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Constitutive Expression of the PhoP Regulon Attenuates *Salmonella* Virulence and Survival within Macrophages

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The *phoP* genetic locus is a two-component regulatory system (*phoP-phoQ*) that controls the expression of genes essential for *Salmonella typhimurium* virulence and survival within macrophages. Strains with a *phoP* constitutive mutation (phenotype PhoP^C) showed up to 10-fold greater expression of *phoP*-activated genes (*pag* loci) than did strains with a wild-type *phoP* locus (phenotype PhoP$^+$). While the *phoP* constitutive mutation resulted in increased expression of *pag* loci, it also dramatically reduced the expression of other protein species. Comparison of the protein content of PhoP$^+$ and PhoP^C strains by two-dimensional protein gel electrophoresis demonstrated that at least 40 separate protein species were changed in expression as a result of this mutation. The PhoP^C *S. typhimurium* were found to be attenuated for virulence and survival within macrophages. This finding suggests that a balanced PhoP-PhoQ regulatory response, which allows expression of *phoP*-repressed as well as -activated genes, is required for full virulence of *S. typhimurium*. We have further shown that small numbers of PhoP^C bacteria can be used as a live attenuated vaccine to protect against mouse typhoid. As few as 15 PhoP^C bacteria protected mice against challenge with 10^5 50% lethal doses of wild-type organisms, suggesting that important protective antigens are regulated by the PhoP-PhoQ virulence regulon.

The *phoP* regulatory locus is composed of two genes, *phoP* and *phoQ*, essential for full virulence, survival within macrophages, and defensin resistance of *Salmonella typhimurium* (4, 15, 17; S. I. Miller, W. Pulkkinen, M. Selsted, and J. J. Mekalanos, submitted for publication). The PhoP (transcriptional activator) and PhoQ (sensor/kinase) proteins have amino acid similarity to other bacterial regulatory proteins (two-component regulators) that control the synthesis of many proteins in response to environmental signals (6, 15, 17, 20). The PhoP and PhoQ gene products are essential for the transcriptional activation of a number of unlinked *phoP*-activated genes (*pag* loci), suggesting that this system comprises a regulon (6, 9, 17). One *pag* locus is essential for full virulence and survival within macrophages (*pagC*), whereas others (*pagA*, *pagB*, and *psiD*), including the *phoN* gene that encodes a periplasmic acid phosphatase, do not as single mutations alter virulence in the BALB/c mouse model of typhoid fever (6, 17). *S. typhimurium* strains with *pagC* mutations are not as attenuated in virulence as *phoP* or *phoQ* mutants, suggesting that other *pag* loci are essential for full virulence (17).

We have previously hypothesized that the PhoQ protein recognizes specific environmental factors in the phagolysosome that lead to activation of the PhoP regulon (17). Previous work by Ames and co-workers, as well as past work in our laboratory, identified starvation for essential nutrients (phosphate, carbon, sulfur, and nitrogen), as well as low pH, as signals for induction of *pag* gene expression (8, 17). Because of the difficulties involved in obtaining specific expression of *pag* gene products under starvation conditions, we have characterized *S. typhimurium* strains with a mutation in the *phoP* locus (*pho-24* [9]) that renders these strains constitutive in the expression of *pag* loci in rich media (phenotype PhoP^C). We report here that in addition to an expected global change in protein expression, PhoP^C strains show a surprisingly pronounced defect in mouse virulence and survival within macrophages.

MATERIALS AND METHODS

Bacterial strains and genetic methods. American Type Culture Collection (ATCC) strain 14028, a smooth virulent strain of *S. typhimurium*, was the parent strain for all virulence studies. Strain TT13208 was a gift from Nang Zhu and John Roth. Strain TA2367 was a generous gift of Gigi Stortz and Bruce Ames (9). Bacteriophage P22HT *int* was used in transductional crosses to construct strains isogenic except for *phoP* locus mutations (2). Luria broth was used as rich medium, and minimal medium was M9 (2). The chromogenic phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate (XP) was used to qualitatively access acid and alkaline phosphatase production in solid media.

Derivatives of *S. typhimurium* ATCC 14028 with the *pho-24* mutation were constructed by use of strain TA2367 as a donor of the *purB* gene in a P22 transductional cross with strain CS003 Δ*phoP* Δ*purB* (17). Colonies were then selected for the ability to grow on minimal medium. A transductant designated CS022 (phenotype PhoP^C) that synthesized 1,750 U of acid phosphatase in rich medium (a ninefold increase over the wild-type level in rich medium; Table 1 and Fig. 1) was used in further studies.

Derivatives of strains CS022 and CS023 *pho-24 phoN2 zxx*::6251Tn*10*d-Cam, an acid phosphatase-negative derivative of CS022, containing *pag* gene fusions were constructed by bacteriophage P22 transductional crosses, using selection of Tn*phoA*- or Mu dJ-encoded kanamycin resistance. Strains were checked for the intact *pag* gene fusion by demonstration of appropriate loss of fusion protein activity on introduction of a *phoP105*::Tn*10*d or *phoP102*::Tn*10*d-Cam allele.

Biochemical analysis. Assays of acid phosphatase, alkaline phosphatase, and β-galactosidase were performed as previously described (17) and are reported in units as defined by Miller (16).

Mouse virulence and vaccination studies. Bacteria grown

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TABLE 1. Bacterial strains and properties

Strain	Genotype	Enzyme activity (U)[a]	Reference or source
10428	Wild type	180 (A)	ATCC; 17
TA2367	*pho-24*	1,925 (A)	9
CS003	Δ*phoP* Δ*purB*	<10 (A)	17
CS022	*pho-24*	1,750 (A)	This work
CS023	*pho-24 phoN2 zxx*::6251Tn*10*d-Cam	25 (A)	This work
CS012	*pagA1*::Mu dJ	45 (B)	17
CS013	*pagB1*::Mu dJ	120 (B)	17
CS119	*pagC1*::Tn*phoA phoN2 zxx*::6251Tn*10*d-Cam	85 (C)	17
SC024	*pagA1*::Mu dJ *pho-24*	450 (B)	This work
CS025	*pagB1*::Mu dJ *pho-24*	980 (B)	This work
CS026	*pagC1*::Tn*phoA pho-24 phoN2 zxx*::6251Tn*10*d-Cam	385 (C)	This work
CS015	*phoP102*::Tn*10*d-Cam	<10 (A)	17
TT13208	*phoP105*::Tn*10*d	<10 (A)	—[b]

[a] A, Acid phosphatase; B, β-galactosidase; C, alkaline phosphatase.
[b] Gift of Ning Zhu and John Roth.

overnight in Luria broth were washed and diluted in normal saline. The wild-type parent strain of CA022 (ATCC 10428) was used for all live vaccine challenge studies. This strain has a 50% lethal dose (LD_{50}) for naive adult BALB/c mice of less than 20 organisms when administered by intraperitoneal (i.p.) injection and 5×10^4 when administered orally in $NaHCO_3$. Mice were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, Mass.) and were 5 to 6 weeks of age at initial challenge. All i.p. inoculations were performed as previously described (17). Oral challenge experiments were performed with bacteria grown in LB broth and concentrated by centrifugation. The bacteria were resuspended in 0.1 M $NaHCO_3$, to neutralize stomach acid, and administered as a 0.5-ml bolus to animals under ether anesthesia. Colony counts were performed to accurately access the number of organisms administered. All challenge

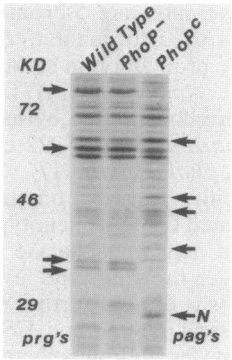

FIG. 1. Denaturing polyacrylamide gel electrophoresis of whole-cell proteins of strains ATCC 10428 (wild-type virulent parent; PhoP$^+$), CS015 (PhoP$^-$) (17), and CS022 (PhoP^C). The gel (12.5% polyacrylamide–sodium dodecyl sulfate) was run in buffer conditions as described by Laemmli (11). The gel was fixed and stained with Coomassie brillant blue R250 in 10% acetic acid–10% methanol. Arrows on the right indicate activated species (*pag*'s); arrows on the left indicate repressed species (*prg*'s). The numbers indicate the positions of molecular size standards (in kilodaltons [KD]) run in adjacent lanes. The whole-cell protein extracts were from stationary-phase cultures grown in Luria broth. N, The 26-kilodalton nonspecific acid phosphatase that is the product of the *phoN* gene.

experiments were performed 1 month after i.p. inoculation and 6 weeks after oral challenge. Challenge inocula were administered by the same route as vaccinations. The care of all animals was under institutional guidelines as set by the animal care committees at the Massachusetts General Hospital and Harvard Medical School.

Protein electrophoresis. One-dimensional protein gel electrophoresis was performed by the method of Laemmli (11) on whole-cell protein extracts of stationary-phase cells grown overnight in Luria broth. The gels were fixed and stained with Coomassie brilliant blue R250 in 10% acetic acid–10% methanol. Two-dimensional protein gel electrophoresis was performed by the method of O'Farrell (18) on the same whole-cell extracts. Isoelectric focusing using 1.5% pH 3.5 to 10 ampholines (LKB Instruments, Baltimore, Md.) was carried out for 9,600 V · h (700 V for 13 h 45 min). The final tube gel pH gradient extended from pH 4.1 to pH 8.1 as measured by a surface pH electrode (BioRad Laboratories, Richmond, Calif.) and colored acetylated cytochrome pI markers (Calbiochem-Behring, La Jolla, Calif.) run in an adjacent tube. The slab gels were silver stained (14).

Macrophage survival assays. Experiments were performed as previously described (17) by the method of Buchmeiher and Heffron (1) as modified from the method of Lissner et al. (12). Stationary-phase cells were opsonized for 30 min in normal mouse serum before exposure to the cultured bone marrow-derived macrophages harvested from BALB/c mice. One hour after infection, gentamicin sulfate (8 μg/ml) was added to kill extracellular bacteria. All time points were done in triplicate and repeated on three separate occasions.

RESULTS

The *phoP* constitutive allele, *pho-24*, results in derepression of *pag* loci. Using diethyl sulfate mutagenesis of *S. typhimurium* LT-2, Ames and co-workers isolated strain TA2367 *pho-24*, which contained a *phoP* locus mutation that resulted in constitutive production of acid phosphatase in rich media (9). This *phoP*-regulated acid phosphatase is encoded by the *phoN* gene, a *pag* locus (9, 17). We wished to analyze whether the *pho-24* allele increased the expression of other *pag* loci. This was done by scoring the effect of the *pho-24* allele on the expression of other *pag* loci recently identified as transcriptional (e.g., *pagA* and *pagB*) and translational (e.g., *pagC*) fusion proteins that required *phoP* and *phoQ* for expression (17). Therefore, we constructed *pag* gene fusion strains, isogenic except for the *pho-24* allele, and assayed fusion protein activity. PhoP^C derivatives of the *pagA*::Mu dJ and *pagB*::Mu dJ strains produced 480 and 980 U, respectively, of β-galactosidase in rich medium, an increase of 9- to 10-fold over values for the fusion strains with a wild-type *phoP* locus (Table 1). The *pagC*::Tn*phoA* gene fusion produced 350 U of alkaline phosphatase, an increase of three- to fourfold over that produced in strain CS119, which is isogenic except for the *pho-24* mutation (17). These results compare with a ninefold increase in the acid phosphatase activity in strain CS022 on introduction of the *pho-24* allele. Therefore, these available assays for *pag* gene expression document that the *pho-24* mutation causes constitutive expression of other *pag* loci besides *phoN*.

Identification of protein species that are repressed as well as activated in the PhoP^C mutant strain. We wished to analyze whole-cell proteins of strain CS022 to estimate the number of protein species that could be potentially regulated by the PhoP regulon. Remarkably, analysis by one-dimensional polyacrylamide gel electrophoresis of the proteins produced

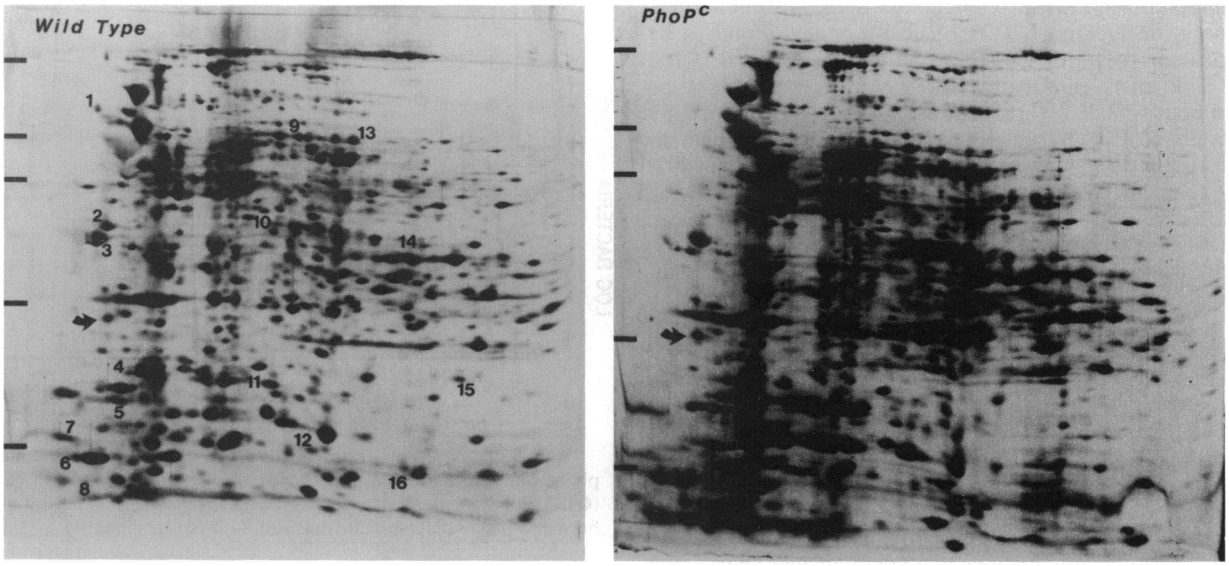

FIG. 2. Two-dimensional electrophoresis performed on wild-type and PhoP^C whole-cell protein samples according to the method of O'Farrell (18) The numbers indicate some of the most easily seen changes in species. Odd numbers indicate species repressed in the PhoP^C strain (potential *prg* loci), and even numbers indicating species activated by the PhoP^C mutation (*pag* loci). Vitamin D-dependent calcium-binding protein (40 ng; pI 5.2) was added to the samples; this standard is indicated by the arrow on the stained gel. The final tube gel pH gradient extended from pH 4.1 to 8.1 (from right to left). Molecular weight standards are marked by bars and correspond (from top to bottom) to myosin (230,000), phosphorylase A (94,000), catalase (60,000), actin (43,000), and lysozyme (14,000). The 10% acrylamide slab gels are silver stained (14).

by strains with the PhoP^C phenotype indicated that some protein species were decreased in expression when many presumptive *pag* gene products were fully induced by the *pho-24* mutations (Fig. 1). The proteins decreased in the PhoP^C strain might represent products of genes that are repressed by the PhoP regulator (see Discussion). To simplify discussion, we have tentatively designated the genes encoding proteins decreased by the *pho-24* allele *prg* loci, for *phoP*-repressed genes. Comparison of wild-type, PhoP$^-$, and PhoP^C mutant strain proteins shows that growth in LB medium at 37°C represents repressing conditions for *pag* gene products and derepressing conditions for *prg* gene products.

To estimate the total number of potentially PhoP-regulated gene products, the total cell proteins of wild-type and PhoP^C mutant strains grown in LB were analyzed by two-dimensional gel electrophoresis. At least 40 species underwent major fluctuation in expression in response to the *pho-24* mutation (Fig. 2), although we have indicated only 15 well-resolved species in this figure.

Virulence defects of the PhoP^C strain. The marked changes in protein expression of the PhoP^C strain led us to investigate its virulence characteristics. Remarkably, strains with the single *pho-24* mutation were markedly attenuated for virulence in mice (Table 2). The number of PhoP^C organisms (2×10^5) that killed 50% of BALB/c mice challenged (LD_{50}) by the i.p. route was near that (6×10^5) of PhoP$^-$ bacteria (17). The PhoP^C strains had growth comparable to wild-type organisms in rich and minimal media (9; data not shown). We also tested PhoP^C mutants for alteration in lipopolysaccharide, which could also explain the virulence defect we observed. Strain CS022 had normal sensitivity to phage P22, normal group B reactivity to antibody to O antigen, and a lipopolysaccharide profile identical to that of the parent strain by polyacrylamide gel electrophoresis and staining (data not shown).

Since the TA2367 *pho-24* strain was constructed by chemical mutagenesis and could have another linked mutation responsible for its virulence defect, we wished to isolate revertants of the PhoP^C phenotype to document that the *pho-24* allele was responsible for the attenuation of virulence we observed. Phenotype PhoP^C revertants, identified by their normal levels of acid phosphatase in rich medium, were isolated among the bacteria recovered from the livers of

TABLE 2. Virulence and protective efficacy of PhoP^C and PhoP$^-$ *Salmonella* strains

Immunizing dose	No. of initial survivors/ total	No. of survivors/total after wild-type challenge dose of:			
		5×10^7[a]	5×10^5	5×10^4	5×10^3
PhoP^C organisms					
15	13/13		5/5	4/5	
50	4/4				4/4
1.5×10^2	11/11		4/4	3/3	
5×10^2	16/16				4/4
1.5×10^3	5/5		3/3	2/2	
5×10^3	4/4				4/4
1.5×10^4	5/5		3/3	2/2	
5×10^4	19/23				4/4
1.5×10^5	5/5		3/3	2/2	
5×10^5	1/4				1/1
5×10^6	0/6				
3×10^9[a]	5/5	5/5			
3×10^{10}[a]	5/5	5/5			
1.5×10^{11}[a]	5/5	5/5			
PhoP$^-$ organisms					
6×10^3	36/36		0/12	0/12	0/12
6×10^4	36/36		0/12	0/12	3/12
6×10^5	19/36		0/6	0/6	4/7
5×10^{10}[a]	7/7	3/7			

[a] Organisms were administered by the oral route. In all other experiments, organisms were administered by i.p. challenge.

mice infected with strain CS022. Six separate phenotypic revertants, designated CS122 to CS128, were found to be fully virulent (LD_{50} of less than 20 organisms for BALB/c mice). The locus responsible for the reversion phenotype was mapped in all six revertants tested for virulence by bacteriophage P22 cotransduction and had linkage characteristics consistent with the *phoP* locus (greater than 90% linkage to *purB*). We therefore conclude that these reversion mutations are not extragenic suppressors but are intragenic suppressors or true revertants of the *pho-24* mutation. Thus, the virulence defect of $PhoP^C$ mutants is probably the result of a single revertable mutation in the *phoP* locus and not the result of a second unrelated mutation acquired during mutagenesis.

Reversion frequency of the $PhoP^C$ phenotype. We investigated the reversion frequency of the $PhoP^C$ mutation in vivo in mice to assess whether reversion could reduce the LD_{50} of this strain. We tested for the presence of revertants of strain CS022 by administering 10^6, 10^4, and 10^2 challenge organisms to each of eight animals by i.p. injection. On day 7, three animals died that received 10^6 $PhoP^C$ organisms. On that day, the livers and spleens of all animals were harvested and homogenized in saline. After appropriate dilution, 10% of the tissue was plated on LB plates containing the chromogenic phosphatase substrate XP. Revertants were identified by their lighter blue colonies compared with $PhoP^C$ bacteria and were confirmed by quantitative acid phosphatase assays. An estimated 10^7, 10^5, and 10^3 organisms per organ were recovered from animals at each of the three respective challenge doses. Revertants were identified only at the highest dose and comprised 0.5 to 1%, or 10^5 organisms per organ, at the time of death. It is likely that revertants are able to compete more effectively for growth in these macrophage-containing organs, since strain CS022 is deficient in survival within macrophages (see below). However, revertants were not identified if fewer than 10^5 organisms were administered in the challenge dose, suggesting that the reversion frequency must be approximately 10^{-5}. The reversion rate of the $PhoP^C$ phenotype for CS022 bacteria grown in LB is in fact 6×10^{-4} when scored by the same colony phenotypes. The percentage of revertants recovered from animals near death suggests that pressure is applied in vivo that selects for revertants of the $PhoP^C$ phenotype and implies that the virulence defect we observed could be much greater quantitatively for a strain with a nonrevertable $PhoP^C$ mutation.

The $PhoP^C$ strain is deficient in survival within macrophages. Because of the importance of survival within macrophages to *Salmonella* virulence (5), we tested $PhoP^C$ bacteria for this property. Strain CS022 was defective in the ability to grow and persist in macrophages as compared with wild-type organisms (Fig. 3). $PhoP^-$ bacteria seemed to have a macrophage survival defect qualitatively similar to that of $PhoP^C$ bacteria but survived consistently better by two- to threefold in side-by-side experiments (data not shown). The increased recovery of organisms that reverted the $PhoP^C$ phenotype in mouse organs rich in macrophage content is consistent with the reduced macrophage survival of $PhoP^C$ mutants in vitro.

Use of the $PhoP^C$ strain as a live vaccine. We previously reported that $PhoP^-$ strains were useful as live vaccines in protecting against mouse typhoid (17). Therefore, we wished to compare the $PhoP^C$ and $PhoP^-$ strains for immunogenicity when used as live attenuated vaccines in mice. This was done by simultaneous determination of survival, after graded challenge doses with the wild-type strain ATCC 10428, in

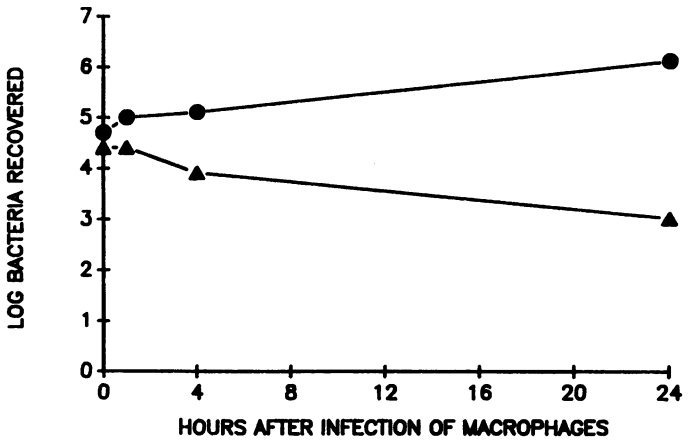

FIG. 3. Survival of strain CS022 ($PhoP^C$) (▲) in cultured macrophages as compared with wild-type *S. typhimurium* ATCC 10428 (●). The experiment shown is a representative one. The difference between the two strains at four and 24 h is significant ($P < 0.05$).

mice previously immunized with graded doses of the two live vaccine strains, CS015 *phoP*::Tn*10*d-Cam and CS022 *pho-24*, as well as a saline control. The results obtained (Table 2) suggest the following conclusions: (i) small i.p. doses of the $PhoP^C$ strain (e.g., 15 organisms) effectively protect mice from challenge doses as large as 5×10^5 bacteria (a challenge dose that represents greater than 10^4 i.p. LD_{50}s), (ii) large doses of $PhoP^C$ organisms given orally completely protect mice from an oral challenge consisting of 5×10^7 wild-type bacteria (over 200 oral wild-type LD_{50}s) and (iii) by comparison, a large dose of $PhoP^-$ organisms (5×10^5) does not provide similar protection. The reversion of the $PhoP^C$ mutation in vivo somewhat complicates the analysis of the use of these strains as vaccines, since revertants of the CS022 strain (i.e., wild-type cells) could increase immunogenicity. However, we were unable to identify revertants by examining 10% of the available spleen and liver tissue from those mice that received 10^4 or fewer organisms.

DISCUSSION

In this report, we show that a *phoP* locus mutation (*pho-24*) results in constitutive expression of genes activated by the PhoP-PhoQ two-component regulatory system and in attenuation of virulence and survival within cultured macrophages (Table 2 and Fig. 3). Previously, we (17) as well as others (4) have shown that strains with null mutations in *phoP* or *phoQ* have reduced survival within macrophages, increased sensitivity to the NP-1 defensins, and reduced lethality for mice (4, 17; Miller et al., submitted). Null mutations in *phoP* and *phoQ* decrease the expression of the same genes whose expression is derepressed by the *pho-24* mutation (i.e., *phoN*, *pagA*, *pagB*, and *pagC*). Therefore, it is apparent that mutations that inactivate or activate the PhoP-PhoQ regulon can attenuate virulence. Given that insertion mutations in at least one *phoP*-activated gene, *pagC*, result in decreased virulence and survival within macrophages, the attenuation of $PhoP^-$ and $PhoQ^-$ strains seems logical.

The molecular basis for attenuation of virulence of $PhoP^C$ strains may be related to the observation that in addition to the expected increase in expression of *pag* gene products, $PhoP^C$ strains show reduced expression of many other proteins detectable by two-dimensional gel electrophoresis.

For purposes of simplicity of discussion, we have termed the genes encoding these repressed proteins *prg* loci, for *phoP*-repressed genes. If one or more of these *prg* gene products are essential to virulence, this may explain the virulence attenuation of PhoP^C *S. typhimurium*.

Several possibilities exist for the mechanism of repression of protein species by the *phoP* constitutive mutation. The PhoP protein may bind to DNA and function as a transcriptional repressor at *prg* loci. The ability to function as a repressor as well as an activator is consistent with the alternate expression of the porin genes *ompF* and *ompC* by the analogous two-component regulator OmpR (7), as well as the identification of both repressed and activated gene products controlled by the coordinate virulence regulators of *Staphylococcus aureus* (19), *Vibrio cholerae* (21), and *Bordetella pertussis* (10). Another possibility is that a *pag* locus encodes a transcriptional repressor of *prg* loci and the system thus functions as a cascade. If the *pho-24* mutation is in *phoQ* and results in full activation of PhoQ kinase activity, this could lead to activation, through phosphorylation, of another PhoP-like transcriptional regulator that functions as a repressor. It is also possible that the activation of *pag* loci leads to posttranscriptional protein effects, such as degradation or modification, that cause a change in the protein expression pattern observed. Identification of *phoP*-repressed gene fusions should help resolve the mechanism of these global changes in protein expression.

The demonstration that both PhoP$^-$ and PhoP^C bacteria are defective in survival within macrophages suggests that both induction and repression of the PhoP regulon may be necessary for survival in the intracellular environment. This demonstration, coupled with the fact that PhoP and PhoQ are environmentally responsive regulators, further suggests that the products of *pag* and *prg* loci may contribute to intracellular survival either in different environments or in a different temporal sequence. The fact that PhoP$^-$ *Salmonella* strains are markedly sensitive to defensins (4), microbicidal peptides of mammalian phagocytes (13), may provide a clue to the intracellular environment in which *pag* gene products are required. We have recently shown that unlike PhoP$^-$ strains, PhoP^C bacteria are as resistant to defensins as are wild-type bacteria, indicating that defensin resistance is likely encoded by a *pag* gene product (Miller et al., submitted). Defensins are active in vitro only at neutral pH, and the killing effect of these molecules has been postulated to be most important in the immediate postphagocytic period, when the pH of the phagosome has been documented to be transiently neutral to alkaline (pH 7.8) (13). It is possible that *pag* gene products, including those necessary for defensin resistance, are most important in the early stages of phagocytosis, perhaps before phagolysosome fusion. Other factors encoded by *prg* loci may be more important to survival within macrophages at a time when defensins are not active (i.e., after acidification of the phagolysosome). Thus, a switch in the PhoP regulon from *pag* to *prg* gene expression might be essential to efficient macrophage survival after *salmonellae* are phagocytosed.

The pathogenesis of mouse typhoid involves passage of bacteria through multiple environments, including gut lumen, endothelial cells, phagocytes, lymphatics, and bloodstream. The macrophage survival defect of PhoP^C bacteria may be sufficient to explain the attenuation we observed, and this may be due to the inability of PhoP^C organisms to synthesize *prg* gene products. However, it is also possible that the reduced virulence of the PhoP^C phenotype demonstrates how important balanced regulation is to the pathophysiologic process. Full virulence is dependent not simply on the synthesis of virulence factors but also on the ability to regulate their expression in the appropriate environment (3, 15). The attenuation observed after overexpression of virulence properties as a result of constitutive mutations in virulence regulators provides evidence that the ability to turn off the expression of loci encoding essential virulence factors may be as important as the ability to turn them on.

Further analysis of the PhoP-PhoQ regulon should lead to greater understanding of the molecular basis of *S. typhimurium* virulence and survival within macrophages. The marked immunogenicity of strains with the PhoP^C phenotype suggests that *pag* genes may be important antigens for the induction of protective immunity against *Salmonella* species and that PhoP^C strains with nonrevertable mutations could be useful as live vaccines in humans and other animals.

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LITERATURE CITED

1. **Buchmeier, N. A., and F. Heffron.** 1989. Intracellular survival of wild-type *Salmonella typhimurium* and macrophage-sensitive mutants in diverse populations of macrophages. Infect. Immun. **57**:1–7.
2. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics, p. 78, 87. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
3. **Dirita, V. J., and J. J. Mekalanos.** 1989. Genetic regulation of bacterial virulence. Annu. Rev. Genet. **23**:455–482.
4. **Fields, P. I., E. A. Groisman, and F. Heffron.** 1989. A *Salmonella* locus that controls resistance to microbicidal proteins from phagocytic cells. Science **243**:1059–1062.
5. **Fields, P. I., R. V. Swanson, C. G. Haidairis, and F. Heffron.** 1986. Mutants of *Salmonella typhimurium* that cannot survive with the macrophage are avirulent. Proc. Natl. Acad. Sci. USA **83**:5189–5193.
6. **Groisman, E. A., E. Chiao, C. J. Lipps, and F. Heffron.** 1989. *Salmonella typhimurium phoP* virulence gene is a transcriptional regulator. Proc. Natl. Acad. Sci. USA **86**:7077–7081.
7. **Hall, M. N., and T. J. Silhavy.** 1981. Genetic analysis of the major outer membranes of *Escherichia coli*. Annu. Rev. Genet. **15**:91–142.
8. **Kier, L. K., R. Weppleman, and B. N. Ames.** 1977. Regulation of two phosphatases and a cyclic phosphodiesterance of *Salmonella typhimurium*. J. Bacteriol. **130**:420–428.
9. **Kier, L. K., R. M. Weppleman, and B. N. Ames.** 1979. Regulation of nonspecific acid phosphatase in *Salmonella*: *phoN* and *phoP* genes. J. Bacteriol. **138**:155–161.
10. **Knapp, S., and J. J. Mekalanos.** 1988. Two *trans*-acting regulatory genes (*vir* and *mod*) control antigenic modulation in *Bordetella pertussis*. J. Bacteriol. **170**:5059–5066.
11. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
12. **Lissner, C. R., R. Swanson, and A. O'Brien.** 1983. Genetic control of the innate resistance of mice to *Salmonella typhimurium*: expression of the *Ity* gene in peritoneal and splenic macrophages isolated *in vitro*. J. Immunol. **131**:3006–3013.
13. **Lehrer, R. I., M. E. Selsted, D. Szklarek, and J. Fleischmann.** 1983. Antibacterial activity of microbicidal cationic proteins 1 and 2, natural peptide antibiotics of rabbit lung macrophages.

Infect. Immun. **42**:10–14.
14. **Merril, C. R., D. Goldman, and M. L. Van Keuren.** 1984. Gel protein strains: silver stain. Methods Enzymol. **104**:441–447.
15. **Miller, J. F., J. J. Mekalanos, and S. Falkow.** 1989. Coordinate regulation and sensory transduction in the control of bacterial virulence. Science **243**:916–922.
16. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Miller, S. I., A. M. Kukral, and J. J. Mekalanos.** 1989. A two component regulatory system (*phoP* and *phoQ*) controls *Salmonella typhimurium* virulence. Proc. Natl. Acad. Sci. USA **86**: 5054–5058.
18. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
19. **Recesi, P., B. Kreiswirth, M. O'Reilly, P. Schlievert, and A. Gruss.** 1986. Regulation of exoprotein gene expression in *Staphylococcus aureus* by *agr*. Mol. Gen. Genet. **202**:58–61.
20. **Ronson, C. W., B. T. Nixon, and F. M. Ausubel.** 1987. Conserved domains in bacterial regulatory proteins that respond to environmental stimuli. Cell **49**:579–581.
21. **Taylor, R. K., V. L. Miller, D. B. Furlong, and J. J. Mekalanos.** 1987. Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc. Natl. Acad. Sci. USA **84**:2833–2837.