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Escherichia coli and *Salmonella enterica* Are Protected against Acetic Acid, but Not Hydrochloric Acid, by Hypertonicity▽

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Chapman et al. (B. Chapman, N. Jensen, T Ross, and M. B. Cole, Appl. Environ. Microbiol. 72:5165–5172, 2006) demonstrated that an increased NaCl concentration prolongs survival of *Escherichia coli* O157 SERL 2 in a broth model simulating the aqueous phase of a food dressing or sauce containing acetic acid. We examined the responses of five other *E. coli* strains and four *Salmonella enterica* strains to increasing concentrations of NaCl under conditions of lethal acidity and observed that the average "lag" time prior to inactivation decreases in the presence of hydrochloric acid but not in the presence of acetic acid. For *E. coli* in the presence of acetic acid, the lag time increased with increasing NaCl concentrations up to 2 to 4% at pH 4.0, up to 4 to 6% at pH 3.8, and up to 4 to 7% (wt/wt of water) NaCl at pH 3.6. *Salmonella* was inactivated more rapidly by combined acetic acid and NaCl stresses than *E. coli*, but increasing NaCl concentrations still decreased the lag time prior to inactivation in the presence of acetic acid; at pH 4.0 up to 1 to 4% NaCl was protective, and at pH 3.8 up to 1 to 2% NaCl delayed the onset of inactivation. Sublethal injury kinetics suggest that this complex response is a balance between the lethal effects of acetic acid, against which NaCl is apparently protective, and the lethal effects of the NaCl itself. Compared against 3% NaCl, 10% (wt/wt of water) sucrose with 0.5% NaCl (which has similar osmotic potential) was found to be equally protective against adverse acetic acid conditions. We propose that hypertonicity may directly affect the rate of diffusion of acetic acid into cells and hence cell survival.

We previously observed that inactivation of *Escherichia coli* O157 SERL 2 by acetic acid at adverse pH in a broth model simulating the aqueous phase of acidic sauces and dressings was reduced by the presence of NaCl (4). Specifically, the time to a 3-$\log_{10}$-unit reduction (t_{3D}) of *E. coli* SERL 2 as function of NaCl concentration was significantly nonmonotonic; that is, the t_{3D} initially increased when NaCl was increased (from 1 to 3% [wt/wt] of solution), but the t_{3D} decreased upon a further increase in NaCl concentration (to 8% [wt/wt] of solution) (4). The statistical significance of this "nonmonotonic" response increased with increasing exposure time from 24 to 72 h (at 23°C), primarily due to a proportionally greater increase in inactivation at 1% (wt/wt) NaCl with increasing treatment time than that which was observed at higher NaCl concentrations (4).

The combination of acid and NaCl is a common example of the food industry's "hurdle" approach, which is used to preserve a large and diverse range of foods, including acidic dressings and sauces, fermented meats, cheeses, and preserved vegetables. Given the widespread use of this hurdle combination in food manufacturing, the first aim of this study was to determine whether the observed protection of *E. coli* SERL 2 from acid inactivation by NaCl is common among *E. coli* and *Salmonella enterica* and at what NaCl concentration maximum protection is achieved. A second aim was to determine whether NaCl protection is specific against acid pH in general or against acetic acid in particular. Third, possible protection against acid inactivation by another osmolyte, sucrose, was assessed to resolve whether the effect is solute specific.

When cells are placed in hypertonic environments, plasmolysis occurs as the cytoplasmic volume reduces due to water loss by osmosis. The thin peptidoglycan layer of gram-negative microorganisms is anchored to the cytoplasmic membrane and can be distended by plasmolysis or even ruptured when plasmolysis is more extreme. Decad and Nikaido (5) observed that the cytoplasmic volume in gram-negative microorganisms was reduced to ~50% at ~0.3 M NaCl but that the plasmolysis-induced cell wall damage was minimal. At 0.5 M (2.9%, wt/wt) NaCl, however, they observed cell wall damage in a large fraction of cells. Thus, in the experiments described here we explored the mechanism of the protective effect of NaCl, and specifically cell wall damage in *E. coli* populations simultaneously exposed to NaCl and either acetic or hydrochloric acid (HCl), by enumeration of both injured and noninjured survivors by culture on media with and without bile salts.

MATERIALS AND METHODS

Cultures and culture conditions. Five nonpathogenic strains of *E. coli* were used in these studies, namely, FRRB (Food Research Ryde Bacterial culture collection) 2697, 2698, 2699, 2700, and 2701. In addition, four strains of *Salmonella* were used: FRRB 2742 (*S. enterica* serovar Montevideo), 2743 (*S. enterica* serovar Poona), 2746 (*S. enterica* serovar Typhimurium), and 2747 (*S.* Typhimurium). Cultures were maintained as glycerol stocks at −80°C and activated by transferring a loopful from the stock into 10 ml of nutrient broth (NB) (CM0001; Oxoid, United Kingdom) prepared according to the manufacturer's instructions. NB cultures were then incubated at 37°C (±1°C) for 22 h. For the preparation of experimental inocula, 10 μl of 22-h NB cultures was transferred to 10 ml

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tryptone soy broth (CM0129; Oxoid) with 1% total glucose (TSB1%G) and incubated with shaking at 200 rpm for 22 h at 37°C (±1°C). At the conclusion of incubation, the pH of TSB1%G cultures was determined using pH indicator papers (type CS, pH 3.8 to 5.5; Whatman International Ltd., United Kingdom) to ensure that it was approximately 4.2, indicating that acid conditioning of the cells was promoted during growth.

Experimental matrix design. For *E. coli*, the effect of increasing NaCl concentration on cell survival in NB was assessed for each of five acid treatments: in the presence of acetic acid at pH 3.6, 3.8, and 4.0 and in the presence of HCl at pH 3.6 and 3.8. For *Salmonella* isolates, the effect of increasing NaCl concentration on cell survival in NB was assessed for each of four acid treatments: in the presence of acetic acid at pH 3.6, 3.8, and 4.0 and in the presence of HCl at pH 3.6 and 3.8. No assessment of inactivation in the presence of HCl at pH 4.0 was attempted because preliminary experiments (data not shown) showed that the selected *E. coli* and *Salmonella* isolates were able to grow in NB under this condition. Similarly, no assessment of inactivation of *Salmonella* in the presence of acetic acid at pH 3.6 was undertaken because preliminary experiments (data not shown) indicated that *Salmonella* was too rapidly inactivated to enable reliable enumeration.

The survival response of *E. coli* and *Salmonella* in acidified NB was assessed at eight concentrations of NaCl: 0.5, 1, 2, 3, 4, 5, 6, and 7% (wt/wt of water) (i.e., 0.5 to 7 g NaCl in 100 g of water used to prepare the broth, or equivalent to 0.5, 1.0, 1.9, 2.9, 3.8, 4.7, 5.6, and 6.5% [wt/wt] of prepared NB, taking into account other broth ingredients). In addition, for all isolates, the effect of sucrose on survival was assessed in NB containing 0.5% (wt/wt of water) NaCl and 10% (wt/wt of water) sucrose (equivalent to 9.0% [wt/wt] of NB) and in NB containing 3% (wt/wt of water) NaCl and 10% (wt/wt of water) sucrose (equivalent to 8.8% [wt/wt] of NB).

Survival experiments. Twenty grams of NB was dispensed aseptically into sterile 28-ml screw-cap polypropylene containers. Twenty-microliter volumes of TSB1%G cultures were used to inoculate each container to achieve an initial concentration of cells of ~10^6 CFU/g. Inoculated NB was incubated statically at 23°C (±1°C). Within 30 min of inoculation, a 1-ml sample of each inoculated NB culture was withdrawn and decimally diluted twice in buffered peptone water (CM0509; Oxoid). Dilutions were surface plated (0.1 ml) onto duplicate tryptone soy agar (TSA) (CM0131; Oxoid) plates. Plates were incubated aerobically at 37°C (±1°) for 48 h prior to counting. Inoculated containers continued to be incubated at 23°C (±1°C) and were sampled at predetermined time intervals for up to 79 h, with a maximum of nine time points (0.5, 1, 7, 24, 31, 48, 55, 72, and 79 h [±0.5 h]). Survivors at each sampling time were enumerated as described above. Each treatment was duplicated using individually grown inocula.

Curve fitting. Viable cells counts were expressed as $\log_{10}$ of the survival fraction, $\log [N(t)/N_0]$, where $N(t)$ and N_0 are the momentary and initial concentrations of cells, respectively. The detection threshold of the experiment was approximately 50 CFU/g. Using the Solver function in Microsoft Excel (Microsoft Corporation), data were fitted to the log logistic model, $\log [N(t)/N_0] = -\ln \{1 + \exp[k_1 \times (t - t_c)]\}$, where t_c (h) is a measure of the shoulder period ("lag") or delay time before inactivation was detected, and k_1 is a constant representing the semilogarithmic inactivation rate when $t \gg t_c$.

Assessment of relative severity of acid conditions. Survival in the presence of 0.5% (wt/wt of water) NaCl was used to assess the relative severity of acid treatment conditions against *E. coli* and *Salmonella*. The effects of acid treatments were compared by single-factor analysis of variance (ANOVA) ($P < 0.05$) of average t_c and k_1 (At_c and Ak_1) values using the single-factor ANOVA tool of Microsoft Excel. For *E. coli* there were generally 10 estimates, comprised of five *E. coli* isolates tested in duplicate, while for *Salmonella* there were generally 8 values, derived from four *Salmonella* isolates tested in duplicate.

Effect of NaCl at different concentrations. The effect of NaCl concentration was assessed by statistical analysis of average relative t_c (At_cR) values for *E. coli* and *Salmonella* as follows. Based on previous observations of the response of *E. coli* SERL 2 (4), it was assumed that the greatest protection would occur in formulations with 3% (wt/wt of water) NaCl. Thus, for each acid treatment and each *E. coli* or *Salmonella* isolate, and for each duplicate, t_cR values were calculated by dividing the t_c at each NaCl concentration by the t_c at 3% (wt/wt of water) NaCl. At_cR values for *E. coli* and *Salmonella* for each acid treatment at each NaCl level were then determined by averaging the t_cR values. The effect of NaCl concentration was then evaluated by single-factor ANOVA ($P < 0.05$) of At_cR using the single-factor ANOVA tool of Microsoft Excel.

Effect of sucrose versus NaCl. At_cR values were statistically analyzed to compare protection against acid by sucrose, and by sucrose in combination with NaCl, with protection by NaCl alone. For each acid treatment and each *E. coli* or *Salmonella* isolate, and separately for each duplicate, t_cR values were calculated as follows. For comparison of t_c values in formulations containing 0.5% NaCl without sucrose (formulation J) and formulations containing 0.5% NaCl and 10% (wt/wt of water) sucrose (formulation I), t_cR values were calculated relative to those in formulations containing 3% NaCl without sucrose (formulation C). For comparison of formulation I and formulation C, t_cR^1 values were calculated relative to t_c values in formulations containing 3% NaCl and 10% (wt/wt of water) sucrose (formulation H). For comparison of formulation C and formulation H, t_cR^2 values were calculated relative to t_c values in formulations I. At_cR values for *E. coli* and *Salmonella* for each treatment were determined by averaging the relevant estimates The effect of NaCl and sucrose treatments on At_cR were compared by single-factor ANOVA ($P < 0.05$) using the single-factor ANOVA tool of Microsoft Excel.

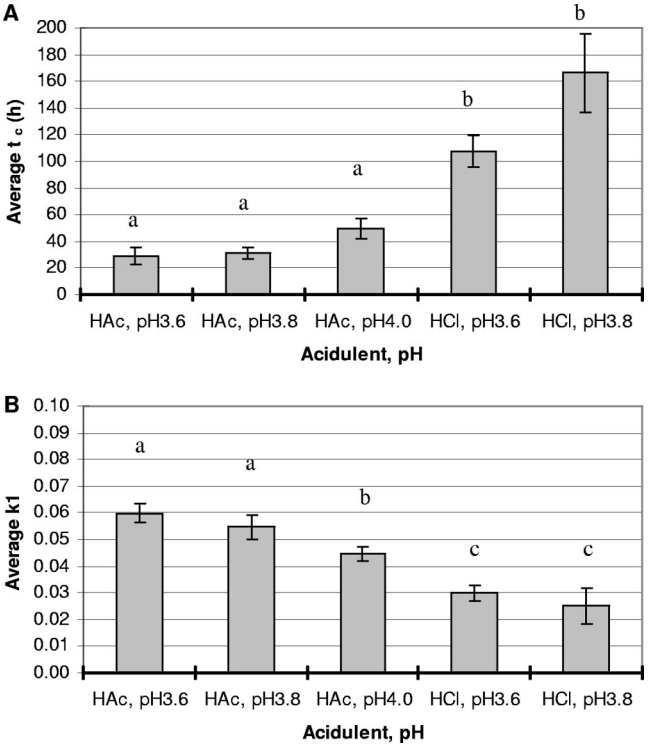

FIG. 1. Average delay time (At_c) before inactivation was detected (A) and average inactivation rate (Ak_1) (B) for *E. coli* in response to adverse acidulent and pH in the presence of 0.5% (wt/wt of water) NaCl. HAc, acetic acid. Error bars indicate standard errors of the means ($n = 10$, except for HCl at pH 3.8, where $n = 9$). Different letter codes (a to c) indicate significant differences ($\alpha = 0.05$) between successive acid treatments. Acid treatments not significantly different are given the same letter.

Assessment of cell wall damage. Cell wall/cell membrane damage was investigated using *E. coli* FRRB 2701 inoculated into NB at pH 3.6, acidified with either HCl or glacial acetic acid and containing 0.5 to 7% NaCl as previously described. At 24, 48, and 72 h, samples were withdrawn from the inoculated NB and plated onto both TSA and TSA plus 0.15% bile salts. Plates were incubated as described above. The percentage of sublethally injured cells after different treatment times was calculated by comparing numbers of CFU on TSA to those on TSA plus 0.15% bile salts (13).

RESULTS

Relative severity of acid conditions. For the five strains of *E. coli* in NB containing 0.5% NaCl, the At_c was not significantly different ($\alpha = 0.05$) among broths acidified with acetic acid to pH 3.6, 3.8, or 4.0, and the At_c was not significantly different between NB acidified with HCl to pH 3.6 or 3.8 (Fig. 1A). However, the At_c was significantly different ($\alpha = 0.05$) between NB acidified with acetic acid and NB acidified with HCl. The

TABLE 1. ANOVA of At_{cR} for *E. coli* in response to NaCl concentration

NaCl concn (%, wt/wt of water)	At_{cR}[a]				
	Acetic acid			HCl	
	pH 3.6	pH 3.8	pH 4.0	pH 3.6	pH 3.8
0.5	0.55 (0.05)a	0.47 (0.06)a	0.58 (0.05)a	*0.99 (0.18)a*	***1.19*** *(0.23)a*
1	0.74 (0.05)bS	0.61 (0.05)aN	0.81 (0.06)bS	***1.37*** *(0.24)aN*	*1.10 (0.26)aN*
2	0.82 (0.05)bS	0.88 (0.07)bS	***1.01*** *(0.04)cS*	*0.94 (0.10)aN*	*1.14 (0.14)aN*
3	1.00	1.00	*1.00*	*1.00*	*1.00*
4	*1.12 (0.03)cS*	*1.02 (0.02)bS*	*0.95 (0.02)cS*	*0.92 (0.06)aN*	*0.84 (0.05)aN*
5	*1.15 (0.08)cS*	***1.04*** *(0.02)bS*	0.85 (0.02)dS	*0.96 (0.11)aN*	*0.81 (0.06)aN*
6	***1.26*** *(0.15)cS*	*1.00 (0.04)bS*	0.79 (0.02)eS	0.82 (0.07)aN	*0.73 (0.06)aN*
7	*1.04 (0.14)cS*	0.91 (0.04)bS	0.67 (0.04)fN	0.69 (0.07)aN	0.54 (0.04)bS

[a] Values are normalized against t_c for the 3% NaCl formulations. Values in parentheses are standard errors of the means with $n = 10$, except for the following treatments, for which $n = 9$: acetic acid at pH 3.8 with 4% NaCl, acetic acid at pH 4.0 with 2 and 4% NaCl, and HCl at pH 3.8 (all NaCl concentrations). Maximum At_{cR} values are in boldface; At_{cR} values in italic are not significantly different ($\alpha = 0.05$) from the maximum At_{cR}. a to f indicate significant ($\alpha = 0.05$) differences of successive samples (with increasing % NaCl) within an acid treatment, where samples not significantly different are given the same letter; At_{cR} values at 2 and 4% NaCl are compared. S and N indicate significantly different ($\alpha = 0.05$) and not significantly different ($\alpha = 0.05$), respectively, from the At_{cR} with 0.5% NaCl.

Ak_1 for *E. coli* was significantly different ($\alpha = 0.05$) between NB acidified with acetic acid at a pH of <4.0 and pH 4.0 and between NB acidified with acetic acid and broths acidified with HCl, but not between NB acidified with HCl to pH 3.6 or 3.8 (Fig. 1B). In general, for *E. coli* the trend in relative severity of acid conditions as assessed by At_c or Ak_1 was acetic acid at pH 3.6 > acetic acid at pH 3.8 > acetic acid at pH 4.0 > HCl at pH 3.6 > HCl at pH 3.8 (Fig. 1). In NB acidified with HCl to pH 3.8, growth of *E. coli* FRRB 2701 (only) occurred at 0.5% NaCl in one of the duplicate samples and in the presence of 1% NaCl in both samples (data not included in analyses).

The same trend in the relative severity of the acid treatments for *E. coli* was also observed for *Salmonella*, but the effect was more extreme. Much more rapid inactivation of *Salmonella* than of *E. coli* occurred when broths were acidified with acetic acid, and growth of some *Salmonella* strains occurred at pH 3.8 when NB was acidified with HCl, at up to 1% (wt/wt of water) NaCl.

TABLE 2. ANOVA of At_{cR} for *Salmonella* in response to NaCl concentration

NaCl concn (%, wt/wt of water)	At_{cR}[a]			
	Acetic acid		HCl	
	pH 3.8	pH 4.0	pH 3.6	pH 3.8
0.5	0.60 (0.08)a	0.68 (0.09)a	Growth[b]	Growth
1	*0.92 (0.07)bS*	*0.90 (0.12)aN*	***2.48*** *(0.52)a*	Growth
2	***1.07*** *(0.04)bS*	***1.06*** *(0.11)aS*	*1.52 (0.22)a*	***1.89*** *(0.22)a*
3	1.00	*1.00*	1.00	1.00
4	0.72 (0.04)cN	*0.92 (0.09)aN*	0.95 (0.14)a	0.72 (0.07)b
5	0.63 (0.05)cN	0.76 (0.07)aN	0.74 (0.13)a	0.48 (0.06)c
6	0.46 (0.09)cN	0.50 (0.07)bN	0.60 (0.16)a	0.41 (0.08)c
7	0.33 (0.11)cN	0.39 (0.05)bS	0.54 (0.13)a	0.31 (0.05)c

[a] Values are normalized against t_c for the 3% NaCl formulations. Values in parentheses are standard errors of the means with $n = 8$, except for the following treatments: acetic acid at pH 3.8 with 5 and 6% NaCl, for which $n = 6$; acetic acid at pH 3.8 with 7% NaCl, for which $n = 5$; and HCl at pH 3.6 with all NaCl concentrations except 0.5% NaCl, for which $n = 6$ and $n = 5$, respectively. Maximum At_{cR} values are in boldface; At_{cR} values in italic are not significantly different ($\alpha = 0.05$) from the maximum At_{cR}. a to c indicate significant ($\alpha = 0.05$) differences of successive samples (with increasing % NaCl) within an acid treatment, where samples not significantly different are given the same letter; At_{cR} values at 2 and 4% NaCl are compared. S and N indicate significantly different ($\alpha = 0.05$) and not significantly different ($\alpha = 0.05$), respectively, from the At_{cR} with 0.5% NaCl.

[b] Growth was observed in at least one sample.

Effect of NaCl at different concentrations. The ANOVAs of the At_cR for *E. coli* and *Salmonella* are shown in Tables 1 and 2, respectively. A summary of the statistically significant ($\alpha = 0.05$) trends in the At_cR responses of *E. coli* and *Salmonella* to increasing NaCl concentration, as a function of acidulent type and pH, is shown in Table 3.

When NB was acidified with HCl, irrespective of pH, a monotonic (consistent and systematic) decrease in the At_cR of both *E. coli* and *Salmonella* occurred in response to increasing NaCl concentration (Table 3). In contrast, in the presence of acetic acid at pH 3.8 and 4.0, for both *E. coli* and *Salmonella*, a nonmonotonic (i.e., initially increasing and then decreasing) response of the At_cR to the increasing NaCl concentration was observed. The inflection point in the nonmonotonic At_cR response was dependent on pH and was different for *E. coli* and *Salmonella*. For *E. coli*, the maximum At_cR occurred in the range of 4 to 6% NaCl at pH 3.8 and at 2 to 4% NaCl at pH 4.0 (Tables 1 and 3), with no significant differences ($\alpha = 0.05$) in the At_cR at different NaCl concentrations in those ranges. For *Salmonella*, the maximum At_cR in the presence of acetic acid occurred at 1 to 2% NaCl at pH 3.8, and at 1 to 4% at pH 4.0 (Tables 2 and 3). For *E. coli* in the presence of acetic acid at pH 3.6, the response of the At_cR to the increasing NaCl

TABLE 3. Trend in At_{cR}[a] responses of *E. coli* and *Salmonella* to increasing NaCl concentration as a function of acidulent type and pH

Organism	Response[b]				
	Acetic acid			HCl	
	pH 3.6	pH 3.8	pH 4.0	pH 3.6	pH 3.8
E. coli	A	NM (5, 4–6)	NM (2, 2–4)	M	M
Salmonella	NT	NM (2, 1–2)	NM (2, 1–4)	M	M

[a] Normalized against t_c for 3% (wt/wt of water) NaCl formulations.

[b] A, antitonic response; NM, nonmonotonic (initially increasing and then decreasing) response; M, monotonic response; NT, not tested. Values in parentheses indicate the percent (wt/wt of water) NaCl and range (not significantly different at $\alpha = 0.05$) at which the At_{cR} inflection point (i.e., maximum At_{cR}) occurs.

TABLE 4. ANOVA of At_{cR} of *E. coli* and *Salmonella* with combinations of 0.5 or 3% (wt/wt of water) NaCl and 0 or 10% (wt/wt of water) sucrose

Organism and formulations compared	At_{cR}[a]				
	Acetic acid			HCl	
	pH 3.6	pH 3.8	pH 4.0	pH 3.6	pH 3.8
E. coli					
J/I[b]	0.55/0.81S	0.47/0.84S	0.58/1.00S	0.99/1.02N	1.09/1.36N
I/C[c]	0.75/0.90N	0.77/0.91N	1.11/1.11N	1.04/1.18N	1.58/1.55N
C/H[d]	1.33/1.55N	1.22/1.39N	1.02/0.94N	1.46/1.13N	2.08/1.00N
Salmonella					
J/I	NT	0.60/1.10S	0.68/1.04S	Growth	Growth
I/C	NT	1.05/0.94N	1.06/1.07N	Growth	Growth
C/H	NT	0.93/1.02N	1.10/0.99N	Growth	Growth

[a] S, significantly different at $\alpha = 0.05$; N, not significantly different at $\alpha = 0.05$; Growth, growth observed in at least one "formulation I" sample; NT, not tested.
[b] J (0.5% NaCl, 0% sucrose) and I (0.5% NaCl, 10% sucrose) normalized relative to C (3% NaCl, 0% sucrose).
[c] I (0.5% NaCl, 10% sucrose) and C (3% NaCl, 0% sucrose) normalized relative to H (3% NaCl, 10% sucrose).
[d] C (3% NaCl, 0% sucrose) and H (3% NaCl, 10% sucrose) normalized relative to I (0.5% NaCl, 10% sucrose).

concentration was unexpectedly antitonic; i.e., as the NaCl concentration increased, the At_cR systematically increased for all NaCl concentrations tested (Table 3).

Effect of sucrose versus NaCl. Comparison of At_cR values for formulation J (0.5% NaCl without sucrose) and formulation I (0.5% NaCl and 10% sucrose) showed that the addition of sucrose to NB acidified with acetic acid significantly ($\alpha = 0.05$) extended survival of *E. coli* and *Salmonella* (i.e., increased the At_cR) at all pH values (Table 4). The addition of sucrose, though, had no effect on survival of *E. coli* in the presence of HCl (Table 4). *Salmonella* sometimes grew in NB acidified with HCl and containing sucrose.

In the presence of either acetic acid or HCl, the At_cR^1 in formulation I (containing 0.5% NaCl and 10% sucrose) was not significantly different from the At_cR^1 in formulation C (containing 3% NaCl only). These results suggest that 10% sucrose (with 0.5% NaCl) and 3% NaCl are equally effective at protecting *E. coli* and *Salmonella* from inactivation by acetic acid. For *E. coli* in the presence of HCl, the lack of protection by 10% sucrose (with 0.5% NaCl) is consistent with the lack of protection observed for 3% NaCl alone.

The addition of 10% sucrose (formulation H) to formulations containing 3% NaCl (formulation C) did not significantly affect the At_cR^2 in the presence of either HCl or acetic acid (Table 4).

Assessment of cell wall damage. The proportion of sublethally injured *E. coli* FRRB 2701 as a function of time of exposure to lethal pH (3.6) due to HCl or acetic acid and in the presence of various concentrations of NaCl is shown in Fig. 2. Sublethal injury was observed earlier (i.e., 24 h) at higher concentrations of NaCl, suggesting that damage to the outer membrane/cell wall by NaCl (in the presence of acid) occurred at an early stage of exposure. As the exposure time increased, injury generally increased at all concentrations of NaCl in the presence of either acid. However, for cells exposed to acetic acid, a higher proportion of sublethally injured cells was observed in the presence of "low" (<2%) and "high" (>5%) concentrations of NaCl than at intermediate levels (2 to 5%) as exposure time increased to 72 h.

DISCUSSION

The results of this study indicate that our previous observation (4) of protection of *E. coli* against inactivation by acetic acid by moderate NaCl concentrations is widely observed among strains of both *E. coli* and *Salmonella* and is not unique to *E. coli* SERL 2. However, such protection is not observed under conditions where HCl is the acidulent. For *E. coli*, higher NaCl concentrations protected against inactivation by more severe acetic acid conditions. For *Salmonella*, the trend was less clear, due to the greater sensitivity of this organism to adverse acetic acid and NaCl treatments. It is important to note that in this study we have expressed the severity of acid treatment in terms of pH. However, it is possible that the severity of the acid treatment, and hence the observation of a nonmonotonic or antitonic response, is actually a function of the concentration of total or undissociated acetic acid present. This aspect remains to be explored.

Weak acid theory is the predominant mechanistic explanation of inactivation of cells by acetic acid. According to this theory, protection against acetic acid could be expected to involve a slowing of acidification of the cytoplasm. Slowing of acidification could be achieved by more effective removal of H^+ from the cytoplasm. In the presence of NaCl, it has been postulated that coupling of Na^+ import to H^+ export might facilitate *E. coli* to maintain internal pH, thus extending survival times (3). However, our comparison of inactivation kinetics in the presence of acetic acid or HCl indicates that the protection afforded by moderate NaCl concentrations is specific to acetic acid and not to protection against low pH per se. If more effective removal of H^+ from the cytoplasm alone explained the protective effect of moderate NaCl concentrations, the protective effect should be observed regardless of whether HCl or acetic acid was employed as an acidulent.

Alternatively, and again according to weak acid theory, the rate of acidification of the cytoplasm could also be dependent on the rate of ingress of the undissociated weak acid species into the cytoplasm. The ability of undissociated acetic acid to passively traverse bilayer membranes is usually attributed to its lipophilicity. Bulk solubility-diffusion theory, which equates bi-

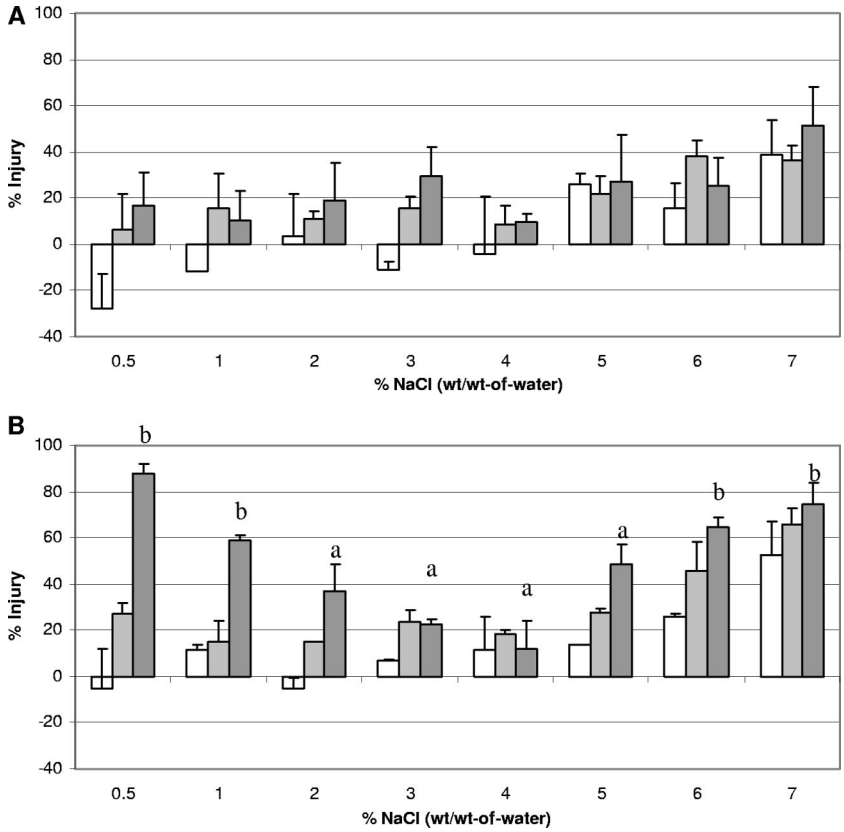

FIG. 2. Average percent injury of *E. coli* 2701 at pH 3.6 as assessed by recovery on TSA plus 0.15% bile salts compared to TSA only. Acidification was with HCl (A) or acetic acid (B) after 24 (white bars), 48 (light gray bars), and 72 (dark gray bars) hours of exposure. Error bars indicate standard errors of the means ($n = 2$). In panel B, different letters indicate significant ($\alpha = 0.05$) differences compared with percent injury in the presence of 3% (wt/wt of water) NaCl after 72 h of exposure; samples not significantly different are given the same letter.

layer membranes with a bulk lipid solvent (that is, a homogenous "oil" phase such as olive oil or octanol), predicts that undissociated acetic acid will rapidly permeate cells under all conditions. However, acetic is only weakly lipophilic (12), since the polar acid functional group dominates over the nonpolar character of the short hydrocarbon chain, and bulk solubility-diffusion theory does not consider the effect of bilayer chain packing or free surface area in the lipid bilayer on the permeability coefficients of weak organic acids (16). Size selectivity in partitioning of weak organic acids has been shown to be amplified with increases in bilayer chain packing, with deviation of the experimental permeability coefficients from those predicted by the bulk solubility-diffusion model being most marked for densely packed gel-state bilayers (17). The sensitivity of the permeability coefficient to membrane chain ordering has been found to be particularly marked for acetic acid, with a significant increase in sensitivity observed between formic and acetic acids in dipalmitoylphospatidylcholine bilayer membranes (17).

At physiological (isotonic) levels of hydration, the phospholipid bilayer of *E. coli* is in a fluid, lamellar, liquid-crystalline phase, but the phospholipids undergo phase transition to a gel state with increasing osmotic pressure (6), which may result from alterations in fatty acid structure or changes in packing geometry (7). It has been determined that for *E. coli*, membrane fluidity is greatly decreased with increasing osmotic pressures up to 40 MPa (2). Plasmolysed cells of *E. coli* have been found to synthesize phospholipid at a greater rate than nonplasmolysed cells (10), and an increase in synthesis of saturated phospholipids in *Lactobacillus delbrueckii* subsp. *bulgaricus* has been shown to lead to the development of a more rigid membrane in response to increased osmotic pressure (14).

In minimal medium the growth rate of *E. coli* has been observed to be maximal at ~0.3 osM (external), and when the osmolarity was varied with a nonpermeable solute such as NaCl, the growth rate decreased with both decreasing and increasing osmolarity around this optimum (9). This suggests that ~0.3 osM is isotonic for *E. coli*. At 23°C, 0.3 osM corresponds to an osmotic pressure, Π, of 0.7 MPa, calculated using the equation $\Pi = 0.1013(iMR'T)$, where i is the dimensionless van't Hoff factor (assumed to be 2 for NaCl), M is the molarity, R' is the gas constant expressed in terms of liters and atmospheres and equal to 0.0821 liters atm/K, T is the absolute temperature expressed as Kelvin, and 0.1013 is a multiplier to convert atm to MPa.

In this study, NB containing 2 and 3% NaCl alone and containing 10% sucrose with 0.5 or 3% NaCl are all hypertonic formulations, with calculated osmolarities of ~0.68, 1.02, 0.43, and 1.21, respectively (ignoring contributions by acids and other components of the NB base, apart from NaCl, and ignoring potential for acid inversion of sucrose) and calculated osmotic pressures of 1.7, 2.5, 1.1, and 3.0 MPa, respectively. All

of these formulations were more protective than 0.5% NaCl alone, suggesting that the mechanism of protection by NaCl and by sucrose may be related to the induction of a slightly plasmolysed physiological state as a consequence of the hypertonic environment, but one which does not result in gross physical damage to the cell wall. Others have observed slight plasmolysis (usually occurring at only one end of the cell) to occur among *E. coli* cells at 0.2 M (~7%, wt/wt) sucrose, while extensive plasmolysis was observed at 0.4 M (~14%, wt/wt), and cell wall collapse was evident at concentrations of >0.4 M (11).

Under more severe acid treatments, significantly increased protection of *E. coli* against acetic acid inactivation was observed up to 4 to 7% NaCl (compared with that at 0.5% NaCl only), corresponding to osmotic pressures of 3.3 to 5.7 MPa. However, no protection by increased osmotic pressure was observed for *E. coli* or *Salmonella* in the presence of HCl (Tables 1 and 2). Thus, we hypothesize that if sustained plasmolysis in the presence of hypertonic concentrations of osmolytes occurs, diffusion of undissociated acetic acid into the cytoplasm may be slowed by the more rigid membrane that results from the increased osmotic pressure. In contrast, diffusion of free protons (e.g., from HCl or dissociated acetic acid in the bulk environment) may be largely unaffected by the increase in membrane rigidity because the slower diffusion of acetic acid into plasmolysed (but not grossly damaged) cells will enable cells to maintain cytoplasmic membrane energization longer, thus enhancing survival of plasmolysed cells compared with nonplasmolysed cells. Protection against acetic acid by increased membrane rigidity might also help to explain the improved resistance of the *Enterobacteriaceae* to inactivation by organic acids observed at lower incubation temperatures (8, 15); we acknowledge, however, that improved survival may be a function simply of the reduced kinetic rate of all reactions at reduced temperature. The effect of temperature on acetic acid diffusion into cells as a function of membrane rigidity requires elucidation.

The results presented here suggest that acetic acid, like lactic acid (1), has a direct effect on the integrity and function of the cell envelope. At low concentrations of NaCl, cell envelope damage (as measured by susceptibility to bile salts) was not apparent in the presence of HCl, but it was inferred to increase over time in the presence of acetic acid. At high concentrations of NaCl, analysis of sublethal injury among survivors indicated that NaCl may also cause membrane damage in the early stages of exposure to a combined NaCl and acid stress, regardless of the acidulent type. Injury of cells at high concentrations of NaCl could be due to extreme plasmolysis, resulting in indirect damage to the cell wall/outer membrane as the cytoplasmic membrane pulls on anchor points at the cell wall. In contrast, injury by acetic acid is inferred to occur more slowly. As NaCl protects *E. coli* against injury by acetic acid, survival is optimal at intermediate concentrations of NaCl, when balanced against the lethal effects of NaCl itself.

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