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The Acid Tolerance Response of *Salmonella typhimurium* Involves Transient Synthesis of Key Acid Shock Proteins

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Although *Salmonella typhimurium* prefers neutral-pH environments, it can adapt to survive conditions of severe low-pH stress (pH 3.3). The process, termed the acid tolerance response (ATR), includes two distinct stages. The first stage, called pre-acid shock, is induced at pH 5.8 and involves the production of an inducible pH homeostasis system functional at external pH values below 4.0. The second stage occurs following an acid shock shift to pH 4.5 or below and is called the post-acid shock stage. During this stage of the ATR, 43 acid shock proteins (ASPs) are synthesized. The present data reveal that several ASPs important for pH 3.3 acid tolerance are only transiently produced. Their disappearance after 30 to 40 min of pH 4.4 acid shock coincides with an inability to survive subsequent pH 3.3 acid challenge. Clearly, an essential feature of inducible acid tolerance is an ability to synthesize these key ASPs. The pre-acid shock stage, with its inducible pH homeostasis system, offers the cell an enhanced ability to synthesize ASPs following rapid shifts to conditions below pH 4.0, an external pH that normally prevents ASP synthesis. The data also address possible signals for ASP synthesis. The inducing signal for 22 ASPs appears to be internal acidification, while external pH serves to induce 13 others. Of the 14 transient ASPs, 10 are induced in response to changes in internal pH. Mutations in the *fur* (ferric uptake regulator) locus that produce an Atr$^-$ acid-sensitive phenotype also eliminate induction of six transiently induced ASPs.

Salmonella typhimurium is a neutralophilic organism capable of growth in minimal medium at pH values between 5 and 9. However, its encounters with low pH can extend well below pH 5. Sites such as the acidic stomach, chemically compromised pond water, and the macrophage phagolysosome represent examples of low-pH environments endured by this facultative intracellular parasite. Our laboratory has demonstrated the existence of an adaptive response, termed the acid tolerance response (ATR), which can effectively protect this organism from the lethal effects of acid conditions below pH 4 (5). Others have demonstrated similar responses in *Escherichia coli* and *Listeria*, *Streptococcus*, and *Enterococcus* spp. (2, 8, 10, 12). The response in *S. typhimurium* was shown to involve two stages, a pre-acid shock stage induced at pH 5.8 and a post-acid shock stage induced at or below pH 4.5 (3). The preshock stage induces an ATR-specific pH homeostasis system which helps maintain the internal pH as the external pH decreases below 4 (6). The postshock phase involves the dramatic induction of 43 acid shock proteins (ASPs) with predicted importance in the prevention or repair of acid damage to macromolecules. The data originally supported a model in which both stages were absolutely required for the ATR. Induction of either stage alone appeared insufficient to elicit protection.

Further analysis of the ATR now reveals that, while both stages are required for maximum protection against a low pH, a single brief period (15 min) of acid shock (pH 4.3) alone will enable cells to tolerate a challenge pH of 3.3. Acid shock (pH 4.3) exposures of >30 min do not afford this protection. Results show that a key subset of ASPs is synthesized transiently at pH 4.3 and then disappears, leaving the cell nearly defenseless against extreme low-pH stress.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. typhimurium* LT2 or its derivatives were used throughout this study. JF1638 (*aniG*::MudJ Δ*earA234*) constitutively produces β-galactosidase and was described previously (1, 4). Culture media included minimal E glucose medium (14) and Luria broth (11). Foster and Hall (5) provide a detailed description of the ATR protocol. Briefly, cultures were grown in pH 7.7 minimal E glucose under semianaerobic conditions to 10^8 cells per ml, adapted to pH 5.8 for one doubling, and then challenged at pH 3.30. Unadapted cells were grown directly to a density of 2×10^8 cells per ml at pH 7.7 and then challenged at pH 3.30.

The acid shock procedure was also described in detail previously (5). Cells were grown in pH 7.7 minimal E glucose medium to a density of 2×10^8 cells per ml, and the pH was adjusted to pH 4.3 (acid shock) for various lengths of time, depending upon the experiment. A previous study has shown that viability at pH 4.3 remains at 100% for at least 3 h (3). The cells were labeled after pH 4.3 shock with ^{35}S-Trans (40 μCi/ml) (ICN Biochemicals) for 2 min, and 100 μg of chloramphenicol per ml was added prior to harvest. Alternatively, if the cells were to be tested for acid tolerance, the pH 4.3 acid-shocked cells would be challenged at pH 3.30 and viability would be determined. Some experiments involved monitoring the irreversible denaturation of β-galactosidase activity as a measure of acid tolerance. In these instances, aliquots of cells undergoing pH 3.30 challenge were rescued to a neutral pH prior to measurement of β-galactosidase activity (6).

Two-dimensional SDS-PAGE. The method for conducting O'Farrell two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was presented by Spector et al. (13). The first dimension was a pH 5 to 7 (right to left) isoelectric focusing gel containing 1.6% (pH 5 to 7) and 0.4% (pH 3 to 10) ampholytes (Bio-Rad). The second dimension was an SDS–11.5% polyacrylamide gel. Acid shock-inducible proteins are indicated by number and are described in detail by Foster (3). Figures shown are representative of duplicate gels of two or three independent

experiments. Results shown in Table 2 are based on high-resolution two-dimensional laser densitometry performed and analyzed with a Biomed Instruments, Inc., two-dimensional scanning system.

RESULTS

Acid tolerance is induced by short-term but not long-term acid shock. There are two useful assays for measuring the ATR. The first involves monitoring viability at pH 3.3. This is appropriate for measuring large differences (10-fold or more) between adapted and unadapted cells. The second technique is more sensitive to smaller differences (2- to 10-fold). Its basis is the irreversible acid denaturation of intracellular β-galactosidase activity that occurs as the cell dies and internal pH decreases to pH 5.5 or below. A strain which constitutively produces β-galactosidase is used for this purpose. A series of experiments designed to examine the stability of β-galactosidase during various acid exposures revealed that, contrary to the working model, preshock treatment was not absolutely required for acid tolerance (Fig. 1). Unadapted cells that subsequently underwent short-term acid shock (pH 4.3 for 10 to 20 min) were able to prevent β-galactosidase denaturation when challenged at pH 3.3. Surprisingly, an acid shock (pH 4.3) for more than 30 to 40 min did not afford this pH 3.3 protection (Fig. 1A). The short-term acid shock phenomenon observed for β-galactosidase also translated to enhanced cell viability as shown in Fig. 1B.

The earlier model stating that the ATR required both pre- and post-acid shock stages (3) was based upon control experiments in which 60 min of acid shock failed to induce acid tolerance. Controls involving shorter periods of acid shock were not tested at that time. It was expected that if acid shock alone elicited acid tolerance, a longer period of acid shock would be just as successful as a short-term acid shock in producing acid tolerance. This was not the case.

The combination of preshock adaptation and short-term acid shock is necessary for maximal acid tolerance. The data presented in Fig. 1 clearly indicate that short-term acid shock can, by itself, trigger an ATR. Nevertheless, maximum acid tolerance still required both pre-acid shock (pH 5.8) adaptation and post-acid shock protein synthesis. This is obvious when the levels of β-galactosidase protection in the second and fifth sets of bars in Fig. 1A as well as the long-term viability measurements in Fig. 1C are compared. In both cases, protection is better and of longer duration when cells undergo both stages of the ATR prior to severe acid challenge (pH 3.3). Previous data have shown that to become acid tolerant, adapted (pH 5.8) cells still require a 15-min pH 4.3 shock prior to severe acid challenge conducted in the presence of chloramphenicol (3). I have found that continuing acid shock beyond 30 min still eliminated subsequent pH 3.3 survival even in preshock-adapted cultures (data not shown). Consequently, preadapting cells at pH 5.8 does not extend the length of time cells can be acid shocked at pH 4.3 and subsequently retain tolerance to pH 3.3.

Transient induction of key ATR ASPs. The data above suggested a transient induction of key ASPs important to acid tolerance. This was confirmed by pulse-labeling pH 4.3 acid-shocked cells at various times following a pH transition from 7.7 to 4.3. The results are presented in Fig. 2. Most of the acid shock-induced proteins reached maximum levels by 20 min and remained expressed without change even after 60 min. However, 14 proteins, marked by arrowheads in Fig. 2, were only transiently induced. All 14 proteins were expressed at high levels by 20 min after acid shock and then decreased or disappeared by 40 to 60 min. Since eliminating

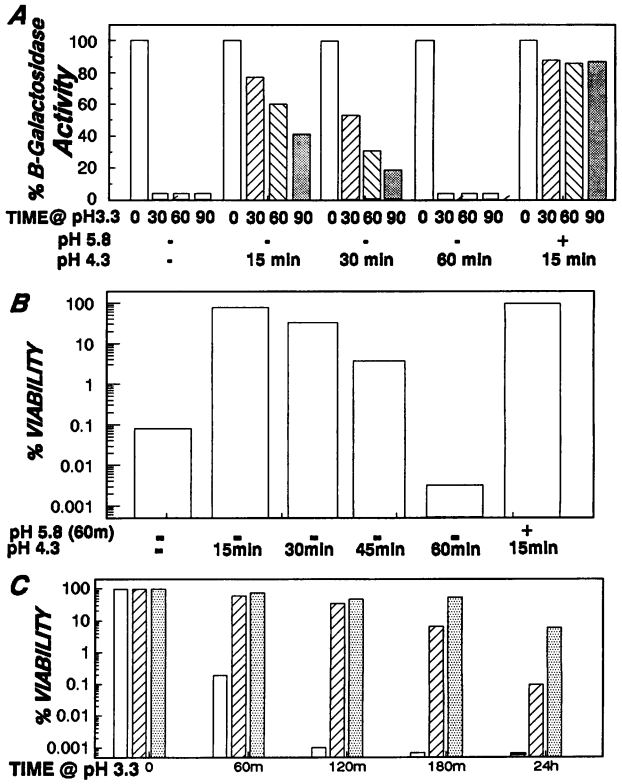

FIG. 1. ATR. (A) JF1638 (constitutively Lac$^+$). Cells were pre-acid shock adapted (pH 5.8 for 1 h) or acid shocked (pH 4.3) as indicated before being challenged at pH 3.30. Samples were taken at the indicated times, the pH was adjusted to 7, and β-galactosidase activity was measured. One hundred percent activity is approximately equal to 1,500 Miller units. Zero time was immediately before the pH was adjusted to 3.30. (B) LT2 treated as indicated and then challenged at pH 3.30 for 90 min. (C) LT2 cells were unadapted (open bars), pH 5.8 adapted (60 min) (hatched bars), or pH 5.8 adapted (60 min) and exposed to 15 min of pH 4.3 acid shock (shaded bars). All cultures were challenged at pH 3.30.

ASP synthesis prevents acid tolerance, these transiently expressed proteins in whole or in part are likely to play an integral role in the protection against acid afforded by acid shock. If the acid-shocked (pH 4.3) cell does not subsequently encounter potentially lethal acid levels (pH 3.3) within a 20- to 30-min period after acid shock, these key proteins are no longer synthesized and the cell becomes vulnerable to a subsequent low pH.

Decreasing pH_i alone will induce many but not all ASPs. One aspect of acid tolerance not previously addressed is whether acid shock genes respond to the internal pH (pH_i) or the external pH (pH_o) environment. An external pH of 4.0 to 4.5 produces an intracellular pH_i of 6 to 7. Consequently, two approaches were used to explore whether acid shock genes respond to the external pH 4 to 4.5 or the internal pH 6 to 7 signals. Both approaches involved artificially lowering the pH_i to between 6 and 7 while maintaining external pH conditions above 5.5. Table 1 summarizes the treatments used and their effects upon pH_i.

The first method involved using the protonophore 2,4-dinitrophenol (DNP) to nearly equilibrate internal pH and external pH. The technique was used previously to illustrate that cell death occurs as the pH_i approaches 5.5 (6). In that study, a concentration of 200 μM DNP still allowed the generation of a small ΔpH (0.3 U) when used at pH_0 5.1. In

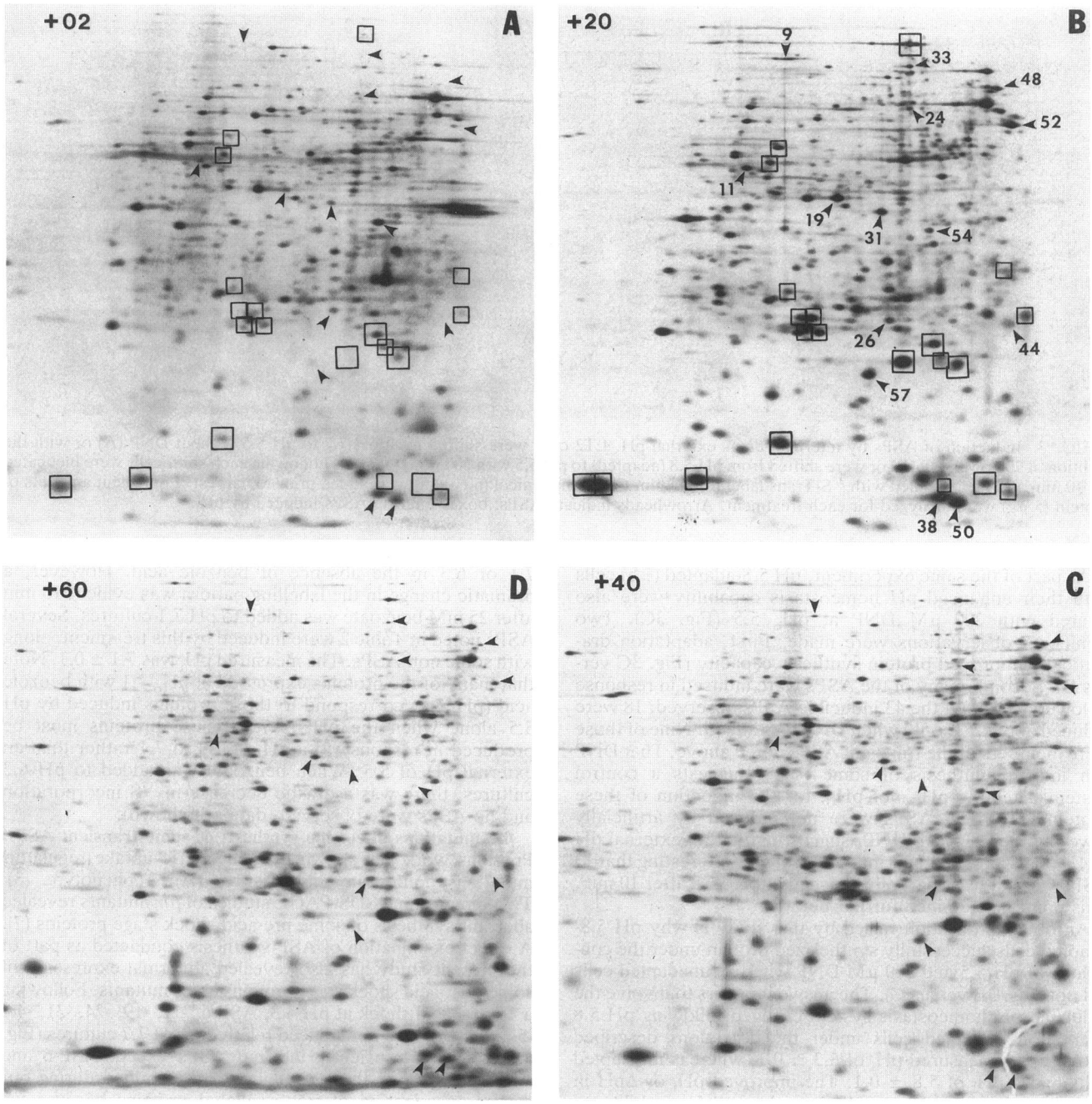

FIG. 2. Transient induction of ASPs. LT2 was grown to 2×10^8 cells per ml in minimal medium, pH 7.7; then the pH of the medium was adjusted to 4.3. At 2 (A), 20 (B), 40 (C), and 60 min (D), a sample was labeled for 2 min. After the labeling period, chloramphenicol (60 μg/ml) was added and the cells were prepared for two-dimensional SDS-PAGE. Transient (arrowheads) and stable (squares) ASPs are indicated.

the current study, when 200 μM DNP was added to a pH 5.5 culture, the pH_i dropped only to 5.9. Thus, a small ΔpH remained, which was consistent with the earlier results. However, protein synthesis in these DNP-treated cells was greatly impaired, as witnessed by poor incorporation of [^{35}S]methionine (Fig. 3B). This result suggested that pH_i values below 6 severely diminish protein synthesis. Even so, some ASPs were synthesized and observed at this pH_i (arrowheads in Fig. 3B). Adding 200 μM DNP to pH 7.7 cells did not alter the polypeptide profile, indicating that DNP itself does not directly affect protein synthesis. Only when DNP could cause a decrease in pH_i was there a negative effect on protein synthesis.

TABLE 1. Response of internal pH of unadapted cells to various treatments used

External pH	Treatment	Internal pH
7.7	None	7.7
5.5	None	7.3
7.1	25 mM benzoate	7.1
5.5	200 μM DNP	5.9
4.3	None	6.2

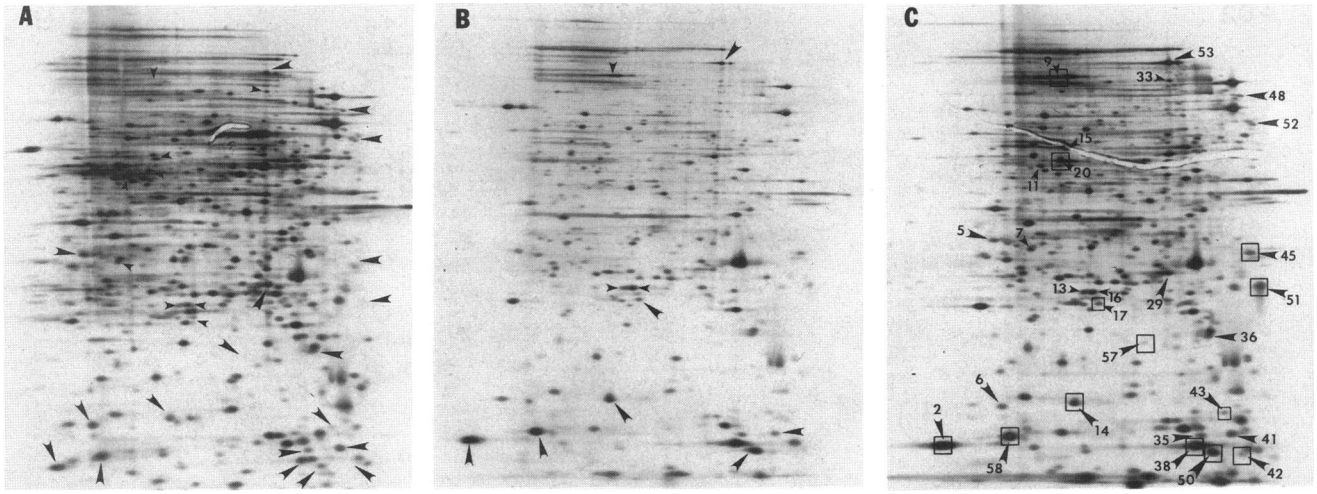

FIG. 3. Induction of ASPs by internal versus external pH. LT2 cells were shifted from pH 7.7 to pH 5.5 without DNP (A) or with the addition of 200 μM DNP (B) or were shifted from pH 5.8 (adapted) to pH 5.5 with 200 μM DNP (C). Following each shift, cells were incubated for 10 min and then labelled with ^{35}S-Trans label for 10 min. Chloramphenicol (60 μg/ml) was added prior to harvest. Equivalent amounts of protein (5 μg) were analyzed for each treatment. Arrowheads indicate ASPs; boxes indicate ASPs induced by pH_i.

As part of the same experiment, pH 5.8-adapted (1 h) cells with their enhanced pH homeostasis capability were also treated with 200 μM DNP at pH_o 5.5 (Fig. 3C). Two significant observations were made. First, adaptation dramatically improved protein synthesis capacity (Fig. 3C versus B). Second, many of the ASPs were induced in response to lowered pH_i. Of the 43 inducible ASPs observed, 18 were induced at pH_o 5.5 only when DNP was added. Nine of those were members of the transient ASPs noted above. That DNP can induce synthesis of some ASPs suggests a control system detecting pH_i, not pH_o, for the induction of these proteins. Thirteen ASPs were not induced by artificially lowering the pH_i with DNP, suggesting that an external pH of 4.4 plays a role in their induction. It was interesting that 13 ASPs were at least partially induced at pH_o 5.5 after 10 min. This point is examined further below.

An obvious question raised by this study is why pH 5.8-adapted cells successfully synthesized protein under the conditions of pH 5.5 and 200 μM DNP whereas unadapted cells did not (Fig. 3B versus C). The answer appears to involve the inducible pH homeostasis system engaged following pH 5.8 adaptation. Adapted cells under the conditions described above had a measured pH_i of 6.3 ± 0.1, whereas unadapted cells had a pH_i of 5.8 ± 0.1. The improved pH_i or ΔpH in adapted cells, therefore, enhances the cells' capability to continue protein synthesis. Differences between unadapted and adapted cells in terms of pH_i and protein synthesis capacity disappeared when 300 μM DNP was used at pH 5.5 (data not shown), supporting this explanation.

To determine whether a somewhat higher pH_i, closer to 7, might induce some ASPs, 25 mM benzoate was added to growing cultures. Weak acids such as benzoic acid will cross the cellular membrane only in their neutral, protonated form. Once inside the cell, the acid will equilibrate between its protonated and ionized states, releasing H^+ in the process, which will then lower pH_i. Consequently, cells were radiolabeled with [^{35}S]methionine at two different external pH values in the presence and absence of 25 mM benzoate. The labeled cells were then subjected to two-dimensional SDS-PAGE analysis. Although the gels are not shown, the results based upon relative densitometry are tabulated in Table 2. A normal polypeptide pattern was evident after labelling at pH 7.1 or 6.3 in the absence of benzoic acid. However, a dramatic change in the labelling pattern was evident 10 min after 25 mM benzoate was added to pH 7.1 cultures. Several ASPs noted in Table 2 were induced by this treatment, along with some non-ASPs. The measured pH_i was 7.1 ± 0.1. Note that many of the proteins expressed at pH_o 7.1 with benzoic acid (pH_i 7.1) correspond to those proteins induced by pH 5.5 alone when the pH_i is 7.3. These proteins must be produced in response to a pH_i of 7.3 to 7.1 rather than an external pH of 5.5. When benzoate was added to pH 6.3 cultures, there was a drastic decrease in ^{35}S incorporation and no ASPs were observed (data not shown).

***fur* mutations affect the synthesis of some transient ASPs.** Previous work has shown that *fur* (ferric uptake regulator) mutations confer an acid-sensitive (Atr^-) phenotype (3). Two-dimensional SDS-PAGE studies of *fur* mutants revealed aberrant synthesis of some pre-acid shock stage proteins (7). A direct examination of ASP synthesis conducted as part of the present study has also revealed abnormal expression of some post-acid shock stage proteins in *fur* mutants. Following a 15-min acid shock at pH 4.3, ASP-6, -11, -19, -24, -31, -48, -54, and -55 were not induced relative to pH 7.7 cultures (Fig. 4). All were transiently induced ASPs except ASP-6 and ASP-55. None of these proteins were identified during our earlier examination of iron-regulated proteins because we examined only protein synthesis with or without iron limitation (7). The earlier study did identify ASP-33, another transient ASP, as regulated by iron and overexpressed at pH 7 in a *fur* mutant. Consequently, *fur* mutations appear to affect both stages of the ATR.

Can post-acid shock generate acid tolerance in some pre-acid shock adaptation-defective mutants? Our laboratory has identified several mutations which confer an acid-sensitive phenotype on *S. typhimurium*. Among these are mutations in *atp* and *fur*. Neither the *atp* nor the *fur* mutants could generate an ATR when adapted at pH 5.8 (pre-acid shock). The current model for adaptive acid tolerance involves pH 5.8 adaptation as primarily enabling ASP synthesis to occur at pH_o values below 4 (see Fig. 6). Thus, successful ASP synthesis appears to be the important determinant in adaptive acid tolerance. This model predicts two specific classes of *atr* mutants, those defective in preshock adaptation ver-

TABLE 2. Effect of pH_i on ASP synthesis

ASP	Densitometric increase (fold) with pH[a]:			Probable signal
	7.3[b]	7.1[c]	6.2[d]	
2			+ (7)	pH_i
5	+ (3.5)	+ (4.2)	+ (5)	pH_i and/or pH_o
6[e]	+ (5.5)		+ (6)	pH_o
7	+ (7)	+ (6.7)	+ (7.5)	pH_i and/or pH_o
9[f]			+	pH_i
11[e,f]		+ (3.2)	+ (3)	pH_i
12				pH_o
13	+ (5)	+ (9)	+ (4)	pH_i and/or pH_o
14	+ (>10)		+ (>10)	pH_o
15	+ (>10)		+ (>10)	pH_o
16	+ (3)	+ (5)	+ (7)	pH_i and/or pH_o
17		+ (4.5)	+ (6)	pH_i
18		+ (>10)		pH_i
19[e,f]				pH_o
20			+ (>10)	pH_i
24[e,f]		+ (3.5)		pH_i
26[f]				pH_o
27				pH_o
28	+ (3.5)	+ (4)		pH_i and/or pH_o
29	+ (4)	+ (3)	+ (5)	pH_o and/or pH_i
31[e,f]				pH_o
33[f]		+ (>10)	+ (>10)	pH_i
34				pH_o
35	+ (5.0)	+ (6.0)	+ (>10)	pH_o and/or pH_i
36	+ (3)		+ (3)	pH_o
38[f]		+ (4)	+ (7)	pH_i
41	+ (6)	+ (9)	+ (>10)	pH_i and/or pH_o
42		+ (4)	+ (5)	pH_i
43			+ (>10)	pH_i
44[f]		+ (3)		pH_i
45			+ (3)	pH_i
48[e,f]		± (2.5)	+ (>10)	pH_i
50[f]			+ (>10)	pH_i
51		+ (5)	+ (>10)	pH_i
52[f]			+ (4.3)	pH_i
53	± (2.8)	+ (4)	+ (5.2)	pH_i
54[e,f]				pH_o
55[e]				pH_o
56				pH_o
57[f]			+ (>10)	pH_i
58		+ (6)	+ (5)	pH_i
DnaK		+ (4)	± (2.6)	pH_i
GroEL		+ (3)	± (2.6)	pH_i

[a] +, ≥3-fold increase relative to untreated (pH 7.7) cells; ±, <3-fold increase relative to untreated (pH 7.7) cells.
[b] The measured pH_i of 7.3 was obtained in cells grown at pH 5.5.
[c] The measured pH_i of 7.1 was obtained at pH_o 7.1 with 25 mM benzoate.
[d] The measured pH_i of 6.2 was obtained at pH_o 5.5 with 200 μM DNP with pH 5.8-adapted cells.
[e] Synthesis required a functional Fur protein.
[f] Transient ASPs.

sus those defective in post-acid shock adaptation. If this prediction is true, then acid shock at pH 4.4 should enable ASP synthesis to occur in mutants specifically defective in preshock adaptation and thereby confer effective acid tolerance. To test this prediction, both the *atp* and the *fur* mutants were examined for acid tolerance following either pH 5.8 (1 h) or pH 4.4 (20 min) adaptation. The results in Fig. 5 confirm that the *atp* mutant is defective in preshock adaptation and dies when challenged directly at pH_o 3.3. However, these cells will mount an ATR when acid shocked at pH 4.4. The extent of tolerance was diminished relative to that of the wild type, but there remained a 100-fold difference between unadapted and pH 4.4-adapted cells. This suggests that the main defect in *atp* mutants involves preshock adaptation. In contrast, the *fur* mutant was severely defective both in preshock and postshock adaptations. This agrees with the data presented above showing defective ASP synthesis in *fur* mutants and with earlier data indicating defects in pre-acid shock protein synthesis (7).

DISCUSSION

The results described above allow modification of the working model for the ATR (3). Figure 6 summarizes the effects of various treatments on induction of the ATR and survival. It is now apparent that in order for *S. typhimurium* to mount a successful ATR, the organism must synthesize a set of key ASPs. The preshock stage, originally thought to be essential to acid tolerance, now proves dispensable under certain conditions. Preshock adaption clearly contributes to the magnitude of protection but is not an absolute requirement for the ATR. Rather, the ATR-specific pH homeostasis system induced by preshock adaptation primarily enables the organism to synthesize ASPs while suspended at an external pH (e.g., pH 3.3) that would ordinarily compromise protein synthesis capability (Fig. 6, pre-shock). The translational apparatus is very sensitive to internal pHs below 6 due to a direct effect either on ribosomes or on energy state, and since the measured pH_i of unadapted cells at pH_o 3.3 is 4.5 to 5, little protein synthesis is likely (Fig. 6). Preshock-adapted cells shifted to pH_o 3.3 are better than unadapted cells at maintaining a pH_i near 6. As a result, the preshocked organism can successfully make the protective ASPs during severe acid exposure. The fact that the pH_i of the adapted cell is higher than that of the unadapted cell also contributes to survival by minimizing acid damage.

The data presented in Fig. 3 further support the presence of a preshock inducible pH homeostasis system. Two important points are illustrated. First, preshock-adapted cells can handle DNP stress better than unadapted cells in that adapted cells generate a ΔpH of 0.6 compared with 0.3 for unadapted cells. Second, this enhanced homeostasis salvages the protein synthesis capacity of the DNP-stressed organism. The mechanism for this inducible system is still not known but could involve the production of a new homeostasis mechanism or the enhanced synthesis or modification of a preexisting system. It is clear that a major proton pump, the Mg^{2+}-dependent proton-translocating ATPase, is required for this inducible system to work but is not required for constitutive housekeeping pH homeostasis (6). However, the acid inducibility of this system has not been explored. It is also clear from the data in Fig. 4 that the H^+-translocating ATPase is not essential to acid tolerance if the second stage of the ATR is already engaged. This result is also consistent with inducible pH homeostasis being required for ASP synthesis below pH_o 4.0.

The transient induction of acid tolerance at pH 4.3 is explained in part by the temporally limited expression of a subset of ASP genes. These genes are expressed only for 15 to 20 min at pH_o 4.3. Beyond that time, no new synthesis of the transient ASPs can be detected and the acid-shocked cells lose their pH 3.3 tolerance. The fact that pH 4.3-induced acid tolerance could be measured only if the cells were shifted from pH 4.3 to 3.3 within 15 to 20 min further suggests that one or more of the key transient acid tolerance proteins are themselves labile unless promptly engaged in their protective role(s) at a severely acid pH. Alternatively, the cell at pH 4.3 could progress into such a severe state of unbalanced growth, as would occur following capricious synthesis of unneeded proteins, that beyond the 20-min acid shock period the cell may become exquisitely sensitive to severe acidity.

A major question addressed in this study was whether

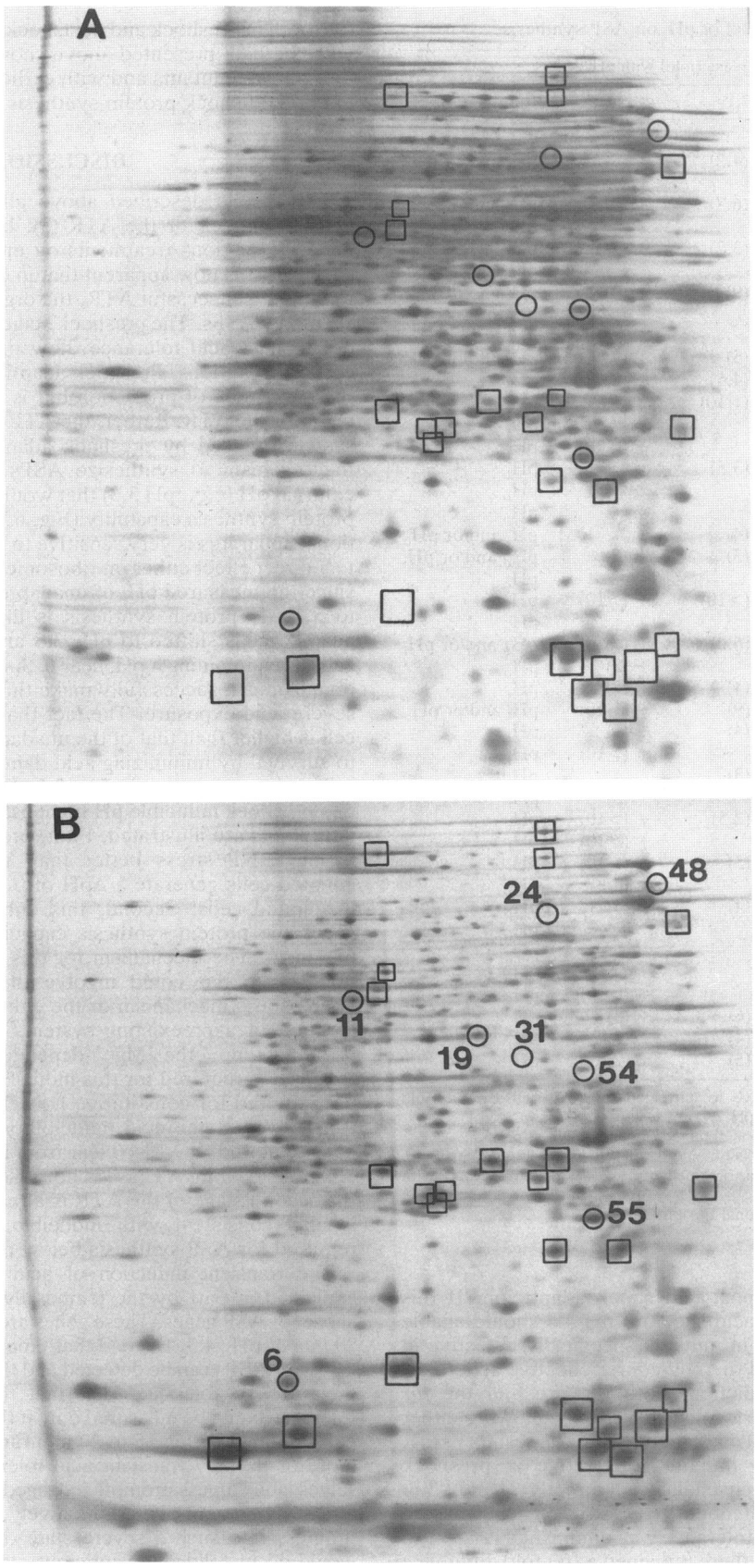

FIG. 4. ASP synthesis in a *fur* mutant (JF2023). pH 7.7-grown cells (A) were shifted to pH 4.4 for 20 min and then labeled for 2 min (B). Proteins still induced by acid shock (boxed) and those not induced by acid shock (circled and numbered) are indicated.

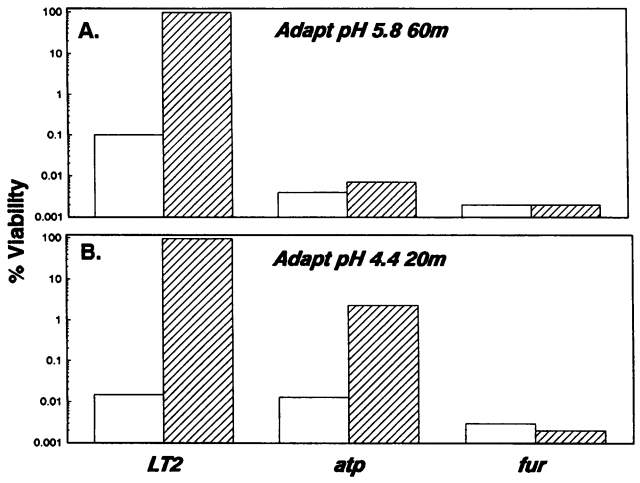

FIG. 5. Acid shock-generated acid tolerance in *atp* but not *fur* mutants. Cells (as indicated below panel B) were either unadapted (open bars) (pH 7.7) or adapted (hatched bars) (pH 5.8 [A] or 4.4 [B]) for the indicated numbers of minutes and then challenged at pH 3.30 for 2 h.

internal or external pH marks the signal for ASP synthesis. Of the 43 ASPs examined, 22 were induced by pH_i on the basis of experiments which artificially lower pH_i. Thirteen others appear responsive to a pH_o of ≤4.5, while the inducing signal for eight remains unknown. Consequently, there are at least two different classes of ASP genes and regulator systems based upon a response to internal or external pH. It appears that there is a pH gradient effect for the series of genes responding to pH_i as an inducing signal. Some ASP genes were induced as the internal pH fell to 7.1; others were not induced until a much lower pH_i. This suggests a series of regulatory proteins (DNA binding or sensor-transmitter) with thresholds for activation or inactivation occurring at progressively lower pH values. On the basis of the results of Heyde and Portalier (9) with *E. coli*, at least one regulator, sigma-32, must remain or become active during acid shock since several heat shock proteins (GroEL, Dnak, HtpG, and HtpM) are induced during acid shock and require sigma-32 to do so. Of the transiently induced ASPs, 10 responded to pH_i and 4 responded to pH_o.

The ASP loci induced by pH_i may be analogous to the

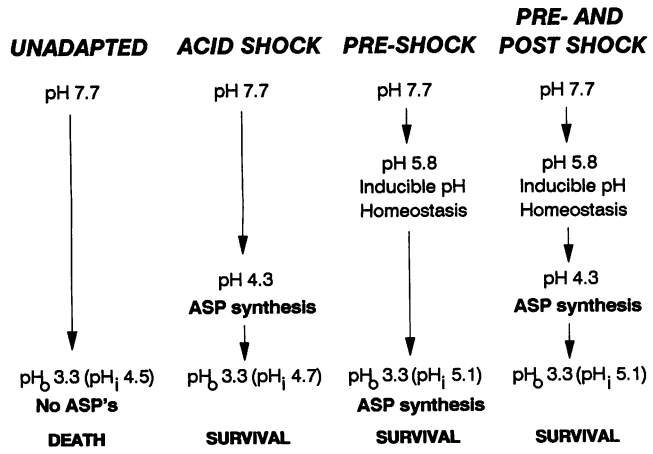

FIG. 6. Diagrammatic representation of ATR.

inaA gene of *E. coli* described by White et al. (15). This locus encodes a potential 168-amino-acid polypeptide of unknown function and appears to be transcriptionally regulated by internal acidification. When pH_i was modulated by benzoate, maximum induction of *inaA* was noted at pH_i 7.0. This gene, therefore, is an intriguing candidate for an acid shock gene.

The fact that pH_i rather than pH_o is a major signal for ASP synthesis is logical in the context of an acid stress response system. Internal proteins required for acid protection would not be needed unless the internal pH begins to approach levels that could cause damage. However, it is not clear whether pH_i is sensed directly by regulatory proteins or instead may damage nucleic acids and/or proteins which in turn induce the system.

The effect of *fur* mutations on transient ASP synthesis confirms the importance of these proteins to acid tolerance and further implicates Fur as contributing to ATR regulation. How Fur mediates this control is not clear but is under investigation.

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