Tolerance to Stress and Ability of Acid-Adapted and Non–Acid-Adapted *Salmonella enterica* Serovar Typhimurium DT104 To Invade and Survive in Mammalian Cells In Vitro†

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**ABSTRACT**

The ability of acid-adapted (AA) and non–acid-adapted (NA) *Salmonella enterica* serovar Typhimurium definitive type 104 (DT104) strains to invade and multiply in mammalian cells in vitro and to survive stress conditions was examined. DT104 and non-DT104 strains were grown in tryptic soy broth without glucose (NA) or in tryptic soy broth containing 1% glucose (AA) for 18 h at 37°C. The invasiveness of DT104 strains in J774A.1 macrophage and Int407 intestinal cell lines was not more extensive than that of non-DT104 strains. In most cases, AA bacteria were less invasive than NA bacteria in both cell lines. Confocal microscopy showed that both DT104 and non-DT104 strains replicated in the two cell lines. In related studies, the survival levels of three strains of AA and NA DT104 and a non-DT104 (LT2) strain in 150 and 15 mM H$_2$O$_2$, 170 and 43 mM acetic acid, 2.6 M NaCl, 2.6 M NaCl containing 170 mM acetic acid, synthetic gastric fluid (SGF) at pH 2 and pH 3, and apple cider were compared. For all four strains, acid adaptation did not result in increased survival in apple cider. After 15 days of storage at 4°C, reductions ranged from 1.96 to 4.1 log$_{10}$ CFU/ml for AA bacteria and from 0.48 to 1.34 log$_{10}$ CFU/ml for NA bacteria from a starting level of ca. 7.00 log$_{10}$ CFU/ml. Neither AA nor NA DT104 strains were more resistant to NaCl, acetic acid, H$_2$O$_2$, or SGF solutions than non-DT104 strain LT2. The level of AA bacteria was not appreciably reduced after exposure to SGF; however, the level of NA bacteria decreased to nondetectable levels in SGF at pH 2 within 3 h of exposure. These results indicate that the DT104 strains examined were not more invasive, nor did they display increased survival in mammalian cells or increased resistance to food environment stresses compared with non-DT104 strains. However, acid adaptation resulted in increased resistance to a low-pH gastric environment for all strains tested. These data indicate that DT104 strains are likely not more virulent or resistant to stresses relevant to foods than are non-DT104 *Salmonella* and that procedures used to inactivate or inhibit the growth of *Salmonella* in foods are likely adequate for DT104 strains.

Multiple-antibiotic-resistant *Salmonella enterica* serovar Typhimurium definitive type 104 (DT104) strains and related phage types DT104b and U302 possessing the antibiotic resistance pattern ACSSuT (ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline) are widespread in the United States, Canada, Europe, and Japan (13, 17). Strains exhibiting additional resistance to trimethoprim and fluoroquinolones are also emerging (17, 30). Strains of DT104 cause illness in animals and have been transmitted from animals and foods of animal origin to humans (28, 32). Risk factors for acquiring DT104 infections in England and Wales included contact with ill farm animals and the consumption of chicken, pork sausage, and meat paste (32). Beef and raw milk cheese have also been associated with DT104-associated outbreaks of illness (11, 31).

The results of a study conducted by Wall et al. (32) indicated that *Salmonella* Typhimurium DT104 infections may be associated with higher morbidity and mortality rates than infections caused by other *Salmonella* phage types. Furthermore, a study on a large outbreak linked to raw milk cheese showed that 72% of patients had bloody diarrhea, and 9% were hospitalized (29). A matched cohort study conducted by Helms et al. (16) showed that the 2-year death rate for patients infected with *Salmonella Typhimurium* R-type ACSSuT (predominantly DT104 strains) was 4.8 times as high as for the general population. Multiresistance of DT104 strains renders antibiotic treatment of serious infections difficult (27). There is a concern that in addition to resistance to antibiotics, DT104 strains may also exhibit increased tolerance to other antimicrobial compounds and to treatments and processes commonly used in food production. Further studies are needed to assess whether DT104 strains are more virulent and/or have a greater ability to tolerate stresses encountered in foods and during food production and storage than nonresistant *S. enterica* Typhimurium or other pathogens.

*Salmonella* must endure the acid pH (ca. 2) of the stomach before attaching to intestinal cells. The organism crosses the intestinal barrier by translocating through M cells or columnar epithelial cells and is then phagocyted by resident macrophages (14). Macrophages infected with *Salmonella* eventually die by apoptosis. Therefore, to cause disease, *Salmonella* must tolerate stresses encountered in

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the food environment, during food processing, in the stomach and intestinal tract, and in the environments of intestinal and phagocytic cells. Studies have shown that growth conditions and growth phase influence the invasiveness of Salmonella (20). Carlson et al. (10) found that DT104 strains did not display a “hyperinvasive” phenotype relative to nonresistant strains (nonresistant DT104, nonresistant Salmonella Typhimurium SL1344, and nonresistant DT104 transformed with a multiresistance plasmid), and interestingly, some strains of multiresistant phage types DT104, U302, DT120, and DT193 were less invasive in HEp-2 cells than were controls.

The exposure of Salmonella spp. to mild or moderate acid stress results in the synthesis of proteins that protect the bacterium from a more severe acid challenge and can potentially allow the organism to survive a subsequent lethal stress of a different type (cross-protection) (5, 25). This phenomenon is termed the acid tolerance response (ATR), and distinct ATR systems are induced in the exponential and stationary growth phases (5, 22). In addition, in Salmonella Typhimurium, organic and inorganic acid tolerances are controlled by different mechanisms (5, 6). The ability of Salmonella Typhimurium to adapt to low-pH environments is of interest, since the organism encounters such environments in foods, in the gastrointestinal tract, and in the macrophage phagosome. The exposure of Salmonella to organic acids in foods may allow the pathogen to adapt and acquire the ability to tolerate subsequent stresses that would normally inactivate it. A recent study comparing the ability of DT104 strains to endure exposure to reactive oxygen and nitrogen species and to survive in murine macrophages with that of S. enterica Typhimurium ATCC 14028s failed to show increased virulence for DT104 strains (1). However, studies have not been conducted to examine the effect of acid adaptation on the ability of DT104 strains to survive stresses found in food environments and in the gastrointestinal tract (cross-protection) and to invade and replicate in mammalian cells in vitro. Thus, the objectives of this study were (i) to determine the survival of DT104 strains in apple cider and their tolerance to other stresses such as salt, hydrogen peroxide, acetic acid, and synthetic gastric fluid; (ii) to determine the invasiveness and survival levels of DT104 strains compared with those of non-DT104 strains in macrophage-like and intestinal cell lines in vitro; and (iii) to determine whether acid adaptation can induce cross-protection against subsequent stresses and increase the levels of invasiveness and survival ability of DT104 strains in intestinal and macrophage-like cell lines.

**MATERIALS AND METHODS**

**Bacterial strains and inoculum preparation.** The bacterial strains used in this study and their sources are listed in Table 1. Bacteria from stocks frozen at −70°C in 20% glycerol were plated on MacConkey agar (Difco Laboratories, Sparks, Md.) and tryptic soy agar (Difco). Acid-adapted cells were obtained by the procedure of Buchanan and Edelson (8). Briefly, a colony of S. enterica serovar Typhimurium or Escherichia coli O157:H7 was diluted in tryptic soy broth (TSB; Difco) and used to inoculate 50 ml of TSB without glucose (TSB-G⁰) and 50 ml of TSB with 1% (wt/vol) glucose (TSB-G¹). To determine whether glucose by-products potentially formed by the autoclaving of TSB with 1% glucose had an effect on the survival of the bacteria when the bacteria were challenged with subsequent stresses, the TSB-G was prepared in two ways: (i) by adding the glucose before the medium was autoclaved for 15 min at 121°C and (ii) by adding a filter-sterilized glucose solution to autoclaved TSB to a final concentration of 1%. All of the cultures were incubated without agitation at 37°C for 18 h to yield stationary-phase cell (ca. 10⁸ CFU/ml), and colonies were enumerated on tryptic soy agar to determine the actual count.

**Survival of AA and NA cells after exposure to stress.** Survival assays were performed to determine the extent to which acid adaptation affected the resistance of DT104 and non-DT104 strains to subsequent stress conditions. Portions of the acid-adapted (AA) and non–acid-adapted (NA) cultures (250 μl) were added to 25 ml of various solutions in 50-ml-volume sterile plastic tubes to yield about 10⁷ CFU/ml. The solutions used were 150 and 15 mM hydrogen peroxide (H₂O₂) in sterile water, 170 and 43 mM glacial acetic acid (HAc) in sterile water, 170 and 43 mM glacial acetic acid (HAc) in sterile water, 2.6 M (15% solution) sodium chloride (NaCl) in TSB (containing 0.25% dextrose), 2.6 M NaCl–170 mM HAc in TSB (containing 0.25% dextrose), and synthetic gastric fluid (SGF) at pHs of 2 and 3 (HCl was used to lower the pH). The SGF contained 8.3 g of proteose peptone (Difco) per liter, 3.5 g of glucose per liter, 2.05 g of NaCl per liter, 0.37 g of KCl per liter, 0.05 g of porcine bile (Animal Technologies, Inc., Tyler, Tex.) per liter, 0.1 g of lysozyme (Sigma Chemical Co., St. Louis, Mo.) per liter, and 13.3 mg of pepsin (from porcine stomach mucosa, P-6887; Sigma) per liter (7). The tubes were kept at room temperature (ca. 24°C), and samples were taken at 0, 1, and 2 h (for the solutions containing 150 and 15 mM H₂O₂ and 170 and 43 mM HAc) or at 0, 1, 2, and 3 h (for the solutions containing SGF, 2.6 M NaCl, and 2.6 M NaCl–170 mM HAc) for plating on brain heart infusion agar (Difco) with a Spiral Plater (Model D, Spiral Biotech, Inc., Bethesda, Md.). The plates were incubated at 37°C for 20 h, and colonies were enumerated with a Model 500A Bacteria Colony Counter (Spiral Biotech).

<table>
<thead>
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* WSU, Washington State University, Pullman, Wash.; CDC, Centers for Disease Control and Prevention, Atlanta, Ga.; PHLS, Public Health Laboratory Service, Exeter, United Kingdom; UGA, University of Georgia (Michael Doyle), Athens, Ga.; FSIS, Food Safety and Inspection Service, Athens, Ga.
The AA and NA cells were also inoculated into 25 ml of unpasteurized apple cider that was purchased from a local college. The inoculated cider was stored at 4°C, and samples were diluted and plated on brain heart infusion agar daily for 15 days. The number of bacteria surviving the various treatments (log10 CFU/ml reduction) was determined by subtracting the log10 CFU/ml value for bacteria surviving after 2 h, 3 h, or 15 days (for the apple cider) from the log10 CFU/ml value for bacteria at time 0.

**Invasion and intracellular replication assays.** Cell invasion assays were carried out with the use of Int407 human small intestinal epithelial cells (ATCC CCL6; American Type Culture Collection, Rockville, Md.) and J774A.1 murine macrophage-like cells (ATCC TIB-67, American Type Culture Collection). The Int407 cells were grown in basal medium (Eagle) with Earle’s salts (modified Eagle’s medium [MEM]; Invitrogen, Carlsbad, Calif.) supplemented with 15% fetal calf serum, and the J774A.1 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) with 4 mM l-glutamine supplemented with 10% fetal calf serum adjusted to contain 1.5 g of sodium bicarbonate per liter; 4.5 g of glucose per liter; and 1.0 mM sodium pyruvate. The AA and NA bacteria were prepared as described above, 10-ml portions were centrifuged, the cell pellets were resuspended in 5 ml of phosphate-buffered saline (PBS) (pH 7.4), and 400 μl was added to 2 ml of MEM or DMEM. The bacterial suspensions (1 ml, ca. 5 × 10^8 CFU) were added in duplicate to confluent cell monolayers in 12-well tissue culture plates, which were incubated in 5% CO2 at 37°C for 1 h to allow bacterial invasion to occur. The number of J774A.1 and Int407 cells per well, determined with a hemacytometer, was ca. 5 × 10^6; thus, the multiplicity of infection was 10 (10 bacteria per epithelial cell or macrophage). The wells were washed twice with DMEM, and then 2 ml of tissue culture medium containing 100 μg of gentamicin sulfate per ml was added to each well. The plates were incubated for 1.5 h in 5% CO2 at 37°C to kill the remaining extracellular bacteria. Subsequently, the wells were washed twice with 2 ml of DMEM, and 1 ml of 1% Triton X-100 (prepared with sterile water) was added to release intracellular bacteria. The number of bacteria per well was determined by plating dilutions on brain heart infusion agar.

Intracellular multiplication of the DT104 and non-DT104 strains was determined by the procedure of Finlay and Falkow (12). Briefly, Int407 and J774A.1 monolayers were incubated with the bacteria as described for the invasion assays. After the DMEM containing 100 μg of gentamicin per ml was removed, the monolayers were washed with DMEM, and 2 ml of medium containing 10 μg of gentamicin per ml was added to each well. The medium was removed after incubation for 24 h at 37°C in 5% CO2, DMEM containing 100 μg of gentamicin per ml was added, and the plate was incubated for 1 h. The monolayers were washed and lysed with 1% Triton X-100. The number of CFU per well was determined as described above.

**TEM and confocal laser scanning microscopy.** To examine internalized DT104 and non-DT104 bacteria, Int407 and J774A.1 monolayers were formed in glass-bottom dishes (no. 15, part no. P35-G-1.5-14-C, MarTech Corporation, Ashland, Mass.). Monolayers were incubated with the bacteria as described above for the invasion and intracellular replication assays and were then stained with the LIVE/DEAD BacLight Bacterial Viability Kit L7012 (Molecular Probes, Eugene, Oreg.) according to the manufacturer’s instructions. Cells were examined with a TCS-SP confocal microscope (Leica Microsystems, Exton, Pa.). The fluorescent channels were excited at 488 nm, and emission was collected at ca. 500 and 635 nm. For transmission electron microscopy (TEM), glutaeraldehyde (Electron Microscopy Sciences, Fort Washington, Pa.) was added to the dishes to a final concentration of 2.5%. For further processing, the glutaeraldehyde fixative solution was removed and replaced with 0.1 M imidazole buffer, and samples were postfixed in 2% osmium tetroxide solution in imidazole buffer for 2 h. The monolayers were dehydrated in ethanol solutions (50%, 80%, and absolute ethanol) and embedded in an epoxy resin mixture. Thin sections of oriented monolayers were cut with diamond knives, stained with solutions of uranyl acetate and lead citrate, and examined in the bright-field mode with a transmission electron microscope (Model CM12, Philips/FEI, Hillsboro, Oreg.).

**Statistical analyses.** Data from three trials were analyzed by analysis of variance with the SAS statistical software system (SAS Institute, Inc., Cary, N.C.). Individual treatment comparisons were carried out with the use of specific contrasts and mean separation techniques. A significance level of P = 0.05 was used for all significance testing.

**RESULTS**

**Invasion and survival of DT104 and non-DT104 bacteria.** The pHs of the media for stationary-phase cultures were ca. 4.5 and 7.5 for cultures grown in TSB-G1 and TSB-G2, respectively, which produced AA and NA DT104 and non-DT104 bacteria. The DT104 strains were not more capable of invading the Int407 or J774A.1 cells than the non-DT104 strains were (Figs. 1A and 2A). For Int407 cells, the levels of invasion and intracellular multiplication for all NA bacteria were higher than those for AA bacteria (Fig. 1A and 1B). For both NA and AA bacteria, there was a significant increase in the numbers of CFU per well at 24 h compared with the numbers at 1 h, indicating that intracellular multiplication of bacteria occurred. The levels of invasion and multiplication for AA strain 41 were significantly lower than those for the other strains, and the multiplication levels for AA strains 16 and 30 were significantly higher than those for strains 10 and LT2. Confocal microscopy results also showed that more NA bacteria than AA bacteria (strain 30) invaded Int407 cells in vitro (Fig. 3). By 24 h, many of the Int407 cells had detached from the culture dishes; however, infected cells that remained attached contained many bacteria (Fig. 3, NA bacteria). A relatively larger number of Int407 cells incubated with AA bacteria remained attached, although fewer of these cells contained bacteria (Fig. 3G) than did cells incubated with the NA bacteria (Fig. 3E). Salmonella-containing vacuoles were evident in monolayers of Int407 cells exposed to Salmonella Typhimurium strain 30 for 24 h (Fig. 3F). The TEM results showed that DT104 strain 30 bacteria that invaded Int407 cells were found inside phagosomes (Fig. 4A). Some phagosomes had fused and contained two or more intact bacteria. By 24 h, there were fewer Int407 cells attached to the wells than there were at 1 h, and many cells had been destroyed and had released the bacteria (Fig. 4B). There was a relatively larger number of intact Int407 cells, and fewer of these cells contained intracellular bacteria in monolayers containing AA bacteria than in those containing NA bacteria; however, AA bacteria replicated and were visible inside phagosomes (Fig. 4C).

There were significant increases in levels of invasion...
for NA bacteria compared with those for AA bacteria for all strains tested except strain LT2 (Fig. 2A). There were significant increases in the numbers of CFU per well for all AA strains except strain LT2 at 24 h compared with the numbers at 1 h, indicating that the bacteria multiplied in the J774A.1 cells (Fig. 2A and 2B). In contrast, there were no significant increases in the numbers of CFU per well for NA strains at 24 h compared with the numbers at 1 h, and the number of CFU per well for NA strain LT2 had decreased significantly at 24 h compared with the number for this strain at 1 h. The tissue culture plates were observed by light microscopy at 1 and 24 h before and after the washes, and it was observed that wells that contained NA bacteria had fewer J774A.1 cells attached at 24 h than did wells that contained AA bacteria; therefore, during the washes, the dead J774A.1 cells harboring bacteria were removed. This is most likely the reason the numbers of CFU per well were not larger at 24 h than at 1 h for the NA bacteria. For AA bacteria at 1 and 24 h, all strains showed significantly larger numbers of CFU per ml than strain 41 did. Confocal microscopy results showed that both NA and AA bacteria (strain 30) invaded J774A.1 cells; however, microscopic analysis showed that there were fewer AA than NA bacteria in the cells (Fig. 3C and 3D). Intact bacteria were found in membrane-bound phagosomes in J774A.1 cells at 1 h (Fig. 4D and 4E). Possible lysosomal membrane glycoprotein-containing vacuoles were visible in close proximity to phagosomes containing bacteria (Fig. 4D). By 24 h, macrophage damage was evident, and bacteria were visible in large vacuoles (Fig. 4E). DT104 strain 41 replicated inside infected macrophages (Fig. 4F); however, this replication caused less damage to the monolayer, since this strain invaded a smaller number of cells. Results for non-DT104 strain 14028 were similar to those for DT104 strain 30 (data not shown).

FIGURE 1. (A) Int407 invasion (after 1 h of incubation of bacteria with Int407 cells) and (B) intracellular replication (after 24 h of incubation of bacteria with Int407 cells) capabilities of DT104 and non-DT104 strains (see Table 1 for descriptions of strains). The results presented represent means ± standard errors for three replicate experiments.

FIGURE 2. (A) J774A.1 invasion (after 1 h of incubation of bacteria with J774A.1 cells) and (B) intracellular replication (after 24 h of incubation of bacteria with J774A.1 cells) capabilities of DT104 and non-DT104 strains (see Table 1 for descriptions of strains). The results presented represent means ± standard errors for three replicate experiments.

Survival of AA and NA Salmonella in apple cider.

In apple cider (pH 3.46), the survival of NA bacteria was significantly more extensive than that of AA bacteria (Fig. 5). The survival levels for the three DT104 strains were not significantly different from that for strain LT2 (non-DT104) over the 15-day storage period. There were larger log_{10} CFU/ml reductions for both NA and AA DT104 strain 41 than for NA and AA strains 3380, 2380, and LT2 after 15 days of storage at 4°C. After 2 h of challenge in 150 mM H_{2}O_{2}, both NA and AA bacteria showed reductions of ca. 5.7 to 7.0 log units (Fig. 6). However, the NA bacteria were significantly more tolerant to treatment with 15 mM H_{2}O_{2} than the AA cells were. Reductions ranged from 4.8 to 5.7 log_{10} CFU/ml and from 0.9 to 3.3 log_{10} CFU/ml for AA and NA bacteria, respectively. The DT104 strains were not significantly more tolerant to 150 mM H_{2}O_{2} or to 15 mM H_{2}O_{2} than strain LT2 was. Strain 41 (NA) was significantly more susceptible to inactivation with 15 mM H_{2}O_{2} than were the other strains tested. After treatment with H_{2}O_{2}, the levels of survival of bacteria grown in medium prepared by adding the
FIGURE 3. Confocal microscopy images for NA (A, C, E, F, and H) and AA (B, D, G, and I) DT104 strain 30 in Int407 (A, B, E, F, and G) and J774A.1 (C, D, H, and I) cell lines. Panels A, B, C, and D show cells after 1 h of incubation, and panels E, F, G, H, and I show cells after 24 h of incubation. Arrows indicate bacteria inside Int407 cells (E and G), and arrowheads indicate Salmonella-containing vacuoles (F).
glucose before autoclaving did not differ appreciably from the levels for bacteria grown in medium prepared by adding the glucose after autoclaving.

**Treatment of NA and AA Salmonella with HAc.** All of the NA bacteria except strain 41 showed decreases in their ability to survive the HAc treatments (Fig. 7). Reductions produced by 170 mM HAc were larger than those produced by 43 mM HAc. For AA bacteria (grown in TSB to which glucose was added prior to autoclaving) exposed to 43 mM HAc, reductions for strains 41, 3380, 2380, and LT2 were 2.1, -0.2, 0.1, and -0.1 log$_{10}$ CFU/ml, respectively, and for NA bacteria the reductions for these strains were 2.2, 0.7, 0.9, and 0.7 log$_{10}$ CFU/ml, respectively. For bacteria exposed to 170 mM HAc, reductions for AA bacteria were 4.4, 0.9, 0.7, and 0.9 log$_{10}$ CFU/ml for strains 41, 3380, 2380, and LT2, respectively, and reductions for the same strains of NA bacteria were 4.7, 2.0, 2.4, and 2.7 log$_{10}$ CFU/ml, respectively. The reductions for AA DT104 strain 2380 and non-DT104 strain LT2 were statistically different from those for the corresponding NA bacteria after treatment with 170 mM HAc. There were also differences between reductions for NA DT104 strain H3380 and AA DT104 strain H3380, although these differences were not statistically significant. Acid adaptation provided some protection against treatment with HAc for all strains except strain 41.
FIGURE 6. Survival of AA and NA DT104 and non-DT104 strains following treatment with $H_2O_2$ (see Table 1 for descriptions of strains). (A) Bacteria were grown in TSB prepared by adding glucose (1%, wt/vol) before the medium was autoclaved for 15 min at 121°C (AA). (B) Bacteria were grown in TSB without glucose (NA). (C) Bacteria were grown in medium to which a filter-sterilized glucose solution was added to obtain a concentration of 1% glucose (AA) after the TSB was autoclaved. The results presented represent means ± standard errors for three replicate experiments.

FIGURE 7. Survival of AA and NA DT104 and non-DT104 strains following treatment with HAc (see Table 1 for descriptions of strains). (A) Bacteria were grown in TSB prepared by adding glucose (1%, wt/vol) before the medium was autoclaved for 15 min at 121°C (AA). (B) Bacteria were grown in TSB without glucose (NA). (C) Bacteria were grown in medium to which a filter-sterilized glucose solution was added to obtain a concentration of 1% glucose (AA) after the TSB was autoclaved. The results presented represent means ± standard errors for three replicate experiments.

FIGURE 8. Survival of AA and NA DT104 and non-DT104 strains following treatment with 2.6 M NaCl and 2.6 M NaCl–170 mM HAc (see Table 1 for descriptions of strains). (A) Bacteria were grown in TSB prepared by adding glucose (1%, wt/vol) before the medium was autoclaved for 15 min at 121°C (AA). (B) Bacteria were grown in TSB without glucose (NA). (C) Bacteria were grown in medium to which a filter-sterilized glucose solution was added to obtain a concentration of 1% glucose (AA) after the TSB was autoclaved. The results presented represent means ± standard errors for three replicate experiments.

Treatment of NA and AA *Salmonella* with 2.6 M NaCl and 2.6 M NaCl–170 mM HAc. The AA and NA DT104 strains were not more resistant to treatment with 2.6 M NaCl–170 mM HAc and with 2.6 M NaCl than the non-DT104 strain (LT2) was (Fig. 8). Strain 41 (both NA and AA) was less tolerant to treatment with 2.6 M NaCl–170 mM HAc and with 2.6 M NaCl than were the other strains tested. Strain 41 grown in medium in which glucose was added before autoclaving was somewhat more tolerant to 2.6 M NaCl–170 mM HAc than were bacteria that under-
FIGURE 9. Survival of AA and NA DT104 and non-DT104 strains following treatment with SGF at pH 2 and at pH 3 (see Table 1 for descriptions of strains). (A) Bacteria were grown in TSB prepared by adding glucose (1%, wt/vol) before the medium was autoclaved for 15 min at 121°C (AA). (B) Bacteria were grown in TSB without glucose (NA). (C) Bacteria were grown in medium to which a filter-sterilized glucose solution was added to obtain a concentration of 1% glucose (AA) after the TSB was autoclaved. The results presented represent means ± standard errors for three replicate experiments.

went acid adaptation in medium to which glucose was added after autoclaving (reductions of 2.4 and 3.2 log units, respectively); however, these differences were not statistically significant. In general, NA strains 41, 3380, 2380, and LT2 were somewhat more tolerant to 2.6 M NaCl–170 mM HAc than the bacteria that had undergone acid adaptation in medium to which glucose was added either before or after autoclaving. Not surprisingly, log reductions were significantly larger for both NA and AA bacteria exposed to 2.6 M NaCl–170 mM HAc than for bacteria exposed to 2.6 M NaCl alone.

Survival of NA and AA bacteria in synthetic gastric fluid. The NA DT104 and non-DT104 strains were significantly more susceptible to SGF at pH 2 than the AA bacteria were (Fig. 9). In <1 h, the number of surviving NA bacteria was below the detection limit, whereas all AA bacteria except for strain 41 survived in SGF at both pH 2 and pH 3 for at least 3 h; for AA strain 41, there were reductions of 1 to 1.5 log units with exposure to SGF at pH 3. In contrast, both the NA and the AA *E. coli* O157:H7 strains survived in SGF at both pH 2 and pH 3 for at least 3 h.

DISCUSSION

A clearer understanding of the physiology of *S. enterica* serovar Typhimurium DT104 strains and of factors that influence the virulence and survival of this organism are important for the development of effective control measures. *Salmonella* strains can tolerate acid conditions and have caused outbreaks of gastroenteritis associated with acidic foods such as orange juice (18). This organism has evolved intricate mechanisms to survive potentially lethal acidic conditions and other types of stresses (5, 25). The acid adaptation of *Salmonella* spp. via a short period of growth in a synthetic medium at pH 5.8 resulted in enhanced survival in cheese and provided subsequent cross-protection against heat, salt, an activated lactoperoxidase system, and the surface active agents crystal violet and polymyxin B (24, 25). In the present study, the DT104 strains tested did not show an increased ability to invade or grow in *Int407* epithelial cells or *J774A.1* macrophage-like cells compared with non-DT104 strains. DT104 strain 41 was less invasive than the other DT104 and non-DT104 strains tested. These results are in agreement with those of Carlson et al. (10), who identified strains of DT104 that were “hypoinvasive” for *HEp-2* cells. Allen et al. (1) found that DT104 strains were not more invasive for *HEp-2* cells and did not have a greater ability to survive in BALB/c peritoneal macrophages than 14028s, a non-DT104 strain. In the present study, acid adaptation resulted in a lower level of invasion in *Int407* and *J774A.1* cells. However, confocal microscopy of the cells showed that once invasion occurred, both NA and AA bacteria replicated in both cell lines in vitro. *Salmonella* attenuates acidification of the phagosome in macrophages and can activate transcription of the virulence genes necessary for its survival in the acidified phagosome (2, 15). The pH of the phagosome with dead bacteria was ≈4.5, while that of the phagosomes containing live *Salmonella* was >5 up to 4 h postinfection (2). In the present study, it is unclear why AA bacteria were less invasive than NA bacteria in both cell lines. Studies have shown that environmental conditions such as low oxygen levels can influence the invasiveness of *Salmonella* in MDCK cells, and *Salmonella* are less invasive in the stationary phase of growth than in the logarithmic phase (20). One locus, termed *hil* (hyperinvasive locus), encodes a factor that is essential for invasion and is rate limiting for invasion in aerobically grown *Salmonella* (21). Therefore, the invasive ability of *Salmonella* is regulated and modulated by changes in the growth state.

The acidification of foods to control the growth of pathogens is a common practice. Therefore, the ability of *Salmonella* and other foodborne pathogens to adapt to acidic conditions is a food safety concern. Additionally, the exposure of *Salmonella* to sublethal acid stress may cause an adaptive response that increases the ability of the organism to survive a previously lethal stress of the same type or of a different type (24, 25). In the present study, the survival of DT104 strains in unpasteurized apple cider and in 150 and 15 mM H₂O₂, 170 and 43 mM HAc, 2.6 M NaCl, 2.6 M NaCl–170 mM HAc, and SGF at pH 2 and at pH 3 was similar to the survival of non-DT104 strain LT2.
There were reductions of \( \sim 7 \log_{10} \) CFU/ml for the NA DT104 and non-DT104 strains after 2 h of exposure to 150 mM H\(_2\)O\(_2\), and there were reductions of 1 to 3 \( \log_{10} \) CFU/ml for these strains after exposure to 15 mM H\(_2\)O\(_2\). Anrany et al. (3) found that the exposure of DT104 strain 11601 to 10 mM H\(_2\)O\(_2\) resulted in the inactivation of all of the bacteria (10\(^7\) CFU) within 90 min. Interestingly, these investigators found that DT104 expressed a “rugose” phenotype when grown on certain media and under certain conditions, and this phenotype possessed an enhanced tolerance to low pH and H\(_2\)O\(_2\) (a 2-log reduction was achieved in 90 min) and was able to form a biofilm. In the present study, AA bacteria (grown in TSB with autoclaved or filter-sterilized glucose) exhibited a decreased tolerance to 15 mM H\(_2\)O\(_2\) compared with NA bacteria. On the other hand, the AA DT104 strains (except strain 41) and the non-DT104 strains exhibited a notable increase in tolerance to 43 and 170 mM HAC compared with NA bacteria. Roering et al. (29) examined the survival of DT104 in pasteurized and unpasteurized apple cider and found reductions of \(< 4.5 \) and \(\geq 5.5 \log_{10} \) CFU/ml during storage of inoculated pasteurized and unpasteurized apple cider, respectively, at 4 and 10°C. These results are not in agreement with the results of the present study, since counts of NA bacteria decreased by only 0.5 to 1.3 \( \log_{10} \) CFU/ml after 15 days of storage at 4°C. The AA bacteria, however, were less tolerant to unpasteurized apple cider than were the NA bacteria, with reductions after 15 days of storage ranging from ca. 2 to 4 \( \log_{10} \) CFU/ml.

AA DT104 and non-DT104 strains were more resistant to HAC treatments than the corresponding NA bacteria, except for DT104 strain 41 (Fig. 7). Additionally, acid adaptation resulted in an increase in the ability of DT104 and non-DT104 strains to withstand challenge with SGF at pH 2. On the other hand, the three \( E. coli \) O157:H7 strains tested, both NA and AA, survived exposure to SGF at pH 2 and at pH 3 for at least 3 h (Fig. 9). Similarly, Roering et al. (29) reported that DT104 and \( Listeria monocyto genes \) were inactivated in SGF at pH 1.5 within 5 and 30 min, respectively, at 37°C, whereas \( E. coli \) O157:H7 decreased by only 1.6 to 2.8 \( \log_{10} \) CFU/ml within 2 h. Inside the host, \( Salmonella \) and \( E. coli \) encounter inorganic acid in the stomach and organic and inorganic acids in the small intestine; therefore, these bacteria may become adapted to acidic conditions in foods, rendering them more virulent in the host. \( Salmonella \) and \( E. coli \) possess different but overlapping acid resistance systems involving multiple regulators (5). \( Salmonella \) possesses both log-phase and stationary-phase acid stress response systems, and each of these systems involves the induction of different sets of acid shock and regulatory proteins. Acid-induced cross-protection against environmental challenges such as osmotic, heat, or oxidative stress occurs in \( Salmonella \); however, this phenomenon is poorly understood. Regulatory systems that function in response to changes in external and internal pH include the RpoS system, the Fur system, and the two-component PhoPQ and OmpR/EnvZ systems (5). \( Salmonella \) possesses two stationary-phase acid tolerance systems that are regulated independently. One does not require induction by low pH and is dependent on the alternative sigma factor, RpoS, and the second is acid inducible and RpoS independent (23). In \( Salmonella \), stress imposed by organic acids stimulates rpoS translation; therefore, in the present study, bacteria that underwent acid adaptation via growth in TSB-G- may have had increased levels of RpoS, although RpoS levels were not determined. In addition, the acid-inducible (pH <5) RpoS-independent system may also have played a role in the tolerance of AA DT104 and non-DT104 strains to SGF at pH 2. \( E. coli \) has three acid resistance systems not found in \( Salmonella \): the oxidative or glucose-repressed system, the glutamate-dependent system, and the arginine-dependent system (5). The glutamate-dependent system allows cells to survive an extremely low acid challenge (pH 2). Arnold and Kaspar (4) found that the acid tolerance in \( E. coli \) O157:H7 was independent of prior exposure to moderate acid conditions. The organism survived in SGF at pH 2 and in acidified TSB (pH 2). Cultures in the late logarithmic and stationary growth phases displayed enhanced acid resistance compared with mid-logarithmic-phase cultures. In the present study, the levels of the three \( E. coli \) O157:H7 strains tested (both AA and NA) did not decrease after 3 h of exposure to SGF at pH 2 or at pH 3. These results are in agreement with those of Arnold and Kaspar (4).

Byrd et al. (9) have shown that a glucose-phosphate by-product formed during the sterilization of PBS containing glucose was toxic to \( E. coli \) O157:H7. The survival of \( E. coli \) O157:H7 in PBS autoclaved with glucose was less extensive than the survival of the organism in PBS containing filter-sterilized glucose. Log-phase cells were significantly more sensitive to the by-product than were stationary-phase cells, and an rpoS mutant was significantly more sensitive than the parent strain. Byrd et al. (9) found that the glucose-phosphate by-product also affected the survival of \( S. enterica \) Typhimurium. These investigators suggested, however, that \( E. coli \) that have a functional rpoS system and have been exposed to low concentrations of the by-product formed in standard media containing 0.25% glucose may not be severely affected by the glucose-phosphate by-product. In the present study, to eliminate the possibility that a glucose by-product formed during the growth of the bacteria in TSB-G+ (with glucose added prior to autoclaving) may have increased the susceptibility of the bacteria to subsequent stresses, the survival of bacteria grown in TSB to which filter-sterilized glucose was added at a concentration of 1% after autoclaving was also evaluated. The results obtained show that bacteria grown in TSB with filter-sterilized glucose were generally not better able to survive any of the applied treatments than the bacteria grown in TSB to which glucose was added prior to autoclaving. It is possible that if glucose had been used at a concentration of 2 to 5% and if logarithmic-phase cells had been challenged with the stress treatments, an effect of a medium by-product would have been observed.

DT104 strain 41 (both AA and NA) was less invasive and more susceptible to all of the applied stress treatments than the non-DT104 strain and all of the other DT104 strains tested. Although strain 41 was less invasive, infected...
cells contained higher levels of the bacteria after 24 h than they did after 1 h, indicating that the intracellular bacteria had the ability to multiply. Jørgensen et al. (19) also examined strain 41 and found that stationary-phase cultures of this strain had a decreased ability to survive air drying on surfaces, exposure to heat, and acid stress compared with other DT104 strains. Additionally, strain 41 was generally less invasive and was recovered less frequently from the livers, spleens, and ovaries of orally infected chickens than a number of other DT104 strains tested. Stress-sensitive strain 41 harbors a functional rpoS gene (19); therefore, this strain may harbor mutations in stress response/ virulence genes other than rpoS. The genetic typing of isolates by pulsed-field gel electrophoresis (PFGE) in the United States and in Germany has shown that DT104 strains are highly clonal, having similar pulsotypes (26, 33). Preliminary studies examining the PFGE patterns of DNA from strain 41 following XbaI digestion have shown that there is at least a one-band difference between the DNA pattern for this strain and that for the predominant DT104 molecular subtype. Further research to examine the PFGE profiles of strain 41 with the use of different restriction enzymes and to identify genes that may be missing or mutated in this strain is ongoing.

In summary, the results of this study show that DT104 strains are not more resistant to acid, osmotic, and oxidative stresses than a non-DT104 strain (LT2) and do not have an increased ability to invade, survive in, and multiply in INT407 and J774A.1 cell lines compared with non-DT104 strains. Additionally, acid adaptation increases the resistance of the bacteria to HAc treatment and to SGF at pH 2. The results of this and other studies indicate that in terms of resistance to stresses related to the food environment and food-processing conditions, DT104 strains may not pose a greater threat to food safety than non-DT104 strains.

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REFERENCES

STRESS TOLERANCE OF S. ENTERICA SEROVAR TYPHIMURIUM DT104


