APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1997, p. 461–467 Vol. 63, No. 2
0099-2240/97/$04.00+0

Acid Adaptation Sensitizes *Salmonella typhimurium* to Hypochlorous Acid

GREGORY J. LEYER[1]† AND ERIC A. JOHNSON[1,2]*

Departments of Food Microbiology and Toxicology[1] and Bacteriology[2], University of Wisconsin, Madison, Wisconsin 53706

Received 15 July 1996/Accepted 6 November 1996

Acid adaptation of *Salmonella typhimurium* at a pH of 5.0 to 5.8 for one to two cell doublings resulted in marked sensitization of the pathogen to halogen-based sanitizers including chlorine (hypochlorous acid) and iodine. Acid-adapted *S. typhimurium* was more resistant to an anionic acid sanitizer than was its nonadapted counterpart. A nonselective plating medium of tryptose phosphate agar plus 1% pyruvate was used throughout the study to help recover chemically stressed cells. Mechanisms of HOCl-mediated inactivation of acid-adapted and nonadapted salmonellae were investigated. Hypochlorous acid oxidized a higher percentage of cell surface sulfhydryl groups in acid-adapted cells than in nonadapted cells, and sulfhydryl oxidation was correlated with cell inactivation. HOCl caused severe metabolic disruptions in acid-adapted and nonadapted *S. typhimurium*, such as respiratory loss and inability to restore the adenylate energy charge from a nutrient-starved state. Sensitization of *S. typhimurium* to hypochlorous acid by acid adaptation also involved increased permeability of the cell surface because nonadapted cells treated with EDTA became sensitized. The results of this study establish that acid-adapted *S. typhimurium* cells are highly sensitized to HOCl oxidation and that inactivation by HOCl involves changes in membrane permeability, inability to maintain or restore energy charge, and probably oxidation of essential cellular components. This study provides a basis for improved practical technologies to inactivate *Salmonella* and implies that acid pretreatment of food plant environments may increase the efficacy of halogen sanitizers.

Salmonella spp. are the major cause of foodborne disease in the United States and certain other countries. It has been estimated that 6.5 to 33 million people become ill from contaminated food annually in the United States, resulting in an estimated 6,000 deaths and an estimated economic cost of $2.5 billion to $3.4 billion per year (5). Of these illnesses, about half have been reported to be caused by salmonellae (12). A variety of foods have been implicated in salmonellosis, including fermented foods and acidic products (12). Despite extensive control efforts by regulatory agencies and the food industry, the percentage of outbreaks due to *Salmonella* is increasing at a rate greater than that of outbreaks due to several other bacterial pathogens (12).

Salmonellae are widespread in nature and are commonly found as inhabitants of animal intestinal tracts. By virtue of their ubiquity, *Salmonella* spp. are exposed to a variety of chemical and environmental stresses. Salmonellae and other bacteria can adapt to stresses by regulating the expression of specific sets of genes termed stimulons (38). Salmonellae and other species of the family *Enterobacteriaceae* are able to express adaptive responses that allow survival under acid conditions (35). Acid adaptation in *Salmonella typhimurium* enhances the ability to withstand severe acid stress by maintaining internal pH homeostasis (19, 20), 24. In addition to withstanding severe acid stress in media, acid-adapted *S. typhimurium* showed increased survival in acidic and fermented foods (33, 34), and exhibited cross-protection against various environmental stresses (32).

* Corresponding author. Mailing address: Department of Food Microbiology and Toxicology, University of Wisconsin, 1925 Willow Dr., Madison, WI 53706. Phone: (608) 263-7944. Fax: (608) 263-1114. E-mail: eajohnso@facstaff.wisc.edu.

† Present address: Abbott Laboratories, Ross Products Division, Columbus, OH 43219.

The food, medical, water, and environmental industries commonly use chemical sanitizers to eradicate pathogenic and spoilage microorganisms from foods, medical devices, and the environment. Chlorine, in its various forms, is the most widely used of all chemical sanitizers (13, 14, 19). Hypochlorites are especially useful because of their rapid activity against a wide variety of microorganisms and their low cost. However, controversy has arisen in recent years regarding the potential deleterious health effects of chlorine and its by-products (4, 13, 26). This study was undertaken to evaluate the efficacy and mechanisms of commonly used chemical disinfectants against *S. typhimurium* that had previously been exposed to a mild acid stress. Unexpectedly, our results indicate that acid adaptation strongly sensitizes salmonellae to hypochlorous acid.

MATERIALS AND METHODS

Chemicals and reagents. All chemicals were obtained from Sigma Chemical Co., St. Louis, Mo. Tryptic soy broth was purchased from Difco, Detroit, Mich.

Bacterial strains and growth conditions. *S. typhimurium* LT2 (provided by Laszlo Csonka, Purdue University) was used throughout this study, and the procedures for growth and acid adaptation in medium E were the same as previously described (32, 33). Four additional strains of *Salmonella* (KB17, SL1917, SH1906, and 7454) were also tested for HOCl sensitization after acid adaptation. For acid adaptation, *S. typhimurium* was grown statically in medium E (pH 7.6) with 0.4% glucose until the culture reached an absorbance at 600 nm (A_{600}) of ~0.1, at which time the medium was acidified to pH 5.0 to 5.8 with a small volume of 10 N HCl. The cells were grown to an A_{600} of 0.25 to 0.30. The nonadapted control cells were grown to an A_{600} of 0.25 to 0.30 in nonacidified medium E. Then 1 ml each of the acid-adapted and nonadapted cells was harvested by centrifugation, washed once in 1 ml of distilled water (dH_2O), and resuspended in 100 μl of dH_2O. These cells were used to determine resistance to chemical disinfectants. Cellular viability after various treatments was determined by plating onto nonselective tryptose phosphate agar–0.1% pyruvate (33) to allow rescuitation of chemically stressed cells.

Dry weight and protein determination. Bacterial dry weights were determined with 10-fold-concentrated cell suspensions. A 1-ml portion of the concentrated cell suspension was dried in an aluminum dry pan with a 55-mm fiberglass filter for 16 to 24 h at 105°C until a constant weight was obtained (25). Protein was

determined by using the bicinchoninic acid protein assay reagent purchased from Pierce, Rockford, IL.

Sanitizer preparation. Chemical sanitizers were provided by the Diversey Wyandotte Corp., Wyandotte, Mich. The liquid chlorinated sanitizer contained 9.2% (wt/vol) sodium hypochlorite as its active ingredient. The hypochlorite stock solution was routinely titrated by the available-chlorine method (17). The anionic acid sanitizer contained 30% (wt/vol) phosphoric acid as its active ingredient. The iodine-based disinfectant contained organic complexes of iodine, providing 1.75% titratable iodine. The iodine-based and anionic acid disinfectant stock solutions were prepared as specified by the manufacturer. Liquid chlorinated sanitizer dilutions were based on available-chlorine titration results.

Tolerance of *S. typhimurium* toward chemical disinfectants. The tolerance of *S. typhimurium* to sanitizers was determined at ambient temperature by adding 100 μl of cell suspension to 4.0 ml of dH_2O (for anionic acid and iodine sanitizers) or to 4.0 ml of 50 mM sodium phosphate buffer (PB), (pH 6.0) (for HOCl studies). Experiments with HOCl were performed with PB at pH 6.0, at which approximately 94% of HOCl is in its nondissociated state (20). The HOCl concentration is expressed as micromoles of HOCl per gram (where 475 μmol is approximately equivalent to 0.5 ppm HOCl). Viability was determined immediately prior to and at appropriate time intervals after addition of the sanitizers. Samples were diluted in tryptic soy broth to neutralize residual sanitizer (37) prior to plating.

Sulfhydryl determination. The cellular surface sulfhydryl concentration was measured with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (21, 48). Cells (10 ml of suspension) treated with HOCl for 10 min were quenched with 100 μl of 0.1 M sodium thiosulfate, harvested by centrifugation, washed twice with an equal volume of dH_2O, and resuspended in 5 ml of 0.1 M potassium phosphate buffer (pH 6.6) containing 1 mM $MgSO_4$. The cell suspension was added to 10 ml of 0.1 M Tris-HCl buffer (pH 8.0) containing 10 mM EDTA. To this suspension, 250 μl of 10 mM DTNB in 0.1 M potassium phosphate buffer (pH 7.0) and 1.25 ml of 10% (wt/vol) sodium dodecyl sulfate were added. The suspension was incubated at 37°C for 1 h, cooled to 0°C, and centrifuged at 18,000 × *g* for 10 min. The A_{412} of the supernatants was measured with a Response spectrophotometer (Gilford, Oberlin, Ohio). A molar extinction coefficient of 13,600 for 5-thio-2-nitrobenzoic acid was used (21). Results are presented as percent sulfhydryl concentration relative to untreated cells.

Respiration measurement. Respiration was estimated by measuring O_2 consumption with a digital oxygen system oxygen electrode (Rank Brothers Ltd., Cambridge, England) and reported as a percentage of the original rate in untreated cells. Results are expressed as millimoles of O_2 per hour per gram (dry cell weight) based on a concentration of 268 μM O_2 in air-saturated buffer at 25°C (43). A 10-ml portion of an overnight culture was centrifuged and resuspended in 1.2 ml of PB (pH 6.0) and kept on ice until use. Then, 400 μl of cell suspension was combined with 400 μl of 300 mM D-glucose in 3.2 ml of air-saturated PB and held with occasional agitation at ambient temperature for 5 min. After 5 min, respiration was monitored at ambient temperature by monitoring the oxygen electrode response on a Linear 1200 chart recorder (Linear Instruments Corp., Reno, Nev.). Once an initial linear oxygen consumption rate was obtained, 20 μl of an appropriately diluted solution of HOCl was added to the respiring suspension, and the subsequent oxygen consumption rate determined.

Determination of enzyme activities. The activities of various dehydrogenase enzymes in crude cell lysates from HOCl-treated or nontreated cells were determined. The culture (225 ml, $A_{600} \approx 0.25$) was centrifuged and resuspended in 1 ml of dH_2O. The cell suspension was added to 224 ml of PB, treated with 1.125 ml of HOCl with stirring, and allowed to react for 10 min, and the reaction was quenched by adding 200 μl of 0.1 M sodium thiosulfate. The cells were harvested by centrifugation at 12,000 × *g* for 10 min, resuspended in 1 ml of 30 mM Tris-HCl buffer (pH 8.0) containing 10 μl of 100 mM phenylmethylsulfonyl fluoride, and passed twice through a French pressure cell (American Instrument Co., Silver Spring, Md.) at 1,000 lb/in^2. Whole cells and cell wall debris were removed by centrifugation at 12,000 × *g* for 10 min, and crude cell lysates were stored at −80°C until analysis. The activities of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (EC 1.2.1.12), lactate dehydrogenase (LDH) (EC 1.1.1.27), and malate dehydrogenase (MDH) (EC 1.1.1.37), were measured spectrophotometrically by recording NADH oxidation (for LDH and MDH) or NAD reduction (for GAPDH) at 340 nm following substrate addition (8, 50). Glutamate dehydrogenase (GDH) activity (EC 1.4.1.2) was determined by measuring the NADPH oxidation rate following cell extract addition to a solution containing 2-ketoglutarate (6.5 mM), NADPH (0.15 mM), and NH_4Cl (30 mM) in a 1-ml final volume of Tris-HCl (30 mM) buffer (pH 8.0). Enzyme activities are expressed as units per milligram, where 1 U is defined as 1 nmol of NADPH (NADH) oxidized or NAD reduced per min. The protein concentration in all enzyme assays was 50 μg/ml.

EC measurements. Adenylate energy charge (EC) step-up in *S. typhimurium* was determined by quantifying extracted nucleotides from HOCl-treated or nontreated cells (9, 10). Acid-adapted or nonadapted cells (450 ml) were harvested by centrifugation and concentrated 10-fold in dH_2O. The concentrated cell suspension (4.5 ml) was added to 180 ml of 50 mM sodium phosphate buffer (pH 6.0). Diluted HOCl (900 μl) was added, and the mixture was incubated at ambient temperature for 15 min. The reaction was quenched by adding 200 μl of 0.10 M sodium thiosulfate. HOCl-treated cells were centrifuged at 12,000 × *g* for 10 min, resuspended in 4.5 ml of E buffer (medium E without glucose supplementation), and incubated for 30 min at 37°C with shaking to starve the cells for energy. After a 30-min starvation, 500 μl was sampled for determination of adenylate nucleotide concentrations. Then 400 μl of a 2.22 M D-glucose solution was added to the remaining cell suspension, and the cells were incubated at 37°C with shaking and sampled at time intervals for nucleotide concentrations.

Nucleotide concentrations were measured chromatographically by analyzing *S. typhimurium* perchloric acid extracts. Cell suspension (500 μl) was added to 200 μl of ice-cold 3 M perchloric acid. The acid-quenched solution was allowed to stand on ice for 30 min, centrifuged at 12,000 × *g* for 5 min, and neutralized with 200 μl of 3 M KOH and 100 μl of 50 mM potassium phosphate (pH 3.0). The precipitated perchlorate ions were removed by centrifugation, and the supernatant was filtered and stored at −80°C until chromatographic analysis. Nucleotide concentrations were quantified on a high-pressure liquid chromatography Hydropore-AX anion-exchange column (Rainin Instruments Co., Inc., Woburn, Mass.). The solvent system that gave optimum resolution was essentially the same as that described by Brown et al. (15). The initial eluant was 5 mM KH_2PO_4 (pH 3.0) (A), and the final eluant was 500 mM KH_2PO_4 (pH 4.0) (B). A linear gradient from 100% A to 100% B was run over 40 min with a flow rate of 1.0 ml/min. The EC of the adenylate system (7) is defined as follows: ([ATP] + 0.5 [ADP])/([ATP] + [ADP] + [AMP]).

Outer membrane permeabilization by EDTA and HOCl tolerance. Washed acid-adapted or nonadapted cells were suspended in 0.1 M Tris-HCl buffer (pH 8.0) containing 10 mM EDTA. The cell suspensions were incubated for 1 or 2 h at 37°C to permeabilize the outer membrane (49), washed twice with dH_2O, and exposed to HOCl in PB for 10 min as previously described. Cell viability was determined before and after treatments with Tris-EDTA and HOCl by plating on TPAP agar.

Statistical analysis. Each data point in the figures and tables is the mean of triplicate experiments. Additionally, each set of conditions was replicated at least once. The error bars in the figures represent one standard deviation from the mean.

RESULTS

Tolerance of *S. typhimurium* to chemical sanitizers. Acid-adapted or nonadapted cells were treated with three classes of sanitizers. The acid-adapted population survived significantly better than nonadapted cells when challenged with 10 ppm of an anionic acid sanitizer at pH 3.23 (Fig. 1A). After a 20-min exposure, a 1,000-fold difference in the cellular viability was observed, and this differential remained throughout the incubation, although both cell populations lost viability over time.

Acid-adapted and nonadapted cells were also treated with an iodine-based sanitizer (Fig. 1B). Unexpectedly, acid adaptation sensitized the cells to the iodine sanitizer. The acid-adapted population died off extremely rapidly, losing 5 log units of an original 7-log-unit population during the first 10 min of exposure. After this time, the viable cell number remained relatively constant during the 30-min exposure, resulting in a ~1,000-fold difference in viability between the two cell populations.

Acid-adapted populations of *S. typhimurium* were also markedly sensitized to HOCl (Fig. 2A). As observed with cells treated with the iodophor, the acid-adapted cell population experienced a rapid and marked viability loss during the initial exposure for 5 min, resulting in a ~1,000-fold difference in cell viability between the two populations after 5 min. After this time, the viabilities of both cell types remained relatively constant. As expected, cell inactivation was dependent on the concentration of HOCl (Fig. 2B). At low HOCl levels, both cell populations survived equally well. When the HOCl concentration was increased to 100 μmol/g of salmonellae, the acid-adapted population was inactivated rapidly whereas the nonadapted population was inactivated much more slowly. Four additional strains of *Salmonella* also exhibited the characteristic sensitization to HOCl after acid adaptation.

Oxidation of sulfhydryls on whole cells. HOCl is a powerful oxidant, and sulfhydryl groups of cell membranous components are potential targets for oxidation and cell inactivation (20, 31, 46, 47). The sulfhydryl content of acid-adapted and nonadapted cells was 94 and 121 nmol of sulfhydryl/mg (dry

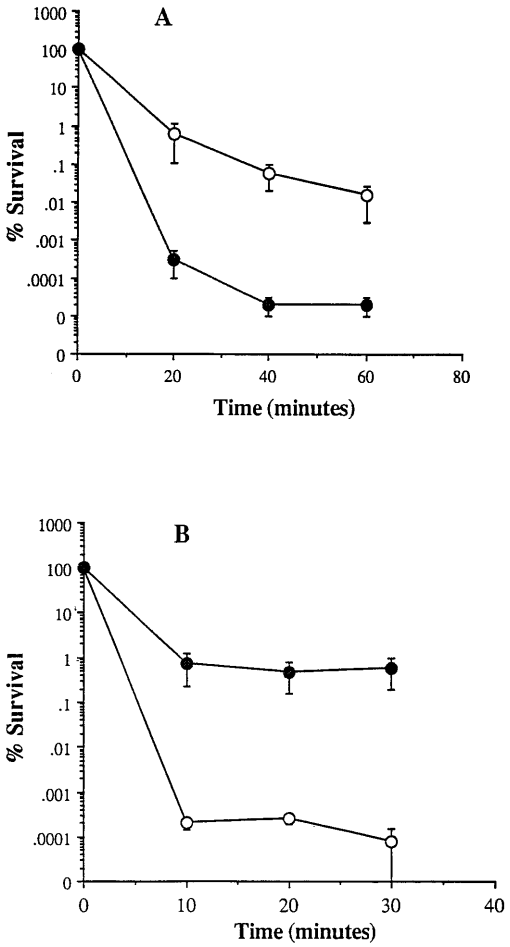

FIG. 1. Survival of acid-adapted (○) and nonadapted (●) *S. typhimurium* during exposure to 10 ppm of anionic acid (pH 3.23) disinfectant (A) or 1.0 ppm of iodine-based disinfectant (B). Error bars in this and subsequent figures indicate standard deviations determined from triplicate experiments; datum points with no error bars indicate that the error is smaller than the symbol.

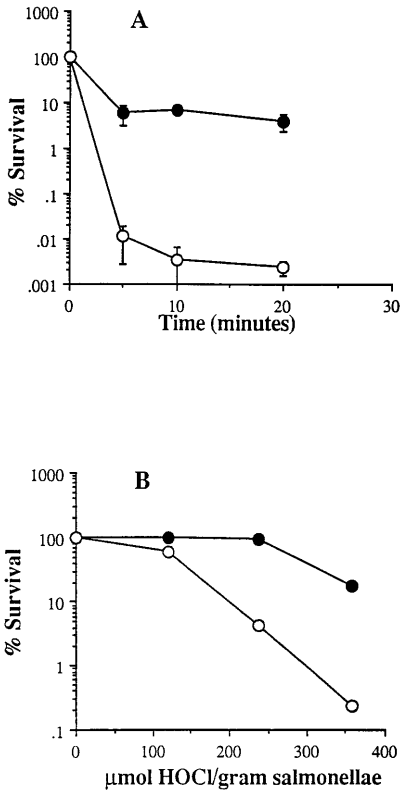

FIG. 2. Survival of acid-adapted (○) and nonadapted (●) *S. typhimurium* during exposure to 475 μmol of HOCl/g (dry weight) of salmonellae (A) or to various HOCl levels (B). Graph B shows HOCl dose-dependent killing kinetics.

weight), respectively. Loss of sulfhydryl groups occurred at approximately the same rate in both cell populations treated with 190 μmol of HOCl/g of cells (Fig. 3). As the HOCl concentration was increased, the sulfhydryls in acid-adapted cells were lost more rapidly than were those in nonadapted cells. At 475 μmol of HOCl/g of salmonellae, only 16% of the original sulfhydryl groups in acid-adapted cells remained unoxidized after 1 h, whereas 61% of the sulfhydryl groups remained in their reduced state in nonadapted cells. Sulfhydryl group oxidation correlated with loss in cell viability.

Effect of HOCl on dehydrogenase enzyme activities. In the experiment described in the previous section, acid-adapted salmonellae showed a greater loss of sulfhydryls than did nonadapted cells. Since oxidation of sulfhydryls has been suggested as a mechanism of cell inactivation by chlorine, and since glutamic acid and related primary metabolites have been implicated in acid resistance (22, 27), the activities of selected sulfhydryl dehydrogenase enzymes involved in primary metabolism were assayed after treatment with HOCl. The enzymes examined varied in their sensitivity to HOCl. GAPDH was inactivated by HOCl to a greater degree in adapted than in nonadapted cells (Table 1). This pattern of enzyme inactivation correlated with loss of viability (Fig. 2B). When treated with 355 μmol of HOCl/g, the adapted and nonadapted extracts retained approximately 52 and 74% of GAPDH activity, respectively. When treated with various levels of HOCl, LDH activity initially increased and then declined in acid-adapted cells (Table 1). This pattern was observed repeatedly. In contrast, LDH was relatively stable in response to HOCl in nonadapted cells. Higher activities of MDH were present in nonadapted than in adapted cell extracts (Table 1). MDH was not inactivated by the HOCl concentration examined, and MDH activity did not correlate with cell viability loss. GDH activity increased in both cell populations during exposure to increas-

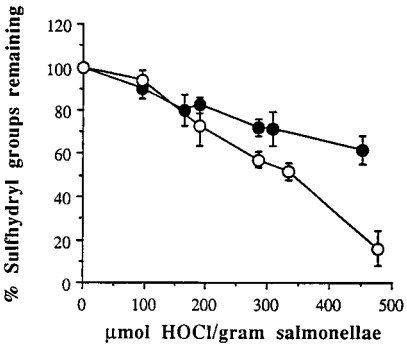

FIG. 3. Loss of cell surface sulfhydryl groups as a function of HOCl dose in acid-adapted (○) and nonadapted (●) *S. typhimurium*. Data are given as the percentage of the original sulfhydryl groups remaining, with 100% representing 121 nmol of sulfhydryl/mg (dry weight) in nonadapted cells and 94 nmol of sulfhydryl/mg (dry weight) in acid-adapted cells.

TABLE 1. Sulfhydryl-dependent dehydrogenase inactivation by HOCl in acid-adapted and nonadapted *S. typhimurium*

HOCl concn (μmol/g of salmonellae)	Activity (U/mg) of[a]:							
	GAPDH		LDH		MDH		GDH	
	Acid adapted	Nonadapted	Acid adapted	Nonadapted	Acid adapted	Nonadapted	Acid adapted	Nonadapted
0	1.921 (0.165)	1.981 (0.127)	0.057 (0.019)	0.065 (0.029)	1.462 (0.026)	3.237 (0.068)	0.029 (0.002)	0.131 (0.005)
119.2	1.875 (0.08)	1.747 (0.145)	0.119 (0.006)	0.036 (0.004)	1.501 (0.081)	3.705 (0.145)	0.689 (0.054)	0.332 (0.019)
238.4	1.131 (0.17)	1.641 (0.087)	0.066 (0.004)	0.039 (0.014)	1.603 (0.037)	3.457 (0.095)	1.499 (0.044)	0.521 (0.008)
357.6	1.032 (0.13)	1.393 (0.074)	0.013 (0.012)	0.033 (0.009)	1.401 (0.088)	3.422 (0.109)	1.01 (0.061)	0.843 (0.052)

[a] Standard deviations of triplicate determinations are given in parentheses.

ing amounts of HOCl (Table 1). These results suggested that the inactivation of sulfhydryl enzymes by HOCl was dependent on the particular enzyme and may contribute to cell killing.

EC step-up in HOCl-treated *S. typhimurium*. Ion-motive ATPase enzymes have been shown to be involved in ATP synthesis and maintenance (39) and have been implicated in the acid tolerance response in *S. typhimurium* (40). Therefore, we investigated the levels of adenylate nucleotides by determining the adenylate EC in acid-adapted and nonadapted cells. The adenylate EC and its recovery from energy starvation have been used to assess the metabolic well-being of cells (6, 9, 11, 18). Healthy bacteria can increase their EC within minutes from a resting-stage EC of 0.1–0.2 to 0.8–0.9 when given an appropriate energy source (9). The ability of acid-adapted and nonadapted *S. typhimurium* cells to undergo an EC step-up after exposure to various HOCl levels was determined (Fig. 4). Energy-starved cells maintained an EC of 0.10 to 0.25. Untreated nonadapted and acid-adapted *S. typhimurium* cells were able to rapidly achieve an EC of >0.60. The ability to produce an EC step-up decreased markedly with increased exposure to HOCl in both adapted and nonadapted cells, and adapted cells were less able to rapidly restore the energy charge. When treated with 77 μmol of HOCl/g, corresponding to a ~30% viability loss in adapted and nonadapted cells, the acid-adapted cells stepped up their EC from 0.13 to 0.27 in 40 min whereas the nonadapted cells stepped up to an EC of 0.55 in 20 min. The inability of acid-adapted *S. typhimurium* to restore their EC was even more pronounced when the HOCl concentration was increased to 191 μmol/g, at which the viability of acid-adapted cells was reduced to 0.03% of the original level and cells were unable to increase their EC above the 0.22 original charge. When the same dose was given to nonadapted cells, the viability was reduced to 2.8% of the original level but the cells were still able to step up the EC from 0.16 to 0.44. These results clearly show that acid-adapted energy-starved cells have a decreased ability to maintain and step up their EC when treated with HOCl.

Inhibition of O_2 uptake by HOCl. Since acid-adapted cells were impaired in energy production and maintenance, we investigated oxygen uptake in adapted and nonadapted cell populations. HOCl treatment affected oxygen uptake differently in acid-adapted and nonadapted *S. typhimurium*. Oxygen uptake was more strongly inhibited by HOCl in acid-adapted *S. typhimurium* cells than in nonadapted cells (Fig. 5). Immediately after HOCl addition, an abrupt decline in O_2 uptake was observed. When acid-adapted cells were treated with 150 μmol of HOCl/g, the oxygen uptake rate decreased to approximately 10% of the original level. At the same HOCl concentration, the nonadapted population retained approximately 50% of the original oxygen uptake rate. Other concentrations of HOCl also differentially affected the O_2 uptake in acid-adapted and nonadapted cell populations (Fig. 5). Hence, acid-adapted cell populations were impaired in oxygen uptake compared to nonadapted cells on treatment with HOCl.

Outer membrane disruption and HOCl tolerance. We previously demonstrated that cell surface characteristics are altered in acid-adapted *S. typhimurium* (32), and therefore we investigated whether disrupting the outer membrane by EDTA treatment (49) would enhance the sensitivity of nonadapted cells to HOCl. Untreated nonadapted and adapted cell populations displayed the characteristic ~10,000-fold difference in sensitivity to HOCl (Fig. 6). The difference in sensitivity was decreased after treatment with Tris-EDTA. After 1 h of treatment with Tris-EDTA, the difference in sensitivity was only

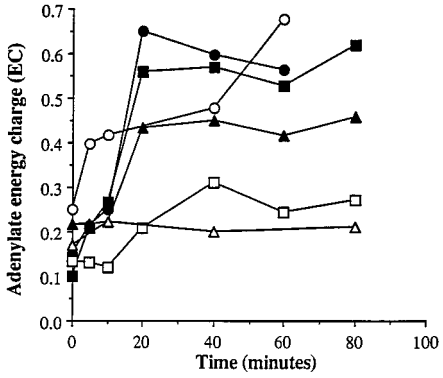

FIG. 4. Inhibition of EC step-up by HOCl oxidation. The EC step-up was measured after HOCl oxidation and energy starvation and was restored by the addition of 222 mM glucose at time zero. Open symbols represent acid-adapted *S. typhimurium*, and solid symbols represent nonadapted cells. ○, ●, 0 μmol of HOCl/g of salmonellae; □, ■, 77 μmol of HOCl/g; △, ▲, 191 μmol of HOCl/g.

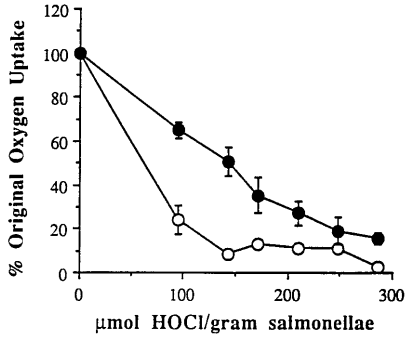

FIG. 5. Inhibition of oxygen uptake by HOCl in acid-adapted (○) or nonadapted (●) *S. typhimurium*. The data represented are the result of three determinations in which 100% control was 7.0 to 9.7 mmol of O_2/h/g (dry weight) in nonadapted cells and 6.4 to 9.7 mmol of O_2/h/g (dry weight) in acid-adapted cells.

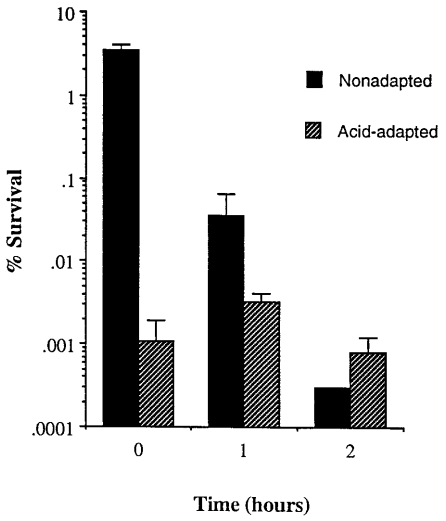

FIG. 6. Sensitization of *S. typhimurium* to HOCl after disruption of the outer membrane by EDTA. The abscissa represents the length of incubation in the presence of 10 mM EDTA in 0.1 M Tris-HCl buffer (pH 8.0). Cells were exposed to 475 μmol of HOCl/g of salmonellae for 10 min before viability determination.

~10-fold. After 2 h of exposure to Tris-EDTA, the nonadapted cells had greater sensitivity to HOCl than the acid-adapted cells did.

DISCUSSION

The ability of microbial pathogens to adapt to environmental stresses such as acid contributes to their survival in the natural environment. Acid adaptation probably plays an important role in food safety, since many foods depend on acidic conditions for preservation (16). We previously showed that acid-adapted *S. typhimurium* and *Escherichia coli* O157:H7 survived better in certain acidic and fermented foods than did their nonadapted counterparts (33, 34). Acid-adapted *S. typhimurium* cells have an enhanced ability to maintain their internal pH (pH_i) at or near a neutral level when exposed to very acidic conditions (pH < 4.0) (23). Therefore, it was not surprising that acid adaptation enhanced their tolerance to an anionic acid disinfectant. However, it was surprising that acid-adapted *S. typhimurium* cells exhibited a dramatic (~10,000-fold) sensitization to halogen sanitizers. In this study, we investigated the physiological mechanisms contributing to the unexpected differential sensitivity to HOCl in acid-adapted and nonadapted *S. typhimurium.*

HOCl is an effective disinfectant partly because most microorganisms do not possess specific enzymatic mechanisms for detoxification of HOCl, as they do for other oxidants such as reactive oxygen species. The mechanism of inactivation of microbial cells by HOCl is not completely elucidated but is believed to involve the oxidation of critical cellular components and depletion of cellular energy. HOCl generated by the activity of myeloperoxidase, H_2O_2, and a halide provides an effective microbiocidal system for mammalian cells (29). Recently, acidification of hypochlorite solutions has been shown to generate ozone, a strong oxidant (30). HOCl causes destruction of cellular components by several mechanisms: (i) sulfhydryl oxidation (46, 47), (ii) inactivation of iron-sulfur centers (3, 28, 44, 45), (iii) inactivation of enzymes involved in respiration (2, 41), (iv) disruption of nutrient transport (1, 9), (v) inhibition of energy generation (9, 11), and (vi) inactivation of chromosomal DNA replication (42). The bactericidal activity of HOCl has not, to our knowledge, been hypothesized to depend on disruption of membrane permeability (1, 36) or inhibition of protein synthesis (36). In this study, we evaluated the inactivation of several cellular targets by HOCl and attempted to identify targets which were disrupted prior to cell death and therefore would contribute to loss of viability.

Cell surface sulfhydryl oxidation correlated with loss in the cell viability of adapted and nonadapted *S. typhimurium*, but nonadapted cells were more resistant on extended exposure. The difference in the resistance of nonadapted and adapted cells to HOCl may be related to the overall sulfhydryl levels in the two populations. Nonadapted cells had a greater quantity of sulfhydryl groups than did acid-adapted cells, which may have bestowed greater protection. To assess the role of sulfhydryl oxidation in cell killing, we also evaluated the inactivation of NAD(P)-linked sulfhydryl enzymes by HOCl. The NAD(P)-linked enzymes examined in this study differed in their activities in acid-adapted and nonadapted cells and also in their susceptibility to HOCl. Inactivation of GAPDH correlated with loss in cell viability. GADPH contains three sulfhydryl groups in the catalytic site, one of which is essential for activity and is also exceptionally reactive with HOCl (3). LDH activity declined during treatment with HOCl, but the decline did not correlate with loss of cell viability. The other two enzymes examined, MDH and GDH, were not inactivated by HOCl. GDH activity increased with increasing dose of HOCl in both acid-adapted and nonadapted cells. Rakita et al. (41) also reported that GDH activity increased on incubation with an activated myeloperoxidase antimicrobial system. These changes may indicate that changes in the redox potential could increase the activity or synthesis of various enzymes. Since we did not observe an obvious correlation between the inactivation of metabolic enzymes, we investigated the role of energy maintenance and oxygen uptake in promoting survival in HOCl-treated cells.

Previous studies indicated that abolition of ATP formation contributes to bacterial killing by HOCl (9–11). In *E. coli*, HOCl-promoted inactivation was accompanied by inhibition of respiration (2, 43). Mutations in the genes in the *atp* operon also have been shown to abolish the acid tolerance response in *S. typhimurium* (22, 40). In our studies, respiration was inactivated by HOCl rapidly in acid-adapted *S. typhimurium* but more slowly in nonadapted cells. Several essential bacterial respiratory components including porphyrins, heme, heme proteins, Fe-sulfur proteins, and cytochromes are sensitive to oxidation by HOCl (3, 44). Since respiration was decreased prior to cell death, its loss could contribute to inactivation, and our data support the hypothesis that maintenance of respiratory ability is especially important for acid-adapted cells exposed to HOCl.

The adenylate nucleotide charge level in bacteria is tightly regulated and is important in maintaining cell viability during energy starvation (18). HOCl has previously been demonstrated to impair the ability of *E. coli* to maintain an adenylate energy charge required for viability (9, 11). Our data support the hypothesis that a useful index of the metabolic well-being of cells during treatment with HOCl is the ability of cells to maintain a suitable EC attainable or to step up the EC when presented with nutrients (9, 11). The difference in the ability of acid-adapted or nonadapted *S. typhimurium* cells to step up the EC after treatment with HOCl indicated that acid-adapted cells were severely impaired in their ability to regenerate energy stores. This conclusion is in accordance with the reports that mutations in the genes encoding Mg^{2+}-dependent proton-

translocating ATPase abolish acid tolerance in *S. typhimurium* (40).

The dramatic sensitization of acid-adapted cells to HOCl may not be entirely explained by differences in their susceptibility to HOCl inactivation of sulfhydryls, ATP production, and energy maintenance. Our data suggest that the ability of HOCl to penetrate through the outer membrane may also be important in sensitization. We previously showed that the outer membrane of acid-adapted *S. typhimurium* has a different composition than that of nonadapted cells (32). In this study, sensitization of nonadapted cells was obtained by treatment with EDTA, which is known to disrupt outer membrane permeability. The actual porin or channel which allows HOCl penetration in gram-negative cells has not been identified. It would be valuable to identify the channels involved, since this would advance our knowledge of the mechanisms of inactivation of salmonellae and other pathogens by halogen sanitizers.

In summary, this communication has demonstrated that acid-adapted *S. typhimurium* is highly sensitized to HOCl action. Acid-adapted salmonellae possess certain fundamental changes in metabolism that predispose them to inactivation. Most importantly, the inability to generate ATP through respiration and to maintain an energy level necessary for survival is important. Also, outer membrane permeability and structure are altered in acid-adapted *S. typhimurium*. This study provides a basis for the development of practical methods to increase the efficacy of halogen sanitizers, such as pretreating the food plant environment with a mild acid prior to sanitization. Our study also suggests that it may be feasible to reduce the quantities of halogen sanitizers used industrially and thereby decrease the possible deleterious impact of chlorine and related compounds on human health.

ACKNOWLEDGMENTS

We thank Marty Krause for excellent technical assistance with experiments.

This research was supported by contributions by the food industry to the Food Research Institute, by the Southeastern Poultry Association, by the Wisconsin Center for Dairy Research through Dairy Management Inc. and the Wisconsin Milk Marketing Board, by NRI Competitive Grants Program/USDA (94-37201-1026), and by the College of Agriculture and Life Sciences, University of Wisconsin, Madison.

REFERENCES

1. **Albrich, J. M., J. H. Gilbaugh III, K. B. Callahan, and J. K. Hurst.** 1986. Effects of the putative neutrophil-generated toxin, hypochlorous acid, on membrane permeability and transport systems of *Escherichia coli*. J. Clin. Invest. **78:**177–184.
2. **Albrich, J. M., and J. K. Hurst.** 1982. Oxidative inactivation of *Escherichia coli* by hypochlorous acid. Rates and differentiation of respiratory from other reaction sites. FEBS Lett. **144:**157–161.
3. **Albrich, J. M., C. A. McCarthy, and J. K. Hurst.** 1981. Biological reactivity of hypochlorous acid: implications for microbicidal mechanisms of leukocyte myeloperoxidase. Proc. Natl. Acad. Sci. USA **78:**210–214.
4. **Amato, I.** 1993. The crusade against chlorine. Science **261:**152–154.
5. **Anonymous.** 1994. Cancer risk fuels search for chlorine substitutes. Food Prot. Rep. **10:**2.
6. **Atkinson, D. E.** 1968. The energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers. Biochemistry **7:**4030–4034.
7. **Atkinson, D. E., and G. M. Walton.** 1967. Adenosine triphosphate conservation in metabolic regulation. Rat liver citrate cleavage enzyme. J. Biol. Chem. **242:**3239–3241.
8. **Banaszak, L. J., and R. A. Bradshaw.** 1975. Malate dehydrogenases, p. 369–396. *In* P. D. Boyer (ed.), The enzymes, vol. XI, Academic Press, Inc., New York, N.Y.
9. **Barrette, W. C., Jr., D. M. Hannum, W. D. Wheeler, and J. K. Hurst.** 1989. General mechanism for the bacterial toxicity of hypochlorous acid: abolition of ATP production. Biochemistry **28:**9172–9178.
10. **Barrette, W. C., Jr., D. M. Hannum, W. D. Wheeler, and J. K. Hurst.** 1988. Viability and metabolic capability are maintained by *Escherichia coli*, *Pseudomonas aeruginosa*, and *Streptococcus lactis* at very low energy charge. J. Bacteriol. **170:**3655–3659.
11. **Barrette, W. C., Jr., J. M. Albrich, and J. K. Hurst.** 1987. Hypochlorous acid-promoted loss of metabolic energy in *Escherichia coli*. Infect. Immun. **55:**2518–2525.
12. **Bean, N. H., and P. M. Griffin.** 1990. Foodborne disease outbreaks in the United States, 1973–1987: pathogens, vehicles and trends. J. Food Prot. **53:**807–814.
13. **Blatchley, E. R. III.** 1994. Disinfection and antimicrobial processes. Water Environ. Res. **66:**361–368.
14. **Block, S. S. (ed.).** 1991. Disinfection, sterilization and preservation, 4th ed. Lea & Febiger, Philadelphia, Pa.
15. **Brown, E. G., R. P. Newton, and N. M. Shaw.** 1982. Analysis of free nucleotide pools of mammalian tissues by high-pressure liquid chromatography. Anal. Biochem. **123:**378–388.
16. **Brown, M. H., and I. R. Booth.** 1991. Acidulants and low pH, p. 22–43. *In* N. J. Russell and G. W. Gould (ed.), Food preservatives. Blackie, Glasgow, Scotland.
17. **Casse, R. A., R. L. Bradley, Jr., and R. R. Williams.** 1985. Chemical and physical methods: available chlorine. *In* G. H. Richardson (ed.), Standard methods for the examination of dairy products, 15th ed. American Public Health Association, Washington, D.C.
18. **Chapman, A. G., and D. E. Atkinson.** 1977. Adenine nucleotide concentrations and turnover rate. Their correlation with biological activity in bacteria and yeast. Adv. Microb. Physiol. **15:**253–306.
19. **Cords, B. R., and G. R. Dychdala.** 1993. Sanitizers: halogens, surface-active agents, and peroxides. *In* P. M. Davidson and A. L. Branen (ed.), Antimicrobials in foods, 2nd ed. Marcel Dekker, Inc., New York, N.Y.
20. **Dychdala, G. R.** 1991. Chlorine and chlorine compounds, p. 131–151. *In* S. S. Block (ed.), Disinfection, sterilization, and preservation, 4th ed. Lea & Febiger, Philadelphia, Pa.
21. **Ellman, G. L.** 1959. Tissue sulfhydryl groups. Arch. Biochem. Biophys. **82:**70–77.
22. **Foster, J. W.** 1995. Low pH adaptation and the acid tolerance response of *Salmonella typhimurium*. Crit. Rev. Microbiol. **21:**215–237.
23. **Foster, J. W., and H. K. Hall.** 1991. Inducible pH homeostasis and the acid tolerance response of *Salmonella typhimurium*. J. Bacteriol. **173:**5129–5135.
24. **Foster, J. W., and H. K. Hall.** 1990. Adaptive acidification tolerance response of *Salmonella typhimurium*. J. Bacteriol. **172:**771–778.
25. **Gerhardt, P.** 1981. Diluents and biomass measurement, p. 504–507. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
26. **Gribble, G. W.** 1995. Chlorine and health. American Council on Science and Health, New York, N.Y.
27. **Hersh, B. M., F. T. Farooq, D. N. Barstad, D. L. Blankenhorn, and J. L. Slonczewski.** 1996. A glutamate-dependent acid resistance gene in *Escherichia coli*. J. Bacteriol. **178:**3978–3981.
28. **Hurst, J. K., W. C. Barrette, Jr., B. R. Michel, and H. Rosen.** 1991. Hypochlorous acid and myeloperoxidase-catalyzed oxidation of iron-sulfur clusters in bacterial respiratory dehydrogenases. Eur. J. Biochem. **202:**1275–1282.
29. **Hurst, J. K., and W. C. Barrette, Jr.** 1989. Leukocytic oxygen activation and microbicidal oxidative toxins. Crit. Rev. Biochem. Mol. Biol. **24:**271–328.
30. **Khan, A. U., and M. Kasha.** 1994. Singlet molecular oxygen evolution upon simple acidification of aqueous hypochlorite: application to studies on the deleterious health effects of chlorinated drinking water. Proc. Natl. Acad. Sci. USA **91:**12362–12364.
31. **Knox, W. E., P. K. Stumpf, D. E. Green, and V. H. Auerbach.** 1948. The inhibition of sulfhydryl enzymes as the basis of the bactericidal action of chlorine. J. Bacteriol. **55:**451–458.
32. **Leyer, G. J., and E. A. Johnson.** 1993. Acid adaptation induces cross-protection against environmental stresses in *Salmonella typhimurium*. Appl. Environ. Microbiol. **59:**1842–1847.
33. **Leyer, G. J., and E. A. Johnson.** 1992. Acid adaptation promotes survival of *Salmonella* spp. in cheese. Appl. Environ. Microbiol. **58:**2075–2080.
34. **Leyer, G. J., L.-L. Wang, and E. A. Johnson.** 1995. Acid adaptation of *Escherichia coli* O157:H7 increases survival in acidic foods. Appl. Environ. Microbiol. **61:**3752–3755.
35. **Lin, J., I. S. Lee, J. Frey, J. L. Slonczewski, and J. W. Foster.** 1995. Comparative analysis of extreme acid survival in *Salmonella typhimurium*, *Shigella flexneri*, and *Escherichia coli*. J. Bacteriol. **177:**4097–4104.
36. **McKenna, S. M., and K. J. A. Davies.** 1988. The inhibition of bacterial growth by hypochlorous acid. Possible role in the bactericidal activity of phagocytes. Biochem. J. **254:**685–692.
37. **Mustapha, A., and M. B. Liewen.** 1989. Destruction of *Listeria monocytogenes* by sodium hypochlorite and quaternary ammonium sanitizers. J. Food Prot. **52:**306–311.
38. **Neidhardt, F. C.** 1987. Multigene systems and regulons, p. 1313–1317. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
39. **Pedersen, P. L., and E. Carafoli.** 1987. Ion motive ATPases. I. Ubiquity,

properties, and significance to cell function. Trends Biochem. Sci. **12:**146–150.
40. **Portillo, F. G., J. W. Foster, and B. B. Finlay.** 1993. Role of acid tolerance response genes in *Salmonella typhimurium*. Infect. Immun. **61:**4489–4492.
41. **Rakita, R. M., B. R. Michel, and H. Rosen.** 1990. Differential inactivation of *Escherichia coli* membrane dehydrogenases by a myeloperoxidase-mediated antimicrobial system. Biochemistry **29:**1075–1080.
42. **Rosen, H., J. Orman, R. M. Rakita, R. Michel, and D. R. VanDevanter.** 1990. Loss of DNA-membrane interactions and cessation of DNA synthesis in myeloperoxidase-treated *Escherichia coli*. Proc. Natl. Acad. Sci. USA **87:** 10048–10052.
43. **Rosen, H., R. M. Rakita, A. M. Waltersdorph, and S. J. Klebanoff.** 1987. Myeloperoxidase-mediated damage to the succinate oxidase system of *Escherichia coli*. J. Biol. Chem. **242:**15004–15010.
44. **Rosen, H., and S. J. Klebanoff.** 1985. Oxidation of microbial iron-sulfur centers by the myeloperoxidase-H202-halide antimicrobial system. Infect. Immun. **47:**613–618.
45. **Rosen, H., and S. J. Klebanoff.** 1982. Oxidation of *Escherichia coli* iron centers by the myeloperoxidase-mediated microbicidal system. J. Biol. Chem. **257:**13731–13735.
46. **Thomas, E. L.** 1979. Myeloperoxidase, hydrogen peroxide, chloride antimicrobial system: nitrogen-chlorine derivatives of bacterial components in bactericidal action against *Escherichia coli*. Infect. Immun. **23:**522–531.
47. **Thomas, E. L.** 1979. Myeloperoxidase-hydrogen peroxide-chloride antimicrobial system: effect of exogenous amines on antibacterial action against *Escherichia coli*. **25:**110–116.
48. **Thomas, E. L., and T. M. Aune.** 1978. Oxidation of *Escherichia coli* sulfhydryl components by the peroxidase-hydrogen peroxide-iodide antimicrobial system. Antimicrob. Agents Chemother. **13:**261–265.
49. **Vaara, M.** 1992. Agents that increase the permeability of the outer membrane. Microbiol. Rev. **56:**395–411.
50. **Worthington, C. C. (ed.).** 1988. Worthington enzyme manual. Worthington Biochemical Corp., Freehold, N.J.