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Adaptive Acidification Tolerance Response of *Salmonella typhimurium*

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***Salmonella typhimurium* can encounter a wide variety of environments during its life cycle. One component of the environment which will fluctuate widely is pH. In nature, *S. typhimurium* can experience and survive dramatic acid stresses that occur in diverse ecological niches ranging from pond water to phagolysosomes. However, in vitro the organism is very sensitive to acid. To provide an explanation for how this organism survives acid in natural environments, the adaptive ability of *S. typhimurium* to become acid tolerant was tested. Logarithmically grown cells (pH 7.6) shifted to mild acid (pH 5.8) for one doubling as an adaptive procedure were 100 to 1,000 times more resistant to subsequent strong acid challenge (pH 3.3) than were unadapted cells shifted directly from pH 7.6 to 3.3. This acidification tolerance response required protein synthesis and appears to be a specific defense mechanism for acid. No cross protection was noted for hydrogen peroxide, SOS, or heat shock. Two-dimensional polyacrylamide gel electrophoretic analysis of acid-regulated polypeptides revealed 18 proteins with altered expression, 6 of which were repressed while 12 were induced by mild acid shifts. An avirulent *phoP* mutant was 1,000-fold more sensitive to acid than its virulent *phoP*$^+$ parent, suggesting a correlation between acid tolerance and virulence. The Mg^{2+}-dependent proton-translocating ATPase was also found to play an important role in acid tolerance. Mutants (*unc*) lacking this activity were unable to mount an acid tolerance response and were extremely acid sensitive. In contrast to these acid-sensitive mutants, a constitutively acid-tolerant mutant (*atr*) was isolated from wild-type LT2 after prolonged acid exposure. This mutant overexpressed several acidification tolerance response polypeptides. The data presented reveal an important acidification defense modulon with broad significance toward survival in biologically hostile environments.**

Salmonella species serve as etiologic agents for a variety of diseases ranging from gastroenteritis to enteric fever. During the life cycle of the normally neutrophilic *Salmonella typhimurium*, exposures to acidic environments are common (20). These encounters can include pond water, stomach acid, and colon contents. But perhaps the most clinically relevant acid exposure occurs after invasion of the intestinal mucosa. During pathogenesis, *S. typhimurium* invade the intestinal epithelial cells, entering phagosomes in which it can continue to grow (7). Penetration of the epithelial cells by these bacteria appears to be a receptor-mediated process. The normal consequence of receptor-mediated endocytosis includes the internal lowering of endosome (phagosome) pH to between 5 and 6 by vacuolar ATPases (14). The organisms eventually proceed from the apical to the basal side of epithelial cells (transcytosis), where they subsequently exit (15). At this point, macrophages will phagocytize *Salmonella* cells which have successfully penetrated the epithelial barrier. Shortly after entering the macrophage, the *Salmonella* cell-containing phagosome will fuse with a lysosome, forming a phagolysosome. It has been reported that *S. typhimurium* can survive within macrophage phagolysosomes (3, 6). The metabolic burst that occurs in the phagolysosome not only produces H_2O_2, superoxide, and other antimicrobial factors, but the acid produced by various metabolic activities lowers the pH of the fused phagolysosome to 3 or 4. This pH alone is normally bactericidal to *S. typhimurium* in vitro, yet the cells manage to survive in this harsh in vivo environment. Clearly, the ability to withstand acidic environments is an important factor in the virulence of *S. typhimurium*. However, virtually nothing is known regarding the genetic determinance of this ability (20). In light of the fact that *S. typhimurium* survives intermittent acid conditions, one would expect this organism to possess an adaptive acidification tolerance mechanism(s). Before this study, no such phenomenon has been reported in the literature, although the ability of *S. typhimurium* to respond adaptively to other stresses is well documented (2, 4, 18, 19).

Several reports of external acid-regulated genes in *S. typhimurium* and *Escherichia coli* have been made in recent years (1, 10, 16). Several examples of *lacZ* operon fusions to pH-regulated genes have shown that maximal induction for each gene occurs in the pH range of 5.5 to 6.0. This pH is similar to that found in nonprofessional phagosomes as noted above. Consequently, a hypothesis was formulated in which *S. typhimurium* grown at pH 5.5 to 6.0 may induce an acid tolerance response (ATR) which ultimately protects the cells from the more severe acid conditions that may be encountered in macrophage phagolysosomes (pH 3.0 to 4.0). This report presents evidence supporting this theory.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains used in this study were all derivatives of *S. typhimurium* LT2 and are listed in Table 1. Minimal E medium supplemented with 0.4% glucose was used because in addition to phosphate buffer, the medium contains a significant amount of citrate which will buffer in the lower pH ranges (21). The adjusted pH values did not change more than 0.05 units during the experiments.

Protocol for observing the ATR. A fresh, overnight culture of cells grown in minimal E glucose medium (pH 7.6) was

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TABLE 1. Bacterial strains used

S. typhimurium strain	Genotype	Source or reference
JF1218	*recA1 rpsL* Δ*srl*	
JF1506	*aniG1072*::Mu dJ Δ*earA305*	1
JF1819	*atr-1*	Spontaneous
JF1842	*ompR*::Tn*5*	SF241 × LT2
JF1892	*unc-102*::Tn*10 atr-1*	SF342 × JF1819
JF1898	*phoP*::Tn*10*	5
JF1912	*phoP*::Tn*10 atr-1*	JF1898 × JF1819
SF236	Δ*oxyR zii-614*::Tn*10*	B. Ames
SF241	Δ*oppBC250 leu-1151*::Tn*10* *ompR*::Tn*5*	C. Higgins
SF342	*unc-102*::Tn*10*	G. Ames

used to inoculate parallel E glucose cultures (pH 7.6) to an initial cell density of 10^6 cells per ml. Both cultures were incubated at 37°C until attaining concentrations of 10^8 viable cells per ml. The culture destined for adaptation was quickly adjusted to pH 5.8 ± 0.1 with HCl. While the pH was adjusted, the culture was maintained at 37°C in a heating block. The time required for pH adjustment was under 1 min. The other culture remained at pH 7.6. After pH adjustment, both cultures were grown to 2×10^8 cells per ml (one doubling) and immediately adjusted to pH 3.3 ± 0.1. This time point was designated t_0. Viable counts were determined at timed intervals by dilution to cold E buffer and plating on LB agar. All experiments involved a minimum of three time points, t_0, 45 min (t_{45}), and 90 min (t_{90}).

O'Farrell two-dimensional analysis of ATR proteins. Cells to be used for two-dimensional electrophoretic analysis of ATR polypeptides were grown under the same conditions outlined above to observe ATR. Cells were labeled with [^{35}S]methionine for 10 min after 1 h of pH 5.8 adaptation. Unadapted cells were also labeled for 10 min at a cell density equivalent to that used for adapted cells. A 1.5-ml sample of labeled cells was pelleted and suspended in 13 μl of a sodium dodecyl sulfate (SDS) lysing solution, boiled, and run in a pH 5 to 7 isoelectric focusing system followed by 11.5% SDS-polyacrylamide gel electrophoresis (PAGE) as described earlier (18). Comparisons were made between adapted and unadapted samples with equivalent protein (5 to 15 μg) and equivalent disintegrations per minute (1×10^6 to 3×10^6). The coordinates given are those provided in a previously published standard two-dimensional profile of *S. typhimurium* polypeptides (18).

RESULTS

Discovery of adaptive ATR. We began studying the effects of acidification as a result of our work on environmental stimulons in *S. typhimurium* (1, 2, 18, 19). Two genes (*aniG* and *hyd*) identified in our laboratory as anaerobiosis inducible were also regulated by the pH of the medium, with maximal expression of both observed at pH 5.8 to 6.0. Exogenous coinduction of the *aniG* locus involves an absolute requirement for both acid and mannose. Similarly, the hydrogenase locus (*hyd*) requires acid, formate, and anaerobiosis as coinducers (1). We also found that pH 5.8 is a mild stress condition that *S. typhimurium* tolerates well. The growth rate of *S. typhimurium* in E glucose medium was slightly slower at pH 5.8 than at pH 7.4. Consequently, it seemed reasonable to suspect that if *S. typhimurium* possesses an inducible ATR, induction would occur at the pH 5.8 to 6.0 level. With this information, we designed the experiment presented in Fig. 1. Cells were grown in duplicate to a density of 10^8 cells per ml at pH 7.4 to 7.6. At that point, the pH of one member of the pair was adjusted to pH 5.8. Both sets were allowed to grow to 2×10^8 cells per ml, at which point the H^+ ion concentration was adjusted to a lethal pH 3.3 level. Minimal E medium was used since the citrate ingredient would help buffer the culture at the lower pH values. At timed intervals after adjustment to lethal acid conditions, viable counts were determined by dilution in E glucose and plating on LB agar. The results (Fig. 1) indeed showed that the adapted cells are protected dramatically from acid death relative to unadapted cells by several orders of magnitude. This phenomenon is referred to as the ATR. We have also observed this phenomenon using complex as well as E medium and with *E. coli* (data not shown).

There are two possible explanations for the development

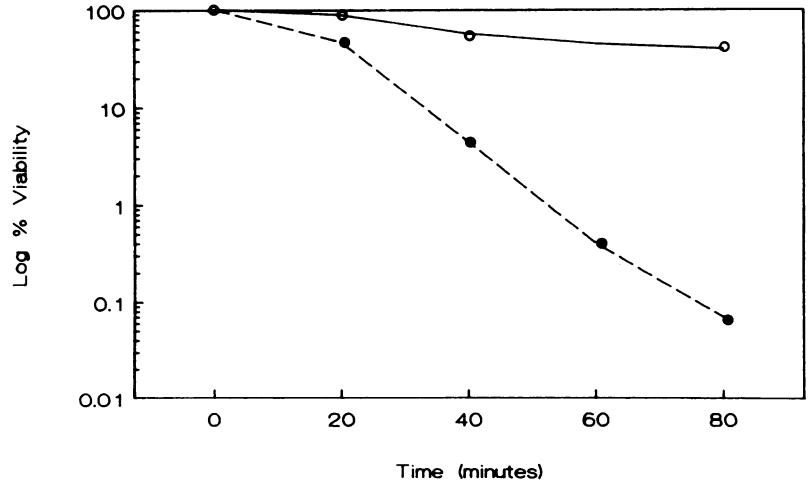

FIG. 1. ATR. Cells grown to 10^8 cells per ml in pH 7.6 minimal glucose medium were adapted by adjusting the medium pH to 5.8 (○). After one doubling, the cells were challenged by readjusting the pH to 3.3 (t_0). Unadapted cultures (●) remained at pH 7.6 until achieving a cell density of 2×10^8 cells per ml and then were directly challenged at pH 3.3 (t_0). Viable counts were determined at timed intervals. The results are expressed in terms of log percent survival.

TABLE 2. Effect of chloramphenicol on adaptation (ATR)

Condition	Chloramphenicol[a]	% Survival[b]
Unadapted	−	0.25
Unadapted	+	<0.01
Adapted[c]	−	26.0
Adapted	+	<0.01

[a] Chloramphenicol was added to a concentration of 40 μg/ml at the point of adaptation.
[b] Percent survival was determined 60 min after pH 3.2 challenge.
[c] Cells were adapted at pH 5.7.

of acid tolerance. Growth in pH 5.8 could result in a physiological adjustment of the cell, making it less susceptible to the lethal effects of acid. Alternatively, acid tolerance could be a molecular process requiring genetic regulation and the synthesis of new proteins. To determine whether protein synthesis was required for adaptive acid tolerance, we added chloramphenicol (40 μg/ml) 15 min before adjusting the culture to the adaptive pH (5.8). The adapted culture without chloramphenicol showed 26% survival after 60 min of challenge at the lethal acid pH. In contrast, the addition of chloramphenicol rendered the culture very susceptible to acid-associated death (Table 2). This experiment was repeated with erythromycin and tetracycline with identical results (data not shown). The results indicate that protein synthesis is required for the ATR system to operate.

The information contained in Table 3 shows that ATR protection increased when the pH used as the adaptive pH decreased from 7.0 to 5.7. This suggested that varying levels of induction probably occur as the cell begins to sense changes in external pH. The induction of ATR did not appear to be an all-or-none phenomenon since measured increases in the external acid stress signal resulted in a graded response in ATR. The most dramatic increase in acid protection occurred at pH 6.0 or below.

Two-dimensional polyacrylamide gel analysis of ATR proteins. Extracts of adapted and unadapted cells were subjected to O'Farrell two-dimensional gel analysis to define the polypeptide components of the ATR system. Figure 2 presents the results of these studies, which are also tabulated in Table 4. The levels of 12 proteins were observed to increase while 6 decreased during the ATR protocol. One of the inducible proteins (coordinates 110 × 78) was identified as the *ompC* product by comigration and the observed loss of the protein in an *ompC* mutant. The level of induction observed for the *Salmonella ompC* appears significantly greater than that noted for the *E. coli ompC* after acid induction (10). A second protein was identified as OmpF by criteria similar to that used for OmpC. In contrast to OmpC, OmpF is repressed by acid. It is interesting that the inverse regulation of these two outer membrane porin genes by acid pH is analogous to their control by medium osmolarity.

TABLE 3. ATR of *S. typhimurium*[a]

Adaptation pH	Challenge pH	% Survival[b]
7.0	3.27	0.25
6.3	3.22	1.6
6.1	3.16	2.8
5.9	3.23	10.6
5.7	3.20	16.0

[a] Cells were grown in E minimal medium to an optical density at 600 nm of 0.2, adjusted to the adaptation pH, and then allowed to grow to an optical density of 0.4, at which point the pH was adjusted to 3.2.
[b] Survival based on viable counts at 45 min after challenge pH.

Our previous two-dimensional PAGE analysis of *Salmonella* environmental stimulons also provided an opportunity to observe whether any of the ATR polypeptides were members of other global response systems (18). Comparisons revealed that five of the ATR proteins did, in fact, respond to other stresses (Table 4). Two were induced by oxygen, two by anaerobiosis, and one by carbon and nitrogen starvation. It was also obvious from comparisons of SDS-boiled versus sonically disrupted extracts that the majority of ATR proteins were membrane associated. This would be expected since the membrane is the most logical site for cellular defense against increasing external H^+ ion concentrations.

The adaptive ATR system does not overlap with oxidative damage, heat shock, or SOS global control systems. There are several adaptive mechanisms that *S. typhimurium* uses as protection against a variety of environmental stresses. These stresses include oxidative damage, such as can occur with exposure to H_2O_2 (4), heat shock (13), and DNA damage (22) among others. Several approaches were taken to discover whether the ATR system overlaps any of these key global response systems. Potential overlap between ATR and oxidative damage was examined by the experiment presented in Table 5. Initial attempts were made to trigger H_2O_2 adaptation by growth at pH 5.8. Secondarily, adaptation to 60 μM H_2O_2 was used to try to mimic ATR. The data indicate that while each control adaptation resulted in protection against homologous challenge (i.e., 60 μM H_2O_2 resulted in protection against 10 mM H_2O_2), no protection was afforded to heterologous challenge (i.e., 60 μM H_2O_2 did not protect against pH 3.3). A similar study was used to show that heat shock did not mimic ATR (data not shown).

Additional efforts to establish a link between ATR and known stress management systems involved the evaluation of a variety of mutants for an adaptive ATR. Mutants defective in the DNA damage SOS system (*recA1*), oxidative stress (*oxyR*), or osmoregulation (*ompR*::Tn*5*) were examined for ATR. In addition, mutations in a known acid-regulated gene, *aniG*, and its regulator, *earA*, were tested (1; J. W. Foster and Z. Aliabadi, Mol. Microbiol., in press). All these mutants exhibited significant ATR (Table 6). These data confirm the uniqueness of the ATR system relative to other global regulatory systems.

Several other systems have been shown to sense or are suspected of responding to external acidification. Mutants defective in these sensing systems were also examined for possible defects in ATR. Exposure to external acid reportedly changes the methylation level of methyl-accepting chemotaxis proteins (17). Consequently, we examined methylation and demethylation mutants (*cheR* and *cheB*) for altered ATR but found no obvious differences relative to wild type (Table 6). Similarly, Heyde and Portalier (10) reported that external acid caused an offsetting increase and decrease in the outer membrane porins OmpC and OmpF, respectively. Mutants defective in the osmoregulatory locus *ompR* prevented this acid regulation. An *ompR*::Tn*5* insertion mutation was subsequently tested for the adaptive ATR. This strain exhibited a normal 2,000-fold increase in percent survival after adaptation, indicating that *ompR* is not required for adaptive acid survival.

Identification of mutants with altered acid tolerance. It seemed reasonable to assume that adaptive resistance to acid stress could result from decreased proton conductance into the cell, increased proton extrusion out of the cell,

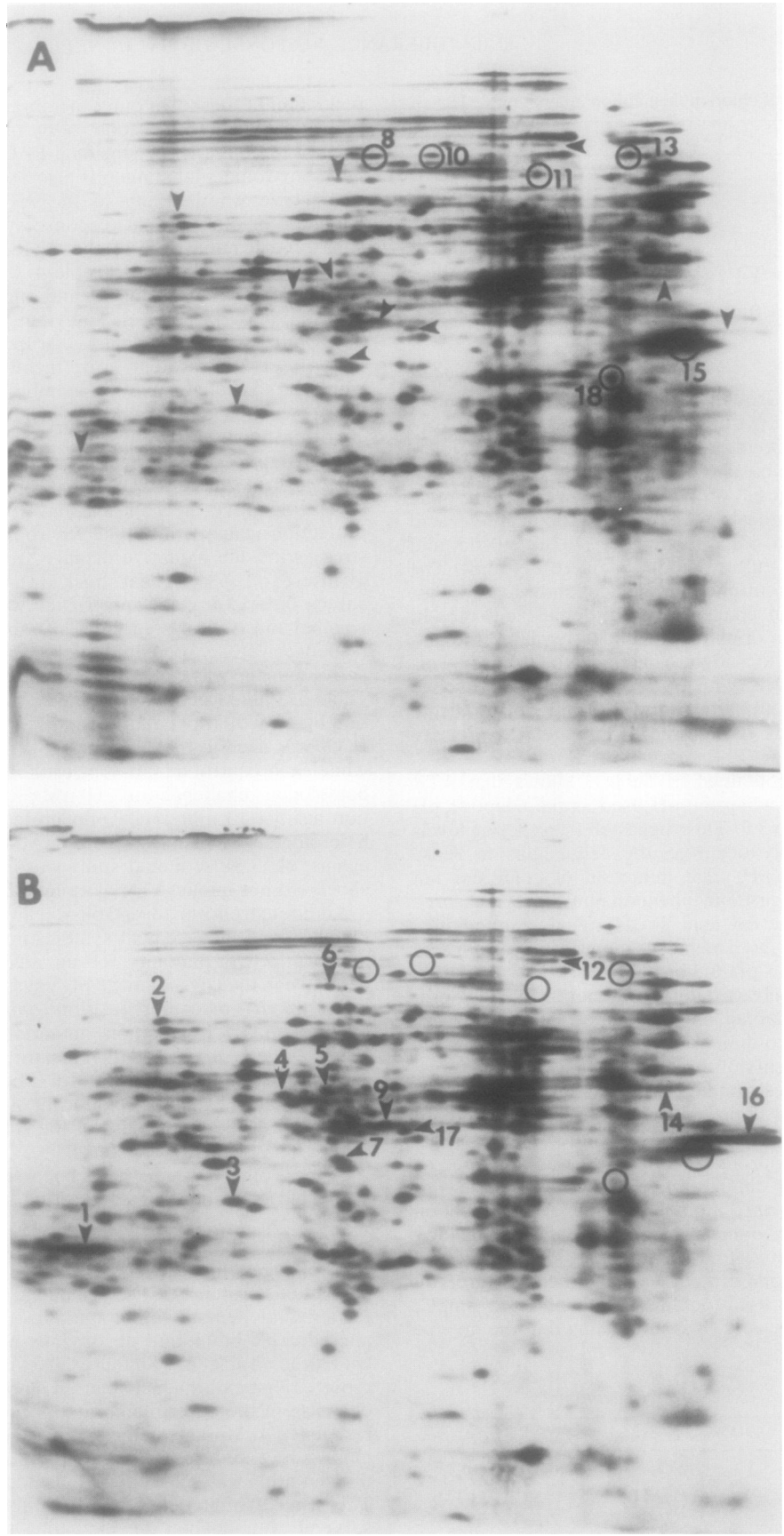

FIG. 2. Two-dimensional SDS-PAGE analysis of ATR polypeptides. Autoradiography profiles are of unadapted (A) and adapted (B) cells labeled for 10 min at equivalent cell densities. SDS-boiled lysates were prepared as described previously (18) and run in a two-dimensional system including a pH 5 to 7 (right to left) isoelectric focusing gel and SDS-11.5% PAGE. Acidic proteins are situated to the right of each gel. Arrowheads indicate acid-induced proteins, while circles designate acid-repressed proteins. Multiple labelings were performed for each condition, and a minimum of two gels were run for each labeling. Tabular results are presented in Table 4.

TABLE 4. Polypeptides induced or repressed during ATR

Protein no.	pH 5 × 7 coordinates[a]	Subcellular fraction[b]	pH control[c]	Other stresses[d]	Altered expt in JF1819[e]
1	10 × 64	Membrane	ACI		
2	21 × 94	Membrane	ACI		
3	33 × 67	Soluble	ACI	Oxygen	+
4	40 × 84	Soluble	ACI		+
5	46 × 85	Membrane	ACI		+
6	46 × 98	Membrane	ACI	Oxygen	
7	47 × 74	Soluble	ACI		+
8	52 × 102	Membrane	ACR		
9	55 × 79	Membrane	ACI		
10	56 × 102	Membrane	ACR		
11	74 × 99	Membrane	ACR	Ammonia, glucose	
12	77 × 103	Membrane	ACI	Anaerobiosis	
13	94 × 102	Membrane	ACR		+
14	97 × 85	Membrane	ACI		
15	103 × 75	Membrane (OmpF)	ACR	Anaerobiosis	
16	110 × 78	Membrane (OmpC)	ACI		
17	55 × 78	Soluble	ACI		
18	89 × 72	Membrane	ACR		+

[a] Coordinates refer to the standard *S. typhimurium* two-dimensional polypeptide map (18).
[b] Subcellular fractions were determined by comparing gels with SDS-lysed cells versus sonically disrupted cells.
[c] pH control refers to acid inducible (ACI) or acid repressible (ACR).
[d] Refers to polypeptides identified as inducible by other environmental stresses.
[e] JF1819 is a constitutively acid-tolerant mutant (*atr-1*).

increased cellular buffer capacity, or any combination thereof. Perusal of the literature revealed two loci, *unc* and *phoP*, which may affect the passage of protons into the cell. These genes were both tested for potential effects on ATR.

The complex Mg^{2+}-dependent proton-translocating ATPase (F_0F_1) catalyzes the synthesis or hydrolysis of ATP coupled with an electrochemical gradient of H^+ (for a review, see reference 8). The genes for the ATPase polypeptides are located in an operon designated *unc*. The gene order is *uncIBEFHAGDC*, with *uncBEF* coding for the F_0 proton pore subunits and *uncHAGDC* coding for the F_1 ATPase subunits. Since this enzyme has an obvious influence on H^+ movement into and out of the cell, it was an obvious candidate for an acid tolerance mechanism. The results presented in Fig. 3 reveal that the *unc*::Tn*10* mutant tested was completely unable to mount an observable ATR. The *unc* mutation also confers an extreme acid-sensitive phenotype, 100- to 1,000-fold more sensitive than LT2. Little is known regarding the *Salmonella unc* operon, and the location of the Tn*10* in the operon is unclear. However, these results clearly suggest that resistance to acid requires a functional H^+-translocating ATPase. The results do not a priori indicate that the ATPase is involved with the adaptive response per se, nor do they rule out the possibility that other mechanisms operate. It is possible, for instance, that the loss of the ATPase in some way leads to overwhelming intracellular acidification in a pH 3 environment through a lack of efficient H^+ efflux.

TABLE 5. Relationship between the H_2O_2 adaptive response and the ATR[a]

Adaptive treatment	Challenge	% Survival (t_{45})
None	10 mM H_2O_2	0.2
60 μM H_2O_2	10 mM H_2O_2	28
pH 5.8	10 mM H_2O_2	0.8
None	pH 3.3	<0.001
pH 5.8	pH 3.3	36
60 μM H_2O_2	pH 3.3	0.01

[a] Cells in each case were LT2. Adaptation was done at an optical density at 600 nm of 0.2 followed by challenge at an optical density at 600 nm of 0.4 or at 1 h postadaptation for H_2O_2.

TABLE 6. ATR in various mutant strains

Strain	Genotype	% Survival (t_{60})		Functional or global system
		Unadapted	Adapted	
LT2	Wild type	0.01	57	None
JF1218	*recA1*	0.1	29	SOS
SF236	Δ*oxyR*	<0.001	33	Oxidative stress
JF1506	*aniG*::Mu dJ Δ*earA*	0.002	35	Senses external mannose in acid
JF1842	*ompR*::Tn*5*	0.03	61	Osmoregulation
SF334	*cheB*::Tn*10*	0.1	63	Chemotaxis
SF339	*cheR*	0.5	21	Chemotaxis
JF1898	*phoP*::Tn*10*	<0.001	0.1	Phosphate regulation, virulence
SF342	*unc*::Tn*10*	<0.001	<0.001	Mg^{2+}-dependent ATPase
JF1819	*atr-1*	41	72	Acidification tolerance

Another locus of potential import to acid resistance is *phoP*. Although *phoP* was first recognized as a positive regulator of nonspecific acid phosphatase, *phoN* (12), Fields et al. (5) have since implicated *phoP* as a virulence locus that is required for the survival of *S. typhimurium* within macrophages. Their studies have shown that *phoP* mutants are more sensitive to defensins, the antimicrobial cationic peptides present in lysosomes of phagocytic cells (9). The defensins are believed to alter membrane integrity by making the cell more permeable to ions. It was reasoned that a *phoP* mutation might also affect the ATR by allowing for increased proton conductance. The results shown in Fig. 3 reveal that a *phoP* mutant was much more sensitive to acid (100- to 1,000-fold) but, nevertheless, still showed adaptive acid tolerance. The level of protection afforded after adaptation of the *phoP* mutant was not as great as wild type (0.1% for *phoP* versus 44% for wild-type survival), but the magnitude of the induction was similar to wild type (100- to 1,000-fold). Thus, while a *phoP* mutant might experience greater proton conductance at pH 3.3 relative to wild type, the increased proton leak appears to be overcome partially but not completely by the ATR. Note that both the *unc* and *phoP* mutants grow well at pH 5.8. They do not appear to be any more sensitive to this mild level of acid than wild type, so they are not generally more sensitive to acid. Their acid sensitivity appears to reside in a reduced capacity to handle massive proton stress.

In contrast to the acid-sensitive mutants described above, we have recently isolated several mutants of *S. typhimurium* that are constitutively acid tolerant. They were isolated as long-term survivors of lethal acid pH challenge. An example of one such mutant, JF1819, is included in Table 6 and Fig. 3. The unadapted culture of JF1819 is extremely resistant to pH 3.3 (41% survival after 60 min) compared with the wild-type LT2 strain (0.01% survival). In fact, these mutants remain viable for several hours after LT2 is completely killed by strong acid. In view of the results obtained with the *phoP* mutant, the *atr-1* mutant was tested for defensin sensitivity (E. Groismann, personal communication). No difference

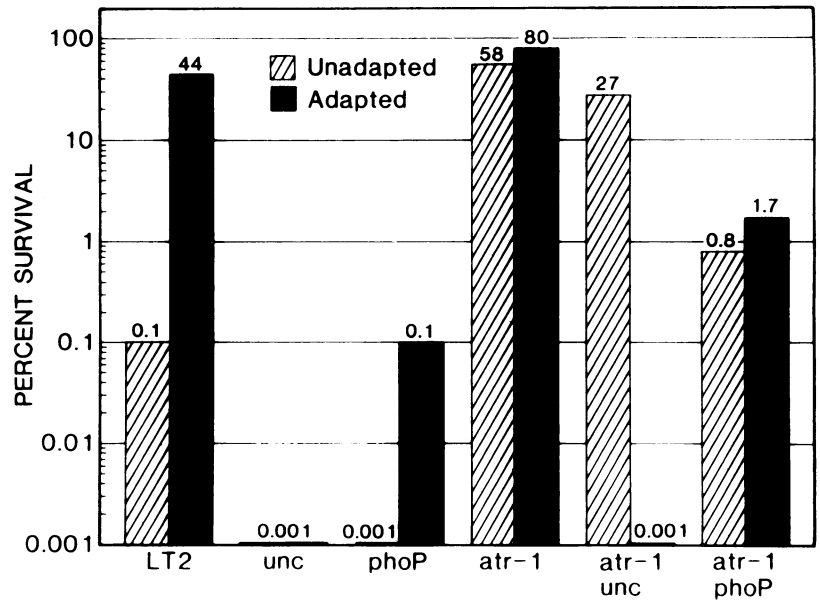

FIG. 3. ATR of *unc*, *phoP*, and *atr* mutants. Values represent percent survival after 60 min of exposure to a pH 3.3 environment. The first and second bars of each pair represent unadapted and adapted cells, respectively. The strains used are LT2, SF342 (*unc*::Tn*10*), JF1898 (*phoP*::Tn*10*), JF1819 (*atr-1*), JF1892 (*unc*::Tn*10 atr-1*), and JF1912 (*phoP*::Tn*10 atr-1*).

was noted compared with wild type. Nevertheless, the isolation of these *atr* mutants provides a means by which molecular investigations of the ATR can proceed.

ATR in *atr-1 unc* and *atr-1 phoP* double mutants. Since the *atr-1* mutation leads to constitutive acid tolerance and the *unc* mutation results in an acid-supersensitive phenotype, an obvious question is which mutation is dominant in an *atr-1 unc*::Tn*10* double mutant (JF1892). The results of an ATR experiment performed on JF1892 revealed that when this strain was unadapted, it displayed the expected Atr-1 acid-tolerant phenotype (Fig. 3). However, if adapted at pH 5.8, the strain assumed an Unc phenotype, which became very sensitive to strong acid. Thus, the combination of *atr-1* and *unc* leads to an inverse ATR, suggesting that treatment of the ATPase-deficient mutant with mild acid increases potential routes of H^+ leak. The subsequent massive proton influx that occurs in strong acid probably overwhelms the *atr-1* constitutive acid tolerance mechanism(s). Alternatively, mild acid may turn off the ATR in the absence of ATPase.

In contrast, a *phoP*::Tn*10 atr-1* mutant did not exhibit an inverse ATR (Fig. 3). This strain was constitutively acid tolerant, i.e., there was no induction in tolerance after mild acid treatment. However, it was still about 50- to 100-fold more sensitive to acid than the *atr-1 phoP*$^+$ control. Clearly, there is something very different about the ways in which *phoP* and *unc* affect the adaptive ATR system.

Two-dimensional PAGE analysis of ATR response in *atr-1* mutant. Some of the proteins identified as acid inducible in the previous two-dimensional PAGE experiment were expected to contribute to acidification tolerance. Therefore, the constitutively acid-tolerant mutant, JF1819, should show altered expression of one or more of these proteins relative to wild type. The autoradiographs displayed in Fig. 4 show that several proteins were altered in their expression during ATR. Six of the ATR polypeptides (shown in Table 4) (five acid inducible and one acid repressible) were overexpressed, as were five non-ATR proteins (indicated with a + in Fig. 4). Clearly, the *atr-1* mutation seems to increase acid tolerance by affecting the expression of these 11 proteins. Which protein(s) is directly responsible for the acid tolerance of JF1819 is not known as yet.

DISCUSSION

The results presented describe a novel global response system whose ultimate physiological role is to protect the cell from external acid stress. The term modulon has recently been proposed to reflect environmental responses that involve increased as well as decreased expression of genes within a system (11). The ATR environmental modulon is unique from other procaryotic stress management controls such as heat shock, oxidative stress, and SOS repair. The conditions which lead to induction and the genes that control these other systems do not significantly affect the ATR system. This fact was also evident from the two-dimensional PAGE analysis of ATR polypeptides in which only minor overlap with other stress-induced sets of proteins was observed (Table 4). As such, the regulatory mechanisms which mediate the ATR are likely to differ from those of classic global controls. Constitutive acid-tolerant mutants have been isolated and are currently being evaluated for their genetic role in the ATR system. It is interesting that while the *atr* mutants are resistant to the lethal effects of external acidification, they still exhibit regulatory control over many pH_0-controlled proteins and genes such as *aniG*. Thus, the resistance mechanism in these mutants does not generally affect the transmembrane signaling required to regulate external acid-regulated genes. Three basic mechanisms can be envisioned as potential contributors toward acid tolerance. These include a decreased membrane conductivity to H^+, increased proton extrusion, or an increased buffering capacity of excess H^+ by cellular constituents. Any one system or combination of systems may participate in the development of acid tolerance.

Current results with the *unc* (H^+-translocating ATPase) and *phoP* (defensin-sensitive) mutants are very intriguing. It is clear that *phoP* mutants are avirulent and more susceptible to defensins. Our results showed that *phoP* also affects the

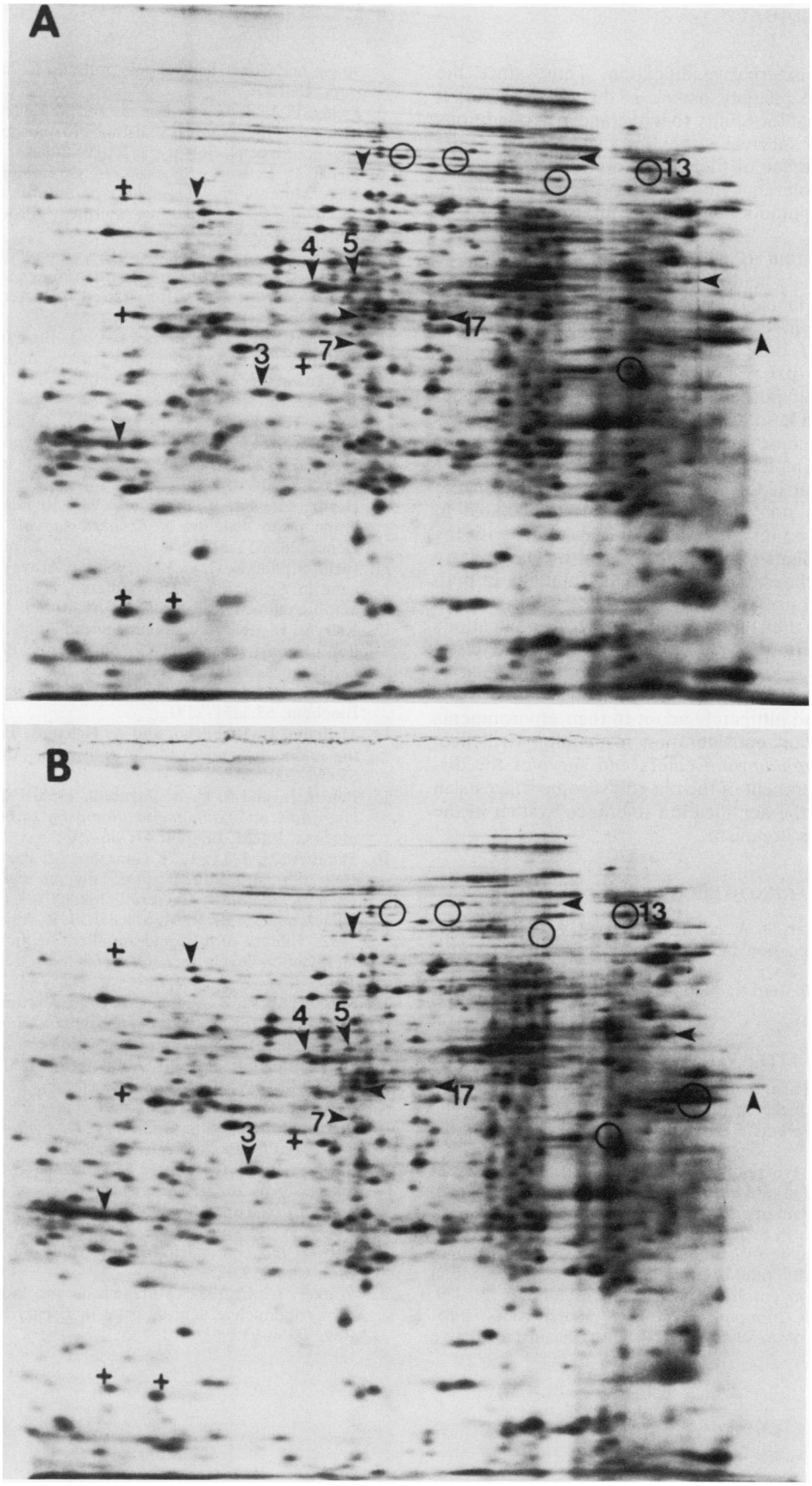

FIG. 4. Two-dimensional SDS-PAGE analysis of the *atr-1* mutant ATR polypeptides. Conditions are identical to those described in the legend to Fig. 2. (A and B) Unadapted and adapted cells, respectively. Numbers correspond to those used in Fig. 2 and are used here to indicate proteins whose expressions are altered in the *atr* mutant. Several non-ATR proteins show increased expression relative to an LT2 control. These proteins are marked with a plus (+) sign.

ability to survive external acidification. Thus, since the environment of the phagolysosome is distinctly acidic, it appears to follow that the ability to withstand acid conditions is also important for survival within this vacuole. Our results when viewed with those of Fields et al. (5) imply a correlation between acid tolerance and virulence. Mouse virulence studies with *atr* mutants will be useful to confirm this correlation.

The *unc*::Tn*10* strain as well as several derivative Δ*unc* strains were extremely acid sensitive. This suggests that the H^+-translocating ATPase is essential for the development of acid tolerance. The inverted ATR phenotype observed with the *unc*::Tn*10* *atr-1* double mutant (Fig. 3) suggests that growth in mild acid (pH 5.8) presensitizes the cell to protons either by increasing potential H^+ leak or by causing an imbalance in the ATR so that the lethal effects of H^+ stress are intensified in some manner. The unadapted *unc atr* double mutant was almost as acid tolerant as an *unc*$^+$ *atr-1* strain, implying that if the ATPase is a component of the ATR system, it is not the only mechanism available to defend against proton influx. In light of our results with the acid-sensitive avirulent *phoP* strain, it will be particularly interesting to test the virulence of the *unc* mutants in both macrophage and mouse systems.

The existence of the ATR system has broad implications concerning the ability of *S. typhimurium* to sense, adapt to, and survive in a variety of environments not the least of which include the phagosome and phagolysosome. How pathogens sense and ultimately adapt to their environments must be an important consideration in defining virulence. The fact that *S. typhimurium* enters and survives the distinctly acidic environment of the phagolysosome suggests an important role for the acidification tolerance system in the pathogenesis of this organism.

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