JOURNAL OF BACTERIOLOGY, May 1998, p. 2409–2417 Vol. 180, No. 9
0021-9193/98/$04.00+0

A Low pH-Inducible, PhoPQ-Dependent Acid Tolerance Response Protects *Salmonella typhimurium* against Inorganic Acid Stress

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Received 6 November 1997/Accepted 27 February 1998

The acid tolerance response enables *Salmonella typhimurium* to survive exposures to potentially lethal acidic environments. The acid stress imposed in a typical assay for acid tolerance (log-phase cells in minimal glucose medium) was shown to comprise both inorganic (i.e., low pH) and organic acid components. A gene previously determined to affect acid tolerance, *atbR*, was identified as *pgi*, the gene encoding phosphoglucoisomerase. Mutations in *pgi* were shown to increase acid tolerance by preventing the synthesis of organic acids. Protocols designed to separate the stresses of inorganic from organic acids revealed that the regulators σ^{38} (RpoS), Fur, and Ada have major effects on tolerance to organic acid stress but only minor effects on inorganic acid stress. In contrast, the two-component regulatory system PhoP (identified as acid shock protein ASP29) and PhoQ proved to be important for tolerance to organic acid stress but had little effect against organic acid stress. PhoP mutants also failed to induce four ASPs, confirming a role for this regulator in acid tolerance. Acid shock induction of PhoP appears to occur at the transcriptional level and requires the PhoPQ system. Furthermore, induction by acid occurs even in the presence of high concentrations of magnesium, the ion known to be sensed by PhoQ. These results suggest that PhoQ can sense both Mg^{2+} and pH. Since *phoP* mutants are avirulent, the low pH activation of this system has important implications concerning the pathogenesis of *S. typhimurium*. The involvement of four regulators, two of which are implicated in virulence, underscores the complexity of the acid tolerance stress response and further suggests that features of acid tolerance and virulence are interwoven.

Neutralophilic bacteria such as *Salmonella typhimurium* prefer to live and grow at a pH near neutrality. However, *S. typhimurium* often encounters a variety of potentially lethal acid stress conditions both in nature and during pathogenesis (10). Acid stress is a complex phenomenon involving the combined biological effects of acidic pH and organic acids that may be present in a given environment. Severe acidic pH (e.g., pH 3) creates a situation whereby protons leak across the membrane faster than housekeeping pH homeostasis systems can remove them. The result is an intracellular acidification to levels that damage or disrupt key biochemical processes. However, even the mild acid stress of a pH 5 medium can become a severe challenge if the medium also contains 200 mM acetate. The reason for this is that even in mildly acidic environments, the protonated form of an organic acid can permeate the cell membrane and dissociate inside the cell, in which the released proton can acidify intracellular pH. After dissociation, the membrane-impermeable, ionized form of the organic acid will accumulate intracellularly, causing further cell damage.

S. typhimurium responds to acidic challenges through a complex adaptive system called the acid tolerance response (ATR), in which adaptation to mild (pH 5.8) or moderate (pH 4.4) acid conditions enables the cell to endure periods of severe acid stress (pH 3). The ATR of *S. typhimurium* requires the synthesis of over 50 acid shock proteins (ASPs) that can be grouped into what appear to be a variety of survival systems. Some of these systems function primarily in exponentially growing cells, while others function in stationary-phase cells. ATR systems operating in stationary phase include those that are dependent on the alternative sigma factor σ^{38} and others that are σ^{38} independent. The σ^{38} protein, which is encoded by *rpoS*, was first recognized as an important transcription factor in stationary-phase bacteria but is now acknowledged to be crucial for many stress responses (15, 19). The stationary-phase, σ^{38}-dependent acid tolerance systems are not induced by acid, probably because σ^{38} levels are already elevated by entry into stationary phase. In contrast, there is a σ^{38}-independent ATR system evident in stationary phase that does require induction by acid (2, 5, 17).

Exponentially growing cells also exhibit σ^{38}-dependent and -independent ATR systems (16). However, while σ^{38}-dependent acid tolerance is not induced by low pH in stationary phase, it is induced by acid shock in exponential-phase cells. Rapidly growing cells that undergo an acid shock will increase σ^{38} production, which will, in turn, increase the expression of a subset of ASPs. The acid shock-induced increase in σ^{38} occurs due to the decreased proteolytic turnover of this sigma factor, a feature of σ^{38} control mediated by MviA (3).

Mutants deficient in σ^{38} have an interesting acid-sensitive phenotype evident in log-phase cells (16). While *rpoS*$^+$ cells become progressively more acid tolerant during acid shock (pH 4.4) adaptations lasting up to 90 min, *rpoS* mutants will induce an ATR only if adaptation does not exceed 20 min (16). Acid shock adaptation for more than 20 min will render *rpoS* mutants extremely acid sensitive. Thus, *rpoS* mutants only transiently induce an ATR. Sustained induction of the ATR is referred to as RpoS-dependent acid tolerance because of its dependence on σ^{38} (16). RpoS-independent systems are also involved in the ATR of log-phase cells. One such system includes a set of ASPs controlled by the major iron regulatory

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TABLE 1. Strains used

Strain	Genotype	Source
SF242	*oxrC (pgi)*::Tn*5*	C. Higgins
SF416 (MM197)	*mgtB11*::Mu*dJ leuBCD485*	M. Maguire
SF530 (UK1, χ3761)		R. Curtiss III
SF588 (χ4971)	UK1 *fur-1 zbf-5123*::Tn*10*	R. Curtiss III
SF627 (YG7153)	*hisG46 rfa*$^+$ Δ*ada*::Km	34
EG1202	*phoQ*::Mu*dJ*	E. Groisman
SF687	14028S *pagA1*::Mu*dJ*	S. Miller
SF720 (EG9671)	14028S *mgtA*::Mu*dJ pmrA1*::Cm	E. Groisman
SF732 (EG5170)	14028S *phoP5090*::Mu*dJ*	E. Groisman
JF1899	*phoP*::Tn*10*	7
JF1915	*psiD19*::Mu*d*1-8	9
JF2471	*atbR (pgi)*::Tn*10*dTc *fabF (atrB)13*::Mu*dJ*	6
JF2585	LT2 *phoQ*::Mu*dJ*	EG1202 × SF1
JF2690	UK1 *rpoS*::*Ap*	16
JF2731	UK1 *rpoS*::*Ap atbR (pgi)*::Tn*10*dTc	JF2471 × JF2690
JF2733	UK1 *atbR (pgi)*::Tn*10*dTc	JF2471 × SF530
JF2811	LT2 *atbR (pgi)*::Tn*10*dTc *atrB (fabF)*::Mu*dJ*/pBF119 *pgi*$^+$	Clone pool × JF2471
JF2812	LT2 *atbR (pgi)*::Tn*10*dTc *atrB (fabF)*::Mu*dJ*/pBF120 *pgi*$^+$	Clone pool × JF2471
JF2938	UK1 *rpoS10*::Mu*dJ*	Mu*dJ* pool × SF530
JF2955	UK1 *rpoS*::*Ap oxrC (pgi)*::Tn*10*	JF2690 × SF242
JF3024	UK1 *ada*::Km	SF627 × SF530
JF3203	UK1 *phoP*::Tn*10*	JF1899 × SF530
JF3204	UK1 *rpoS*::Ap *phoP*::Tn*10*	JF1899 × JF2690
JF3264	UK1 *pmrA508*ΩKm	M. Spector
JF3274	UK1 *mgtB11*::Mu*dJ*	SF416 × SF530
JF3302	UK1 *phoQ*::Mu*dJ rpoS*::Ap	JF2585 × JF2690
JF3303	UK1 *pagA1*::Mu*dJ*	SF687 × SF530
JF3439	UK1 *rpoS10*::Mu*dJ phoP*::Tn*10*	JF1899 × JF2938
JF3529	UK1 *rpoS10*::Mu*dJ phoP*::Tn*10*/pEG9050 (*phoQ* expressed from *lacP*)	JF3439 × pEG9050 (E. Groisman)
JF3530	UK1 *rpoS10*::Mu*dJ phoP*::Tn*10*/pEG9071 (*phoPQ* expressed from *lacP*)	JF3439 × pEG9071 (E. Groisman)
JF3531	UK1 *pagA1*::Mu*dJ phoP*::Tn*10*	JF1899 × JF3303
JF3547	UK1 *pagA1*::Mu*dJ pmrA1*::Cm	SF720 × JF3303
JF3550	UK1 *psiD19*::Mu*d*A	JF1915 × SF530
JF3551	UK1 *psiD19*::Mu*d*A *pmrA*::Km	JF1915 × JF3264
JF3552	UK1 *mgtB11*::Mu*dJ phoP*::Tn*10*	JF1899 × JF3274
JF3553	UK1 *mgtB11*::Mu*dJ pmrA1*::Cm	SF720 × JF3274
JF3554	UK1 *psiD19*::Mu*d*A *phoP*::Tn*10*	JF1915 × JF3203
JF3561	UK1 *psiD19*::Mu*d*A *pmrA1*::Cm	SF720 × JF3550
JF3594	UK1 *phoP5090*::Mu*dJ*	SF732 × SF530
JF3609	UK1 *phoP5090*::Mu*dJ*/pEG9071 (*phoPQ* expressed from *lacP*)	JF3401 × JF3594

protein Fur (8, 14). The present report describes a second RpoS-independent system controlled by PhoPQ, a two-component regulatory system known to sense extracellular magnesium concentrations (28, 30, 32).

Multiple systems of acid tolerance may, in part, provide fail-safe redundancies that ensure survival should one system fail. However, the multifactorial nature of acid stress (i.e., the effects of acidic pH and organic acid concentration) might dictate a need for systems specific for one or the other acid stress component. If this is so, one may be able to classify specific acid response systems with respect to their utility in handling organic (weak acid) versus inorganic (low pH) acid stress. The acid stress experienced by cells exponentially growing in minimal glucose media shifted to pH 3 has been shown to involve both organic and inorganic acid components. The evidence presented indicates that RpoS is essential for surviving the organic acid stress component but two systems, one RpoS dependent and the other PhoPQ dependent, provide partially redundant protection against inorganic acid stress (i.e., low pH). In agreement with its role in inducible acid tolerance, PhoP is shown to be an acid shock protein.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media. The strains used throughout this study are listed in Table 1. Plasmids pEG9050 and pEG9071 were provided by E. Groisman (27). LB complex medium and Vogel and Bonner E minimal medium supplemented with 0.4% glucose were prepared as liquid and solid (1.5% agar) media (31). NCE-lactose medium was prepared as described by Maloy (20). N-minimal medium included 38 mM glycerol and 0.1 or 1% vitamin-free Casamino Acids (23). The following antibiotics were used at the concentrations indicated; ampicillin, 60 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 30 μg/ml; and tetracycline, 10 or 20 μg/ml for minimal and rich media, respectively.

β-Galactosidase and acid tolerance assays. β-Galactosidase was measured according to the method described by Miller (21). Standard ATR assays were conducted with strains grown overnight at 37°C in E glucose (EG) broth containing the appropriate antibiotic. A 1/100 dilution of the overnight broth was inoculated into 3 ml of EG broth (pH 7.7), and the mixture was incubated at 37°C with shaking. The cells were grown to an optical density at 600 nm (OD_{600}) of 0.40 (2×10^8 CFU/ml), at which point cultures destined for adaptation were adjusted to pH 4.4 and incubated for 60 min. Acid challenge of unadapted and adapted cultures involved readjusting the pH to 3.1 for the indicated time. The acid stress in this assay included both organic and inorganic acids. Percent survival was calculated by dividing the number of CFUs on LB agar at time *x* by the number of CFUs at time zero and multiplying by 100.

The fresh medium ATR assay (organic acid free) was performed by a similar method. However, spent medium was removed by centrifugation of the culture for 3 min in a microcentrifuge. The cell pellet was resuspended in fresh EG medium preadjusted to pH 4.4 or 3.1 at 37°C. The pH of the fresh medium culture was adjusted after resuspension to correct for subtle changes in pH. When indicated, 1 mM acetic acid (Fisher Scientific, Norcross, Ga.) was added

to fresh EG medium to mimic spent EG medium. Lengths of adaptation and challenge were the same as those used in the standard ATR assay described above.

Cloning of *atbR*. AtbR was previously identified as a putative negative regulator of *fabF* (initially designated *atrB* [6]). The *fabF-lac atbR* strain (JF2471) grew poorly on NCE-lactose medium, whereas the *fabF-lac* strain grew well. This phenotype was counterintuitive with respect to AtbR being a negative regulator of *fabF*. Nevertheless, this characteristic provided a clue as to the identity of AtbR in that its absence interfered with lactose metabolism and served as a useful selection marker for cloning efforts. Plasmids able to complement the Lac$^-$ phenotype of JF2471 were selected from a clone pool on NCE-lactose (Ap) medium. Two distinct plasmids with this ability were isolated from JF2811 and JF2812 and designated pBF119 and pBF120, respectively. A 3.8-kb *Bgl*I fragment from pBF120 was used as a probe in hybridization experiments against *Bgl*I-digested chromosomal DNA from *S. typhimurium* LT2 and JF2475 (*atbR*::Tn*10*). Differences seen in the hybridization pattern of the two strains indicated that the cloned insert contained *atbR*. A 5.5-kb *Cla*I/*Sal*I insert from pBF120 was subcloned into pMOB (TN1000 kit; Gold Biotechnologies, St. Louis, Mo.) and designated pBF215. A 2.4-kb *Pst*I fragment was removed from pBF215 by digestion with *Pst*I followed by religation of the plasmid. This smaller derivative, designated pBF215-1, was unable to complement the slow growth phenotype of JF2471. Restriction enzymes and T4 DNA ligase were purchased from Gibco BRL (Gaithersburg, Md.). Dideoxy-DNA sequencing of these plasmids was performed with the Sequenase version 2.0 DNA Sequencing kit (U.S. Biochemicals, Cleveland, Ohio). α-^{35}S-dATP for sequencing was purchased from NEN Life Sciences Products (Boston, Mass.). Analyses of nucleotide and amino acid sequences were performed with the Genetics Computer Group Package (version 7).

Two-dimensional SDS-PAGE. Two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of acid shock proteins was performed as described by Spector et al. (29) with cells labeled for 3 min with ^{35}S-Translabel (40 μCi/ml; ICN Pharmaceuticals, Inc., Irvine, Calif.). Approximately 5 μg of protein was analyzed for each sample. Basic and acidic proteins are situated to the left and right, respectively, of each autoradiograph. The first dimension was a pH 5 to 7 isoelectric focusing gel containing 1.6% (pH 5 to 7) and 0.4% (pH 3 to 10) ampholytes (Bio-Rad Laboratories, Melville, N.Y.), and the second dimension was an SDS–11.5% polyacrylamide gel. The results presented are representative of three independent experiments.

N-terminal sequence analysis of ASP29. ASP-29 was purified by using a modified two-dimensional gel protocol. A 0.5-liter volume of minimal E glucose medium was inoculated with a 1/100 dilution of an overnight culture of JF2690 (*rpoS*). At an OD_{600} of 0.40, a portion of the culture (3 ml) was removed for ^{35}S-Translabel labeling. The labeled and unlabeled cultures were combined and centrifuged, and the pellets were resuspended in 1× sonication buffer (100 mM Tris [pH 7.4], 5 mM $MgCl_2$). The sample was sonicated for five 30-s bursts. After sonication, the cellular debris was pelleted by low (12,100 × *g*- and high (380,000 × *g*)-speed spins. The supernatant was loaded onto a Centricon-100 (Amicon) concentrator with a 100,000-molecular-weight protein cutoff. The Centricon-100 concentrator was spun at 2,800 rpm. The filtrate was then loaded onto a Centricon-30 concentrator and centrifuged at 2,800 rpm. Retentates from both centricons were processed for two-dimensional gels. After electrophoresis, the polyacrylamide gels were transferred to polyvinylidene difluoride membranes (Bio-Rad). The blots containing proteins from the Centricon-30 retentate were sent to the WISTAR Institute (Philadelphia, Pa.) for amino-terminal protein sequencing of ASP29.

RESULTS

Mutations in *pgi* suppress the pH 3 acid-sensitive phenotype of *rpoS* mutations. The identity of a Tn*10* insertion mutation (originally called *atbR*::Tn*10*) that increased the acid tolerance of unadapted *S. typhimurium* LT2 in a minimal glucose medium (6) was determined to be *pgi*, the gene encoding phosphoglucoisomerase. GenBank comparison of the nucleotide sequence obtained from pBF215-1 (obtained as described in Materials and Methods) with primer T7 revealed that *atbR* was 81.1% homologous over 169 bp to *Escherichia coli pgi* (GenBank accession no. X15196; data not shown). The *pgi (atbR)*::Tn*10*dTc insertion strain also exhibited the *pgi* phenotype of defective glucose metabolism.

The LT2 strain originally used to analyze the *pgi (atbR)*::Tn*10* insertion produces very little σ^{38}, which in turn limits the ATR (16). Therefore, we suspected that the *pgi* mutation suppressed the acid-sensitive phenotype of *rpoS* mutants. To test this theory, the *pgi (atbR)*::Tn*10* insertion was analyzed for its effect on an *rpoS*$^+$ strain (UK1) and an *rpoS* null mutant. At pH 3, the *rpoS* mutant JF2690 proved to be extremely acid sensitive after 60 min of adaptation, while the *rpoS pgi (atbR)* mutant (JF2731) was very acid tolerant even when unadapted (Fig. 1). The effect of *pgi (atbR)* on the ATR of an *rpoS*$^+$ strain was to increase tolerance even in unadapted cells. Subsequently, a *pgi* mutation obtained from the *Salmonella* Genetic Stock Center was also shown to suppress the acid-sensitive phenotype of an *rpoS* mutant (Fig. 1).

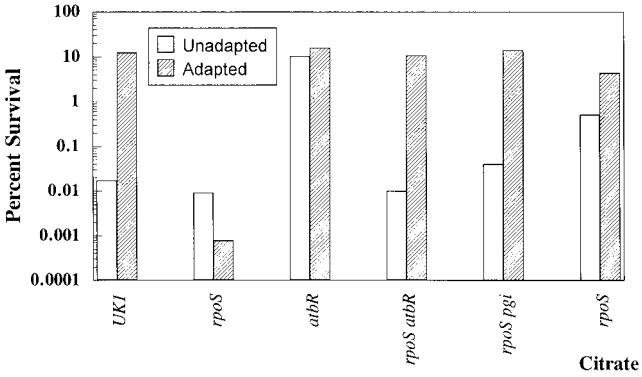

FIG. 1. The influence of *rpoS*, *pgi*, and growth on citrate on the ATR. Cells were grown to mid-log phase (approximately 2×10^8 cells per ml) in minimal EG medium (pH 7.7). When indicated, E medium with citrate was used as the carbon source. Unadapted cell cultures were adjusted to pH 3, and samples were taken for viable counts at time zero and 60 min and after acid challenge. Adapted cultures were adjusted from pH 7.7 to 4.4 for 1 h and then acid challenged for 1 h at pH 3. Wild-type UK1 (SF530), *rpoS*::*Ap* (JF2690), *atbR* (JF2733), *rpoS atbR* (JF2731), and *rpoS pgi* (JF2955) cells were assayed. The data are a representative sample of triplicate experiments.

Organic acids cause the pH 3 acid-sensitive phenotype of *rpoS* mutants. Mutants defective in *pgi* do not utilize glucose, but they can grow on the citrate present in the minimal EG medium used for ATR analyses. Growth on this nonfermentable carbon source and the lack of glucose fermentation end products (organic acids) may explain why *pgi* mutations suppress the pH 3 acid-sensitive phenotype of *rpoS* mutants. Figure 1 reveals that an *rpoS* mutant grown on citrate as the sole carbon source did, as predicted, exhibit an acid-tolerant phenotype similar to that of the *rpoS pgi* mutant. However, when it was grown on glucose, the *rpoS* mutant could not generate an ATR.

Further proof that fermentation end products were responsible for the acid-sensitive nature of *rpoS* mutants at pH 3 was obtained by replacing spent growth medium with fresh medium prior to pH 3 challenge. Figure 2 illustrates that when this experiment was performed, the removal of organic acids prior to acid challenge exposed an inducible acid tolerance in what previously was considered to be an acid-sensitive *rpoS* mutant (Fig. 2, bars 3 and 4). Adding 1 mM acetate to the fresh medium during challenge negated the fresh medium effect, proving that the acid-sensitive *rpoS* phenotype was due to weak acids present in growth medium (Fig. 2, bar 5). This amount of acetate is equivalent to what is produced by a mid-log culture grown on glucose. The addition of 1 mM acetate had no effect on the ability of *rpoS*$^+$ cells to generate an ATR (data not shown). The results indicate that there are at least two systems involved in the log-phase ATR, i.e., an RpoS-dependent system capable of handling organic acid stress and an RpoS-independent system useless against organic acids but effective against inorganic acid stress.

Effects of *fur* and *ada* on organic versus inorganic acid stress survival. Medium exchange experiments were used to analyze two other genes (*fur* and *ada*) that when mutated cause an acid-sensitive phenotype. Fur is a global regulatory protein

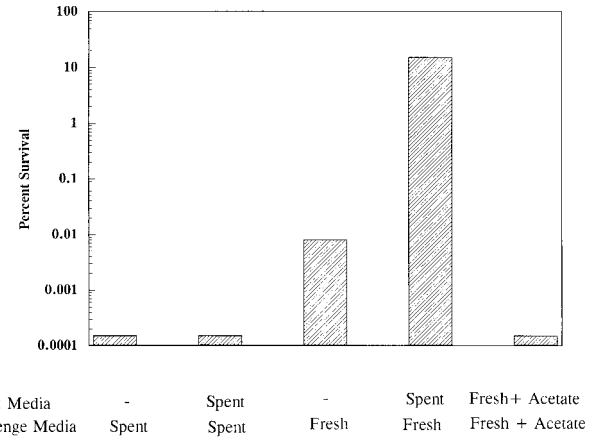

FIG. 2. RpoS-dependent systems are required for effective tolerance to organic acid stress. Cells (JF2690 [*rpoS*::*Ap*]) were grown and treated as indicated in the legend to Fig. 1. Spent, cells were grown, adapted, and challenged in the same EG medium; fresh, cells were removed from growth medium by centrifugation and resuspended in fresh EG medium already adjusted to pH 3 for challenge. In one experiment, the cells were resuspended in fresh medium made to contain 1 mM acetate (as acetic acid). The data are a representative sample of triplicate experiments.

that senses intracellular Fe^{2+} and appears to sense intracellular pH independently of its ability to sense iron (8, 14). The Ada protein is involved in the adaptive response of *E. coli* to alkylating agents (13, 25). We have found that *fur* (14) and *ada* (Fig. 3) mutants are acid sensitive in the standard ATR assay. Using the medium exchange strategy, we decided to examine whether these genes were involved with protection against organic or inorganic acid stresses. The results indicated that an *ada* mutant was acid sensitive in organic acids but acid tolerant in the absence of organic acid (Fig. 3). The results with the *fur* mutant suggest that Fur, too, is required more for protection against organic acid stress, although a minor adaptive response is still evident in spent medium (Fig. 3).

To this point, we have identified only genes which impact the tolerance exhibited to organic acid stress. Identification of a gene that influences inorganic but not organic acid tolerance would provide confirmation that organic and inorganic acid stresses are different and that distinct ATR systems exist for each. One such mutant was identified as described below.

ASP29 is PhoP. One of the ASP proteins targeted for identification was a 26-kDa protein designated ASP29 (4). Its location on two-dimensional SDS-polyacrylamide gels suggested that it was identical to a protein called spot 7 that is induced by growth in macrophages (1). Amino-terminal sequence analysis of ASP29 provided a 20 residue N-terminal sequence identical to the N terminus of the regulatory protein PhoP (data not shown). Figure 4 illustrates the position of ASP29 on a two-dimensional gel, its induction by acid shock (Fig. 4A versus B), and its absence in a *phoP* mutant (Fig. 4C and D). The results indicate that ASP29 is, indeed, PhoP.

PhoP- and PhoQ-dependent systems protect against inorganic but not organic acid stress. PhoP, along with PhoQ, forms a two-component system that is required for the pathogenesis of *S. typhimurium* (22, 30). An early study of acid tolerance suggested that PhoP was involved in the ATR (7). However, that study was unknowingly performed with an *rpoS* mutant background that allowed only transient induction of an ATR. To determine if PhoP was essential for acid tolerance in an *rpoS*$^+$ strain, we examined the effect of *phoP* mutations on the ATR of UK1. As shown in Fig. 5, a mutation in *phoP* (JF3203) had a small but reproducible effect on acid tolerance in an *rpoS*$^+$ cell, reducing it by approximately 10-fold, whether or not organic acids were present. Clearly, RpoS-dependent systems are more important than PhoP-dependent systems in protecting cells against media at pH 3 containing fermentation end products. However, when an *rpoS phoP* mutant was tested for inorganic acid tolerance, it was extremely acid sensitive compared to an *rpoS phoP*$^+$ cell (Fig. 5 [JF3439 versus JF2690]). The data provided in Fig. 5 also illustrate that both PhoP and PhoQ are required for this system of acid tolerance. Strains that were phenotypically PhoP$^+$ Q$^-$ (JF3302), PhoP$^-$ Q$^-$ (JF3439), and PhoP$^-$ Q$^+$ (JF3529) all proved to be acid sensitive. In addition, acid tolerance of the *phoP*::Tn*10* strain (PhoP$^-$ Q$^-$) was rescued by a plasmid expressing wild-type *phoPQ* (JF3530).

PhoPQ also controls the expression of another two-component regulatory system called PmrA (11, 24, 26). Some PhoPQ-dependent genes are regulated both by Mg^{2+} and acid (28). It has been suggested that PhoPQ controls the Mg^{2+}-dependent expression of these genes, while PmrA controls their induction by acidic pH (26). Consequently, we questioned whether the acid-sensitive nature of *phoPQ* mutants could be through PhoPQ control of *pmrA*. Examination of an *rpoS pmrA* mutant revealed a normal inducible tolerance to inorganic acid (data not shown). Thus, the acid-sensitive nature of *phoPQ* mutants is due to a different subset of PhoPQ-regulated genes.

PhoP is required for the low-pH induction of a subset of acid shock proteins. Shifting cells from a slightly alkaline pH (pH 7.7) to a moderately acidic pH (pH 4.4) results in the synthesis of 51 ASPs. The regulatory systems that sense environmental shifts in pH and control the expression of these ASPs are, of course, crucial to the ATR. The alternative sigma factor σ^{38} is required for the synthesis of 8 ASPs, while Fur is required for a separate set of 8 proteins. Since PhoP proved to be an ASP and *phoP* mutants are acid sensitive, it was reasonable to suspect that PhoPQ might also regulate another ASP subset. Figure 4 illustrates that a set of four ASPs, different from the previously mentioned sets, are missing from a *phoP* mutant. As shown above, ASP29 is PhoP, but the identities of the others, i.e., ASP6, -15, and -52, remain unknown. Thus, 20 of 51

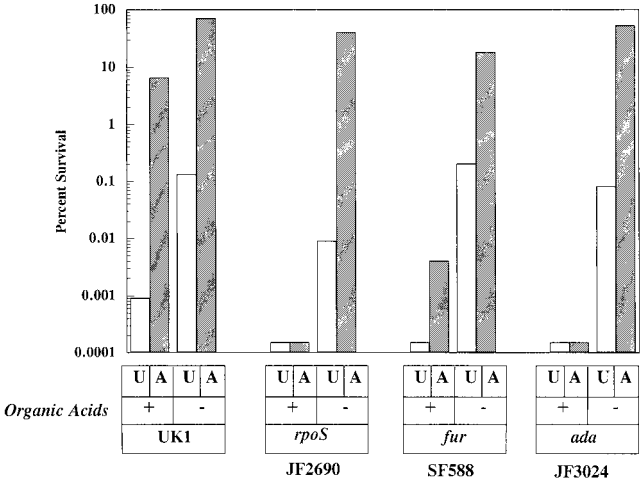

FIG. 3. Roles of *fur* and *ada* mutations on organic versus inorganic acid stress. Cells (UK1 [wild type], JF2690 [*rpoS*::*Ap*], SF588 [*fur-1*], and JF3024 [*ada*]) were grown and treated essentially as indicated in the legend to Fig. 1. Acid challenges (pH 3) were conducted in the presence (+ [spent medium]) or absence (− [fresh medium]) of organic acids. Results are representative of triplicate experiments. U, unadapted; A, adapted.

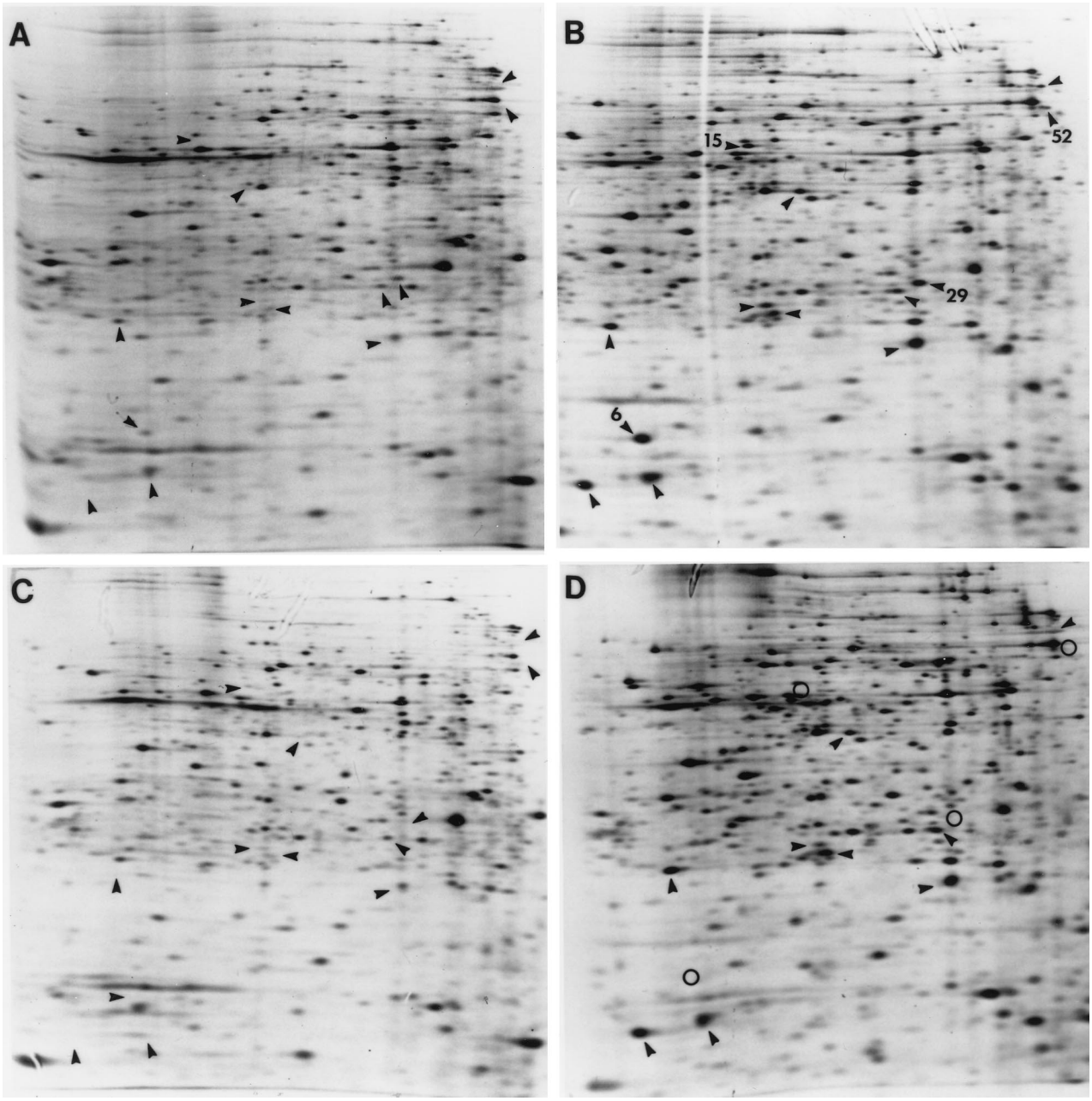

FIG. 4. PhoP controls the acid shock induction of four ASPs. Two-dimensional SDS-PAGE analysis of ASPs produced by JF2690 (*rpoS*) (A and B) and JF3204 (*rpoS phoP*) (C and D). Unadapted cells (A and C) and cells that were shocked with acid (pH 4.4) for 20 min (B and D) are shown. PhoP-dependent ASPs are indicated by numbers in panel B and by open circles in panel D. Several other ASPs are indicated by arrowheads.

identified ASPs fall into one of three different regulatory groups. It is interesting to note that the acid shock induction of these PhoP-dependent ASPs was not dependent on PmrA, the PhoPQ-controlled regulator suspected of sensing pH (data not shown).

The reliance of PhoPQ-dependent gene expression on acidic pH, magnesium, and PmrA. The current model for PhoPQ regulation holds that the membrane-bound sensor kinase PhoQ senses extracytoplasmic Mg^{2+}. Under high Mg^{2+} concentrations, PhoQ does not phosphorylate PhoP so that PhoP is not active as a DNA-binding protein (30). PhoQ is not generally considered a pH sensor. As noted above, it has been proposed that the pH control of a subset of PhoP-regulated genes is due to the PmrAB two-component system, which itself can be activated by PhoP (26). Thus, the pH and Mg^{2+} controls over PhoP-dependent genes are thought to be separate. Loss of PhoP should eliminate the Mg^{2+} control of these genes but not their regulation by pH. Conversely, a *pmrA* mutation should abrogate pH control but not Mg^{2+} regulation. Previous studies indicating that PhoP does not sense pH were done under fairly mild pH conditions (pH 6). We retested the model under more acidic conditions, achieved in a stepwise manner (pH 7.7 to 5.8 to 4.9) thought to mimic more closely what *Salmonella* might experience during pathogenesis. Using a *pagA-lacZ* fusion as a

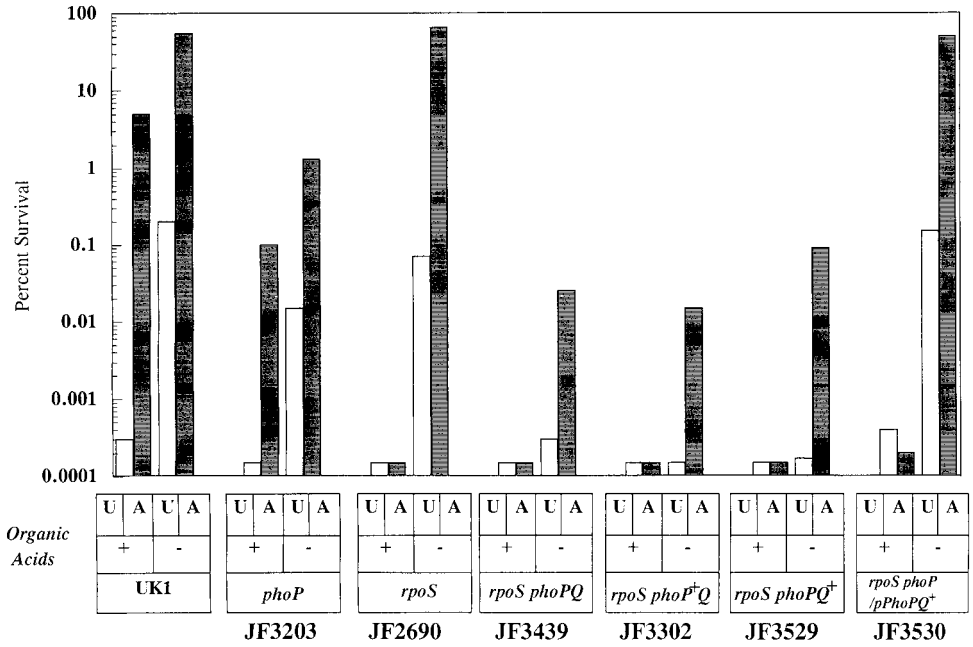

FIG. 5. The PhoPQ two-component signal transduction system is involved in inorganic acid tolerance. Cells (indicated below each set of bars) were grown and treated as indicated in the legends to Fig. 1 and 3. U, unadapted; A, adapted.

reporter for PhoP activity, we found that *pagA* was induced in a PhoP-dependent fashion by acidic pH, even in the presence of repressing concentrations of Mg^{2+} (Fig. 6A). Since PhoP can increase *pmrAB* transcription but is not essential for *pmrAB* expression, the results were contrary to what was expected, which was that *phoP* should have had little effect on acidic pH control of this target gene. When a *pmrA* mutation was tested, it too proved to be essential for the acid induction of *pagA-lacZ* at high Mg^{2+} concentrations as well as for the low Mg^{2+} response.

Because of these findings, other PhoP-dependent genes were analyzed for the effects of *phoP* and *pmrA* mutations on pH and Mg^{2+} regulation. In contrast to *pagA*, a *phoP* mutation did not eliminate the pH 4.9 acid induction of *psiD*, now known to be *pmrC* (10a), but did severely reduce it (Fig. 6B). This result is similar to a previous report which examined the expression of the PhoP-dependent gene *pbgP* under less acidic conditions (26). However, unlike *pbgP*, PmrA was important for both the Mg^{2+} and pH control of *psiD* (*pmrC*) (Fig. 6B).

The *mgtB* gene, encoding a magnesium transport system, is known to be regulated by Mg^{2+} and is PhoPQ dependent (28). Under the conditions shown in Fig. 7, induction of the *mgtB-lacZ* fusion was found to require both low Mg^{2+} and an acidic pH shift. Low magnesium levels alone did not induce this gene in the time frame of this experiment. Consequently, *mgtB* can also be considered an acid-inducible gene. As shown in Fig. 7, the acid and low-Mg^{2+} response of *mgtB* proved to be PhoP dependent but, unlike that of *psiD* (*pmrC*), was PmrA independent. One possible explanation for these results is that PhoQ senses pH in addition to Mg^{2+} and under acidic conditions will phosphorylate PhoP even in the presence of excess Mg^{2+}.

Since PhoPQ autoregulates its own expression (27), we decided to test whether *phoPQ* expression was itself induced by acid, as the two-dimensional gels would suggest. In order to conduct this study, we constructed a strain containing a chromosomal *phoP*::Mu*d*J insertion and a plasmid in which *phoPQ* was placed under the control of the *lac* promoter (27). The results shown in Fig. 8 confirmed that when PhoPQ is produced following isopropyl-β-D-thiogalactopyranoside (IPTG) induction, the expression of *phoP-lacZ* is induced under low Mg^{2+} conditions. However, under high Mg^{2+} conditions, *phoP* was also induced by acidic pH shifts lasting no more than 1 h. The results indicate that PhoQ senses H^+ in addition to Mg^{2+} concentrations.

DISCUSSION

The results presented confirm that acid stress is a combination of low pH (inorganic acid) and the concentration of organic weak acids present in the microbial environment. In addition, the data illustrate that there are distinct tolerance systems for each type of acid stress. The RpoS-dependent system is clearly required for protection against organic acid stress, whereas PhoPQ is used mostly against inorganic acid stress. With respect to inorganic acid stress, the data suggest that the PhoPQ and RpoS systems are somewhat redundant, although both are required for optimum acid tolerance. The minor decrease in organic acid tolerance seen in the *phoP* mutant probably reflects the fact that part of how these acids work is by lowering internal pH, much as inorganic acid does. Alternatively, the loss of PhoP may slightly increase permeability toward organic acids, insofar as some PhoP-regulated genes have been shown to affect the cell surface (12). The results also indicate that the iron regulatory protein Fur and the adaptive response protein Ada are required for organic but not inorganic acid tolerance. The manner in which Fur protein aids in acid tolerance is not clear. It does appear that Fur can sense acid and iron levels independently and that Fur regulates the expression of several acid shock proteins in an iron-independent manner (14). It is predicted that one or more of the Fur-dependent ASPs is involved in this protection.

The Ada protein is involved in the adaptive response of *E. coli* to alkylating agents in two ways, i.e., as an enzyme by

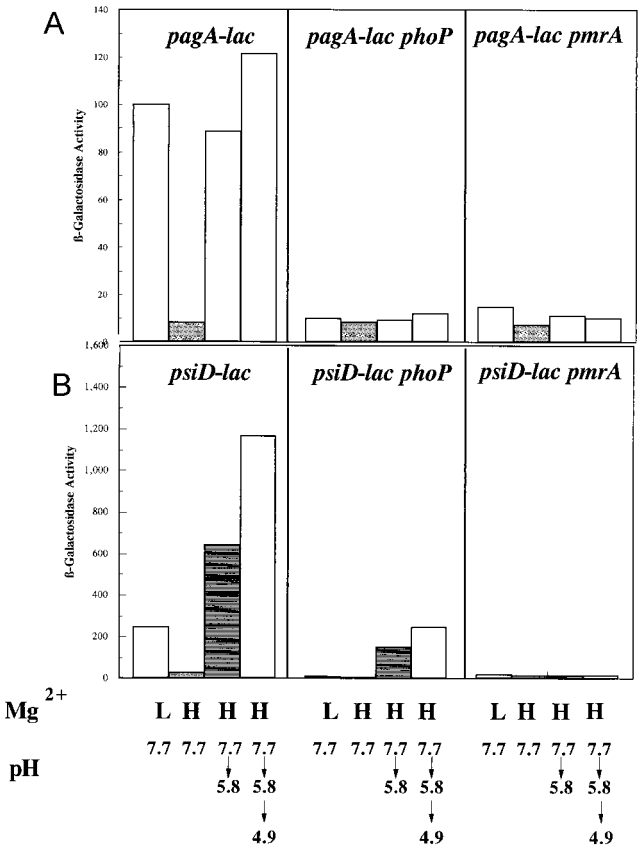

FIG. 6. The effects of Mg^{2+}, acid, *phoP*, and *pmrA* on the expression of *pagA* and *psiD* (*pmrC*). Cells were grown in N medium (pH 7.7) supplemented with 1% vitamin-free Casamino Acids to mid-log phase (2×10^8 cells/ml) in either 10 mM (H) or 10 μM (L) $MgSO_4$. As indicated below each bar, the cells were adapted at pH 5.8 for 60 min or underwent a stepwise adaptation at pH 5.8 for 30 min followed by 30 min at pH 4.9. The results are representative of triplicate experiments. (A) *pagA-lac* (JF3303), *pagA-lac phoP* (JF3531), and *pagA-lac pmrA* (JF3547) cells; (B) *psiD-lac* (JF3550), *psiD-lac phoP* (JF3554), and *psiD-lac pmrA* (JF3561) cells.

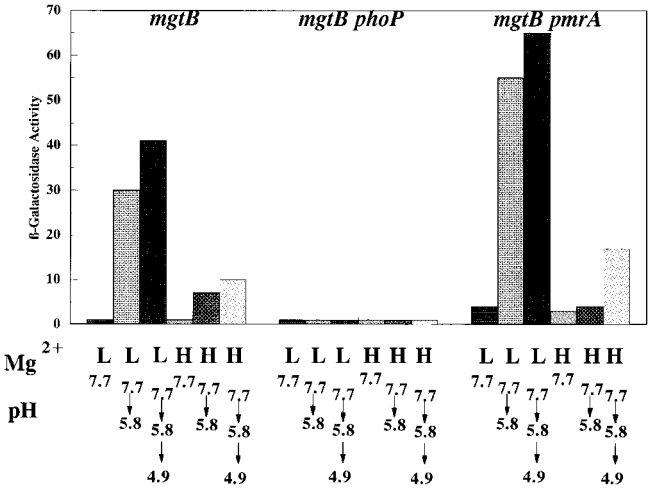

FIG. 7. The effects of Mg^{2+}, acid, *phoP*, and *pmrA* on the expression of *mgtB*. Cells were grown in N medium (pH 7.7) supplemented with 1% vitamin-free Casamino Acids to mid-log phase (2×10^8 cells/ml) in either 10 mM (H) or 10 μM (L) $MgSO_4$. As indicated below each bar, the cells were adapted at pH 5.8 for 60 min or underwent a stepwise adaptation at pH 5.8 for 30 min followed by 30 min at pH 4.9. The results are representative of triplicate experiments. Results for *mgtB-lac* (JF3274), *mgtB-lac phoP* (JF3552), and *mgtB-lac pmrA* (JF3551) cells are shown.

removing methyl groups from O^6 methyl guanine residues and as a potent transcriptional activator of its own gene and several others involved in the response (25). *S. typhimurium* appears to produce a defective Ada protein that has lost the ability to induce its own expression but still can activate other target genes involved in DNA repair (13, 33, 34). The fact that *ada* expression falls under RpoS control in *E. coli* suggests that one of the reasons that *rpoS* mutants in *Salmonella* are acid sensitive is because they cannot induce *ada*. Why an *ada* mutant would be sensitive to organic acids is not intuitively obvious, since organic acids probably do not cause methylation damage to DNA. However, low pH can facilitate the inappropriate methylation of DNA by *S*-adenosyl methionine (18). Acid shock-stimulated accumulation of RpoS may lead to the induction of Ada in *Salmonella*. Upon removing methyl groups from O^6 methyl guanine residues, Ada may then induce other components of the Ada regulon (other than *ada* itself). The products of these other genes may deal with damage caused by organic acids. Confirmation of this model awaits more detailed analysis of *ada* expression in *S. typhimurium.*

Consistent with a role for PhoP in acid tolerance was the finding that PhoP is itself an acid shock protein that is required for the expression of several other ASPs. The role that acidic pH plays in the expression of PhoP-dependent genes is complex. While the acid-induced expression of some PhoP-dependent genes requires PmrA [e.g., *pbgP* and *psiD* (*pmrC*)], acid-induced expression of others is PmrA independent (e.g., *mgtB* and *phoP*) or requires both PhoP and PmrA (e.g., *pagA*). The observation that the transcription of *phoPQ* is induced by acid shock in a PhoPQ-dependent manner certainly suggests that PhoQ can sense pH; thus, both PhoPQ and PmrAB appear to sense acid stress. It is not apparent, at this point, whether PhoQ senses pH independently of Mg^{2+} or whether pH affects the interaction between Mg^{2+} and the Mg^{2+}-sensing site on PhoQ. If the latter is the case, then acidic conditions could be translated by the cell as low Mg^{2+}, possibly triggering an influx of this ion. Increasing intracellular magnesium concentrations

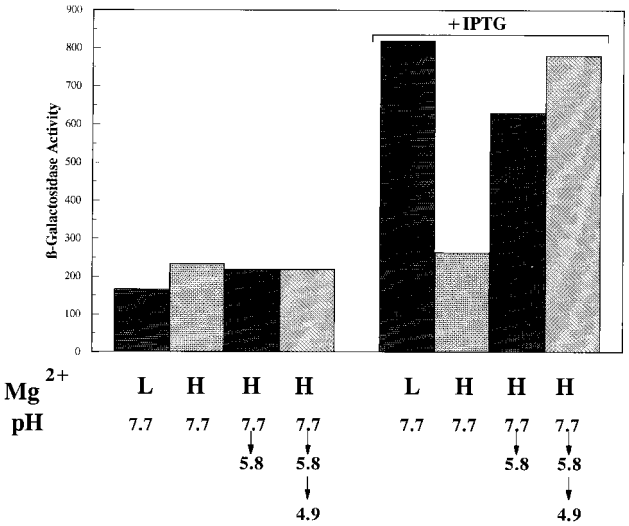

FIG. 8. The effects of Mg^{2+} and acid on the expression of *phoP*. Cells were grown as described in the legends to Fig. 6 and 7 either in the presence or in the absence of 0.2 mM IPTG to induce the plasmid-borne *phoPQ* operon.

through the acid induction of Mg^{2+} transport via PhoP might serve a protective function under acidic conditions. The observation that *mgtB* mutations do not confer an acid-sensitive phenotype (data not shown) may simply reflect a redundancy in Mg transport systems or that Mg^{2+} transport is not an essential component of acid tolerance.

The induction of *phoPQ* by low pH also has important implications in terms of virulence. Mutants defective in this system are avirulent. Since the environmental condition implicated in the control of PhoP-regulated genes is Mg^{2+}, it has been reasoned that in vivo conditions in which Mg^{2+} levels are low are important in initiating the activation of this system (28, 30). However, the present studies suggest that even when Mg^{2+} concentrations in vivo are high, low pH environments can also trigger activation of PhoP. Therefore, the environmental cue within the macrophage that triggers the PhoPQ cascade remains undetermined but probably reflects a combination of Mg^{2+} and H^+ ion concentrations. The following is a working model that attempts to integrate the available data concerning how Mg^{2+}, H^+, PhoPQ, and PmrAB might cooperatively regulate gene expression. The model predicts at least two basic sets of PhoP-regulated genes. In this model, one set of PhoP-regulated genes needs only PhoP phosphate (PhoP-P) for induction, while the second set requires both PhoP-P and PmrA-P. Individual members of the first set will vary in their responses to the different levels of PhoP-P achieved through PhoQ sensing different levels of Mg^{2+}. Higher or lower H^+ concentrations will influence the level of Mg^{2+} that is required to produce a given level of PhoP-P. Thus, genes that require little PhoP-P for induction could respond to either low pH or low Mg^{2+}. Genes that require high PhoP-P will be induced under extremely low Mg^{2+} concentrations at neutral pH or under more moderate Mg^{2+} levels in an acidic environment.

The second set of PhoP-regulated genes could be regulated by a PhoP-PmrA cascade that amplifies the pH response. In this case, acidic environments (even under high Mg^{2+} conditions) would generate enough PhoP-P to induce the PmrA system (represented by *psiD* [*pmrC*] in Fig. 6). The PmrA system subsequently undergoes autoinduction, which would serve to amplify the pH signal. The resultant high level of PmrA-P generated either through the PmrB sensor kinase sensing pH directly or through some other signal will then fully induce the PmrA subset of PhoP-regulated genes.

The data presented underscore the complexity of acid tolerance and pH sensing systems in *S. typhimurium*. The number of regulatory systems involved with acid survival now numbers four, including Fur, σ^{38}, PhoPQ, and Ada. Clearly, several more regulatory systems are involved, since there are at least 20 or so ASPs for which regulatory systems have not been revealed. The elaborate response raised against acid stress suggests that many different facets of cellular physiology are impacted by low pH and that an elaborate, interwoven regulatory network is critical for survival.

ACKNOWLEDGMENTS

We thank M. Maguire, S. Miller, E. Groisman, and M. Yamada for their generous gifts of various strains and plasmids and M. Moreno and J.-C. Giard for critically reading the manuscript. Various discussions with M. Maguire and M. Spector were especially helpful and gratefully acknowledged.

This work was supported by an award (GM48017) from the National Institutes of Health.

REFERENCES

1. **Abshire, K. Z., and F. C. Neidhardt.** 1993. Analysis of proteins synthesized by *Salmonella typhimurium* during growth within a host macrophage. J. Bacteriol. **175:**3734–3743.
2. **Bearson, S., B. Bearson, and J. W. Foster.** 1997. Acid stress responses in enterobacteria. FEMS Microbiol. Lett. **147:**173–180.
3. **Bearson, S. M. D., W. H. Benjamin, Jr., W. E. Swords, and J. W. Foster.** 1996. Acid shock induction of RpoS is mediated by the mouse virulence gene *mviA* of *Salmonella typhimurium*. J. Bacteriol. **178:**2572–2579.
4. **Foster, J. W.** 1991. *Salmonella* acid shock proteins are required for the adaptive acid tolerance response. J. Bacteriol. **173:**6896–6902.
5. **Foster, J. W.** 1995. Low pH adaptation and the acid tolerance response of *Salmonella typhimurium*. Crit. Rev. Microbiol. **21:**215–237.
6. **Foster, J. W., and B. Bearson.** 1994. Acid sensitive mutants of *Salmonella typhimurium* identified through a dinitrophenol selection strategy. J. Bacteriol. **176:**2596–2602.
7. **Foster, J. W., and H. K. Hall.** 1990. Adaptive acidification tolerance response of *Salmonella typhimurium*. J. Bacteriol. **172:**771–778.
8. **Foster, J. W., and H. K. Hall.** 1992. The effect of *Salmonella typhimurium* ferric-uptake regulator (*fur*) mutations on iron and pH-regulated protein synthesis. J. Bacteriol. **174:**4317–4323.
9. **Foster, J. W., and M. P. Spector.** 1986. Phosphate-starvation regulon of *Salmonella typhimurium*. J. Bacteriol. **166:**666–669.
10. **Foster, J. W., and M. Spector.** 1995. How *Salmonella* survives against the odds. Annu. Rev. Microbiol. **49:**145–174.

10a. **Groisman, E.** Personal communication.

11. **Gunn, J. S., and S. I. Miller.** 1996. PhoP-PhoQ activates transcription of *pmrAB*, encoding a two-component regulatory system involved in *Salmonella typhimurium* antimicrobial peptide resistance. J. Bacteriol. **178:**6857–6864.
12. **Guo, L., K. B. Lim, J. S. Gunn, B. Bainbridge, R. P. Darveau, M. Hackett, and S. I. Miller.** 1997. Regulation of lipid a modifications by *Salmonella typhimurium* virulence genes *phoP-phoQ*. Science **276:**250–253.
13. **Hakura, A., K. Morimoto, T. Sofuni, and T. Nohmi.** 1991. Cloning characterization of the *Salmonella typhimurium ada* gene, which encodes O^6-methylguanine-DNA methyltransferase. J. Bacteriol. **173:**3663–3672.
14. **Hall, H. K., and J. W. Foster.** 1996. The role of Fur in the acid tolerance response of *Salmonella typhimurium* is physiologically and genetically separable from its role in iron acquisition. J. Bacteriol. **178:**5683–5691.
15. **Hengge-Aronis, R.** 1996. Back to log phase: σ^s as a global regulator in the osmotic control of gene expression in *Escherichia coli*. Mol. Microbiol. **21:**887–893.
16. **Lee, I. S., J. Lin, H. K. Hall, B. Bearson, and J. W. Foster.** 1995. The stationary-phase sigma factor σ^s (RpoS) is required for a sustained acid tolerance response in virulent *Salmonella typhimurium*. Mol. Microbiol. **17:**155–167.
17. **Lee, I. S., J. L. Slonczewski, and J. W. Foster.** 1994. A low-pH inducible stationary phase acid tolerance response in *Salmonella typhimurium*. J. Bacteriol. **176:**1422–1426.
18. **Lindahl, T., and B. Nyberg.** 1972. Rate of depurination of native deoxyribonucleic acid. J. Biochem. **11:**3610–3617.
19. **Loewen, P. C., and R. Hengge-Aronis.** 1994. The role of the sigma factor σ^s (KatF) in bacterial global regulation. Annu. Rev. Microbiol. **48:**53–80.
20. **Maloy, S. R.** 1990. Experimental techniques in bacterial genetics. Jones and Bartlett Publishers, Boston, Mass.
21. **Miller, J. H.** 1992. A short course in bacterial genetics. A laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
22. **Miller, S. I., A. M. Kukral, and J. J. Mekalanos.** 1989. A two-component regulatory system (*phoPphoQ*) controls *Salmonella typhimurium* virulence. Proc. Natl. Acad. Sci. USA **86:**5054–5058.
23. **Nelson, D. L., and E. P. Kennedy.** 1972. Transport of magnesium by a repressible and a nonrepressible system in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **69:**1091–1093.
24. **Roland, K. L., L. E. Martin, C. R. Esther, and J. K. Spitznagel.** 1993. Spontaneous *pmrA* mutants of *Salmonella typhimurium* LT2 define a new two-component regulatory system with a possible role in virulence. J. Bacteriol. **175:**4154–4164.
25. **Saget, B. M., and G. C. Walker.** 1994. The Ada protein acts as both a positive and a negative modulator of *Escherichia coli*'s response to methylating agents. Proc. Natl. Acad. Sci. USA **91:**9730–9734.
26. **Soncini, F. C., and E. A. Groisman.** 1996. Two-component regulatory systems can interact to process multiple environmental signals. J. Bacteriol. **178:**6796–6801.
27. **Soncini, F. C., E. G. Véscovi, and E. A. Groisman.** 1995. Transcriptional autoregulation of the *Salmonella typhimurium phoPQ* operon. J. Bacteriol. **177:**4364–4371.
28. **Soncini, F. C., E. G. Véscovi, F. Solomon, and E. A. Groisman.** 1996. Molecular basis of the magnesium deprivation response in *Salmonella typhimurium*: identification of PhoP-regulated genes. J. Bacteriol. **178:**5092–5099.
29. **Spector, M. P., Z. Aliabadi, T. Gonzalez, and J. W. Foster.** 1986. Global control in *Salmonella typhimurium*: two-dimensional electrophoretic analysis of starvation-, anaerobiosis-, and heat shock-inducible proteins. J. Bacteriol. **168:**420–424.
30. **Véscovi, E. G., F. Soncini, and E. A. Groisman.** 1996. Mg^{2+} as an extracel-

lular signal: environmental regulation of salmonella virulence. Cell **84:**165–174.
31. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218:**97–106.
32. **Waldburger, C. D., and R. T. Sauer.** 1996. Signal detection by the PhoQ sensor-transmitter. J. Biol. Chem. **271:**26630–26636.
33. **Yamada, M., A. Hakura, T. Sofuni, and T. Nohmi.** 1993. New methods for gene disruption in *Salmonella typhimurium*: construction of an *ada*-deletion derivative of *Salmonella typhimurium* TA 1535. J. Bacteriol. **175:**5539–5547.
34. **Yamada, M., B. Sedgwick, T. Sofuni, and T. Nohmi.** 1995. Construction and characterization of mutants of *Salmonella typhimurium* deficient in DNA repair of O^6-methylguanine. J. Bacteriol. **177:**1511–1519.

ERRATA

Promoter Selectivity of the *Bradyrhizobium japonicum* RpoH Transcription Factors In Vivo and In Vitro

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Volume 180, no. 9, p. 2398: Fig. 6 should appear as shown below.

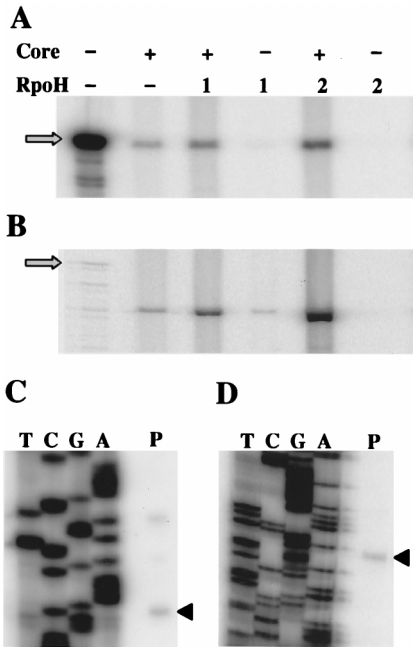

FIG. 6.

A Low pH-Inducible, PhoPQ-Dependent Acid Tolerance Response Protects *Salmonella typhimurium* against Inorganic Acid Stress

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Volume 180, no. 9, p. 2409, abstract, line 9: "tolerance to organic acid stress" should read "tolerance to inorganic acid stress." Page 2414, column 1, line 18: "However, unlike *pbgP*" should read "Like *pbgP*."