JOURNAL OF BACTERIOLOGY, July 1993, p. 4475–4484
0021-9193/93/144475-10$02.00/0

Vol. 175, No. 14

A PhoP-Repressed Gene Promotes *Salmonella typhimurium* Invasion of Epithelial Cells

IRMGARD BEHLAU AND SAMUEL I. MILLER*

Infectious Disease Unit, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114

Received 28 January 1993/Accepted 17 May 1993

The *Salmonella typhimurium* transcriptional regulators, PhoP/PhoQ, induce *phoP*-activated gene (*pag*) expression to promote virulence and intracellular survival within macrophages. This response to the macrophage intracellular environment is simulated by *phoP/phoQ* constitutive mutations (phenotype PhoP^c) that increase the expression of *pag* genes and repress the synthesis of approximately 20 proteins encoded by *phoP*-repressed genes (*prg* genes) (S. I. Miller and J. J. Mekalanos, J. Bacteriol. 172:2485–2490, 1990). PhoP^c bacteria are attenuated for mouse virulence, suggesting that *prg* genes are virulence genes. We now report the identification of five unlinked *prg* loci by use of the transposon Tn*phoA*. In general, medium conditions (i.e., starvation) that activate *pag* expression repress *prg* expression. However, variable effects on the PhoP regulon were observed when bacteria were grown under different oxygen tensions (*pag* and *prg* genes) or exposed to low pH (*prg* genes), suggesting heterogenous control of the regulon. One *prg* locus, *prgH*, was demonstrated to contribute to mouse virulence by both the oral and the intraperitoneal routes. *prgH* was located at 59 min on the *Salmonella* chromosome, a region where other genes essential to invasion of epithelial cells are clustered. The *prgH* locus was highly linked to one invasion locus, *hil* (C. A. Lee, B. D. Jones, and S. Falkow, Proc. Natl. Acad. Sci. USA 89:1847–1851, 1992), although transcription of *prgH* was opposite that of the Tn*5*B50-encoded promoters that result in a hyperinvasive or *hil* phenotype. Both PrgH and PhoP^c mutant *S. typhimurium* were found to be defective in induction of endocytosis by Madin-Darby canine kidney (MDCK) epithelial cells. The invasion defect of PrgH but not that of PhoP^c mutant bacteria was complemented by plasmids containing *prgH* (*hil*) DNA. Therefore, two virulence properties of *Salmonella* species, induction of endocytosis by epithelial cells and survival within macrophages, are oppositely modulated by the PhoP/PhoQ virulence regulators.

Salmonella species cause a spectrum of diseases, including gastroenteritis, enteric fever, and septicemia (15). After oral ingestion, *Salmonella typhimurium* crosses the small intestinal mucosa and disseminates to colonize the spleens, livers, and bone marrow of mice (6). The exposure of *Salmonella* species to these different extracellular (stomach, intestine, lymphatic system, and bloodstream) and intracellular (epithelial cell, polymorphonuclear leukocyte, and monocyte-macrophage) environments should expose the bacterium to varied pH and oxygen tensions and other complex environments. These environments stimulate the bacterium to express genes that encode proteins which facilitate coexistence. Such virulence factors in a wide number of gram-positive and -negative bacteria are controlled by environmentally responsive regulators (26, 29).

In *S. typhimurium*, one such regulon is modulated by the PhoP (transcriptional activator) and PhoQ (sensor kinase) proteins, which are essential to *S. typhimurium* pathogenesis and survival within macrophages (9, 32). PhoP/PhoQ are similar to other known two-component systems in which signal transduction is accomplished by a phosphotransferase mechanism (14, 31, 32). Previously reported phenotypes of *phoP* and *phoQ* mutants include attenuated mouse virulence, decreased survival within macrophages, and sensitivity to low pH and defensins (9, 10, 32–34). A number of loci that require an intact *phoP* locus for transcriptional activation have been defined previously (14, 31, 32). The transcription of *phoP*-activated genes (*pag* genes) is induced when *Salmonella* species are within acidified macrophage but not epithelial cell phagosomes (3). The induction of *pagC* transcription results in the synthesis of an 18-kDa envelope protein that promotes virulence and survival within macrophages (32, 37) but is not required for epithelial cell invasion (35). Therefore, the regulation and virulence function of *pag* genes may be limited to survival within macrophages (31).

Constitutive mutations in the *phoP* locus (phenotype PhoP^c) that result in increased expression of *pag* genes in an unregulated fashion also markedly attenuate *S. typhimurium* virulence and survival within macrophages (33). The virulence defect of PhoP^c strains may be explained by their decreased expression of approximately 20 polypeptides encoded by *phoP*-repressed genes (*prg* genes) (33). *prg* loci are expected to be repressed when *Salmonella* species are within acidified macrophage phagosomes (pH < 5.0) and maximally expressed in environments where *pag* genes are repressed. Therefore, we undertook to identify *phoP*-repressed loci to further define the virulence defect of PhoP^c strains and to identify new genes essential to *Salmonella* pathogenesis.

MATERIALS AND METHODS

Bacterial strains and plasmids. All bacterial strains used in this study are described in Table 1.

Media and chemical reagents. Luria-Bertani (LB) broth (40) was used as the rich medium. Antibiotics were used in the following concentrations (in micrograms per milliliter) in growth media or agar: ampicillin, 100; chloramphenicol, 25; gentamicin, 30; kanamycin, 45; and tetracycline, 25. The

* Corresponding author.

TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype and/or description	Reference and/or source[a]
S. typhimurium ATCC 14028s derivatives		
14028s	Wild type	ATCC
CS002	*phoP12*	32
CS003	Δ*phoP* Δ*purB*	32
CS012	*pagA1*::*Mu dJ*	32
CS013	*pagB1*::*Mu dJ*	32
CS119	*pagC1*::Tn*phoA phoN2 zxx*::6251Tn*10*d-Cm	32
CS015	*phoP-102*::Tn*10* d-Cm	32
CS019	*phoN2 zxx*::6251Tn*10*d-Cm	32
CS022	*pho-24*	33
CS023	*pho-24 phoN2 zxx*::6251Tn*10*d-Cm	33
CS030	*phoN2 zxx*::6251Tn*10*d-Cm *phoP12*	This work
AD154	*phoP12 purB1744*::Tn*10*	Gift from E. Eisenstadt
CS031	*pho-24 purB1744*::Tn*10*	This work
IB001	*phoN2 zxx*::6251Tn*10*d-Cm Δ*phoP* Δ*purB*	This work
IB002	CS030 with *prgA1*::Tn*phoA*	This work
IB003	IB002 with *pho-24 purB1744*::Tn*10*	This work
IB004	IB002 with *phoP12 purB1744*::Tn*10*	This work
IB005	CS019 with *prgA1*::Tn*phoA*	This work
IB006	CS015 with *prgA1*::Tn*phoA*	This work
IB007	CS030 with *prgB1*::Tn*phoA*	This work
IB008	IB007 with *pho-24 purB1744*::Tn*10*	This work
IB009	IB007 with *phoP12 purB1744*::Tn*10*	This work
IB010	CS019 with *prgB1*::Tn*phoA*	This work
IB011	CS015 with *prgB1*::Tn*phoA*	This work
IB012	CS030 with *prgB2*::Tn*phoA*	This work
IB013	IB012 with *pho-24 purB1744*::Tn*10*	This work
IB014	IB012 with *phoP12 purB1744*::Tn*10*	This work
IB015	CS019 with *prgB2*::Tn*phoA*	This work
IB016	CS015 with *prgB2*::Tn*phoA*	This work
IB017	CS030 with *prgC1*::Tn*phoA*	This work
IB018	IB017 with *pho-24 purB1744*::Tn*10*	This work
IB019	IB017 with *phoP12 purB1744*::Tn*10*	This work
IB020	CS019 with *prgC1*::Tn*phoA*	This work
IB021	CS015 with *prgC1*::Tn*phoA*	This work
IB022	CS030 with *prgE1*::Tn*phoA*	This work
IB023	IB022 with *pho-24 purB1744*::Tn*10*	This work
IB024	IB022 with *phoP12 purB1744*::Tn*10*	This work
IB025	CS019 with *prgE1*::Tn*phoA*	This work
IB026	CS015 with *prgE1*::Tn*phoA*	This work
IB027	CS030 with *prgE2*::Tn*phoA*	This work
IB028	IB027 with *pho-24 purB1744*::Tn*10*	This work
IB029	IB027 with *phoP12 purB1744*::Tn*10*	This work
IB030	CS019 with *prgE2*::Tn*phoA*	This work
IB031	CS015 with *prgE2*::Tn*phoA*	This work
IB032	CS030 with *prgE3*::Tn*phoA*	This work
IB033	IB032 with *pho-24 purB1744*::Tn*10*	This work
IB034	IB032 with *phoP12 purB1744*::Tn*10*	This work
IB035	CS019 with *prgE3*::Tn*phoA*	This work
IB036	CS015 with *prgE3*::Tn*phoA*	This work
IB037	IB001 with *prgH1*::Tn*phoA*	This work
IB038	IB037 with *pho-24 purB1744*::Tn*10*	This work
IB039	IB037 with *phoP12 purB1744*::Tn*10*	This work
IB040	CS019 with *prgH1*::Tn*phoA*	This work
IB041	CS015 with *prgH1*::Tn*phoA*	This work
IB042	Tn*5*B50-380 in IB040	This work
IB043	pWKSH5 in IB040	This work
IB044	pWKSH5 in CS022	This work
CS032	*oxiA1049*::Mu d1-8 *supD10*	This work
CS033	*oxiC1048*::Mu d1-8 *supD10*	This work
CS034	*oxiE4*::Mu d1 Δ*nadA100*	This work
Other *S. typhimurium* derivatives		
AK3011-AK3314	Collection of randomly spaced Tn*10*Δ16Δ17 insertions	19
TT520	*srl-202*::Tn*10*	41; SGSC
TT2979	*srl-211*::Tn*5*	41; SGSC
TN3061	*zcf-845*::Tn*10 dcp-1 zhg-1635*::Tn*10*d-Cm	41; SGSC

Continued on following page

TABLE 1—*Continued*

Strain	Relevant genotype and/or description	Reference and/or source[a]
SH7782	*ompD*::Tn*5*	41; SGSC
χ4115	*invA*::*cat*	13; J. Galan
EE517	Δ*hil-517* (Tn*5*B50-380)	22; C. Lee
JF897	*oxiA1049*::Mu d1-8 *supD10*	2; SGSC
JF896	*oxiC1048*::Mu d1-8 *supD10*	2; SGSC
JF739	*oxiE4*::Mu d1 Δ*nadA100*	2; SGSC
S. enteritidis		
CDC5	Clinical wild-type isolate	45; V. Miller
SM7	Str^r *smb*	45; V. Miller
Escherichia coli		
SM10(pRT291)	Contains plasmid pRT291 (Tn*phoA*) derived from pRK290 selecting for Tc^r and Km^r	49
MM294(pPH1JI)	Contains Gm^r plasmid pPH1JI, which is incompatible with pRK290	49
VV42(pWKSH5)	Contains plasmid pWKSH5, a derivative of pSC101 (51) that contains a 5.1-kb *Hin*dIII fragment of *hil* DNA including *prgH*	V. Bajaj and C. Lee

[a] ATCC, American Type Culture Collection; SGSC, *Salmonella* Genetic Stock Center.

chromogenic substrate BCIP (5-bromo-4-chloro-3-indolylphosphate [*p*-toluidine salt]) was used to detect phosphatase activity on agar at a final concentration of 40 μg/ml. *p*-Nitrophenyl phosphate was used as a substrate for quantitative measurement of alkaline phosphatase (AP) activity. Media were buffered to various pH ranges with 1 M sodium citrate. E medium (Vogel-Bonner minimal) was prepared as described previously (7). Nitrogen-, carbon-, and phosphate-free medium ($N^-C^-P^-$) was prepared as described by Kier et al. (17). This starvation medium was supplemented with 0.04% (wt/vol) glucose as the carbon source, 10 mM NH_4Cl as the nitrogen source, and 1 mM $NaH_2PO_4 \cdot H_2O$ as the phosphate source. The carbon concentration is 1 log less than that described by Kier et al. (17) and was likely the most growth limiting.

Measurement of AP activities. AP activities of strains which were isogenic except for mutations in the *phoP* locus were measured in cultures grown from a single-colony inoculum under various oxygen tensions with or without shaking at 37°C. Anaerobic cultures were grown in an anaerobic chamber (Coy Laboratory Products, Inc.) with a gas mixture of 80% N_2, 10% O_2, and 10% CO_2 at 37°C. For acid regulation, aliquots of mid-logarithmic cultures were removed to measure initial pH and AP activity. Sodium citrate (1 M; pH > 6.0) or citric acid (1 M; pH 4.7) was added to equivalent amounts of culture to a final concentration of 50 mM citrate. Cultures were grown aerobically for 2 h at 37°C, and then pH and AP measurements were taken. AP activity was measured as described previously (27). AP units were calculated by the following formula: units = $\{OD_{420}/[\text{time (minutes)} \times \text{volume} \times OD_{600}]\} \times 1{,}000$ as defined by Miller for β-galactosidase (30), where OD_{420} and OD_{600} are the optical densities at 420 and 600 nm, respectively.

Bacterial genetic techniques. Bacteriophage P22HT*int*-mediated transduction was performed as described previously (7). Tn*phoA* mutagenesis was performed with a broad-host-range plasmid (pRT291) to deliver Tn*phoA* (49). Transpositions of Tn*phoA* into *Salmonella* DNA were identified by use of the incompatibility plasmid pPH1JI (49).

Screening for *phoP*-repressed genes was performed with CS031, the donor strain of the *pho-24* allele. CS031 was constructed by a P22 bacteriophage transductional cross between strains AD154 and CS022, which contain the *purB*::Tn*10* allele and the *pho-24* allele, respectively. The linkage of *pho-24* and *purB*::Tn*10* was 70%, similar to the linkage of *purB* to other *phoP* alleles. Therefore, when P22 bacteriophage transductional crosses were performed between CS031 and the strains containing active gene fusions to *phoA*, strains could be screened for loss of fusion protein activity upon acquisition of tetracycline resistance. The initial screening involved detection of a loss of AP activity in approximately 70% of colonies that acquired tetracycline resistance, as they were presumed to contain the *pho-24* allele. In addition, controls were performed with strain AD154, which contains the same *purB*::Tn*10* allele linked to a *phoP* null allele, *phoP12*.

Plasmid DNA was transformed into *S. typhimurium* LB5010 by the calcium chloride and heat shock procedures (24).

Linkage analysis of *phoP*-repressed genes. The chromosomal location of Tn*phoA* insertions in *phoP*-repressed genes (*prg*::Tn*phoA*) was determined by linkage analysis to a bank of strains with Tn*10*Δ16Δ17 insertions (19). Cells with Tn*phoA* insertions were spread on LB agar plates containing 10 μg of tetracycline per ml and 40 μg of BCIP per ml. Then P22 lysates grown on strains with Tn*10*Δ16Δ17 insertions were spotted onto plates with a multiprong inoculator. After being inoculated overnight, plates were reviewed for linkage by looking for mixed blue-and-white colonies. Linkage was confirmed and quantitated by carrying out individual transductional crosses between the strains containing Tn*10*Δ16Δ17 and the strain with the Tn*phoA* insertion. After selection for the Tn*10*Δ16Δ17-encoded tetracycline resistance, strains were scored for a loss of blue color and Tn*phoA*-encoded kanamycin resistance. Some Tn*phoA* strains were found to be linked to Tn*10*Δ16Δ17 strains with no known map location. Two of these Tn*10*Δ16Δ17 insertions were physically mapped by Liu and Sanderson by pulsed-field gel electrophoresis following *Xba*I restriction endonuclease digestion (23). On the basis of physical mapping, linkage analysis to other transposon insertions by P22 bacteriophage transduction was determined as necessary.

DNA analysis. Chromosomal DNA was prepared as described by Mekalanos with proteinase K instead of pronase

(25). Purification of plasmid DNA was performed by standard methods (40). Restriction endonuclease digestion was performed according to the recommendations of the manufacturer (New England Biolabs). DNA, size fractionated in agarose gels, was transferred to GeneScreen Plus membranes (New England Nuclear/Dupont, Boston, Mass.) for blot hybridization by the method of Southern (44). DNA probes were purified from agarose gels by the freeze-squeeze method (48) and radiolabelled with [^{32}P]dCTP by the random primer method (8).

Protein analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (20).

Acid sensitivity. Acid sensitivity was tested by the method of Foster and Hall (10). Strains were grown aerobically in E medium–0.4% glucose at 37°C to an OD_{600} of 0.5. The pH of the bacterial culture was decreased to nearly 3.3 by the addition of 1 M hydrochloric acid. An aliquot was taken immediately; the remainder of the culture was incubated further at 37°C, with subsequent aliquots removed at 40- and 80-min time points. The pH of the cultures remained near 3.3. The aliquots were diluted 1:10 in cold phosphate-buffered saline (PBS), washed, and resuspended in normal saline prior to serial dilutions being plated for CFU.

Growth of tissue culture cells. J774.2 macrophages were cultured in Dulbecco's minimal essential medium with 10% fetal bovine serum (DMEM–10% FBS) as previously described (11, 38, 47). Cultured bone marrow macrophages were harvested from BALB/c mice purchased from the Charles River Breeding Laboratories (Wilmington, Mass.) and cultured as described previously (46). Madin-Darby canine kidney (MDCK) epithelial cells were used between passages 40 to 58 to maximize bacterial adherence and invasion (21). Epithelial cell lines were cultured in DMEM–10% FBS and 1% penicillin-streptomycin solution at 37°C in a 5% CO_2 atmosphere as described previously (11, 38, 47).

Bacterial survival within macrophages. Experiments were performed as previously described (5). Bacteria grown to stationary phase were opsonized for 30 min in normal mouse serum before being exposed to cultured bone marrow-derived macrophages harvested from BALB/c mice. One hour after infection, gentamicin (10 μg/ml) was added to kill extracellular bacteria. Procedures at all time points (1, 4, and 24 h) were done in triplicate and were repeated on three separate occasions.

Bacterial adherence and uptake by epithelial cells. Bacterial strains were grown at 37°C without shaking (microaerophilic conditions) to a final density of approximately 2×10^8 CFU/ml. Assays were performed by seeding 10^5 MDCK cells per well in 24-well tissue culture plates. Cells were incubated overnight at 37°C in a 5% CO_2–95% air atmosphere in DMEM–10% FBS without antibiotics until they were >80% confluent. The adherence and invasion assays are modified versions of the protocol of Lee and Falkow (21). The confluent MDCK monolayers were washed three times with PBS, and then 0.9 ml of cold DMEM–10% FBS was added to each well. Bacteria were washed in LB broth and resuspended in an equivalent volume of DMEM–10% FBS. Approximately 5×10^7 bacteria per well was added. The plates were spun at 500 rpm in a Sorvall RT6000B at 4°C for 10 min and then incubated at 4°C for 30 min. Adherent bacteria were recovered by washing the plates three times with PBS, lysing the epithelial cells in 0.5 ml of 1% Triton X-100–PBS, and plating for CFU/ml on LB agar. A morphologic assessment of adherence was also performed by staining bacterially infected epithelial cell monolayers grown overnight on coverslips for 7 min in 1 μg of 4′,6-diamidino-2-phenylindole. These 4′,6-diamidino-2-phenylindole-stained coverslips were examined by both fluorescence and phase-contrast microscopy with a Leitz Laborlux 12 microscope.

Invasion or bacterium-mediated endocytosis (BME) was assessed by allowing bacteria to adhere as described above. Plates containing bacteria and epithelial cells were incubated for 2 h at 37°C in a 5% CO_2–95% air atmosphere. Each well was washed three times with PBS to remove non-cell-associated bacteria. DMEM–10% FBS supplemented with 10 μg of gentamicin per ml was then added to kill extracellular bacteria (50). After 90 min of incubation, the cell monolayers were washed three times with PBS and the viable intracellular bacteria were released by vigorous pipetting with 0.5 ml of 1% Triton X-100–PBS. Viable bacteria were quantitated by plating for CFU/ml on LB agar medium. All assays were done in triplicate and were repeated on at least three different occasions.

SM7, an invasion-deficient *Salmonella enteritidis* mutant, and CDC5, an invasive clinical wild-type isolate of *S. enteritidis* (gifts from V. Miller), were used as controls for BME (45).

Bacterial defensin sensitivity. NP-1 defensin was purified from rabbit peritoneal neutrophils (gift from M. Selsted) as described previously (42, 43). Typically, 10^5 bacteria in 0.5% tryptone in a 100-μl volume (9, 43) was exposed to 50 to 100 μg of defensin per ml at 37°C for 2 h. The reactions were stopped by diluting the reaction mixture with 0.9% NaCl (43). Appropriate dilutions were plated to determine the CFU of surviving bacteria per ml. Assays were performed in duplicate at least twice for each strain. Appropriate assays with sensitive ($PhoP^-$) and resistant (wild-type) strains were performed as controls.

Mouse virulence studies. Bacteria were grown aerobically at 37°C to stationary phase, washed with LB broth, and diluted in normal saline. Thirty-five-day-old (16- to 18-g) female BALB/c mice were purchased from the Charles River Breeding Laboratories, Inc. Diluted bacterial samples in saline were injected intraperitoneally with an inoculum of 0.1 to 0.15 ml. Bacteria were administered orally as a 0.5-ml bolus to mice that had fasted for 2 h via a 2-in. (ca. 5.1-cm) straight, 18-gauge, stainless-steel animal oral feeding needle (Harvard Apparatus, Inc., South Natick, Mass.) under mild 2-bromo-2-chloro-1,1,1-trifluoroethane (Halothane) anesthesia. The number of organisms administered was quantitated by plating for CFU/ml on LB agar. The care of all animals was under institutional guidelines as set by the animal care committee at the Massachusetts General Hospital and Harvard Medical School. Mouse 50% lethal doses (LD_{50}s) were determined by the method of Reed and Muench (39). The LD_{50} determinations were repeated on three separate occasions.

Competition assays were performed after bacteria were administered orally to mice as described above. Bacteremia was assessed on days 1 to 4 from tail bleeds or intracardiac punctures with 50 μl of blood plated immediately and after growth in LB broth at 37°C overnight. Splenic and intestinal harvests were performed on days 1 to 6 with organs homogenized in 3 ml of 0.9% sodium chloride. Samples and cultures were plated in serial dilutions. *S. typhimurium* was confirmed by characteristic growth (black colonies) on Hektoen Enteric Agar (Difco Laboratories) and by the macroscopic slide agglutination test with *Salmonella* group B rabbit serum (antigens 4, 5, and 12) (Fisher Scientific).

TABLE 2. Comparison of the effects of *phoP* locus mutations on Prg-PhoA fusion protein activity

Allele	Activity[a] (U of AP)		Fold repression
	PhoP$^-$	PhoP^c	
prgA1::Tn*phoA*	29	7	4
prgB1::Tn*phoA*	137	27	5
prgB2::Tn*phoA*	77	19	4
prgC1::Tn*phoA*	14	1	14
prgE1::Tn*phoA*	21	5	4
prgE2::Tn*phoA*	34	6	6
prgE3::Tn*phoA*	25	6	4
prgH1::Tn*phoA*	92	2	46

[a] Values were calculated from stationary-phase cultures. The numbers denote representative values of experiments performed on three separate occasions and represent activity as defined by Miller for β-galactosidase (30). PhoP$^-$ indicates that the strain assayed contains the *phoP12* allele (CS030). PhoP^c indicates that the strain assayed contains the *pho-24* allele (CS031).

RESULTS

Isolation of strains with Tn*phoA* insertions in *phoP*-repressed genes. *phoP*-repressed genes were expected to be highly expressed in strains deleted for *phoP/phoQ* and grown in *pag*-repressing conditions (rich medium). A PhoP$^-$ PhoN$^-$ strain (IB001) was constructed by a P22 transductional cross between CS019 and CS003. IB001 was chosen for Tn*phoA* mutagenesis so that background acid phosphatase, encoded by *phoN*, would not interfere with the measurement of fusion protein activity upon alteration of the *phoP* locus. A total of 1,800 individual blue colonies with PhoA fusion protein activity were isolated on LB agar plates containing BCIP. These colonies were the product of 18 separate matings, with approximately 20 pools in each. These strains were tested for reduction of fusion protein activity upon acquisition of the *pho-24* allele (CS031), which resulted in a PhoP^c phenotype. AP assays were then performed on strains which were isogenic except for the *phoP* locus (see Materials and Methods).

The PhoP^c phenotype was confirmed in these strains by preparation of whole-cell protein extracts and SDS-PAGE analysis. All strains with a PhoP^c phenotype demonstrated the expected distinctive pattern of protein expression in PhoP^c strains, i.e., repressed protein species of specific sizes (data not shown) (33).

Eight strains with gene fusions to *phoP*-repressed genes were identified. As shown in Table 2, the synthesis of most *prg*::Tn*phoA* fusion proteins was fully repressed by the *pho-24* allele; however, some were only partially repressed. Of note, values for *prgB-phoA* fusions in strains with a wild-type *phoP* locus (Table 3) lower than those in PhoP$^-$ strains (Table 2) may represent some degree of repression in the presence of PhoP.

Chromosomal location of *prg*::Tn*phoA* loci. *prg*::Tn*phoA* linkage analysis was performed with a bank of strains with randomly spaced Tn*10*Δ16Δ17 insertions (19) to determine chromosomal locations and whether *prg*::Tn*phoA* alleles were unlinked loci. The *prg*::Tn*phoA* insertions were in five distinct linkage groups. Three alleles, *prgE1-3*::Tn*phoA*, were identically linked to the Tn*10*Δ16Δ17 insertion of AK3091 (26%), and two other alleles, *prgB1-2*::Tn*phoA*, were similarly linked to the Tn*10*Δ16Δ17 insertions of AK3190 (94%), AK3249 (89%), and AK3186 (50%). Another allele, *prgH1*::Tn*phoA*, was found to be 37% linked to the Tn*10*Δ16Δ17 insertion of strain AK3304. The other two *prg* alleles did not demonstrate linkage to the bank of strains tested. The chromosomal DNA of these two strains was analyzed by Southern hybridization analysis with a portion of Tn*phoA* as a probe, and a rough physical map of the sites located adjacent to the Tn*phoA* insertion was determined. These alleles, *prgA* and *prgC*, had different restriction endonuclease sites surrounding the Tn*phoA* insertions (data not shown). In addition, the repression of *prgA* and *prgC* fusion protein activity in strains with the *pho-24* mutation was different; *prgC* was completely repressed, while *prgA* was only partially repressed. These data suggested that these loci are different. Therefore, we identified five unlinked loci encoding envelope proteins repressed in the PhoP^c phenotype.

Although three *prg* loci that were linked to transposon insertions were identified, none of the Tn*10*Δ16Δ17 insertions had a known map location. The physical map locations of two of these transposon insertions, AK3249 and AK3304, were graciously analyzed by S. Liu and K. Sanderson by use of *Xba*I restriction endonuclease digestion and pulsed-field gel electrophoresis. Since Tn*10*Δ16Δ17 contains a single *Xba*I site, these Tn*10*Δ16Δ17 insertions can be assigned to a specific *Xba*I fragment with a known map location (23). AK3249 was assigned to 28 to 32 min, while AK3304 was assigned to either end of the 58- to 70-min fragment. Further P22 transduction to known markers in those regions was performed. The Tn*10*Δ16Δ17 insertion of strain AK3249 and *prgB1*::Tn*phoA* were found not to be linked to the Tn*10* insertion of strain TN3061 (6% linked to *dcp*), which has a transposon insertion at 28 min, or to the *ompD*::Tn*5* insertion of strain SH7782 at 32 min. Therefore, we are unable to assign a map location more definite than the 28- to 32-min region. *prgH1*::Tn*phoA* was found to be very weakly linked to the *srl202*::Tn*10* insertion of strain TT520 (<0.1%) at 59 min. Therefore, *prg* genes are widely located and unlinked on the *Salmonella* chromosome, consistent with the function of PhoP/PhoQ as global regulators.

Environmental regulation of *prg* loci. Since PhoP/PhoQ are environmentally responsive regulators, the effects of different growth conditions on *prg*::Tn*phoA* expression were tested. The growth rate of strains with *prg*::Tn*phoA* insertions was comparable to that of wild-type organisms under all conditions (data not shown). The expression of all *prg* loci was maximal in late logarithmic growth phase, when bacteria were grown in rich (LB) medium (data not shown). An example of this is the comparison of values of *prgH*::Tn*phoA* expression in Table 3. Since the expression of *pag* loci was maximal in starvation conditions (which only reaches a maximal OD_{600} of 0.5) and stationary-growth phase, this was consistent with a reciprocal relationship between the expression of *pag* and *prg* genes. Further analysis of *prg* locus expression under starvation conditions confirmed this reciprocal relationship (Table 3). *prgH* expression was repressed (Table 3) and other *prg* genes were minimally affected under starvation conditions (data not shown), in contrast to the induction of *pag* expression when bacteria were starved (Table 3) (16).

The effects of oxygen tension in rich medium on *pag* and *prg* expression were also tested (Table 3), as these have recently been defined as an important signal for BME (21). Interestingly, different but not reciprocal regulation of *pag* and *prg* loci was found upon growth at different oxygen tensions. Although *pagA* and *pagB* loci were minimally affected by growth at different oxygen tensions (data not shown), the *pagC* virulence locus was approximately fivefold repressed when bacteria were grown anaerobically rather than aerobically (Table 3). Interestingly, variability

TABLE 3. In vitro regulation of *prg* loci

Strain	Allele	Activity[a] (U of AP)						
		Medium[b]		Conditions[c]			pH[d]	
		Starvation	Rich	Aerobic	Microaerophilic	Anaerobic	4.5	7.0
IB010	*prgB1*::Tn*phoA*	21	26	33	777	1,521	332	26
IB040	*prgH1*::Tn*phoA*	7	181	142	85	41	8	18
CS119	*pagC1*::Tn*phoA*	1,263	102	431	173	81	145	27

[a] As defined by Miller for β-galactosidase (30).

[b] The effect of starvation on *prg* and *pag* expression. Starvation medium ($N^-C^-P^-$) (17) contained 0.04% glucose, 10 mM NH_4Cl, and 1 mM $NaH_2PO_4 \cdot H_2O$. The fusion protein activity for starvation medium was measured after 48 h of growth (OD_{600} = 0.5), while that in rich medium (LB) was measured in late-logarithmic-growth phase (OD_{600} = 1.0). All cultures were grown aerobically.

[c] The effect of oxygen tension on expression of *phoP*-activated and *phoP*-repressed genes. Expression in rich medium under aerobic conditions at stationary phase (OD_{600} > 1.4) and microaerophilic (OD_{600} = 0.8) and strict anaerobic conditions with 80% N_2–10% O_2–10% CO_2 (OD_{600} = 0.6) after 24 h of growth is compared.

[d] The effect of pH on the expression of fusion protein activity of *prg* and *pag* loci. Expression was measured from cultures grown to logarithmic phase (OD_{600} = 0.5) in LB medium buffered to various pHs with sodium citrate.

was also noted in the expression of *prg* loci in response to growth conditions in the absence of oxygen. One locus, *prgH*, was repressed 3-fold in anaerobic growth, while another locus, *prgB*, was induced almost 50-fold when grown anaerobically (Table 3). Other *prg* loci had minimal changes in fusion protein expression as a result of different oxygen tensions in the growth media (data not shown).

Low pH conditions also had a variable effect on *prg* expression (Table 3). The expression of *pagC* fusion protein activity was induced under acid conditions, as previously known (3, 33). When bacteria were grown to mid-logarithmic phase, no significant induction of the relative repression of *prgH* expression was noted in media of low pH, while *prgB* expression was induced upon exposure of bacteria to low pH (Table 3). Hence, loci maximally expressed under diverse environmental conditions can all be repressed by the $PhoP^c$ phenotype.

***prgH* is a virulence locus for *S. typhimurium*.** Since the $PhoP^c$ phenotype resulted in virulence attenuation and repressed the synthesis of approximately 20 proteins, we wished to test the virulence of strains with single mutations in *prg* loci (Table 4). Strains with *prg*::Tn*phoA* insertions were screened for virulence defects by intraperitoneal injection of approximately 150 organisms into BALB/c mice. Control experiments were also performed with wild-type bacteria. When strains containing the *prgH1*::Tn*phoA* insertion were injected into mice intraperitoneally, it took significantly longer (approximately 10 to 14 days) for the mice to develop clinical signs of typhoid fever, such as a scruffy phenotype (fever and piloerection) and hepatosplenomegaly, although the mice eventually died (data not shown). All other strains with *prg*::Tn*phoA* insertions showed a pattern of illness similar to that of wild-type bacteria (data not shown). More extensive testing of the LD_{50}s of strains containing *prgH* mutations was performed. *prgH* mutants were determined to have an LD_{50} of approximately 60 organisms, compared with a value of <10 for wild-type bacteria. Because of the difficulty of accurately delivering organisms in small doses to mice, we constructed a strain with a mutation in both *prgH* and *phoP*. The $PrgH^-$ $PhoP^-$ strain had a greater than 10-fold increase in LD_{50} compared with that for CS015, an isogenic $PhoP^-$ strain (Table 4). This additive effect further documented that the *prgH1*::Tn*phoA* mutation attenuated *S. typhimurium* virulence. This also indicated that mutations which affected two phases of PhoP/PhoQ-regulated gene expression were additive in their effects on virulence. Strains with *prgH1*::Tn*phoA* insertions were also tested for virulence when administered by the oral route. A 10-fold decrease in virulence (increase in LD_{50}) was observed (Table 4).

TABLE 4. Effects of *prgH1*::Tn*phoA* mutation on *Salmonella* mouse virulence[a]

Method and strain	Genotype or description	LD_{50} (no. of animals)[b]
Intraperitoneal injection		
14028s	Wild type	<10
IB040	*prgH1*	5.6×10^1 (16)
CS015	*phoP-102*	6.7×10^5 (29)
IB041	*prgH phoP-102*	1.2×10^7 (31)
Oral inoculation		
14028s	Wild type	6.5×10^4 (35)
IB040	*prgH1*	6.5×10^5 (21)

[a] Strains were isogenic, and 35-day-old BALB/c mice were used.

[b] LD_{50}s were determined by the method of Reed and Muench (39). Number of animals refers to the number used at bacterial dilutions near the LD_{50} for each allele. The LD_{50} determinations were repeated on three separate occasions.

Further analysis of the efficiencies of strains with *prgH1*::Tn*phoA* insertions in crossing the mucosal barrier was by competition experiments with wild-type bacteria. During the first 72 h after oral inoculation, *prgH1*::Tn*phoA* mutants were never found in the bloodstream of mice, while wild-type organisms always were (data not shown). Other strains with *prg* mutations were also tested for virulence defects by the oral route, but none were dramatically changed in virulence (data not shown).

Mutations in oxygen-induced genes do not affect mouse virulence. The findings that both *prgH* and *pagC* loci were repressed by anaerobic growth and required for full virulence suggested that a shift from anaerobic to aerobic conditions might serve as a general signal for virulence gene induction. Therefore, we constructed strains with mutations in loci previously described as induced by oxygen (2). ATCC 14028s derivatives with *oxiA*, *oxiC*, and *oxiE* mutations were constructed (CS032, CS033, and CS034), and they had virulence similar to that of wild-type bacteria (data not shown). Although these gene fusions could still mark operons containing virulence genes, these data suggest that these loci are not essential to full virulence and that oxygen induction is not always correlated with virulence function.

***prgH* mutants have normal survival within macrophages.**

Since the PhoP^c phenotype resulted in a defect in bacterial survival within macrophages, we tested whether this defect was due to a lack of synthesis of a *prgH*-encoded protein. A strain with the *prgH1*::Tn*phoA* insertion was tested for intracellular survival within bone marrow-derived macrophages from BALB/c mice and J774.2 cells, a macrophage-derived cell line. No defect in intracellular survival was observed (data not shown), indicating either that this assay was insensitive to defects in intracellular survival that result in modest mouse virulence defects or that *prgH* functions in a different site to promote virulence. A strain with a *prgB1*::Tn*phoA* insertion was also tested and found not to have a defect in survival within macrophages (data not shown).

***prg*::Tn*phoA* insertions do not suppress the phenotypes of PhoP mutants.** Several phenotypes of *phoP* mutants, including defensin and acid sensitivities as well as mouse virulence attenuation, were tested for suppression upon the addition of a *prg*::Tn*phoA* mutation. It was reasoned that some of the effect of a *phoP* mutation could be due to an inability to suppress the synthesis of *prg* products. Therefore, PhoP mutant strains which were isogenic except for *prg*::Tn*phoA* mutations were constructed and tested for mouse virulence, for which suppression would involve an increase in virulence, or decreased acid and defensin sensitivities. However, *prg*::Tn*phoA* insertions had no effect on the virulence phenotypes of PhoP$^-$ bacteria (data not shown). Therefore, we concluded that the *prg*::Tn*phoA* mutations that we have identified did not, as single mutations, suppress the PhoP null phenotype.

PrgH and PhoP^c mutants are defective in BME by cultured epithelial cells. The following several observations led us to test for BME of strains with *prg*::Tn*phoA* insertions: (i) Lee and Falkow have previously demonstrated that factors necessary for bacterial uptake by epithelial cells were induced upon anaerobic growth (21) and that the expression of one *prg* locus, *prgB*, was dramatically induced under these growth conditions, (ii) *prgH* mutants were defective in competition with wild-type organisms upon reaching the bloodstream of mice in the first 72 h after oral ingestion, and (iii) *prgH1*::Tn*phoA* was located at 59 min on the bacterial chromosome, a location where other genes essential to invasion are clustered. Therefore, the BME of *prg*::Tn*phoA* and PhoP^c *S. typhimurium* strains was tested. Strains with *prgH* and *pho-24* mutations had a significant reduction ($P < 0.01$) in their abilities to induce uptake by MDCK polarized epithelial cells compared with wild-type bacteria. Other *prg* strains with Tn*phoA* insertions did not demonstrate a statistically significant defect in BME by epithelial cells (Table 5). The adherence of strains defective in BME was unaffected by the *prgH*::Tn*phoA* insertion when determined by cell-associated CFU/ml before the administration of gentamicin (Table 5) and by microscopy (data not shown).

***prgH* is within the *hil* locus.** With the findings that *prgH* was located at 59 min and that *prgH1*::Tn*phoA* insertions resulted in decreased BME, we wished to determine the location of *prgH* relative to those of other invasion loci at 59 min. P22 transduction linkage analysis indicated that the Tn*10*Δ16Δ17 of strain AK3304 had linkage similar to those of *invA* (40%) (12) and *prgH* (37%); however, *invA* was not linked to sorbitol. The *prgH1*::Tn*phoA* insertion was found to be highly linked (99.6%) to the transposon insertion of EE517, a strain with an 8.5-kb deletion adjacent to the Tn*5*B50-378 insertion of *hil* (22). This demonstrated that *prgH* was highly linked to *hil*.

A rough physical map of the restriction endonuclease sites surrounding the Tn*phoA* insertion of strain IB037 was constructed (Fig. 1). The published restriction endonuclease map of the *invA-E* region did not indicate any similarities. Plasmids containing the cloned *inv* and *hil* DNA were then used as probes in Southern hybridization analysis of chromosomal DNA from wild-type ATCC 10428s and IB040 bacteria containing the *prgH1*::Tn*phoA* insertion. When plasmid pSB300, which contains other invasion loci highly linked to *invA-E* (*invH*, *invF*, and part of *invG*) (11a), was used as a probe, no differences in hybridization patterns between wild-type bacteria and strain IB040 were found. This indicated that *prgH* was not located within the *inv* region. However, when plasmid pVB3 (kindly provided by C. Lee and V. Bajaj), comprising a 5-kb region immediately downstream of the Tn*5*B50-380 insertion of *hil*, was used as a probe, the *prgH1*::Tn*phoA* insertion was demonstrated to be located within this region (data not shown). By use of the known restriction map of the *hil* locus (22) and the known restriction endonuclease sites of Tn*phoA*, the physical map of this area and the relationship of *prgH1*::Tn*phoA* within it were further defined (Fig. 1). Interestingly, the *prgH1*::Tn*phoA* insertion was oriented so that the direction of transcription of the *phoA* fusion protein was opposite that of the Tn*5*B50 insertions that confer the *hil* phenotype and contain a constitutive neomycin promoter that is transcribed out of the transposon (Fig. 1).

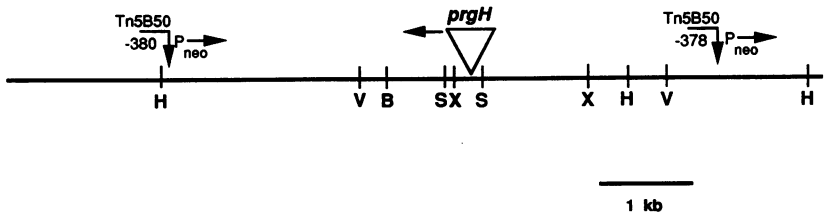
Since it was possible that a protein whose expression was altered by the Tn*5*B50-380 insertion might alter the expression of *prgH*, strains containing both insertions were constructed and the *prgH-phoA* fusion protein activities were compared under different environmental conditions. Interestingly, derepression of fusion protein activity when bacteria were starved or grown anaerobically was observed (Fig. 1). This demonstrated that the Tn*5*B50-380 insertion increased *prgH* expression, even though *prgH* transcription was opposite that of the Tn*5*B50-380-encoded neomycin promoter.

To rule out the possibility that the BME defect of the *prgH* mutant was an artifact of the PhoA fusion protein produced, complementation analysis was performed with plasmid

TABLE 5. Effects of *prg*::Tn*phoA* insertions on *Salmonella*-mediated endocytosis by MDCK epithelial cells

Strain	Genotype and/or description	% Adherence[a]	% Invasion[a]
14028s	Wild type	4.2	3.8
SM7	Str^r *smb*		0.6*
CS119	*pagC1*::Tn*phoA*		1.9
IB005	*prgA1*::Tn*phoA*		7.6
IB010	*prgB1*::Tn*phoA*		2.9
IB020	*prgC1*::Tn*phoA*		1.5
IB025	*prgE1*::Tn*phoA*		1.9
IB040	*prgH1*::Tn*phoA*	5.7	0.1*
CS022	*pho-24*	1.9	0.06*
IB043	pWKSH5 in IB040		17.5*
IB044	pWKSH5 in CS022		0.09*

[a] Microaerophilically grown bacterial strains were assessed for changes in adherence and invasion. Adherence was determined as the percentage of bacteria adhered to the cells after centrifugation and 30 min of incubation at 4°C/total number of bacteria added to each well. Invasion was determined as the percentage of bacteria that had invaded after a 2-h incubation with gentamicin/total number of bacteria added to each well. There was no difference between *S. typhimurium* wild-type and *S. enteritidis* CDC5 wild-type strains with respect to adherence and invasion frequencies. Asterisks represent statistical significance by variance analysis of the invasion data done in triplicate compared with those for the wild type ($P < 0.01$).



Strain	Allele	Starvation	LB (aerobic)	LB(anaerobic)
IB040	*prgH1*::Tn*phoA*	5	142	41
IB042	Tn*5*B50-380 *prgH1*::Tn*phoA*	46	248	227

FIG. 1. Location of *prgH* within *hil* locus. The arrows indicate the direction of orientation of the neomycin promoter of Tn*5*B50 insertions within the *hil* locus and the direction of transcription of the *prgH1*::Tn*phoA* fusion protein. Restriction endonuclease sites are as follows: B, *Bam*HI; H, *Hin*dIII; X, *Xho*I; S, *Sac*I; V, *Eco*RV. The effect of the Tn*5*B50-380 insertion on expression of *prgH* fusion protein activity is shown below the diagram. Starvation (repressing conditions for *prg* genes), bacteria were grown aerobically for 48 h in starvation medium ($N^-C^-P^-$) (17) containing 0.04% glucose, 10 mM NH_4Cl, and 1 mM $NaH_2PO_4 \cdot H_2O$; LB (aerobic), bacteria were grown in LB broth (rich medium) to late logarithmic phase (nonrepressing conditions) (OD_{600}, >1.0); LB (anaerobic), bacteria were grown under strict anaerobic conditions for 24 h (OD_{600}, 0.6). All of the numbers represent activity in units of AP as defined by Miller for β-galactosidase (30).

pWKSH5, containing a 5.1-kb *Hin*dIII fragment composed of *hil* and *prgH* (courtesy of C. Lee and V. Bajaj). Plasmid pWKSH5 was crossed into PrgH (IB040) and PhoP^c (CS022) mutant bacteria to create strains IB043 and IB044, respectively. The BME phenotype of the PrgH mutant was similar to that of the wild type with the insertion of pWKSH5, while that of the PhoP^c mutant was not complemented by pWKSH5 (Table 5). Therefore, we conclude that a gene product altered in synthesis as a result of the *prgH*::Tn*phoA* insertion was necessary for BME.

DISCUSSION

Pathogenic bacteria possess regulatory mechanisms to control the expression of virulence factors in response to environmental signals (26, 29). In *S. typhimurium*, the synthesis of virulence factors encoded by *phoP*-activated genes (*pag* genes) is induced by the PhoP/PhoQ regulators in response to the acidic environment of macrophage phagosomes (3). In this work, by the use of a strain with a *phoP/phoQ* locus mutation that constitutively simulates the environmental activation of *pag* genes (phenotype PhoP^c), five unique *phoP*-repressed loci encoding envelope proteins were defined. Consistent with PhoP/PhoQ functioning as global transcriptional regulators, *phoP*-repressed genes (*prg* genes) were found to be widely spaced on the chromosome. One would predict that *prg* genes will be repressed in environmental conditions that activate *pag* expression. Consistent with this prediction, the expression of *prg* loci was repressed under starvation conditions, when *pag* loci were induced (Table 6).

Some variability in repression of *prg*::Tn*phoA* activity was observed with strains with the PhoP^c phenotype. While two loci demonstrated complete repression of fusion protein activity, others demonstrated only partial repression (Table 2). It is possible that the degree of repression of some *prg* loci may be greater when *pag* genes are maximally activated within acidified macrophage phagosomes. Consistent with this possibility is the observation that the expression of *pag* genes in PhoP^c strains is 5- to 10-fold less than that observed after bacteria are phagocytosed by macrophages (3).

One *prg* locus, *prgH*, was found to contribute to mouse virulence when *S. typhimurium* was administered by both the oral and the intraperitoneal routes. PrgH as well as PhoP^c mutants were further found to be defective in bacterium-mediated uptake by epithelial cells, suggesting that an inability to cross epithelial barriers might contribute to the attenuation of virulence observed. Competition studies with mice after oral ingestion of bacteria further supported the hypothesis that *prgH* mutants were defective in transcytosis across the intestinal epithelial barrier. Therefore, this work defined at least two phases of PhoP/PhoQ-regulated protein expression essential to bacterial virulence. In one phase, *prg* expression promotes BME by epithelial cells (Table 6), while in another phase, *pag* expression promotes survival within macrophages (32).

Other virulence regulators with two alternating phases of protein expression have been described previously (18, 36). In *Vibrio cholerae*, growth conditions that maximize the expression of the major ToxR-regulated virulence factors, cholera toxin and the toxin-coregulated pili, also repress the expression of a number of proteins, including the major

TABLE 6. Summary of PhoP/PhoQ environmental regulation[a]

Environment	Type or characteristic for the following gene:	
	pag	*prg*
Media	Starvation	Rich
O_2	Aerobic (*pagC*)	Aerobic (*prgH*) and anaerobic (*prgB*)
pH	3.3–5.5	3.3–5.5 (*prgB*) and >6.0 (*prgH*)
Mammalian cells	Macrophage	Epithelial

[a] Comparison of the different environments where *pag* and *prg* genes are expressed.

outer membrane protein OmpT (36). In *Bordetella pertussis*, mutations in BvgS, the environmental sensor component of the *vir* two-component system which activates the syntheses of pertussis toxin, adenylate cyclase, and filamentous hemagglutinin, can also repress the expression of flagellin (1, 28) and virulence factors encoded by *vir*-repressed genes (*vrg* genes) (4). Although the many microenvironments that an organism experiences are likely to be complex and constantly changing, the concept of two alternating phases of expression of virulence determinants has not only resulted in the isolation of virulence factors but also provided a working model for the study of in vivo regulation.

For systemic pathogens such as *Salmonella* species, the environments the organism experiences may be more complex and varied than those encountered by mucosal pathogens. The achievement of intermediate states of *pag* and *prg* expression could be essential to virulence at some stage of the infectious cycle. Consistent with this possibility was the lack of uniformity in the expression of *pag* and *prg* genes on growth observed at different oxygen tensions and pH conditions. This may also indicate that not all regulation of *pag* and *prg* genes is mediated directly through PhoP and PhoQ. Regardless of this possibility, it seems likely, given the function of PhoP as a transcriptional regulator, that *prg* locus repression occurs at the level of transcription. However, all *prg* loci were defined as protein fusions to AP, and posttranscriptional effects could be responsible for the regulation which we have observed.

Regardless of the mechanism of repression of *prg* loci, the approach of defining genes repressed by the *pho-24* mutation has led to the discovery of at least one virulence locus, *prgH*. The attenuation of *prgH* mutants was much less than that observed in strains with the PhoP^c phenotype, in which many proteins are repressed simultaneously. Therefore, it seems likely that other *prg* virulence loci remain to be identified and that a loss of *pag* and *prg* regulation contributes to the defect of the PhoP^c strain. Nevertheless, the discovery of *prgH* demonstrates that virulence genes are oppositely regulated by PhoP/PhoQ and further documents that *pag* and *prg* regulation in an appropriate environment is necessary for virulence.

prgH was found to be located at 59 min on the *Salmonella* chromosome; this is a site where a number of other loci essential to epithelial cell invasion have been defined. Linkage analysis and Southern hybridization data indicate that *prgH* is highly linked to the hyperinvasive locus (*hil*). This locus was defined by Lee et al. as Tn*5*B50 insertions that result in strains that do not require microaerophilic growth to be invasive (22). We have demonstrated that *prgH* lies between two of these Tn*5*B50 insertions that confer a *hil* phenotype (Fig. 1). Since deletion mutants in this region have been previously demonstrated to also have defects of BME (22) and since we have been able to complement the BME defect of *prgH* mutants with a plasmid containing this locus, this further supports the hypothesis that a protein not synthesized as a result of the *prgH1*::Tn*phoA* insertion promotes BME (Fig. 1).

One would predict that genes essential to the *hil* phenotype would be induced under microaerophilic conditions (22), similar to what was found for *prgB*. Paradoxically, *prgH* expression was maximal during aerobic growth, and the Tn*5*B50-380 insertion, which results in a *hil* phenotype, derepressed expression of *prgH*. In addition, the direction of transcription predicted by the *prgH1*::Tn*phoA* insertion is opposite that of the Tn*5*B50-380-encoded neomycin promoter associated with the *hil* phenotype. Although other interpretations of this result are possible, this could mean that a regulatory protein interrupted by or transcribed from the Tn*5*B50-380 insertion affects the expression of *prgH*.

It will be interesting to see whether other invasion genes are also regulated by PhoP/PhoQ. In this respect, the observation that pWKSH5, a plasmid containing *prgH* (*hil*), did not complement PhoP^c bacteria for BME is interesting. If *prgH* were expressed from pWKSH5, despite the presence of the *pho-24* mutation, this would support the possibility that other genes repressed as part of the PhoP^c phenotype are necessary for BME.

In this work, the identification and characterization of *prgH* have shown that PhoP/PhoQ oppositely regulate factors necessary for bacteria to enter or to survive within mammalian cells. This further documents the importance of gene regulation to bacterial virulence. The identification of *prg* loci will allow us to further study the regulation of bacterial genes after infection of mammalian cells. Understanding the regulation of virulence genes should aid in the development of new live vaccines for typhoid fever and increase our knowledge of the complex interactions between bacteria and mammalian cells.

ACKNOWLEDGMENTS

We thank R. K. Taylor, J. J. Mekalanos, V. Miller, A. Kukral, K. Sanderson from the *Salmonella* Genetic Stock Center, J. Galan, and J. Foster for the generous gifts of strains. We especially thank C. Lee and V. Bajaj for the gift of strains and plasmids and the sharing of unpublished data to define the location of *prgH*. We thank S. Liu and K. Sanderson for graciously analyzing the physical map locations of AK3249 and AK3304. We thank E. Ortega-Barria for his gift of MDCK epithelial cells, J. A. Swanson for J774.2 macrophage line culture, and M. Selsted for NP-1 defensins. We thank the MGH bacteriology laboratory for use of its anaerobic chamber. We also thank W. P. Loomis, C. Alpuche-Aranda, and other members of our laboratory for helpful discussions and review of the manuscript.

I.B. was supported by an Individual National Research Service Award from the National Institute of Allergy and Infectious Diseases (AI08280) and the Searle Scholar's Postdoctoral Fellowship in Gram-Negative Bacterial Infections awarded by the Infectious Diseases Society of America. This work was supported by grants from the National Institutes of Health (AI30479 and AI00917) to S.I.M.

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