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Genetics

A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence

(gene regulation/pathogenesis/macrophage survival)

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ABSTRACT **We have determined that *Salmonella typhimurium* strains with mutations in the positive regulatory locus *phoP* are markedly attenuated in virulence for BALB/c mice. The DNA sequence for the *phoP* locus indicates that it is composed of two genes present in an operon, termed *phoP* and *phoQ*. The deduced amino acid sequences of the *phoP* and *phoQ* gene products are highly similar to other members of bacterial two-component transcriptional regulators that respond to environmental stimuli. *S. typhimurium* strains with transposon insertions that create transcriptional and translational gene fusions that require *phoP* and *phoQ* for expression have been isolated and have different chromosomal locations, indicating that this system is a regulon. One of these fusion strains, containing a mutation in a gene termed *pagC*, has a virulence defect. Other strains, including those containing mutations in the *phoN* gene, encoding an acid phosphatase, have wild-type virulence. Strains with *pagC*, *phoP*, or *phoQ* mutations have decreased survival in cultured mouse macrophages. When used as live vaccines in mice, strains with *phoP* or *phoQ* mutations afford partial protection to subsequent challenge by wild-type *S. typhimurium*.**

The bacterial genus *Salmonella* contains species that cause a spectrum of diseases in humans and animals that includes enteric fever, septicemia, and gastroenteritis (1). *Salmonella typhi*, the cause of typhoid (enteric) fever, infects only humans. This narrow host specificity has led to the extensive use of *Salmonella typhimurium* infection of BALB/c mice as an experimental model for typhoid fever (2).

The survival and growth of *Salmonella* within the macrophage phagolysosome is felt to be essential for typhoid pathogenesis (1–3). Fields and Heffron (4) obtained evidence supporting this hypothesis by demonstrating that *S. typhimurium* transposon insertion mutants that did not survive intracellularly in cultured macrophages had reduced virulence in mice.

The genetic study of bacterial virulence in a number of organisms has demonstrated that the expression of many virulence factors is coordinately regulated in response to environmental signals (5). In this paper, we report that a two-component regulatory system (6), *phoP*/*phoQ*, regulates the expression of genes involved in virulence and macrophage survival of *S. typhimurium*.

MATERIALS AND METHODS

Strains and Genetic Methods. American Type Culture Collection (ATCC) strain 14028, a smooth virulent strain of *S. typhimurium*, was the parent strain for all virulence studies. Various mutant strains were constructed by bacteriophage P22 HT*int* transduction of transposon insertions (ref. 7, pp. 78 and 87). Bochner selection (8) was used to excise precisely Tn*10* insertions (CS002) and to generate deletion mutations in *purB* and *phoP* (CS003). DNA cloned by complementation was obtained from wild-type plasmid libraries of *S. typhimurium* LT2 DNA obtained as gifts from Charles Miller (Case Western Reserve, Cleveland, OH) and Nicholas Kredich and Robin Monroe (Duke University, Durham, NC) (9). Map positions of mutations were determined by transposon linkage analysis as previously described (10). Strains are listed in Table 1.

Media. Luria broth (ref. 7, p. 201) was used unless otherwise indicated. M9 was used as minimal medium (ref. 7, p. 203). 5-Bromo-4-chloro-3-indolyl phosphate [*p*-toluidine salt (X-*P*)] was used in plates at 40 μg/ml to screen for phosphatase activity. Medium with X-*P* as a sole phosphate source was as described (14) and was used in cloning the *phoN* gene by complementation of the *phoN2* allele. Low-carbon, low-phosphate, low-nitrogen medium was prepared as described by Kier *et al.* (11) and contained 0.4% succinate as a carbon source and 10 mM NH_4Cl as a nitrogen source.

Transposon Mutagenesis. Random mutagenesis of *S. typhimurium* was accomplished by using MudJ (15), Tn*10d*-*Cam* (16, 17), and Tn*phoA* (18). *Escherichia coli* strain CC118 was used as a *phoA*-negative recipient for Tn*phoA* mutagenesis of plasmid DNA with Tn*phoA* as described (19). Plasmid pSM001 containing the cloned *phoN* gene was mutagenized with a Tn*10d*-Kan as described by Way *et al.* (20). Insertions into the *Salmonella phoN* gene (CS008) were obtained by cloning a 3.9-kilobase (kb) *Eco*RI fragment containing the *phoN104*::Tn*10d*-Kan insertion in the vector pGP704 (21). The resultant plasmid (pSM002) was mobilized into *S. typhimurium* and cells that had undergone exchange events were selected (21). The resultant strains showed loss of acid phosphatase activity and a transposon insertion that mapped to 95 min. Southern blot analysis confirmed recombination into the correct chromosomal DNA restriction enzyme fragment.

A plasmid containing the cloned *phoP* locus (pSM003) was mutagenized with MudJ in *S. typhimurium* TT1028 (15) by growth at permissive temperature for MudJ transposition. Recombination of one of these insertions into the *S. typhimurium* chromosome (CS053) was accomplished by use of a polymerase A mutant of ATCC 14028 followed by P22 bacteriophage transduction into wild-type ATCC 14028.

Mouse Virulence and Vaccination Studies. Bacteria grown overnight in Luria broth were washed and diluted in saline. Five-week-old male BALB/c mice were obtained from Charles River Breeding Laboratories; they were used before 7 weeks of age. For LD_{50} determinations, bacteria diluted in saline were injected intraperitoneally and the mice were scored for viability for a period of 1 month. For vaccination studies, survivors were challenged 1 month after initial

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Abbreviation: X-*P*, 5-bromo-4-chloro-3-indolyl phosphate.

Table 1. Strains and their properties

Strain	Genotype	LD_{50}	Challenge LD_{50}	MSI	Enzyme activity
10428	Wild type	<20		6.13	180 (A)
CS002	*phoP12*	5×10^5	3×10^3		20 (A)
CS009	*phoQ101*::MudJ	4×10^5	5×10^3	0.32	25 (A)
CS015	*phoP102*::Tn*10d-Cam*	7×10^5	7×10^3	0.40	<10 (A)
CS003	*phoP*del*purB*del	5×10^6	<20		<10 (A)
CS053	*phoP103*::MudJ	6×10^5	6×10^3		30 (B)
CS008	*phoN104*::Tn*10d-Kan*	<20			<10 (B)
CS019	*phoN2* zxx::6251Tn*10d-Cam*	<20			<10 (A)
CS120	*phoN105*::Tn*phoA*	<20			55 (C)
CS012	*pagA1*::MudJ	<20			45 (B)
CS013	*pagB1*::MudJ	<20			120 (B)
CS119	*pagC1*::Tn*phoA*	7×10^3		0.42	85 (C)
CS018	*pagC1*::Tn*phoA* *phoP*::Tn*10d-Tet* *phoN2* zxx::6251Tn*10d-Cam*				<5 (C)
CS015	*phoN105*::Tn*phoA* *phoP102*::Tn*10d-Cam*	5×10^5			<5 (C)
CS016	*pagA1*::MudJ*phoP12* *purB1744*::Tn*10*				<5 (B)
CS017	*pagB1*::MudJ*phoP12* *purB1744*::Tn*10*				<5 (B)
CS020	*pagA1*::MudJ *purB1744*Tn*10*				54 (B)
CS021	*pagB1*::MudJ *purB1744*::Tn*10*				110 (B)

LD_{50} is the dose at which 50% of the animals are killed. All values were determined from at least 20 animals at the indicated dose as well as doses 10-fold greater and lower. MSI is macrophage survival index, and the values for CS009, CS015, and CS119 are significantly different than the value for ATCC 10428 ($P < 0.05$). Enzyme activities in units as described in the references are A, acid phosphatase (11); B, β-galactosidase (12); and C, alkaline phosphatase (14). All assays shown were done on cells grown in rich media (LB) at 37°C. In genotype del indicates a deletion of the gene.

inoculations with various doses of wild-type strain 10428 to assess an LD_{50}.

Biochemical and Molecular Analysis. Southern blot analysis, chromosomal DNA preparation, restriction enzyme digestion, purification of plasmid DNA, and subcloning of DNA fragments was performed by standard methods (22). DNA sequencing was performed on both strands of single-stranded fragments cloned in M13mp18 and -19 by use of the Sequenase kit (United States Biochemical). β-Galactosidase (12), alkaline phosphatase (14), and acid phosphatase (11) assays were performed as described.

Macrophage Survival. Intracellular survival of bacteria was measured by the method of Buchmeier and Heffron (23), which is a modification of the method of O'Brien and colleagues (24), using bone marrow-derived BALB/c macrophages. All strains were tested in triplicate on three different occasions. The macrophage survival index (MSI) was the mean bacterial count at 24 hr divided by that obtained 1 hr after gentamicin exposure.

RESULTS

Mutations in the *phoP* Locus Reduce the Virulence of *S. typhimurium* in Mice and Its Survival in Macrophages. A number of mutations in *S. typhimurium* are known to dramatically affect virulence, but each causes one or more metabolic defects reflected in new growth requirements *in vitro* (25–28). Recognizing that many bacterial virulence factors are coordinately regulated (5), we wished to identify a regulatory mutation that reduced the virulence of *S. typhimurium* but did not dramatically alter its growth properties. We studied strains with mutations in *oxyR*, *ntrA*, *ompR*, and *envZ* and found that these mutations [*oxyR*del2 (29), *ntrA209* (30), *ompR1009* (31), and *envz1005* (31)] had no measurable effect on the virulence of *S. typhimurium* ATCC 14028 (data not shown). In contrast, any of four different mutations in the *phoP* locus caused a marked reduction in the virulence of strain ATCC 14028 as measured by LD_{50} analysis (Table 1).

The *phoP* is one of two loci (*phoP* and *phoN*) found by Ames and colleagues (32) to be necessary for the production of an acid phosphatase by *S. typhimurium*. These investigators postulated that *phoP* was a regulatory locus and *phoN* was the phosphatase structural gene.

We have confirmed these conclusions by DNA cloning of the *phoN* and *phoP* loci and DNA sequencing of the *phoP* locus (see below). We have further determined that there are at least two genes, which we term *phoP* and *phoQ*, present in this regulatory locus. The data in Table 1 indicate that mutations in either of these two genes cause over a 10,000-fold reduction in virulence of *S. typhimurium*. *phoP*$^-$ strains grow as well as parental strains in rich and minimal media (refs. 31 and 33; data not shown), suggesting that their virulence defect resulted in reduced expression of either the *phoN*-encoded acid phosphatase or another virulence factor whose expression was regulated by the *phoP* locus.

To assess the role of the acid phosphatase as a virulence factor, a transposon insertion was made in a cloned copy of the *phoN* locus and recombined into the chromosome of the mouse virulent strain ATCC 14028. A second insertion mutation in *phoN* was obtained as a result of Tn*phoA* mutagenesis. Table 1 shows that three *phoN* mutants (strains CS008, CS019, and CS120) have the same LD_{50} as the parental strain for BALB/c mice. These data indicate that the attenuation induced by *phoP* and *phoQ* mutations is not the result of reduced acid phosphatase production but is probably associated with another defect.

Recently, Fields *et al.* (34) have reported that one class of *S. typhimurium* mutants that are defective in macrophage survival have mutations that map to the *phoP* locus. Accordingly, we used an intracellular survival assay (24) adapted to *S. typhimurium* by Buckmeier and Heffron (23) to assess the survival of *phoP* mutants in bone marrow-derived mouse macrophages. As shown in Table 1, *phoP* and *phoQ* mutants do not survive as well as the parental strain in bone marrow-derived macrophages from BALB/c mice, confirming the conclusions of Fields *et al.* (34).

A Virulence Gene (*pagC*) Requires *phoP* for Expression. We have identified and mapped three loci that require *phoP* for their expression. These were identified by two methods, both involving transposons that generate either transcriptional

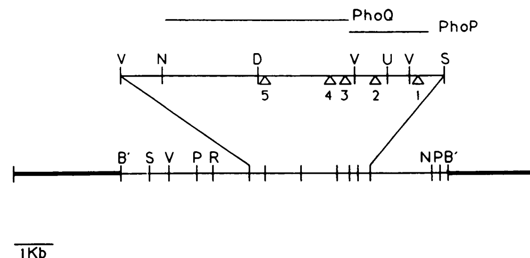

FIG. 1. Restriction map of pSM003. The locations of the PhoP- and PhoQ-encoding sequences are shown at the top and the direction of transcription is right to left. The thick lines represent pBR328 vector sequences and the thin lines represent insert sequences. Triangles show the location of the following transposon insertions: 1, *phoP103*::MudJ; 2, *phoP102*::Tn*10d-Cam*; 3, *phoQ106*::Tn*phoA*; 4, *phoQ107*::Tn*phoA*; 5, *phoQ101*::MudJ. Restriction enzyme sites are as follows: B, *Bam*HI; D, *Dra* I; N, *Nco* I; P, *Pst* I; R, *Eco*RI; S, *Sal* I; U, *Pvu* II; V, *Eco*RV.

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         10         20         30         40         50         60         70
TCGACGAAC TTAAATAATG CCTGCCTCAC CCTCTTTTCT TCAGAAAGAG GGTGACTATT TGTCTGGTTTA

         80         90        100        110        120        130        140
TTAACTGTT TATCCCCAAA GCACCATAAT CAACGCTAGA CTGTTCTTAT TGTTAACACA AGGGAGAAGAG

ATG ATG CGC GTA CTG GTT GTA GAG GAT AAT GCA TTA TTA CGC CAC CAC CTG AAG  194
Met Met Arg Val Leu Val Val Glu Asp Asn Ala Leu Leu Arg His His Leu Lys   18

GTT CAG CTC CAG GAT TCA GGT CAC CAG GTC GAT GCC GCA GAA GAT GCC AGG GAA  248
Val Gln Leu Gln Asp Ser Gly His Gln Val Asp Ala Ala Glu Asp Ala Arg Glu   36

GCT GAT TAC TAC CTT AAT GAA CAC CTT CCG GAT ATC GCT ATT GTC GAT TTA GGT  302
Ala Asp Tyr Tyr Leu Asn Glu His Leu Pro Asp Ile Ala Ile Val Asp Leu Gly   54

CTG CCG GAT GAA GAC GGC CTT TCC TTA ATA CGC CGC TGG CGC AGC AGT GAT GTT  356
Leu Pro Asp Glu Asp Gly Leu Ser Leu Ile Arg Arg Trp Arg Ser Ser Asp Val   72

TCA CTG CCG GTT CTG GTG TTA ACC GCG CGC GAA GGC TGG CAG GAT AAA GTC GAG  410
Ser Leu Pro Val Leu Val Leu Thr Ala Arg Glu Gly Trp Gln Asp Lys Val Glu   90

GTT CTC AGC TCC GGG GCC GAT GAC TAC GTG ACG AAG CCA TTC CAC ATC GAA GAG  464
Val Leu Ser Ser Gly Ala Asp Asp Tyr Val Thr Lys Pro Phe His Ile Glu Glu  108

GTA ATG GCG CGT ATG CAG GCG TTA ATG CGC CGT AAT AGC GGT CTG GCC TCC CAG  518
Val Met Ala Arg Met Gln Ala Leu Met Arg Arg Asn Ser Gly Leu Ala Ser Gln  126

GTG ATC AAC ATC CCG CCG TTC CAG GTG GAT CTC TCA CGC CGG GAA TTA TCC GTC  572
Val Ile Asn Ile Pro Pro Phe Gln Val Asp Leu Ser Arg Arg Glu Leu Ser Val  144

AAT GAA GAG GTC ATC AAA CTC ACG GCG TTC GAA TAC ACC ATT ATG GAA ACG CTT  626
Asn Glu Glu Val Ile Lys Leu Thr Ala Phe Glu Tyr Thr Ile Met Glu Thr Leu  162

ATC CGT AAC AAC GGT AAA GTG GTC AGC AAA GAT TCG CTG ATG CTT CAG CTG TAT  680
Ile Arg Asn Asn Gly Lys Val Val Ser Lys Asp Ser Leu Met Leu Gln Leu Tyr  180

CCG GAT GCG GAA CTG CGG GAA AGT CAT ACC ATT GAT GTT CTC ATG GGG CGT CTG  734
Pro Asp Ala Glu Leu Arg Glu Ser His Thr Ile Asp Val Leu Met Gly Arg Leu  198

CGG AAA AAA ATA CAG GCC CAG TAT CCG CAC GAT GTC ATT ACC ACC GTA CGC GGA  788
Arg Lys Lys Ile Gln Ala Gln Tyr Pro His Asp Val Ile Thr Thr Val Arg Gly  216

CAA GGA TAT CTT TTT GAA TTG CGC TA  ATG AAT AAA TTT GCT CGC CAT TTT CTG  841
Gln Gly Tyr Leu Phe Glu Leu Arg     Met Asn Lys Phe Ala Arg His Phe Leu    9

CCG CTG TCG CTG CGG GTT CGT TTT TTG CTG GCG ACA GCC GGC GTC GTG CTG GTG  895
Pro Leu Ser Leu Arg Val Arg Phe Leu Leu Ala Thr Ala Gly Val Val Leu Val   27

CTT TCT TTG GCA TAT GGC ATA GTG GCG CTG GTC GGC TAT AGC GTA AGT TTT GAT  949
Leu Ser Leu Ala Tyr Gly Ile Val Ala Leu Val Gly Tyr Ser Val Ser Phe Asp   45

AAA ACC ACC TTT CGT TTG CTG CGC GGC GAA AGC AAC CTG TTT TAT ACC CTC GCC 1003
Lys Thr Thr Phe Arg Leu Leu Arg Gly Glu Ser Asn Leu Phe Tyr Thr Leu Ala   63

AAA TGG GAA AAT AAT AAA ATC AGC GTT GAG CTG CCT GAA AAT CTG GAC ATG CAA 1057
Lys Trp Glu Asn Asn Lys Ile Ser Val Glu Leu Pro Glu Asn Leu Arg Met Gln   81

AGC CCG ACC ATG ACG CTG ATT TAC GAT GAA ACG GGC AAA TTA TTA TGG ACG CAG 1111
Ser Pro Thr Met Thr Leu Ile Tyr Asp Glu Thr Gly Lys Leu Leu Trp Thr Gln   99

CGC AAC ATT CCC TGG CTG ATT AAA AGC ATT CAA CCG GAA TGG TTA AAA ACG AAC 1165
Arg Asn Ile Pro Trp Leu Ile Lys Ser Ile Gln Pro Glu Trp Leu Lys Thr Asn  117

GGC TTC CAT GAA ATT GAA ACC AAC GTA GAC GCC ACC AGC ACG CTG TTG AGC GAA 1219
Gly Phe His Glu Ile Glu Thr Asn Val Asp Ala Thr Ser Thr Leu Leu Ser Glu  135

GAC CAT TCC GCG CAG GAA AAA CTC AAA GAA GTA CGT GAA GAT GAC GAT GAT GCC 1273
Asp His Ser Ala Gln Glu Lys Leu Lys Glu Val Arg Glu Asp Asp Asp Asp Ala  153

GAG ATG ACC CAC TCG GTA GCG GTA AAT ATT TAT CCT GCC ACG GCG CGG ATG CCG 1327
Glu Met His Ser Val Ala Val Asn Asn Ile Tyr Pro Ala Thr Ala Arg Met Pro  171

CAG TTA ACC ATC GTG GTG GTC GAT ACC ATT CCG ATA GAA CTA AAA CGC TCC TAT 1381
Gln Leu Thr Ile Val Val Val Asp Thr Ile Pro Ile Glu Leu Lys Arg Ser Tyr  189

ATG GTG TGG AGC TGG TTC GTA TAC GTG CTG GCC GCC AAT TTA CTG TTA GTC ATT 1435
Met Val Trp Ser Trp Phe Val Tyr Val Leu Ala Ala Asn Leu Leu Leu Val Ile  207

CCT TTA CTG TGG ATC GCC GCC TGG TGG AGC TTA CGC CCT ATC GAG GCG CTG GCG 1489
Pro Leu Leu Trp Ile Ala Ala Trp Trp Ser Leu Arg Pro Ile Glu Ala Leu Ala  225

CGG GAA GTC CGC GAG CTT GAA GAT CAT CAC CGC GAA ATG CTC AAT CCG GAG ACG 1543
Arg Glu Val Arg Glu Leu Glu Asp His His Arg Glu Met Leu Asn Pro Glu Thr  243

ACG CGT GAG CTG ACC AGC CTT GTG CGC AAC CTT AAT CAA CTG CTC AAA AGC GAG 1597
Thr Arg Glu Leu Thr Ser Leu Val Arg Asn Leu Asn Gln Leu Leu Lys Ser Glu  261

CGT GAA CGT TAT AAC AAA TAC CGC ACG ACC CTG ACC GAC CTG ACG CAC AGT TTA 1651
Arg Glu Arg Tyr Asn Lys Tyr Arg Thr Thr Leu Thr Asp Leu Thr His Ser Leu  279

AAA ACG CCG CTC GCG GTT TTG CAG AGT ACG TTA CGC TCT TTA CGC AAC GAA AAG 1705
Lys Thr Ala Leu Ala Val Leu Gln Ser Thr Leu Arg Ser Leu Arg Asn Glu Lys  297

ATG AGC GTC AGC AAA GCT GAA CCG GTG ATG CTG GAA CAG ATC AGC CGG ATT TCC 1759
Met Ser Val Ser Lys Ala Glu Pro Val Met Leu Glu Gln Ile Ser Arg Ile Ser  315

CAG CAG ATC GGC TAT TAT CTG CAT CGC GCC AGT ATG CGC GGT AGC GGC GTG TTG 1813
Gln Gln Ile Gly Tyr Tyr Leu His Arg Ala Ser Met Arg Gly Ser Gly Val Leu  333

TTA AGC CGC GAA CTG CAT CCC GTC GCG CCG TTG TTA GAT AAC CTG ATT TCT GCG 1867
Leu Ser Arg Glu Leu His Pro Val Ala Pro Leu Leu Asp Asn Leu Ile Ser Ala  351

CTA AAT AAA GTT TAT CAG CGT AAA GGG GTG AAT ATC AGT ATG GAT ATT TCA CCA 1921
Leu Asn Lys Val Tyr Gln Arg Lys Gly Val Asn Ile Ser Met Asp Ile Ser Pro  369

GAA ATC AGT TTT GTC GGC GAG CAA AAC GAC TTT GTC GAA GTG ATG GGC AAC GTA 1975
Glu Ile Ser Phe Val Gly Glu Gln Asn Asp Phe Val Glu Val Met Gly Asn Val  387

CTG GAC AAC GCT TGT AAA TAT TGT CTG GAG TTT GTC GAG ATT TCG GCT CGC CAG 2029
Leu Asp Asn Ala Cys Lys Tyr Cys Leu Glu Phe Val Glu Ile Ser Ala Arg Gln  405

ACC GAC GAT CAT TTG CAT ATT TTC GTC GAA GAT GAC GGC CCA GGC ATT CCC CAC 2083
Thr Asp Asp His Leu His Ile Phe Val Glu Asp Asp Gly Pro Gly Ile Pro His  423

AGC AAA CGT TCC CTG GTG TTT GAT CGC GGT CAG CGC GCC GAT ACC CTA CGA CCA 2137
Ser Lys Arg Ser Leu Val Phe Asp Arg Gly Gln Arg Ala Asp Thr Leu Arg Pro  441

GGA CAA GGC GTG GGG CTG GCT GTC GCG CGC GAG ATT ACG GAA CAA TAC GCC GGG 2191
Gly Gln Gly Val Gly Leu Ala Val Ala Arg Glu Ile Thr Glu Gln Tyr Ala Gly  459

CAG ATC ATT GCC AGC GAC AGT CTG CTC GGT GGC GCC CGT ATG GAG GTC GTT TTT 2245
Gln Ile Ile Ala Ser Asp Ser Leu Leu Gly Gly Ala Arg Met Glu Val Val Phe  477

GGC CGA CAG CAT CCC ACA CAG AAA GAG GAA TAA ACATTTCTGT GCGTTCTTCC       2298
Gly Arg Gln His Pro Thr Gln Lys Glu Glu                                  487
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FIG. 2. DNA sequence of the *phoP* and *phoQ* genes. Shown are 2298 nucleotides located between the *Sal* I and *Nco* I sites of plasmid pSM003. The deduced amino acid sequences of PhoP and PhoQ are the first and second open reading frames shown, respectively. A potential ribosome-binding site preceding the *phoP* sequence is indicated by a thin underline, and two proposed hydrophobic transmembrane stretches in PhoQ are indicated by the thick underlines.

fusions to the the *E. coli lacZ* gene [i.e., MudJ (15)] or translation fusions to the *E. coli phoA* gene [i.e., Tn*phoA* (13)]. We constructed a bank of MudJ insertion mutants in strain ATCC 10428 and screened several thousand colonies for β-galactosidase production on a starvation medium (i.e., limited in carbon, nitrogen, and phosphate), which had previously been shown to increase the production of acid phosphatase 4- to 7-fold (32, 35, 36). Several hundred transcriptionally active fusion strains were screened for the requirement of an intact *phoP* locus for *lacZ* expression by introduction of the *phoP12* allele (32). Table 1 (strains CS012, -013, -016, -017, -020, and -021) shows that a functional *phoP* locus was required for high expression of MudJ fusions to two of these loci, termed *pagA* and *pagB* (for PhoP-activated gene). Chromosomal mapping of *pagA1*::MudJ insertion by P22 transduction shows it is located at 42 min and is 98% linked to a Tn*10* insertion in AK3255 (10). The *pagB1*::MudJ fusion was not determined to be linked to a bank of transposon insertions that have been used extensively for mapping in *S. typhimurium* (10). Strains with *pagA* and *pagB* insertions have normal growth rate in rich media, are not auxotrophic, and do not show a virulence defect in mouse LD_{50} assays (Table 1, CS012 and CS013).

We also screened a bank of *S. typhimurium* mutants with Tn*phoA* insertions (13, 18). An *S. typhimurium* strain with a *phoN* mutation (CS019) was used as the parent strain for this insertion bank to suppress background X-*P* hydrolysis. The fusion bank was screened on LB agar for $PhoA^+$ colonies, and 225 strains so obtained were screened for expression of their Tn*phoA* fusions after introduction of the *phoP12* allele. This approach identified two strains carrying *phoP*-activated gene fusions of which one (CS120) was identified as an insertion in the *phoN* locus by mapping and Southern blot hybridization (data not shown). The other strain (CS119), carrying the *pagC1*::Tn*phoA* insertion, had a map position of 25 min and was 37% linked to the Tn*10*Δ16Δ17 of strain AK3140, 75% linked to the Tn*10*Δ16Δ17 of strain AK3233, and not linked to the *phoP103*::*Tn10d-Cam* allele.

As can be seen in Table 1, strain CS119 shows a pronounced virulence defect in BALB/c mice and also survives less well in mouse bone marrow-derived macrophages *in vitro*. The *pagC1*::Tn*phoA* insertion of strain CS119 was transduced into a new CS019 background and confirmed to be responsible for the virulence defect, although polarity effects of the transposon on expression of a downstream gene might also contribute to this phenotype. These data indicate that the *phoP* locus is required for the expression of the *phoN* gene and at least three other unlinked loci, one of which, *pagC*, is likely to encode a virulence factor required for intracellular survival in macrophages.

Use of *phoP* Mutants as Live *Salmonella* Vaccines. Survivors of a LD_{50} challenge of *S. typhimurium* strains with *phoP* mutations were infected with wild-type organisms 1 month after their original inoculation. There was over a 1000-fold increase in the LD_{50} dose in animals previously immunized with *phoP* mutants (Table 1). These data document that prior exposure to avirulent *phoP* mutants affords significant protection to challenge with wild-type organisms.

Cloning and Sequencing of the *phoP* Locus and the Identification of *phoP* and *phoQ* Genes. The *phoP* genetic locus was cloned by complementation of a *S. typhimurium* deletion mutant lacking *phoP* and *purB* function (CS003) from a pBR328-based gene bank of *S. typhimurium* strain LT2 DNA. The plasmid obtained (pSM003) contained an 8.1-kb insert, whose restriction map is shown in Fig. 1. Deletion analysis by restriction enzyme digestion and ligation, or insertion analysis with MudJ, showed that a 3-kb region of this insert (Fig.

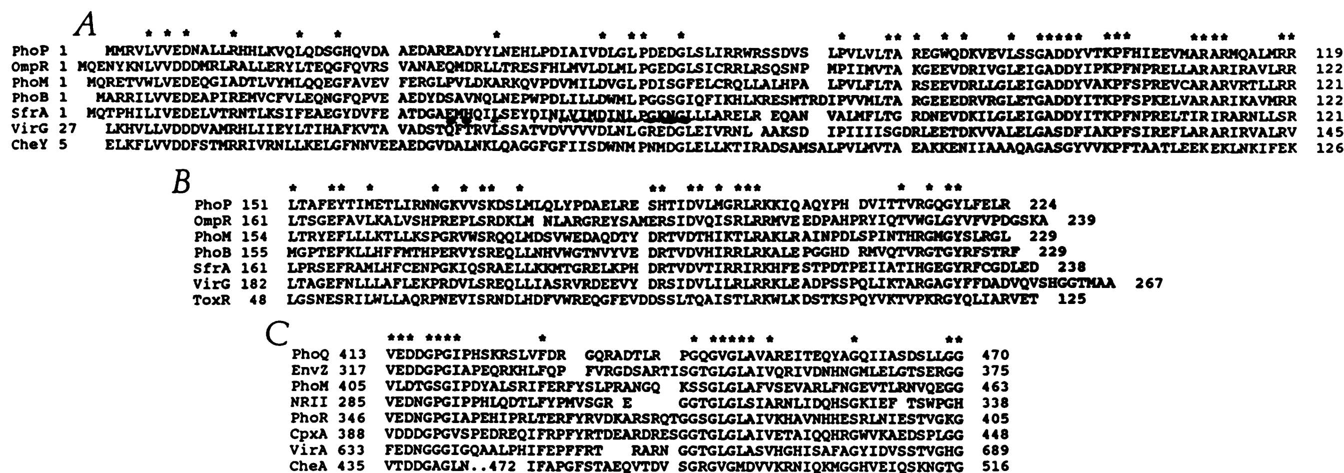

FIG. 3. Similarity between members of bacterial two-component regulators and PhoP and PhoQ. The deduced amino acid sequences of PhoP and PhoQ, in the standard one-letter code, are partially aligned with the deduced amino acid sequences of OmpR (31), PhoM-ORF2 (39), PhoB (37), SfrA (38), VirG (40), ToxR (41), CheY (42), EnvZ (31), PhoM-ORF3 (39), NRII (43), PhoR (37), CpxA (38), VirA (40), and CheA (42). Gaps were introduced to maximize similarities relative to PhoP and PhoQ. Asterisks denote residues that are identical in at least six members of each group of proteins. (*A*) Alignment of the first 119 residues of PhoP; (*B*) alignment of the last 74 residues of PhoP; (*C*) alignment of residues 413–470 of PhoQ.

1) contained sequences necessary for complementation of the *phoP* mutation as scored by acid phosphatase production. The transcriptional orientation of the *phoP* and *phoQ* genes shown in Fig. 1 was inferred from the orientation of MudJ insertions that eliminated complementation activity but expressed β-galactosidase. One of these insertions was recombined into the chromosome, resulting in strain CS053, which is as avirulent as other *PhoP* mutants (Table 1). Linkage analysis by cotransduction of known markers using P22HT*int* bacteriophage confirmed that the cloned DNA recombined into the chromosome at 25 min and hence was the *phoP* locus.

The DNA sequence of this region is shown in Fig. 2.* It contains two major open reading frames (the *phoP* and *phoQ* genes) encoding predicted polypeptides of 224 and 487 amino acid residues, respectively. Because both these open reading frames were in the same orientation and overlap by a single base pair, we tentatively conclude that both genes are in the same transcriptional unit (i.e., the *phoPQ* operon).

The deduced amino acid sequences of PhoP and PhoQ were used to search the Protein Identification Resource Database, using the FASTP algorithm of Lipman and Pearson (33). The PhoP search was conducted on Nov. 10, 1988, and the PhoQ search on Jan. 3, 1989. The PhoP sequence showed a marked similarity at its amino and carboxyl termini to bacterial transcriptional activators such as OmpR (31), PhoB (37), SfrA (38), PhoM-ORF2 (39), VirG (40), and other proteins which are known to be members of a family of two-component regulators that respond to environmental signals (6) (Fig. 3). PhoP also showed a strong similarity in its amino-terminal domain to CheY (Fig. 3*A*) and NRII (not shown), proteins that are known to be phosphorylated (42). The carboxyl-terminal domain of PhoP is also homologous to the amino-terminal region of ToxR (Fig. 3*B*), a domain that has been implicated in the transcriptional activation and DNA-binding activity of this *Vibrio cholerae* virulence regulator (41).

Analogously PhoQ showed a high similarity in its carboxyl-terminal portion to proteins such as EnvZ (31), PhoR (37), CpxA (38), PhoM-ORF3 (39), NRI (43), and VirA (40) (Fig. 3*C*) that represent the second components in the two-component regulators noted above (6), as well as the kinase CheA (42). These proteins are thought to be membrane-associated (except CheA and NRI) protein kinases that detect environmental signals and then phosphorylate themselves and the amino-terminal domains of the transcriptional regulators they are paired with (6, 44–46). Consistent with this model, PhoQ has two hydrophobic transmembrane segments located at amino acid residues 17–44 and 191–218 (Fig. 2) that are predicted to be transmembrane segments by the method of Engelman *et al.* (47). Moreover, we have isolated several PhoA$^+$ Tn*phoA* fusions to *phoQ* that all map to the intervening amino acid sequence between these two putative transmembrane segments (Fig. 1), suggesting that this domain represents a periplasmic loop and giving PhoQ a transmembrane topology similar to other sensor-kinase proteins such as EnvZ (48). The predicted sequence of the PhoQ protein also carries a sequence (residues 274–279, including histidine-276) that is homologous to the site of CheA autophosphorylation (45) and the histidine residue proposed to be utilized in NRI autophosphorylation (44) as well as a region of similarity (residues 413–484) to the proposed ATP-binding domain of other members of the sensor family (6). Together, these data argue strongly that the PhoP and PhoQ gene products are members of the family of bacterial two-component regulatory systems.

DISCUSSION

Several regulons in bacteria, including those responding to nitrogen limitation, phosphate limitation, sugar transport, and osmolarity, are controlled by two-component regulators (sensor kinases/transcriptional activators) (5, 6). We describe here a two-component regulatory system of *S. typhimurium* that is required for full virulence in BALB/c mice and intracellular survival in cultured macrophages. The PhoP and PhoQ proteins are necessary for the expression of a number of genes, some of which, like *pagC*, are involved in virulence.

In regard to the virulence defect associated with mutations in the *phoP* locus, we were able to confirm the observations of Fields *et al.* (34) that *phoP* locus mutants are defective in macrophage intracellular survival. These investigators also demonstrated that *phoP* mutants show increased sensitivity to small cationic peptides derived from neutrophil granules known as defensins (49). We have identified an insertion in a PhoP-regulated gene termed *pagC* which reduces the survival of *S. typhimurium* in macrophages. On the basis of the properties of Tn*phoA* (13) and the *pagC*::Tn*phoA* fusion, we propose that *pagC* encodes a secreted, periplasmic, or mem-

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M24424).

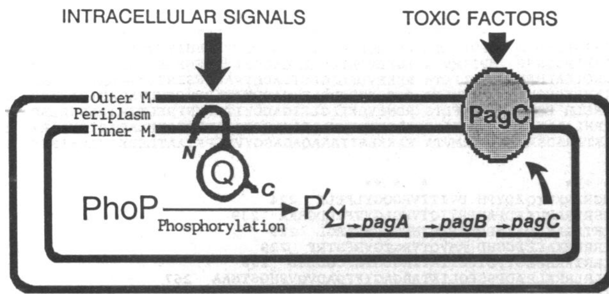

FIG. 4. Hypothetical model for the PhoP regulon. The phagolysosome surrounds the schematically drawn *S. typhimurium* cell. Environmental signals present in the phagolysosome are received by the periplasmic domain of PhoQ. The carboxyl-terminal portion (C) of PhoQ then catalyzes the phosphorylation of PhoP in the bacterial cytoplasm. Modified PhoP (P′) activates promoters of *PhoN* (not shown) and other PhoP-activated loci (*pag* genes). One of these (*pagC*) encodes an envelope protein that promotes resistance to toxic factors present in the phagolysome.

brane protein involved in resistance to defensins or other microbiocidal host factors in the phagolysosome (Fig. 4).

The structures of the predicted PhoP and PhoQ gene products together with the virulence defects and decreased intracellular survival of *phoP*, *phoQ*, and *pagC* mutants suggest a working model for the PhoP regulon (Fig. 4). The predicted amino acid sequence of PhoQ indicates that it has regions of similarity with the sensor class homologs, suggesting that PhoQ functions as a membrane-associated protein kinase that phosphorylates PhoP in response to environmental signals. Phosphorylated PhoP could then activate promoters for *pag* genes. By analogy to sensors of the two-component regulator family (6), the periplasmic domain of PhoQ may be involved in sensing environmental parameters present in the phagolysosome. In regard to this proposed role, the predicted periplasmic domain of PhoQ has a remarkable stretch of 20 amino acids (residues 135–154) that contains 10 negatively charged amino acids and only 4 positively charged residues. This anionic region may be involved in the recognition of intracellular signals (defensins, cations, low pH, etc.) unique to the phagolysosome. We have noted the derepression of *pagA*, *pagB*, *pagC*, and *phoN* expression approximately 2- to 3-fold by growth of *S. typhimurium* in media of low pH (data not shown), while Ames and colleagues (35, 36) have previously reported that PhoN expression is induced by limitation of carbon, nitrogen, and phosphorus. These data suggest that starvation and low pH may represent signals that PhoQ might sense in the phagolysosome.

Strains of *S. typhimurium* with *phoP* mutations afforded some protection when used as live vaccines by the intraperitoneal route. We have also shown that *phoP* and *phoQ* mutants are avirulent for BALB/c mice when administered orally, yet they still efficiently colonize the mouse gut and confer substantial protective immunity (data not shown). Thus, analysis of this virulence regulon should lead to a better understanding of the molecular basis of *Salmonella* pathogenesis and may lead to the development of safe, attenuated *Salmonella* strains suitable for use as carrier vaccines for the delivery of heterologous antigens to the mucosal immune system.

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