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Population-Wide Survey of *Salmonella enterica* Response to High-Pressure Processing Reveals a Diversity of Responses and Tolerance Mechanisms

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ABSTRACT High-pressure processing is a nonthermal method of food preservation that uses pressure to inactivate microorganisms. To ensure the effective validation of process parameters, it is important that the design of challenge protocols consider the potential for resistance in a particular species. Herein, the responses of 99 diverse *Salmonella enterica* strains to high pressure are reported. Members of this population belonged to 24 serovars and were isolated from various Canadian sources over a period of 26 years. When cells were exposed to 600 MPa for 3 min, the average reduction in cell numbers for this population was 5.6 $\log_{10}$ CFU/ml, with a range of 0.9 $\log_{10}$ CFU/ml to 6 $\log_{10}$ CFU/ml. Eleven strains, from 5 serovars, with variable levels of pressure resistance were selected for further study. The membrane characteristics (propidium iodide uptake during and after pressure treatment, sensitivity to membrane-active agents, and membrane fatty acid composition) and responses to stressors (heat, nutrient deprivation, desiccation, and acid) for this panel suggested potential roles for the cell membrane and the RpoS regulon in mediating pressure resistance in *S. enterica*. The data indicate heterogeneous and multifactorial responses to high pressure that cannot be predicted for individual *S. enterica* strains.

IMPORTANCE The responses of foodborne pathogens to increasingly popular minimal food decontamination methods are not understood and therefore are difficult to predict. This report shows that the responses of *Salmonella enterica* strains to high-pressure processing are diverse. The magnitude of inactivation does not depend on how closely related the strains are or where they were isolated. Moreover, strains that are resistant to high pressure do not behave similarly to other stresses, suggesting that more than one mechanism might be responsible for resistance to high pressure and the mechanisms used may vary from one strain to another.

KEYWORDS RpoS, *Salmonella enterica*, serovars, high-pressure processing, outer membrane

High-pressure processing (HPP) is a method of nonthermal food processing in which microorganisms are inactivated by pressures exceeding 300 MPa (2,961 atm). Pressure is transmitted by a fluid medium surrounding the food and hence is applied evenly and instantaneously throughout the food matrix. Because the process does not require added heat or chemicals, it is an attractive method to control the levels of spoilage organisms in minimally processed foods. Examples of HPP-treated foods that are commercially available include fruit juices and jams, guacamole, oysters, and ready-to-eat sliced meats such as chicken, turkey, and ham (1).

The kinetics of microbial inactivation by high pressure are complex (2). Cellular inactivation is not proportional to the magnitude or holding time of the applied pressure (3, 4). Increased holding times, especially at sublethal pressures, may contribute to tailing effects, in which a subpopulation of cells survive the inactivation process

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(5). The effects of the food matrix on pressure inactivation of cells are variable. Some physiochemical properties of food (e.g., low water activity) may exert protective effects, whereas others (e.g., low pH) may increase sensitivity to high pressure (1, 5).

High pressure exerts its effects through the disruption of noncovalent bonds. Hence, its effects on cells are manifold. The cell membrane is the most pressure-sensitive cellular structure (5, 6). Physical damage to the membrane upon exposure to pressure has been examined microscopically, and the loss of barrier function has been inferred through the loss of membrane proteins, the leakage of cellular materials, and the increased uptake of membrane-impermeable dyes such as propidium iodide (PI) (7–9). Secondary pressure targets include multimeric protein and nucleic acid structures, which can affect diverse cellular processes, including replication and metabolism (6). Consequently, the responses of cells to high pressure vary considerably among and within bacterial species (6, 10–12).

Studies of pressure-resistant bacterial strains have identified a number of adaptations that may be involved in pressure resistance. Piezophilic and pressure-tolerant organisms isolated from high-pressure environments exhibit structural adaptations, such as increased levels of membrane fatty acid unsaturation, that enable life in those environments (13). Foodborne bacteria are exposed to high-pressure environments intermittently, if at all, and demonstrate more dynamic responses to high pressure. A number of studies have implicated members of the *rpoS* regulon in coordinating the response of *Escherichia coli* to high pressure (14–16). In particular, mutants that are defective in the synthesis of cyclopropane fatty acids demonstrate increased sensitivity to pressure (7, 17). It has also been suggested that genes involved in heat shock and cold shock responses are involved in pressure tolerance, despite the observation of little to no correlation between temperature and pressure stress (18–20).

Salmonella enterica is an important foodborne pathogen (21). The strains within this species are taxonomically diverse and can belong to one of >2,500 serovars, based on their antigenic properties (22). Members within a serovar may be closely related or genetically diverse (23–25). The response of *Salmonella enterica* to high-pressure processing has been studied in a variety of experimental systems and food matrices, including milk, juice, almonds, seeds, meat, and peanut butter (10, 12, 26–30). The reported responses are variable and must be examined in the context of process parameters, the food matrix, and strain physiology. Studies investigating the responses of multiple strains of *S. enterica* to high pressure within the same experimental system demonstrate the inherent variability within the species. Sherry et al. (29) reported an average reduction of 3.3 $\log_{10}$ CFU/ml for 40 *S. enterica* strains from 33 serovars (1 strain each for 30 serovars, 2 strains each for 2 serovars, and 6 strains for 1 serovar) in spent culture medium exposed to a pressure of 350 MPa for 10 min at 20°C. The reported range was 2.5 $\log_{10}$ CFU/ml, with many strains exhibiting a difference from the most resistant strain of <1 $\log_{10}$ CFU/ml. Alpas et al. (10) investigated the responses of 6 strains (2 strains each for 3 serovars), in culture broth diluted with 1% peptone water, to exposure to 345 MPa for 5 min at 25°C; this population was much more sensitive to pressure, with an average reduction in cell number of 7 $\log_{10}$ CFU/ml and a difference of at least 2.9 $\log_{10}$ CFU/ml between the most pressure-tolerant strains and the most pressure-sensitive strains. Whitney et al. (12) also observed variability among 5 strains (1 strain each for 5 serovars) associated with foodborne outbreaks. When exposed to a pressure of 300 MPa for 2 min at 6°C in tryptic soy broth (TSB), the 5 strains exhibited an average decrease of 2.4 $\log_{10}$ CFU/ml, ranging from 0.53 $\log_{10}$ CFU/ml for the most pressure-resistant isolate to 3.0 $\log_{10}$ CFU/ml for the most pressure-sensitive isolate. These differences were lessened at 550 MPa, at which the strains exhibited an average cell decrease of 4.7 $\log_{10}$ CFU/ml, with a range of 3.8 to 5.4 $\log_{10}$ CFU/ml.

Continued testing of *S. enterica* strains will establish baseline levels of pressure resistance within the species and allow comparisons to be made between and within serovars; the strains with the greatest potential for resistance could be used as appropriate strains for challenge studies and simulation of worst-case scenarios for risk assessment. To achieve this goal, we examined the responses of a diverse population

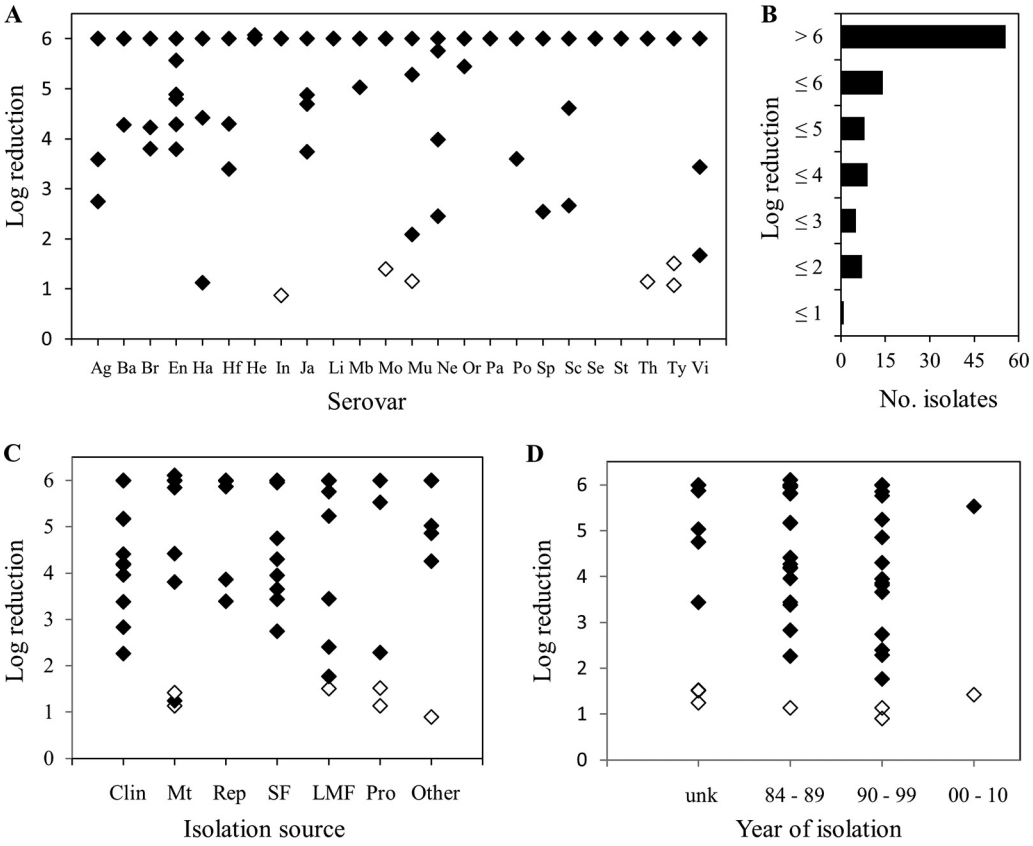

FIG 1 Decreases in cell numbers for 99 *S. enterica* strains exposed to 600 MPa for 3 min. (A, C, and D) Decreases in cell numbers according to serovar (A), isolation source (C), and date of isolation (D). (B) Frequency of decreases observed for this population of strains. Open symbols indicate the 6 pressure-tolerant strains selected for further characterization. The limit of detection was 1 log unit below the initial cell concentration. Ag, Agona; Ba, Bareilly; Br, Braenderup; En, Enteritidis; Ha, Hadar; Hf, Hartford; He, Heidelberg; In, Infantis; Ja, Javiana; Li, Litchfield; Mb, Mbandaka; Mo, Montevideo; Mu, Muenchen; Ne, Newport; Or, Oranienburg; Pa, Panama; Po, Poona; Sp, Saintpaul; Sc, Schwarzengrund; Se, Senftenberg; St, Stanley; Th, Thompson; Ty, Typhimurium; Vi, Virchow; Clin, clinical; Mt, meat; Rep, reptile; SF, seafood; LMF, low-moisture food; Pro, produce; unk, unknown.

of 99 strains (3 to 6 strains each for 24 serovars) of *S. enterica* to a pressure of 600 MPa, and 11 strains with low or high tolerance to pressure were further characterized to elucidate the physiological basis of pressure tolerance in *S. enterica*.

RESULTS

***S. enterica* strains demonstrate a range of levels of tolerance to pressure.** The responses of 99 *S. enterica* strains to exposure to a pressure of 600 MPa for 3 min involved reductions ranging from 0.9 to 6 $\log_{10}$ CFU/ml (Fig. 1A; also see Table S1 in the supplemental material). Approximately one-half of the strains (55/99 strains) were completely eliminated by the treatment, resulting in a median reduction of 6 $\log_{10}$ CFU/ml and an average reduction of 5.1 $\log_{10}$ CFU/ml for this population of *S. enterica* strains. The numbers of strains demonstrating reductions of $\leq$4, $\leq$3, $\leq$2, and $\leq$1 $\log_{10}$ CFU/ml were 22, 13, 7, and 1, respectively (Fig. 1B). The most pressure-tolerant strain was a *S. enterica* serovar Infantis isolate originally cultured from a noodle salad in 1995; this strain demonstrated a reduction of 0.9 $\log_{10}$ CFU/ml under the conditions of the experiment.

The responses of the isolates within 20 of the 24 serovars were heterogeneous, with no one serovar exhibiting exclusively pressure-tolerant strains. Only members of *S. enterica* serovars Litchfield, Panama, Senftenberg, and Stanley demonstrated uniform responses (reductions of $>$6 $\log_{10}$ CFU/ml) under the test conditions. Similarly, there did not appear to be any relationship between levels of pressure tolerance observed

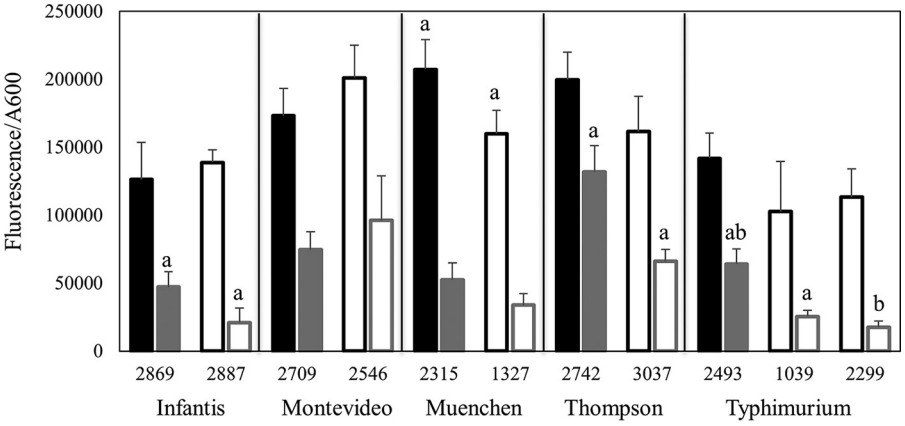

FIG 2 Propidium iodide uptake by pressure-sensitive (closed bars) and pressure-tolerant (open bars) strains of *S. enterica* during (black) and after (gray) exposure to 600 MPa for 3 min. Within each panel, bars that share a lowercase letter are significantly different ($P < 0.05$).

among the strains and either their source or their year of isolation (Fig. 1C and D). The most pressure-resistant strains, demonstrating decreases of <2 $\log_{10}$ CFU/ml, were originally isolated from meat, low-moisture foods, produce, or a Greek noodle salad (Fig. 1C, other) over the span of $\geq$26 years.

The membrane barrier is affected during and after exposure to high pressure. To characterize the nature of pressure resistance in *Salmonella*, 6 pressure-tolerant strains belonging to the *S. enterica* serovars Infantis, Montevideo, Muenchen, Thompson, and Typhimurium (2 strains) were selected for further characterization. Five pressure-sensitive strains belonging to those serovars were randomly selected for comparison.

The membrane damage sustained by the 11 strains during exposure to pressure of 600 MPa for 3 min was assessed by exposing cells to the membrane-impermeable fluorescent dye propidium iodide (PI) prior to pressure treatment (Fig. 2). Cells that were not exposed to pressure had low baseline fluorescence values, ranging from 4,130 to 12,500 fluorescence units (average, 7,350 fluorescence units), suggesting that the influx of PI was low or negligible. In contrast, the fluorescence values of cells exposed to high pressure increased by >20-fold, ranging from 82,300 to 236,000 fluorescence units (average, 164,000 fluorescence units). The increase in fluorescence suggested considerable uptake of PI by the cells during pressurization. With the exception of *S. enterica* serovar Muenchen, the fluorescence values, and hence the permeability of the membranes of the pressure-tolerant strains to PI, were comparable to those of the pressure-sensitive strains. Strain 1327, the pressure-tolerant representative strain from *S. enterica* serovar Muenchen, demonstrated a fluorescence value of 160,000 fluorescence units, whereas its pressure-sensitive counterpart (strain 2315) demonstrated a modestly but significantly higher fluorescence value of 207,000 fluorescence units.

To examine the restoration of the membrane barrier after pressurization, cells were exposed to pressure and then were exposed to PI for 10 min following pressure removal (Fig. 2). The fluorescence values for the cells under these conditions ranged from 16,500 to 154,000 fluorescence units (average, 64,700 fluorescence units). These values were lower than those for cells exposed to PI prior to pressurization, suggesting that the membrane barrier had been restored to some degree during the interval between the additions of the dye to the two sets of cells. Comparison of the fluorescence values for the pressure-tolerant strains with those for their respective pressure-sensitive counterparts revealed that the members of *S. enterica* serovars Infantis, Thompson, and Typhimurium demonstrated significantly less PI uptake 10 min after the removal of pressure.

Membrane characteristics of pressure-tolerant *S. enterica* strains. The growth of the 11 pressure-tolerant or pressure-sensitive strains in membrane-active detergents

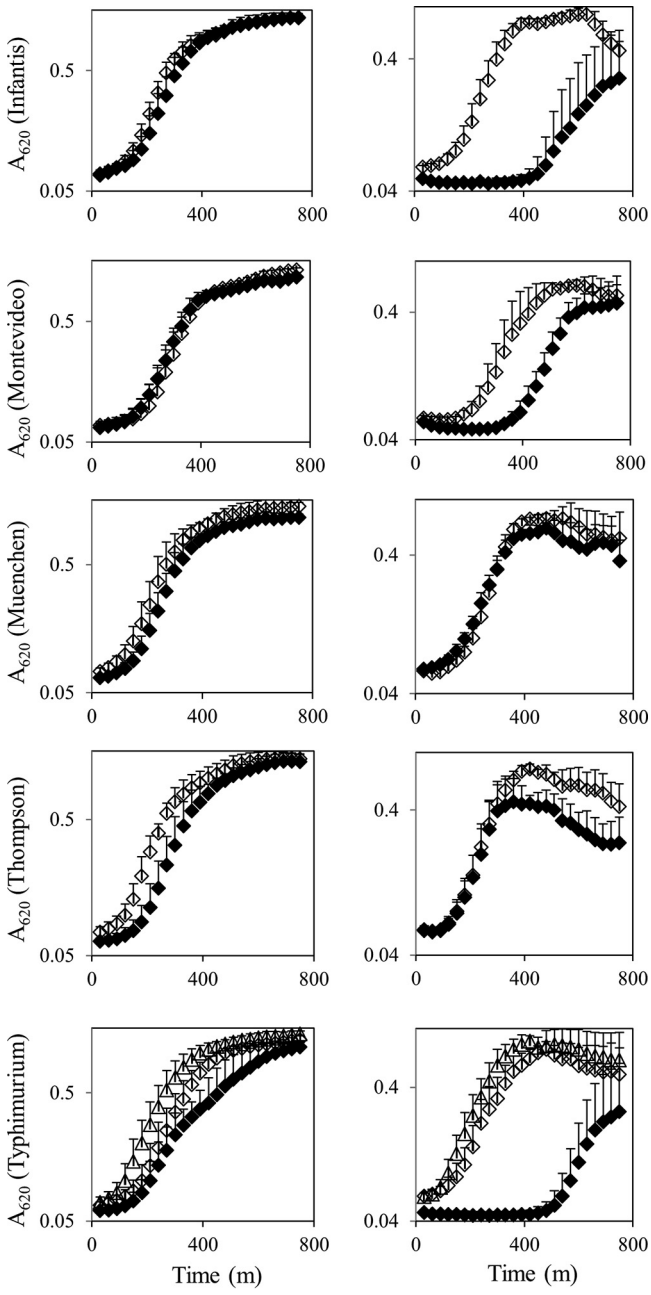

FIG 3 Growth of pressure-tolerant (open symbols) and pressure-sensitive (closed symbols) strains of *S. enterica* in Luria-Bertani (LB) broth (left) or LB broth with 1% SDS (right). For *S. enterica* serovar Typhimurium: open triangles, *S. enterica* serovar Typhimurium strain 1039; open diamonds, *S. enterica* serovar Typhimurium strain 2299.

was determined, to assess the baseline membrane characteristics of these organisms. In comparison with their pressure-sensitive counterparts, the pressure-tolerant strains belonging to *S. enterica* serovars Infantis, Montevideo, and Typhimurium demonstrated more robust growth in 1% SDS (Fig. 3). Specifically, these strains exhibited a shorter lag time in the detergent (130 min versus 500 min, 225 min versus 360 min, and 80 min and 130 min versus 710 min for the pressure-tolerant versus pressure-sensitive *S. enterica* strains belonging to *S. enterica* serovars Infantis, Montevideo, and Typhimurium, respectively). The *S. enterica* serovar Thompson pair exhibited similar growth kinetics; however, the turbidity of the pressure-sensitive strain appeared to peak and then to decrease gradually, in comparison with the pressure-tolerant strain. The growth of the

TABLE 1 Membrane fatty acid composition of *S. enterica* strains with different levels of pressure tolerance

Strain	Pressure tolerance	Composition (% of total membrane lipids)[a]			
		SFA	HFA	UFA	CFA
S. enterica serovar Infantis 2869	Sensitive	49.72	8.81	12.64	25.48
S. enterica serovar Infantis 2887	Resistant	49.1	9.15	10.91	26.93
S. enterica serovar Montevideo 2709	Sensitive	48.75	10.59	20.11	13.42
S. enterica serovar Montevideo 2546	Resistant	46.04	9.58	27.09	10.47
S. enterica serovar Muenchen 2315	Sensitive	52.99	9.71	10.95	21.04
S. enterica serovar Muenchen 1327	Resistant	52.08	9.08	10.05	24.78
S. enterica serovar Thompson 2742	Sensitive	50.94	8.97	11.09	25.08
S. enterica serovar Thompson 3037	Resistant	53.52	9.59	9.48	21.91
S. enterica serovar Typhimurium 2493	Sensitive	53.47	9.86	14.61	17.01
S. enterica serovar Typhimurium 1039	Resistant	52.75	10.09	13.38	17.88
S. enterica serovar Typhimurium 2299	Resistant	51.97	9.16	7.34	27.89

[a]SFA, saturated fatty acids, i.e., lauric acid ($C_{12:0}$), myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$), and steric acid ($C_{18:0}$); HFA, hydroxylated fatty acids, i.e., 3-hydroxy-myrisitc acid (3-OH-C_{14}); UFA, unsaturated fatty acids, i.e., palmitoleic acid (9-*cis*-$C_{16:1}$) and *cis*-vaccenic acid (11-*cis*-$C_{18:1}$); CFA, cyclopropane fatty acids, i.e., *cis*-9,10-methylene-hexadecanoic acid (ΔC_{17}) and lactobacillic acid (ΔC_{19}).

11 strains in other membrane-active agents, including Triton X-100, sodium deoxycholate, EDTA, and $MgCl_2$, was indistinguishable (data not shown). All 11 strains were susceptible to polymyxin B sulfate, gentamicin, and kanamycin.

The membrane fatty acid compositions of stationary-phase cultures of the 5 pressure-sensitive strains and 6 pressure-resistant strains grown in TSB were determined. For all strains, approximately one-half of the membrane lipids were saturated fatty acid methyl esters, with palmitic aid ($C_{16:0}$) predominating (Table 1). The second most predominant species were the unsaturated fatty acids palmitoleic acid (9-*cis*-$C_{16:1}$) and *cis*-vaccenic acid (11-*cis*-$C_{18:1}$) for *S. enterica* serovar Montevideo and the cyclopropane fatty acids *cis*-9,10-methylene-hexadecanoic acid (ΔC_{17}) and lactobacillic acid (ΔC_{19}) for *S. enterica* serovars Infantis, Muenchen, Thompson, and Typhimurium. Comparison of the fatty acid profiles of the pressure-sensitive strains and the pressure-resistant strains revealed that the resistant counterparts of *S. enterica* serovars Infantis, Muenchen, Thompson, and Typhimurium had smaller proportions of unsaturated fatty acids than did their pressure-sensitive counterparts (10.9%, 10.1%, 9.48%, and 13.4% and 7.34% for the pressure-tolerant strains versus 12.6%, 11.0%, 11.1%, and 14.6% for the pressure-sensitive strains, respectively). Resistant members of *S. enterica* serovars Infantis, Muenchen, and Typhimurium were further distinguished from their sensitive counterparts by possessing larger proportions of cyclopropane fatty acids in their membranes (26.9%, 24.8%, and 17.9% and 27.9% for the pressure-tolerant strains versus 25.5%, 21.0%, and 17.0% for the pressure-sensitive strains, respectively). Although the difference in membrane composition was striking for some strains (e.g., *S. enterica* serovar Typhimurium strains 2493 and 2299), when the findings were viewed collectively there were no significant differences in fatty acid composition between the pressure-resistant and pressure-sensitive strains.

General stress resistance of pressure-tolerant *S. enterica* strains. To determine whether pressure resistance in *S. enterica* could be generalized to resistance to other forms of stress, the 11 pressure-tolerant or pressure-sensitive strains were exposed to stress-inducing conditions, including heat, nutrient deprivation, desiccation, and citric acid. When the cell numbers of the pressure-tolerant strains were compared with those of their pressure-sensitive counterparts, significant differences were not observed with respect to survival at 60°C, with nutrient deprivation, or with desiccation (Fig. 4). Significant differences were observed, however, when the 11 strains were exposed to 50 mM citric acid (pH 2.2) for 2 h (Fig. 4A and 5). Under those conditions, the pressure-tolerant strains from *S. enterica* serovars Muenchen, Thompson, and Typhimurium had greater numbers of survivors than did the pressure-sensitive strains from

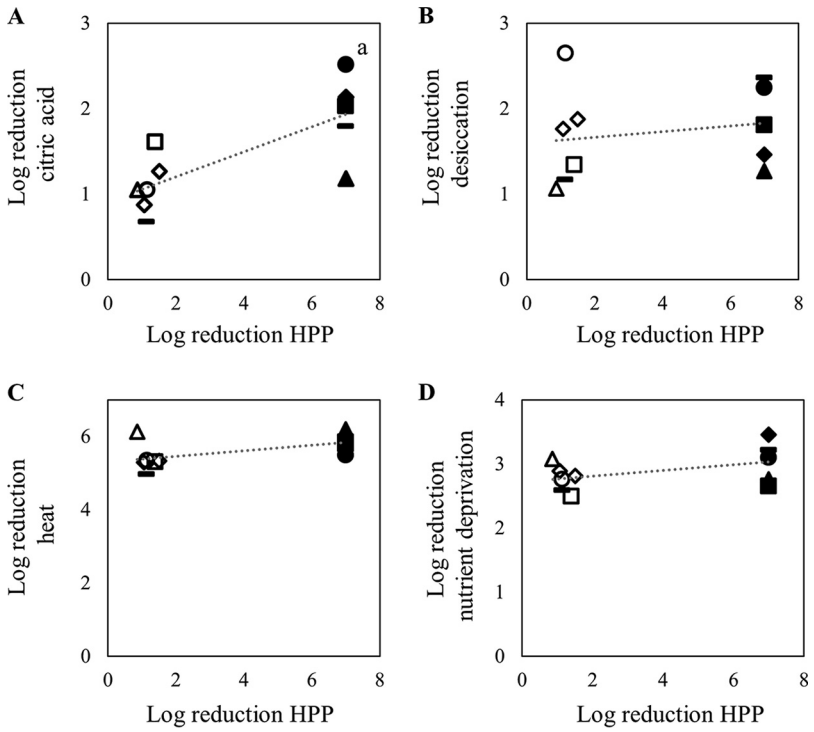

FIG 4 Correlations between inactivation by high-pressure processing (HPP) (600 MPa for 3 min) and exposure to 50 mM citric acid (pH 2.2) for 2 h (A), desiccation (B), heat (60°C for 5 min) (C), or nutrient deprivation (D) for pressure-tolerant (open symbols) and pressure-sensitive (closed symbols) *S. enterica* strains. Triangles, *S. enterica* serovar Infantis; squares, *S. enterica* serovar Montevideo; circles, *S. enterica* serovar Muenchen; horizontal bars, *S. enterica* serovar Thompson; diamonds, *S. enterica* serovar Typhimurium. a, significant difference between the two groups of bacteria ($P < 0.05$).

the same serovars. These differences were not observed for the members of *S. enterica* serovars Infantis and Montevideo.

Catalase activity of pressure-tolerant isolates. The phenotypic differences for some of the pressure-tolerant strains versus the pressure-sensitive strains in response to citric acid suggested the involvement of RpoS. To gauge the levels of RpoS activity in the cells, a catalase assay was performed (Fig. 6). All 11 strains were positive for catalase activity. No differences in catalase activity were observed between the 2 *S. enterica* serovar Infantis strains. However, the pressure-tolerant strains from *S. enterica* serovars Montevideo, Muenchen, Thompson, and Typhimurium had significantly higher levels of catalase activity than did their pressure-sensitive counterparts.

DISCUSSION

The responses of individual *S. enterica* strains to high hydrostatic pressure depend on a number of factors related to both the process and the organism (1, 4, 5). In order

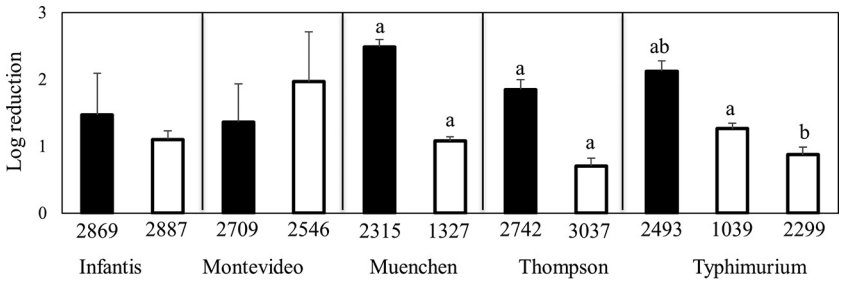

FIG 5 Average reductions for pressure-sensitive (closed bars) and pressure-tolerant (open bars) strains of *S. enterica* after exposure to 50 mM citric acid (pH 2.2) for 2 h. Within each panel, bars that share a lowercase letter are significantly different ($P < 0.05$).

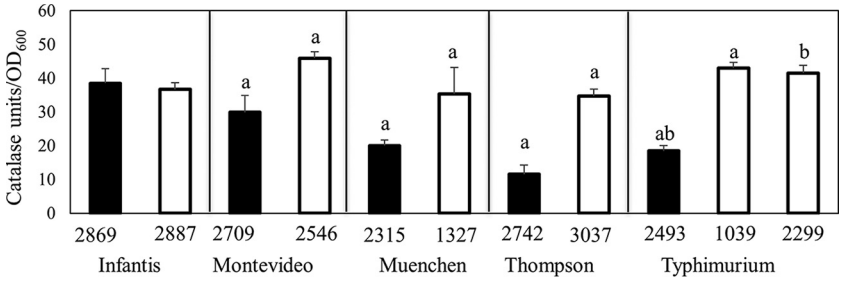

FIG 6 Average catalase activities of pressure-sensitive (closed bars) and pressure-tolerant (open bars) strains of *S. enterica*. Within each panel, bars that share a lowercase letter are significantly different ($P < 0.05$).

to define the basic levels of pressure resistance in *Salmonella*, 99 *S. enterica* strains from 24 serovars, isolated from diverse sources over a period of ≥26 years, were subjected to high pressure under identical experimental conditions. Upon exposure to 600 MPa for 3 min, a difference of at least 5 $\log_{10}$ CFU/ml between the most pressure-tolerant strain and the most pressure-sensitive strains was observed. Despite the close genetic relationships among strains in some serovars, there did not appear to be any relationship between the degree of pressure resistance and the serovar classification; the isolation source and the date of isolation also did not correlate with pressure resistance. Similar trends were observed in a study investigating the pressure resistance of 100 *E. coli* strains (11). However, the distributions of the resistance patterns differed between the bacterial species. The *E. coli* strains demonstrated reductions that appeared to be evenly distributed between 3 $\log_{10}$ CFU/ml and 5.5 $\log_{10}$ CFU/ml, with a median of 3.9 $\log_{10}$ CFU/ml. In contrast, the median reduction observed for the *S. enterica* strains was 6 $\log_{10}$ CFU/ml, largely due to the complete inactivation of 56% of the strains upon exposure to 600 MPa. The *S. enterica* population harbored more strains that demonstrated reductions of ≤2 $\log_{10}$ CFU/ml, compared to the *E. coli* population (8 strains and 3 strains, respectively). Therefore, although the majority of *S. enterica* strains may be highly sensitive to high-pressure processing, significant proportions may exhibit unusual levels of pressure tolerance. This conclusion contrasts with previous reports that suggested more uniform responses to high pressure (10, 29). These differences may be a result of differences in process parameters and experimental conditions used in the studies or may reflect inherent differences between the tested strains.

The membrane characteristics of 5 *S. enterica* serovars were investigated to determine the nature of pressure resistance in *Salmonella*. The pressure-tolerant strains of some serovars demonstrated reduced levels of propidium iodide uptake (*S. enterica* serovars Infantis, Muenchen, Thompson, and Typhimurium) and higher levels of cyclopropane fatty acids (*S. enterica* serovars Infantis, Muenchen, and Typhimurium), as reported for some *E. coli* strains (Table 2) (7, 17). Two exceptions to this trend were the pressure-tolerant strains belonging to *S. enterica* serovars Thompson and Montevideo.

TABLE 2 Summary of phenotypic properties associated with pressure-tolerant strains in five *S. enterica* serovars

	Membrane property[a]			RpoS regulon	
***S. enterica* serovar**	**PI uptake**	**CFA**	**Growth in SDS**	**Acid tolerance**	**Catalase activity**
Infantis	+	+	+	−	−
Montevideo	−	−	+	−	+
Muenchen	+	+	−	+	+
Thompson	+	−	−	+	+
Typhimurium	+	+	+	+	+

[a]+, a difference between the pressure-tolerant and pressure-sensitive strains within the serovar was observed; −, a difference between the pressure-tolerant and pressure-sensitive strains within the serovar was not observed; CFA, cyclopropane fatty acids.

It may be that, in the case of the pressure-tolerant *S. enterica* serovar Montevideo strain, 10 min was not a suitable time point at which to observe differences in propidium iodide uptake. However, neither strain demonstrated elevated levels of cyclopropane fatty acids, in comparison to their pressure-sensitive counterparts. The *S. enterica* serovar Thompson strain exhibited reduced propidium iodide uptake, and the *S. enterica* serovar Montevideo strain showed increased growth in 1% SDS. These results suggest that, at least in these 2 *S. enterica* serovars, the membrane bilayer may be involved but may not play as critical a role in pressure resistance as has been observed for other *S. enterica* serovars and *E. coli*. For example, membrane repair after the removal of pressure is an energy-dependent process requiring the synthesis of both RNA and protein (31). Thus, it may be that those systems were differentially affected in the pressure-sensitive strains upon the application of pressure. Alternatively, other features of the membrane that were not investigated in this study, such as membrane proteins, may play a more critical role in pressure resistance, as has been observed for some strains of *S. enterica* (8, 9).

Resistance to pressure in *E. coli* is correlated with RpoS activity, and exposure to high pressure selects for variants with increased RpoS activity (15, 16). The role of RpoS in maintaining the integrity of the *E. coli* cell membrane during exposure to stressors such as pressure and SDS has been established, and there are a number of downstream targets that may also mediate pressure resistance independent of the cell membrane (7, 17, 32–35). The differences observed among the 11 *S. enterica* strains with respect to acid resistance and catalase activity suggested a role for RpoS in coordinating the response to pressure in this organism as well. The overall responses of the 11-strain panel were heterogeneous. Compared to their pressure-sensitive counterparts, the pressure-tolerant strains of some serovars (*S. enterica* serovars Muenchen, Thompson, and Typhimurium) exhibited phenotypes associated with enhanced RpoS activity (Table 2). In contrast, the pressure-tolerant *S. enterica* serovar Montevideo strain exhibited only enhanced catalase activity, and the pressure-tolerant *S. enterica* serovar Infantis strain did not exhibit higher levels of any activity associated with RpoS. These results suggest that the role of RpoS in mediating pressure resistance may be more critical in some *S. enterica* serovars than in others. At present, there are few data on the contributions of RpoS to stress resistance in different serovars of *S. enterica*, and this is an area that warrants further study.

The 5 serovars investigated in this study demonstrated unique patterns of membrane properties and stress responses that are correlated with pressure resistance in *E. coli* (Table 2). There did not seem to be a uniform coordinated response to high pressure among the strains investigated in this study. The strains of some serovars, such as *S. enterica* serovar Infantis, seemed to rely solely on membrane phenotypes to mediate pressure resistance, whereas other serovars, such as *S. enterica* serovars Muenchen and Typhimurium, might have also used RpoS to coordinate responses to pressure. Therefore, based on this data set, it appears that there may be multiple paths to pressure resistance in *S. enterica*.

The responses of *S. enterica* to high pressure are heterogeneous with respect to both the degree of inactivation and the mechanisms used to overcome it. The unpredictable nature of these responses should be considered during the design of challenge studies and the selection of surrogate strains. This data set will be useful for the identification of the most pressure-resistant populations of *S. enterica* and the simulation of worst-case scenarios for risk assessment.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The 99 strains used in this study were from the Bureau of Microbial Hazards *Salmonella* culture collection and comprised 24 serovars. Three to 6 strains from each serovar were tested; the strains are listed in Table S1 in the supplemental material. Prior to each set of experiments, strains were freshly cultured, from storage at −80°C, on tryptic soy agar (TSA) at 35°C for approximately 24 h. Two or 3 colonies from TSA were inoculated into the specified culture medium and incubated for 18 h at 37°C, with shaking at 250 rpm, on the day preceding each experiment. All procedures were carried out at ambient temperatures unless indicated otherwise.

High-pressure treatment of bacterial cells. Cells were cultured in TSB, collected by centrifugation, washed twice in phosphate-buffered saline (PBS) (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl), and resuspended in PBS to a cell density of 1×10^7 CFU/ml. A portion of the cell suspension (4 ml) was used to fill sterile sampling bags (7.5 by 18.5 cm; Fisher Scientific, Ottawa, Canada). The bags were sealed without any headspace by using a Midwest Pacific Impulse heat sealer and were trimmed of excess plastic to form pouches (approximately 2.5 by 6 cm). The pouches were placed and sealed in a larger sampling bag (11.5 by 23 cm; Fisher) containing 1% bleach, to inactivate pathogens in the event of a leak during pressure treatment. Pressure treatment was administered using a 1-liter-capacity pressure vessel (Dustec Hochdrucktechnik GmbH, Wismar, Germany), with water as the transmission fluid. A pressure of 600 MPa was applied for 3 min, with a 1-min pressurization/30-s depressurization cycle. The pressure treatment was conducted at 24°C, and adiabatic heating of the unit during pressurization increased the temperature of the water in the pressure cell to a maximum of 39°C. Cells were immediately enumerated by direct TSA plate counting. Plates were incubated at 35°C for 72 h. The limit of detection for the surviving cells was 10 CFU/ml, or a reduction of 1 log unit below the starting concentration. If no surviving cells were recovered, then the reduction was recorded as >6 log units.

To assess membrane permeability during the pressurization/depressurization cycle, bacterial suspensions were prepared to an optical density at 600 nm (OD_{600}) of 0.2 and propidium iodide (Molecular Probes, Eugene, OR) was added to a final concentration of 3 μM prior to pressure treatment. Cells were collected immediately after exposure to pressure, washed twice, and resuspended in PBS.

The degree of membrane permeabilization following pressure treatment was assessed by incubating pressure-treated cells with 3 μM propidium iodide for 10 min at room temperature and processing the cells as described above. Cellular fluorescence was measured in a fluorescence microplate reader (BioTek, Winooski, VT), with an excitation wavelength of 495 nm and an emission wavelength of 615 nm. Fluorescence units were calculated by normalizing the fluorescence readings to the absorbance of the cell suspensions. The background fluorescence of the untreated cells has been subtracted from the reported fluorescence values. Differences between means were calculated using Student's *t* test, with a significance of 0.05.

Analysis of tolerance to stress. Stationary-phase cells cultured in TSB were harvested by centrifugation, washed twice in PBS, and resuspended to a final cell concentration of 2×10^9 CFU/ml PBS. Heat tolerance was determined by incubating cells in a heated circulating water bath (VWR, Mississauga, Canada) set to 60°C. After 5 min, the cells were immediately placed in an ice water bath. Surviving cells were enumerated by direct plating on TSA and incubation at 35°C for 24 h. Acid tolerance was determined by incubating cells in an equal volume of 100 mM citric acid (resulting in a final pH of 2.2) for 2 h at room temperature prior to enumeration. Tolerance to nutrient deprivation and desiccation was determined as described previously (36, 37). Reported results are the average and standard deviation of three independent experiments. Differences between means were calculated using Student's *t* test, with a significance of 0.05.

Catalase assays. The catalase activity of cells was determined using the method described by Iwase et al. (38). Briefly, stationary-phase cells were collected from TSB, washed twice, and resuspended in PBS. A 100-μl aliquot of this bacterial suspension was added to a borosilicate glass tube (13 mm by 100 mm) containing 100 μl of 1% Triton X-100 (Sigma-Aldrich, Oakville, Canada) and 100 μl of 30% hydrogen peroxide (Sigma-Aldrich). After 15 min of incubation at room temperature, the height of the foam was measured using a metric ruler. Catalase activity was estimated using a standard curve prepared by using catalase from bovine liver (product no. C1345; Sigma), and findings were normalized to the optical density of the cell preparation. Results are reported as the average and standard deviation of three independent experiments. Statistical differences between sample means were calculated using Student's *t* test, with a significance level of 0.05.

Growth assays. Overnight cultures of cells grown in Luria-Bertani (LB) broth (1% tryptone peptone, 0.5% yeast extract [wt/vol]) were diluted 100-fold in either LB broth or LB broth containing supplements. Supplements included the detergents SDS, Triton X-100, and sodium deoxycholate, used at concentrations of 1%. EDTA was used at a concentration of 1 mM. For some experiments, 10 mM $MgCl_2$ was added to the detergent solution. Cells were aliquoted in duplicate in a 96-well flat-bottomed polystyrene plate and incubated in a microplate reader (BioTek) set at 37°C, with continuous shaking. The instrument measured and recorded the absorbance at 620 nm every 30 min. Growth parameters (lag time and doubling time) were calculated according to the procedures described by Hall et al. (39). Results reported are the average and standard deviation of six trials conducted with three independent cultures.

Antibiotic sensitivity assays. The sensitivity of selected strains to antibiotics was assessed with the disk diffusion assay, according to the procedures described by the Clinical and Laboratory Standards Institute (40).

Fatty acid methyl ester analysis. Strains were grown in TSB to stationary phase and washed with PBS as described previously. Cell pellets were shipped on dry ice to a certified testing laboratory, where they were analyzed by gas chromatography (41).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01673-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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