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Salmonella Acid Shock Proteins Are Required for the Adaptive Acid Tolerance Response

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***Salmonella typhimurium*, as well as other enteric bacteria, experiences significant fluctuations in H^+ ion concentrations during growth in diverse ecological niches. In fact, some pH conditions which should kill cells rapidly, such as stomach acidity, are nevertheless tolerated. The complete mechanism for this tolerance is unknown. However, I have recently demonstrated that *S. typhimurium* has the ability to survive extreme low pH (pH 3.0 to 4.0) if first adapted to mild pH (pH 5.5 to 6.0). This phenomenon has been referred to as the acidification tolerance response (ATR). The exposure to mild acid is referred to as preshock, and the proteins involved are called preshock ATR proteins. A second type of encounter with acid, called acid shock, involves shifting cells directly from alkaline conditions (pH 7.7) to acid conditions (pH 4.5 or below). During acid shock, the organism immediately ceases reproduction and dramatically changes the expression of at least 52 proteins. All but four are distinct from the preshock ATR proteins. Surprisingly, acid shock alone did not afford significant protection against strong acid challenge in minimal medium. Furthermore, inhibiting protein synthesis prior to acid shock revealed that the acid shock proteins do not appear to contribute to acid survival in minimal medium even at pH 4.3. Constitutive cellular pH homeostatic mechanisms seem sufficient to protect cells at this pH. The data suggest that the induction of acid shock and preshock ATR proteins are separate processes requiring separate signals. However, for *S. typhimurium* to survive extreme acid conditions, it must induce both the preshock and acid shock systems. Preventing the expression of one or the other eliminates acid tolerance. I propose a two-stage process that allows *S. typhimurium* to phase in acid tolerance as the environmental pH becomes progressively more acidic.**

Neutralophilic bacteria, such as *Salmonella typhimurium*, can grow over a wide range of pH conditions (pH 5 to 9) because of physiologically triggered pH homeostasis mechanisms. Probable mechanisms of pH homeostasis include the use of H^+ antiport systems to maintain internal pH (pH_i) at a relatively constant level (~7.6) over a wide range of external pH conditions (pH_o). There are several excellent reviews on this aspect of pH homeostasis (3, 17). While *S. typhimurium* is capable of growing in minimal media with a pH as low as 5.0, below pH 4.0 the cells undergo a rapid acid death apparently due to an inability to maintain a pH_i suitable for viability (see below and references 7, 8, and 12). However, this laboratory has recently demonstrated that *S. typhimurium* can be adapted to survive this harsh environment. This organism possesses a novel system of acid stress management including an inducible pH homeostasis system that functions beyond normal constitutive pH homeostasis (7). The acidification tolerance response (ATR) occurs if cells are preincubated (preshock adapted) at pH levels near 6 (optimum, pH 5.8) for 1 h prior to challenge exposure at pH 3.3 (acid shock). Under these conditions, survival of adapted cells is 100- to 1,000-fold better than that of unadapted cells. The ATR system was found to require protein synthesis and as such represents a newly described genetic response to environmental stress. Analysis of polypeptide profiles utilizing polyacrylamide gel electrophoresis (PAGE) has revealed that 18 polypeptides change during pH 5.8 preshock exposure. Of these, 12 are induced and 6 are repressed during adaptation. This system appears to be controlled by the ferric uptake regulator, Fur (9). Mutations in the *fur* locus eliminate induction of several acid pH-inducible genes. They also prevent synthesis of the inducible pH homeostasis system and thus confer an extremely acid-sensitive phenotype on the cell. Surprisingly, the role of Fur in the system does not appear to depend on iron availability; Fur may sense internal pH directly. This adaptation response appears quite specific for pH as determined through a variety of genetic, physiologic, and biochemical means. Furthermore, a similar phenomenon has also been described for *Escherichia coli*, indicating that the phenomenon is not limited to *S. typhimurium* (7, 11).

Recently, there have been other reports of two-dimensional polyacrylamide gel analyses of acid-inducible protein synthesis in both *E. coli* and *S. typhimurium*. Each study utilized a different low pH condition. Hickey and Hirshfield (12) examined protein synthesis during a shift from pH 7.0 to 5.0 and found that 19 proteins in *S. typhimurium* and 13 proteins in *E. coli* were increased 2- to 14-fold over the controls. Hyde and Portalier (13) identified 16 proteins of *E. coli* that are produced during a shift from pH 6.9 to 4.3. They refer to these proteins as acid shock proteins (ASPs). An attractive hypothesis based on these observations is that the ASPs could contribute to low pH survival. However, their actual role in surviving acid stress was not examined. Consequently, I studied the ASPs in *S. typhimurium* with specific emphasis on their contribution to successful survival in low pH. I found that neither the preshock ATR nor ASP system alone will provide acid tolerance but that both must be induced for *S. typhimurium* to survive severe acid conditions.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All strains used in this study are derivatives of *S. typhimurium* LT2. SF342 (*atp-102*::Tn*10*) was a gift from G. Ames. JF1819 (*atr-1*) is an

acid-tolerant mutant whose isolation was described previously (7). JF2023 is a spontaneous *fur* mutant whose isolation is described elsewhere (9).

Culture media included E medium supplemented with 0.4% glucose (23) and LB medium (5). The ATR was demonstrated as described previously (7). Briefly, an overnight culture of *S. typhimurium* was used to inoculate parallel E glucose broths (pH 7.7). Cells to be adapted to pH 5.8 were grown with moderate aeration until an optical density at 600 nm (OD_{600}) of 0.2, at which point the pH of the medium was adjusted with HCl. Once the cell density doubled (OD_{600} = 0.4), the pH was readjusted to the challenge pH of 3.3. Aliquots were taken, diluted into cold E medium, and plated onto LB agar. Cultures to be treated at pH 4.3 were grown directly to an OD_{600} of 0.4 (pH 7.7), at which point the pH was adjusted.

Labeling and two-dimensional analysis of ASP. Cells were grown in minimal E medium (pH 7.7) to an OD_{600} of 0.4 (approximately 2×10^8 cells per ml). One-half of the culture was labeled with 50 μCi of Trans ^{35}S (ICN Biomedicals, Inc.) per ml for 15 min. The other half of the culture was adjusted rapidly to pH 4.3 with HCl, allowed to incubate for 15 min, and then labeled as described for the pH 7.7 culture. Labeled cells were harvested and processed for two-dimensional PAGE as described earlier (1, 22). Coordinates provided in Table 1 correspond to the standard polypeptide map presented in reference 22.

Measurement of internal pH. The method for measuring pH_i involved the distribution of radiolabeled weak acids across the cellular membrane. The procedure was described in detail earlier (8). Measurements were made in duplicate within 15 min of a pH shift.

RESULTS

Survival of cells in intermediate-strength acid conditions. *S. typhimurium* can grow in conditions as low as pH 5 and will rapidly lose viability in a matter of hours if shifted below pH 4 (7, 12). However, little is known regarding the kinetics of survival in the intermediate range from pH 4 to pH 5. Consequently, to investigate the relationship between acid shock and the ATR system, it was necessary to determine survival kinetics in the intermediate pH range. Questions could then be asked concerning whether cells that are preshock adapted at pH 5.8 will survive a pH 4 to 5 environment better than unadapted cells. Also, I could determine whether the preshock ATR proteins are identical to the ASP or whether shifting cells from pH 7.7 to 4.3 or 3.3 induces unique ASP.

Figure 1 illustrates the survival rate of *S. typhimurium* following shifts from pH 7.7 to pH 5, 4.3, 3.8, and 3.3. The results indicate that some growth occurred at pH 5, while a logarithmic death was evident at pH 3.8 or below. However, in the intermediate range of H^+ ion concentration (pH 4.3), the cells remained nearly 100% viable for several hours. After 24 h, they did eventually lose viability (data not shown). I next examined whether the ATR system, triggered at pH 5.8, could increase long-term survival or perhaps allow for some growth at pH 4.3. The results, summarized in Fig. 2, reveal that preadapting cells at pH 5.8, while successfully enhancing viability below pH 4 (first bar pair), had no influence upon short (second bar pair)- or long (third bar pair)-term survival at pH 4.3. The long-term decline in viability at pH 4.3 does not seem to be a simple manifestation of stationary-phase effects since pH 7.7 cultures that were allowed to continue into the stationary phase do not lose any viability for several days (21). Thus, survival between pH 4 and 5 (moderate acid conditions) seems distinct from what is required at pH 3.3 (severe acid conditions).

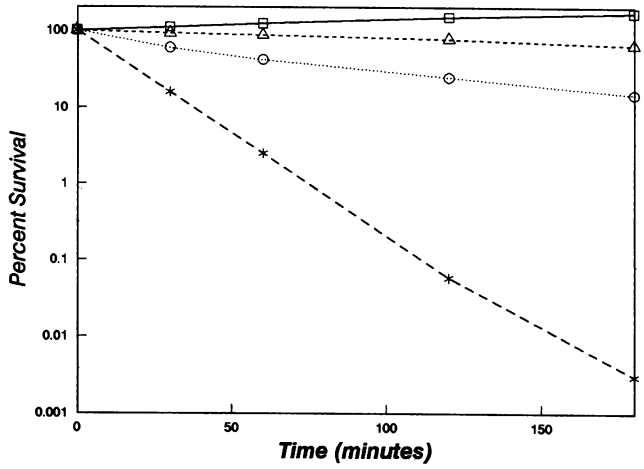

FIG. 1. Survival of *S. typhimurium* under low pH conditions. Cells were grown in E glucose medium (pH 7.7) to an OD_{600} of 0.4, and the pH was adjusted to 5.0 (□), 4.3 (△), 3.8 (○), or 3.3 (×). Viability was measured as colony-forming ability on LB agar plates. One hundred percent viability equals approximately 2×10^8 cells per ml.

Acid shock-inducible proteins. As shown above, cells shifted to intermediate acid conditions survive for a considerable period of time whether or not they are adapted to pH 5.8. Consequently, it seemed logical to assume that unique ASPs may be produced at pH 4.3 whose function is to extend viability during moderate acid stress. To examine this possibility, *S. typhimurium* was grown at pH 7.5 to a cell density of approximately 2×10^8 cells per ml, shifted to pH 4.3 for

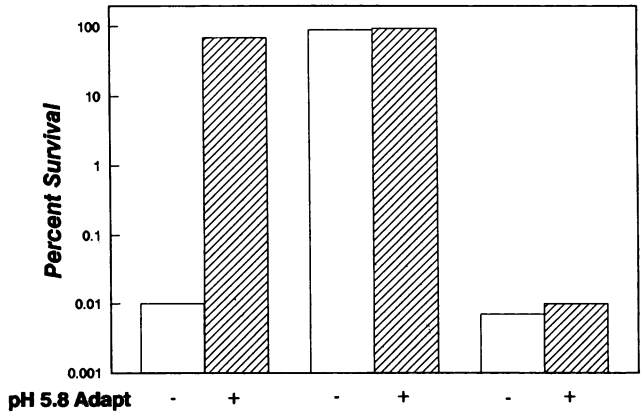

FIG. 2. Effect of pH 5.8 adaptation on pH 4.3 survival. Cultures were grown in minimal E glucose medium (pH 7.7) to an OD_{600} of 0.4 unless they were destined for adaptation. Cultures to be adapted were grown to an OD_{600} of 0.2, the pH was adjusted to 5.8, and the cultures were then grown to an OD_{600} of 0.4. At an OD_{600} of 0.4, all cultures were readjusted to the challenge pH indicated and incubated for the time indicated. One hundred percent survival equals the viable counts obtained immediately before the challenge pH adjustments.

TABLE 1. ASPs

ASP	Coordinates[a] ($x \times y$)	Regulation[b]	Other modulons[c]
1	14 × 36	I	
2	18 × 30	I	
3	23 × 94	R	
4	35 × 88	R	
5	36 × 71	I	Heat shock
6	38 × 36	I	
7	43 × 71	I	Anaerobiosis, iron
8	43 × 86	R	
9	52 × 101	I	
10	46 × 98	R	Oxygen, iron, ATR
11	44 × 84	I	Anaerobiosis
12	50 × 65	I	
13	48 × 60	I	
14	47 × 38	I	Nitrogen, phosphate, NAD
15	47 × 87	I	
16	50 × 60	I	
17	56 × 62	I	Phosphate, anaerobiosis
18	53 × 57	I	Heat shock
19	56 × 79	I	
20	46 × 85	I	Oxygen, ATR
21	57 × 91	R	
22	58 × 97	R	
23	63 × 84	R	
24	68 × 92	I	Nitrogen
25	65 × 64	R	
26	70 × 60	I	Heat shock
27	66 × 42	I	Anaerobiosis
28	73 × 53	I	
29	73 × 64	I	
30	76 × 63	R	Anaerobiosis, nitrogen
31	75 × 74	I	
32	77 × 93	R	
33	74 × 99	I	Iron, ATR
34	89 × 51	I	
35	90 × 31	I	Heat shock
36	90 × 51	I	
37	83 × 35	R	
38	88 × 29	I	Anaerobiosis
39	95 × 104	I	
40	47 × 96	R	
41	103 × 30	I	Heat shock
42	107 × 30	I	
43	104 × 35	I	
44	100 × 60	I	
45	107 × 67	I	
46	105 × 77	R	ATR
47	101 × 92	R	
48	103 × 97 (DnaK)	I	Heat shock
49	112 × 27	R	
50	95 × 28	I	
51	112 × 64	I	
52	100 × 94 (GroEL)	I	Oxygen, heat shock

[a] Coordinates refer to the standard polypeptide map published earlier (22).
[b] Regulation of a given protein is indicated as inducible (I) or repressible (R) during a shift from pH 7.7 to 4.3.
[c] Other stress modulon polypeptides were catalogued in reference 22 (heat shock, anaerobiosis inducible, phosphate limitation, oxygen inducible, nitrogen limitation), reference 7 (ATR), or reference 9 (iron limitation).

15 min, and labeled with [^{35}S]methionine for 10 min. Two-dimensional sodium dodecyl sulfate (SDS)-PAGE analysis of these proteins revealed a dramatic change in the levels of 52 proteins (Fig. 3). This remarkable molecular realignment in the protein profile contrasts to what was reported previously at pH 5.8 (the ATR adaptive pH), at which only modest changes were observed in 18 polypeptides (7). Table 1 lists these pH 4.3 ASPs and indicates where significant overlap occurs with other stress modulons of *S. typhimurium*. It is significant that all but four of the ASPs are different from the preshock ATR proteins described previously (7). ASP10, ASP20, ASP33, and ASP46 correspond to ATR-6, ATR-5, ATR-11, and ATR-15 in the previous report. Conspicuous is the induction of DnaK and GroEL during acid shock. Identity was made on the basis of size and position matches with earlier reports (4). The potential significance of this finding will be discussed below.

ASPs alone do not contribute to short-term severe acid survival in minimal medium. An obvious theory one may derive from the acid shock results noted above is that one or more of these proteins may be required for survival at pH 4.3. Might these proteins also protect cells at the pH 3.3 level? Experiments in which cells were preadapted for 1 h at pH 4.3 revealed that exposure to this H^+ ion concentration, while sufficient to induce ASP synthesis, did not afford any significant protection at pH 3.3 relative to unadapted cells (Fig. 4A). In contrast, adaptation to pH 5.8 successfully protected cells at pH 3.3 when compared with the unadapted control (Fig. 4A). These results indicated that the ASPs alone do not contribute to the adaptive ATR of *S. typhimurium*.

The ASPs, however, may be important for viability at pH 4.3 and could represent a second mechanism for acid survival. To examine this possibility, I adapted cells at pH 4.3 or 5.8 with and without chloramphenicol. This drug has a bacteriostatic effect on cell growth by blocking protein synthesis. Subsequently, growth inhibition can be reversed by removing the drug. If the ASPs are needed for pH 4.3 survival, then inhibiting protein synthesis should make cells sensitive to pH 4.3. Chloramphenicol was added as indicated in Fig. 4B, 15 min prior to adjusting the culture pH to 4.3 or 5.8. Note that in this experiment pH 4.3 really represents both the adaptive and challenge pH conditions. Surprisingly, I found that the inhibition of protein synthesis during a shift from pH 7.7 to pH 4.3 did not decrease survival over a 3-h period (Fig. 4B). In contrast, protein synthesis was absolutely required during the adaptive ATR since preventing protein synthesis during pH 5.8 adaptation eliminated survival at pH 3.3 (Fig. 4A). The effectiveness of chloramphenicol in preventing protein synthesis at pH 4.3 was demonstrated by radiolabeling chloramphenicol-treated cells incubating at pH 4.3. Subsequent SDS-PAGE analysis of labeled proteins revealed that chloramphenicol-treated cells exhibited minimal labeling of proteins relative to untreated pH 4.3 cells (<5%). Furthermore, none of the ASPs that were clearly observed in untreated cells were found in chloramphenicol-treated cells (data not shown). Thus, the mechanism for survival at pH 4.3 is dramatically different from that employed at pH 3.3. Further, it appears that the pH 4.3 survival strategy over the 3-h period does not even require the synthesis of the ASPs noted above and is probably a purely physiologic response most likely involving normal constitutive pH homeostasis mechanisms.

Do known ATR acid-sensitive mutants survive pH 4.3 acid stress? Two types of mutants that exhibit a nonadaptable acid-sensitive phenotype (Atr^-) have been identified in this laboratory. The mutations occur in the *atp* operon encoding the Mg^{2+}-dependent H^+-translocating ATPase and the *fur* locus, whose product is the ferric uptake regulator (7, 8). These mutants do not exhibit an ATR during adaptation at pH 5.8 (i.e., they do not show enhanced survival at pH 3.3 following pH 5.8 adaptation). Since the data obtained thus

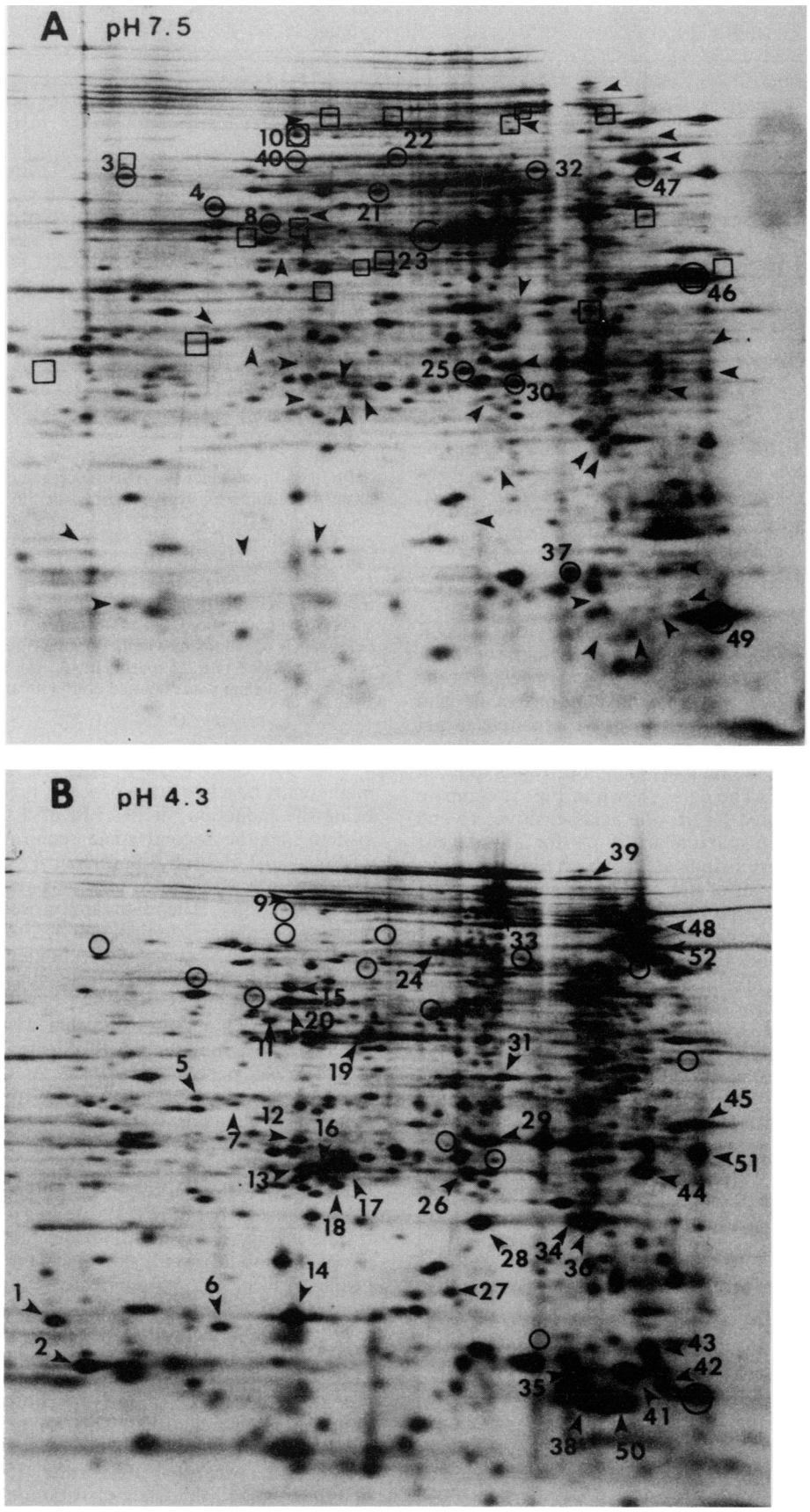

FIG. 3. Two-dimensional PAGE analysis of ASP. Circles and arrows represent proteins that decreased and increased, respectively, following acid shock treatment. Squares mark the positions of the preshock ATR proteins identified in reference 7. (A) Cells grown at pH 7.7. (B) Cells grown at pH 7.7, shifted to pH 4.3 for 15 min, and then labeled for 15 min.

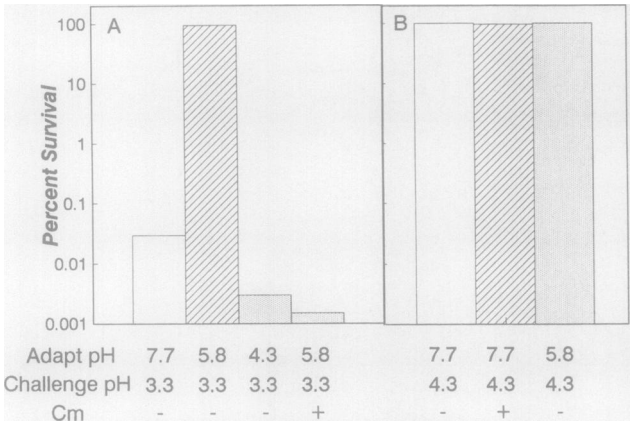

FIG. 4. Effect of pH 4.3 adaptation and chloramphenicol (cm) on acid survival. The basic procedure used was similar to that described in the legend to Fig. 2. Cultures shifted to pH 3.3 (A) and pH 4.3 (B) were incubated for 90 min and 3 h, respectively. Where indicated, chloramphenicol (68 μg/ml) was added 15 min before pH 5.8 adaptation or pH 4.3 challenge.

far indicated that pH 4.3 survival does not rely on the ATR system, it was important to determine whether the acid-sensitive phenotype of *atp* or *fur* mutants was specific for the adaptive ATR. If these mutations cause a nonspecific acid sensitivity, then the mutants should prove sensitive to pH 4.3. On the other hand, if the mutations specifically disrupt the ATR system, then the mutants should prove as tolerant of pH 4.3 as wild type. The data, shown in Fig. 5, compare the survival of unadapted LT2 to the acid-sensitive *atp* and *fur* and acid-tolerant *atr* mutants at pH 4.3 (for 3 h) and pH 3.3 (for 90 min). The results indicate that both the *atp* and the *fur* mutants were tolerant of pH 4.3 conditions but not pH 3.3 even after adaptation at pH 5.8, confirming the significance of *atp* and *fur* specifically to the ATR system.

Survival in extreme acid requires both the acid shock and the ATR systems. The results obtained so far indicated that ASP were not required for survival in moderate acid (pH 4.3) and that acid shock alone will not result in acid tolerance during severe acid exposure (pH 3.3). Why, then, does the cell produce ASPs? I questioned whether acid tolerance

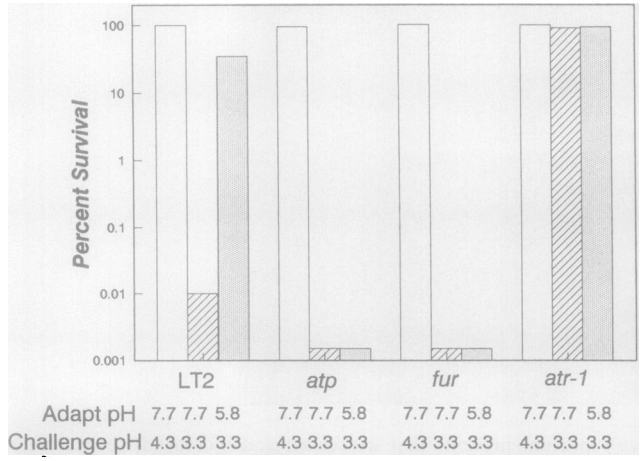

FIG. 5. Low pH susceptibility of *atp*, *fur*, and *atr-1* mutants. The basic procedure was that described in the legends to Fig. 2 and 4.

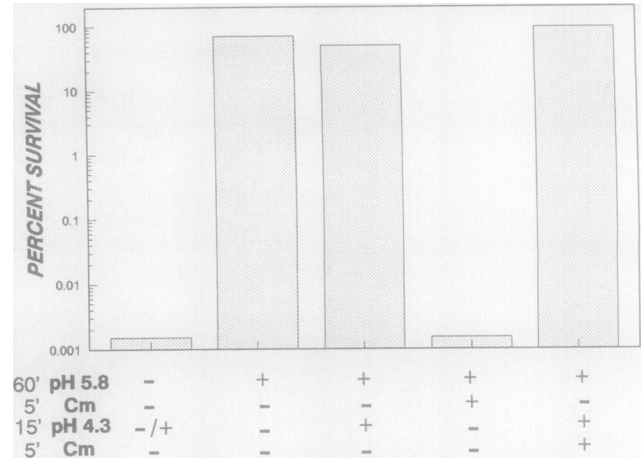

FIG. 6. Involvement of ASP in surviving severe acid stress. The basic procedure was that described in the legend to Fig. 2. Cells were adapted at pH 5.8 for 60 min. Some adapted cultures received chloramphenicol (Cm) (50 μg/ml) for 5 min before a subsequent pH_o shift. Cultures that were acid shocked were incubated at pH 4.3 for 15 min. Some of those also received a 5-min chloramphenicol treatment before a final pH adjustment to pH 3.3. In each case, survival was measured 2 h after shifting to pH 3.3. The columns below each bar indicate whether a given treatment was (+) or was not (−) included in the experiment. A −/+ indicates that the result depicted in the bar was obtained both with and without the indicated treatment.

might really be a two-stage process, the first stage (preshock) being the induction, at pH 5.8, of a new pH homeostasis system and the second stage requiring the production of ASPs at pH 4.3 or 3.3. When I tested this theory, I discovered that tolerance to severe acid did indeed require both conditions. The data are presented in Fig. 6. The addition of chloramphenicol (bar 4) after allowing cells 1 h to adapt at pH 5.8 completely eliminated pH 3.3 survival (compare with bar 2). These cells still possessed the inducible pH homeostasis system (see below) but did not survive pH 3.3. This alone suggested that ASPs were required for survival. Were they the same ASPs that are produced at pH 4.3? The last bar in Fig. 6 indicates that they are. In this experiment, cells were adapted for 1 h at pH 5.8 to induce the preshock homeostasis system and acid shocked at pH 4.3 for 15 min to induce the ASPs, and chloramphenicol was added 5 min before shifting to pH 3.3 to prevent further protein synthesis. Survival was equivalent to that observed in the controls (bars 2 and 3). Clearly, when viewed with the results of Fig. 4, the data indicate that adaptive acid tolerance is a two-stage process requiring the preshock induction of the ATR-specific pH homeostasis system and postshock synthesis of acid shock response survival proteins.

Inducible pH homeostasis system does not require ASP synthesis. Our model of the ATR includes the induction, at pH 5.8, of an ATR-specific pH homeostasis system. Could the ASPs induced at pH 4.3 or below be required for this homeostasis mechanism? The following data argue that ASPs are not required for the pH homeostasis system. ΔpH ($pH_i - pH_o$) was measured within 15 min of the shift by determining [^{14}C]salicylate distribution. All cells had a ΔpH of 1.6 at pH_o 4.3. At pH_o 3.3, unadapted cells (pH 7.7) had a ΔpH of 0.9, pH 5.8-adapted (1 h) cells had a ΔpH of 1.9, and cells adapted for 1 h at pH 5.8 and then treated with chloramphenicol (50 μg/ml) 5 min before the shift had a ΔpH

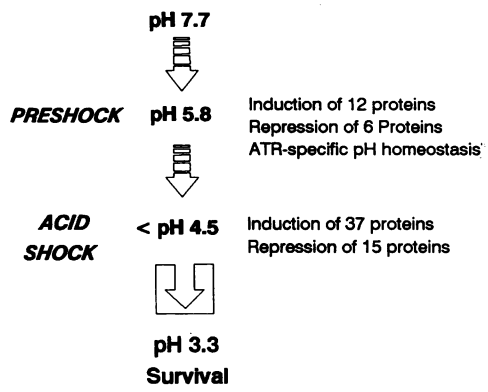

FIG. 7. Schematic representation depicting the two stages of the ATR.

of 1.7. As long as the cells were allowed to adapt at pH 5.8, the inducible pH homeostasis system was evident. Preventing protein synthesis after adaptation but before acid shock did not prevent homeostasis. The evidence supports the two-stage model of ATR encompassing preshock induction of ATR-specific pH homeostasis and a subsequent acid shock to induce additional protection proteins.

DISCUSSION

The data presented expand on the initial discovery of the adaptive ATR of *S. typhimurium*. Clearly, there are two acid tolerance mechanisms which operate in *S. typhimurium*. One, a constitutive system which functions down to a pH of approximately 4, does not require protein synthesis and probably relies on the physiologically controlled housekeeping pH homeostasis mechanisms that include Na^+/H^+ and K^+/H^+ antiporters (14). The second acid tolerance mechanism requires induction and is referred to as the ATR. In contrast to the first system, the ATR will protect cells below pH 4 and requires protein synthesis to be effective. Furthermore, the ATR requires the Mg^{2+}-dependent proton-translocating ATPase (*atp*) as well as the *fur* gene product, while nonadaptive acid tolerance requires neither (7–9).

Further evidence for two distinct acid tolerance systems comes from measuring the internal pH levels of pH 5.8-adapted and unadapted cells at a variety of external pH values (8, 9). There are no significant differences in the internal pH of adapted versus unadapted cells down to pH_o values of 4, reflecting the operation of a noninducible housekeeping pH homeostatic system. Below pH_o 4.0, however, adapted cells maintain pH_i in a range 0.4 to 0.9 units above that of unadapted cells. This inducible pH_i enhancement is a clear manifestation of the ATR system (8). The ATR adaptive system seems to serve, at least in part, as a second, more stringent level of proton defense that operates at very low pH.

However, the ATR is more complex than initially perceived. My results strongly indicate that the induction of acid tolerance is a two-stage process (Fig. 7). The first stage, triggered at pH_o below 6.0, induces synthesis of the ATR-specific homeostasis mechanism discussed above. I refer to this as preshock adaptation. The second stage, triggered below pH_o 4.5, induces a different set of proteins which by themselves will not afford protection against extreme low pH. This is the acid shock stage of the ATR. This stepwise process is logical considering that in nature gradient transitions in pH are more likely to be encountered by an organism than sharp, severe changes. With this in mind, how might the ASPs protect the cell from low pH? The answer is not known, as yet. However, one potentially important role for these proteins could be to minimize the acid denaturation of internal proteins that occurs when internal pH falls below 5.5 (8). We have shown previously that cell death begins at pH_i 5.0 to 5.5 and accelerates as pH_i falls below that range. Consistent with this protein protection model is the observation that several of the chaparonin class of stress proteins are induced during acid shock (13). There is evidence that the chaparonins such as DnaK and GroE are capable of refolding heat-denatured proteins into their native state (15, 18, 19). I suggest they are also capable of refolding acid-denatured proteins. Consequently, in my model of acid tolerance during severe acid stress, the preshock ATR-specific pH homeostasis system would, during severe acid encounters, maintain internal pH at a level that minimizes acid denaturation of proteins, as well as any other acid-induced damage. This system must be in place before entering severe acid and should be induced whenever the cell senses a transition to acid (i.e., preshock). The second stage encompassing the ASPs need not be started until the severe acid encounter. Based on my results, this will occur around pH 4.5, a nonlethal pH stress. The cell is now fully armed to combat proton stress and acid damage should it encounter potentially lethal acid levels (<pH 4). Preshock-adapted cells are able to synthesize ASPs at least down to pH_o 3.3 when the pH_i is still reasonably compatible with protein synthesis. This is why adapted cells will survive a shift directly from pH 5.8 to 3.3.

Are there other possible functions for the ASPs produced at pH 4.3? *S. typhimurium* does find itself in situations in which acid is only one of several hazardous conditions it must deal with. For example, *S. typhimurium* can survive in the phagolysosomes of macrophages where the pH can drop to 4.8 to 4.5 (4, 6, 16). Also present in the phagolysosome are reactive oxygen intermediates and antimicrobial proteins or peptides, all of which conspire to destroy an invading organism. Perhaps the drop to pH_o 4.5 serves as a signal for *S. typhimurium* to produce proteins required for a variety of survival strategies within the macrophage. That signal could be the significant change in pH_i from 7.8 to 6.4 that occurs following a shift to pH_o 4.5 under semianaerobic conditions. Certainly, vacuolar pH levels will significantly influence microbial interactions with the host eukaryotic cell. It is important to note that Buchmeier and Heffron (4) have reported that GroEL and DnaK are induced during *Salmonella* infection of macrophages. This is a condition that does not involve thermoinduction. Since the normal phagolysosome environment is considered to be acid, it is conceivable that vacuolar acid shock contributes to the induction of these proteins.

Even though *S. typhimurium* can survive pH 4.3 (minimal medium) or pH 3.8 (complex medium) for an extended period, it cannot multiply (Fig. 1 and unpublished observation). However, as demonstrated previously by Gale and Epps (10) as well as others (2, 20), there are a variety of decarboxylases and deaminases that respond to low or high pH, respectively. In the presence of low pH and amino acids, the induction of decarboxylases does change the pH of poorly buffered media toward neutrality. That ASPs may play a role in readjusting medium pH, at least in complex media, was demonstrated by exposing untreated and chloramphenicol-treated cells to pH 3.8 in unbuffered LB medium. The rapid neutralization of the medium catalyzed by

untreated cells was completely prevented by the addition of chloramphenicol (data not shown). Of course, this phenomenon is inconsequential with respect to the ATR in minimal medium. The medium is well buffered and lacks the presence of amino acids required to induce the catabolizing enzymes. Nevertheless, it is another approach the organism has to survive in certain acid environments.

Another intriguing question concerns what possible transcriptional signal mechanisms could be utilized by the cell when shifting to pH 4.3? Some ASPs arising at pH_o 4.3 could be the result of conformational changes or even denaturation of specific repressors as a result of an acid pH_i. Even if this theory proves correct in some cases, denaturation certainly cannot be the only signal. For example, Hyde and Portalier (13), in their study of *E. coli*, found that a subset of the proteins induced at pH 4.3 were *rpoH* dependent. RpoH is a sigma factor isomer that is required for the transcription of the heat shock regulon. Consequently, this factor must be active and not denatured at pH_o 4.3. Alternatively, novel signal transduction components could sense pH_o directly or measure changes in ΔpH. Subsequent signals could then be transmitted to the transcriptional apparatus in the form of phosphorylation or other protein modification. Whatever the regulatory scenario, it is evident that the ASPs, in concert with the preshock ATR proteins, play a significant role in acid tolerance both in nature and during pathogenesis.

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