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Transcriptional Regulation of *Salmonella* Virulence: a PhoQ Periplasmic Domain Mutation Results in Increased Net Phosphotransfer to PhoP

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A mutation in the *phoP*/*phoQ* locus (*pho-24*) that results in unregulated expression of PhoP-activated genes (phenotype PhoP constitutive [PhoP^c]) was mapped to *phoQ*. Change of a Thr to Ile at position 48 of PhoQ was responsible for the PhoP^c phenotype (attenuation of mouse virulence, defects in epithelial cell invasion, and macrophage spacious phagosome formation). PhoP phosphorylation by membrane extracts required PhoQ, and PhoQ Ile-48-containing membranes demonstrated increased net phosphorylation of PhoP.

Two-component regulatory systems allow bacteria to sense and respond to environmental cues by altering gene expression. One such system, PhoP/PhoQ, regulates proteins required for *Salmonella typhimurium* and *S. typhi* virulence (8, 15, 21). By analogy to similar proteins, PhoQ is a membrane-bound sensor histidine kinase that transfers phosphate to an aspartate(s) in the amino terminus of PhoP to either activate or repress target gene transcription. PhoP and PhoQ either positively or negatively regulate several pathogenic properties of *Salmonella* spp., including mouse and human virulence (8, 16, 21), resistance to antimicrobial peptides and low pH (7, 9, 23), survival within cultured macrophages (8, 21), formation of spacious phagosomes within macrophages (1, 2), entrance into epithelial cells (4, 17, 25), and bacterially induced immunosuppression (31).

Genes whose transcription is regulated by PhoP have been identified, and these genes have been termed PhoP-activated (*pag*) and PhoP-repressed (*prg*) genes. *pag* products include PagA to PagP (5, 21), PsiD (11), MgtABC (10), PhoN (nonspecific acid phosphatase) (20), PmrA/PmrB (12), and PhoP (28), which regulates its own transcription. Two conditions that are most potent in regulating *pag* through the PhoQ sensor are the divalent cations Mg^{2+} and Ca^{2+}, which repress *pag* (10), and the macrophage phagosome environment, which activate *pag* (3). Because *pag* genes are transcriptionally induced within macrophage phagosomes, it is possible that low cation concentration may be the signal sensed by PhoQ in vivo. Given the phenotypes known to be effected by PhoP/PhoQ and the fact that *pag* genes are expressed intracellularly, it is likely that *pag* genes play a role in *Salmonella* intracellular survival. *prg*-encoded products, which include secreted proteins and proteins that form part of a type III secretion apparatus (similar to those found in *Shigella* and *Yersinia* spp.), stimulate macropinocytosis and entrance into nonphagocytic eucaryotic cells by a ruffling mechanism (17, 25).

An *S. typhimurium* strain with a *phoP* locus mutation (*pho-24*) that constitutively expressed *pag* and repressed *prg* was isolated after diethyl sulfate mutagenesis (19, 22). The *pho-24* allele has a pleiotropic effect on *S. typhimurium* virulence. Altered phenotypes include attenuation of mouse virulence and lack of survival within cultured macrophages (22), loss of the ability to induce macropinocytosis and spacious phagosome formation in macrophages (2), and abolished epithelial cell invasion (17) and a marked decrease in protein secretion (25). In this work, we sought to define the mutation that caused these phenotypic alterations and the effect of this mutant protein on the activation of PhoP by phosphorylation.

To characterize the *pho-24* locus, the DNA encoding PhoP/PhoQ was cloned from CS022. PCR was used to amplify a fragment containing both *phoP* and *phoQ* from CS022 chromosomal DNA, and this fragment was cloned into pBR328, using restriction sites engineered into the primers (Table 1). The resulting clone, pPC01, was shown to complement a *phoPQ* deletion by the restoration of β-galactosidase activity in a strain containing the deletion and a *pagB*::MudJ insertion (data not shown). The *phoP* and *phoQ* genes of pPC01 were sequenced with various primers by the dideoxy method of Sanger et al. (27) as modified for use with Sequenase (U.S. Biochemical, Cleveland, Ohio). When the sequences of the *phoP* and *phoQ* genes from wild-type (WT) strain 14028s and pPC01 were compared, a one-base change (C to T) at position 957 of the published sequence was identified. This mutation resulted in a Thr-to-Ile change and is predicted to be in a periplasmic region of PhoQ that immediately follows a hydrophobic and likely membrane-spanning domain (Fig. 1). Mutagenesis of pPC01 with λTn*phoA* (13) was used to determine if this region of PhoQ was likely to be periplasmic. Several of the active fusions were sequenced, and all were found to be located between the two hydrophobic domains (amino acids 17 to 44 and 191 to 218) of PhoQ, suggesting that PhoQ likely contains one periplasmic region in which the PhoQ Ile-48 residue is located (data not shown).

CS022 is attenuated for virulence in mice (4-log increase in 50% lethal dose). However, Miller and Mekalanos (22) showed that following intraperitoneal inoculation of BALB/c mice with a dose well above the 50% lethal dose, virulent organisms could be and were, upon the onset of clinical illness, recovered from the livers of the some of the infected mice. These organisms were thought to be revertants of the original mutation because the locus responsible for the reversion phenotype mapped to *phoP* (22). To confirm that the *pho-24* mutation had reverted in these organisms, the *phoP* and *phoQ* genes of five independent revertants were PCR amplified, and

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype or relevant phenotype	Source or reference
Salmonella strains[a]		
ATCC 14028s	WT	ATCC
CS015	*phoP102*::Tn*10*d-cam	21
CS022	*pho-24*	22
CS204–CS208	CS022 revertants from mouse livers	22
CS019	*phoN2 zxx*::*6251*Tn*10*d-cam	21
Plasmids		
pGP704	Pir-dependent suicide vector	24
pMLB1109	Transcriptional reporter vector used to construct pLB02	3
pLB02	Firefly luciferase transcriptional reporter vector	This study
pLB222	pLB02 with the cloned *tet* promoter	This study
pLB5354	pLB02 with the cloned *phoP* promoter	This study
pPC01	pBR328 with the cloned *phoP* and *phoQ* from strain CS022	This study
pQE30	His tag vector	Qiagen
pQP05	pQE30 with the cloned *phoP*	This study
E. coli strains		
DH5α	F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *endA1 recA1 hsdR17 deoR thi-1 supE441 gyrA96 relA*1	Bethesda Research Laboratories
SM10λPir	*thi-1 thr-1 leuB6 supE44 tonA21 lacY1 recA*::RP4-2-Tc::Mu	
M15/pREP4	*E. coli* host for pQP05 that carries the *lac* repressor on pREP4	Qiagen
JSG024	SM10λPir with pLB222	This study
JSG071	SM10λPir with pLB5354	This study
JSG057	M15/pREP4 with pQP05	This study

[a] All are derivatives of 14028s.

the PCR products were directly sequenced. All five organisms recovered from the livers of infected mice had a reversion of the point mutation in *phoQ*. These data confirmed that the C-to-T point mutation in *phoQ* is the mutation responsible for the attenuated virulence of strain CS022.

Because a phosphorelay was predicted to be the mechanism of communication between PhoP and PhoQ, it was likely that constitutive activation of the PhoP regulon in CS022 was caused by increased net phosphotransfer from PhoQ Ile-48 to PhoP. To test this possibility, it was first necessary to purify PhoP. PhoP was tagged with six amino-terminal His residues and purified by metal chelate affinity chromatography. The *phoP* gene was PCR amplified without its native promoter or

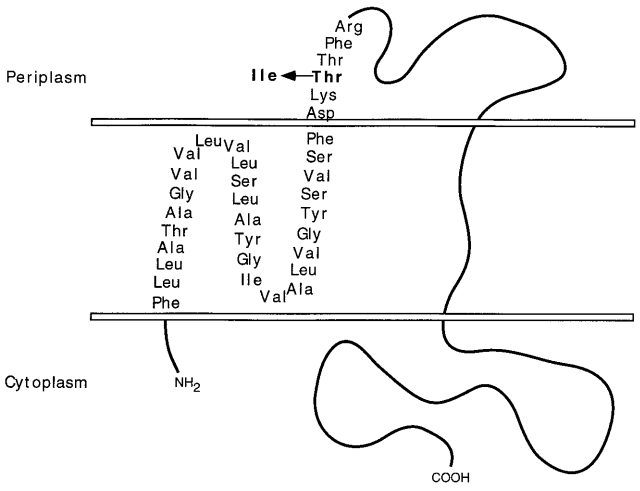

FIG. 1. Diagram of the membrane-bound sensor-kinase PhoQ depicting the first hydrophobic and likely membrane-spanning domain and the mutation at position 48 that results in increased expression of *pag* and repression of *prg*.

ribosome binding site and cloned into the vector pQE30 (under control of a lactose-inducible promoter) to produce plasmid pQP05. The *phoP* gene in pQP05 was sequenced to confirm that no mutations were introduced by PCR. The *phoP* gene, when cloned with *phoQ* in a low-copy-number vector with both genes under control of the *lac* promoter, was able to restore *pag* expression in a *phoP/Q* deletion strain (data not shown). Transcription of *phoP* on pQP05 was induced in logarithmic growth by the addition of isopropylthiogalactopyranoside (IPTG; 2 mM, final concentration). After 2 h, cells were harvested and lysed by sonication in sonication-wash buffer (50 mM sodium phosphate, [pH 7.8], 100 mM NaCl, 0.1% Triton X-100). The cleared lysate was passed over a 2-ml packed column of Ni-nitrilotriacetic acid (NTA) agarose and washed with 50 ml of wash buffer and 40 ml of wash buffer plus 10% glycerol. After one wash with 20 mM imidazole (in wash buffer plus 10% glycerol), PhoP-His was eluted in 3 ml of 150 mM imidazole. The eluate was dialyzed overnight against several changes of 10 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES, pH 8.0)–10% glycerol–0.25 mM dithiothreitol. Figure 2 shows a Coomassie blue-stained sodium dodecyl sulfate (SDS)-polyacrylamide gel of cell lysates and of the various steps that resulted in PhoP-His purification. By observation of the gel, the PhoP-His protein appears highly purified, as demonstrated by the presence of only minor bands in addition to PhoP by visual inspection, and was shown by Western blotting (immunoblotting) to react with an anti-PhoP polyclonal antibody (data not shown).

Membrane extracts from *S. typhimurium* 14028s (WT), CS022 (PhoQ Ile-48), and CS015 (PhoPQ$^-$) were isolated by a spheroplast-sonication method. Briefly, spheroplasts were isolated as described by Hantke (14), sonicated, and pelleted in a microcentrifuge for 30 min at 4°C. The pellet, containing fragmented membranes, was resuspended in 10% glycerol. This membrane extract was unreactive by Western blotting to the anti-PhoP polyclonal antibody (data not shown). Membranes

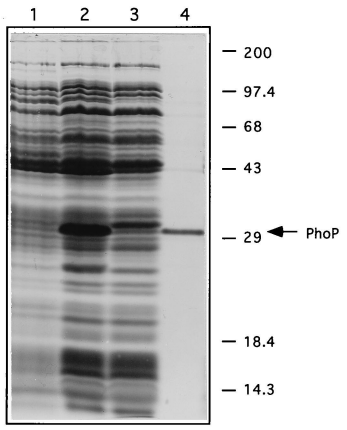

FIG. 2. Coomassie blue-stained SDS-polyacrylamide gel showing purification of the PhoP-His protein. Lane 1, whole-cell lysate of strain JSG057 (uninduced); lane 2, whole-cell lysate of strain JSG057 (induced with IPTG); lane 3, proteins from induced whole-cell lysate that did not bind to the Ni-NTA resin; lane 4, proteins from the Ni-NTA resin after extensive washing and elution with imidazole. The PhoP-His protein appears highly purified, as demonstrated by the presence of only minor bands in addition to PhoP. Sizes are indicated in kilodaltons.

(5 μg), PhoP-His (0.6 μg), and [γ-^{32}P]ATP (3.3 pmol) were incubated at 37°C for 30 min in reaction buffer (50 mM Tris-HCl [pH 7.5], 100 mM KCl, 5 mM $MgCl_2$). Samples were separated on 10% SDS-polyacrylamide gels and dried. Labeling of PhoP was detected by autoradiography. Figure 3A shows the results of phosphotransfer between CS015, which makes no detectable PhoQ by Western blot analysis (data not shown), or CS022 membranes and PhoP-His. Specific labeling can be seen with CS022 membranes (lane 1), while no labeling of PhoP-His was observed with CS015 membranes (lane 2). Thus, these data support the supposition that PhoQ functions as a kinase. Lanes 3 (CS022 membranes) and 4 (CS015 membranes) are controls in which no PhoP-His is added, which demonstrate that the observed band corresponds to the PhoP-His protein.

Figure 3B shows a phosphotransfer assay with membranes from logarithmically grown 14028s or CS022 cultures. A six-fold increase in PhoP-His labeling can be seen with CS022 membranes (lane 2) compared with 14028s membranes (lane 1) as measured by densitometry using NIH Image (version 1.59).

Previous evidence with PhoP/PhoQ produced from a multicopy plasmid indicated that it was likely that PhoP regulated its own expression (28). This finding raised the possibility that more PhoQ could be present in membrane preparations from CS022 than in those from WT and therefore result in increased PhoP-His phosphorylation. To confirm that *phoP* could regulate its own expression in the absence of potential multicopy effects, we constructed a transcriptional fusion of the *phoP* promoter to the firefly luciferase gene. A 281-bp region of the *phoP* promoter was PCR amplified (using JG53 [5′ GGAAT TCGAGTTGACCCGTGGCAAG 3′] and JG54 [5′ GGGGT ACCCTTGTGTTAACAATAAG 3′]) and cloned, using restriction sites engineered into the primers, into the firefly luciferase transcriptional reporter vector pLB02 to create plasmid pLB5354. Plasmid pLB02 is a combination of the suicide vector pGP704 (24) and the transcriptional reporter vector pMLB1109 (3), requires the Pir protein for plasmid maintenance, and contains a 700-bp chromosomal region (located downstream from *pagC* [26]) where recombination of the vector onto the chromosome can occur. Strain SM10λPir containing pLB5354 was mated with strain CS019 with selection for plasmid-encoded antibiotic resistance. Southern blot analysis confirmed that the vector had recombined downstream of *pagC* (data not shown). P22HT *int* transduction (6) was used to move the chromosomal region containing the recombined plasmid into CS022 (PhoQ Ile-48) and CS015 (PhoPQ$^-$). Luciferase activities of CS019::*phoP-luc*, CS022::*phoP-luc*, and CS015::*phoP-luc* were assayed as previously described (12). As shown in Table 2, a 22-fold induction of luciferase activity directed by the *phoP* promoter was observed between CS022 and CS015 backgrounds and a 3-fold induction was seen between CS022 (PhoQ Ile-48) and CS019. A constitutive promoter (from the *tet* gene of pBR322) was not affected in any of the three

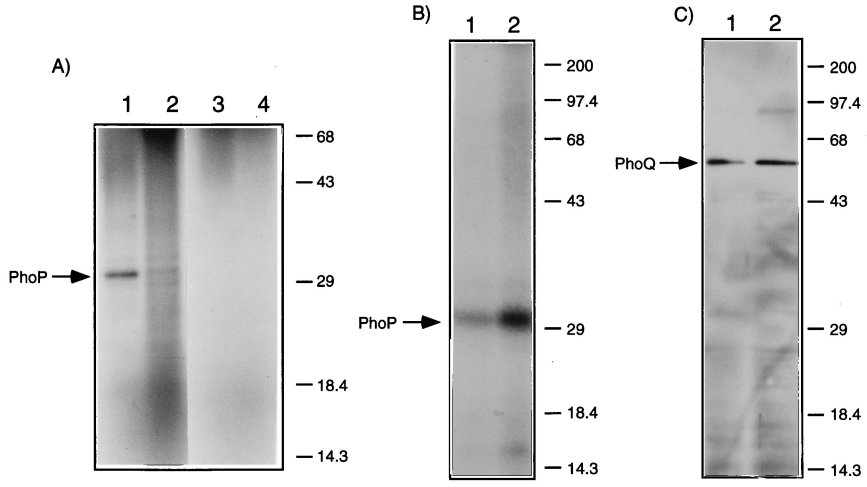

FIG. 3. Phosphotransfer assays demonstrating phosphorylation of the PhoP-His protein by *Salmonella* membrane preparations. Panels A and B are autoradiograms of phosphotransfer assays that included membrane preparations, PhoP-His, and [γ-^{32}P]ATP. (A) Membrane preparations from overnight cultures. Lane 1, CS022 membranes plus PhoP-His; lane 2, CS015 membranes plus PhoP-His; lane 3, CS022 membranes without PhoP-His added; lane 4, CS015 membranes without PhoP-His added. (B) Membranes isolated from logarithmically grown cultures. Lane 1, ATCC 14028s membranes plus PhoP-His; lane 2, CS022 membranes plus PhoP-His. (C) Western blot analysis of lanes 1 and 2 from panel B blotted to nitrocellulose and reacted with a PhoQ polyclonal antibody (rabbit antipeptide from amino acids 135 to 152 of PhoQ) demonstrated that equal amounts of PhoQ were present in both membrane extracts. The cross-reacting band seen at 90 kDa was variably observed in both 14028s and CS022 membrane preparations. Positions of molecular weight markers (in kilodaltons) are indicated at the right of each panel.

TABLE 2. Analysis of *phoP*/*phoQ* autoregulation

Promoter::*luc* fusion	RLU[a] (mean ± SD)			Fold induction	
	CS015	CS019	CS022	P^c/P^{-b}	P^c/WT[c]
phoP	15,099 ± 865	110,557 ± 6,513	335,742 ± 11,325	22	3
tet	88,975 ± 2,479	76,652 ± 2,133	72,165 ± 1,466		

[a] Luciferase activity is expressed in relative light units (RLU) for each promoter::*luc* CS015, CS019, or CS022 chromosomal fusion.
[b] Expressed as RLU of CS022 divided by the RLU of CS015. P^c, CS022; P^-, C5015.
[c] Expressed as RLU of CS022 divided by the RLU of CS019.
[d] NI, no induction.

backgrounds tested. Therefore, these data confirmed previous findings in multicopy constructs which suggested that *phoP* regulated its own expression. Because *phoP* (and therefore *phoQ* because they form an operon) transcription is induced threefold in CS022 compared with WT, a Western blot analysis was performed with an anti-PhoQ polyclonal antibody on the membrane preparations used for Fig. 3B. The result (Fig. 3C) shows that despite differences in transcription, equal amounts of PhoQ are present in membrane preparations. This may be due to the simple fact that translation efficiency and or protein stability is much lower for PhoQ than PhoP as is typical of two-component systems in operons (29). Therefore, these data indicate that a net increase in phosphotransfer to PhoP-His by CS022 membranes was not due to an increase in the relative amount of the PhoP-specific kinase, PhoQ. The observed net phosphotransfer increase could be due to an increased rate of phosphotransfer or a decrease in PhoQ phosphatase activity, a sensor-kinase function that has been demonstrated for proteins of the PhoQ family (18).

The ability of PhoQ to sense the macrophage phagosome environment and to activate transcription through PhoP is vital to the ability of *S. typhimurium* to cause disease. In this work, we provide evidence that a phosphorylation cascade mediates communication between PhoQ and PhoP. PhoP phosphorylation required PhoQ, and membranes from the PhoQ Ile-48 mutant (CS022) had increased net phosphorylation of PhoP. The PhoQ Ile-48 mutation is located three amino acids from the end of the first hydrophobic domain of PhoQ; therefore, this residue is likely to be periplasmic. Activation of the PhoP regulon by the PhoQ Ile-48 mutation is predicted to be caused by a conformational change in PhoQ that inactivates phosphatase activity or to activates kinase activity. Alternatively, this conformational change, or possibly a direct interaction involving the amino acid at position 48, could increase PhoQ dimerization, which is likely necessary for sensor-kinase intermolecular histidine phosphorylation and kinase activity (30).

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