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Treatment of *Salmonella enterica* Serovar Enteritidis with a Sublethal Concentration of Trisodium Phosphate or Alkaline pH Induces Thermotolerance

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The responses of *Salmonella enterica* serovar Enteritidis to a sublethal dose of trisodium phosphate (TSP) and its equivalent alkaline pH made with NaOH were examined. Pretreatment of *S. enterica* serovar Enteritidis cells with 1.5% TSP or pH 10.0 solutions resulted in a significant increase in thermotolerance, resistance to 2.5% TSP, resistance to high pH, and sensitivity to acid and H_2O_2. Protein inhibition studies with chloramphenicol revealed that thermotolerance, unlike resistance to high pH, was dependent on de novo protein synthesis. Two-dimensional polyacrylamide gel electrophoresis (PAGE) of total cellular proteins from untreated control cells resolved as many as 232 proteins, of which 22 and 15% were absent in TSP- or alkaline pH-pretreated cells, respectively. More than 50% of the proteins that were either up- or down-regulated by TSP pretreatment were also up- or down-regulated by alkaline pH pretreatment. Sodium dodecyl sulfate-PAGE analysis of detergent-insoluble outer membrane proteins revealed the up-regulation of at least four proteins. Mass spectrometric analysis showed the up-regulated proteins to include those involved in the transport of small hydrophilic molecules across the cytoplasmic membrane and those that act as chaperones and aid in the export of newly synthesized proteins by keeping them in open conformation. Other up-regulated proteins included common housekeeping proteins like those involved in amino acid biosynthesis, nucleotide metabolism, and aminoacyl-tRNA biosynthesis. In addition to the differential expression of proteins following TSP or alkaline pH treatment, changes in membrane fatty acid composition were also observed. Alkaline pH- or TSP-pretreated cells showed a higher saturated and cyclic to unsaturated fatty acid ratio than did the untreated control cells. These results suggest that the cytoplasmic membrane could play a significant role in the induction of thermotolerance and resistance to other stresses following TSP or alkaline pH treatment.

The U.S. Department of Agriculture has approved the use of trisodium phosphate (TSP) as a postchill processing aid to reduce bacteria on raw poultry carcasses (5, 23). TSP treatment has been shown to significantly reduce the populations of *Escherichia coli* O157:H7 (15, 73), *Salmonella enterica* serovar Enteritidis (27), *S. enterica* serovar Typhimurium (15, 74), *Clostridium sporogenes* (14, 15), *Campylobacter jejuni* (58, 66), and *Listeria monocytogenes* (13, 58, 66) on animal carcasses. TSP, when dissolved, results in an alkaline solution with a pH of 12.3 in water and 10.0 in tryptic soy broth (TSB) for a 1.5% (wt/vol) solution. Studies to understand the antimicrobial mode of action of TSP have shown that the alkaline pH of TSP is responsible for its antimicrobial activity and that *S. enterica* serovar Enteritidis can survive exposure to 1.5% TSP or its equivalent alkaline pH of 10.0 made with NaOH without any significant reduction in cell numbers for 1 h (59). Enterobacteria like *E. coli* and *Salmonella* species are commonly exposed to alkaline pH. Environmental alkalinization can follow exposure to polluted waters, for example, from extreme alkaline sewage, chemical industry, and agricultural effluents (52). Enterobacteria can also be subjected to alkalinity in some foods (e.g., egg whites) (32) and some food processing treatments (31). The response to alkaline pH can be characterized as alkaline shock (exposure to alkaline pH for up to 30 min) and alkaline adaptation (exposure to alkaline pH for periods of more than 60 min). Adaptation of *Salmonella* or *E. coli* to alkaline conditions is accompanied by induced thermotolerance, increased resistance to bile salts, and increased resistance to high pH (17, 24, 32, 71). Conversely, alkaline adaptation of *S. enterica* serovar Enteritidis or *E. coli* sensitizes the cells to acid stress (51) and vice versa (53).

The ability to grow at, or withstand, alkaline pHs could require the acquisition of enzymes capable of remaining active at high extracellular pHs (36) and could also be accomplished by modifications in gene expression. Although alkaline adaptation increases thermotolerance in *E. coli* and salmonellas, it has not been correlated with the expression of heat shock proteins (HSPs) (30). On the contrary, studies have shown the induction of HSPs by a mild pH upshift from 7.0 to 8.8 (5 to 10 min; alkaline stress) (71). In other specific studies, an unknown alkaline shock protein (23 kDa) in *Staphylococcus aureus* (37) and some genes whose expression changes as a function of alkaline stress (6, 19, 54) have been described. However, the relationship between alkaline stress and the heat shock response is not very clear. In *Enterococcus faecalis*, an alkaline shift in the external medium induces the synthesis of HSPs but did not induce maximal thermotolerance (17). Thus, the mechanism underlying thermotolerance due to alkaline adaptation is not clear and is hypothesized to involve changes in the physiology of alkali-adapted cells.

In addition to the role of HSPs in the induction of thermo-

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tolerance, modifications in membrane fluidity have also been shown to be involved in the adaptation to thermal stress. Bacterial cytoplasmic membranes consist mainly of lipids and have been shown to be a site for thermal injury (12, 28, 75). The cytoplasmic membrane represents the boundary between the cytoplasm and the external environment and regulates the flow of nutrients and metabolic products in and out of the cell, thereby permitting homeostasis of the cytoplasmic environment (10, 35). Growth conditions such as the composition of the growth medium (2, 3, 35, 60), the growth phase (age) of the cells (35, 42, 50, 75), the incubation temperature at which the bacteria were cultured (2, 35, 55, 56, 60, 70), and the pH (10, 56, 60) markedly affect the composition of the membrane lipid. *E. coli* cells subjected to an abrupt temperature shift from 30 to 45°C and held at the high temperature for various periods of time revealed a gradual decrease in the total unsaturated to saturated fatty acid ratio in the cytoplasmic membrane, resulting in reduced membrane fluidity and a higher melting point (43). This reduction in membrane fluidity correlated with the cellular heat shock response, as detected by the change in the induction levels of GroEL and DnaK (40, 43). In *Pediococcus* sp. a decrease in the unsaturated to saturated and cyclic fatty acid ratio during the transformation from log- to stationary-phase growth was accompanied by an increase in the D values, suggesting a relationship between thermotolerance and reduced membrane fluidity (4). However, to our knowledge there exists no published report suggesting that thermotolerance due to TSP or alkaline pH treatment results from changes in membrane fatty acid composition. In this study stationary-phase cells were used to examine the effect of pretreatment of *S. enterica* serovar Enteritidis with a sublethal concentration of TSP or alkaline pH on tolerance to lethal stresses, protein synthesis, and membrane fatty acid composition.

MATERIALS AND METHODS

Media and chemicals. Tryptic soy agar (TSA), TSB, magnesium chloride ($MgCl_2$), phenylmethylsulfonyl fluoride, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), DNase, RNase A, chloramphenicol, and bromophenol blue were purchased from Sigma Chemical Co. (St. Louis, Mo.). Peptone water was obtained from Difco Laboratories (Detroit, Mich.); TSP was from Rhône-Poulenc (Cranbury, N.J.); sodium hydroxide (NaOH) was from Fisher Scientific (Nepean, Ontario, Canada); sodium chloride (NaCl) was from Merck (Darmstadt, Germany); EDTA was from J. T. Baker Chemical Co. (Phillipsburg, N.J.); dithiothreitol (DTT), HEPES, glycerol, lysozyme, mercaptoethanol, sodium dodecyl sulfate (SDS), sodium *N*-lauroyl sarcosinate (Sarkosyl), tris base, and urea were from Life Technologies (Grand Island, N.Y.); 6 N HCl was from BDH Chemicals (Toronto, Ontario, Canada); and immobilized pH gradient (IPG) buffer (pH 4.0 to 7.0) and Immobiline DryStrip gels were from Amersham Bioscience Corp. (Uppsala, Sweden).

Bacteria and culture conditions. *S. enterica* serovar Enteritidis (ATCC 4931) was obtained from the American Type Culture Collection (Manassas, Va.). The stock culture was grown on TSA at 37°C for 18 h and maintained for a maximum period of a month at 4°C, after which a new stock culture was prepared from the frozen stock. Cells in the stationary phase of growth were prepared as described previously (59) and used for various treatments.

Treatment with a sublethal dose of TSP or alkaline pH. Stationary-phase cells were harvested by centrifugation at 12,000 × *g* for 10 min at 4°C (Sorvall RC-2B SuperSpeed refrigerated centrifuge), washed twice with phosphate-buffered saline (pH 7.0), and resuspended in 100 ml of TSB containing 1.5% (wt/vol) TSP or TSB adjusted to pH 10.0 (pH equivalent to 1.5% TSP in TSB) with NaOH prior to use. Fresh TSB (pH 7.2) was used for control experiments. TSP or pH 10.0 treatment was carried out for 1 h at room temperature (RT; 23 ± 2.0°C) in a rotary shaker (200 rpm). The sublethal dose of TSP used was determined from preliminary experiments. Protein synthesis inhibition experiments were conducted by adding 50 μg of chloramphenicol per ml to the TSP or alkaline pH treatment solution.

Challenge conditions. Cell pellets of control and TSP- or alkaline pH-treated serovar Enteritidis were resuspended in TSB and exposed to (i) pH 11.0 (adjusted with NaOH) at 37°C, (ii) pH 2.6 (adjusted with 6 N HCl) at 37°C, (iii) 30 mM H_2O_2 at 37°C, (iv) 2.5% (wt/vol) TSP at RT, or (v) heat (55°C). The concentration of each lethal factor was determined by preliminary experiments in which several concentrations of each lethal factor were tested. At predetermined time intervals, 1.0-ml sample aliquots were removed and diluted in 0.1% peptone water and appropriate dilutions were plated on TSA plates and incubated for 24 h at 37°C. CFU were then enumerated.

In the case of heating at 55°C, the pellets were resuspended in 100 ml of TSB preheated to 55°C in a standard 160-ml screw-cap milk dilution bottle. Each bottle contained a sterile magnetic stir bar, and a water-driven magnetic stirrer was operated under each heating vessel to ensure that a uniform temperature was maintained throughout the vessel. An uninoculated control bottle containing a thermometer was used to monitor the heating vessel temperature.

Data analysis. Survivor curves (log CFU per milliliter versus time) were constructed with Excel (Microsoft, Redmond, Wash.), and the negative inverse of the slope of a linear best-fit line for each survivor curve was used to calculate the D value (time at a given temperature or treatment that resulted in a 90% reduction in the number of surviving cells). The Student *t* test for samples of unequal size (65) was used to test for significant ($P < 0.05$) differences between average D values.

Analysis of total cellular proteins by 1D SDS-PAGE and immunoblotting of total cellular proteins. Total cellular protein extracted by sonication (10 bursts, 10 s each at 60 W, Sonifier Cell Disruptor; Branson Ultrasonic Corp., Danbury, Conn.) of the cells in 50 mM HEPES (pH 7.4) were separated by one-dimensional (1D) SDS-polyacrylamide gel electrophoresis (PAGE) (38). Two micrograms of the protein preparation was loaded per lane on an SDS-polyacrylamide gel (4.0% stacking gel and 12.0% resolving gel) and run at a 100-V constant voltage for 2 h at RT with a Bio-Rad Mini-Protean II system. The gels were silver stained in accordance with the manufacturer's (Amersham Biosciences Corp., Quebec City, Quebec, Canada) instructions. Another SDS-polyacrylamide gel was loaded with 25 μg of protein per lane and used for Western blotting. The proteins were transferred to a nitrocellulose membrane with a Bio-Rad semidry system at 20 V for 18 min. Mouse monoclonal antibodies against the DnaK and GroEL HSPs were purchased from StressGen Biotechnologies Corp. (Victoria, British Columbia, Canada) and used in accordance with the manufacturer's instructions for immunoblotting.

Sample preparation for 2D PAGE of total cellular proteins. Total cellular protein was extracted by resuspending washed cell pellets in 1.0 ml of lysis buffer (40 mM Tris [pH 7.0], 0.1 mg of lysozyme per ml, 1.5 mM phenylmethylsulfonyl fluoride, 9 M urea, 4.0% [wt/vol] CHAPS, 100 mM DTT) for 1 h at RT. The extracts were centrifuged at 16,000 × *g* for 10 min (Eppendorf 5415C benchtop centrifuge) to remove cell debris. The resulting supernatant was treated with 0.1 volume of buffer containing 50 mM $MgCl_2$, 1 mg of DNase I per ml, and 0.25 mg of RNase A per ml. The reaction was stopped after 15 min at 4°C with 3 volumes of ice-cold acetone. Proteins were then precipitated for 2 h at −20°C. The precipitate was collected by centrifugation at 4,500 × *g* for 15 min (Eppendorf 5804R benchtop centrifuge), resuspended in 2.0 ml of protein-solubilizing solution (9.0 M urea, 4.0% [wt/vol] CHAPS, 100 mM DTT, 2% [vol/vol] pH 4.0 to 7.0 IPG buffer), and held at RT for 1 h. The dye-binding assay of Bradford was then performed to quantify the protein concentration (9).

Isoelectric focusing. Protein extracts were subjected to high-resolution 2D PAGE as described by O'Farrell (47) and modified by Görg et al. (26). Equal amounts of proteins (8 μg) were loaded onto a first-dimension gel strip. Isoelectric focusing was performed with a Multiphor II electrophoresis unit with the Immobiline DryStrip kit (Amersham Biosciences Corp.) as follows. Immobiline DryStrip gels (pH 4.0 to 7.0) were rehydrated in 125 μl of rehydration solution (9.0 M urea, 2.0% [wt/vol] CHAPS, 100 mM DTT, 2.0% [vol/vol] pH 4.0 to 7.0 IPG buffer, 0.01% [wt/vol] bromophenol blue) containing 8 μg of protein for 16 h at RT. Isoelectric focusing was achieved when the total running time yielded 60 kVh at 20°C. Following isoelectric focusing, the gels were equilibrated twice for 10 min each time in fresh isoelectric focusing gel equilibration buffer (50 mM Tris-HCl [pH 8.8], 6 M urea, 30% [vol/vol] glycerol, 2.0% [wt/vol] SDS, 65 mM DTT, 0.01% [wt/vol] bromophenol blue).

SDS-PAGE. Equilibrated isoelectric focused strips were placed on top of a uniform SDS–14% polyacrylamide gel for second-dimension electrophoresis with the Mini-Protean II electrophoresis system (Bio-Rad, Richmond, Calif.). Second-dimension separation was carried out at a constant 100 V for 2 h at RT. After electrophoresis, the gels were silver stained in accordance with the manufacturer's (Amersham Biosciences Corp.) instructions.

TABLE 1. D values of pretreated *S. enterica* serovar Enteritidis ATCC 4931 challenged with different lethal stresses

Treatment	D value (min)[a]				
	55°C	2.5% (wt/vol) TSP	pH 11.0	pH 2.6	30 mM H_2O_2
Control	2.53 (A)	1.37 (A)	0.85 (A)	1.65 (A)	4.12 (A)
1.5% TSP	6.46 (B)	2.86 (B)	1.75 (B)	1.29 (B)	3.70 (B)
1.5% TSP + 50 μg of chloramphenicol/ml	4.12 (C)	2.44 (C)	1.66 (B)	0.57 (C)	2.95 (C)
pH 10.0	4.04 (B)	4.65 (B)	2.39 (B)	1.08 (B)	3.61 (B)
pH 10.0 + 50 μg of chloramphenicol/ml	2.39 (C)	1.52 (C)	2.12 (B)	0.43 (C)	2.4 (C)

[a] Values in the same column followed by different letters are significantly different ($P < 0.05$) between the following treatments: (i) control and 1.5% TSP, (ii) control and 1.5% TSP plus chloramphenicol, (iii) 1.5% TSP and 1.5% TSP plus chloramphenicol, (iv) control and pH 10.0, and (v) pH 10.0 and pH 10.0 plus chloramphenicol. Data from each treatment are the mean from three separate replications. Standard deviations were in the range of 10 to 15% of the given values.

Analysis of protein spots on 2D gels. Individual gels were scanned on an Epson 1200C scanner with a transparency adapter as 8-bit grayscale 300-dot/in. images and then stored. Differentially expressed proteins were then detected and quantified from the stored images with PDQuest 2-D Analysis Software (Bio-Rad).

Extraction of detergent-insoluble OM proteins for SDS-PAGE. Detergent-insoluble outer membrane (OM) proteins were obtained from the cell pellets by a modification of the procedure described by Filip et al. (16) and Portnoy et al. (49). Cell pellets were resuspended in 0.5 ml of 50 mM HEPES buffer (pH 7.4) and, while kept on ice, sonicated at the same settings as mentioned previously. The sonicated cells were then centrifuged at 16,000 × *g* for 2 min at 4°C to remove cell debris. The supernatant was transferred to a 1.5-ml centrifuge tube, and the cell membranes were sedimented from the supernatant by centrifugation at 16,000 × *g* for 30 min at 4°C. The supernatant was decanted, and the cell membrane pellets were thoroughly resuspended in 0.2 ml of 50 mM HEPES (pH 7.4) by repeated pipetting. The cytoplasmic membranes were solubilized by addition of an equal volume of detergent (2% Sarkosyl in 50 mM HEPES, pH 7.4) and incubated at RT for 30 min with intermittent mixing. The detergent-insoluble OM proteins were then pelleted by centrifugation (setting mentioned above), and the protein pellet was washed once (without resuspending the pellet) with 0.5 ml of 50 mM HEPES buffer. The washed protein pellets were then suspended in 50 μl of 50 mM HEPES buffer, and the protein concentration was quantified with the dye-binding assay of Bradford (9).

SDS-PAGE of detergent-insoluble OM proteins. The detergent-insoluble OM protein preparation was separated by 1D SDS-PAGE (38). Three micrograms of the protein preparation was loaded per lane on an SDS-polyacrylamide gel (4.0% stacking gel and 12.0% resolving gel) and run at a constant 100 V for 2 h at RT with a Bio-Rad Mini-Protean II system. The gels were then silver stained in accordance with the manufacturer's (Amersham Biosciences Corp.) instructions. The gels were scanned as mentioned earlier, and densitometric analysis of differentially expressed proteins was performed with an IS-1000 Digital Imaging System (Alpha Innotech Corp., San Leandro, Calif.). Different amounts (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 μg) of bovine serum albumin run on the same gel were used as standards for densitometric analysis of differentially expressed proteins.

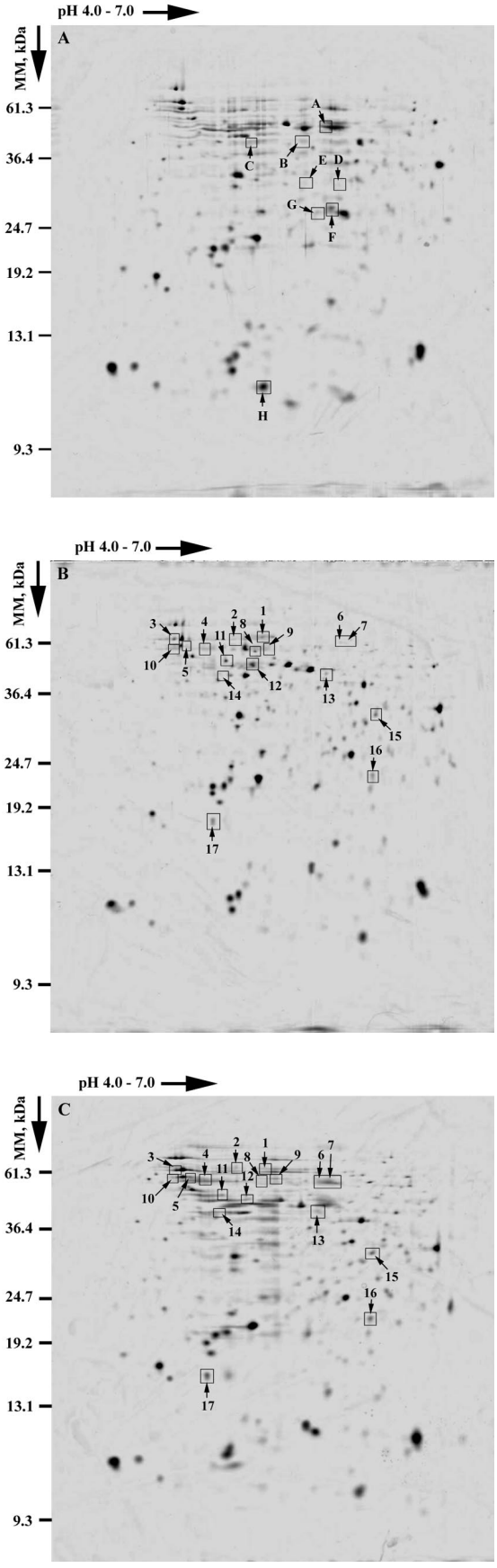
MS of proteins. Protein spots of interest were excised from the gel, destained, and in gel digested with trypsin in accordance with the established protocols for the MassPrep robotic workstation (Water/Micromass, Manchester, United Kingdom). The samples were then dried in a Speed-Vac and reconstituted in 0.1% trifluoroacetate (TFA). The samples were then desalted and concentrated with ZipTips C18 (Millipore Corp., Bedford, Mass.) before analysis by matrix-assisted laser desorption ionization (MALDI)–time of flight mass spectrometry (MS). Briefly, the ZipTips were wetted twice with 75% acetonitrile (10 μl) and then equilibrated four times with 0.1% TFA (10 μl). The samples were then aspirated and dispensed five times through the ZipTips to bind the peptides to the ZipTips. TFA (0.1%) was then aspirated five times through the ZipTips to remove salts, and the sample was then eluted directly onto the MALDI plate with 75% acetonitrile containing 5 mg of α-cyano-4-hydroxycinnamic acid per ml.

The MALDI-time of flight mass spectrometer (Voyager DE-STR; Applied Biosystems, Foster City, Calif.) was operated in the positive-ion reflectron mode. Four hundred laser shots were averaged and processed with Data Explorer software (Applied Biosystems). The samples were internally calibrated with trypsin autolytic fragments, and database searches were carried out with Protein Prospector (University of California at San Francisco MS facility). Sample preparation and MS analysis were performed by the MS facility at the National Research Council Plant Biotechnology Institute (Saskatoon, Saskatchewan, Canada).

Fatty acid analysis. The total fatty acids were extracted from 40 to 50 mg (wet weight) of cell pellet and methyl esterified as described by Annous et al. (2). A Hewlett Packard 5890 series 2 gas-liquid chromatograph (Hewlett Packard, Avondale, Pa.) equipped with a flame ionization detector and a capillary column (Ultra 2; Hewlett Packard catalog no. 19091B-102; cross-linked 5% phenylmethyl silicone; 25 m by 0.22 mm [inside diameter]; film thickness, 0.33 mm; phase ratio, 150) with hydrogen as the carrier gas was used for separation and detection of fatty acid methyl esters (FAMEs). The FAME peaks were automatically integrated by Hewlett Packard 3365 ChemStation software, and FAMEs were identified with the MIDI Microbial Identification Software (Sherlock TSBA Library version 3.80; Microbial ID, Inc., Newark, Del.).

RESULTS AND DISCUSSION

Influence of TSP or alkaline pH pretreatment on thermotolerance and sensitivity to other stresses. In a previous study to elucidate the mechanism of antimicrobial activity of TSP we reported that stationary-phase cells of *S. enterica* serovar Enteritidis ATCC 4931 can survive exposure to 1.5% TSP or its equivalent alkaline pH of 10.0, made with NaOH, for 1 h (59). In the same study we also reported that the effect of TSP on serovar Enteritidis was similar to that of an equivalent alkaline pH treatment and the antimicrobial activity of TSP was lost when the pH of the treatment solution containing TSP was adjusted to 7.0. These results suggest that serovar Enteritidis cells would respond in a similar fashion when exposed to a sublethal concentration of TSP or its equivalent alkaline pH. In the control treatment from the previous study (59), stationary-phase cells were held in fresh TSB for 1 h and no significant increase in cell numbers was observed. This confirmed that the stationary-phase cells in the control treatment had not entered the exponential log phase and would therefore be suitable for use in stress tolerance induction studies. Table 1 shows the D values of 1.5% TSP- or pH 10.0-pretreated cells of *S. enterica* serovar Enteritidis challenged with either 55°C, 2.5% TSP, pH 11.0 (pH equivalent to a treatment solution containing 2.5% TSP), pH 2.6, or 30 mM H_2O_2. Pretreatment with 1.5% TSP or pH 10.0 significantly ($P < 0.05$) induced thermotolerance and resistance to TSP and high pH while sensitizing serovar Enteritidis cells to acid and H_2O_2 (Table 1). Chloramphenicol inhibition of protein synthesis during TSP or alkaline pH exposure significantly ($P < 0.05$) reduced the acquisition of induced thermotolerance or resistance to an increased TSP concentration (Table 1), suggesting that a mechanism involving de novo protein synthesis is necessary for increased thermotolerance and resistance to TSP. However, the resistance to pH 11.0 was independent of de novo protein synthesis as no significant ($P < 0.05$) difference in resistance was observed (Table 1) even when protein synthesis was inhibited with chloramphenicol during TSP or alkaline pH pretreatment. Similar reports of induced thermotolerance and resistance to high pH in *S. enterica* serovar Enteritidis, *E. coli*, and *E. faecalis* following alkaline adaptation (durations ranging from



30 to 60 min) have been made by a number of workers (17, 25, 32, 33, 53). In *E. coli* (51, 54) and *E. faecalis* (17), pH adaptation resulted in the induction of a cross-response between alkaline and acid adaptation (i.e., alkaline pH-adapted cells became sensitive to acid and vice versa). A similar response of acid sensitivity was observed in 1.5% TSP- or pH 10.0-pretreated cells of serovar Enteritidis (Table 1). The induction of thermotolerance following alkaline adaptation has previously been shown to be dependent on protein synthesis (33), whereas resistance to high pH appeared to be due to a phenotypic change (25, 32) during alkaline adaptation. Our results also confirm that the induction of thermotolerance, unlike the resistance to high pH, was dependent on protein synthesis (Table 1). Therefore, it would be expected that the proteins induced during alkaline adaptation would belong to the family of HSPs responsible for induction of thermotolerance. However, to our knowledge, there are no reports of increased thermotolerance following alkaline adaptation correlated with the induction of HSPs. Alkaline stress (5 to 10 min) in *E. coli* induced the heat shock response, which involved a sixfold induction of the expression of the HSPs DnaK and GroEL (71). A similar response was also observed in *E. faecalis*, where alkaline stress (30 min) caused 2.9- and 8.5-fold increases in DnaK and GroEL expression, respectively, but did not induce maximum thermotolerance (17). Therefore, the mechanism of induction of thermotolerance during alkaline adaptation is not clear and does not seem to involve the induction of HSPs.

Effect of TSP or alkaline pH pretreatment on protein synthesis. Total cellular and detergent-insoluble OM proteins extracted from untreated and 1.5% TSP- or pH 10.0-pretreated serovar Enteritidis cells were analyzed by 2D PAGE and SDS-PAGE, respectively. Total cellular proteins separated by 1D SDS-PAGE were transferred to nitrocellulose membranes and probed with anti-DnaK and anti-GroEL monoclonal antibodies. No significant difference in the expression of either of the HSPs tested was observed in TSP- or alkaline pH-pretreated cells compared to the untreated control (data not shown), suggesting that TSP or alkaline pH adaptation does not induce the global stress response observed during heat shock. This led to the hypothesis that thermotolerance induced by TSP or alkaline pH pretreatment could involve other stress proteins and/or could be due to some physiological changes that occur during TSP or alkaline pH pretreatment.

To examine differential expression of proteins due to TSP or alkaline pH pretreatment, total cellular proteins extracted from untreated control and 1.5% TSP- or pH 10.0-pretreated cells were separated by 2D PAGE. 2D gels for each treatment were run in duplicate three times to confirm the reproducibility of the protein pattern. Only protein spots that appeared in four out of six gels were selected for analysis. Of the approximately 232 proteins expressed in control cells (Fig. 1A), 22% were not

FIG. 1. 2D PAGE profile of total cellular proteins from untreated control (A), 1.5% TSP-pretreated (B), and pH 10.0-pretreated (C) cells of *S. enterica* serovar Enteritidis ATCC 4931. Proteins labeled in panel A are proteins that were down-regulated by both 1.5% TSP and pH 10.0 pretreatments. Proteins labeled in panels B and C are proteins that were up-regulated during both 1.5% TSP and pH 10.0 pretreatments. MM, molecular mass.

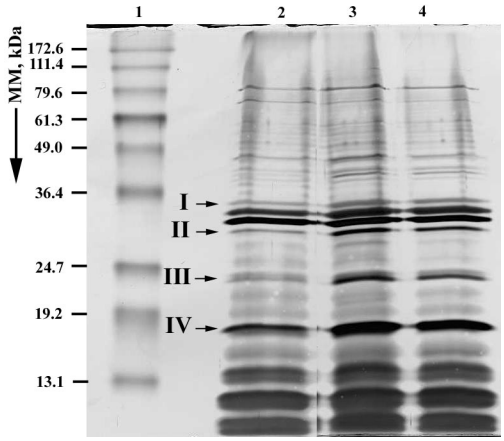


FIG. 2. 1D SDS-PAGE profile of detergent-insoluble OM proteins from untreated control (lane 2), 1.5% TSP-pretreated (lane 3), and pH 10.0-pretreated (lane 4) cells of *S. enterica* serovar Enteritidis ATCC 4931. Lane 1 is a prestained protein molecular mass ladder. Arrows point to proteins that were differentially expressed following the different treatments. MM, molecular mass.

present in 1.5% TSP-treated cells (Fig. 1B) compared to the 15% absent in pH 10.0-treated cells (Fig. 1C). Thirty-one proteins were up-regulated more than twofold by TSP pretreatment. Out of these, four proteins were up-regulated by more than fourfold. An alkaline up-shift of pH 7.0 to 10.0 up-regulated 54 proteins by more than twofold, out of which 11 and 5 proteins were up-regulated more than four- and eightfold, respectively. However, what is more significant is that more than 50% of the proteins that were either up-regulated (Fig. 1B) or down-regulated (Fig. 1A) by 1.5% TSP pretreatment were also up-regulated (Fig. 1C) or down-regulated (Fig. 1A), respectively, by pH 10.0 pretreatment. Differential expression of proteins by *E. coli* and salmonellas due to changes in their growth environment (nutrient-limiting conditions, anaerobiosis) (7, 67) or exposure to various stresses has been reported by a number of workers (1, 8, 20, 30, 39, 67).

The cytoplasmic membrane, being the boundary between the cytoplasm and the external environment, is the first site in the bacterial cell that is exposed to various stresses. Accordingly, it could be expected that a response to stress would start from the cytoplasmic membrane. Thus, detergent-insoluble OM proteins from untreated control and 1.5% TSP- or pH 10.0-pretreated cells were examined by 1D SDS-PAGE. The SDS-PAGE profile of these proteins showed differential expression of as many as four proteins compared to the untreated controls (Fig. 2). Densitometric analysis of these differentially expressed proteins revealed at least a 1.5-fold increase in expression compared to the untreated control.

Acids and bases have been shown to have multiple effects on gene expression in *E. coli* (for a review, see references 18, 48, and 63). At pH extremes, several enzymes of amino acid catabolism are induced. Foster and Hall (20) have shown differential expression of several proteins following mild acid shifts. At an acidic external pH, amino acid decarboxylases generate amines, which are exported and thereby reverse acidification (22, 41, 45, 46, 69). At an alkaline pH, catabolism of amino acids could release ammonia, which deprotonates and volatilizes while channeling the carbon skeleton into acids (7, 22). Slonczewski and coworkers have examined proteins induced in *E. coli* during growth in acidic and alkaline environments (7, 68). They reported that at high pH, tryptophan deaminase (TnaA), and *o*-acetylserine sulfhydrylase A (CysK) were induced to a high level. In addition to these enzymes, genes involved in arginine (AstD) and glutamate (GabT) catabolic pathways channel carbon into acids instead of producing alkaline amine and thereby prevent alkalinization of *E. coli* cytoplasm. TnaA is thought to reverse alkalinization by metabolizing amino acids to produce acid products. Also induced at high pH, but only in anaerobiosis, was glutamate decarboxylase (GadA), which neutralizes acidity and enhances survival in extreme acid. GadA induction during anaerobic growth may help protect alkali-grown cells from acidification resulting from anaerobic fermentation.

Mass spectrometric analysis of selected differentially expressed detergent-insoluble OM proteins (Table 2) identified these proteins as those involved in the transport of small hydrophilic molecules across the cytoplasmic membrane and chaperone proteins that aid in the export of newly synthesized proteins by keeping them in open conformation. The survival of *S. enterica* serovar Enteritidis during TSP or alkaline pH exposure decreases in a TSP concentration- and pH-dependent manner. However, *S. enterica* serovar Enteritidis can survive 1.5% TSP or pH 10.0 treatment for 1 h without any significant loss in cell viability (59). This suggests that *S. enterica* serovar Enteritidis is capable of maintaining essential cellular activities under these conditions. Mass spectrometric analysis of selected differentially expressed proteins in TSP- or alkaline pH-pretreated cells revealed up-regulation of proteins involved in amino acid biosynthesis, nucleotide metabolism, and aminoacyl-tRNA biosynthesis (Table 2). These proteins play an important role in regulating the housekeeping functions of the cells. The observation of up-regulation of proteins involved in amino acid biosynthesis during TSP or alkaline pH treatment is in agreement with observations of Slonczewski and coworkers (7, 68). The up-regulation of proteins involved in amino acid biosynthesis suggests that accumulation of acids could be happening within the cell thereby preventing alkalinization of the internal pH. While this provides a possible explanation for *S. enterica* serovar Enteritidis survival in 1.5% TSP or pH 10.0 treatment solutions, pretreatment with TSP or alkaline pH resulted in the down-regulation of a translational elongation factor (Table 2) involved in the binding of aminoacyl-tRNA to the A site of ribosomes during protein synthesis. This would mean that protein synthesis in TSP- or alkaline pH-pretreated cells would be inhibited compared to that of untreated controls, thus explaining the observed reduction in the number of proteins expressed during these pretreatments.

Fatty acid analysis. A number of studies have suggested a relationship between membrane fluidity and stress tolerance. The composition of membrane fatty acids is responsible for the maintenance of membrane fluidity. One of the most important consequences of membrane fatty acid changes in microorganisms is modulation of the activity of intrinsic proteins that perform functions such as ion pumping and nutrient uptake (57). Thermotolerance in *E. coli* and *Salmonella* induced by prior heat shock is accompanied by decreased membrane fluidity (40, 44), in addition to the transient induction of the HSPs. This decrease in membrane fluidity is brought about by

TABLE 2. Identification of selected proteins that were differentially expressed by *S. enterica* serovar Enteritidis ATCC 4931 cells following TSP or alkaline pH pretreatment[a]

Protein identification (regulation)[b]	Protein	Function/family	pI (pH)	Molecular mass (kDa)
A (↓)	Translational elongation factor	Promotes GTP-dependent binding of aminoacyl-tRNA to A site of ribosomes during protein biosynthesis	5.3	43.3
1 (↑)	Arginyl-tRNA synthetase	ATP + L-arginine + tRNA (ARG) = AMP + diphosphate + L-arginyl-tRNA(ARG); belongs to class I aminoacyl-tRNA synthetase family	6.3	72.2
3 (↑)	Arginosuccinate lyase	*N*-(L-Argino)succinate = fumarate + L-arginine; arginine biosynthesis	5.1	50.8
11 (↑)	Trigger factor	Involved in protein export; acts as chaperone by maintaining newly synthesized protein in open conformation	4.5	48.9
I (↑)	Aspartokinase 2	First step in common biosynthetic pathway leading from ASP to diaminopimelate and LYS	4.8	44.3
II (↑)	OM protein C	Forms passive diffusion pores that allow low-molecular-weight hydrophilic materials to across OM	4.6	41.2
III (↑)	OM protein W	Belongs to OM OMPW/ALKL family	5.6	22.9
IV (↑)	Xanthine phosphoribosyltransferase	Nucleotide metabolism; purine metabolism	5.2	17.4

[a] The protein spots were identified by MS analysis. Function and family analyses were based on search results from the National Center for Biotechnology Information database.
[b] Protein identifications correlate with spot and band identifications in Fig. 1 and 2. Arrows in parentheses beside protein identifications indicate whether the proteins were up- or down-regulated relative to the control.

a reduction in the ratio of unsaturated to saturated fatty acids in the membranes. In *Pediococcus* sp., a decrease in the unsaturated to saturated and cyclic fatty acid ratio during transformation from log to stationary phase was accompanied by an increase in thermotolerance (4). In *E. coli*, thermotolerance induced by acid adaptation does not induce the heat shock response but is accompanied by either conversion of unsaturated fatty acids present in the phospholipids to their cyclopropane derivatives or their replacement with saturated fatty acids (10). In the present study, pretreatment of serovar Enteritidis cells with 1.5% TSP or pH 10.0 resulted in a significant ($P < 0.05$) reduction in the concentration of unsaturated fatty acids (16:1 Ω7c, 18:1 Ω7c) and was accompanied by a significant ($P < 0.05$) increase in the concentration of saturated (16:0) and cyclic (17:0, 19:0) fatty acids (Table 3). Such a change in membrane fatty acid composition has been associated with decreased membrane fluidity and increased thermotolerance (4). Although these previous studies have correlated similar changes in the composition of the membrane fatty acids during heat shock or acid adaptation to the induction of thermotolerance, this is the first report, to our knowledge, linking thermotolerance induced during alkaline adaptation to changes in membrane fatty acid composition.

The results of this study indicate that *S. enterica* serovar Enteritidis responds similarly to exposure to TSP and alkaline conditions. Exposure of *S. enterica* serovar Enteritidis to a sublethal dose of TSP or its equivalent alkaline pH made with NaOH induces thermotolerance, resistance to high pH, and resistance to increased concentrations of TSP and sensitizes serovar Enteritidis cells to acid and H_2O_2. While protein inhibition studies suggested that thermotolerance induced by TSP or alkaline adaptation was dependent on protein synthesis, none of the common HSPs attributed to thermotolerance were detected. However, analysis of total cellular and detergent-insoluble OM proteins from TSP- or alkaline pH-pretreated cells revealed the differential expression of a number of proteins involved in transport and housekeeping functions, findings that fail to explain induced thermotolerance. The relationship between membrane fatty acid composition and thermotolerance has been shown in *E. coli* following heat shock or acid adaptation. Thermotolerance due to acid adaptation does not involve the induction of HSPs but seems to involve changes in membrane fluidity. Alkaline adaptation or TSP pretreatment results in a significant reduction in the ratio of unsaturated to saturated and cyclic fatty acids, similar to the changes observed during heat shock or acid adaptation. Therefore, the results of this study suggest that while TSP or alkaline pH adaptation does not induce the DnaK and GroEL HSPs, the induction of thermotolerance and resistance to other lethal stresses in *S. enterica* serovar Enteritidis may be the consequence of changes to the cytoplasmic membrane like reduction in membrane fluidity and expression of OM transport proteins.

As bacteria enter stationary phase, growth slows down and morphological and genetic changes occur to prolong survival and increase resistance to a variety of stress conditions (for a review, see reference 62). RpoS is an alternative sigma factor of bacterial RNA polymerase (34, 72) that mediates the in-

TABLE 3. Fatty acid composition of *S. enterica* serovar Enteritidis ATCC 4931 exposed to different treatments

Fatty acid	Avg fatty acid composition (%) ± SD[a]		
	Control	1.5% (wt/vol) TSP	pH 10.0
SFA			
12:0	5.17 ± 0.51	5.52 ± 0.66	5.84 ± 0.70
14:0	10.80 ± 1.40	13.11 ± 1.44	12.38 ± 1.48
16:0	25.07 ± 3.25	32.10 ± 3.53	31.59 ± 3.16
USFA			
16:1 ω7c	12.66 ± 1.39	2.40 ± 0.31	2.27 ± 0.27
18:1 ω7c	11.50 ± 1.61	4.19 ± 0.50	5.11 ± 0.61
CFA			
17:0	10.69 ± 1.38	16.85 ± 2.02	17.92 ± 2.32
19:0	6.11 ± 0.67	8.85 ± 1.06	8.95 ± 1.07

[a] Data from each treatment are the mean from three separate replications. SFA, saturated fatty acid; USFA, unsaturated fatty acid; CFA, cyclic fatty acid.

crease in the expression of many genes during the transition to stationary phase (29). The *rpoS* gene has been reported to a play an important role in the survival of *E. coli* upon exposure to chemical and physiological stresses. *E. coli* O157:H7 cells deficient in the expression of the *rpoS* gene were more susceptible to acidic, osmotic, and heat stresses than were wild-type cells (11, 21, 64). Sharma and Beuchat (61) studied the sensitivity of the wild type and an *rpoS*-deficient mutant of *E. coli* O157:H7 to alkaline cleaners and their subsequent resistance to heat and sanitizers. They did not observe any difference in the sensitivity of log- and stationary-phase cells to these alkaline cleaners. However, they observed induction of thermotolerance following exposure to the alkaline cleaners, which was higher in the stationary-phase wild-type strain than in the *rpoS*-deficient mutant. In the present study we used stationary-phase cells to understand the responses of *S. enterica* serovar Enteritidis to TSP or alkaline pH treatment. We would expect log-phase cells of *S. enterica* serovar Enteritidis to be more susceptible to TSP or alkaline pH treatment compared to cells in the stationary phase. We have not examined whether this is true or if TSP or alkaline pH pretreatment would induce *rpoS* gene expression in log-phase cells; however, these studies remain to be completed.

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