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

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Received 27 October 2008/Accepted 29 March 2009

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-log10-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 decreases due to water loss by osmosis. The thin peptidoglycan layer of gram-negative microorganisms is anchored to the cytoplasmic membrane and can be detached 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
trypotene 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 ~10^8 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 immediately diluted twice in buffered peptone water (CM0199; 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°C) 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 72 h, with a maximum of nine time points (0.5, 1, 7, 24, 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 (t + t_{0.5})]), where t (h) is a measure of the shoulder period (delay) or time before inactivation was detected, and k_1 (h) is a constant representing the semilogarithmic inactivation rate when t > t_{0.5}.

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_{0.5} and k_1 (Atc, AtcR, and AtcR 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_{0.5} (Atc, R) 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 containing 3% (wt/wt of water) NaCl. Thus, for each acid treatment and each E. coli or Salmonella isolate, and for each duplicate, t_R values were calculated by dividing the t_{0.5} at each NaCl concentration by the t_{0.5} at 3% (wt/wt of water) NaCl. At_R values for E. coli and Salmonella for each acid treatment at each NaCl level were then determined by averaging the t_R values. The effect of NaCl concentration was then evaluated by single-factor ANOVA (P < 0.05) of At_R using the single-factor ANOVA tool of Microsoft Excel.

Effect of sucrose versus NaCl. At_R 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 for each duplicate, t_R values were calculated as follows. For comparison of t_{0.5} 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_R values were calculated relative to those in formulations containing 5% NaCl without sucrose (formulation C). For comparison of formulation I and formulation C, t_R values were calculated relative to t_{0.5} values in formulations containing 3% NaCl and 10% (wt/wt of water) sucrose (formulation H). For comparison of formulation C and formulation H, t_R values were calculated relative to t_{0.5} values in formulations I. At_R 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_R were compared by single-factor ANOVA (P < 0.05) using the single-factor ANOVA tool of Microsoft Excel.

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 was not significantly different (α = 0.05) among broths acidified with acetic acid to pH 3.6, 3.8, or 4.0, and the At was not significantly different between NB acidified with HCl to pH 3.6 or 3.8 (Fig. 1A). However, the At was significantly different (α = 0.05) between NB acidified with acetic acid and NB acidified with HCl. The
Ak for E. coli was significantly different (α = 0.05) between NB acidified with acetic acid at a pH < 4.0 and at 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 Ak or AAc 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.

Effect of NaCl at different concentrations. The ANOVAs of the AkR for E. coli and Salmonella are shown in Tables 1 and 2, respectively. A summary of the statistically significant (α = 0.05) trends in the AkR 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 AkR, 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 AkR to the increasing NaCl concentration was observed. The inflection point in the nonmonotonic AkR response was dependent on pH and was different for E. coli and Salmonella. For E. coli, the maximum AkR 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 (α = 0.05) in the AkR at different NaCl concentrations in those ranges. For Salmonella, the maximum AkR 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 AkR to the increasing NaCl

### Table 1. ANOVA of AkR for E. coli in response to NaCl concentration

<table>
<thead>
<tr>
<th>NaCl concen (%)</th>
<th>Acetic acid</th>
<th>HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 3.6</td>
<td>pH 3.8</td>
</tr>
<tr>
<td>0.5</td>
<td>0.55 (0.05)a</td>
<td>0.47 (0.06)a</td>
</tr>
<tr>
<td>1</td>
<td>0.74 (0.05)bS</td>
<td>0.61 (0.05)aN</td>
</tr>
<tr>
<td>2</td>
<td>0.62 (0.05)bS</td>
<td>0.57 (0.07)bS</td>
</tr>
<tr>
<td>3</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>4</td>
<td>1.12 (0.03)cS</td>
<td>1.02 (0.02)S</td>
</tr>
<tr>
<td>5</td>
<td>1.15 (0.08)cS</td>
<td>1.04 (0.02)bS</td>
</tr>
<tr>
<td>6</td>
<td>1.26 (0.15)cS</td>
<td>1.00 (0.04)bS</td>
</tr>
<tr>
<td>7</td>
<td>1.04 (0.14)cS</td>
<td>0.91 (0.04)bS</td>
</tr>
</tbody>
</table>

- Values are normalized against t, for the 3% NaCl formulations. Values in parentheses are standard errors of the means with n = 10, except for the following treatments: acetic acid at pH 3.8 with 5% NaCl, acetic acid at pH 4.0 with 2% and 4% NaCl, and HCl at pH 3.8 (all NaCl concentrations). Maximum AkR values in boldface; values in italic are not significantly different (α = 0.05) from the maximum AkR to indicate significant (α = 0.05) differences of successive samples (with increasing % NaCl) within an acid treatment, where samples not significantly different are given the same letter; AkR values are at 2% and 4% NaCl are compared. S and N indicate significantly different (α = 0.05) and not significantly different (α = 0.05), respectively, from the AkR with 0.5% NaCl.

### Table 2. ANOVA of AkR for Salmonella in response to NaCl concentration

<table>
<thead>
<tr>
<th>NaCl concen (%)</th>
<th>Acetic acid</th>
<th>HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 3.6</td>
<td>pH 4.0</td>
</tr>
<tr>
<td>0.5</td>
<td>0.60 (0.08)a</td>
<td>0.68 (0.09)a</td>
</tr>
<tr>
<td>1</td>
<td>0.92 (0.07)bS</td>
<td>0.90 (0.12)μN</td>
</tr>
<tr>
<td>2</td>
<td>1.07 (0.04)bS</td>
<td>1.06 (0.11)μN</td>
</tr>
<tr>
<td>3</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>4</td>
<td>0.72 (0.04)cN</td>
<td>0.92 (0.09)μN</td>
</tr>
<tr>
<td>5</td>
<td>0.63 (0.05)cN</td>
<td>0.76 (0.07)μN</td>
</tr>
<tr>
<td>6</td>
<td>0.46 (0.09)cN</td>
<td>0.50 (0.07)μN</td>
</tr>
<tr>
<td>7</td>
<td>0.33 (0.11)cN</td>
<td>0.39 (0.05)bS</td>
</tr>
</tbody>
</table>

- Values are normalized against t, 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% 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 AkR values are in boldface; AkR values in italic are not significantly different (α = 0.05) from the maximum AkR to indicate significant (α = 0.05) differences of successive samples (with increasing % NaCl) within an acid treatment, where samples not significantly different are given the same letter; AkR values are at 2% and 4% NaCl are compared. S and N indicate significantly different (α = 0.05) and not significantly different (α = 0.05), respectively, from the AkR with 0.5% NaCl.

### Table 3. Trend in AkR responses of E. coli and Salmonella to increasing NaCl concentration as a function of acidulent type and pH

<table>
<thead>
<tr>
<th>Organism</th>
<th>Acetic acid</th>
<th>HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 3.6</td>
<td>pH 3.8</td>
</tr>
<tr>
<td>E. coli</td>
<td>A</td>
<td>NM (5, 4–6)</td>
</tr>
<tr>
<td>Salmonella</td>
<td>NT</td>
<td>NM (2, 1–2)</td>
</tr>
</tbody>
</table>

- Normalized against t, for 3% (wt/wt of water) NaCl formulations.
- 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 α = 0.05) at which the AkR inflection point (i.e., maximum AkR) occurs.
concentration was unexpectedly antitonic; i.e., as the NaCl concentration increased, the \( \text{At}_{\text{R}} \) systematically increased for all NaCl concentrations tested (Table 3).

**Effect of sucrose versus NaCl.** Comparison of \( \text{At}_{\text{R}} \) 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 \( \text{Salmonella} \) (i.e., increased the \( \text{At}_{\text{R}} \)) 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). \( \text{Salmonella} \) sometimes grew in NB acidified with HCl and containing sucrose.

In the presence of either acetic acid or HCl, the \( \text{At}_{\text{R}} \) in formulation I (containing 0.5% NaCl and 10% sucrose) was not significantly different from the \( \text{At}_{\text{R}} \) 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 \( \text{Salmonella} \) 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 \( \text{At}_{\text{R}} \) 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 increase, 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 \( \text{Salmonella} \) 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 \( \text{Salmonella} \), 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 undisassociated 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 undisassociated weak acid species into the cytoplasm. The ability of undisassociated acetic acid to passively traverse bilayer membranes is usually attributed to its lipophilicity. Bulk solubility-diffusion theory, which equates bi-
layer membranes with a bulk lipid solvent (that is, a homoge-
nous “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 perme-
ability coefficients of weak organic acids (16). Size selectivity in
partitioning of weak organic acids has been shown to be am-
plified with increases in bilayer chain packing, with deviation of
the experimental permeability coefficients from those pre-
dicted by the bulk solubility-diffusion model being most
marked for densely packed gel-state bilayers (17). The sensi-
tivity of the permeability coefficient to membrane chain order-
ing has been found to be particularly marked for acetic acid,
with a significant increase in sensitivity observed between for-
mic and acetic acids in dipalmitoylphosphatidylcholine bilayer
membranes (17).

At physiological (isotonic) levels of hydration, the phospho-
lipid bilayer of \textit{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 \textit{E. coli}, mem-
brane fluidity is greatly decreased with increasing osmotic pres-
sures up to 40 MPa (2). Plasmolysed cells of \textit{E. coli} have been
found to synthesize phospholipid at a greater rate than non-
plasmolysed cells (10), and an increase in synthesis of saturated
phospholipids in \textit{Lactobacillus delbrueckii} subsp. \textit{bulgaricus}
has been shown to lead to the development of a more rigid mem-
brane in response to increased osmotic pressure (14).

In minimal medium the growth rate of \textit{E. coli} has been
observed to be maximal at \(-0.3 \text{ osM (external)}, \) 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 \(-0.3 \text{ osM is isotonic for } \textit{E. coli}. \) At 23°C, 0.3 osM corre-
sponds to an osmotic pressure, II, of 0.7 MPa, calculated using
the equation

\[
II = 0.1013(iMRI/T), 
\]

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 atmo-
spheres 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 ig-
noring potential for acid inversion of sucrose) and calculated
osmotic pressures of 1.7, 2.5, 1.1, and 3.0 MPa, respectively. All

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{Average percent injury of \textit{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.}
\end{figure}
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 to 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 aciculent 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.

ACKNOWLEDGMENTS

We thank I. Ebersdorfer, L. Brown, and D. Procter, as well as M. Begum and M. Bradbury of Food Science Australia, for their technical assistance.

We acknowledge partial financial support of this research by The Australian Food Safety Centre of Excellence, an initiative of the Australian Federal Government’s National Food Industry Strategy and a consortium of the Tasmanian Institute of Agricultural Research and Food Science Australia.

REFERENCES