Induction of Acid Resistance of *Salmonella typhimurium* by Exposure to Short-Chain Fatty Acids

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Exposure to short-chain fatty acids (SCFA) is one of the stress conditions *Salmonella typhimurium* encounters during its life cycle, because SCFA have been widely used as food preservatives and SCFA are also present at high concentrations in the gastrointestinal tracts of host animals. The effects of SCFA on the acid resistance of the organism were examined in an attempt to understand the potential role of SCFA in the pathogenesis of *S. typhimurium*. The percent survival of *S. typhimurium* at pH 3.0 was determined after exposure to SCFA for 1 h at pH 7.0. The percent acid survival, which varied depending on the SCFA species and the concentration used, was 42 after exposure to 100 mM propionate at pH 7.0 under aerobic incubation conditions, while less than 1% could survive without exposure. The SCFA-induced acid resistance was markedly enhanced by anaerobiosis (64%), lowering pH conditions (138% at pH 5.0), or increasing incubation time (165% with 4 h) during exposure to propionic acid. When protein synthesis during exposure to propionate was blocked by chloramphenicol, the percent acid survival was less than 1, indicating that the protein synthesis induced by exposure to propionate is required for the induction of the acid resistance. The percent acid survival determined with the isogenic mutant strains defective in acid tolerance response revealed that AtrB protein is necessary for the full induction of acid resistance by exposure to propionate, while unexpectedly, inactivation of PhoP significantly increased acid resistance over that of the wild type (*P* < 0.05). The results suggest that the virulence of *S. typhimurium* may be enhanced by increasing acid resistance upon exposure to SCFA during its life cycle and further enhanced by anaerobiosis, low pH, and prolonged exposure time.

*Salmonella* spp. are among the major foodborne pathogens which are of public concern with respect to food safety (22). Since *Salmonella* is a facultatively anaerobic bacterium and does not require strict conditions for its growth, this pathogen is able to proliferate and survive in diverse environmental niches, including most environmental ecosystems, food production and processing systems, and intestinal tracts of the host animals. During its life cycle, *Salmonella* can encounter various environmental stress conditions, such as nutrient starvation, pH extremes, oxidative stress, osmotic shock, and heat shock (12), which may have dramatic effect(s) on its survival and virulence (1).

Depending on the severity and duration of the exposure to the stressors, either the growth or survival of *Salmonella* is inhibited, or the cells lose viability. This organism also has the capability of sensing the stress conditions as signals for inducing dramatic changes in gene expression and protein synthesis (12). Although the mechanisms of how *Salmonella* sense the stress conditions are not well understood, the general function of the stress response is to enable the cells to be more tolerant or resistant to the stress conditions encountered.

One important adaptation mechanism of *Salmonella typhimurium* is the acid tolerance response (ATR), where the acid resistance of *S. typhimurium* is greatly enhanced when the cells are exposed to conditions considered mildly acidic (pH 5.8) (9). Acid adaptation of *S. typhimurium* appears to have an important role in the survival in various stress conditions. Leyer and Johnson (24) reported that acid adaptation induces cross-protection against several environmental stresses, and Wilmes-Riesenberg et al. (33) showed that mutants which were more sensitive to acid were highly attenuated, suggesting a strong correlation in *S. typhimurium* between the ability to mount an ATR and virulence.

One of the potential stress conditions frequently encountered by *S. typhimurium* is the presence of short-chain fatty acids (SCFA), such as acetate, propionate, and butyrate. SCFA are produced as fermentation products by native intestinal microflora and can be present at high concentrations in gastrointestinal ecosystems possessing large numbers of highly fermentative anaerobic bacteria. In humans, for example, the concentrations of SCFA are 35 mmol/kg in the small intestine and 134 mmol/kg in the large intestine (4). *Salmonella* spp. may also encounter the SCFA acetate and propionate in food products, such as meat carcasses, salad dressing, and mayonnaise, where they are widely used as preservatives (100 to 300 mM) due to their antibacterial activities (3, 5, 16, 20).

In this study we report that the acid resistance of *S. typhimurium* is greatly increased after exposure of this organism to SCFA, and this SCFA-induced acid resistance is further enhanced by acid pH, anaerobiosis, and prolonged exposure to SCFA. The results suggest that the exposure of *S. typhimurium* to SCFA increases the virulence of this pathogen by increasing the acid resistance.

**MATERIALS AND METHODS**

**Bacterial strains.** *S. typhimurium* strains used are listed in Table 1. Generalized transduction with P22 HT105/1 net-201 was conducted by the method of Maloy et al. (27). Transductants selected on Luria-Bertani (LB) agar plates containing relevant antibiotics were purified on Evans Blue-Uranine (EBU) (27) plates and subsequently cross-streaked with P22 H5 for identification of and subsequent elimination of pseudolysogens and lysogens. A spontaneous mutant of *S. typhimurium* ATCC 14028s resistant to nalidixic acid (NA) was used in all of the experiments, except for Fig. 2 and 5, where wild-type and ATR mutants of *S. typhimurium* ATCC 14028s and SL1344 were used. Using the antibiotic marker strain allowed the use of broth and agar media containing the antibiotics, so that...
TABLE 1. S. typhimurium strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype*</th>
<th>Source or construction</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>14028s</td>
<td>Wild type</td>
<td>ATCC</td>
<td>This work</td>
</tr>
<tr>
<td>14028s Na+ mutant</td>
<td>ATCC</td>
<td>This work</td>
<td></td>
</tr>
<tr>
<td>SL1344</td>
<td>Wild type</td>
<td>ATCC</td>
<td>18</td>
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<tr>
<td>JF1898 phosphatase and tol</td>
<td>JF1898(P22) × 14028s</td>
<td>This work</td>
<td></td>
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<td>JF2560 region-specific phosphatase</td>
<td>JF2560(P22) × 14028s</td>
<td>23</td>
<td></td>
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<tr>
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<td>JF1898(P22) × 14028s</td>
<td>This work</td>
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<td>SF342(P22) × 14028s</td>
<td>This work</td>
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<tr>
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<td>SF342(P22) × SL1344</td>
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* Na+, naldixic acid resistant; Ap+, ampicillin resistant.

** ATCC, American Type Culture Collection, Rockville, Md.; SGSC, Salmonella Genetic Stock Center, Calgary, Alberta, Canada.

The potential problems by contamination could be avoided. We confirmed that the results were not affected by the Na+ marker of the mutant strain.

**Preparation of media.** Tryptic soy broth (TSB; Difco Laboratories, Detroit, Mich.) and E medium (0.02% MgSO4·7H2O, 0.2% citric acid (C6H8O7·2H2O), 1% K2HPO4, 0.35% NaH2PO4·2H2O) containing 0.2% glucose (27) were used for the SCFA adaptation (pH 7.0) and acid survival assay (pH 3.0). For the preparation of anaerobic TSB medium, aerobically prepared TSB medium were dispensed into test tubes (13 by 100 mm) (4 ml/tube) and placed in an anaerobic chamber (Coy Laboratories, Ann Arbor, Mich.) with a mixed-gas atmosphere consisting of 10% H2, 10% CO2, and 80% N2 gas. After 4 to 5 h of equilibrium, the tubes were sealed with butyl rubber stoppers and autoclaved for 20 min. The initial oxidation-reduction (O/R) potential of the medium was measured with an Orion pH meter 240 (Corning Inc., Corning, N.Y.), which was kept in an anaerobic chamber. The electrode was stored in a standard ferrous-ferric solution and calibrated with a reference electrode by the method of Light (25).

**Conditions for SCFA adaptation and acid resistance assay.** The SCFA adaptation assay was conducted as follows, unless described otherwise. Ten microliters of a fresh culture of S. typhimurium grown overnight in each test tube containing 4 ml of TSB medium (pH 7.0), and grown in a water bath at 37°C until the optical density (A600) reached 0.2 on a Spectronic 20D spectrophotometer (Milton Roy Co., Rochester, N.Y.) was followed by addition of filter-sterilized SCFA stock solution (1.0 M; pH 7.0, adjusted with NaOH) to the specified final concentration and incubated at 37°C for 1 h. To determine the acid resistance of the cells, 100 μl of the adapted culture was transferred to 4 ml of phosphate-buffered saline (PBS) buffer (pH 7.2) and of TSB medium (pH 3.0, adjusted with HCl). The CFU/ml of the adapted culture was determined by plating serial dilutions in PBS buffer (pH 7.2) on tryptic soy agar (TSA; Difco Laboratories) plates containing NA (25 μg/ml) and used as initial cell populations. The TSB medium (pH 3.0) inoculated with adapted S. typhimurium was incubated for an additional hour at 37°C, and the CFU/ml in TSB medium (pH 3.0) was determined in the same way and used as final cell populations. The percent acid survival was then calculated as (initial population/final population) × 100.

**Agar well diffusion assay.** Growth inhibition by SCFA was measured by an agar well diffusion assay. A fresh culture of S. typhimurium grown overnight in TSB was diluted 100-fold in PBS buffer (pH 7.2), and approximately 105 cells in 100 μl were spread on an M9 (0.6% Na2HPO4, 0.3% KH2PO4, 0.05% NaCl, 0.1% NH4Cl, 2 mM MgSO4, 0.1 mM CaCl2) agar plate containing 0.2% glucose. The well was made using a Pasteur pipette, and 80 μl of 1.0 M SCFA stock solution (pH 7.0) was added to the well. After 24 h of incubation in a 37°C incubator, the diameter of the inhibitory zone was measured. For the assay under anaerobic growth conditions, the plates were transferred and incubated at 37°C in an anaerobic chamber.

**Statistical analysis.** The diameters of inhibition zones and the percent survival were analyzed by least-squares mean separations which were accomplished by the pdff routine option of the General Linear Models (GLM) procedure in SAS statistical analysis software program, version 6.11 (SAS). Each mean was the average of three independent trials, and means were considered significantly different at P < 0.05.

**RESULTS AND DISCUSSION**

**Acid resistance after adaptation to SCFA.** In our preliminary study we found that adaptation to propionate markedly increased the acid resistance of an S. typhimurium strain isolated from poultry (21). Guilfoyle and Hirshfield (14) also reported that the acetic acid resistance of Escherichia coli can be induced by exposure to SCFA at neutral pH. In order to investigate the observations in detail, we determined the acetic acid resistance of S. typhimurium after adaptation to various SCFA as described in Materials and Methods. The percent survival of S. typhimurium after 1-h exposure to pH 3.0 was conveniently used to represent the acid resistance of the cells by the method of Foster and Hall (11). We also compared their effects under both aerobic and anaerobic incubation conditions (Fig. 1A). The levels of acid resistance induced by acetate and propionate were significantly greater than the levels induced by other SCFA or NaCl under both aerobic and anaerobic conditions (P < 0.05). NaCl (100 mM, pH 7.0) was used as a negative control because NaOH was used to adjust the pH of the SCFA stock solutions (1.0 M, pH 7.0). No survivors were recovered when acid resistance was determined before adaptation. We also found that the acid resistance induced by the various SCFA was significantly enhanced by anaerobiosis (P < 0.05). Greater than 60% survival was seen after 1 h at pH 3.0 after adaptation to either acetate or propionate. The measured O/R potentials of the aerobic and anaerobic TSB media were 378.0 ± 5.0 and −62.2 ± 16.7 mV, respectively. This result may have an important implication in the sense that anaerobic...
conditions resulting in a negative O/R potential can be found in the gastrointestinal tracts of host animals and also in vacuum-packaged meat products (20). The O/R potential in the cecum of a conventional rat with native microflora was $-206 \pm 40$ mV when it was read 5 min after insertion of the electrode in the cecum (34), and Broberg (2) reported the O/R of the ruminal contents was approximately $-150$ mV. However, it remains to be determined if there is a correlation between O/R potential and SCFA-induced acid resistance while the O/R potential reaches the levels observed in the gastrointestinal tract or rumen.

It was previously shown by Lin et al. (26) that the induction of acid resistance by adaptation to mild pH requires components of complex medium in E. coli and Shigella flexneri, but not in S. typhimurium, suggesting that nutrient composition also plays an important role in SCFA-induced acid resistance. Therefore, we determined the acid resistance of S. typhimurium in complex (TSB) and minimal (E) media at pH 3.0 after exposure to SCFA in the medium at pH 7.0 (Fig. 1B). We found that the acid resistance after adaptation to the same SCFA was not significantly different between minimal and complex media, except when butyrate was used for adaptation ($P < 0.01$). When butyrate was added, the acid resistance in minimal medium increased approximately 2.7-fold over that in complex medium.

**Growth inhibition by SCFA.** S. typhimurium is able to use SCFA, such as acetate or propionate, as its sole carbon and energy source under aerobic growth conditions but requires considerable adaptation time (18 to 20 h) to grow on propionate (7, 19). On the other hand, SCFA have been widely used as food preservatives because of their antibacterial activity (3, 5, 16) and also have been suggested to be a major factor inhibiting the colonization by S. typhimurium by competitive exclusion of native microflora in the mouse intestine (17, 28).

Therefore, we reasoned that the acid resistance of S. typhimurium induced by exposure to SCFA shown in Fig. 1 may be the result of growth inhibition by SCFA rather than specific activities of SCFA. To answer this question, we quantitatively measured the growth-inhibitory activities of various SCFA species at pH 7.0 and compared them under both aerobic and anaerobic growth conditions. We used an agar well diffusion assay for convenient screening, and the growth inhibition of S. typhimurium was represented by the diameters (in millimeters) of the inhibitory zones (Fig. 2). Under both aerobic and anaerobic conditions, the growth inhibition by propionate was significantly higher than those by other SCFA and NaCl ($P < 0.01$). No inhibition of S. typhimurium was observed with acetate or NaCl. We also found that the growth-inhibitory effects of all SCFA species tested were significantly lower under anaerobic growth conditions than those under aerobic growth conditions ($P < 0.05$).

The acid resistance was induced by acetate, although acetate did not inhibit the growth of S. typhimurium. Also, the acid resistance induced by SCFA was greatly enhanced by anaerobiosis, while the growth inhibition by SCFA was suppressed under anaerobic conditions. Therefore, the above results indicate that the induction of acid resistance of S. typhimurium by exposure to SCFA was not mediated by a growth-inhibitory effect. **Effects of various adaptation conditions on SCFA-induced acid resistance.** In order to examine the effects of various adaptation conditions on the induction of acid resistance, we chose to use propionate for further study, because it appears to be the most effective in terms of both growth inhibition and induction of acid resistance.

To examine the dose response of SCFA-induced acid resistance, we determined the acid resistance of S. typhimurium after adaptation to propionate at various concentrations (0 to 100 mM) at pH 7.0 (Fig. 3A). As expected, the percent survival increased from $<0.1$ to 54 as the propionate concentration was increased from 0 to 100 mM in 20 mM increments. The effect of adaptation time on the level of acid resistance was also examined by increasing the time of adaptation to 100 mM propionate at pH 7.0 from 0 to 4 h (Fig. 3B). Without adaptation, only $<0.1\%$ survived, while the acid resistance was significantly increased by increasing the adaptation time to longer than 1 h ($P < 0.01$). When the adaptation time was extended to 4 h, 165% of the cells survived, which means that the adapted cells of S. typhimurium were able to multiply even at pH 5.0. One possibility is that the cells present as clumps were separated upon exposure to acidic pH, which then resulted in increased cell numbers, as determined by CFU. As the exposure time of S. typhimurium to SCFA in environmental niches is likely to be much longer than 1 h, the enhancement of SCFA-induced acid resistance by prolonged exposure to SCFA appears to be relevant in its life cycle. However, whether SCFA-induced acid resistance could be enhanced further by exposure to SCFA for much longer times (e.g., several days) remains to be determined.

It is known that SCFA enter bacterial cells only in undissociated forms, and as extracellular pH decreases, the portion of the undissociated SCFA increases, increasing their activities to bacterial cells (3). Although the mode of action by which SCFA increase the acid resistance of S. typhimurium is not known, we assumed that pH level may be one critical factor controlling SCFA-induced acid resistance. Therefore, we determined the acid resistance of S. typhimurium after adaptation to 100 mM propionate at different pHs (Fig. 3C). For this study, the adaptation protocol was partially modified so that the adaptation to 100 mM propionate could be done exactly at pH 5.0, 6.0, 7.0, and 8.0. The cells of S. typhimurium were grown in TSB medium (pH 7.0) overnight, and 400 μl of the culture was transferred to TSB medium at pH 5.0, 6.0, 7.0, and 8.0 containing 0 or 100 mM propionate. After incubation for 1 h, the adapted cultures were assayed for acid survival as described in the Materials and Methods. When adapted to propionate at pH 8.0, only 2% survived. However, as the pH was decreased, the percent survival greatly increased, leading to 138% survival when adapted at pH 5.0, which indicates the capability of the adapted cells to multiply at pH 3.0. However, the percent survival of the cells without exposure to propionate
was significantly lower than the cells adapted to propionate at the same pH ($P < 0.05$). The enhanced survival in acidic pH conditions might be explained either by combined effects of both ATR and SCFA-induced acid resistance or increased concentrations of propionate anion in acidic conditions. According to Cummings et al. (4), the pHs of different sections of the human intestine are $6.3 \pm 0.1$ in the ileum, $5.6 \pm 0.2$ in the cecum, and $6.2 \pm 0.1$ in the colon. Therefore, it seems reasonable to suppose that the mildly acidic conditions in the human intestine play a role in enhancing SCFA-induced acid resistance in the intestinal environment.

**Role of protein synthesis in SCFA-induced acid resistance.** We reasoned that the SCFA-induced acid resistance of *S. typhimurium* described thus far should be conferred by the synthesis of a specific set of proteins. Therefore, we determined the acid resistance of *S. typhimurium* after adaptation to 100 mM propionate at pH 7.0 while protein synthesis was blocked by addition of chloramphenicol (50 $\mu$g/ml) at different time points during the adaptation (Fig. 4). This concentration (50 $\mu$g/ml) was previously shown to block the protein synthesis of *S. typhimurium* completely (10).

![FIG. 4. Effect of blocking protein synthesis on the percent acid survival of *S. typhimurium* ATCC 14028s after adaptation to propionate (100 mM). Chloramphenicol (50 $\mu$g/ml) was added at different time points during the adaptation procedure.](http://aem.asm.org/)

The results show that the addition of chloramphenicol 5 min before the addition of propionate completely abolished the acid resistance, whereas the addition of the antibiotics 30 min after the addition of propionate and 5 min before the acid challenge did not have a significant effect on the induction of acid resistance compared to that of the control ($P < 0.05$). The results indicate that a set of proteins newly synthesized within 30 min after the cells are exposed to propionate was responsible for the induction of acid resistance by adaptation to propionate. However, protein synthesis after the acid challenge at pH 3.0 did not appear to have an essential role in the acid resistance. By using two-dimensional gel electrophoresis, Guilfoyle and Hirshfield (15) identified six proteins which are specifically synthesized in response to butyrate (11 mM) at pH 5.5, including inducible arginine decarboxylase, lipomamide acetyltransferase, inducible lysyl-tRNA synthetase. Although it is not known if these proteins are also synthesized in *S. typhimurium* by exposure to other SCFA, they may play an important role in expressing the SCFA-induced acid resistance phenotype.

**SCFA-induced acid resistance of isogenic ATR mutant strains.** The best-characterized mechanism for the induction of acid resistance in *S. typhimurium* is the ATR, which occurs when the acid resistance of this microorganism is greatly in-
duced after the cells are adapted to mildly acidic pH conditions (9). Numerous genes of *S. typhimurium* that are necessary to express ATR phenotypes have been identified and characterized, including *rpoS*, *phoP*, *fur*, *mvic*, *atp*, and *atrB* (9). It is reasonable to speculate that there may be an overlap between the genetic systems for ATR and SCFA-induced acid resistance. Therefore, we examined the effects of the ATR mutations on SCFA-induced acid resistance (Fig. 5). To compare the ATR mutations in different genetic backgrounds, the mutations linked to antibiotic markers were transferred by P22 transduction from the ATR mutant strains obtained from Foster and Hall (11) and Lee et al. (23) to the wild-type *S. typhimurium* ATCC 14028s and SL1344 strains, and the resulting transductants were selected on LB plates containing relevant antibiotics. Among the four ATR mutations tested, only the mutants harboring a mutation in *atrB* appeared to be defective in inducing acid resistance after adaptation to 100 mM propionate at pH 7.0 in both strain backgrounds but not statistically significant (*P > 0.05*) compared to that of the wild type. The mutation in the *rpoS* and *atp* gene did not change the acid resistance after adaptation to propionate significantly (*P > 0.05*), whereas unexpectedly, the *phoP* mutation in ATCC 14028s background significantly increased the acid survival of *S. typhimurium* over that of the wild type (*P < 0.05*), Wilmes-Reisenberg et al. (33) reported that the *atrB* mutation in SL1344 strain just slightly increased the 50% lethal dose (LD$_{50}$) in mouse infection study over that of the wild type. It is possible that the partial defect of the *atrB* mutant in SL1344 background in SCFA-induced acid resistance is the reason for the minor role of the gene in virulence during mouse infection. If that is correct, it may be interesting to determine the LD$_{50}$ of *atrB* mutant in ATCC 14028s background, because this mutation almost completely abolished the SCFA-induced acid resistance (0.8% survival) in this strain background. Although it was not shown in Fig. 5, we also found that a mutant strain of *S. typhimurium* SFI with a mutation in the *fur* gene (JF2391 [23]) could not mount SCFA-induced acid resistance.

**Concluding remarks.** Given that *S. typhimurium* is a major foodborne pathogen and also an enteropathogen which can be found in the gastrointestinal tracts of host animals, such as acidic pH and low redox potential, and prolonged exposure to SCFA, markedly enhance the SCFA-induced acid resistance. There are two stages during the process of infection in which acid resistance would be required for successful pathogenesis of *S. typhimurium*. One is the gastric acidity (pH 3.0) in the stomach that *Salmonella* ingested with contaminated food materials would pass through (32), and the other one is the acidification (pH 4.0 to 5.0) of the phagolysosome which *Salmonella* would encounter when phagocytosed by macrophages (30).

To probe the hypothesis that the SCFA-induced acid resistance reported in this study is required for *Salmonella* to cause systemic disease in a host animal, we will need to isolate mutant strains of *S. typhimurium* defective in SCFA-induced acid resistance and determine the LD$_{50}$ of the mutant strains in an infection study.

In addition to the results in this study, we also found that the resistance of *S. typhimurium* to other stress conditions including reactive oxygen and high osmolarity was increased by exposure to the mixture of SCFA species at the concentrations comparable to those in large intestines of animals (data not shown). The capability of SCFA to induce cross-protection to various stress conditions indicates that SCFA could have a more profound effect on the survival or virulence of *S. typhimurium* in a host animal. This could have biological and practical implications for controlling *Salmonella* spp. in food production systems.

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**FIG. 5.** Percent acid survival of wild-type (**WT**) and ATR mutant strains of *S. typhimurium* ATCC 14028s and SL1344 after adaptation to propionate (100 mM). The percent acid survival determined before adaptation was <0.0001% for all the strains tested (not shown on the graph).
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