

Cold Shock Induction of Thermal Sensitivity in *Listeria monocytogenes*

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Cold shock at 0 to 15°C for 1 to 3 h increased the thermal sensitivity of *Listeria monocytogenes*. In a model broth system, thermal death time at 60°C was reduced by up to 45% after *L. monocytogenes* Scott A was cold shocked for 3 h. The duration of the cold shock affected thermal tolerance more than did the magnitude of the temperature downshift. The *Z* values were 8.8°C for controls and 7.7°C for cold-shocked cells. The *D* values of cold-shocked cells did not return to control levels after incubation for 3 h at 28°C followed by heating at 60°C. Nine *L. monocytogenes* strains that were cold shocked for 3 h exhibited *D*₆₀ values that were reduced by 13 to 37%. The *D*-value reduction was greatest in cold-shocked stationary-phase cells compared to cells from cultures in either the lag or exponential phases of growth. In addition, cold-shocked cells were more likely to be inactivated by a given heat treatment than nonshocked cells, which were more likely to experience sublethal injury. The *D* values of chloramphenicol-treated control cells and chloramphenicol-treated cold-shocked cells were no different from those of untreated cold-shocked cells, suggesting that cold shock suppresses synthesis of proteins responsible for heat protection. In related experiments, the *D* values of *L. monocytogenes* Scott A were decreased 25% on frankfurter skins and 15% in ultra-high temperature milk if the inoculated products were first cold shocked. Induction of increased thermal sensitivity in *L. monocytogenes* by thermal flux shows potential to become a practical and efficacious preventative control method.

Listeria monocytogenes remains a perplexing risk management problem for the food industry, as well as for regulatory and public health agencies. Despite decreased human incidence in the United States (44), food-borne listeriosis outbreaks continue. Isolation of *Listeria* spp. during product quality control testing and isolation of *L. monocytogenes* from ready-to-eat products due to mandatory inspection continues to impart significant economic losses to food processors. For example, the Food and Drug Administration reported that *L. monocytogenes* contamination accounted for 16% (90 of 569) of all recalled products between October 1991 and 30 September 1992 and for 57% (90 of 158) of class I recalls during that time (46).

L. monocytogenes is frequently isolated from food because of its widespread occurrence in the environment and its ability to grow at refrigerated temperatures (39). Furthermore, it possesses constitutive resistance to heat inactivation that is at least as great as those of most vegetative food-borne pathogens, such as the common *Salmonella enterica* serotypes (12, 21, 33). Like many bacteria, *L. monocytogenes* may respond to several sublethal stress factors by increasing its heat tolerance. Modulators include starvation (24), growth temperature (27, 37, 42), growth on surfaces (15), solutes that lower water activity or oxidants (2, 19, 31, 38), acid shock (13), heat shock (6, 30), and the heating menstrum (5, 10). Among these, induction by heat shock is the best characterized (11, 36). Understanding the mechanism of thermal tolerance modulation is an important approach that could reveal strategies to increase the thermal sensitivity of *L. monocytogenes*.

Data from a broad group of microorganisms suggests that the application of a cold shock or cold acclimation may in-

crease heating sensitivity in *L. monocytogenes*. For example, exposure to cold before heating increased the heat sensitivity of *Escherichia coli* (25, 26) and *S. enterica* serovar Enteritidis phage type 4 (20). In addition, *L. monocytogenes* was more heat sensitive when previously grown at cold temperatures (37, 42).

Since an easily applied and cost-effective approach to eliminate *L. monocytogenes* from ready-to-eat foods is needed, our objective was to test the hypothesis that exposure to cold temperatures before heating reduces *L. monocytogenes* thermal tolerance. We also performed initial investigations on the mechanism of action and the application of thermal flux in food products.

MATERIALS AND METHODS

Bacterial strains. *L. monocytogenes* strains V7, Scott A, S9V5, and H2NG and *Listeria innocua* strain 2340 were obtained from the culture collection of the U.S. Department of Agriculture (USDA), Agricultural Research Service (ARS), Eastern Regional Research Center (ERRC; Wyndmoor, Pa.). *L. monocytogenes* strains 20169, 20306, 20389, 418, and 65102 were gifts from S. Greene (Food Safety & Inspection Service [FSIS], USDA, Washington, D.C.). The FSIS strains included strains from a variety of meat and poultry products. Each culture was prepared by inoculating thawed cells from previously frozen stocks into 50 ml of brain heart infusion (BHI; Difco Laboratories, Detroit, Mich.) broth and incubating for 16 h at 37°C with shaking at 250 rpm. Ten-milliliter samples of cells were harvested by centrifugation at 16,000 × *g* at room temperature. Cell pellets were resuspended in 10 ml of BHI containing 10% glycerol (Sigma Chemical Co., St. Louis, Mo.), transferred (200 μl) to 1.2-ml sterile cryogenic vials (model 5000-0012; Nalgene Company, Rochester, N.Y.), and then frozen and stored at -70°C. Before each experiment, one frozen tube was thawed at room temperature, and the 200 μl was transferred into Luria-Bertani (LB) broth (40) and then incubated at 5, 19, 26, or 37°C with agitation at 250 rpm until the desired growth phase (lag, exponential, or stationary) was obtained. The growth phase of the cultures was estimated using the USDA Pathogen Modeling Program (<http://www.arserrc.gov>) and periodically verified by plate counts on BHI agar (BHIA).

Cold shock. Ten-milliliter samples of a 24-h stationary-phase culture, previously grown at 37°C in BHI, were transferred to 16-by-150-mm sterile glass tubes. One tube was held at 37°C as a control while the remaining tubes were simultaneously submerged into an ice bath to a depth sufficient to insure that all of the culture was below the ice line. Temperature shifts were monitored by a model 115 thermocouple thermometer (Barnant Company, Barrington, Ill.), equipped with a type E thermocouple (Omega, Stamford, Conn.) inserted into an unin-

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TABLE 1. Effect of cold shock duration and magnitude on *D*-value estimates of *L. monocytogenes* Scott A grown at 37°C

Cold shock (°C)	<i>D</i> -value estimates and reductions				
	0-h cold shock, <i>D</i> value	1-h cold shock		3-h cold shock	
		<i>D</i> value	% Reduction	<i>D</i> value	% Reduction
Control	1.26 (0.10) a				
15		0.98 (0.04) b	22	0.71 (0.08) c	44
10		0.93 (0.07) c	26	0.69 (0.01) e	45
5		0.89 (0.02) c	29	0.71 (0.06) c	44
0		0.85 (0.01) d	33	0.79 (0.03) e	37

^a Data are means (SD) of decimal reduction times (in minutes) at 60°C ($n = 3$). Values that are not followed by identical letters are significantly different ($P < 0.05$). The percent reduction is that from the control *D* value.

oculated BHI tube that was placed into the ice bath along with the inoculated samples. When the target temperatures of 15, 10, 5, or 0°C were obtained, the tubes were transferred to water baths inside incubators (Model G27; New Brunswick Scientific, New Brunswick, N.J.) equilibrated to 15, 10, 5, or 0°C, respectively. Samples were stored at each temperature for 1 to 3 h and then were diluted and heat treated as described below. For some experiments, the cold-shocked cells were held at 28°C prior to thermal challenge.

Thermal inactivation. Except for the frankfurter experiments, heating data were obtained using a Colworth House submerged-coil heating apparatus (Pro-trol Limited Surrey, United Kingdom) (7), equilibrated at 60, 65, and 68°C. In all model system experiments, control and cold-shocked cultures were diluted 10-fold (final concentration, approximately 10^8 CFU/ml) in sterile 0.1 M pH 7.0 Butterfield's phosphate buffer immediately prior to thermal inactivation. Heat-treated samples (200 to 1,000 μ l) were collected at timed intervals into 15-by-45-mm 1 dram glass vials (model 60910-L; Kimble Glass Co., Vineland, N.J.), immediately cooled in an ice water bath, diluted using 0.1% peptone (pH 6.85; Difco), and then plated in duplicate using a spiral plater (Model D; Spiral Systems, Cincinnati, Ohio) or directly spread plated onto BHIa plates. Inoculated plates were incubated for 48 h at 37°C, and colonies were counted either manually or electronically (Model 500A; Spiral Systems).

Injury determination. Cells from stationary-phase cultures previously grown at 37°C and cold shocked at 0°C for 3 h were heat treated at 60 or 68°C. The heat-treated samples were spiral or streak plated onto duplicate BHI and BHI plus 5% NaCl (wt/vol) agar plates (42), incubated at 37°C for 48 h, and then enumerated. Injury was defined as the CFU on BHIa minus the CFU on BHI plus 5% NaCl (wt/vol) agar plates. The experiment was performed in duplicate.

Chloramphenicol treatment. Ten milliliters of a 24-h *L. monocytogenes* Scott A culture was centrifuged at $16,070 \times g$ for 10 min. The supernatant fluid was discarded, the pellet was resuspended in Butterfield's phosphate buffer, and the centrifugation was repeated. After discarding the supernatant fluid, cells were resuspended in Butterfield's buffer both with and without 100 μ g of chloramphenicol (Sigma)/ml (final concentration). Exposure was for 30 min at room temperature. Subsequently, cells were either thermally challenged directly or cold shocked for 3 h at 0°C and then were thermally challenged. Experiments were performed in duplicate.

***D*- and *Z*-value estimation and statistical analyses.** For comparison of treatment and control effects on thermal death time, preliminary data analyses revealed that similar results were attained using either time to a 4-log population density reduction, a modified logistic equation, or linear regression (47). Thus, *D* values were estimated by linear regression of the log survivors as a function of time at a specific temperature. The *Z* values were estimated by linear regression of the log of the *D* values at 60, 65, and 68°C against these challenge temperatures. For both estimates, the regression line that best fit the survivor curve was determined and the negative reciprocal of the slope was used to calculate the *D* and *Z* values. Statistical evaluations were performed using SAS, especially the General Linear Model procedure (version 6.08; SAS Institute, Cary, NC).

Cold shock effects in foods. Frankfurters (pork-beef-water and other ingredients, 67:10:23, respectively) were gifts from A. Oser (Hatfield Quality Meats, Hatfield, Pa.). The frankfurters were collected from a continuous processing system immediately after the cooking-smoking cycle and before entrance into the cooling brine shower. Frankfurters were transported in a chilled state to the ERRC within 30 min and used immediately. After aseptic casing removal, 3-cm² square portions of the frankfurter meat outer layer (approximately 1 mm thick; hereafter referred to as "skin") were aseptically separated and removed with the aid of a sterile scalpel. The meat squares were sterilized by immersion in 70% ethanol for 10 min followed by evaporation under a biological safety hood (SteriGARD Hood model 56-600; Baker Co. Inc., Sanford, Maine) at room temperature for 1 h. Fifty microliters of a 24-h *L. monocytogenes* Scott A culture, previously grown at 37°C, was applied directly to the surface of the skin and then spread evenly using a sterile inoculating loop to yield approximately 10^6 CFU/cm². The inoculated skins were dried at room temperature for 1 h in the biological safety hood and then aseptically transferred to stomacher bags (Spiral Biotech, Bethesda, Md.). Bags were folded and placed in oxygen barrier film bags

(model 01 46 09; Koch Supplies Inc., Kansas City, Mo.) which were evacuated at -10^5 Pa (model A 300/16; Multivac, Kansas City, Mo.) and heat sealed. Sealed bags were immersed into a 60°C water bath (model EX 251-HT; Neslab, Newington, N.H.) either immediately or after cold shock by immersion in 0°C water for 3 h. Sampling began when the skins reached 60°C, which generally took about 2 min, and continued for 10 min. Skin temperature was continuously monitored on control samples by insertion of a type E thermocouple (Omega) into the center of the sample. The thermocouple was attached to a data logger (model 4021; Keithley Metrabyte, Taunton, Mass.), and data were collected using data logging software (Labtech Notebook; Labtech Technologies Corp., Wilmington, Mass.). Samples from the 60°C water bath were transferred to ice, diluted 10-fold (wt/vol) in Butterfield's buffer, then blended in a Stomacher Lab-Blender 400 (model BA602i; Seward, Ltd., London, England) for 2 min. After blending, samples were enumerated in the same manner as the model system studies. The experiment was replicated in quadruplicate.

One milliliter of an *L. monocytogenes* Scott A culture previously grown at 37°C for 24 h was added to 9 ml of 2% fat ultra-high temperature (UHT) milk (Parmalat USA, Moonachie, N.J.) at room temperature to yield a final level of approximately 10^8 CFU/ml. The milk was then either heat challenged at 60°C or cold shocked for 3 h at 0°C before heating. Samples were collected and enumerated as above. The experiment was replicated in triplicate.

RESULTS

Cold shock induction and magnitude on thermal sensitivity. Cold shock of *L. monocytogenes* Scott A before heating resulted in reductions of up to 45% in thermal death times (Table 1). Specifically, stationary-phase cells grown at 37°C and heated at 60°C yielded an estimated *D*₆₀ value of 1.26 min. Cold shock at 0, 5, 10, or 15°C for 1 to 3 h resulted in *D*-value reductions from 22 to 33% that varied directly with the magnitude of the temperature downshift. The analysis of variance and Bonferroni *t*-test analyses showed that a 1-h cold shock was significantly ($P < 0.05$) associated with *D*-value reductions compared to that of the control. The analyses also demonstrated that cold shock duration was more important than the magnitude of the temperature downshift. For example, all *D* values were lower for the samples that were cold shocked for 3 h (range, 0.69 to 0.79 min) than any of the samples that were cold shocked for 1 h (range, 0.85 to 0.98 min). The *D* values reached a minimum if the cold shock temperature was maintained for 3 h ($P > 0.05$), regardless of the magnitude of temperature downshift (from 37 to 15°C or below). Additional experimentation (data not presented) showed no further *D*-value reduction after holding samples at $\leq 15^\circ\text{C}$ for 24 h. In related experiments, stationary-phase *L. monocytogenes* Scott A cells, previously grown at 37°C in LB broth, exhibited a *Z* value of 8.84°C ($R^2 > 0.99$, $n = 3$), while cold shocking these cells for 3 h at 0°C reduced the *Z* value by 15% to 7.71°C ($R^2 > 0.99$, $n = 3$). Also, transfer of cold-shocked stationary-phase Scott A cells (*D*₆₀ = 0.72, standard deviation [SD] = 0.05 min, $n = 2$) to 28°C for either 1 or 3 h (*D*₆₀ = 0.96, SD = 0.00 min, $n = 2$; same for both) did not restore the thermal tolerance to control levels (*D*₆₀ = 1.30, SD = 0.04 min, $n = 2$).

TABLE 2. Changes in thermal resistance of *Listeria* strains grown to stationary phase at 37°C as a result of cold shock at 0°C for 3 h

Species	Strain	Origin	Source ^a	<i>D</i> ₆₀ value (min) [mean (SD)]		<i>D</i> -value decrease (%)	No. of trials
				Control	Cold shock		
<i>L. innocua</i>	2340	Beef	ERRC	1.52 (0.01)	1.20 (0.01)	26	2
<i>L. monocytogenes</i>	V7	Dairy	ERRC	1.31 (0.03)	0.93 (0.02)	30	2
<i>L. monocytogenes</i>	Scott A	Clinical	ERRC	1.26 (0.10)	0.79 (0.03)	37	27
<i>L. monocytogenes</i>	S9V5	Sausage	ERRC	1.01 (0.05)	0.76 (0.05)	25	2
<i>L. monocytogenes</i>	H2NG	Beef	ERRC	0.91 (0.03)	0.73 (0.04)	20	2
<i>L. monocytogenes</i>	65102	Poultry	FSIS	1.37 (0.03)	0.97 (0.00)	29	2
<i>L. monocytogenes</i>	20306	Pheasant/pork paté	FSIS	1.27 (0.03)	0.88 (0.01)	30	2
<i>L. monocytogenes</i>	20389	Sliced ham	FSIS	1.19 (0.01)	0.91 (0.00)	24	2
<i>L. monocytogenes</i>	418	Cooked poultry	FSIS	1.20 (0.12)	1.05 (0.00)	13	2
<i>L. monocytogenes</i>	20169	Beef/pork frankfurter	FSIS	1.29 (0.02)	0.91 (0.01)	30	2

^a Sources of strains were the Microbial Food Safety Research Unit, ERRC, USDA, and the Microbiology Division, Office of Public Health & Science, FSIS, USDA.

Response to cold shock among *Listeria* isolates. The nine *L. monocytogenes* isolates that were grown to stationary phase at 37°C and then heat challenged at 60°C yielded results that are shown in Table 2. Strains V7, Scott A, S9V5, and H2NG from the ERRC yielded a mean *D* value of 1.12 min that was not statistically different ($P > 0.05$) than the mean *D* value of 1.26 min obtained for the five *L. monocytogenes* strains from the FSIS. The single *L. innocua* strain evaluated exhibited a 27% higher *D* value than the mean *D* value of all the *L. monocytogenes* strains tested. After cold shock at 0°C for 3 h, *D* values of the *L. monocytogenes* strains decreased from a mean of 1.2 min (nonshocked) to a mean of 0.88 min, a 26.4% mean reduction in thermal tolerance. The data also revealed 28.0 and 25.2% *D*-value reductions for the strains that had been stored long-term at -70°C and the FSIS strains, respectively, from those of cells that had not been cold shocked. Thermal tolerance of cold-shocked *L. innocua* cells similarly decreased 26% compared to nonshocked controls.

Cell culture phase and growth temperature effects on cold shock-induced thermal sensitivity. Analysis of variance indicated highly significant ($P < 0.01$) effects on *D* values of *L. monocytogenes* Scott A due to growth phase and/or preheating treatment (control versus cold shock). The Bonferroni mean separation analysis (35) indicated that nonshocked stationary-phase cells (control) were the most thermally resistant, exponential-phase cells were the most thermally sensitive, and lag-phase cells were intermediate between the two. Table 3 shows that cells grown at 37°C to stationary phase ($D = 1.30$ min) were 33% more thermally resistant than counterpart exponential cells ($D = 0.98$ min) and 23% more resistant than lag-phase cells ($D = 1.05$ min). It is interesting to note that while there was no statistically significant effect of growth tempera-

ture on the *D* value of control cells within any growth phase, lower *D* values were generally observed when lag-, exponential-, or stationary-phase cells were grown at 5 or 19°C compared to those grown at 26 or 37°C. Also, the range of *D*₆₀ values determined for cells grown at different temperatures showed the least variation for stationary-phase cells. For example, thermal resistance of control (non-cold-shocked) stationary-phase cells varied only 5% (range, 1.27 to 1.30 min) over the experimental-growth temperature range. In contrast, thermal resistance of non-cold-shocked exponential-phase cells varied by about 22% (0.80 to 0.98 min) over the experimental growth temperature range.

Two effects were noted when cells were cold shocked at 0°C for 3 h. First, there was a highly significant ($P < 0.01$) *D*-value reduction across all growth phases and growth temperatures from those of nonshocked controls. For example, the *D*₆₀s of cold-shocked stationary-, exponential-, and lag-phase cells, across all growth temperatures, were reduced by means of 38, 15, and 26%, respectively, compared to those of the controls. There was also a significant association ($P < 0.05$) between colder growth temperature and smaller cold shock-induced *D*-value reductions for exponential-phase but not stationary- or lag-phase cells. Second, among cells grown at different temperatures to different growth phases, cold-shocked cells exhibited a smaller range of *D* values (0.69 to 0.90 min) compared to the range of *D* values determined for non-cold-shocked cells (0.80 to 1.34 min).

Role of injury. Injury was examined using stationary-phase cells of *L. monocytogenes* Scott A previously grown at 37°C and cold shocked at 0°C for 3 h prior to heat challenge at 60 or 68°C and enumeration on selective and nonselective agar plates incubated aerobically. As shown in Table 4, thermal

TABLE 3. Combined effect of growth temperature and growth phase on induced thermal sensitivity of *L. monocytogenes* Scott A before and after cold shock for 3 h at 0°C^a

Growth temp (°C)	<i>D</i> value [mean (SD)] of cells in:								
	Lag phase			Exponential phase			Stationary phase		
	Control	Cold shocked	% Decrease	Control	Cold shocked	% Decrease	Control	Cold shocked	% Decrease
37	1.05 (0.15)	0.81 (0.11)	23	0.98 (0.19)	0.78 (0.05)	20	1.30 (0.02)	0.84 (0.00)	35
26	1.16 (0.06)	0.78 (0.02)	33	0.92 (0.06)	0.77 (0.04)	16	1.34 (0.01)	0.90 (0.09)	33
19	0.99 (0.09)	0.71 (0.06)	28	0.80 (0.06)	0.69 (0.04)	14	1.29 (0.00)	0.77 (0.06)	40
5	0.98 (0.05)	0.79 (0.02)	19	0.80 (0.04)	0.73 (0.01)	9	1.27 (0.00)	0.72 (0.03)	43
Mean ^c	1.04 (0.11) ^b	0.77 (0.06) cd		0.88 (0.12) c	0.74 (0.05) d		1.30 (0.03) a	0.81 (0.08) cd	

^a The Bonferroni least significant difference is 0.13.

^b Values for control and cold-shocked cells are means (SD) of duplicate trials of *D* values (in minutes) at 60°C.

^c Values that are not followed by identical letters are significantly different ($P < 0.05$).

TABLE 4. Use of selective and nonselective plating media to estimate injured cells of *L. monocytogenes* Scott A after growth to stationary phase at 37°C, cold shock at 0°C for 3 h, and heat challenge

Heating temp (°C)	Treatment	D value ^a	
		BHI	BHI + 5% NaCl
60	Control	1.16 (0.02)	0.79 (0.07)
60	Cold shock	0.80 (0.06)	0.78 (0.08)
68	Control	0.13 (0.02)	0.12 (0.02)
68	Cold shock	0.11 (0.02)	0.11 (0.02)

^a Values are means (SD) of two replicate trials.

death times for *L. monocytogenes* were predictably shorter at 68°C (ca. 0.1 min) than at 60°C (ca. 1.0 min). There was evidence that sublethal injury occurred in control (non-cold-shocked) cells at 60°C, since estimated *D* values were 1.16 and 0.79 min for nonselective and selective media, respectively. In contrast, there was no evidence of sublethal injury in cold-shocked cells heated at this temperature or in control or cold-shocked cells subsequently heated to 68°C. The increased lethality of the 68°C heat treatment may overwhelm the ability to distinguish sublethally injured cells by using selective and non-selective media plating, thus explaining the lack of detecting sublethal injury in control cells heated at 68°C.

Chloramphenicol effects on thermal resistance. Chloramphenicol at 100 µg/ml reduced the *D* value of *L. monocytogenes* Scott A by 27% to 0.93 (SD = 0.02 min) from a control *D* value of 1.29 (SD = 0.00 min). This reduction was not significantly different ($P > 0.05$) from the cold shock ($D = 0.94$, SD = 0.00 min) nor the cold shock plus chloramphenicol treatment ($D = 0.91$, SD = 0.03 min) (Table 5).

Cold shock effects on *L. monocytogenes* D values on frankfurter skins and in UHT milk. Table 6 shows that the thermal resistance of *L. monocytogenes* Scott A was 25% lower on vacuum-packaged frankfurter skins that were cold shocked at 0°C and then heated at 60°C ($D = 1.67$ min) compared to non-shocked controls ($D = 2.22$ min). Similarly, Table 6 shows that the thermal death time of *L. monocytogenes* Scott A was 15% lower in UHT 2% milk that was cold shocked at 0°C ($D = 1.03$ min) compared to non-cold-shocked controls ($D = 1.26$ min).

In general, *D* values for control and cold-shocked cells were higher for cells inactivated in foods than for cells inactivated in the broth system. Cold shock lowered the *D* values for cells inactivated in foods; however, the *D* values were still greater than the *D* values obtained with cold-shocked cells in a broth system. Data obtained using one system should be used with great caution when applied to a dissimilar system.

TABLE 5. The effect of chloramphenicol treatment and/or cold shock at 0°C for 3 h on the mean *D*₆₀ values of *L. monocytogenes* Scott A previously grown to stationary phase at 37°C

Treatment ^a	D value ^b
Control.....	1.29 (0.00) a
3-h cold shock at 0°C.....	0.94 (0.00) b
3-h chloramphenicol treatment.....	0.93 (0.02) b
3-h chloramphenicol treatment and cold shock at 0°C.....	0.91 (0.03) b

^a Final concentration of chloramphenicol, 100 µg/ml.

^b Values are means (SD) of two replicate trials. Values that are not followed by identical letters are significantly different ($P < 0.05$).

TABLE 6. Effect of cold shock at 0°C for 3 h on mean *D*₆₀ values of *L. monocytogenes* Scott A previously grown to stationary phase at 37°C and inoculated onto frankfurter skin or into UHT 2% milk

Treatment	D value ^a	D-value decrease (%)
Control cells inoculated onto frankfurter meat	2.22 (0.14)	
Cells inoculated onto frankfurter meat and cold shocked for 3 h at 0°C	1.67 (0.13)	25
Control cells inoculated into UHT milk	1.26 (0.00)	
Cells inoculated into UHT milk and cold shocked for 3 h at 0°C	1.03 (0.02)	15

^a Values are means (SD) of two replicate trials.

DISCUSSION

The aim of this study was to determine if increased thermal sensitivity could be induced by cold shocking *L. monocytogenes* cells prior to heat challenge. Findings supported this hypothesis, particularly for stationary-phase cells. Compared to a 1-h cold shock at 0°C, a 3-h cold shock was more effective in reducing thermal resistance, 22 and 44% reductions, respectively (Table 1). Additionally, thermal death time determinations had a lower SD after cold shock.

The *D*₆₀ and *Z* values reported herein were generally consistent with those values reported elsewhere for *L. monocytogenes*. For example, *D*₆₀ values were reported as 1.46 to 16.7 min and *Z* values as 4.6 to 8.4 for meat, chicken, and fish (12). Similarly, *Z* values were reported as 4.3 to 9.9 in various foods (33). In the present study, the heating coil apparatus yielded results that were in the general range of those reported in the above-mentioned studies and that were very reproducible from run to run.

Unlike the heat shock response, which is short lived in *L. monocytogenes* (6), the current study revealed that cold shock-induced thermal sensitivity was sustained, even after returning cells to 28°C for up to 3 h prior to thermal challenge. This suggests that the cellular changes or damage that invoked the thermal sensitivity persisted as well. While maintenance of thermal sensitivity at cold temperatures is expected because of a temperature-dependent lowering of reaction rates, carryover at the warmer temperature was unanticipated and remains unexplained. Further experimentation is warranted to elucidate the persistence of the increased thermal sensitivity induced by cold shock. The 27% higher *D* value of the *L. innocua* strain compared to the mean *D* value of the *L. monocytogenes* strains tested supports a previous report (14) of greater thermal resistance in *L. innocua* compared to that in *L. monocytogenes*.

Cold shock induction of thermal sensitivity in nine *L. monocytogenes* strains isolated from a variety of sources and an *L. innocua* strain provides good evidence for the broader application of this process to foods in which *Listeria* is a problem. Further research is warranted to reveal the scope and limits of cold shock induction of thermal sensitivity, including whether similar phenotypes can be induced in other food-borne pathogens. There is some evidence that cold-induced thermal sensitivity is more pronounced for gram-positive bacteria and yeasts than for gram-negative bacteria (26). The results with *Listeria* also support observations regarding thermal tolerance changes in *S. enterica* serovar Enteritidis phage type 4 (20) as a result of prechallenge incubation temperature. The present study also supports the generally accepted notion that stationary-phase cells are more resistant to stress than lag- or exponential-phase cells (Table 3). The *D* values were growth tem-

perature independent for all phases of growth tested. These findings, in part, agree with those of Pagán et al. (37), who also observed that stationary-phase cells possessed maximum thermal tolerance. In contrast, they concluded that heat resistance depended on growth temperature. The observation that lag-phase cells had a thermal resistance intermediate between the thermal resistance of stationary- and exponential-phase cells may be indicative of lag-phase cells containing a mixture of stationary- and exponential-phase cells (34).

Cold shock reverted the stationary-phase-induced thermal tolerance to the more sensitive exponential-phase (non-cold shock) levels. It is unclear, however, if a stationary-phase-specific mechanism is suppressed or if a more general cellular response is activated. The lowered and less variable *D* values of cold-shocked cells from all growth phases and growth temperatures suggests the practical benefits of this potential application. The observation that injury occurred in control cells heat treated at 60°C, but not in cells that were either cold shocked and heat treated at 60°C or treated at 68°C, suggests that cold shock increased killing. Furthermore, the observation that both chloramphenicol and cold shock lowered *D* values compared to those of control cells (Table 5) suggests that cold shock suppression of protein synthesis might be responsible for the thermal tolerance loss.

Cold shock to *L. monocytogenes* cells is associated with an initial cessation of growth, resumption of growth after an adaptive period, and changes in protein synthesis (3). Total protein synthesis is dramatically decreased and a set of stress proteins is induced after cold shock; in *E. coli*, this is due to cellular changes that affect the translation machinery (22). These observations have had investigators suggest that ribosomes may be acting as a temperature sensor (45). More important, in addition to a decrease in total protein synthesis there is a concomitant suppression of heat shock proteins and induction of cold shock proteins that occurs after cold shock (17). This led to the conclusion that heat and cold shock responses are antagonistic in *Bacillus subtilis* (18). The hypothesis is supported by the observation that the expected *D*-value increase after heat shock was eliminated in *E. coli* O157:H7 if chilling was performed immediately post-heat shock (48). Considering that ribosome function is relatively consistent among Eubacteria, it is reasonable to suggest that similar functionality can be assigned to the ribosomes in *L. monocytogenes*. There is evidence that the ribosome is the molecular sensor for thermal tolerance in *L. monocytogenes*. Protection of the 30S ribosomal subunit was proposed as a critical mechanism for thermal tolerance (43). This hypothesis was recently tested by using differential scanning calorimetry (DSC) to show that cold shock induced changes in the ribosomes that changed their thermal tolerance (4). Additional experiments showed that antibiotics that caused prominent shifts in DSC thermogram peaks, corresponding to ribosomes and their subunits, also resulted in alterations of thermal tolerance (4).

The demonstration that *D*-value reductions occurred in milk and on frankfurter skins in this study shows that cold shock sensitization may be used to reduce the prevalence of *L. monocytogenes* in ready-to-eat foods. One potential application of this technique may be as a post-processing pasteurization step for ready-to-eat meats, such as frankfurters. The concept of post-process pasteurization for frankfurters has been demonstrated with other technologies, such as surface steam pasteurization (8); however, additional methods will expand the potential application of these technologies. Moreover, cold shock may be useful to reduce pathogens in fruit and vegetable juices, which are collected at room temperature. If quickly chilled, then moderately heated, there may be improved safety without

sensory or nutrient losses. In addition, this observation has important implications for predictive microbiology, especially on development of heat processing schemes, since this research shows that the temperature history of cells can affect thermal death time requirements.

The so-called "hurdle" or "barrier concept" (28) was proposed in the 1970's. The use of combination treatments has been viewed as advantageous from a variety of food quality and food utilization standpoints (16). Initially, there was little understanding that one sublethal barrier, if applied first, could generate tolerance toward a second, unrelated control measure. More recently, such cross-protective effects have been demonstrated for *L. monocytogenes*. For example, heat shock increased the resistance of *L. monocytogenes* to the subsequent stresses of ethanol and NaCl (32). In another study, surface-adherent growth of *L. monocytogenes* increased resistance to various sanitizers and to heat (15). Finally, high temperature tolerance increased after *L. monocytogenes* was acid shocked (13). While the goal of the hurdle concept is to inhibit pathogen growth through the use of a combination of inhibitory factors, only a few studies have demonstrated this result. In one study, *L. monocytogenes* cells grown in NaCl-containing media resulted in either decreased thermal tolerance or no effect, depending upon the strain (9). Another study showed that osmotic "down shock" caused rapid loss of thermal tolerance (24). In another study, acid adaptation sensitized *S. enterica* serovar Typhimurium to hypochlorous acid oxidation (29). Therefore, failure to fully consider the stresses that a food-borne pathogen may encounter either prior to contaminating food or during food processing may result in suboptimal pathogen elimination from the food.

A model for cross-sensitization can be proposed from concepts derived from various sources (17, 18, 41, 48). Initiation may occur through application of a sublethal stress, which results in a bacterium misinterpreting the signal from its environment. This stimulates genes that permit survival in the continued presence of the primary stress. The application of a secondary stress, such as the cold/heat shock regime described herein, may result in enhanced lethality of the overall process.

In conclusion, the present study demonstrates the efficacy of cold shock prior to heating as a pathogen intervention strategy for *L. monocytogenes*. Additional research needs to be conducted for its further development. Exploitation of cold shock induction of increased thermal sensitivity could help reduce the economic and public health impact of *L. monocytogenes* contamination on foods.

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