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The Response Regulator PhoP Is Important for Survival under Conditions of Macrophage-Induced Stress and Virulence in *Yersinia pestis*

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The two-component regulatory system PhoPQ has been identified in many bacterial species. However, the role of PhoPQ in regulating virulence gene expression in pathogenic bacteria has been characterized only in *Salmonella* species. We have identified, cloned, and sequenced PhoP orthologues from *Yersinia pestis*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica*. To investigate the role of PhoP in the pathogenicity of *Y. pestis*, an isogenic *phoP* mutant was constructed by using a reverse-genetics PCR-based strategy. The protein profiles of the wild-type and *phoP* mutant strains, grown at either 28 or 37°C, revealed more than 20 differences, indicating that PhoP has pleiotrophic effects on gene expression in *Y. pestis*. The mutant showed a reduced ability to survive in J774 macrophage cell cultures and under conditions of low pH and oxidative stress in vitro. The mean lethal dose of the *phoP* mutant in mice was increased 75-fold in comparison with that of the wild-type strain, indicating that the PhoPQ system plays a key role in regulating the virulence of *Y. pestis*.

Yersinia pestis is the etiological agent of bubonic plague, a serious and often fatal disease in humans. The organism is usually transmitted through the bite of an infected flea. Each case of plague has public health significance, since patients with subsequent pulmonary involvement may act as sources of pneumonic plague, a fatal and highly transmissible form of the disease (31). The expression of many virulence genes in *Y. pestis* is upregulated at 37°C. For example, bacteria transmitted from a flea, where they have been growing at ambient temperatures of 28°C or below, do not express the F1 capsular antigen or many of the low-calcium-response plasmid products, which are thought to allow the bacteria to resist phagocytosis (6, 31, 36). Many of the bacteria delivered into the host are phagocytosed by polymorphonuclear leukocytes or macrophages. The trafficking of phagocytes to local lymph nodes and the multiplication of bacteria within the lymph nodes, give rise to a bubo (the characteristic sign of the infection). The bacteria within polymorphonuclear leukocytes are destroyed, but those within macrophages survive and express various virulence determinants, allowing their growth and eventual release from the macrophages, resulting in a bacteremia (2). Thus, during infection, the bacteria undergo an intracellular phase in phagocytic cells and a later extracellular phase. This change in the site of colonization must involve changes in the biochemical makeup of the cell to allow survival and growth in these different environments, and it is likely that the bacteria possess mechanisms which allow sensing of environmental changes and corresponding modulation of gene expression.

The modulation of the expression of virulence determinants is a recurrent theme in the pathogenicity of a variety of organisms. This modulation is often controlled at the transcriptional level by a twin superfamily of sensor and regulator proteins (13, 22, 37). In the case of *Salmonella enterica* serovar Typhimurium, a two-component regulatory system involving PhoP (the transcriptional regulator) and PhoQ (the sensor kinase) has been shown to play a key role in adaptation of the bacteria to intracellular environments and for survival within macrophages (14, 19, 23). PhoP has been shown to regulate more than 40 polypeptides; the PhoP-activated genes are induced when *Salmonella* serovar Typhimurium is in an intracellular environment, while other genes are repressed by PhoP in this environment. Many of the PhoP-repressed genes are maximally expressed in extracellular environments encountered by *Salmonella* serovar Typhimurium, such as during the invasion of epithelial cells (4). Therefore, two virulence properties of *Salmonella* serovar Typhimurium, induction of endocytosis by epithelial cells and intramacrophage survival, are oppositely regulated by the PhoPQ regulon.

Given the importance of the PhoPQ signal transduction system in *Salmonella* serovar Typhimurium, we set out to determine whether a PhoPQ system is present in pathogenic *Yersinia* and to analyze the genes encoding this system at a molecular level. This information has allowed us to construct an isogenic *Y. pestis phoP* mutant and thereby determine the role of the PhoPQ system in survival within macrophages and the effect of the *phoP* mutation on the virulence of *Y. pestis*.

MATERIALS AND METHODS

Bacterial strains, plasmids, growth conditions, and chemicals. Bacterial strains and plasmids used in this study are listed in Table 1. *Y. pestis* was routinely cultured aerobically at 28°C in blood agar base (BAB) broth, on BAB agar (30), on Congo red agar (including 25 g of heart infusion broth/liter) (33), or on *Yersinia* selective agar (Oxoid, Basingstoke, United Kingdom). Defined media were prepared as described by Straley and Bowmer (39). *Escherichia coli* strains were cultured and stored as described by Sambrook et al. (34). All chemicals were purchased from Sigma-Aldrich (Poole, United Kingdom). Ampicillin and tetracycline were used at final concentrations of 55 and 20 μg/ml, respectively.

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TABLE 1. Bacterial strains and plasmids used in this study[a]

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
Y. pestis		
GB	Virulent wild-type strain, bv. orientalis	33
SAI2.2	*Y. pestis* GB *phoP* mutant	This study
Y. pseudotuberculosis YPIII pIB1	Virulent wild-type strain, serotype III	5
Y. enterocolitica 8081	Virulent wild-type strain, serotype O8	5
E. coli		
XL2-Blue MRF′	Cloning strain	Stratagene
CC118 λpir	Cloning strain	29
S17 λpir pNJ5000	Triple mating strain with Tet^r helper plasmid	18
Plasmids		
pUC19	Ap^r	Pharmacia
pCVD442	Ap^r Suc^s, suicide vector	9
pNJ5000	Tet^r, for triple mating	18
pYP5	pUC19 containing 262-bp PCRDOP gene fragment of *Y. pestis phoP*	This study
pYPTB7	pUC19 containing 262-bp PCRDOP gene fragment of *Y. pseudotuberculosis phoP*	This study
pDA5	pUC19 plus 4-kb *Dra*I chromosomal fragment containing *phoP*	This study
pSA2	pUC19 and 1.5-kb *Ssp*I chromosomal fragment containing *phoP*	This study
pSAI1	pSA2 with 31-bp deletion in *phoP*	This study
pSAI2	Donor plasmid for conjugation; Ap^r Suc^s, pSA1 *Xba*I-*Pvu*II fragment containing the mutated *phoP* gene cloned into *Xba*I-*Sma*I pCVD442	This study

[a] Abbreviations: Ap^r, ampicillin resistant; Tet^r, tetracycline resistant; Suc^s, sucrose sensitive.

Unless otherwise stated, plasmid and genomic extractions, restriction enzyme digestions, DNA ligations, and transformations into *E. coli* were performed by standard procedures (34) using enzymes provided by Promega Ltd. (Southampton, United Kingdom) or Boehringer Mannheim (Lewes, United Kingdom).

PCR and cloning procedures. Oligonucleotide primers used for PCR are summarized in Table 2. The *Y. pestis*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica phoP* gene fragments were amplified using PCR with degenerate oligonucleotide primers (PCRDOP) (42) with primers P3 and P4, based on known PhoP sequence data for *E. coli* and *Salmonella* serovar Typhimurium (27). The PCR was performed with 40 cycles of 1 min at 94°C, 1 min at 40°C, and 1 min at 72°C. The 262-bp products were digested with *Pst*I and *Hin*dIII, ligated into similarly digested pUC19, and transformed in *E. coli* XL2-Blue MRF′ cells (Stratagene Europe, Amsterdam, The Netherlands). The cloned fragments were sequenced using the dideoxynucleotide chain termination method with an Applied Biosystems (Warrington, United Kingdom) PRISM sequencing kit, and the data were compared with other PhoP sequences using BLASTX software (1).

To determine the entire *phoP* sequence, the cloned *Y. pseudotuberculosis phoP* gene fragment was used with Southern blotting to identify a 4-kbp *Dra*I fragment and a 1.5-kbp *Ssp*I fragment from digests of *Y. pseudotuberculosis* chromosomal DNA. The fragments were isolated from a SeaPlaque GTG agarose gel (Flowgen, Sittingbourne, United Kingdom), ligated into pUC19/*Sma*I/BAP (Amersham Pharmacia Biotech, St. Albans, United Kingdom), and transformed into *E. coli* XL2-Blue MRF′ cells to generate plasmids pDA5 and pSA2, respectively. The nucleotide sequences of these cloned fragments allowed the design of primers P41 and P42, which were used to amplify a 1.5-kb product containing the entire *Y. pestis* or *Y. pseudotuberculosis phoP* genes. Both PCR products were purified through S-300HR (Amersham Pharmacia Biotech) and nucleotide sequenced. The complete *phoP* gene sequences from *Y. pestis* and *Y. pseudotuberculosis* were determined from sequence data from three independent PCR reactions.

A 31-bp deletion and unique *Bgl*II site were engineered into the *phoP* gene in plasmid pSA2, using inverse-PCR mutagenesis (IPCRM) (10, 43), with primers P9 and P10. Fifty picograms of alkali-denatured pSA2 was added to a PCR mixture containing primers P9 and P10 and subjected to PCR with 40 cycles of 1 min at 94°C, 1 min at 50°C, and 4 min at 72°C. PCR products were digested with *Bgl*II, self ligated, and transformed into *E. coli* XL2-Blue MRF′ to generate plasmid pSAI1. Primers P27 and P24, which flank the deletion site, were used to screen for mutants by PCR, using 1 μl of boiled cell supernatant added to a standard reaction mixture, which was subjected to 40 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C.

Construction of a *Y. pestis phoP* mutant. Plasmid DNA from pSAI1 was digested with *Xba*I and *Pvu*II, and the excised fragment containing the mutated *Y. pseudotuberculosis phoP* gene was ligated with pCVD442, which had been previously digested with *Xba*I and *Sma*I. This ligation was electroporated into *E. coli* CC118 λpir cells to form pSAI2. pSAI2 was introduced into *Y. pestis* strain GB by conjugation in a three-way mating (25), using a *Y. pestis* GB wild-type recipient, *E. coli* CC118 λpir pSAI2, and *E. coli* S17 λpir pNJ5000 (18). Equal volumes (50 μl) of the three strains were mixed, and 100 μl was spotted onto an L agar plate. After incubation (28°C for 8 h), the bacteria were recovered, resuspended in 1 ml of Luria-Bertani (LB) broth, washed twice in LB broth, and

TABLE 2. Oligonucleotides used for PCR

Primer	PCR method	Strand	Sequence (5′–3′)[a,b]
P3	PCRDOP	+	AATCTGCAGYTNMGNCAYCAYYTNAANGT
P4	PCRDOP	−	CCAAGCTTNARNACYTCNACYTTPTCYTG
P9	IPCRM	+	CCAGATCTGGATGGCTTAAGCCTTATC
P10	IPCRM	−	AAAGATCTTATCTGGGCCATGTTCCTG
P24	*phoP* specific	−	ACTTTATCTTGCCAGCTTT
P27	*phoP* specific	+	CGCGTTGTTGCGTCACCAT
P41	*phoP* amplification	+	ACCTATCACCAGATATTGGCGTG
P42	*phoP* amplification	−	CCCATCATCATTCTACTGATGTGCG

[a] Underlined nucleotides represent *Pst*I (P3), *Hin*dIII (P4), and *Bgl*II (P9 and P10) restriction endonuclease sites.
[b] R = A or G; Y = C or T; M = A or C; N = A, C, G, or T.

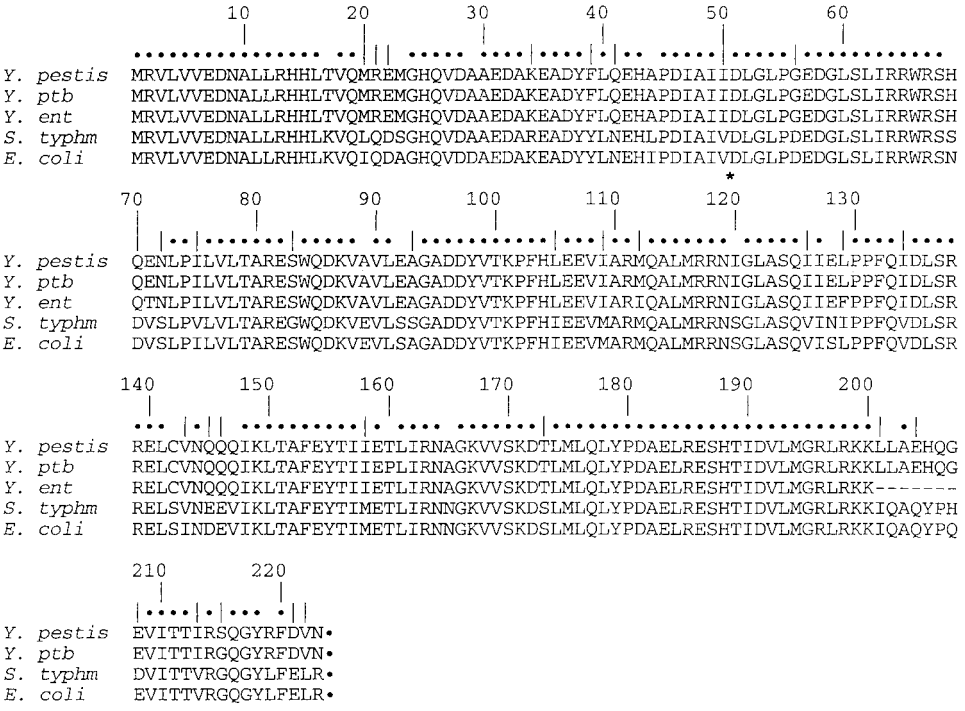

FIG. 1. Comparison of the deduced PhoP amino acid sequences of *Y. pestis*, *Y. pseudotuberculosis* (*Y. ptb*), *Y. enterocolitica* (*Y. ent*), *E. coli*, and *Salmonella* serovar Typhimurium (*S. typhm*), aligned by using Clustal V multiple sequence alignment software. Identical amino acids (●), conserved changes (|), and the essential aspartic acid residue (*) are shown.

cultured on *Yersinia* selective agar containing ampicillin at 28°C. Individual ampicillin-resistant *Y. pestis* colonies were inoculated into BAB broth, grown overnight at 28°C, and plated onto BAB agar or BAB agar supplemented with 5% (wt/vol) sucrose (9). Sucrose-tolerant revertants occurred at a rate of 1 in 3×10^4, and these colonies were screened using PCR with primers P27 and P24, followed by digestion of the PCR product with *Bgl*II. An ampicillin-sensitive *Y. pestis phoP* double-crossover mutant, termed SAI2.2, was identified and used for further studies.

Survival following environmental stresses and exposure to defensins. The effect of low pH, high osmolarity, and oxidative stress on *Y. pestis* was determined as described by Badger and Miller (3). *Y. pestis* wild-type and *phoP* mutant strains were grown overnight at 28°C, and the cells were pelleted and resuspended appropriately. For oxidative stress experiments, cells were incubated for 1 h at 28°C in 15 mM H_2O_2 in deionized water. For high-osmolarity stress, cells were incubated in 2.4 M NaCl in deionized water for 1 h at 28°C. For low-pH stress, cells were incubated in LB broth, adjusted to pH 3 with HCl, for 5 min at ambient temperature (22°C). Control bacteria were incubated in sterile phosphate-buffered saline (PBS) (pH 7.4). After exposure, the bacteria were pelleted at $12,000 \times g$ for 5 min at 22°C, resuspended in 0.9% (wt/vol) NaCl, and enumerated after growth at 28°C for 48 h on Congo red agar. All survival experiments were repeated in triplicate.

Survival in J774 macrophage cell cultures. *Y. pestis* cells from 1-ml volumes of an 18-h culture grown at 28°C were diluted with 9 ml of PBS, centrifuged at $10,000 \times g$ for 10 min at 22°C, and resuspended in 10 ml of PBS. The number of viable cells was determined after culturing dilutions of the cell suspensions on Congo red agar. J774 macrophage cells were seeded at a density of 5×10^5 ml^{-1} in Dulbecco's modified essential medium (Sigma-Aldrich) into 24-well tissue culture dishes and cultured until confluent. The tissue culture medium was removed, 150 μl (10^6 cells) of the bacterial suspension in PBS was added, and the cells were incubated at 37°C for 30 min. The suspension above the cell monolayer was removed, and the cells were washed three times with PBS. One milliliter of 2% L15 medium (Sigma-Aldrich) containing 10 μg of gentamicin/ml was added, and the cells were incubated for 30 min at 37°C. The cells were washed twice with PBS, and 1 ml of 2% L15 medium containing 2 μg of gentamicin/ml was added to the cells. The cells were incubated at 37°C, and at various time points the growth medium was removed, the cells were washed with PBS, and 200 μl of 0.1% sodium deoxycholate was added to the cells, which were lysed by aspiration. The lysate was diluted in PBS, and the number of viable cells was determined after growth at 28°C for 48 h on Congo red agar. Duplicate samples were taken at all time points, and the assay was repeated four times.

2D gel electrophoresis. Protein profiles of *Y. pestis* wild-type and *phoP* mutant strains were compared by two-dimensional (2D) gel electrophoresis. Bacteria were grown for 6 h at 28°C or 37°C in BAB broth. Cells were washed once with PBS and then boiled for 10 min in a 0.1% (wt/vol) sodium dodecyl sulfate (SDS) solution. Cellular debris was removed by centrifugation, and then the proteins were concentrated using Amicon Centriplus concentrators (Millipore, Watford, United Kingdom) and stored at −20°C. Culture supernatants were treated with protease inhibitors (Complete; Roche Diagnostics Ltd., Lewes, United Kingdom), 9 mM urea, 65 mM dithiothreitol, and 2% (vol/vol) Triton X-100 to obtain completely denatured and reduced proteins. Proteins were separated using 2D gel electrophoresis as described by Gorg et al. (17). Immobiline dry strips pH 4-7 linear (18 cm; Amersham Pharmacia Biotech) were rehydrated with each protein sample (30 to 40 μg) as recommended by the manufacturers. Isoelectric focusing was carried out for 44,900 V · h at 20°C. After equilibration, horizontal SDS electrophoresis was carried out using Excel Gel SDS, with a gradient of 12 to 14% T (Amersham Pharmacia Biotech) at 15°C. The separated proteins were then visualized by a silver staining method (21). Molecular weight standards (Bio-Rad, Hemel Hempstead, United Kingdom) were also applied to the gel.

Determination of virulence in mice. The median lethal doses (MLD) of the *Y. pestis* wild-type and *phoP* mutant were assessed by subcutaneous injection of groups of five female 6-week-old BALB/c mice (Charles River Laboratories, Margate, United Kingdom) with serial dilutions of exponential-phase broth cultures grown at 28°C (33). Humane endpoints were strictly observed, and animals deemed incapable of survival were humanely killed by cervical dislocation. Times to humane death (typically 3 to 5 days) were recorded, and the MLD of the *phoP* mutant was determined by the method of Reed and Muench (32).

Nucleotide sequence accession numbers. The nucleotide sequences of the *Y. pestis* and *Y. pseudotuberculosis phoP* genes have been submitted to the EMBL database under accession numbers Y08758 and X66587, respectively.

RESULTS

Analysis of the *Y. pestis phoP* gene. PCRDOP experiments consistently amplified a single band of 262 bp from *Y. pestis* (GB), *Y. pseudotuberculosis* (YPIIIpIBI), and *Y. enterocolitica* (8081). Southern blot analysis with the 262-bp *Y. pseudotuberculosis phoP* gene fragment used as a probe against *Y. pseudotuberculosis* chromosomal DNA digested with *Dra*I and *Ssp*I revealed single hybridization bands of 4.0 and 1.5 kb, respectively (data not shown). These chromosomal fragments were cloned into pUC18 and sequenced. These sequence data allowed the design of PCR primers and the subsequent amplification of the entire *phoP* genes from both *Y. pestis* and *Y.*

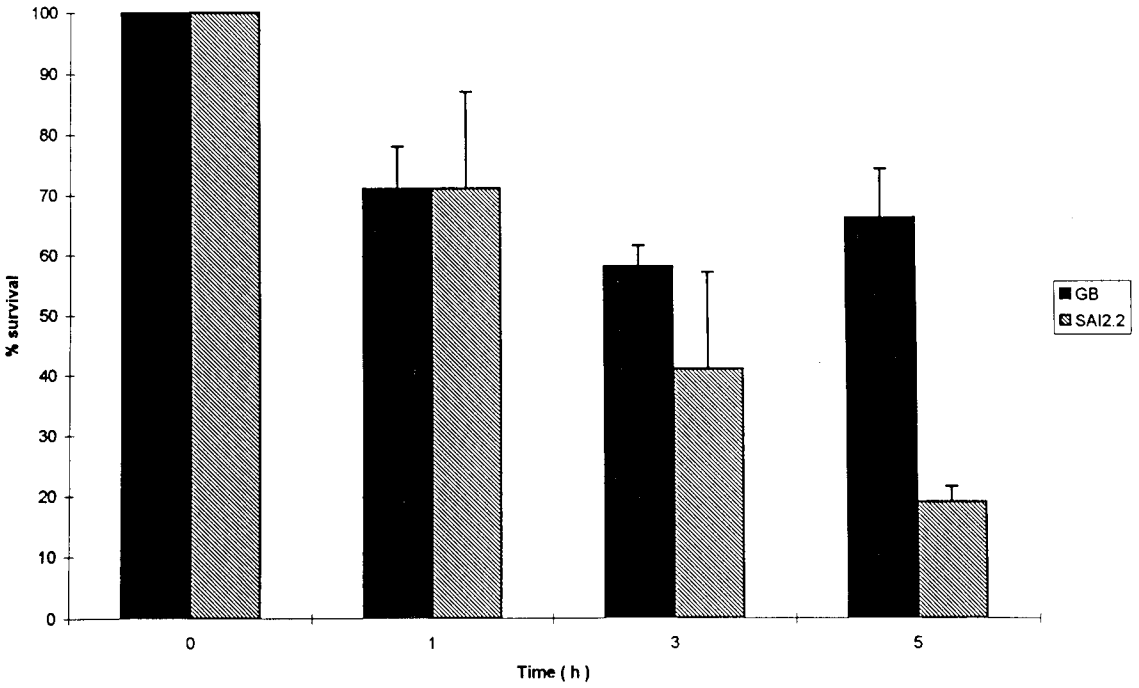

FIG. 2. Survival of *Y. pestis* wild-type (GB) and *phoP* mutant (SAI2.2) strains after uptake by J774 macrophages. Results shown are the means of duplicate determinations in two separate experiments, with standard errors.

pseudotuberculosis. Sequence analysis of amplified 1.5-kbp sequences revealed the presence of a *phoQ* orthologue 5 bp downstream of *phoP* (data not shown). The *phoP* open reading frame encoded a protein with an M_r of 25,586. The deduced amino acid sequence of the *Y. pestis* PhoP was aligned with reported sequences of PhoP from *E. coli* and *Salmonella* serovar Typhimurium and with the PhoP sequence from *Y. pseudotuberculosis* and *Y. enterocolitica*, using the Clustal V multiple sequence alignment software (Fig. 1). The *Y. pestis* PhoP revealed 99.6, 99.5, 94.2, and 93.3% similarity and 99.1, 98, 82.3, and 80.4% identity to the respective *Y. pseudotuberculosis*, *Y. enterocolitica*, *E. coli*, and *Salmonella* serovar Typhimurium counterparts. The *Y. pestis* PhoP contained the conserved aspartate (D) residue at position 51, which is the likely site of phosphorylation by PhoQ. Analysis of the *Y. pestis* (CO92) genome sequence recently released by the Sanger Centre (http://www.sanger.ac.uk/Projects/Y_pestis/) revealed a single copy of the *phoP* gene and confirmed the presence of the *phoQ* gene immediately downstream of *phoP*.

Construction of the *Y. pestis phoP* mutant. An isogenic *Y. pestis phoP* mutant was constructed by allelic replacement using the *Y. pseudotuberculosis phoP* gene, which had a deletion of the conserved aspartate residue at position 51 that is required for phosphorylation of response regulators. There was sufficient homology between the *phoP* genes from *Y. pseudotuberculosis* and *Y. pestis* to allow recombination to occur between the *Y. pseudotuberculosis* gene carried by pSAI2 and the gene on the *Y. pestis* chromosome. The mutation was confirmed by PCR and subsequent digestion of the PCR product to show the presence of the unique *Bgl*II site within the amplified region that was absent in the wild-type gene. Further confirmation that the second crossover event had occurred was the loss of the ampicillin resistance gene marker in the newly constructed mutant.

Contribution of PhoP to survival under stresses. The effect of the *phoP* mutation on the phenotype was examined by subjecting the mutant and wild-type strains to a range of environmental stresses. The mutation in *phoP* rendered the bacteria slightly more sensitive to low pH and oxidative killing ($P < 0.1$, using the Student *t* test with unpaired sets) and significantly more sensitive to high osmolarity ($P < 0.05$, using the Student *t* test with unpaired sets).

Survival in J774 macrophages. J774 macrophage cell cultures were infected with either *Y. pestis* GB or *Y. pestis* SAI2.2. After incubation to allow uptake of bacteria, the extracellular bacteria were killed with gentamicin. At 0, 1, 3, or 5 h postinfection, infected cells were lysed, and the number of viable bacteria within the cells was determined. The results showed that in the case of both the wild-type and the *phoP* mutant strains, the number of viable bacteria declined during the first hour (Fig. 2). However, whereas 34% of the wild-type bacteria were killed by macrophages 5 h postinfection, 81% of the *phoP* mutants had been killed by this time.

Protein expression by a *Y. pestis phoP* mutant. The protein expression profiles of *Y. pestis* wild-type and *phoP* mutant strains grown in BAB broth at both 28 and 37°C were studied (Fig. 3 and 4). The 2D patterns of both strains at both temperatures investigated are highly similar and comparable. Only obvious differences recognizable by visual evaluation are reported.

Virulence of the *phoP* mutant in mice. The *Y. pestis phoP* mutant was less virulent in mice than the wild-type strain. The reported MLD for the GB wild-type strain is 1 CFU (33). The *phoP* mutant was determined to have an MLD of 75 CFU.

DISCUSSION

Many pathogenic bacteria colonize a variety of niches within the host, and the differential expression of bacterial genes is necessary to allow the bacterium to survive and proliferate in these different environments. The rapid adaptation of pathogens to these different niches is often achieved via two-

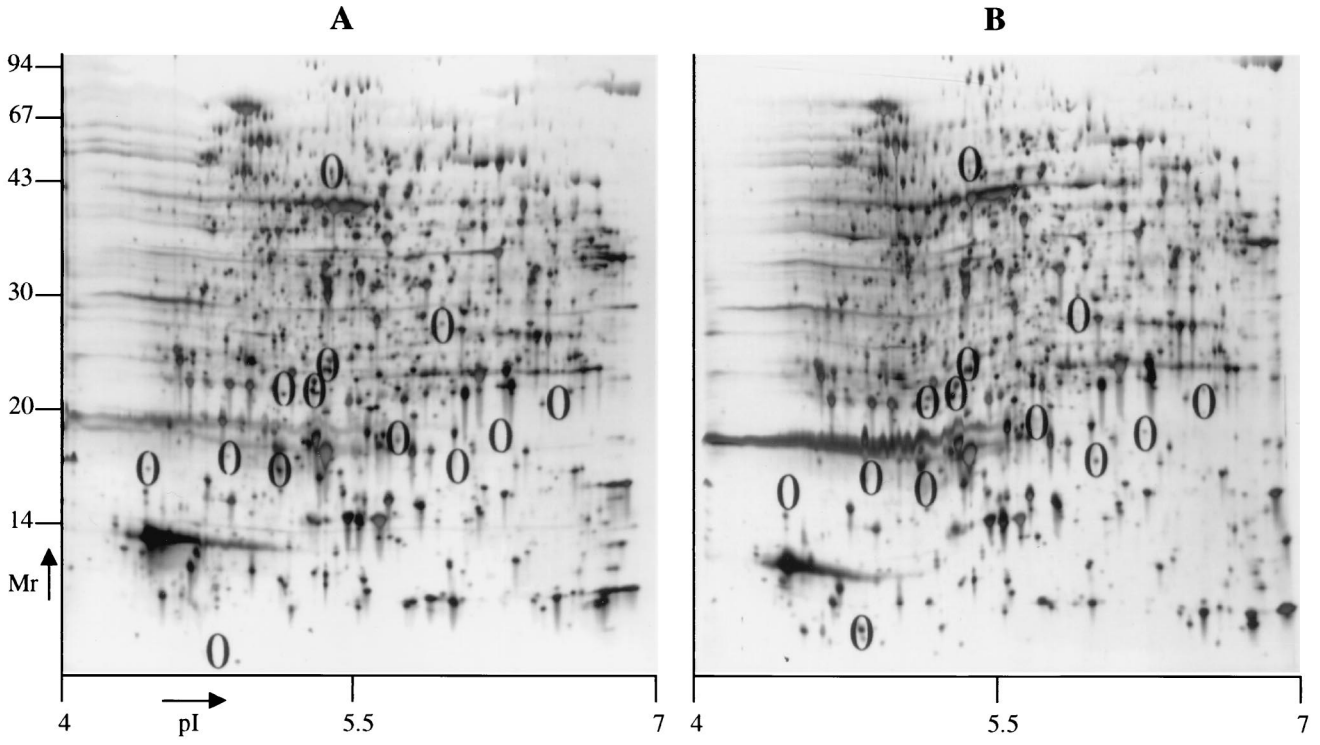

FIG. 3. 2D gel electrophoresis protein expression profiles for the *Y. pestis* wild-type strain (a) and *Y. pestis phoP* mutant strain (b) grown at 28°C. Differences in protein expression are highlighted (0). Mr, molecular weights in thousands.

component regulatory systems, which enable changes in the environment to be detected and thus regulate bacterial gene expression accordingly. One such system is the PhoPQ regulon, which has been characterized for *E. coli* and *Salmonella* serovar Typhimurium. In this system, PhoQ senses changes in the environment, which results in autophosphorylation of a histidine residue and the subsequent phosphorylation of an aspartate residue in PhoP, which is then able to coordinate gene expression. Different sets of genes are differentially regulated by this system. The *Salmonella* serovar Typhimurium PhoPQ regulon plays a central role in the regulation of several determinants that are required to be differentially expressed during infection (13, 14, 23). The pleiotropic role for the PhoPQ regulon in *Salmonella* serovar Typhimurium has been confirmed by mutational analysis of *phoP* and is related to several phenotypic changes, including deficiency in epithelial cell invasion (4), increased susceptibility to cationic peptides (12) and low pH (28), reduced survival rates in macrophages (11), and altered presentation of antigens (41).

Hybridization studies have suggested that PhoPQ is widely distributed among enteric bacteria (19), but only the *Salmonella* serovar Typhimurium and *E. coli* genes have been characterized in detail. In this study, we have shown that a PhoPQ signal transduction system operates in *Y. pestis*. Alignment of the deduced amino acid sequences of the *Y. pestis* PhoP with those from *Salmonella* serovar Typhimurium and *E. coli* revealed extensive amino acid identity. Given the amino acid similarity between the *Y. pestis* and *Salmonella* serovar Typhimurium PhoP proteins (93.3%) and the identification of a PhoQ orthologue downstream, we are confident that the equivalent *Y. pestis phoP* gene was characterized in this study.

Both *Y. pestis* and *Salmonella* serovar Typhimurium are capable of surviving and multiplying in one of the most hostile environments bacterial pathogens encounter, the phagolysosomes within macrophages (38). Following phagocytosis of bacteria, macrophages induce a series of events in an attempt to kill the pathogen. In our studies, macrophages were infected at 37°C with bacteria which had been cultured at 28°C to mimic the events which would follow delivery of the bacteria into the host via a flea. Thus, these bacteria may not initially be protected from phagocyte killing. This may explain why the number of wild-type and *phoP* mutant bacteria initially declined after infection of macrophages. However, 5 h after infection of macrophages, it was clear that the *phoP* mutant had been killed much more efficiently than the wild-type strain.

Mechanisms of macrophage killing include the generation of reactive oxygen intermediates and the acidification of the phagolysosome (2). The *phoP* locus of *Salmonella* serovar Typhimurium is essential for virulence and survival within macrophages (15, 28). Exposing the *Y. pestis phoP* mutant to a range of environmental stresses which might be encountered in the macrophage showed it to be more sensitive to oxidative stress and low pH than the wild-type strain. Another environmental signal that has been shown to be important in moderating the expression of the PhoP/PhoQ signal transduction system is levels of Mg^{2+} ions (16). In low-Mg^{2+}-ion environments, PhoP has been shown to regulate high-affinity magnesium transporters (16). Preliminary data suggest that a similar system may be operating in *Y. pestis*, since the PhoP mutant was unable to grow on Mg^{2+}-depleted solid medium, in contrast to the wild-type strain (P. Oyston, unpublished observation). A further important role for PhoP in the virulence of *Salmonella* serovar Typhimurium is that PhoP seems to indirectly play a role in lipid A modification (20). Analysis of wild-type and PhoP mutant polysaccharides on Tricine gels reveal no differences in their respective lipopolysaccharide profiles. However, further sophisticated analysis of the PhoP mutant lipid A composition and structure is required before

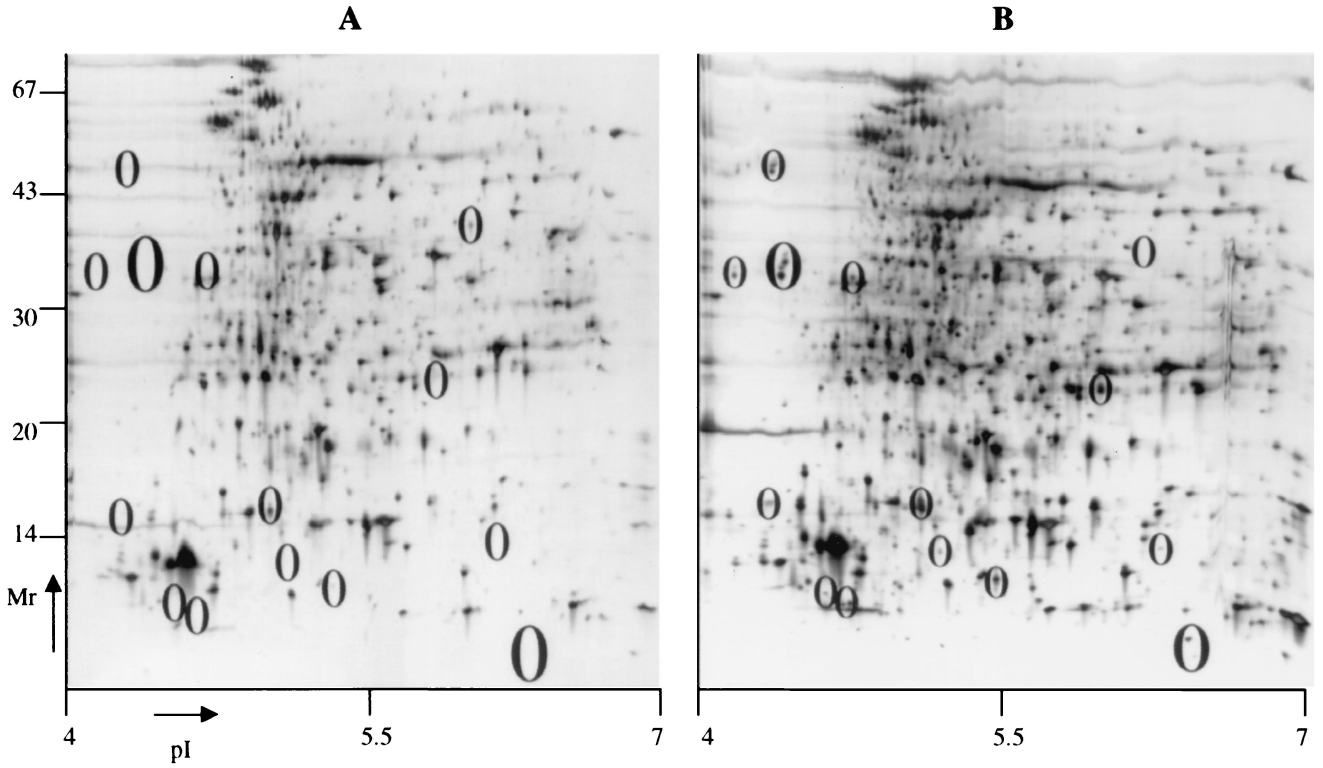

FIG. 4. 2D gel electrophoresis protein expression profiles for the *Y. pestis* wild-type strain (A) and *Y. pestis phoP* mutant (B) grown at 37°C. Differences in protein expression are highlighted (0). Mr, molecular weight in thousands.

firm conclusions on the possible role of PhoP on lipid A modification in *Y. pestis* can be made.

Numerous differences were observed between the total protein profiles of *Y. pestis* wild-type and *phoP* mutant strains which had been cultured in vitro, indicating that the PhoPQ system has pleiotropic effects on gene expression. Protein spots that are absent in the *phoP* mutant but present in the wild type probably represent proteins whose expression is activated by PhoP. Thus, mutation of the *phoP* gene results in these genes not being expressed. Conversely, protein spots absent in the wild type but present in the *phoP* mutant are probably proteins whose expression is normally repressed by PhoP. Therefore, in the *phoP* mutant strain these proteins are expressed. The absence of a large number of *phoP*-activated proteins observed when the cells were grown at 37°C was surprising. However, the pI range of proteins examined was restricted to 4 to 7 in order to obtain comparable, reproducible gels. Thus, changes in the expression of alkaline proteins would not have been observed. The molecular and biochemical characterization of these *phoP*-activated and repressed proteins could provide insight into the mechanisms by which *Y. pestis* survives the increased temperature encountered when it is transmitted from fleas to humans and how the pathogen avoids macrophage killing and causes disease.

Rationally attenuated strains of a wide range of bacteria have been produced by inactivation of genes of various biosynthetic pathways, such as *aro*, *pur*, *gal*, and *cya* (7, 26, 30, 35). Inactivation of the genes encoding response regulators, such as PhoP and OmpR, has been shown to be attenuating for *Salmonella* serovar Typhimurium (8, 14). Whereas the inactivation of *phoP* in *Salmonella* serovar Typhimurium is severely attenuating (12, 14, 27), the mutation in *Y. pestis* is only partially attenuating. The differences in orders of magnitude of virulence between the *Salmonella* PhoP and *Yersinia* PhoP in murine infection models suggest possible differences in the roles of PhoP in these organisms. This may be a reflection of the fact that PhoP regulates different sets of loci in these species. Alternatively, this may be explained by the different sites occupied by the bacteria in vivo. *Salmonella* serovar Typhimurium is primarily an intracellular pathogen. By contrast, *Y. pestis* is dependent on an initial intracellular phase, followed by the widespread growth of bacteria extracellularly (40). Therefore, the influence of the mutation of *phoP*, rendering an organism more sensitive to oxidative stress and extremes of pH, would have a larger impact on the intracellular *Salmonella* and would thus be more attenuating than for the transiently intracellular *Y. pestis*. Detailed knowledge of virulence-associated genes aids in the design of novel live vaccines. A *Salmonella enterica* serovar Typhi *phoP*-attenuated mutant has been shown to be effective in human volunteers (24). Although the *Y. pestis phoP* is only partially attenuating, the method used to prepare the *Y. pestis phoP* mutant in this study did not rely on the incorporation of an antibiotic resistance marker. Thus, it should be possible to graduate attenuation further in *Y. pestis* by the introduction of further mutations in key genes. The mutation of *phoP* in combination with the mutation of other genes could produce a *Y. pestis* strain with potential as a live vaccine candidate.

In conclusion, we have identified, cloned, and sequenced a PhoPQ orthologue from *Y. pestis* and constructed an isogenic mutant. Characterization of the *phoP* mutant has shown that the PhoPQ two-component regulatory system plays a role in the intracellular survival of *Y. pestis* similar to that of its orthologue in *Salmonella* serovar Typhimurium. However, due to the predominantly extracellular location of *Y. pestis* during infection, the effect of a *phoP* mutation on virulence is weaker

than with *Salmonella* species. Of greater interest is the number of proteins that appear to be activated by the PhoPQ system at 28°C and repressed at 37°C, suggesting an important role for this two-component regulatory system in the survival of *Y. pestis* in the flea and/or during transmission.

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