JOURNAL OF BACTERIOLOGY, Aug. 1995, p. 4364–4371 Vol. 177, No. 15
0021-9193/95/$04.00+0

Transcriptional Autoregulation of the *Salmonella typhimurium phoPQ* Operon

FERNANDO C. SONCINI, ELEONORA GARCÍA VÉSCOVI, AND EDUARDO A. GROISMAN*

Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110

Received 17 October 1994/Accepted 22 May 1995

The *Salmonella typhimurium* PhoP-PhoQ two-component regulatory system controls the expression of several genes, some of which are necessary for virulence. During a screening for PhoP-regulated genes, we identified the *phoPQ* operon as a PhoP-activated locus. β-Galactosidase activity originating from *phoPQ-lac* transcriptional fusions required the presence of both the transcriptional regulator PhoP and its cognate sensor-kinase PhoQ. At low concentrations, PhoQ stimulated expression of *phoPQ-lac* transcriptional fusions. However, larger amounts of PhoQ protein without a concomitant increase in PhoP failed to activate *phoPQ-lac* fusions. Two different transcripts are produced from the *phoPQ* operon during exponential growth. These transcripts define two promoters: $phoPp_1$, which requires both PhoP and PhoQ for activity and which is environmentally regulated, and $phoPp_2$, which remains active in the absence of PhoP and PhoQ but which is slightly stimulated by these proteins. The pattern of transcriptional autoregulation was also observed at the protein level with anti-PhoP antibodies. In sum, autoregulation of the *phoPQ* operon provides several levels of control for the PhoP-PhoQ regulon. First, environmental signals would stimulate PhoQ to phosphorylate the PhoP protein that is produced at basal levels from the PhoP-PhoQ-independent promoter. Then, phospho-PhoP would activate transcription of $phoPp_1$, resulting in larger amounts of PhoP and PhoQ and increased expression of PhoP-activated genes. A return to basal levels could be mediated by a posttranscriptional mechanism by which translation of the mRNA produced from $phoPp_1$ is inhibited.

Salmonellae are facultative intracellular pathogens responsible for several disease syndromes in a wide variety of animal species. In humans, they have been implicated in four pathological conditions: typhoid fever, gastroenteritis (food poisoning), bacteremia, and the asymptomatic carrier state (14). Certain *Salmonella* serotypes have a very narrow host range, while others are poorly host adapted and cause distinct diseases in different hosts. For example, typhoid fever is primarily caused by the human-adapted *Salmonella typhi*, while *Salmonella typhimurium*, the leading serotype associated with gastroenteritis in humans, causes a typhoid-like disease in susceptible mice (16). The ability to genetically manipulate *S. typhimurium* and the availability of excellent models of infection have allowed the identification of many of the virulence determinants that enable *Salmonella* spp. to adapt and prosper within different host environments.

In *S. typhimurium*, virulence is controlled at the transcriptional level by several proteins, including the RpoS sigma factor (7), the cyclic AMP-binding protein CRP (4), and the two-component regulatory systems OmpR-EnvZ (6) and PhoP-PhoQ (8, 11, 13, 28). The PhoP-PhoQ system was originally identified as a virulence determinant by the intramacrophage survival defect of *S. typhimurium phoP* mutants (8, 9). A virulence role for *phoP* was independently demonstrated by others investigating regulatory loci necessary for virulence (11, 28). The *phoP* locus encodes two proteins, PhoP and PhoQ, with homology to the regulators-receivers and the sensors-transmitters of the two-component family, respectively (13, 28). PhoQ is predicted to be an inner membrane protein that phosphorylates and dephosphorylates the putative DNA-binding protein PhoP in response to environmental changes. The PhoP-PhoQ system is peculiar in that both null mutations in either *phoP* or *phoQ* as well as a constitutive allele mapping to *phoQ* result in attenuation for virulence (9, 29).

As expected for the pleiotropic role of the PhoP-PhoQ system, several phenotypes have been associated with mutations in the *phoPQ* operon, including hypersusceptibility to antimicrobial peptides (8, 15, 30, 32) and acid pH (10), deficiency in epithelial cell invasion (1), and the inability to survive within macrophages (9) and to alter antigen presentation (41). It has been estimated that some 40 polypeptides are regulated by PhoP-PhoQ (29) and that at least 9 of these proteins are induced within the macrophage (2). Only two PhoP-activated genes have been cloned and sequenced: *phoN* (17, 22) and *pagC* (35), encoding a nonspecific acid phosphatase and outer membrane protein, respectively. Upstream of the *phoN* open reading frame, there is a region in which 13 of 16 nucleotides are identical to a DNA segment present upstream of the *phoP* coding region (13, 17). If this sequence corresponded to a PhoP-binding site, then PhoP could be involved in controlling transcription of the *phoPQ* operon. For example, the homologous system PhoB-PhoR is positively autoregulated at the level of transcription. PhoB binds to the Pho box, a sequence present in the promoter regions of several PhoB-regulated genes, including the *phoBR* operon (25, 26).

During a search for PhoP-regulated genes, we identified the *phoPQ* operon as a PhoP-regulated locus. In this paper, we report a molecular genetic analysis of autoregulation of *phoPQ* in *S. typhimurium*. We establish that full expression of *phoPQ* requires both PhoP and PhoQ, that two promoters are used to transcribe the *phoPQ* operon, and that these promoters differ in their response to and dependence on PhoP-PhoQ.

* Corresponding author. Mailing address: Department of Molecular Microbiology, Washington University School of Medicine, 660 S. Euclid Ave., Campus Box 8230, St. Louis, MO 63110. Phone: (314) 362-3692. Fax: (314) 362-1232. Electronic mail address: groisman@borcim.wustl.edu.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. Strains and plasmids used in this study are listed in Table 1. The physical maps of plasmids pUHE21-2*lacI*^{q}

TABLE 1. Strains and plasmids

Strain or plasmid	Genotype	Reference or source
S. typhimurium		
14028s	Wild type	ATCC[a]
TT10288	*hisD9953*::MudJ *hisA9944*::MudI	21
MS7953s	*phoP7953*::Tn*10*	8
MS5996s	*phoQ5996*::Tn*10*	8
EG5170	*phoP5170*::MudJ	This work
EG5172	*phoQ5172*::MudJ	This work
EG9252	*phoP9252*::MudJ *phoP7953*::Tn*10*	This work
EG9266	*phoP5170*::MudJ *phoQ5996*::Tn*10*	This work
EG9267	*phoQ5996*::Tn*10 phoQ5172*::MudJ	This work
EG9315	*phoP5170*::MudJ *phoP7953*::Tn*10*	This work
EG9316	*phoP7953*::Tn*10 phoQ5172*::MudJ	This work
E. coli		
JM109	F′ *traD36 lacI*^{q} Δ(*lacZ*)*M15 proA*$^+$*B*$^+$/e14$^-$ (McrA$^-$) Δ(*lac-proAB*) *thi gyrA96* (Nal^r) *endA1 hsdR17* ($r_K^-m_{K^+}$) *relA1 supE44 recA1*	43
TB1	F′ *ara* Δ(*lac-proAB*) *rpsL* (Str^r) [ϕ*80* d*lac*Δ(*lacZ*)*M15*]	43
Plasmids		
pUHE21-2*lacI*^{q}	rep$_{pMB1}$ Ap^r *lacI*^{q}	23
pEG5381	rep$_{pMB1}$ Ap^r *phoPQ*$^+$	13
pEG5433	pEG5381 Δ*Sal*I	13
pEG9014	rep$_{pMB1}$ Ap^r *lacI*^{q} *phoP*$^+$	This work
pEG9050	rep$_{pMB1}$ Ap^r *lacI*^{q} *phoQ*$^+$	This work
pEG9071	rep$_{pMB1}$ Ap^r *lacI*^{q} *phoPQ*$^+$	This work

[a] ATCC, American Type Culture Collection.

(23), pEG9014, pEG9050, and pEG9071 are shown in Fig. 1. Luria-Bertani (LB) and green agar plates as well as LB broth were prepared as described previously (27). Ampicillin and kanamycin (both from Sigma) were used at 50 μg/ml each, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Jersey Lab Supply) was used at 64 μg/ml, and isopropyl-β-D-thiogalactopyranoside (IPTG; U.S. Biochemicals) was used at 0.1 mM.

Bacterial genetic techniques. Phage P22 lysates were prepared and used as described previously (5). Plasmid DNA was introduced into the different strains by phage P22-mediated transduction with lysates prepared in plasmid-containing strains or by electroporation with a Bio-Rad apparatus according to the manufacturer's recommendations. MudJ is the mini Mu derivative originally designated MudI1734 by Castilho et al. (3). MudJ insertions in the *phoPQ* operon were isolated with a P22 lysate grown in strain TT10288 to infect either 14028s or MS7953s/pEG9014. EG9266 and EG9267 were constructed by transducing *phoQ5996*::Tn*10* from MS5996s into EG5170 and EG5172, respectively. EG9315 and EG9316 were constructed by transducing *phoP7953*::Tn*10* from MS7953s into EG5170 and EG5172, respectively.

DNA biochemistry and molecular biological techniques. Plasmid DNA was prepared by the boiling method (20). Other molecular biological techniques were taken from Sambrook et al. (37). To construct pEG9014, a PCR-generated fragment harboring the *phoP* coding region was cloned into the *Hin*dIII and filled-in *Bam*HI sites of pUHE21-2*lacI*^{q} (23) (Fig. 1). Plasmid pEG9050 was made by cloning a PCR-generated fragment harboring the *phoQ* coding region into the *Hin*dIII and filled-in *Bam*HI sites of pUHE21-2*lacI*^{q}. Plasmid pEG9071 was generated by ligating the *phoPQ*-containing 3.8-kb *Nsi*I-*Eco*RI fragment from pEG5433 (14) with pEG9014 DNA that had been digested with *Nsi*I and *Hin*dIII. *Eco*RI and *Hin*dIII were filled in with the Klenow fragment before ligation. That plasmid pEG9014 harbored an insert whose sequence is identical to that of wild-type *phoP* was confirmed by DNA sequence analysis.

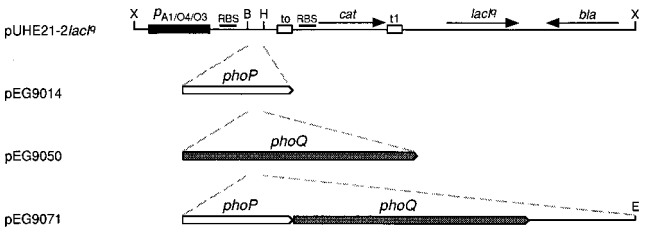

FIG. 1. Structure of plasmids carrying *phoP*, *phoQ*, and *phoPQ*. The pUHE21-2*lacI*^{q} plasmid is shown linearized at the *Xho*I site (drawing is not to scale). The positions of the A1-O4-O3 promoter, multicloning site, ribosome-binding sites (RBS), the promoterless chloramphenicol acetyltransferase gene (*cat*), the *lac* repressor gene (*lacI*^{q}), the β-lactamase gene (*bla*), and the transcriptional terminators (to and t1) are indicated. Plasmids pEG9014, pEG9050, and pEG9071 were constructed by inserting *phoP*- and *phoQ*-derived fragments into pUHE21-2*lacI*^{q} as described in Materials and Methods. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; X, *Xho*I.

To localize the site of insertion of the MudJ elements in the *phoPQ* operon, we used PCR to amplify the chromosomal DNA with primers 312 (5′-GTGGATC CGGTACCTGGTCGACGAACTTA-3′) and 11838 (5′-CGTGAAACGCTT TCGCG-3′), which correspond to the 5′ promoter region of *phoP* and to the right (*attR*) end of MudJ, respectively. The precise position of MudJ in EG9252 was determined by DNA sequencing of the PCR product by the dideoxynucleotide chain-termination method with Sequenase version 2.0 (U.S. Biochemicals), α-^{35}S-dATP (Amersham), and primer 312.

To determine the site of transcription initiation of the *phoPQ* operon, we conducted primer extension analysis with total RNA and two different primers, 366 (5′-ATCCTCTACAACCAGTACGCGCATCAT-3′) and 369 (5′-GAATC CTGGAGCTGAACCTTCAGGTGG-3′). The primers were end labeled with polynucleotide kinase and hybridized at 42°C overnight with 15 μg of total RNA. Super Script II RNase H$^-$ reverse transcriptase (Gibco BRL) was used to extend the mixture. The cDNA products were examined by electrophoresis through 6% polyacrylamide–8 M urea gels. To map the exact transcriptional start sites, sequencing reactions were performed on the *phoPQ*-containing plasmid pEG5381 with the same ^{32}P-labeled primer that was used for the primer extension reactions.

Enzymatic determinations and Western blot (immunoblot) analysis. β-Galactosidase activity (27) was determined with overnight cultures grown in LB broth containing 50 μg of ampicillin per ml and either 0 or 0.7 mM IPTG. Detection of PhoP in Western blots was carried out by loading sodium dodecyl sulfate (SDS)–10% polyacrylamide gels with 30 μg of protein corresponding to whole-cell extracts prepared from overnight cultures grown in LB broth. Western blot analysis was performed as described previously (34) with rabbit antibodies raised against a maltose-binding protein–PhoP hybrid protein and purified with purified PhoP protein.

RESULTS

Identification of *phoPQ* as a PhoP-regulated locus. During a screening for PhoP-regulated loci, we identified a strain harboring a MudJ insertion that mapped to the *phoPQ* operon and whose β-galactosidase activity was dependent on PhoP. MudJ is a derivative of bacteriophage Mu that carries a pro-

moterless *lac* operon segment near its right end, and insertions in the correct transcriptional orientation may result in the production of β-galactosidase (3). MudJ insertions were originally isolated in strain MS7953s/pEG9014, harboring a chromosomal *phoP*::Tn*10* and a plasmid with *phoP* under the control of a derivative of the *lac* promoter (Fig. 1). Mutants were patched onto two LB X-Gal plates, one of which contained the gratuitous inducer of the *lac* promoter, IPTG. Colonies that exhibited differences in β-galactosidase activity were candidates for harboring fusions to PhoP-regulated genes. These candidates were purified, the MudJ was transduced into both wild-type and *phoP*::Tn*10* strains, and the β-galactosidase activities exhibited by the new pair of strains were compared. Differences in β-galactosidase activities for isogenic wild-type and *phoP* mutant strains were observed for the majority of these mutants.

One MudJ insertion exhibited anomalous behavior in that the transductants of wild-type and *phoP* mutant strains displayed the same levels of β-galactosidase. This was in contrast to the phenotype of the original MudJ mutant (strain EG9252), which exhibited differences upon induction of plasmid-encoded *phoP*. Moreover, the MudJ transductant of the wild-type strain was sensitive to the antimicrobial peptide protamine and failed to produce nonspecific acid phosphatase activity, phenotypes that are characteristic of strains with null alleles of *phoP* or *phoQ* (12). That this mutant harbored an insertion in the *phoP* locus was confirmed in phage transduction experiments. Using a phage P22 lysate grown in EG9252/pEG9014 as the donor and the wild-type strain 14028s as the recipient, we established a tight linkage of the MudJ with *phoP*::Tn*10*: 98% of the kanamycin-resistant transductants (49 of 50) were also resistant to tetracycline. Therefore, we had isolated a *lac* gene fusion to the *phoPQ* locus whose activity was modulated by PhoP (strain EG9252 in Table 2).

Analysis of different *phoPQ*::*lac* gene fusion strains. Over the last few years, our laboratory has isolated several MudJ insertions in the *phoPQ* operon on the basis of their inability to produce nonspecific acid phosphatase and their linkage to *purB*. We investigated whether two such mutants (EG5170 and EG5172), harboring MudJ insertions in the same transcriptional orientation as that of the *phoPQ* genes, exhibited the same regulatory behavior as strain EG9252 (Fig. 2). Mutants EG5170 and EG5172 were transformed with the *phoP*$^+$-containing plasmid pEG9014, and β-galactosidase activity was determined in extracts prepared from cells grown under both inducing and noninducing conditions for *lacp*-controlled PhoP (0.7 and 0 mM IPTG, respectively). In contrast to the results obtained with EG9252, no differences in β-galactosidase activity could be detected for derivatives of EG5170 and EG5172 harboring the *phoP*$^+$ plasmid upon induction for PhoP expression (Table 2).

To establish the molecular basis for the dissimilar results obtained with strains EG9252, EG5170, and EG5172, we determined the site of MudJ insertion within the *phoPQ* operon in the three mutants. We used PCR to amplify the DNA segment between a region upstream of the *phoPQ* promoter and the right end of MudJ and ran the PCR products on an agarose gel (Fig. 2C). The MudJ was localized to the *phoP* and *phoQ* open reading frames in EG5170 and EG5172, respectively, and upstream of the *phoP* start codon in EG9252. Apart from the particular position of each MudJ insertion, EG9252 differed from EG5170 and EG5172 in that it harbored a Tn*10* insertion at the 3′ end of *phoP*. A promoter within Tn*10* (24) could provide a low level of *phoQ* transcription sufficient to mediate phosphorylation of PhoP, which could then activate transcription at the *phoPQ* promoter.

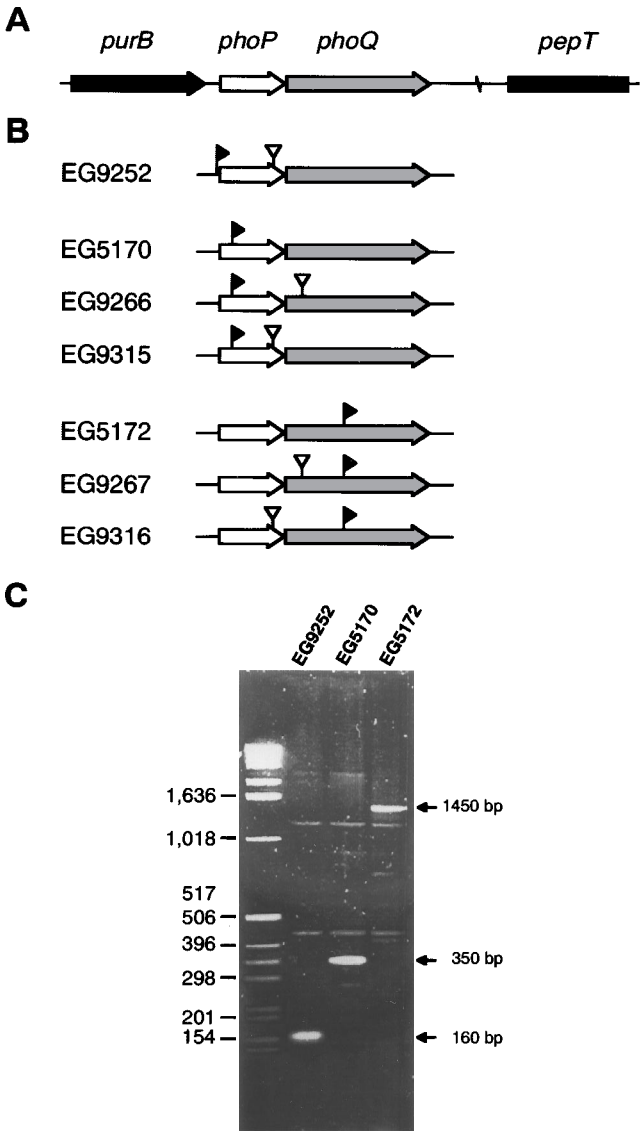

FIG. 2. Structure of the *phoP* locus in strains harboring insertions in the *phoPQ* operon. (A) Genetic map of the *phoPQ* operon in the *S. typhimurium* 25-min region. (B) Location of MudJ (black arrowheads) and Tn*10* (open triangles) insertions in EG9252, EG5170, EG5172, and their derivatives. (C) Localization of MudJ insertions in EG9252, EG5170, and EG5172. Shown is an ethidium bromide-stained, 1.5% agarose gel of the products obtained after PCR amplification of EG9252, EG5170, and EG5172 with primers complementary to the 5′ promoter region of *phoP* and to *attR* of MudJ. The numbers at the left are base pairs.

PhoQ is required for PhoP-mediated activation of *phoPQ*. To investigate the role of PhoQ in autoregulation, we constructed a series of isogenic derivatives of EG5170 and EG5172 harboring Tn*10* insertions in either *phoP* or *phoQ*. We used the *phoP7953*::Tn*10* present in EG9252, which had been localized to the 121-bp *Pvu*II-*Eco*RV fragment in the 3′ end of the *phoP* coding region, and the *phoQ5996*::Tn*10*, which had been mapped to the 5′ end of *phoQ* (13). The resulting strains harbored a MudJ and a Tn*10* within the *phoPQ* operon (Fig. 2B). These strains were transformed with the *phoP*$^+$-containing plasmid pEG9014 or the plasmid vector, and β-galactosidase activities were determined under both inducing and noninducing conditions (Table 2). Strain EG9315, an EG5170 derivative harboring the same *phoP7953*::Tn*10* insertion as

TABLE 2. β-Galactosidase activity of *phoPQ-lac* transcriptional fusions of strains expressing different amounts of PhoP and PhoQ

Strain	Structure of chromosomal *phoPQ* operon[a]	Plasmid	Structure of plasmids harboring *phoP* and *phoQ*[a]	β-galactosidase activity[b] 0 mM	β-galactosidase activity[b] 0.7 mM	Induction coefficient[c]
EG9252		pUHE21-2*lacI*^{q}		4.6	5.1	1.1
		pEG9014		4.8	110.4	23.0
		pEG9050		5.0	4.6	0.9
		pEG9071		4.3	156.7	36.4
EG5170		pUHE21-2*lacI*^{q}		243.0	225.3	0.9
		pEG9014		241.5	270.0	1.1
		pEG9050		250.0	226.8	0.9
		pEG9071		260.5	2736.7	10.5
EG9266		pUHE21-2*lacI*^{q}		252.0	218.0	0.9
		pEG9014		245.6	209.2	0.9
		pEG9050		252.6	237.7	0.9
		pEG9071		258.8	2817.5	10.9
EG9315		pUHE21-2*lacI*^{q}		155.1	178.5	1.2
		pEG9014		182.2	1146.1	6.3
		pEG9050		156.4	147.6	1.0
		pEG9071		167.9	1066.9	6.3
EG5172		pUHE21-2*lacI*^{q}		126.9	118.2	0.9
		pEG9014		132.2	110.2	0.8
		pEG9050		818.7	156.4	0.2
		pEG9071		1062.2	952.3	0.9
EG9267		pUHE21-2*lacI*^{q}		6.3	5.2	0.8
		pEG9014		5.9	5.9	1.0
		pEG9050		5.8	6.1	1.0
		pEG9071		5.4	5.6	1.0
EG9316		pUHE21-2*lacI*^{q}		13.3	17.5	1.3
		pEG9014		15.5	22.0	1.4
		pEG9050		17.4	15.9	0.9
		pEG9071		21.0	20.6	1.0

[a] Genes and transposons are shown as follows: , *phoP*; ▨, *phoQ*; , MudJ; and ■, Tn*10*.

[b] β-Galactosidase specific activity is expressed in Miller units and was determined as described in Materials and Methods. Values are the averages of two independent experiments done in duplicate. The values 0 mM and 0.7 mM refer to the final concentrations of IPTG in the assay.

[c] Induction coefficient refers to the ratio of β-galactosidase activities obtained with 0.7 and 0 mM IPTG.

EG9252, exhibited an increase in β-galactosidase activity upon induction of the *lac* promoter when harboring pEG9014 but not when carrying the plasmid vector. This result suggested that a promoter within the Tn*10* in *phoP* was transcribing *phoQ*. Indeed, no induction could be observed in EG9266, an isogenic derivative harboring a Tn*10* within *phoQ* rather than *phoP*. As expected, the *phoP*::Tn*10* and *phoQ*::Tn*10* derivatives of EG5172 (EG9316 and EG9267, respectively), which harbors a MudJ in *phoQ*, could not express β-galactosidase. Cumulatively, these results indicate that autoregulation requires both *phoP* (provided by the pEG9014 plasmid) and *phoQ* (transcribed by a promoter within the Tn*10* present in *phoP*).

To further examine the role of PhoQ in autoregulation, we studied the behavior of strains harboring pEG9050, a plasmid isogenic to pEG9014 but with *phoQ* under the control of the *lac* derivative promoter (Fig. 1). The *phoQ* defect of strain EG5172 could be rescued upon transformation with pEG9050: this strain produced 6.5 times more β-galactosidase activity relative to that of derivatives containing pEG9014 or the plasmid vector. These data were obtained with uninduced cells because induction of *phoQ* in pEG9050 decreased the β-galactosidase activity fivefold relative to that of the uninduced culture. The activation effect of PhoQ was at the *phoPQ* promoter because derivatives of EG5172 with Tn*10* insertions upstream of the MudJ (EG9267 and EG9316) expressed the same low levels of β-galactosidase in pEG9050-transformed cells as in cells transformed with the plasmid vector (Table 2). Plasmid pEG9050 did not stimulate or repress production of β-galactosidase in strains harboring insertions within *phoP* (EG9252, EG5170, EG9266, and EG9315) (Table 2). These results indicate that low levels of PhoQ stimulate expression of *phoPQ*, probably by phosphorylating PhoP, and confirm the requirement of PhoP for autoregulation.

Regulation of *phoPQ*::*lacZ* gene fusions by a plasmid harboring the *phoPQ* operon. The experiments described in the previous sections were conducted with strains in which *phoP* was expressed from the chromosome and *phoQ* was expressed from a plasmid, or vice versa. We examined the behavior of the seven mutant strains carrying plasmid pEG9071, a pUHE21-2*lacI*^{q} derivative harboring the whole *phoPQ* operon under control of the *lac* derivative promoter (Fig. 1). Induction of *phoPQ* resulted in an increase in β-galactosidase activity in EG9252, EG5170, EG9266, and EG9315 (Table 2), confirming that both PhoP and PhoQ are necessary for autoregulation of the *phoPQ* operon. As was observed with EG5172/pEG9050, introduction of pEG9071 into EG5172 resulted in 8 to 10 times higher β-galactosidase activity relative to that of strains harboring the plasmid vector. However, whereas induction of *phoQ* in EG5172/pEG9050 resulted in the repression of β-galactosidase activity, expression remained high in EG5172/pEG9071 upon induction of *phoPQ*.

Two promoters transcribe the *phoPQ* operon. To identify the transcription start site(s) of the *phoPQ* operon, we performed primer extension experiments with RNA prepared from cells harvested in late exponential phase or following overnight growth. Two RNA species were detected in an exponentially growing wild-type *Salmonella* culture (Fig. 3). These RNAs correspond to two distinct transcription start sites and are not artifacts of primer extension because they were detected with two different primers and in S1 mapping experiments (data not shown). The transcripts differ in 11 nucleotides and display distinct regulatory features. First, both transcripts were detected in cells harvested in logarithmic phase, but their levels were very much reduced in stationary-phase cells. Second, the shorter transcript was produced in wild-type cells and *phoP* and *phoQ* mutants, whereas the longer transcript could be detected only in wild-type cells. This set of experiments demonstrated that two promoters transcribe the *phoPQ* operon: $phoPp_1$, which is dependent on PhoP and PhoQ for activity, and $phoPp_2$, which does not require PhoP or PhoQ but which is slightly stimulated by their presence.

The relative strengths and the regulation of the two promoters were evaluated by analyzing the levels of β-galactosidase activity in different mutants. In strain EG9252, the MudJ is present between the transcription start sites for the two *phoPQ* promoters (Fig. 3), and so the low levels of β-galactosidase reflect the activity of $phoPp_1$. In contrast, the *lac* operon in the isogenic EG9315 strain which is expected to be transcribed from the two *phoPQ* promoters expressed higher levels of β-galactosidase activity (Table 2). Interestingly, the activation ratios for PhoP- and PhoP-PhoQ-induced cells were much higher for EG9252 (23 to 36 times) relative to that for EG9315 (6.3 times) (Table 2). We investigated the β-galactosidase activity produced by strains bearing *phoP*$^+$ or *phoPQ*$^+$ plasmids and incubated with different amounts of IPTG. In strains transcribing *lac* from the two promoters (EG5170, EG9315, and EG5172), maximum activity was detected at 70 μM IPTG and no further increases were found upon growth in the presence of 0.7 or 2.5 mM IPTG (Fig. 4A). On the other hand, the β-galactosidase activity originating from the single promoter in

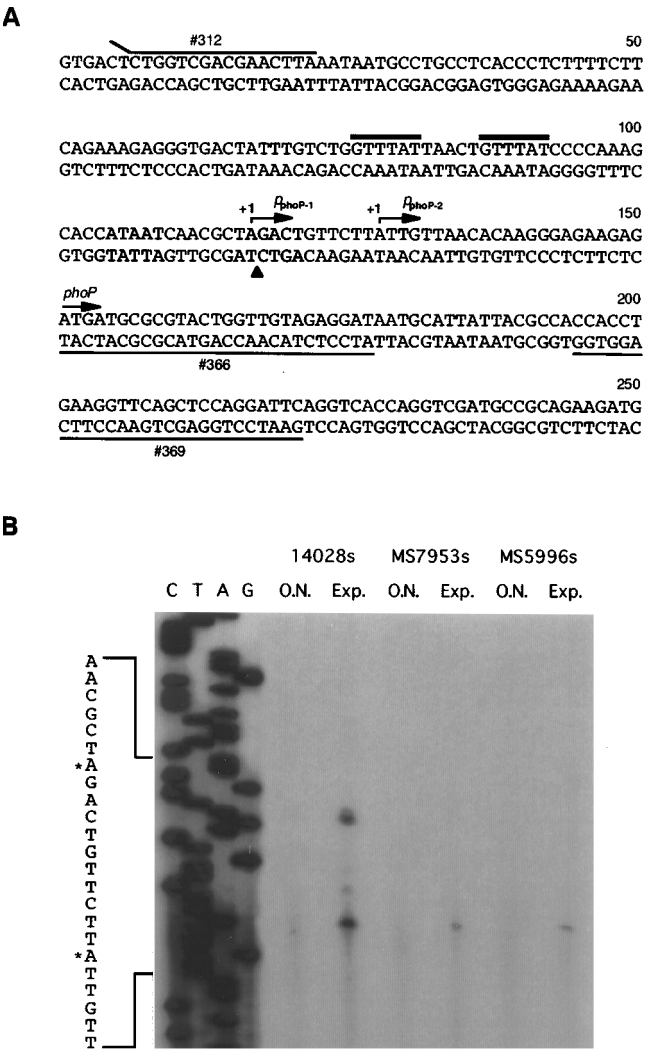

FIG. 3. The transcription start sites of the *phoPQ* operon. (A) Nucleotide sequence of the *phoPQ* promoter region. The arrows indicate the starts and directions of transcription and the position of the phoP open reading frame. The start site of the $phoPp_1$ transcript is numbered as +1, the −10 regions for $phoPp_1$ and $phoPp_2$ are shaded, and the region harboring a direct repeat is overlined. The sequences corresponding to primers 312, 366, and 369 are underlined. The arrowhead indicates the location of the MudJ insertion in EG9252. (B) Primer extension analysis (with the 366 primer) of *phoPQ* mRNA extracted from overnight (O.N.) or late-exponential-phase (Exp.) cultures of the wild type (14028s), the *phoP7953*::Tn*10* mutant (MS7953s), and the *phoQ5996*::Tn*10* mutant (MS5996s). The CTAG lane corresponds to dideoxy chain termination sequence reactions in the region encompassing the promoters. The asterisks indicate the transcription initiation sites.

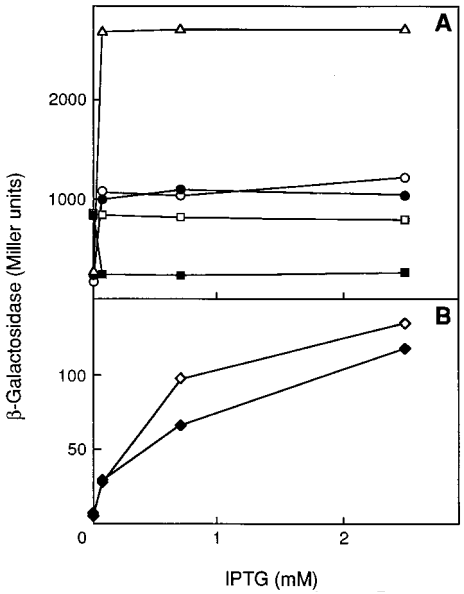

FIG. 4. Expression of chromosomal *phoPQ-lac* transcriptional fusions in strains expressing different levels of PhoP and PhoQ. (A) β-Galactosidase activities of EG5170/pEG9071 (open triangles), EG9315/pEG9014 (solid circles), EG9315/pEG9071 (open circles), EG5172/pEG9071 (open squares), and EG5172/pEG9050 (solid squares) cells grown with the indicated concentrations of IPTG. (B) β-Galactosidase activities of EG9252/pEG9014 (solid diamonds) and EG9252/pEG9071 (open diamonds) cells grown with the indicated concentrations of IPTG. β-Galactosidase activity was determined with overnight cultures as described in Materials and Methods. These results correspond to one of two independent experiments performed in duplicate.

strains EG9252/pEG9014 and EG9252/pEG9071 climbed as the concentration of IPTG increased from 70 μM to 2.5 mM (Fig. 4B). These results reflect the activities of cells grown in LB broth to stationary phase. The relative strengths of the two promoters may be different under other growth conditions.

As was stated above, the transcripts produced from $phoPp_1$ and $phoPp_2$ differ in 11 nucleotides (Fig. 3). Secondary structure predictions for the corresponding mRNAs indicate that the longer transcript could generate a stem-loop structure that would include the Shine-Dalgarno region for *phoP* (Fig. 5).

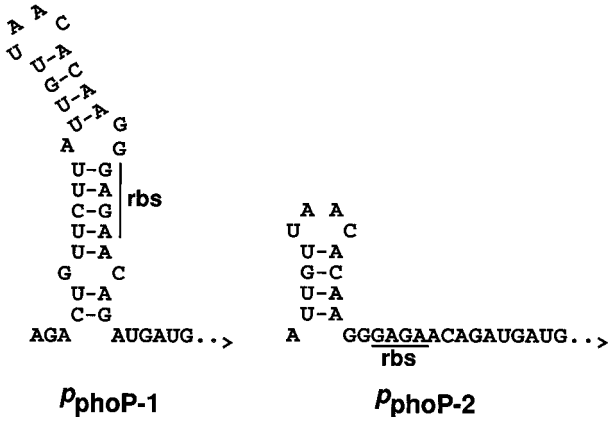

FIG. 5. Predicted secondary structures corresponding to the 5′ regions of the mRNAs produced from the two *phoP* promoters (p_{phoP-1} and p_{phoP-2}), as predicted by the FOLD and SQUIGGLES programs (Genetics Computer Group). The predicted stem in the transcript produced from $phoPp_1$ (p_{phoP-1}) would occlude the ribosome-binding site (rbs) for *phoP*.

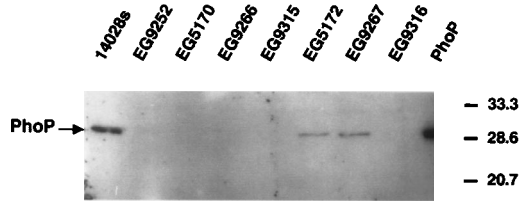

FIG. 6. Regulation of expression of PhoP in different mutant strains. Whole-cell extracts were prepared from the wild type (14028s) and the following mutants: EG9252, EG5170, EG9266, EG9315, EG5172, EG9267, and EG9316. PhoP indicates a lane loaded with purified PhoP. Thirty micrograms of extract was loaded per lane, run in an SDS-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane; Western blotting was performed as described in Materials and Methods. Molecular mass standards (Bio-Rad) are indicated in kilodaltons at the right.

The predicted structure, which would have a ΔG of −3.9 kcal/mol, could prevent translation of *phoP* and also potentially of *phoQ* since the latter does not have a ribosome-binding site and its translation appears to be coupled to that of PhoP. The stabilization and destabilization of these stem-loop structures could have a regulatory effect on the expression of PhoP and PhoQ.

Autoregulation detected at the protein level. We investigated whether the regulatory pattern of *phoPQ* observed at the transcriptional level was also seen at the protein level. Using purified anti-PhoP antibodies, we performed Western blot analyses of protein extracts prepared from the different mutant strains described above. PhoP was detected in wild-type cells and at much lower levels in the *phoQ* mutant strains EG5172 and EG9267 (Fig. 6). The reduced levels of PhoP are probably due to the absence of PhoQ to activate the system. As expected, no PhoP could be found in EG9316 (an EG5172 derivative with *phoP*::Tn*10*) or in other strains with insertions in *phoP* (EG9252, EG5170, EG9266, and EG9315).

DISCUSSION

We investigated the regulation of the *phoPQ* operon of *S. typhimurium*, which encodes the transcriptional regulator PhoP and the putative kinase-phosphatase PhoQ. We established that *phoPQ* is transcribed from two promoters, one of which is positively autoregulated by both PhoP and PhoQ. Autoregulation was originally detected in EG9252, a strain with properties that made the discovery of this phenomenon possible. These properties include the presence of two appropriately located transposons: a Tn*10* in the 3′ end of *phoP* that provided a promoter for low levels of PhoQ expression (24) and a MudJ downstream of the PhoP-dependent promoter $phoPp_1$ but upstream of the start site for $phoPp_2$, a promoter that is still expressed in the absence of PhoP and PhoQ. Transcription originating from Tn*10* was low (compare 15 to 20 U of β-galactosidase in EG9316 with 110 to 130 U coming from the *phoPQ* promoter in EG5172), but sufficient PhoQ was produced to appropriately modulate PhoP. Our discovery of autogenous regulation of *phoPQ* is in contrast to proposals by Miller and coworkers that *phoP* is not autoregulated (19).

Autoregulation requires both PhoP and PhoQ. In strains harboring *phoP-lac* fusions, increased β-galactosidase activity was detected only upon transformation with plasmids carrying $phoP^+$ or $phoPQ^+$ (Table 2). Similarly, in mutants with *phoQ-lac* gene fusions, induction was observed only in strains carrying $phoQ^+$ or $phoPQ^+$ plasmids (Table 2). On the basis of the similarity of PhoP-PhoQ to other two-component systems (13, 28), we predict that PhoQ will phosphorylate-dephosphorylate

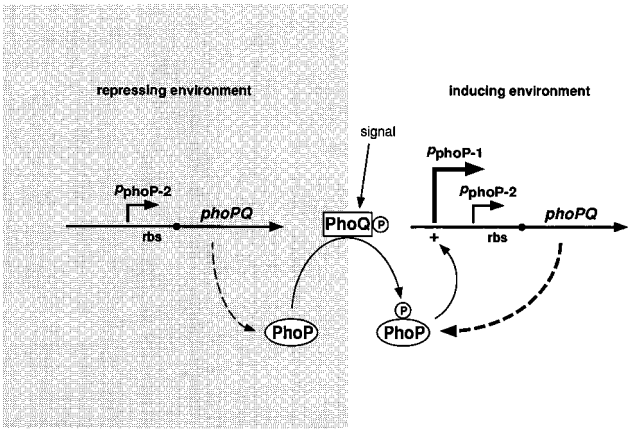

FIG. 7. Model for autoregulation of the *phoPQ* operon. In a repressing environment, the *phoPQ* operon is transcribed from $phoPp_2$ (p_{phoP-2}). When the activating signal is present, the sequential phosphorylation of PhoQ and PhoP takes place on proteins translated from the mRNA made from $phoPp_2$. This results in the production of phospho-PhoP, which can activate transcription from $phoPp_1$ (p_{phoP-1}) and to a lower extent from $phoPp_2$. A return to basal levels of expression could be achieved by inhibiting the translation of the mRNA produced from $phoPp_1$ or by the activation of the phosphatase activity of PhoQ as described in the text. rbs, ribosome-binding site.

PhoP in response to environmental changes (33). Then, our results are consistent with a model that predicts the phosphorylated form of PhoP to be the actual transcriptional activator of *phoPQ*. The unphosphorylated form of PhoP is unlikely to compete with phospho-PhoP for a PhoP-binding site(s) in the *phoPQ* promoter because the transcription levels remained unchanged upon induction of PhoP expression in a *phoQ* mutant carrying a $phoP^+$ plasmid.

Phenotypically, PhoQ behaved as both an activator and a repressor of the *phoPQ* operon. Small amounts of PhoQ were sufficient to induce the PhoP-dependent expression of *phoPQ*. However, overexpression of PhoQ without a concomitant increase of PhoP resulted in levels of *phoPQ* transcription observed in a *phoQ* mutant (Table 2 and Fig. 4A). These results could reflect a titration of PhoP by the overexpressed PhoQ. Alternatively, they can be rationalized in terms of the predicted kinase and phosphatase activities of PhoQ (33). When the inducing signal is limiting, overexpression of PhoQ might lead to an increase in its phosphatase activity, resulting in the displacement of the equilibrium between the phosphorylated and unphosphorylated forms of PhoP. The levels of β-galactosidase in a *phoQ* mutant harboring the $phoPQ^+$ plasmid (EG5172/pEG9071) remained high upon induction of *phoPQ*, perhaps reflecting the fact that both PhoP and PhoQ are being overexpressed from this plasmid. On the other hand, activation of *phoPQ-lac* in a *phoP* mutant required induction of $phoP^+$ or $phoPQ^+$. These results suggest that PhoQ acts catalytically while PhoP is required in stoichiometric amounts with respect to its target sites in the chromosome.

We have identified two promoters, $phoPp_1$ and $phoPp_2$, transcribing the *phoPQ* operon. Transcription from $phoPp_1$ requires phospho-PhoP because no transcript could be detected in either *phoP* or *phoQ* mutants, which should mimic noninducing conditions in the wild-type bacterium. On the other hand, $phoPp_2$ was active in the absence of PhoP or PhoQ, but its activity was slightly stimulated by PhoP-PhoQ. The promoter $phoPp_1$ harbors a cATAAT Pribnow box and a GTT TAT direct repeat 12 bp and 22 bp upstream. DNA motifs recognized by other regulators, such as PhoB (25, 40) and OmpR (31, 36, 39), of the two-component family are arranged similarly in the promoters of the genes they regulate. Analysis of $phoPp_2$ showed a TAgAcT −10 region and a weak −35 region (TGTTtAtc) which could be responsible for the PhoP-PhoQ-independent transcription. The GTTTAT repeat proximal to $phoPp_2$ is 24 bp upstream of its −10 region. The promoter activity of $phoPp_1$ (measured as β-galactosidase in EG9252/pEG9014) was proportional to the level of PhoP expression (Fig. 4B). On the other hand, small amounts of PhoP were sufficient to achieve maximum levels of β-galactosidase in strains transcribed from the two promoters (i.e., EG5170/pEG9071 and EG9315 and EG5172 transformed with either pEG9014 or pEG9071) (Fig. 4A).

Our data suggest that the PhoP-PhoQ regulon is subjected to several levels of regulation (Fig. 7). First, environmental changes may promote phosphorylation of PhoQ and the subsequent phosphorylation of PhoP on proteins made from the mRNA produced from $phoPp_2$. Then, a positive feedback loop would stimulate transcription from $phoPp_1$, resulting in increased levels of both PhoP and PhoQ and a concomitant activation of PhoP-activated genes. Positive autoregulation has been observed in other two-component regulatory systems, including those of the homologous *phoBR* operon of *Escherichia coli* (18), the *virA* and *virG* genes of *Agrobacterium tumefaciens* (42), and the *bvgAS* operon in *Bordetella pertussis*. Interestingly, *bvgAS* is also transcribed from several promoters, two of which respond to environmental signals via BvgA-BvgS (38).

One can envision several scenarios that could mediate the return of the PhoP-PhoQ regulon to basal levels of expression. One possibility is for PhoQ to have phosphatase activity upon phospho-PhoP and for this property to be stimulated by the disappearance of the stimulating signal. Alternatively, PhoP or phospho-PhoP may be intrinsically unstable, requiring continuous synthesis to achieve high levels of PhoP-activated determinants. A third possibility is that PhoP or another protein may help stabilize the stem-loop structure that may be formed when *phoPQ* is transcribed from $phoPp_1$ (Fig. 5). The formation of this stem would occlude the Shine-Dalgarno region and prevent translation of *phoP*. Further experiments are required to determine whether these or other models explain the regulatory features of this locus.

ACKNOWLEDGMENTS

We thank A. Aspedon, G. Blanco, R. Kranz, and C. Parra-Lopez for comments on the manuscript and F. Solomon for technical assistance.

This work is supported by a grant from the National Institutes of Health (AI29554) to E.A.G. F.C.S. is a Career Investigator of the Consejo de Investigaciones de la Universidad Nacional de Rosario (CIUNR), Rosario, Argentina, and is partially supported by the Pew Charitable Trusts. E.A.G. is a recipient of a Junior Faculty Research Award from the American Cancer Society.

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