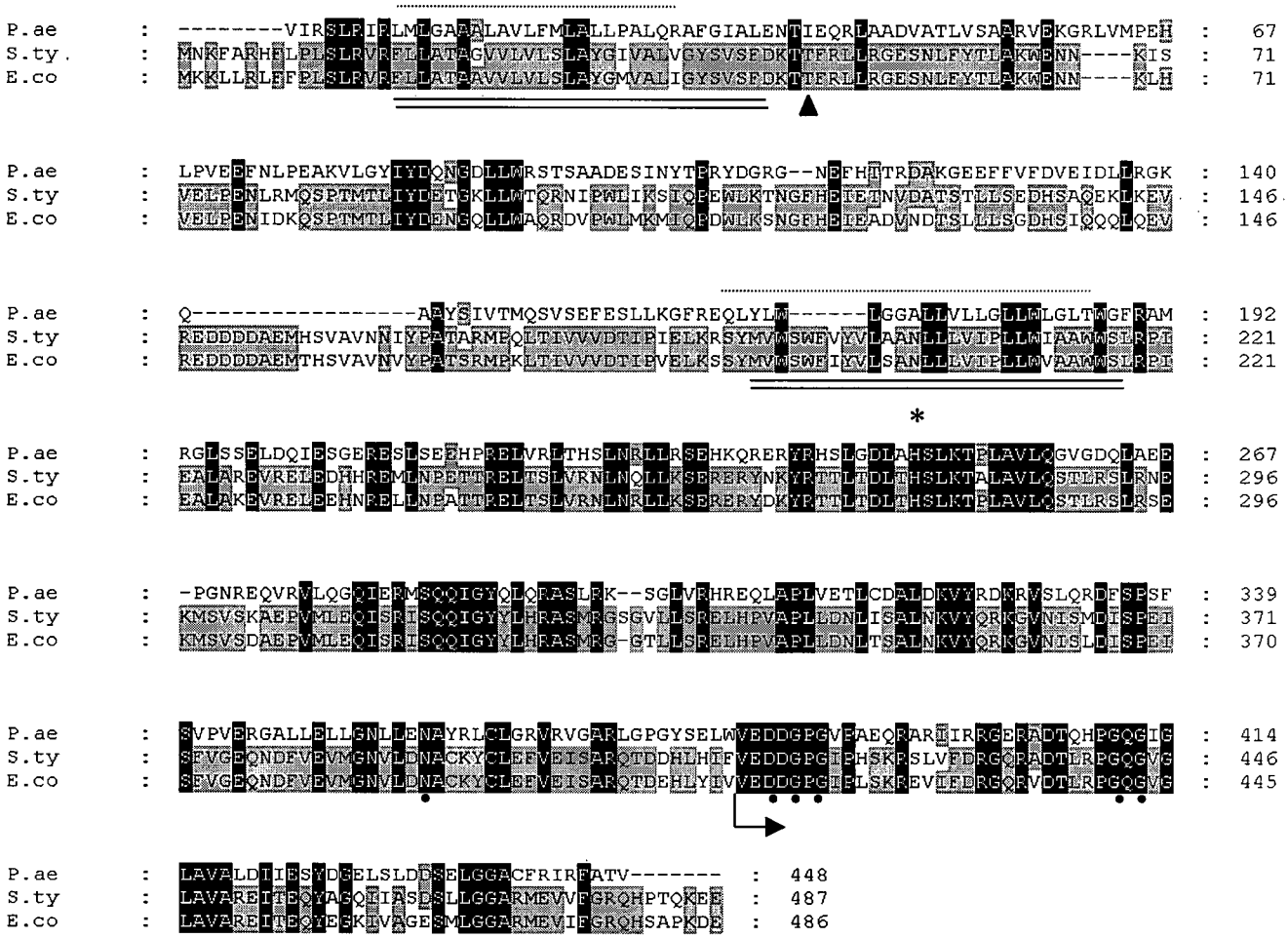
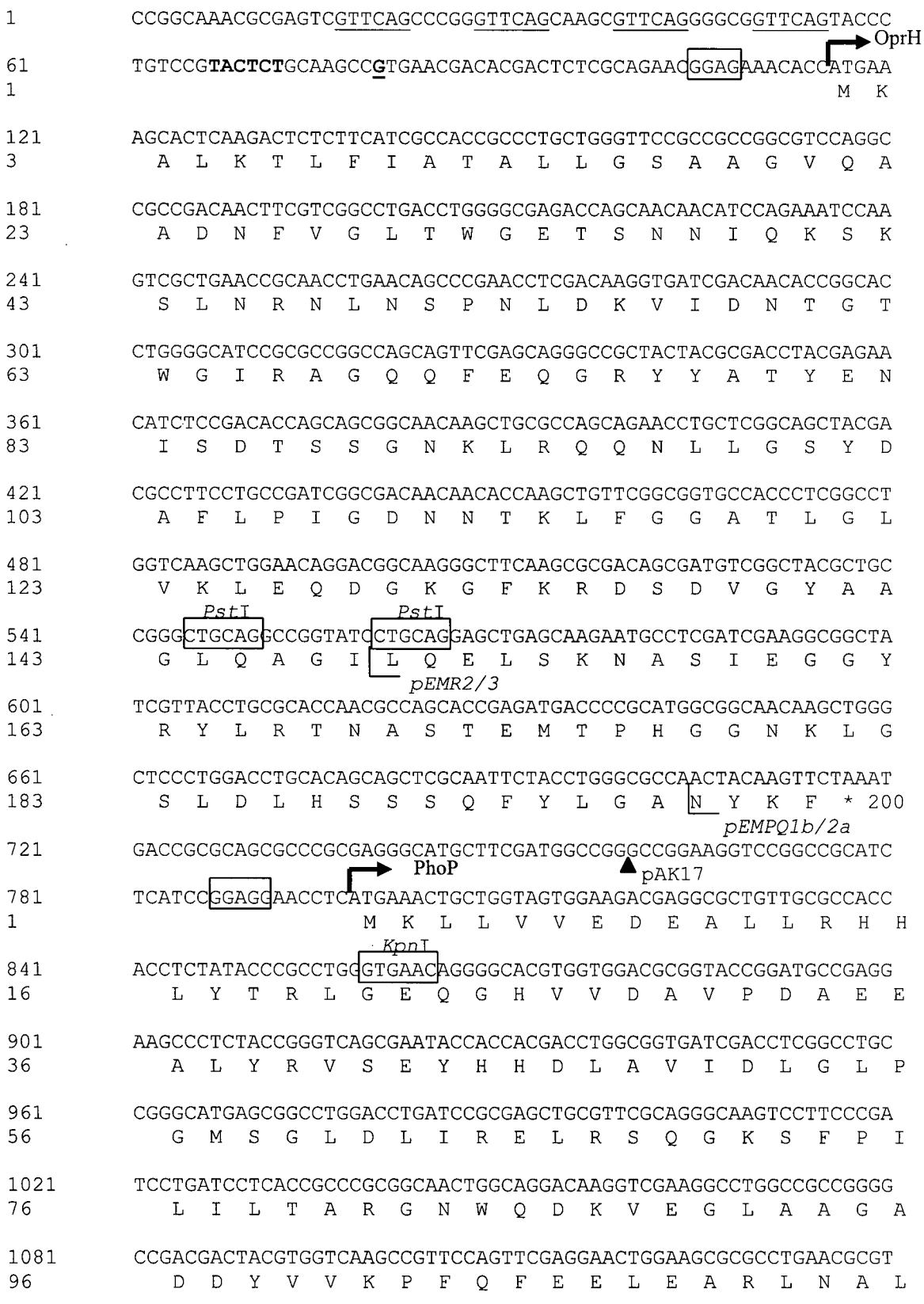
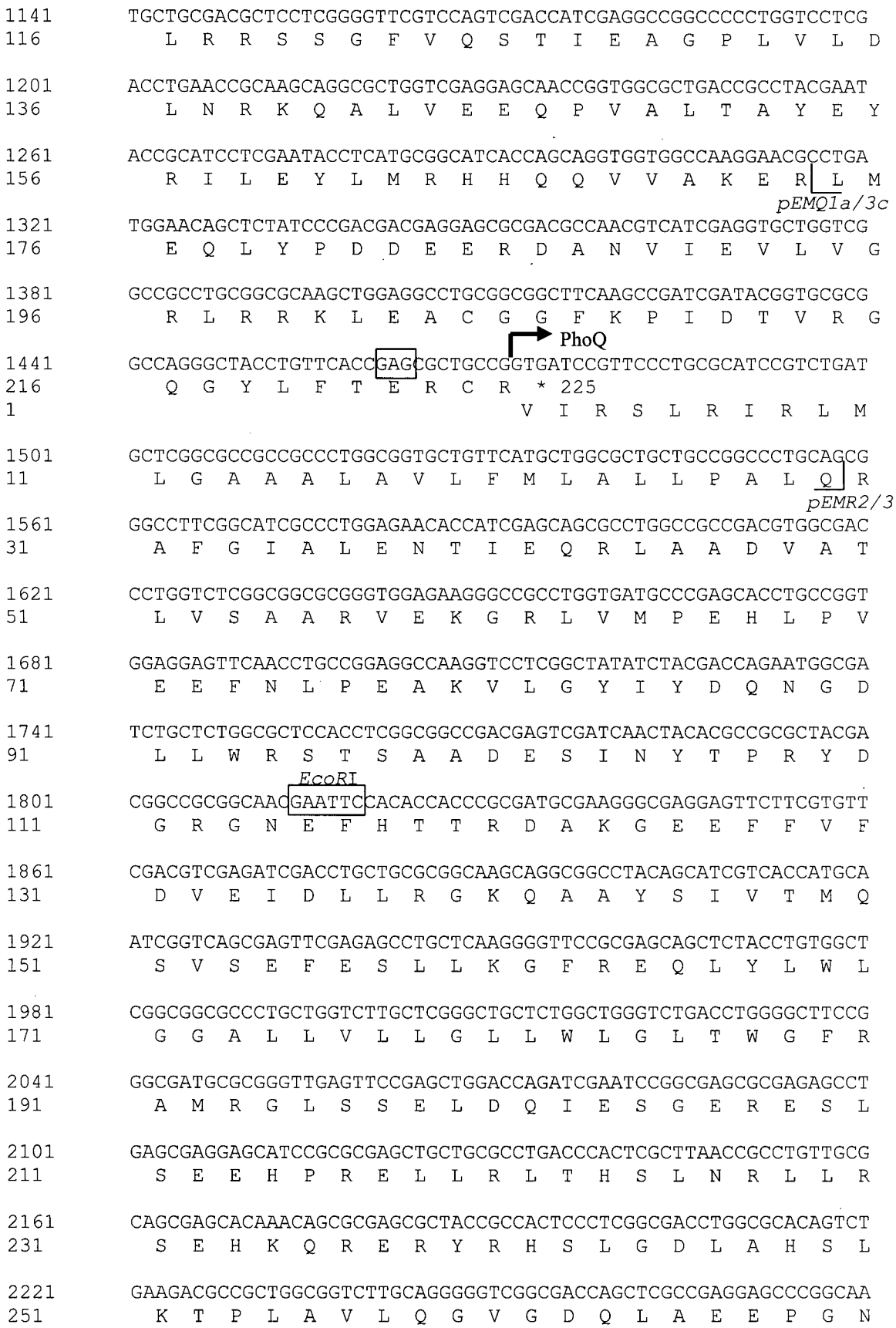


Figure 3. Amino acid sequence alignment of the PhoQ proteins
Translation of the *phoQ* gene from of *P. aeruginosa* (P.ae) was aligned with the protein sequence from *E. coli* (E.co) and *S. typhimurium* (S.ty). *P. aeruginosa* PhoQ shows 33% sequence identity to PhoQ proteins of both *S. typhimurium* and *E. coli*. Predicted transmembrane domains are indicated by underlines for the *S. typhimurium* protein and by dashed overhead lines for the *P aeruginosa* PhoQ. Residues conserved among sensor kinases are indicated by dots below the sequence and the histidine resicue believed to be the site of autophosphorylation is marked by an asterisk. The start of the putative ATP binding domain is indicated by a directional arrow below the sequence. The amino acid substitution Thr48→Ile in a *S. typhimurium* mutant, that constitutively expresses PhoP, activated genes in indicated by an arrowhead below.


OprH
PstI
PstI
pEMR2/3
pEMPQ1b/2a
PhoP
pAK17
KpnI


1141 TGCTGCGACGCTCCTCGGGGTTCGTCCAGTCGACCATCGAGGCCGGCCCCCTGGTCCTCG
116 L R R S S G F V Q S T I E A G P L V L D
1201 ACCTGAACCGCAAGCAGGCGCTGGTCGAGGAGCAACCGGTGGCGCTGACCGCCTACGAAT
136 L N R K Q A L V E E Q P V A L T A Y E Y
1261 ACCGCATCCTCGAATACCTCATGCGGCATCACCAGCAGGTGGTGGCCAAGGAACGCCTGA
156 R I L E Y L M R H H Q Q V V A K E R L M
pEMQ1a/3c
1321 TGGAACAGCTCTATCCCGACGACGAGGAGCGCGACGCCAACGTCATCGAGGTGCTGGTCG
176 E Q L Y P D D E E R D A N V I E V L V G
1381 GCCGCCTGCGGCGCAAGCTGGAGGCCTGCGGCGGCTTCAAGCCGATCGATACGGTGCGCG
196 R L R R K L E A C G G F K P I D T V R G
PhoQ
1441 GCCAGGGCTACCTGTTCACCGAGCGCTGCCGGTGATCCGTTCCCTGCGCATCCGTCTGAT
216 Q G Y L F T E R C R * 225
1 V I R S L R I R L M
1501 GCTCGGCGCCGCCGCCCTGGCGGTGCTGTTCATGCTGGCGCTGCTGCCGGCCCTGCAGCG
11 L G A A A L A V L F M L A L L P A L Q R
pEMR2/3
1561 GGCCTTCGGCATCGCCCTGGAGAACACCATCGAGCAGCGCCTGGCCGCCGACGTGGCGAC
31 A F G I A L E N T I E Q R L A A D V A T
1621 CCTGGTCTCGGCGGCGCGGGTGGAGAAGGGCCGCCTGGTGATGCCCGAGCACCTGCCGGT
51 L V S A A R V E K G R L V M P E H L P V
1681 GGAGGAGTTCAACCTGCCGGAGGCCAAGGTCCTCGGCTATATCTACGACCAGAATGGCGA
71 E E F N L P E A K V L G Y I Y D Q N G D
1741 TCTGCTCTGGCGCTCCACCTCGGCGGCCGACGAGTCGATCAACTACACGCCGCGCTACGA
91 L L W R S T S A A D E S I N Y T P R Y D
EcoRI
1801 CGGCCGCGGCAACGAATTCCACACCACCCGCGATGCGAAGGGCGAGGAGTTCTTCGTGTT
111 G R G N E F H T T R D A K G E E F F V F
1861 CGACGTCGAGATCGACCTGCTGCGCGGCAAGCAGGCGGCCTACAGCATCGTCACCATGCA
131 D V E I D L L R G K Q A A Y S I V T M Q
1921 ATCGGTCAGCGAGTTCGAGAGCCTGCTCAAGGGGTTCCGCGAGCAGCTCTACCTGTGGCT
151 S V S E F E S L L K G F R E Q L Y L W L
1981 CGGCGGCGCCCTGCTGGTCTTGCTCGGGCTGCTCTGGCTGGGTCTGACCTGGGGCTTCCG
171 G G A L L V L L G L L W L G L T W G F R
2041 GGCGATGCGCGGGTTGAGTTCCGAGCTGGACCAGATCGAATCCGGCGAGCGCGAGAGCCT
191 A M R G L S S E L D Q I E S G E R E S L
2101 GAGCGAGGAGCATCCGCGCGAGCTGCTGCGCCTGACCCACTCGCTTAACCGCCTGTTGCG
211 S E E H P R E L L R L T H S L N R L L R
2161 CAGCGAGCACAAACAGCGCGAGCGCTACCGCCACTCCCTCGGCGACCTGGCGCACAGTCT
231 S E H K Q R E R Y R H S L G D L A H S L
2221 GAAGACGCCGCTGGCGGTCTTGCAGGGGGTCGGCGACCAGCTCGCCGAGGAGCCCGGCAA
251 K T P L A V L Q G V G D Q L A E E P G N

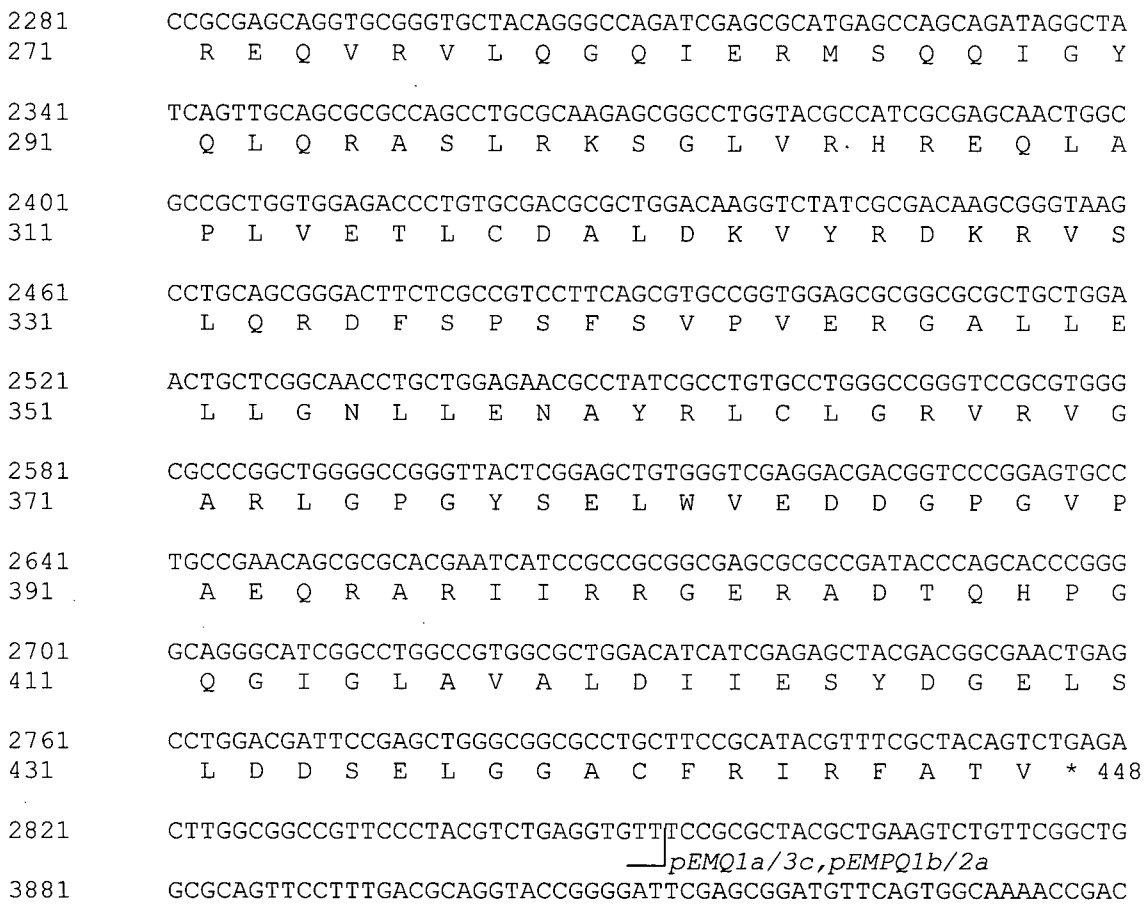


Figure 4. Genetic organization of the *oprH*, *phoP* and *phoQ* genes
The nucleotide sequence and the translation of the *oprH*, *phoP* and *phoQ* genes are shown. An arrow indicates the start of the coding region of each gene and putative Shine-Dalgarno sequences are boxed. The start of transcription from the *oprH* promoter is in bold and underlined. A putative –10 sequence is also indicated in bold and the hexanucleotide repeats are underlined. The restriction enzyme sites for insertion of *xylE*-Gm^R cassettes are boxed and named above (*Pst*I for *oprH*, *Kpn*I for *phoP* and *Eco*RI for *phoQ*). The regions cloned into plasmids carrying the *phoP* and *phoQ* genes (Table 2) are bound by brackets with the name of the plasmids in italics below. An arrowhead indicates the extent of deletion in plasmid pAK17.

(GTTTAT) have also been identified upstream of the *phoP* genes in *S. typhimurium* (Groisman *et al.*, 1989) and *E. coli* (Groisman *et al.*, 1992).

To aid the future study of the transcriptional behavior of these genes, their chromosomal context was determined. Starting 224 bp upstream of the *oprH* gene and oriented in the opposite direction, six ORFs were identified. Their putative gene products showed amino acid sequence similarity to the Nap family of proteins (NapEFDABC) found in various Gram negative organisms (Figure 5). In *Rhodobacter sphaeroides* these proteins are involved in denitrification (Liu *et al.*, 1999). One hundred bases downstream of *phoQ* is a large ORF (1120 aa) coding for a putative protein that shows similarity only to a hypothetical protein from *E. coli*.

3.2 Construction of the *phoP* Mutant H851

To investigate the role of PhoP and PhoQ in regulation of *oprH, phoP* and *phoQ* transcription, a *P. aeruginosa* mutant was constructed in which the *phoP* gene was interrupted by a *xylE*-Gm^R cassette. To construct this mutant, the plasmid pEXP was created by cloning the *phoP*::*xylE*-Gm^R fusion into plasmid pEX100T. Plasmid pEXP was then conjugally transferred into wildtype *P. aeruginosa* PAO1 to allow integration of the gene fusion into the chromosome by homology mediated crossover. Many cointegrates were obtained by selection on BM2 media containing gentamicin and carbenicillin. The *sacB* positive selection was used to select for double crossover events by streaking cointegrates on LB media containing gentamicin and sucrose. Approximately 20 clones identified by this method were checked for excision of plasmid

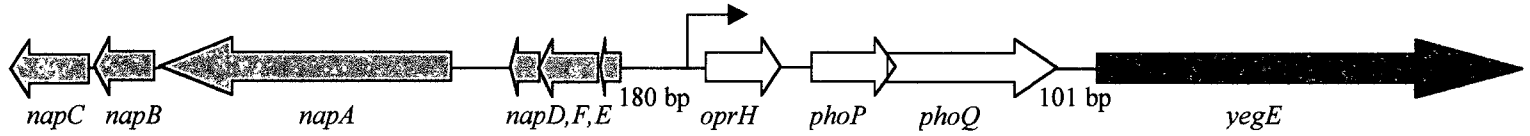


Figure 5. Chromosomal context of the *oprH*, *phoP* and *phoQ* genes
Open reading frames (ORFs) surrounding the genes are indicated by coloured arrows. Each gene is named for the protein that its translation show greatest homology to. *napC*: cytochrome C-type protein of *Pseudomonas* species G-179, *napB*: cytochrome C-type protein *napB* precursor of *Ralstonia eutropha*, *napA*: periplasmic nitrate reductase precursor of *Pseudomonas* species G-179, *napD*: *Rhodopseudomonas* species protein, *napF*: *Rhidobacter sphaeroides* protein, *napE*: *Rhidobacter sphaeroides* protein, *yegE*: hypothetical 123.9 kD protein in the *udk-alkA* intergenic region of *E. coli*. The number of bases between the *napE* and the *oprH* promoter and between *phoQ* and the *yegE* are indicated.

sequences by testing for growth on carbenicillin. Four carbenicillin sensitive clones were randomly selected for Southern blot analysis.

Genomic DNA from the above clones was digested with both *Pst*I and *Bbs*I, which allowed verification of the insertion of the cassette in the correct location and the excision of plasmid sequences (Figure 6A). Digests were resolved on an agarose gel, blotted and probed with DNA complementary to both the *xylE* and *phoP* genes. A fragment of the correct size was identified in all four mutants when the blot was probed with the *xylE* fragment, compared to the absence of any signal in the wild type (Figure 6B). Similarly, when compared to wild type, all four mutants showed a 3.4 kb increase in the *Pst*I fragment hybridizing to the *phoP* probe which corresponded to the insertion of the *xylE*-Gm^R cassette into the *phoP* gene (Figure 6B). When genomic DNA from the mutants was digested with *Bbs*I, a change from 1.4 kb to 985 bp for the fragment hybridizing to the *phoP* probe was observed due to the additional *Bbs*I site within the cassette (Figure 6B).

Based on these results, one of these positive mutants (H851) was selected for further study. In addition to being *phoP* null, this mutant contains a *phoP*::*xylE* chromosomal gene fusion. In this construct omega fragments flank the gentamicin resistance gene, *aacC1*, preventing read-through from the *aacC1* promoter into downstream genes (Schweizer and Hoang, 1995). The *phoP* mutant H851 was, therefore, assumed to be both *phoP* and *phoQ* negative. This *phoP* mutant was not defective for growth on both rich media (LB) and minimal media with low magnesium (BM2, 0.02mM $MgSO_4$) in contrast to a *S. typhimurium phoP*::Tn*10* insertional mutant which was deficient for growth in low magnesium liquid media (Soncini *et al.*, 1996)

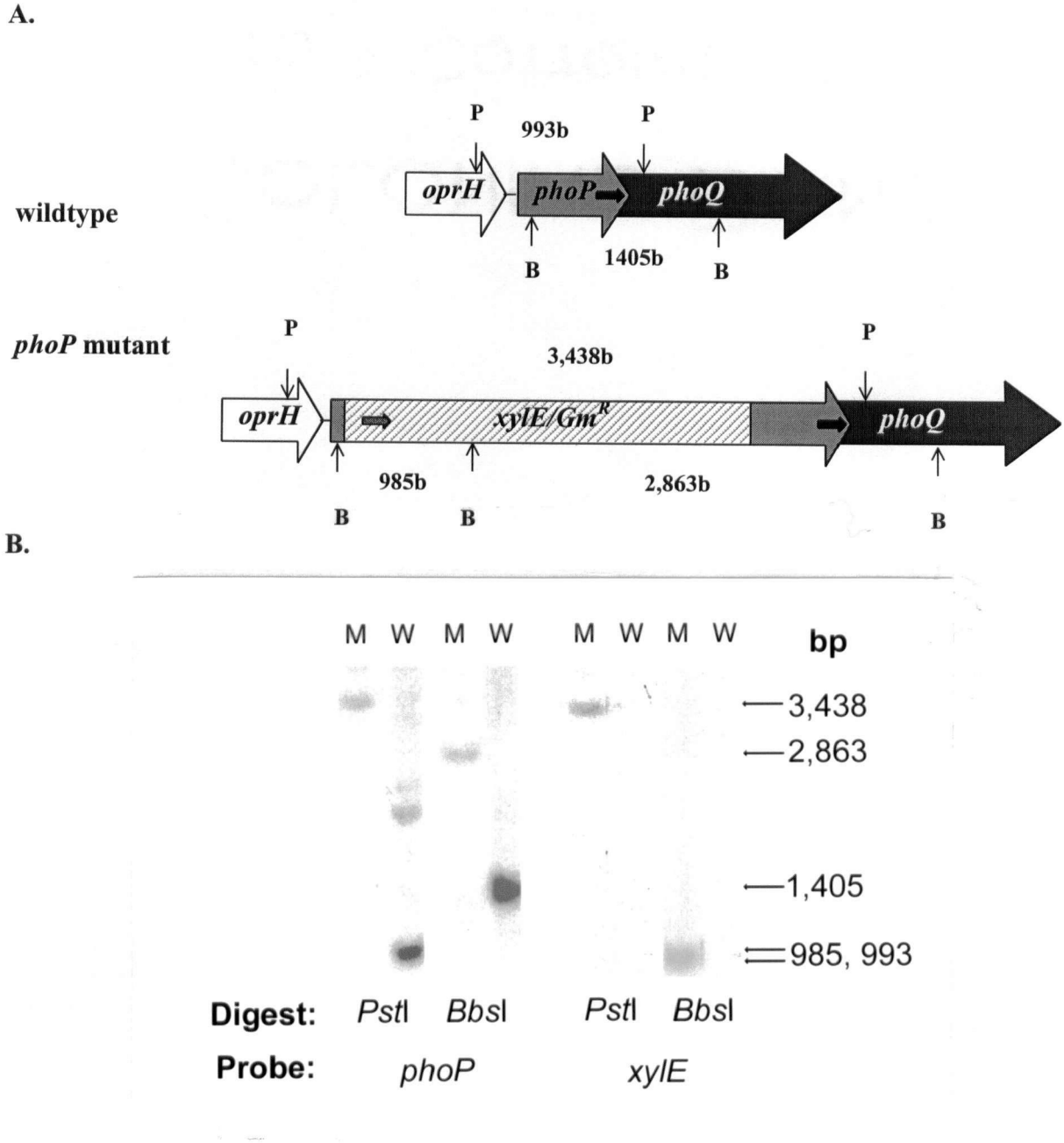


Figure 6. Confirmation of the genotype of the *phoP* mutant, H851, by Southern blot hybridization.

A. *Pst*I (P) and *Bbs*I (B) sites are indicated above and below the genes in wild type and the *phoP* mutant. The expected size of digest fragments in base pairs are shown between the sites. The *xylE*-gentamicin resistance cassette is represented by the hatched box. Approximate sites of *phoP* and *xylE* probe binding are indicated by small arrows within the genes.

B. Enzymes used to digest genomic DNA from wild type (W) and the *phoP* mutant (M) and the probes used for hybridization are indicated below each blot. The fragment sizes are indicated to the right of the blots.

3.3 Magnesium Regulated Expression of OprH in the *phoP* and *phoQ* mutants

To determine the role of PhoP and PhoQ in expression of the outer membrane protein OprH, a *phoQ* mutant (H854), constructed in a similar manner to that described above for the *phoP* mutant (Macfarlane *et al.*, 1999), was studied. In H854, the *xylE*-Gm^R cassette had been inserted into the unique *Eco*RI site in *phoQ* and the mutant was confirmed by Southern blot analysis. Multicopy plasmids encoding PhoP and PhoQ, cloned individually or together (Table 2), were transformed into both the *phoP* and *phoQ* mutants and OprH expression was assessed by Western immunoblotting. As previously reported (Nicas and Hancock, 1980), OprH expression was induced in wildtype *P. aeruginosa* under low magnesium conditions (0.02mM $MgSO_4$) but was almost completely absent under high magnesium conditions (2mM $MgSO_4$) (Figure 7, lanes 1 and 2). In contrast, OprH was not expressed in the *phoP* mutant under either high or low magnesium conditions (Figure 7, lanes 3 and 4). Transformation of this mutant with a plasmid encoding PhoP under control of the *lac* promoter (pEMR3, Table 2) restored OprH expression irrespective of magnesium concentration and to a higher level than that seen in wildtype *P. aeruginosa* grown in low magnesium (Figure 7, lanes 7 and 8 versus lane 2). The *phoP* mutant could be complemented with a plasmid carrying both genes in reverse orientation to the *lac* promoter (pEMPQ2a, Table 2). This plasmid both restored OprH expression and the regulation of this expression by magnesium (Figure 7, lanes 15 and 16). Surprisingly, a plasmid encoding both PhoP and PhoQ expressed from the *lac* promoter (pEMPQ1b, Table 2) did not restore OprH expression in the *phoP* mutant (Figure 7, lanes 13 and 14). Plasmids carrying *phoP* in the reverse orientation to the *lac* promoter (pEMR2), *phoQ* in either orientation (pEMQ1a and pEMQ3c) or the control

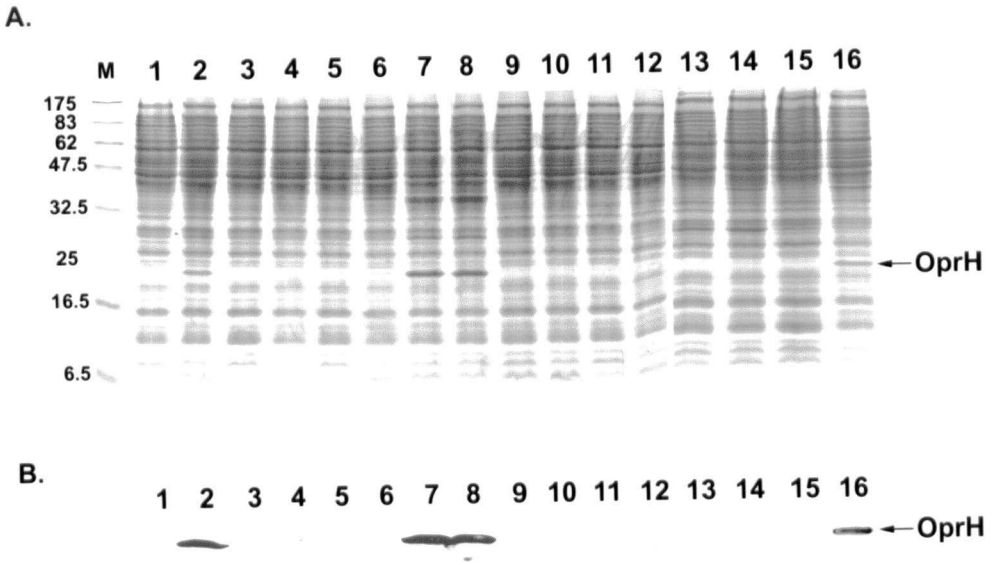


Figure 7. Effect of magnesium and PhoP and PhoQ expressing plasmids on OprH expression in the *phoP* mutant.

A. 15% SDS-polyacrylamide gel and **B.** Western immunoblot of whole cell lysates from *P. aeruginosa* cells with PhoP and PhoQ expressing plasmids grown in high and low magnesium. Lane M. molecular weight marker; lane 1. H103 (wild type *P. aeruginosa*) in low Mg^{2+}; lane 2. H103 in high Mg^{2+} ; lane 3. H851(*phoP* mutant) in high Mg^{2+}; lane 4. H851 in low Mg^{2+}; lane 5. H851/pEMR2 in high Mg^{2+}; lane 6. H851/pEMR2 in low Mg^{2+}; lane 7. H851/pEMR3 in high Mg^{2+}; lane 8. H851/pEMR3 in low Mg^{2+}; lane 9. H851/pEMQ1a in high Mg^{2+}; lane 10. H851/pEMQ1a in low Mg^{2+}; lane 11. H851/pEMQ3c in high Mg^{2+}; lane 12. H851/pEMQ3c in low Mg^{2+}; lane 13. H851/pEMPQ1b in high Mg^{2+}; lane 14. H851/pEMPQ1b in low Mg^{2+}; lane 15. H851/pEMPQ2a in high Mg^{2+}; lane 16. H851/pEMPQ2a in low Mg^{2+}.

plasmid (pUCP19) did not affect OprH expression in the *phoP* mutant (Figure 7, lanes 5 and 6, 9 and 10, and 11 and 12; data not shown for pUCP19).

In contrast to the *phoP* mutant, OprH expression in the *phoQ* mutant H854 was both constitutive and high (Figure 8, lanes 2 and 3). A plasmid carrying *phoQ* behind the *lac* promoter (pEMQ1a) decreased expression of OprH in both high and low magnesium, but to a greater extent in high magnesium (Figure 8, lanes 9 and 10). Regulated expression of OprH was also restored in the *phoQ* mutant when the plasmid encoding PhoP and PhoQ (pEMPQ2a) was used to complement (Figure 8, lanes 13 to 16). Similar to the *phoP* mutant, expression of PhoP and PhoQ from the *lac* promoter on the multicopy plasmid pEMPQ1b suppressed expression of OprH (Figure 8, lanes 13 and 14). Plasmids carrying *phoP* or *phoQ* in the reverse orientation to the *lac* promoter (pEMR2 and pEMQ2a) and the control plasmid (pUC19) did not affect OprH expression in the *phoQ* mutant (Figure 8, lanes 5 and 6 and 11 and 12; data not shown for pUCP19).

3.4 The Role of PhoP-PhoQ in Transcription of the *oprH-phoP-phoQ* Operon

3.4.1 Transcriptional Linkage of *oprH* and *phoP-phoQ*

The proximity of the *oprH* and *phoP* genes (79 bp apart) prompted us to investigate whether cotranscription of these two genes occurs. Reverse transcription followed by PCR (RT-PCR) was performed using total RNA from wildtype *P. aeruginosa* and primers complementary to basepairs 475-494 of *oprH* and basepairs 120-139 of *phoP*. The presence of both these genes on a single transcript would give a 346 bp product after RT-PCR. When RNA from *P. aeruginosa* grown in low magnesium (to

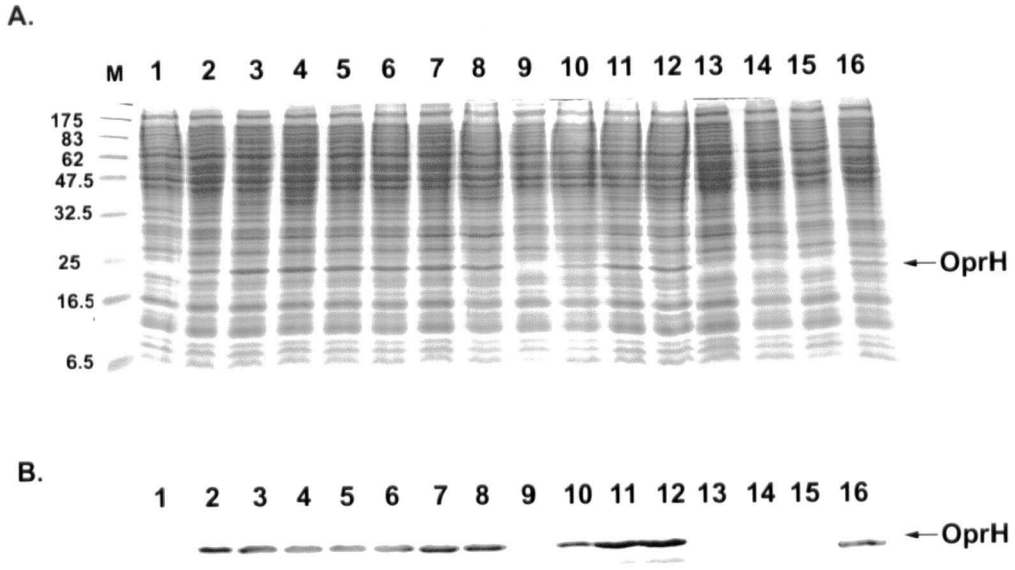


Figure 8. Effect of magnesium and PhoP and PhoQ expressing plasmids on OprH expression in the *phoQ* mutant.

A. 15% SDS-polyacrylamide gel and **B.** Western immunoblot of whole cell lysates from *P. aeruginosa* cells with PhoP and PhoQ expressing plasmids grown in high and low magnesium. Lane M. molecular weight marker; lane 1. H103 (wild type *P. aeruginosa*) in low Mg^{2+}; lane 2. H103 in high Mg^{2+}; lane 3. H854 (*phoQ* mutant) in high Mg^{2+}; lane 4. H854 in low Mg^{2+}; lane 5. H854/pEMR2 in high Mg^{2+}; lane 6. H854/pEMR2 in low Mg^{2+}; lane 7. H854/pEMR3 in high Mg^{2+}; lane 8. H854/pEMR3 in low Mg^{2+}; lane 9. H854/pEMQ1a in high Mg^{2+}; lane 10. H854/pEMQ1a in low Mg^{2+}; lane 11. H854/pEMQ3c in high Mg^{2+}; lane 12. H854/pEMQ3c in low Mg^{2+}; lane 13. H854/pEMPQ1b in high Mg^{2+}; lane 14. H854/pEMPQ1b in low Mg^{2+}; lane 15. H854/pEMPQ2a in high Mg^{2+}; lane 16. H854/pEMPQ2a in low Mg^{2+}.

increase the abundance of *oprH* transcript) was used as the template for RT-PCR, a product of this size was observed (Figure 9, lane 1). In the absence of an RNA template or of reverse transcriptase enzyme, no product was observed, but a fragment of the same size was obtained in the control PCR reaction using genomic DNA as the template (Figure 9, lanes 2, 3 and 4). As the *phoP* and *phoQ* genes overlap by four nucleotides and a promoter for *phoQ* would have to lie within the *phoP* gene, it was assumed that *phoP* and *phoQ* were also transcriptionally linked. Thus the three genes, *oprH*, *phoP* and *phoQ* form a small operon. This gene arrangement was confirmed by Northern blot experiments described below.

3.4.2 Magnesium Regulated Transcription of *oprH*, *phoP* and *phoQ*

Transcription of the *oprH*, *phoP* and *phoQ* genes was compared in wild type *P. aeruginosa* and the *phoP* and *phoQ* mutants by Northern blot analysis. RNA isolated from wild type *P. aeruginosa* grown in high (2mM $MgSO_4$) and low (0.02mM $MgSO_4$) magnesium media was probed with DNA complementary to each of these genes. These experiments revealed not only magnesium regulated transcription of *oprH*, but also magnesium regulated *phoP* and *phoQ* transcription (Figure 10). After growth in low magnesium, a major mRNA 0.7 kb in size was observed that represented *oprH* alone (Figure 10A). Additional transcripts were observed, 1.3 and 2.7 kb in size (Figure10A), that were an appropriate length to represent *oprH-phoP* and *oprH-phoP-phoQ* respectively. The hybridization pattern of these transcripts to probes complementary to *phoP* and *phoQ* (Figure 10B and C) confirmed the presence of these genes on *oprH* transcripts. Both these larger transcripts were present at lower concentrations than the

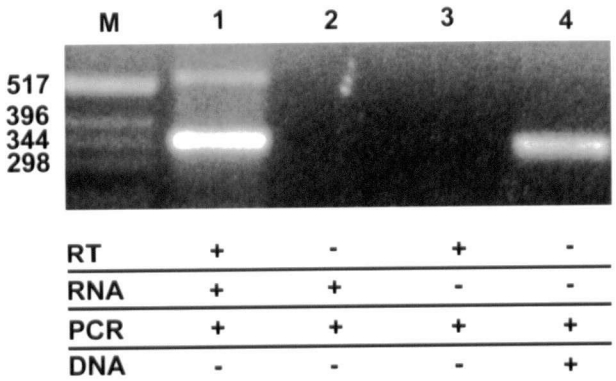


Figure 9. Cotranscription of the *oprH* and *phoP* genes as shown by RT-PCR. Lane M. molecular weight marker; lane 1. whole cell RNA from H103 (wild type *P. aeruginosa*) grown in low Mg^{2+} after RT-PCR with primers RT-forward and RT-reverse (Table 3); lane 2. RNA submitted to PCR without prior reverse transcription; lane 3. as lane 1 without RNA template; lane 4. control PCR with H103 genomic DNA and RT-forward and RT-reverse primers. The results shown are one of three independent experiments.

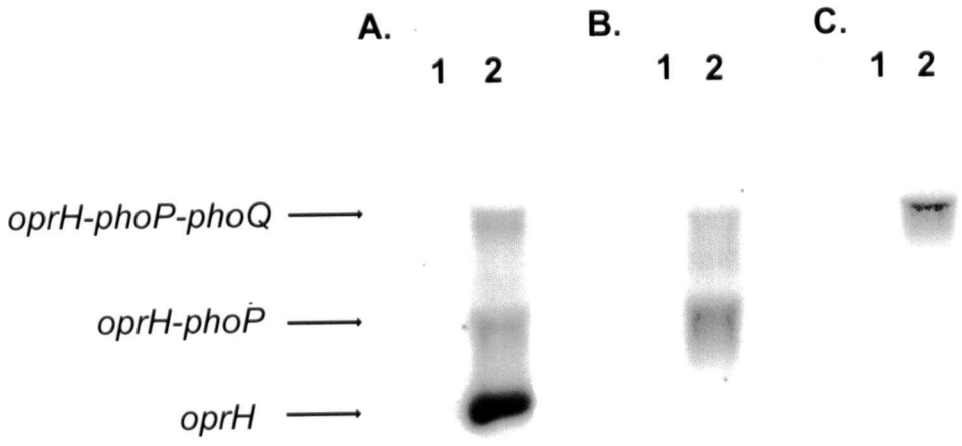


Figure 10. Northern blot analysis of *oprH*, *phoP* and *phoQ* transcription in wild type *P. aeruginosa* in response to magnesium.
RNA isolated from H103 (wild type *P. aeruginosa*) grown in high **1.** and low **2.** Mg^{2+} and probed with DNA complementary to **A.** *oprH*, **B.** *phoP* and **C.** *phoQ*. Transcript sizes corresponding to *oprH* (0.7 kb), *oprH-phoP* (1.3kb) and *oprH-phoP-phoQ* (2.7 kb) are shown on the left.

oprH transcript. After growth in high magnesium, transcripts containing either *phoP* or *phoQ* were completely absent (Figure 10B, and C, lane 1), but a low level of *oprH* transcription was observed in overexposed Northern blots (data not shown). Under either growth conditions no transcripts carrying *phoP* alone (0.7 kb), *phoQ* alone (1.3 kb) or *phoP-phoQ* (2.0 kb) were observed (Figure 10B and C).

No transcription of *oprH*, *phoP* or *phoQ* was seen in Northern blot analysis of the *phoP* mutant (data not shown). The absence of an *oprH* transcript was consistent with the previously observed absence of OprH protein. In addition, the absence of *phoQ* containing transcripts was consistent with the hypothesis that *phoP* and *phoQ* were transcriptionally linked.

In contrast to the *phoP* mutant, the levels of *phoP* and *oprH* transcripts in the *phoQ* mutant were approximately the same in both high and low magnesium (Figure 11, lane 1 is slightly under-loaded). Transcripts corresponding to *oprH* alone, *oprH-phoP* and *oprH-phoP-phoQ*::*xylE* fusion were observed (Figure 11, A and B). The levels of *oprH* and *phoP* transcription in both high and low magnesium appeared to be greater in H854 than those seen in wildtype *P. aeruginosa* under low magnesium growth conditions (Figure 10A, lane 2 compared with Figure 11A, lanes 1 and 2).

To quantitatively compare the amount of *oprH*, *phoP* and *phoQ* transcription in the mutants and the wildtype, RNA dot blots were performed. An *oprH* mutant, with the *xylE*-Gm^R cassette inserted into the *Pst*I site (Figure 3) of the *oprH* gene (Macfarlane and Hancock, unpublished data) was also assessed for *phoP* and *phoQ* transcription. The probes used in the RNA dot blot experiments were identical to those used in the Northern blots. All signals were standardized by comparison to blots probed with *rpoB*, the gene

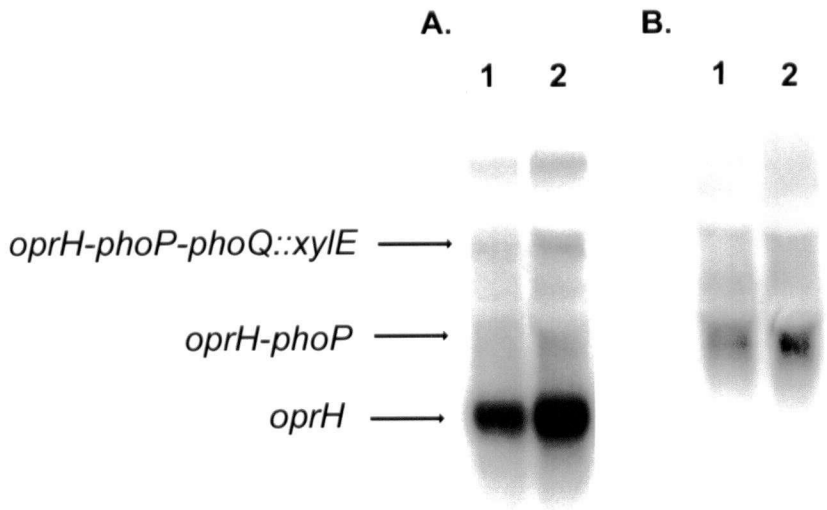


Figure 11. Northern blot analysis of *oprH*, *phoP* and *phoQ* transcription in *phoQ* mutant in response to magnesium.
RNA isolated from H854 (*phoQ* mutant) grown in high **1.** and low **2.** Mg^{2+} and probed with DNA complementary to **A.** *oprH* and **B.** *phoP*. Lane 1 in both panels is slightly underloaded.Transcript sizes corresponding to *oprH* (0.7 kb), *oprH-phoP* (1.3kb) and *oprH-phoP-phoQ::xylE* (2.7 kb) are shown on the left.

encoding the beta subunit of RNA polymerase, which was assumed to be constitutively expressed. The level of each gene transcript relative to that in wildtype *P. aeruginosa* in high magnesium is given in Table 4.

Quantitative analysis of *oprH*, *phoP* and *phoQ* transcription confirmed many of the observations made by Northern blot analysis. Transcription of these genes was not observed in the *phoP* mutant and the *phoQ* mutant showed constitutive and high levels of *phoP* and *oprH* transcripts. The levels of *phoP* transcripts were about 1.7-fold higher and transcription of *oprH* was 1.5-fold higher in the *phoQ* mutant compared to the wildtype grown in low magnesium (Table 4). The levels of induction of *phoQ* transcription in wildtype under conditions of low magnesium were higher than that seen for the *phoP* gene (13.6 ± 0.4 compared with 9.4 ± 1.0), whereas the levels of *phoP* and *phoQ* transcripts appeared equal in Northern blots (Figure 10A). This might have been due to experimental error or differing concentrations or affinities of labeled *phoP* and *phoQ* probes. The latter was evident in the comparison of Northern blots probed with *phoP* and *phoQ* (Figure 10B and C). The *oprH* mutant appeared to be negative for *phoP* and *phoQ* transcription in both high and low magnesium (Table 4).

The probe used to assess *oprH* transcription hybridized to the region upstream of the *xylE*-Gm^R cassette insertion in the *oprH* mutant, hence a transcript could be observed in RNA dot blots (Table 4). Transcription of *oprH* in this mutant was still regulated by magnesium to approximately the same extent as in wildtype *P. aeruginosa*, despite our inability to detect any *phoP* or *phoQ* transcripts by RNA dot blot (Table 4) and Northern blot analysis (data not shown).

Table 4. Relative levels of *oprH*, *phoP* and *phoQ* transcription in wildtype *P. aeruginosa* and the *oprH*, *phoP* and *phoQ* mutants as determined by quantitative RNA dot blots

Gene	Strain	High Mg^{2+}	Low Mg^{2+}
oprH	wildtype (H103)	1.00	52.1 ± 6.0
	oprH mutant (H855)	1.59 ± 0.32[a]	46.0 ± 12.2[a]
	phoP mutant (H851)	0.66 ± 0.25	1.16 ± 0.44
	phoQ mutant (H854)	80.1 ± 19.4	76.3 ± 10.4
phoP	wildtype (H103)	1.00	9.4 ± 1.0
	oprH mutant (H855)	0.45 ± 0.02	0.63 ± 0.04
	phoP mutant (H851)	0.70 ± 0.03	1.00 ± 0.11
	phoQ mutant (H854)	16.2 ± 3.7	15.6 ± 4.8
phoQ	wildtype (H103)	1.00	13.6 ± 0.4
	oprH mutant (H855)	0.37 ± 0.17	0.46 ± 0.05
	phoP mutant (H851)	0.15 ± 0.01	0.23 ± 0.02
	phoQ mutant (H854)	0.38 ± 0.01	0.47 ± 0.07

[a] *oprH* transcription in the *oprH* mutant represents transcription of the region upstream of the *xylE*-Gm^R cassette.

3.4.3 Reporter Gene Transcription in the *oprH*, *phoP* and *phoQ* Mutants

In order to further determine the effect of PhoP and PhoQ on *oprH*, *phoP* and *phoQ* transcription, assays were performed to quantitate the amount of catechol-2,3-dioxygenase expressed from the *xylE* chromosomal fusions in each mutant in the presence of plasmid encoded PhoP and PhoQ. Catechol-2,3-dioxygenase activity was determined in log-phase cells grown in high and low magnesium media. No activity was observed in wildtype *P. aeruginosa* (data not shown).

Low level enzyme activity was observed in strain H851 (*phoP*::*xylE*-Gm^R) without a vector control (Table 5). This activity was only slightly higher (1.39-fold) in low magnesium. Whereas a plasmid carrying PhoQ alone (pEMQ1a) had no effect on catechol-2,3-dioxygenase activity, the PhoP plasmid (pEMR3) caused a strong increase in activity in both high and low magnesium (a 350-fold increase over mutant alone). Similar to the effect of plasmid pEMPQ2a (*phoP* and *phoQ* in reverse orientation to the *lac* promoter) on the production of OprH, this plasmid imposed regulation of catechol-2,3-dioxygenase expression by magnesium. The level of catechol-2,3-dioxygenase activity with plasmid pEMPQ2a, after growth in low magnesium, was much lower than seen with plasmid pEMR2. The control plasmid pUCP19, and plasmids carrying *phoP* in the reverse orientation, *phoQ* on either orientation or *phoP* and *phoQ* in the same orientation relative to the *lac* promoter had no effect on catechol-2,3-dioxygenase activity (Table 5 or data not shown).

In contrast to the *phoP* mutant, strain H854 (*phoQ*::*xylE*-Gm^R) showed a greater level of catechol-2,3-dioxygenase activity without PhoP and PhoQ encoding plasmids (Table 6). The observed difference in activity between high and low magnesium was less

Table 5. Catechol-2,3-dioxygenase activity in strain H851 (*phoP*:: *xylE*-Gm^R) harbouring PhoP and PhoQ encoding plasmids

	Catechol-2,3-dioxygenase Activity / pmol min^{-1} μg^{-1} protein		
	Low Mg^{2+}	**High Mg^{2+}**	**Fold Difference**
Plasmid[a]	Mean activity[b] ± S.E.M (relative amount)	Mean activity[b] ± S.E.M (relative amount)	Low/High[d]
none	60.3 ± 5.0 (1.00)	43.5 ± 3.6 (1.00)	1.4
pEMR3 (*phoP*^{F})	17,427 ± 4,554 (289)	15,545 ± 4,675 (357)	1.1
pEMQ1a (*phoQ*^{F})	61.9 ± 4.9 (1.03)	43.8 ± 3.7 (1.00)	1.4
pEMPQ1b (*phoPQ*^{F})	66.1 ± 9.2 (1.10)	43.6 ± 9.2 (1.00)	1.5
pEMPQ2a (*phoPQ*^{R})	3,428 ± 194[c] (56.8)	55 ± 7[c] (1.26)	62.3

[a] Genes cloned behind (F) or in reverse orientation (R) to the *lac* promoter

[b] Values are the mean of three independent experiments

[c] The values for H851/pEMPQ2a, with an independent evaluation of H851 without plasmid as a positive control, were performed at a later date than all other values. Catechol dioxygenase activities for H851 in these trials 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

[d] Low/High gives the increase in expression in low magnesium compared to high

Table 6. Catechol-2,3-dioxygenase activity in strain H854 (*phoQ*::*xylE*-Gm^R) harbouring PhoP and PhoQ encoding plasmids

	Catechol-2,3-dioxygenase Activity / pmol min^{-1} μg^{-1} protein		
	Low Mg^{2+}	**High Mg^{2+}**	**Fold Difference**
Plasmid[a]	Mean activity[b] ± S.E.M (relative amount)	Mean activity[b] ± S.E.M (relative amount)	Low/High[c]
none	2,039 ± 206 (1.00)	1,165 ± 10 (1.00)	1.8
pEMR3 (*phoP*^{F})	1,771 ± 166 (0.87)	1,178 ± 128 (1.01)	1.5
pEMQ1a (*phoQ*^{F})	327 ± 51 (0.16)	24.0 ± 2.8 (0.02)	13.6
pEMPQ1b (*phoPQ*^{F})	10.1 ± 1.4 (0.005)	5.2 ± 0.5 (0.004)	1.9
pEMPQ2a (*phoPQ*^{R})	207 ± 33 (0.10)	4.9 ± 0.2 (0.004)	42.3

[a] Genes cloned behind (F) or in reverse orientation (R) to the *lac* promoter
[b] Values are the mean of three independent experiments
[c] Low/High gives the increase in expression in low magnesium compared to high

than two-fold. Plasmids carrying *phoQ* in the same orientation, or *phoP* and *phoQ* in reverse orientation to the *lac* promoter decreased the level of catechol-2,3-dioxygenase expression from the fusion by as much as 250-fold. Complementation of the mutant with the PhoQ encoding plasmid (pEMQ1a) imposed regulation by magnesium to give a 13.6-fold difference in catechol-2,3-dioxygenase activity between high and low magnesium growth conditions, whereas the plasmid encoding both PhoP and PhoQ (pEMPQ2a) resulted in an even greater difference (42.3-fold). Although the plasmid pEMPQ1b (*phoP* and *phoQ* in the forward orientation) decreased expression from the *xylE* gene, the difference in catechol-2,3-dioxygenase activity between high and low magnesium was less than two-fold.

To confirm results obtained in RNA dot blots, which suggested that transcription of *oprH* was still regulated in strain H855 (*oprH*::*xylE*-Gm^R), catechol-2,3-dioxygenase activity from this chromosomal fusion was assessed. Enzyme activities were 155.6 ± 33.2/pmol min^{-1} μg^{-1} protein in high magnesium and 3,001.5 ± 64.8/pmol min^{-1} μg^{-1} protein in low magnesium. This 19.3-fold difference was consistent with the difference in *oprH* transcription observed in RNA dot blots, though it was not as high (compare with a 52.1 ± 6.0-fold increase in low magnesium).

3.5 Analysis of the *oprH-phoP-phoQ* Promoter

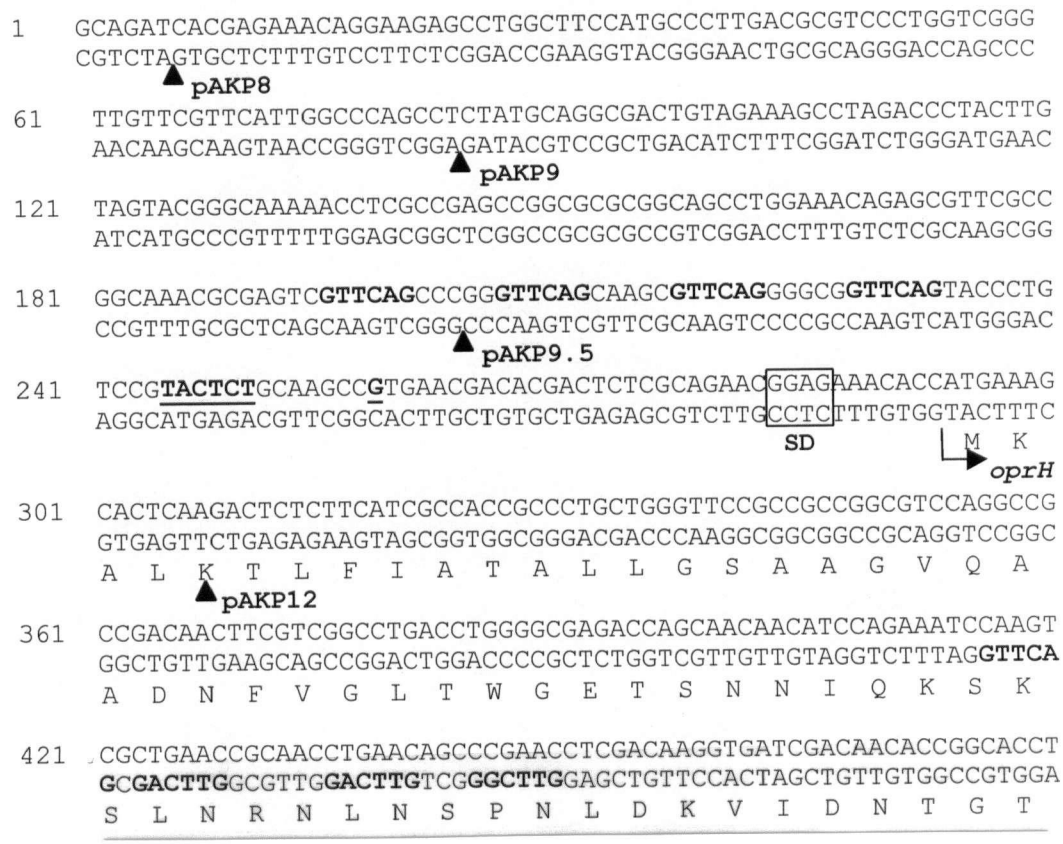
3.5.1 Sequences Necessary for Regulated Expression of OprH

Since magnesium regulated expression of OprH was assumed to be PhoP-PhoQ dependent, the ideal background for expression of *oprH* promoter deletion constructs

would be PhoP-PhoQ positive but negative for OprH expression. My transcriptional studies indicated that the *P. aeruginosa oprH* mutant was PhoP-PhoQ negative and was unsuitable for this purpose. Therefore expression of deletion constructs was attempted in *E. coli* as this organism is known to contains PhoP and PhoQ and hexanucleotide repeats have been found upstream of the autoregulated *phoP-phoQ* genes in this organism (Groisman *et al.*, 1992).

Location of the *oprH* promoter was achieved through transforming *E. coli* with a set of ExoIII digested and re-circularized portions of plasmid pGB22 and assessing OprH expression. The smallest plasmid still allowing OprH expression contained 211 bp of sequence upstream of the ATG codon (data not shown). To further investigate the sequences necessary for OprH expression and regulation by magnesium, an additional deletion was made in this 211 bp region that eliminated one of the hexanucleotide repeats and left only 90 bp of upstream sequence (pAKP9.5, Table 2, Figure 12A). After ExoIII digestion, the region containing the *oprH* gene and upstream sequences from each deletion was subcloned into pUCP19 to remove the *phoP* gene that was present in the original plasmid pGB22 (Table 2). Analysis of OprH expression from *E. coli* containing the deletion constructs indicated that even with only 90 bp of sequence upstream of the ATG codon, magnesium regulated expression of OprH was observed (Figure 12B).

A.



B.

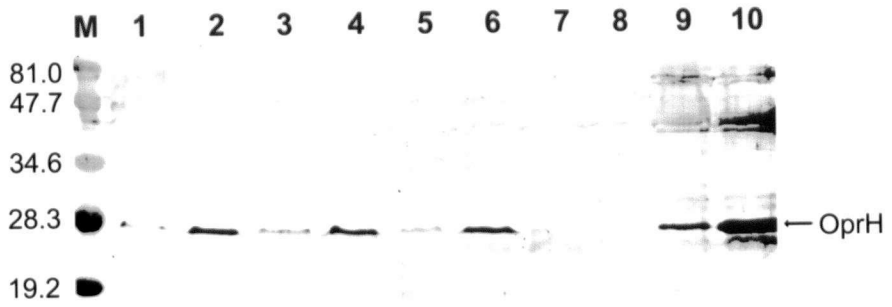


Figure 12. Expression of OprH from promoter deletions constructs in *E. coli* in response to magnesium levels.

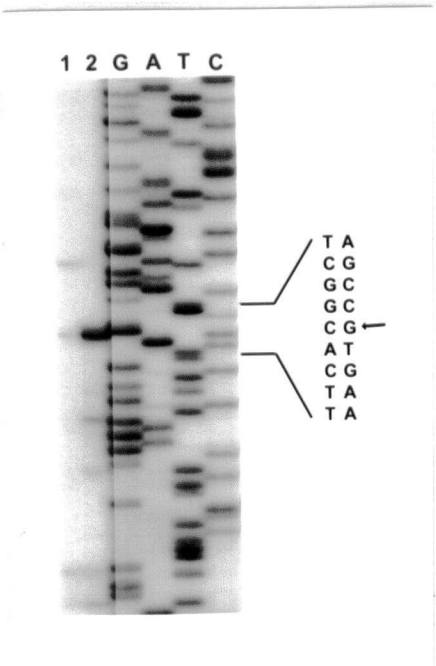
A. The start of each deletion construct is indicated by an arrowhead below the sequence. The Shine-Dalgarno sequence is boxed. Hexanucleotide repeats are in bold. The start of the *oprH* transcript (as determined by primer extension, Figure 12) and the putative –10 sequence are in boldface and underlined. **B.** Western immunoblot of OprH expression in *E. coli* DH5α harbouring *oprH* promoter deletion constructs and grown in high or low Mg^{2+}. Lane M. molecular weight marker; lane 1. *E. coli*/ pAKP8 in high Mg^{2+}; lane 2. *E. coli*/pAKP8 in low Mg^{2+}; lane 3. *E coli*/pAKP9 in high Mg^{2+}; lane 4. *E. coli*/ pAKP9 in low Mg^{2+}; lane 5. *E. coli*/ pAKP9.5 in high Mg^{2+}; lane 6. *E. coli*/ pAKP9.5 in low Mg^{2+}; lane 7. *E. coli*/ pAKP12 in high Mg^{2+}; lane 8. *E. coli*/ pAKP12 in low Mg^{2+}; lane 9. H103 (wild type *P. aeruginosa*) control in high Mg^{2+}; lane 10. H103 in low Mg^{2+}.

3.5.2 Determination of the Transcription Start Site for the *oprH-phoP-phoQ* Operon

Given the fact that there was no discernable σ^{70} promoter sequence upstream of *oprH*, an attempt was made to determine the transcription start site for this gene by primer extension analysis. A primer complementary to basepairs 96-115 of *oprH* (OprH-rev2, Table 3) was used to generate cDNA from RNA transcripts containing *oprH*. One primer extension product, which appeared strongly in low magnesium and to a lesser extent in high magnesium, was assumed to be the magnesium inducible *oprH* transcript (Figure 13). Analysis of this product on a denaturing acrylamide gel placed the start site at a guanine residue 36 bp upstream of the ATG codon. A weak –10 consensus sequence (TActcT) was identified 7 bp upstream of this transcriptional start, but no sequence similar to a –35 sequence was observed.

Although previous experiments suggested the *oprH-phoP-phoQ* operon was transcribed from a single promoter located upstream of *oprH*, an attempt was made to identify any *phoP-phoQ* transcripts by primer extension. Two primers that bound within *phoP* gave a variety of extension products, but no consistent start site. A number of large extension products were observed from RNA isolated under both high and low magnesium growth conditions (data not shown).

A.



B.

GGCAAACGCGAGTC**GTTCAG**CCCGG**GTTCAGC**AAGC**GTTC**

tataat

AGGGGCG**GTTCAG**TACCCTGTCCG**TACTCT**GCAAGCC**G**TG

oprH

AACGACACGACTCTCGCAGAACGGAGAAACACCATGAAAG

M K

Figure 13. Determination of the start of *oprH* transcription by primer extension.
A. Primer extension products using primer OprH-rev2 (Table 3)and RNA isolated from wild type *P.aeruginosa* grown in either lane 1. high Mg^{2+} or lane 2. low Mg^{2+} and. Lanes GATC represent sequencing reactions performed with OprH-rev2 and plasmid pGB22 (Table 2). The start of the transcript is indicated by an arrow to the right of the figure.
B. Sequence upstream of *oprH* is shown with the transcription start site in bold and underlined. A putative -10 sequence is also shown in bold with the consensus sequence above. Hexanuceotide repeats upstream of the -10 region are highlighted.

4 Discussion

In *S. typhimurium*, the PhoP-PhoQ two-component regulatory system regulates expression of over forty genes in response to extracellular magnesium ion levels (García Véscovi et al., 1996). The *P. aeruginosa* outer membrane protein, OprH, is preferentially expressed in low magnesium conditions (Nicas and Hancock, 1980). This prompted investigation of whether PhoP and PhoQ homologues exist in *P. aeruginosa* that regulate expression of OprH. Not only were candidate *phoP* and *phoQ* open reading frames (ORFs) identified, but these putative genes were located 79 nucleotides downstream of the *oprH* gene. Furthermore, the *phoQ* gene overlaps the upstream *phoP* gene by four nucleotides suggesting the genes encoding these proteins are transcriptionally linked. It was postulated that these three genes in *P. aeruginosa* are cotranscribed from a magnesium regulated promoter upstream of *oprH*.

In support of this hypothesis, RT-PCR revealed that *oprH* and *phoP* were found in the same transcriptional unit (Figure 9). Northern blot analysis confirmed that indeed both these genes and *phoQ* were cotranscribed from a promoter upstream of *oprH* (Figure 10). Thus, *oprH*, *phoP* and *phoQ* form a small operon. Although cotranscription of *phoP* and *phoQ* is seen in *S. typhimurium* and *E. coli*, in these organisms no transcriptionally linked upstream genes have been found (Soncini *et al.*, 1995; Kasahara *et al.*, 1992). In contrast genes encoding a PhoP-regulated two-component regulatory system in *S. typhimurium*, PmrA-PmrB, are cotranscribed with an upstream gene (*pmrC*) which encodes a putative membrane protein (Gunn and Miller, 1996). Both the *pmrC-pmrA-pmrB* and *phoP-phoQ* operons in this organism have two promoters; one regulated promoter that drives transcription of the entire operon and a second constitutive promoter

for transcription of *pmrA-pmrB* alone or *phoP-phoQ* at low levels (Gunn and Miller, 1996; Soncini and Groisman, 1996; Soncini *et al.*, 1995). A magnesium regulated promoter was identified upstream of *oprH* in *P. aeruginosa* by primer extension analysis, but no second promoter in this region or in the *oprH-phoP* intergenic region could be found (Figure 13).

Although no *phoP-phoQ* transcripts were observed by Northern blot analysis (Figure 10), there was some suggestion that low level *phoP-phoQ* transcription occured independently of *oprH* transcription. An *oprH* insertional mutant, in which no *phoP-phoQ* transcripts were detected in Northern or RNA dot blots, still exhibited magnesium regulated *oprH* transcription (Table 4). While this could occur as a result of a second magnesium responsive regulatory mechanism acting on the *oprH* promoter, it seems unlikely as no regulated OprH expression was observed in either the *phoP* or *phoQ* mutant. In addition, a plasmid carrying *phoP* and *phoQ* in the opposite orientation to the *lac* promoter was able to complement *phoP* and *phoQ* mutants for *oprH* and *phoP* transcription and regulation (Figure 7 and 8, Table 5 and 6). Ninety-three bases of sequence upstream of *phoP* are carried on this plasmid (Figure 4) and may include a promoter driving low level expression of *phoP-phoQ*. The *phoP* and *phoQ* genes are probably expressed at very low level from a second promoter to ensure sufficient protein is present to allow the system to respond to the inducing signal.

No other genes except *oprH*, *phoP* and *phoQ* were found to be transcribed from the *oprH* promoter. Upstream ORFs are in the opposite orientation to *oprH* (Figure 5), and thus are probably divergently transcribed. Downstream of *phoQ* there is only a large ORF (Figure 5), and no transcripts of the appropriate size to include this gene were

observed in Northern blots (Figure 10). No strong stem loop structures were identified downstream of the *phoQ* gene, but rho-dependant termination may occur. Possible stem loops structures were located in the *oprH-phoP* intergenic region (6 base stem, 9 base loop, ΔG -8.0 kcal/mol) as well as 66 nucleotides into the *phoQ* gene (6 base stem, 10 base loop, ΔG -6.0 kcal/mol) and could function as rho-independent terminators for the *oprH* and *oprH-phoP* transcripts observed in Northern blots.

Northern blot analysis revealed that *phoP* and *phoQ* transcription was induced in low magnesium (Figure 10). Although a low level of the 0.7 kb *oprH* transcript was observed in high magnesium on over-exposed Northern blots (data not shown), no detectable *phoP* or *phoQ* transcription occurred under these conditions. The second most abundant transcript in low magnesium conditions was one that contained *oprH* and *phoP*, followed by the *oprH-phoP-phoQ* transcript. This differential expression of transcriptionally linked genes could occur through weak termination, read-through from *oprH* and *phoP* or RNA processing of the large 2.7 kb (*oprH-phoP-phoQ*) transcript.

Activation of transcription in response to low magnesium ion levels most likely occurs through transduction of the signal from PhoQ to PhoP, followed by PhoP binding and enhancing transcription from promoters of PhoP-activated genes. In *S. typhimurium* and *E. coli* no consensus PhoP-binding sequence has been identified, though a set of direct hexanucleotide repeats (GTTTAT) spaced four nucleotides apart are present in the *phoP* promoter region 11 bases upstream of the proposed –10 sequence (Groisman *et al.*, 1989 and 1992). Similar repeats (GTTCAG) located the same distance from the Pribnow box in the *oprH* promoter may serve as PhoP binding sites (Figure 4). Despite the sequence difference between the repeats, PhoP from *E. coli* may be able to bind to the

oprH promoter. This is supported by the magnesium regulated expression of OprH in *E. coli* harbouring the promoter deletion constructs (Figure 12). It is noteworthy that only three of the four repeats were sufficient for this regulatory effect. This may be due to the fact that only two repeats are found in the *E. coli* promoter (Groisman *et al.*, 1992) and thus its PhoP protein may bind through a slightly different mechanism.

Although similar repeats found on the non-coding strand within *oprH* are imperfect, they may play a role in regulation of OprH, PhoP and PhoQ expression. For example, in high magnesium they could serve as PhoP binding sites to repress OprH expression, as is seen in the repression of the *Bacillus subtilis spo0A*, *abrB* and *kinA* promoters by Spo0A binding within these genes (Hoch, 1995). They may also function in feedback inhibition when phosphorylated PhoP levels get too high. Equally possible is a positive regulatory role for these sequences, as is seen in the requirement for internal regulator binding sites for PhoP of *B. subtilis* in transcription of *phoA* and *pstS* (Liu *et al.*, 1998). A search was performed to determine if similar repeats were located upstream of *P. aeruginosa* genes homologous to those regulated by PhoP in *S. typhimurium*. No such sequences were found upstream of putative *pmrA/pmrB* or *mgtB* genes (*mgtB* encodes a putative magnesium ion transporter protein). Five equally spaced, identical repeats and one imperfect repeat were identified in the genome, but they occurred upstream of an ORF that showed no sequence similarity to proteins in the non-redundant NCBI database. Downstream of this ORF is a second ORF with homology to a *B. subtilis* protein similar to 3-oxoacyl-acyl-carrier protein reductase (Morbidoni *et al.*, 1996).

The *P. aeruginosa* PhoP protein sequence shows 53 and 54% identity at the amino acid level to *S. typhimurium* and *E. coli* PhoP proteins respectively (Figure 2).

Most importantly, the residues postulated to constitute the acidic pocket for phosphotransfer from PhoQ (Asp-8, Asp-51 and Lys-101; Stock *et al.*, 1989) are conserved. Presumably, as the response regulator, PhoP is a DNA binding protein. No helix-turn-helix DNA binding motif was identified in PhoP, but this is not uncommon. OmpR of *E. coli* has no recognizable DNA binding motif but it has clearly been shown to bind the promoter regions of *ompF* and *ompC* (Pratt and Silhavy, 1995).

P. aeruginosa PhoP is an activator of *oprH*, *phoP* and thus *phoQ* transcription. Disruption of the *phoP* gene completely abolished OprH expression and transcription (Figure 7 and Table 4) indicating that PhoP is necessary for any transcription from the *oprH* promoter. Deletion of the *phoP* gene also disrupts its own and *phoQ* transcription as seen in the analysis of the *phoP::xylE* fusion (Table 5) and mRNA analysis (Table 4). Thus, the PhoP-PhoQ system autoregulates its expression from the *oprH* promoter. Autoregulation has been seen in other two-component regulatory systems (Gunn and Miller, 1996; Seki *et al.*, 1987 and 1988). A low level of *phoP::xylE* transcription was observed in the absence of PhoP (Table 5). This may reflect *phoP* transcription independent of PhoP or it could simply be a result of *xylE* incorporation anywhere into the *P. aeruginosa* genome.

The sensor-kinase of this two-component regulatory system also shows sequence conservation at the amino acid level with the corresponding proteins from *S. typhimurium* and *E. coli* (Figure 3, 33% identity). Although the N-terminal sequence does not show high identity, the region after the second transmembrane segment shows greater similarity. More importantly, both the histidine at position 249 and the region possibly involved in ATP binding (amino acids 381 to 448) are conserved. The protein contains

two putative transmembrane domains and its location is assumed to be in the cytoplasmic membrane. It is notable that the sequence between the two membrane spanning regions, the periplasmic sensing domain, shows little similarity to the PhoQ proteins of *S. typhimurium* and *E. coli*. In addition, an amino acid difference at position 40 in the *P. aeruginosa* protein (isoleucine instead of threonine) is the same as that seen in a *S. typhimurium* mutant (*pho-24*, Thr48→ Ile) that constitutively expresses PhoP-activated genes (Gunn *et al.*, 1996). As the periplasmic region, and specifically Thr48, has been shown to be involved in sensing (García Véscovi *et al.*, 1997), it will be interesting to determine what differences in PhoQ function occur in *P. aeruginosa*.

The role of PhoQ is to modulate expression of PhoP-regulated genes in response to magnesium. This was evident from the constitutive, unregulated *oprH* and *phoP* transcription in the *phoQ* mutant (Figure 7 and 10, Table 4 and 6). Moreover, PhoQ downregulates PhoP mediated transcription, such that its deletion resulted in higher expression of OprH and *phoP* transcription than was observed in wildtype *P. aeruginosa*. In early studies of PhoP-PhoQ in *S. typhimurium*, higher level transcription of *phoP* was observed in a *phoQ* mutant though no negative regulatory effect for this protein has been proposed (Groisman *et al.*, 1989; Soncini *et al.*, 1995). In *P. aeruginosa*, expression of plasmid encoded PhoQ decreased *xylE* reporter gene transcription and OprH expression (Table 6 and Figure 8). It is accepted that most sensor kinases in two-component regulatory systems often possess phosphatase activity towards their cognate response regulators, especially when the phosphorylated response regulator is long lived (Stock *et al.*, 1995). PhoQ may act as a phosphatase towards phosphorylated PhoP and its absence, therefore, would allow a high level of activated PhoP to accumulate. Given that it is the

phosphorylated form of the regulator that is commonly believed to be the DNA binding species, this increase in phospho-PhoP would result in a high level of activation of the *oprH* promoter.

The question of how PhoP could be phosphorylated in the absence of PhoQ remains. The large number of possible two-component regulatory systems identified through homology searches of the *P. aeruginosa* genome allow for the possibility that a second kinase could phosphorylate PhoP through crosstalk between systems. Crosstalk has been demonstrated *in vitro* in *E. coli* between the OmpR-EnvZ, CheY-CheA and NRI-NRII systems (Igo *et al.*, 1989) and has been proposed to occur between PmrB of the PmrA-PmrB system and PhoP in *S. typhimurium* (Soncini and Groisman, 1996). Homologues of PmrA and PmrB have been identified in the *P. aeruginosa* genome by sequence homology searches and could be involved in crosstalk with PhoP-PhoQ. In addition, PhoP could be phosphorylated by a small molecule phosphodonor such as acetyl phosphate, as is the case for CheY in *E. coli* (Stock *et al.*, 1995). Finally, it may be possible that the unphosphorylated form of the response regulator activates transcription if it is present in high enough concentration. Given that PhoP is overexpressed in the *phoQ* mutant, high enough levels of this protein could be achieved to see activation by the unphosphorylated response regulator. Any one of these possibilities could explain the activation of reporter gene transcription in strain H851 (*phoP*::*xylE*-Gm^R) when PhoP was added back on a multicopy plasmid (pEMR3) without concomitant PhoQ expression (Table 5).

Of particular significance in the complementation of the *phoP* and *phoQ* mutants with plasmid borne genes, was the ability of a multicopy plasmid carrying *phoP* and

phoQ in reverse orientation to the *lac* promoter (pEMPQ2a) to restore back to wildtype *oprH* and *phoP* transcription. A similar plasmid carrying these genes behind the *lac* promoter (pEMPQ1b) could not complement. It is possible that the levels of PhoP and PhoQ produced from the latter plasmid were so high that they could not function like they do in wildtype *P. aeruginosa*. In this case it is also possible that PhoQ did not insert into the cytoplasmic membrane. On plasmid pEMPQ2a there are 93 bp of sequence upstream of *phoP* that encompass the entire intergenic region and 14 nucleotides of *oprH*. It is possible that in this region a promoter exists that drives expression of PhoP and PhoQ at low levels which, in multicopy, may be similar to the levels observed in wildtype *P. aeruginosa*. In addition, antisense regulation could occur through transcription of the opposite strand driven from the *lac* promoter. Whether antisense regulation plays a role in normal PhoP-PhoQ regulation, however, remains to be determined.

PhoP-PhoQ in *S. typhimurium* is indirectly involved in resistance to polymyxin B and other cationic antimicrobials through interaction with PmrA-PmrB (Roland *et al.*, 1993; Gunn and Miller, 1996). This second two-component system regulates expression of at least four genes involved in LPS modifications that may disrupt the ability of these positively charged antimicrobials to interact with the negatively charged membrane (Roland *et al.*, 1994; Gunn *et al.*, 1998; Guo *et al.*, 1998). Through regulation of, and interaction with PmrA-PmrB, PhoP-PhoQ activates expression of resistance genes in conditions of low magnesium and calcium. In *P. aeruginosa* PhoP-PhoQ also seems to play a role in regulation of polymyxin B resistance, though its role is not as straightforward as it is in OprH regulation (Macfarlane *et al.*, 1999). Wildtype *P.*

aeruginosa becomes resistant to polymyxin B under magnesium limiting conditions (Brown and Melling, 1969). Both the *phoP* and *phoQ* mutants remain resistant in low magnesium but the *phoQ* mutant also exhibits resistance in high magnesium (Macfarlane *et al.*, 1999). It was demonstrated that whenever a $PhoP^+$ $PhoQ^-$ phenotype occurred, resistance was seen in high magnesium as well as low, establishing the role of PhoP in the resistance phenotype. Moreover, when the phenotype was $PhoP^-$ $PhoQ^+$, susceptibility to polymyxin B occured in low magnesium, establishing a role for PhoQ in modulation of polymyxin B resistance. In this way PhoP may indirectly activate expression of genes necessary for resistance and PhoQ, in the absence of PhoP, may crosstalk to another system, such as PmrA-PmrB, to turn off expression of resistance genes.

A role for OprH in resistance to polymyxin B and EDTA has previously been proposed (Nicas and Hancock, 1980; 1983; Bell *et al.*, 1991). In low magnesium the positively charged protein takes the place of magnesium ions in stabilizing interactions with LPS molecules. Unlike these ions, this integral membrane protein could not be chelated by EDTA or displaced by cationic antimicrobials, and would contribute to resistance. Although complementation of an *oprH*::*tet* mutant with plasmid encoded OprH established the role of this protein in EDTA resistance, resistance to polymyxin B could not be restored by this plasmid alone (Bell *et al.*, 1991; Young *et al.*, 1992). Through work presented in this thesis it is now clear that the *oprH*::*xylE*-Gm^R mutant is deficient in PhoP and PhoQ expression as well. It is logical, therefore, to envision OprH as playing only a secondary role in polymyxin B resistance, while other PhoP-PhoQ regulated genes contribute to the bulk of this phenotype. Nevertheless, the fact that *oprH*

is transcriptionally linked to and regulated by a system responsible at least in part for polymyxin B resistance, suggests it may be important for this phenotype.

In conclusion, the work presented in this thesis has enabled identification and preliminary characterization of a PhoP-PhoQ two-component regulatory system in *P. aeruginosa*. Moreover, I have demonstrated that the genes encoding PhoP, PhoQ and the upstream OprH form an operon that is transcribed from a promoter upstream of *oprH*. The PhoP-PhoQ system regulates expression of both OprH and of itself in response to extracellular magnesium ion concentration. PhoP is necessary for transcription from the *oprH* promoter and PhoQ modulates the response to magnesium and down-regulates expression. Further study will enable us to better understand the role of PhoQ and the repeats in the *oprH* promoter region. This, in turn, may enable the identification of other genes regulated by PhoP-PhoQ in *P. aeruginosa*.

5 References

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

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1987 and updates. Current protocols in molecular biology. John Wiley andSons, Inc.

Baker, S. J., C. Daniels, and R. Morona. 1997. PhoP/Q regulated genes in *Salmonella typhi*: identification of melittin sensitive mutants. Microb. Pathogenesis **22**: 165-179.

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

Bell, A., and R. E. W. Hancock. 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.

Botzenhart, K., and H. Rüden. 1987. Hospital infections caused by *Pseudomonas aeruginosa*. In "Basic Research and Clinical Aspects of *Pseudomonas aeruginosa*", G. Döring, I. A. Holder, and K. Botzenhart (Eds.), Karger, Basal, Switzerland. 1-15.

Brown, M. R. W., and J. Melling. 1969. Role of divalent cations in the action of polymyxin B and EDTA on *Pseudomonas aeruginosa*. J. Gen. Microbiol. **54**: 263-274.

Brown, M. R. W., H. Anwar, and P. A. Lambert. 1984. Evidence that mucoid *Pseudomonas aeruginosa* in the cystic fibrosis lung grows under iron-restricted conditions. FEMS Microbiol. Lett. **21**: 113-117.

Bryan, L. E. 1979. Resistance to antimicrobial agents: the general nature of the problem and the basis of resistance. In "*Pseudomonas aeruginosa*: Clinical Manifestations of Infection and Current Therapy", R. D. Doggett (Ed.), Academic Press, New York, NY. 219-270.

Clarke, P. H., and J. H. Slater. 1986. Evolution of enzyme structure and function in *Pseudomonas*. In "The Bacteria, Volume X. The Biology of *Pseudomonas*", J. R. Sokatch (Ed.), Academic Press, Orlando, FL. 71-75.

Dennis, J. J., and P. A. Sokol. 1995. Electrotransformantion of *Pseudomonas*. Methods Mol. Biol. **47**: 125-133.

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

Döring, G., M. Maier, E. Müller, Z. Bibi, B. Tümmler, and A. Kharazmi.1987. Virulence factors of Pseudomonas aeruginosa. In "Basic Research and Clinical Aspects of *Pseudomonas aeruginosa*", G. Döring, I. A. Holder, and K. Botzenhart (Eds.), Karger, Basal, Switzerland. 136-148.

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

García Véscovi, E., Y. M. Ayala, E. Di Cera, and E. A. Groisman. 1997. Characterization of the bacterial sensor protein PhoQ. J. Biol. Chem. **272**: 1440-1443.

García Véscovi, E., Y. M., F. C. Soncini, and E. A. Groisman. 1996. Mg^{2+} as an extracellular signal: environmental regulation of *Salmonella* virulence. Cell **84**: 165-174.

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

Goldberg, J. B., and D. E. Ohman. 1984. Cloning and expression in *Pseudomonas aeruginosa* of a gene involved in the production of alginate. J. Bacteriol. **158**:1115-1121.

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

Groisman, E. A., F. Heffron, and F. Solomon. 1992. Molecular analysis of the *Escherichia coli phoP* locus. J. Bacteriol. **174**: 486-491.

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

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

Gunn, J. S., and S. L. Miller. 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., E. L. Hohmann, and S. L. Miller. 1996. Transcriptional regulation of *Salmonella* virulence: a PhoQ periplasmic domain mutation results in increased net phosphotransfer to PhoP. J. Bacteriol. **178**: 6369-6373.

Guo, L., K. B. Lim, C. M. Podule, M. Daniel, J. S. Gunn, M. Hackett, and S. I. Miller. 1998. Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. Cell **95**: 189-198.

Guo, L., K. B. Lim, J. S. Gunn, B. Bainbridge, R. P. Darveau, M. Hackett, and S. I. Miller. 1997. Regulation of lipid A modification by *Salmonella typhimurium* virulence genes phoP-phoQ. Science **276**: 250-253.

Hancock,, R. E. W. 1997. The bacterial outer membrane as a drug barrier. Trends in Microbiol. **5**: 37-42.

Hancock, R. E. W., and D. P. Speert. 1996. Antibiotics for Pseudomonas and related infections. In "Cystic Fibrosis-Current Topics, Volume 3", J. A. Dodge, J. H. Brock, and J. H. Widdecombe (Eds.), John Wiley & Sons Ltd., New York, NY. 245-266.

Hancock, R. E. W., R. Siehnel, and N. Martin. 1990. Outer membrane proteins of *Pseudomonas*. Mol. Microbiol. **4**: 1069-1075.

Hancock, R. E. W., V. J. Raffle, and T. I. Nicas. 1981. Involvement of the outer membrane in gentamicin and streptomycin uptake and killing in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **19**: 777-785.

Hancock, R. E. W., and A. M. Carey. 1979. Outer membrane of *Pseudomonas aeruginosa*: heat- and 2-mercaptoethanol-modifiable proteins. J. Bacteriol. **140**: 902-910.

Henihoff, S. 1984. Unidirectional digestion with exonuclease III creates targetd breakpoints for DNA sequencing. Gene. **28**: 351-359.

Hoch, J. A. 1995. Control of cellular development in sporulating bacteria by phosphorelay. In "Two-Component Signal Transduction", J. A. Hoch, and J. S. Thomas (Eds.), ASM Press, Washington, DC. 129-144.

Hofmann, K., and W. Stoffel. 1993. TMBASE - a database of membrane spanning protein segments. Biol. Chem. Hoppe-Seyler. **374**: 166

Høiby, N., G. Döring, P. O. Schiøtz. 1987. Pathogenic mechanisms of chronic Pseudomonas aeruginosa infection in Cystic Fibrosis patients. In "Basic Research and Clinical Aspects of *Pseudomonas aeruginosa*", G. Döring, I. A. Holder, and K. Botzenhart (Eds.), Karger, Basal, Switzerland. 60-76.

Igo, M. M., A. J. Ninfa, J. B. Stock, and T. J. Silhavy. 1989. Phosphorylation and dephosphorylation of a bacterial transcriptional activator by a transmembrane receptor. Gen. Develop. **3**: 1725-1734.

Ingelbreht, I. L., C. I. Mandelbaum, and T. E. Mirkov. 1998. Highly sensitive Northern hybridization using a rapid protocol for downward alkaline blotting of RNA. BioTechniques **25**: 420-498.

Kasahara, A., A. Nakata, and H. Shinagawa. 1992. Molecular analysis of the *Escherichia coli phoP-phoQ* operon. J. Bacteriol. **174**: 492-498.

Kiers, L. D., R. M. Weppelman, and B. N. Ames. Regulation of nonspecific acid phosphatase in *Salmonella*: *phoN* and *phoP* genes. J. Bacteriol. **138**: 155-161.

Korn, L. J., and C. Queen. 1984. Analysis of biological sequences on small computers. DNA. **3**: 421-436.

Kropinski, A. M. , L. C. Chan, and F. H. Milazzo. 1979. The extraction and analysis of lipopolysaccharide from *Pseudomonas aeruginosa* strain PAO, and three rough mutants. Can. J. Microbiol. **25**: 390-398.

Liu, H. P., S. Takio, T. Satoh, and I. Yamamaoto. 1999. Involvement in denitrification of the *napKEFDABC* genes encoding the periplasmic nitrate reductase system in the denitrifying phototrophic bacterium *Rhodobacter sphaeroides* f. sp. *denitrificans*. Biosci. Biotechnol. Biochem. **63**: 530-536.

Liu, P. V. 1974. Extracellular toxins of *Pseudomonas aeruginosa*. J. Infect. Dis. **130** (suppl); S94-S99.

Liu, W., Y. Qi, and F. M. Hulett. 1998. Sites internal to the coding regions of *phoA* and *pstS* bind PhoP and are required for full promoter activity. Mol. Microbiol. **28**: 119-130.

Macfarlane, E. L. A., A. Kwasnicka, M. M. Ochs, and R. E. W. Hancock. 1999. PhoP-PhoQ homologues in *Pseudomonas aeruginosa* regulate expression of the outer-membrane protein OprH and polymyxin B resistance. Mol. Microbiol. in press.

Miller, S. I., W. S. Pulkkinen, M. E. Selsted, and J. J. Mekalanos. 1990. Characterization of defensin resistance phenotypes associated with mutations in the phoP virulence regulon of *Salmonella typhimurium*. Infect. Immun. **58**: 3706-3710.

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

Moore, R. A., W. A. Woodruff, and R. E. W. Hancock. 1987. Antibiotic uptake pathways across the outer membrane of *Pseudomonas aeruginosa*. In "Basic Research and Clinical Aspects of *Pseudomonas aeruginosa*", G. Döring, I. A. Holder, and K. Botzenhart (Eds.), Karger, Basal, Switzerland. 172-181.

Morbidoni, H. R., D. Demendoza, and J. E. Cronan. 1996. *Bacillus subtilis* acyl carrier protein is encoded in a cluster of lipid biosynthesis genes. J. Bacteriol. **178**: 4794-4800.

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

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

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

Nikaido, H., and R. E. W. Hancock. 1986. Outer membrane permiability of *Pseudomonas aeruginosa*. In "The Bacteria, Volume X. The Biology of *Pseudomonas*", J. R. Sokatch (Ed.), Academic Press, Orlando, FL. 154-193.

Nikaido, H., and M. Vaara. 1985. Molecular basis of bacterial outer membrane permiability. Microbiol. Rev. **49**: 1-32.

Ochs, M. M., M. P. McCusker, M. Bains, and R. E. W. Hancock. 1999. Negative regulation of the *Pseudomonas aeruginosa* outer membrane porin OprD selective for imipenem and basic amino acids. Antimicrob. Agents Chemother. **43**: 1085-1090.

Patt, L. A., and Silhavy, T. J. 1995. Porin regulon of *Escherichia coli*. In "Two-Component Signal Transduction", J. A. Hoch, and J. S. Thomas (Eds.), ASM Press, Washington, DC. 105-127.

Pegues, D. A., M. J. Hantman, I. Behlau, and S. I. Miller. 1995. PhoP/PhoQ transcriptional repression of *Salmonella typhimurium* invasion genes: evidence for a role in protein secretion. Mol. Microbiol. **17**: 169-181.

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

Roland, K. L., C. R. Esther, and J. K. Spintznagel. 1994. Isolation and charcterization of a gene, *pmrD*, from *Salmonella typhimurium* that confers resistance to polymyxin when expressed in multiple copies. J. Bacteiol. **176**: 3589-3597.

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

Sambrook, J., E. F. Frisch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbour, NY.

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

Schindler, M., and M. J. Osborn. 1979. Interaction of divalent cations and polymixin B with lipopolysaccharide. Biochem. **18**: 4425-4430.

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

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

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

Seki. T., H. Yoshikawa, H. Tukahashi, H. Saito. 1988. Nucleotide sequence of the *Bacillus subtilis phoR* gene. J. Bacteriol. **170**: 5935-5938.

Seki. T., H. Yoshikawa, H. Tukahashi, H. Saito. 1987. Cloning and nucleotide sequence of *phoP*, the regulatory gene for alkaline phosphatase and phosphdiesterase in *Bacillus subtilis*. J. Bacteriol. **169**: 2913-2916.

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

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

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

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

Stock, J. B., M. G. Surette, M. Levit, and P. Park. 1985. Two-component signal transduction systems: structure-function relationships and mechanisms of catalysis. In "Two-Component Signal Transduction", J. A. Hoch, and J. S. Thomas (Eds.), ASM Press, Washington, DC. 25-51.

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

Young, M. L., M. Bains, A. Bell, and R. E. W. Hancock. 1992. Role of *Pseudomonas aeruginosa* outer membrane protein OprH in polymyxin and gentamicin resistance: isolation of an OprH-deficient mutant by gene replacement techniques. 1992. Antimicrob. Agents Chemother. **36**: 2566-2568.