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The acid tolerance response of *Salmonella typhimurium* provides protection against organic acids

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Salmonella typhimurium encounters a variety of acid stress situations during pathogenesis and in the natural environment. These include the extreme low pH encountered in the stomach and a less acidic intestinal environment containing large amounts of organic weak acids (volatile fatty acids). The acid tolerance response (ATR) is a complex defence system that can minimize the lethal effects of extreme low pH (pH 3). The data presented illustrate that the ATR can also defend against weak acids such as butyric, acetic or propionic acids. Although an acid shock of pH 4·4 induced the ATR, growth in subinhibitory concentrations of weak acids did not. Various mutations shown to affect tolerance to extreme acid conditions (pH 3) were tested for their effects on tolerance to weak acids. An *rpoS* mutant lacking the alternative sigma factor σ^s failed to protect cells against weak acids as well as extreme acid pH. The *fur* (ferric uptake regulator) and *atp* (Mg^{2+}-dependent ATPase) mutants defective in extreme acid tolerance showed no defects in their tolerance to weak acids. Curiously, the *atbR* mutant that exhibits increased tolerance to extreme acid pH proved sensitive to weak acids. Several insertions that rendered cells sensitive to organic acids were isolated, all of which proved to be linked to the *rpoS* locus.

Keywords: *Salmonella typhimurium*, acid tolerance, σ^s, RpoS, volatile fatty acids

INTRODUCTION

Organic acids such as acetic, propionic and butyric acids are major constituents of some of the more common microbial niches. For example, the intestinal contents of humans can contain levels of volatile fatty acids (VFAs) up to 150 mM, produced as a result of fermentation by natural flora (Cummings, 1981; MacFarlane *et al.*, 1992; Cummings *et al.*, 1987). However, organic acids can have deleterious effects on the growth and viability of bacteria and for that reason are commonly used as food preservatives (Salmond *et al.*, 1984; Fay & Farias, 1975; Eklund, 1980; Bergeim, 1940; Freese *et al.*, 1973). The lethal effects of these weak acids are not only concentration-dependent

but are also related to the pH of the environment and to the dissociation constant of the chemical. Benzoic acid, for instance, is more bactericidal at a given concentration when in an acid environment than it is at a neutral or alkaline pH. Consequently, a greater concentration of benzoic acid is required at pH 7 to kill an organism than is required at pH 5. This property is due to the fact that the unionized (protonated) form of a weak acid is more permeable to cell membranes than the ionized form (Cherrington *et al.*, 1990, 1991a). Thus, as an environment containing organic acids acidifies, the proportion of undissociated weak acid will increase, making more organic acid available to penetrate a cell. After the unionized form of the acid is inside the cell, the more alkaline intracellular environment (pH$_i$ 7·5–7·8) will cause dissociation of the weak acid and acidification of the cytoplasm. It is generally accepted that the lethal effects of organic acids occur due to the lowering of internal pH although some argue that the intracellular accumulation of the dissociated weak acid itself plays the most significant role in cell death (Cherrington *et al.*, 1991a; Russell, 1992; Salmond *et al.*, 1984). The reported

Abbreviations: ATR, acid tolerance response; VFA, volatile fatty acid.

effects of short chain organic acids on *Escherichia coli* include inhibition of macromolecular synthesis (Cherrington *et al.*, 1990), dissolution of ΔpH and selective enzyme sensitivity to lowered pH$_i$, (reviewed in Cherrington *et al.*, 1991a). The lethal effects apparently do not involve membrane perturbation (Cherrington *et al.*, 1991b). Very little information is available concerning the resistance of enteric organisms to organic acids (Goodson & Rowbury, 1989; Hentges *et al.*, 1995).

A system that potentially could combat organic acid stress is the acid tolerance response (ATR) of *Salmonella typhimurium*. The ATR is a complex stress response system that enables *S. typhimurium* to survive brief encounters with extreme acid environments as low as pH 3 (Foster & Spector, 1995) There are distinct ATR systems induced in exponential-phase and stationary-phase cells (Lee *et al.*, 1994, 1995; Foster & Hall, 1990). The exponential-phase ATR involves the induction of at least 50 proteins called acid shock proteins (ASPs; Foster, 1991, 1993; Lee *et al.*, 1995). Synthesis of eight of those proteins require an alternative sigma factor called σ^S (Lee *et al.*, 1995). The σ^S protein, encoded by *rpoS*, is important as a regulator of stationary phase physiology and during osmotic shock (Loewen & Hengge-Aronis, 1994). We have recently shown that σ^S levels increase upon an acid shock of exponential-phase cells, thereby partially explaining the acid shock induction of this subset of ASPs (Lee *et al.*, 1995; Bearson *et al.*, 1996). These eight σ^S-dependent proteins are required for sustained induction of acid tolerance. Without them acid tolerance is only transiently induced via acid shock for about 20 min. Stationary-phase cells have, in addition to a general stress resistance induced by stationary phase itself, an acid-pH-inducible system of acid tolerance that is independent of σ^S (Lee *et al.*, 1994). Only 15 stationary-phase ASPs are produced, four of which are also induced during the exponential phase.

S. typhimurium often encounters moderately acidic pH environments (pH 4·5–6) that are spiked with organic acids (e.g. intestinal contents and faeces), yet the organism can survive these potentially lethal situations. Because of this we questioned whether or not the ATR might provide a survival advantage even at non-extreme acid pH values. A previous study examining acid-induced resistance to weak acids in *E. coli* used extremely short exposures at very low pH (pH 3·5) (Goodson & Rowbury, 1989). That study used complex medium, which complicates assigning specific systems of tolerance to weak acid protection. *E. coli* possesses several systems of acid resistance which function in complex medium but which are not active in minimal medium (Lin *et al.*, 1995). *S. typhimurium* does not appear to possess these complex-medium-dependent acid resistance systems. Consequently, we examined organic acid exposures in *S. typhimurium* at more moderate acid pH (pH 4·4) and for periods of time that are more reflective of *in vivo* situations. In addition, we examined the various ATR systems and several mutants in genes known to affect tolerance to hydrochloric acid for their abilities to survive exposure to weak acids.

METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are all derivatives of *S. typhimurium* and are listed in Table 1. The minimal medium used was medium E containing 0·4 % glucose (minimal E glucose) (Vogel & Bonner, 1956). The complex media used were Luria-Bertani (LB) (Davis *et al.*, 1980) and MacConkey (Difco) media. Cultures were grown in semi-aerobic conditions: 3 ml media in 13 × 100 mm test tubes, with shaking at 240 r.p.m., at an angle of 45 ° and at 37 °C. Antibiotics used included kanamycin (Km, 100 μg ml^{-1}) and tetracycline (Tc, 10 or 20 μg ml^{-1}) for minimal and complex media, respectively.

Exponential-phase ATR. This was measured in the present study essentially as described earlier (Lee *et al.*, 1995) but with modification to test the effects of weak acids. Exponential-phase cells were obtained by making a 1:200 dilution of an overnight culture in fresh minimal E glucose (3·0 ml) pH 7·7. When the cultures reached a cell density of 2 × 10^8 c.f.u. ml^{-1}, the weak acid to be tested was added to unadapted cells and the pH adjusted to a value of 4·4 with HCl (lethal challenge). Adapted cells were prepared by acid shock treatment at pH 4·4 for 1 h before adding the weak acid for acid challenge. Viable counts were determined for adapted and unadapted cells at various times after acid challenge. Each experiment was performed a minimum of two and usually three times. The data shown represent mean percentage survival values, with variability for each time point not exceeding 50 % of the stated value (i.e. a 50 % mean survival value ranged between 25 and 75 % upon repetition). Weak acids or their salts were added at the following concentrations unless indicated otherwise: sodium benzoate, 10 mM; sodium propionate, 150 mM; butyric acid, 50 mM; and sodium acetate, 100 mM. The VFA cocktail contained 87 mM acetic acid, 25 mM butyric acid and 37 mM sodium propionate and was designed to approximately reflect measured values in intestinal contents (Cummings, 1981). The cocktail was adjusted to pH 4·4 before addition.

Stationary-phase ATR. This was measured as described earlier (Lee *et al.* 1994). Cells were initially grown to stationary phase in minimal E glucose pH 8 and harvested. Unadapted cells were directly resuspended at a concentration of 2 × 10^8 c.f.u. ml^{-1} in minimal E glucose pH 4·4 containing the weak acid to be tested whereas adapted cells were prepared by resuspension in minimal E glucose pH 4·4 for 2 h before adding the weak acid.

Table 1. *S. typhimurium* strains

Strains	Genotype	Source
UK1 (χ3761)	Wild-type	R. Curtiss, III (Curtiss *et al.*, 1981)
SF261(TT10287)	*hisD9953*::MudJ *his-9941*::Mud1	J. Roth, University of Utah, USA.
JF2690	UK1 *rpoS*::Ap	Lee *et al.* (1995)
JF2733	UK1 *atbR*::Tn10	UK1 × P22(JF2471)
JF2872	UK1 *relA*::Tn10	UK1 × P22(SF137)
JF2918	UK1 *zdg*::MudJ ΔnlpD-rpoS	UK1 × P22(SF261)
JF2921	UK1 *atr-40*::MudJ	UK1 × P22(SF261)
JF2922	UK1 *atr-45*::MudJ	UK1 × P22(SF261)
JF2923	UK1 *atr-46*::MudJ	UK1 × P22(SF261)
JF2925	UK1 *atr-48*::MudJ	UK1 × P22(SF261)

Alternatively, cells grown overnight in minimal E glucose at pH 8 (unadapted cells) and cells grown overnight in minimal E glucose at pH 5·5 (adapted) were diluted to 2×10^6 c.f.u. ml^{-1} in minimal E glucose pH 3·0. Two to three repetitions were performed for each experiment with percentage survival values reproducible to within 50% of the stated value.

Genetic procedures. Transductions were performed using P22 HT 105/1-*int* as described previously (Holley & Foster, 1982; Aliabadi *et al.*, 1988). Mu*d*J transpositions and Tn*10* insertions near Mu*d*J insertions were constructed as described earlier (Foster & Bearson, 1994). General mapping of Tn*10* insertions were made using the Mu*d*-P22 prophage system described by Benson & Goldman (1992).

Organic-acid mutant screening procedure. *S. typhimurium* UK1 was infected with P22 phage propagated on SF261, plated on LB for 5 h and replicated on minimal E glucose Km. His$^+$ Km^R colonies were picked to microtitre dishes containing minimal E glucose Km (100 μl) for growth and storage (plates were stocked by adding 50 μl sterile glycerol per well). Seven thousand colonies were screened as follows for sensitivity to weak acids. Fresh microtitre plates containing 100 μl LB Km were inoculated, grown overnight and 5–10 μl transferred to new microtitre plates containing 100 μl pH 4·4 LB containing 10 mM benzoate. Plates were incubated at 37 °C for 1, 2, 3, 4 and 5 h, and at each time point survivors rescued by transferring 5–10 μl culture to MacConkey Km plates. MacConkey medium was used for rescue because the presence of bile salts and dyes eliminates many acid-damaged cells that are not inviable on LB. Mutants that could not survive a 2 h exposure to benzoate in this assay were reisolated from the master plate and retested. Eight of the retested mutants maintained the organic-acid-sensitive phenotype. Each of these Mu*d*J mutations was transferred to fresh UK1 cells and retested in the microtitre plate assay followed by *in vitro* ATR assays before stocking.

RESULTS AND DISCUSSION

The exponential-phase and stationary-phase ATR can protect against organic acids

As shown in Fig. 1 (square symbols), organic acids are lethal at moderate concentrations in an acid environment. The pH 4·4 condition itself does not affect viability of *S. typhimurium* over the period of time examined (Foster, 1991). However, the addition of various organic acids (Fig. 1a, b, c) and a VFA cocktail mimicking intestinal and faecal conditions (Fig. 1d) clearly proved lethal over a short period of time at this pH. These organic acid concentrations had no effect on viability if added to pH 7-grown cells (data not shown). Consequently, we chose to examine what effect inducing the ATR might have in combating the lethal effects of several weak acids. Shown in Fig. 1 are the results of stressing cells at pH 4·4 with 10 mM benzoate (a), 150 mM propionate (b), 100 mM acetate (c) or a VFA cocktail including 87 mM acetic acid, 25 mM butyric acid and 37 mM propionate (d). In each instance, cells that were acid-shock-adapted for 1 h at pH 4·4 (Fig. 1, triangles) before adding the weak acid were better prepared to survive the toxic effects of organic weak acids. It appears that the exponential-phase ATR can successfully protect against stress imposed by weak acids.

The stationary-phase ATR provided increased resistance to some, but not all weak acids. The data in Table 2
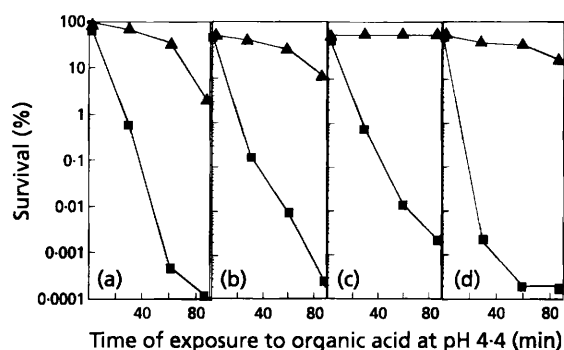
Fig. 1. Effect of the exponential-phase ATR on organic acid resistance. Cells were grown to mid-exponential phase (2×10^8 c.f.u. ml^{-1}) in minimal E glucose pH 7·7 prior to addition of 10 mM benzoate (a), 150 mM propionate (b), 100 mM acetate (c) or a VFA cocktail containing 87 mM acetic acid, 25 mM butyric acid and 37 mM propionate (d). The pH of unadapted cultures (■) was adjusted to 4·4 immediately after addition of organic acids. The pH of adapted cultures (▲) was adjusted to 4·4 for 1 h before adding organic acids. Values represent the means of three experiments and in all cases 100% survival was equivalent to approximately 2×10^8 c.f.u. ml^{-1}.

illustrates that inducing the stationary-phase ATR by overnight growth at pH 5·5 provided protection against propionate and acetate but not against benzoate, even though the benzoate concentration was much lower than the other two weak acids tested. This finding supports the notion that the lethal effects of some weak acids go beyond simply acidifying internal pH (Cherrington *et al.*, 1991a; Russell, 1992; Salmond *et al.* 1984). The nature of the anion appears to have a significant effect, at least in stationary-phase cells. The fact that exponential-phase cells in which acid tolerance had been induced were better able to tolerate weak acids than stationary-phase cells implies different mechanisms of tolerance occur in exponential-phase versus stationary-phase cells.

It is important to note that simple growth arrest did not induce tolerance to acid stress. Previous studies have attempted to induce acid tolerance by osmotic shock, H_2O_2 and temperature shock. None of these conditions successfully induced cross protection against acid conditions (Foster & Hall, 1990). In addition, it is clear that protein synthesis is required for the development of acid tolerance (Foster & Hall, 1990; Foster, 1991, 1993; Lee *et al.*, 1995; Bearson *et al.*, 1996).

Growth in organic acids does not induce organic acid resistance at low pH

As discussed above, acid shock involving growth arrest successfully induced resistance to weak acids. An investigation was conducted to determine if resistance to weak acids could be induced by the weak acids themselves without growth arrest, i.e. will growth in a sublethal concentration of organic acid induce an adaptive response or does acid shock alone induce resistance to organic acids? Cells were grown in minimal E glucose containing either 6 mM acetate, 0·25 mM benzoate or 0·75 mM

Table 2. The effect of stationary-phase ATR on resistance to organic acids

S. typhimurium UK1 was grown overnight in minimal E glucose pH 8 (unadapted) or minimal E glucose pH 5·5 (adapted). Cultures were diluted in minimal E glucose pH 4·4 containing 150 mM acetate, 10 mM benzoate or 100 mM propionate. Results are the means of three experiments.

| Exposure (h)	Survival (%)					
	Acetate		Benzoate		Propionate	
	Unadapted	Adapted	Unadapted	Adapted	Unadapted	Adapted
1	< 0·04	24	< 0·04	< 0·04	0·76	44·4
2	< 0·04	0·11	< 0·04	< 0·04	< 0·03	15·5
3	< 0·04	< 0·04	< 0·04	< 0·04	< 0·03	5·4
4	< 0·04	< 0·04	< 0·04	< 0·04	< 0·03	1·3

propionate and allowed to mid-exponential phase. The concentrations of organic acids were 5- to 10-fold below the minimum inhibitory concentrations at pH 7. Each culture plus a control culture grown in the absence of added organic acid was diluted 1/1000 in a series of media at pH 4·4 containing 150 mM acetate, 5 mM benzoate or 100 mM propionate. Viability was measured over the course of several hours. None of these growth conditions resulted in enhanced resistance to the organic acids at low pH (data not shown); acid shock adaptation was required to develop this type of resistance.

Effect of *rpoS, fur, atp* and *atbR* mutations on organic acid ATR

Several mutations that affect acid tolerance at pH 3 were previously characterized. The genes involved include the alternative sigma factor locus *rpoS* (Lee *et al.*, 1995), the ferric uptake regulator, *fur* (Foster & Hall, 1992; Foster, 1991), the major proton translocating ATPase *atp* (Foster & Hall, 1991) and an apparent regulator of acid tolerance, *atbR* (Foster & Bearson, 1994; B. Bearson & J. W. Foster, unpublished). These mutations were tested for their effects on resistance to weak acids. Fig. 2(b) shows that mutations in *rpoS* have a severe dampening effect on inducible resistance to organic acid similar to the reported effect of *rpoS* on extreme (pH 3) acid tolerance. The *atbR* mutation, however, had an effect opposite to that reported earlier for extreme acid tolerance. This mutation was shown to increase tolerance to pH 3 but, surprisingly, increased sensitivity to organic acids even in acid-shock-adapted cells (Fig. 2c). Mutations in *fur* and *atp* did not have any obvious effect on inducible organic acid resistance (data not shown). This was not unexpected since their effects are seen mostly on the transient ATR observed in *rpoS* mutants (Lee *et al.*, 1995). A *relA* mutation not known to affect the ATR likewise had no effect upon inducible resistance to organic acids (Fig. 2d).

Isolation of organic-acid-sensitive mutants

The protonophore dinitrophenol was previously used to isolate mutants defective in the transient ATR (Foster &
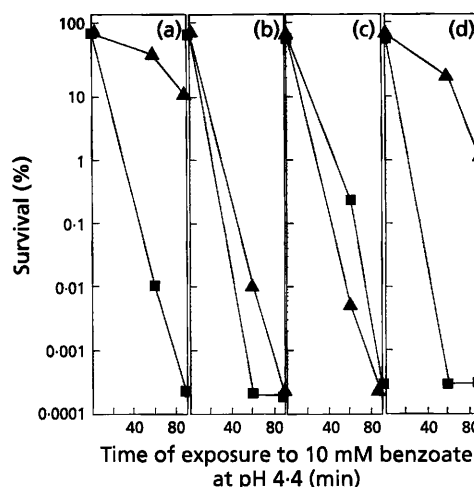
Fig. 2. Effects of *rpoS, atbR* and *relA* mutations on resistance to benzoate. *S. typhimurium* UK1 (a), and *rpoS* (b), *atbR* (c) and *relA* (d) mutants were grown and treated with 10 mM benzoate at pH 4·4 as described in the legend to Fig. 1. ■, Unadapted cultures; ▲, pH 4·4-adapted cultures. Values represent the means of three experiments and in all cases 100% survival was equivalent to approximately 2×10^8 c.f.u. ml^{-1}.

Bearson, 1994) but there has been little success using this technique to isolate mutations that affect the sustained ATR. The observation that the ATR can provide tolerance to organic acids provided a new mutant screening methodology. Microtitre plates containing LB Km were used to prepare a series of random MudJ insertion mutants for testing. Overnight cultures were replicated to a second series of microtitre plates containing LB plus 10 mM benzoate. This is a lethal screening condition. After 2 h, survivors were identified following replication to MacConkey lactose medium. Insertion mutants that could not survive the 2 h in benzoic acid were picked from the master microtitre dish and their insertions transferred to fresh UK1 cells. The tolerance of these mutants to a weak acid stress (benzoate) at pH 4·4 and to an extreme acid stress (pH 3) are shown in Figs 3
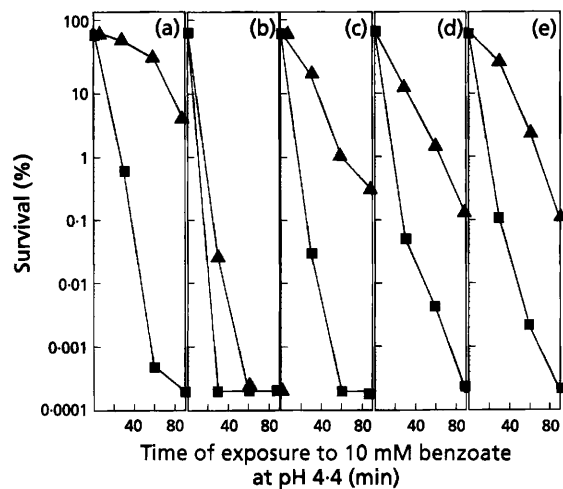
Fig. 3. Organic acid ATR of mutants sensitive to organic acids. *S. typhimurium* strains UK1 (a), JF2918 (b), JF2921 (c), JF2923 (d) and JF2925 (e) were grown and treated with 10 mM benzoate at pH 4·4 as described in the legend to Fig. 1. ■, Unadapted cultures; ▲, pH 4·4-adapted cultures. Values represent the means of three experiments and in all cases 100% survival was equivalent to approximately 2×10^8 c.f.u. ml^{-1}.
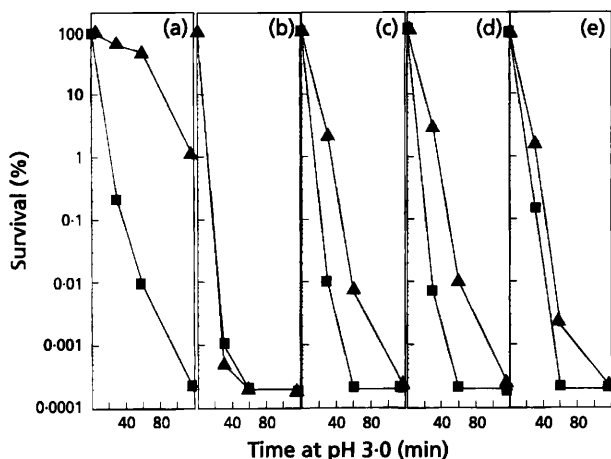
Fig. 4. Strong acid (HCl) ATR of mutants sensitive to organic acids. *S. typhimurium* strains UK1 (a), JF2918 (b), JF2921 (c), JF2923 (d) and JF2925 (e) were treated as described in the legend to Fig. 1 without any addition of organic acids and the pH adjusted to pH 3. ■, Unadapted cultures; ▲, pH 4·4-adapted cultures. Values represent the means of three experiments and in all cases 100% survival was equivalent to approximately 2×10^8 c.f.u. ml^{-1}.

and 4, respectively. The insertion in JF2918 dramatically diminished tolerance to both conditions. The other three insertions eliminated extreme acid tolerance but, surprisingly, had little effect on organic acid tolerance even though the cells were initially isolated as being organic-acid-sensitive. Part of the reason for this apparent paradox appears to be the use of MacConkey medium in the initial mutant selection strategy. The bile salts and

dyes in this medium are known to inhibit recovery of acid-damaged cells (Roth & Keenan, 1971; Przybylski & Witter, 1979). Since the organic acid tolerance assay involves recovery on LB, the effect of these mutations on organic acid damage were minimized. In addition, the results suggest that the acid stress inflicted by pH 3 is more severe than that by pH 4·4 plus organic acids since these three mutants survived the latter but not the former condition.

The acid-sensitive insertion mutations map to the *rpoS* region

Cotransductional analysis with a library of Tn*10* insertions placed the Mu*d*J insertions in strains JF2921, JF2923 and JF2925 near *rpoS*. However, only a 50% reduction in catalase activity was observed (data not shown) indicating these mutations are not in *rpoS* but probably interrupt transcription of *rpoS* from one or more of the multiple promoters for this gene (Takayanagi *et al.* 1994; Lange *et al.* 1995). Cloning and sequence analysis of the Mu*d*J fusion junction in JF2918 revealed that the insertion in this strain occurred just upstream of the *nlpD* gene, which itself is upstream of *rpoS*. The strain does not produce σ^S as determined by Western blotting (data not shown) and could not be transduced with Tn*10* insertions in or near *rpoS*, although transductions with other Tn*10* insertions were normal. The results suggest that a deletion extending through *rpoS* occurred adjacent to the Mu*d*J insertion making JF2918 refractory to recombination in this area of the chromosome. The complete loss of σ^S made this strain sensitive to both organic and HCl acid stress. The residual σ^S present in the other strains permitted a degree of organic acid resistance in liquid culture but did not confer tolerance to strong acid (pH 3). This again suggests that pH 3 acid stress is more severe than the stress imposed by weak acids at pH 4·4. The fact that *rpoS* mutants are sensitive to organic acid stress confirms that growth arrest alone will not confer resistance to organic acids.

The results from this work have revealed that the inducible ATR, in addition to its importance for surviving strong acid environments (pH 3), is an important means of surviving exposures to weak acids prevalent in the gastro-intestinal environment. Thus, the ATR will protect *S. typhimurium* against both types of acid stress that the organism will encounter during pathogenesis. The alternative sigma factor σ^S clearly plays an important role in protecting against the lethal effects of weak acids. Preliminary evidence suggests that the primary role of σ^S in acid tolerance may be to handle the stress imposed by weak acids. It may not be required for acid tolerance in the absence of weak acids (B. Bearson & J. W. Foster, unpublished data).

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