Changes in Barotolerance, Thermotolerance, and Cellular Morphology throughout the Life Cycle of *Listeria monocytogenes*[▽]

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Changes in barotolerance, thermotolerance, and cellular morphology throughout the life cycle of *Listeria monocytogenes* were investigated. For part 1 of this analysis, *L. monocytogenes* ATCC 19115 was grown to log, stationary, death, and long-term-survival phases at 35°C in tryptic soy broth with yeast extract (TSBYE). Cells were diluted in whole milk that had been subjected to ultrahigh temperatures (UHT whole milk) and then high-pressure processed (HPP) at 400 MPa for 180 s or thermally processed at 62.8°C for 30 s. As cells transitioned from the log to the long-term-survival phase, the $D_{400 \text{ MPa}}$ and $D_{62.8^\circ\text{C}}$ values increased 10- and 19-fold, respectively. Cells decreased in size as they transitioned from the log to the long-term-survival phase. Rod-shaped cells transitioned to cocci as they entered the late-death and long-term-survival phases. *L. monocytogenes* strains F5069 and Scott A showed similar results. For part 2 of the analysis, cells in long-term-survival phase were centrifuged, suspended in fresh TSBYE, and incubated at 35°C. As cells transitioned from the long-term-survival phase to log and the stationary phase, they increased in size and log reductions increased following HPP or heat treatment. In part 3 of this analysis, cells in long-term-survival phase were centrifuged, suspended in UHT whole milk, and incubated at 4°C. After HPP or heat treatment, similar results were observed as for part 2. We hypothesize that cells of *L. monocytogenes* enter a dormant, long-term-survival phase and become more barotolerant and thermotolerant due to cytoplasmic condensation when they transition from rods to cocci. Further research is needed to test this hypothesis and to determine the practical significance of these findings.

Listeria monocytogenes is a gram-positive, non-spore-forming, psychrotrophic, facultatively anaerobic, and pathogenic bacterium (12). This microorganism is unique among food-borne pathogens because it can exist both as a saprophyte in natural environments and as a pathogen in animals and humans (15). *L. monocytogenes* is often present in food-processing plants and sometimes contaminates raw or processed foods, such as raw milk and ready-to-eat (RTE) foods (42). Most recently, *L. monocytogenes* was shown to cause a large outbreak due to the consumption of RTE meats in Canada (http://www.phac-aspc.gc.ca/alert-alerte/listeria/listeria_2008-eng.php). The cause of postpasteurization contamination by *L. monocytogenes* may be due to survival of this pathogen inside hard-to-clean equipment in packaging rooms (46). Certain serotypes (4b, 1/2a, and 1/2 b) are known to cause listeriosis in immunocompromised individuals, as well as in infants and the elderly (48), and are responsible for nearly 35% of deaths in the United States due to known food-borne bacterial pathogens (34). Large outbreaks of listeriosis in the U.S. have been reported due to the consumption of RTE dairy and meat products (12). Thus, a zero tolerance policy was established for this pathogen in RTE foods by U.S. Food and Drug Administration and U.S. Department of Agriculture (14).

High-pressure processing (HPP) is a nonthermal process of increasing interest because it causes microbial inactivation at moderate temperatures. Solid or liquid foods are normally subjected to 50 to 1,000 MPa for seconds to minutes (50).

HPP reduces the number of vegetative microbial cells in foods (5, 28, 43), while food attributes, such as color, texture, and vitamins, remain largely unaffected. As a result, high-pressure-processed foods such as guacamole, fruit juices, RTE meats, and oysters have become popular in the marketplace (7).

The effect of growth phase on barotolerance, thermotolerance, and cellular morphology of bacteria has been widely studied. Cells of *L. monocytogenes* in the stationary phase are more barotolerant than log-phase cells (20). The thermotolerance of stationary-phase cells of *Escherichia coli* and *L. monocytogenes* is also higher than that of log-phase cells of these pathogens (25, 32). Rod-shaped cells of *E. coli* and *Arthrobacter* are known to change to cocci when cultures transition from the log phase to the stationary phase (11, 17, 29).

Most microbiology textbooks discuss only four phases of the bacterial growth curve (lag, log, stationary, and death); however, some reports (9, 44) have discussed a fifth phase, in which bacteria exhibit long-term survival. This fifth phase has been termed the “long-term stationary phase” in *E. coli* (9) or the “senescent phase” in *Serratia* and *Sarcinia* spp. (44). Bacteria in the environment are known to enter a starved state and survive for long periods until starvation is relieved (30). However, research describing this long-term-survival phase in *L. monocytogenes* is nonexistent.

Therefore, the purpose of the present study was to investigate changes in barotolerance, thermotolerance, and cellular morphology throughout the life cycle of *L. monocytogenes*. To simulate real-world food processing conditions, the effect of storage time in milk at 4°C on barotolerance and thermotol-

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erance of cells in the long-term-survival phase was also investigated.

MATERIALS AND METHODS

Preparation of microorganisms. *L. monocytogenes* ATCC 19115, which was isolated from human cerebrospinal fluid (4), was obtained from the American Type Culture Collection (ATCC; Manassas, VA). *L. monocytogenes* F5069, which was isolated from raw milk (10), was obtained from Lewis Graves (Food-borne and Diarrheal Disease Branch, Centers for Disease Control and Prevention). *L. monocytogenes* Scott A, which was isolated from a patient in the 1983 Massachusetts milk outbreak (10), was obtained from the Food Science Department at Pennsylvania State University. Glycerol stocks of these strains were maintained at -80°C and streaked on tryptic soy agar with 0.6% yeast extract (TSAYE; Becton Dickinson, Sparks, MD) with incubation at 35°C for 48 h. For each strain, one colony on the plate was inoculated into 10 ml of tryptic soy broth with yeast extract (TSBYE), with incubation at 35°C for 24 h. A 0.1-ml aliquot of the resulting culture was then diluted with 9.9 ml of 0.1% peptone water (Becton Dickinson) to achieve $\sim 10^7$ CFU/ml, and 0.1 ml of the diluted culture was inoculated into 100 ml of TSBYE.

Part 1: transition from log to long-term-survival phase. *L. monocytogenes* ATCC 19115 was inoculated into TSBYE and incubated at 35°C for 12 h, 16 h, 22 h, 28 h, 41 h, and 2 to 30 days to obtain cells in late-log, stationary, early-death, mid-death, late-death, and long-term-survival phases, respectively. *L. monocytogenes* strains F5069 and Scott A were incubated in TSBYE at 35°C for up to 21 days (these two strains were only used in high pressure, thermal processing, and Gram stain experiments). Cells were then pressure or heat treated and observed following the procedures described below.

(i) High-pressure processing. To measure survival after HPP at 400 MPa for 180 s, bottles containing 99 ml of sterile whole milk that had been subjected to ultrahigh temperatures (UHT whole milk) were inoculated with 1 ml of cell cultures in different phases and shaken. To determine the $D_{400\text{ MPa}}$, cell cultures at different phases were diluted in sterile UHT whole milk to achieve similar starting concentrations of 10^6 to 10^7 CFU/ml. Samples were prepared and pressure treated in a 2-liter HPP unit with a volume capacity of 2 liters (Avure Technologies, Kent, WA) according to the procedure described by Hayman et al. (20). Samples in sealed plastic vials were high pressure processed at 400 MPa for various times at room temperature ($\sim 22^{\circ}\text{C}$). The come-up time to reach 400 MPa was ~ 90 s, and the come-down time was ~ 9 s. During processing the temperature inside the vessel increased from $\sim 22^{\circ}\text{C}$ to $\sim 35^{\circ}\text{C}$. After HPP, the sealed plastic vials containing the samples were put in ice water until plating (~ 20 min). The experiment was replicated three times.

(ii) Thermal processing. To measure survival after thermal processing at 62.8°C for 30 s, bottles containing 99 ml of sterile UHT whole milk were inoculated with 1 ml of cell cultures in different phases and shaken. To determine the $D_{62.8^{\circ}\text{C}}$, cell cultures at different phases were diluted in UHT whole milk to achieve a similar starting concentration of 10^4 to 10^5 CFU/ml. Samples were transferred to thermal death time (TDT) tubes, which were sealed by using a type 3 blowpipe, and then heat treated as described by Knabel et al. (27). After the samples were heated at 62.8°C for various times, the TDT tubes were removed from the water bath and placed in ice water for 10 min before plating. The come-up time to reach 62.8°C from room temperature ($\sim 22^{\circ}\text{C}$) was 87 s, and the come-down time (from 62.8 to 4°C) was 130 s. The temperature come-up and come-down times were measured by using a flanged TDT tube equipped with a type T copper-constantan thermocouple (O. F. Ecklund, Inc., Cape Coral, FL) connected to an HH2002AL read-out device (Omega Engineering, Inc., Stamford, CT). The experiment was replicated three times.

(iii) Enumeration after HPP or thermal processing. Pressure- or heat-treated samples were plated on TSAYE with subsequent incubation at 30°C for 48 h before colony enumeration. When necessary, samples were diluted in 0.1% peptone water before plating. The limit of detection was 10 CFU/ml.

(iv) Light microscopy (LM) of Gram stains. Gram stains of cell cultures were examined with a $\times 100$ oil immersion objective lens using a BX40F4 light microscope equipped with a DP71 digital camera (Olympus Optical, Tokyo, Japan).

(v) Scanning electron microscopy (SEM). Cells of *L. monocytogenes* ATCC 19115 in different growth phases were collected on a 0.22- μm -pore-size filter and then fixed with the primary fixative (1.5% glutaraldehyde–2.5% paraformaldehyde in 0.1 M cacodylate buffer [pH 7.4]) at 4°C overnight and refixed with the secondary fixative (1% osmium tetroxide in 0.1 M cacodylate buffer [pH 7.4]) at room temperature ($\sim 22^{\circ}\text{C}$) for 1 h. Fixed cells were dehydrated by washing them with 50, 70, 85, 95, and 100% ethanol sequentially; dried using a CPD 030 critical point dryer (Bal-Tec, Brookline, NH) using liquid CO_2 as the transitional fluid; sputter coated with Au and Pt; and then examined by using a JSM 5400 scanning

electron microscope (JEOL, Tokyo, Japan) at an instrument magnification of $\times 10,000$ to $\times 20,000$.

(vi) Transmission electron microscopy (TEM). Volumes of 1.5 ml of cell cultures of *L. monocytogenes* ATCC 19115 in different growth phases were centrifuged at $13,000 \times g$ for 5 min at room temperature ($\sim 22^{\circ}\text{C}$). Pellets were fixed with the same primary fixative as used in the SEM preparation at 4°C for 3 days, and refixed with the same secondary fixative as used in the SEM preparation at room temperature ($\sim 22^{\circ}\text{C}$) for 1 h. Fixed cells were dehydrated by washing with 50, 70, 90, and 100% ethanol and 100% acetone sequentially. Dehydrated cells were infiltrated at room temperature ($\sim 22^{\circ}\text{C}$) using eponate resin and embedded at 70°C overnight for polymerization. Polymerized resin blocks were sectioned into thin slices, which were examined by using a JEM 1200EX II transmission electron microscope (JEOL) at an instrument magnification of $\times 6,000$ to $\times 25,000$.

Part 2: transition from the long-term-survival phase back to the log and stationary phases in TSBYE at 35°C . *L. monocytogenes* ATCC 19115 was incubated in TSBYE at 35°C for 30 days (long-term-survival), and then 10 ml of the culture was centrifuged at $13,000 \times g$ for 15 min at 20°C using an Avanti-J-26 XPI centrifuge (Beckman Coulter). Pellets were suspended in 10 ml of fresh TSBYE to achieve a starting concentration of $\sim 10^8$ CFU/ml and then incubated at 35°C for 0.3 to 1 h, 2 to 4 h, or 5.5 to 9 h to obtain cells in lag, log or stationary phase, respectively. Cells were then pressure or heat treated and observed using SEM according to the procedures described for part 1 above and observed using slide cultures, as described below.

(i) Slide cultures. A 0.1-ml aliquot of a 30-day-old cell culture of *L. monocytogenes* ATCC 19115 in the long-term-survival phase was mixed with 10 ml of molten TSAYE at $\sim 39^{\circ}\text{C}$. One drop of this mixture was then added onto a sterile microscope slide and covered with a sterile coverslip. After the slide culture solidified, it was examined at room temperature ($\sim 22^{\circ}\text{C}$) every 1 to 1.5 h with a $100\times$ oil immersion objective lens using a BX40F4 phase-contrast microscope (Olympus Optical). The light source was shut off between observations to avoid excessive heat that might kill cells on the slide. The experiment was replicated twice.

Part 3: transition from the long-term-survival phase back to the log and stationary phases in milk at 4°C . Cultures (10 ml) of *L. monocytogenes* ATCC 19115 in the long-term-survival phase were harvested and centrifuged as described for part 2. Pellets were suspended in 10 ml of sterile UHT whole milk and then incubated at 4°C for 2 to 4 days or 6 to 29 days to obtain cells in log or stationary phase. Cells were then pressure or heat treated as described for part 1.

Statistical analysis. Enumeration and cellular morphology data were analyzed by using analysis of variance (ANOVA; general linear model) using Minitab software (version 14.20; Minitab, State College, PA). Pairwise comparisons were made by using Tukey's least significant difference test ($\alpha = 0.05$). To calculate D values, linear regressions of enumeration data from high-pressure and thermal inactivation experiments were performed by using Microsoft Excel (version 2003; Microsoft, Redmond, WA). The coefficient of determination (R^2) of the linear regression of log reductions versus cell length was calculated to evaluate the correlation between cell length and barotolerance or thermotolerance.

RESULTS

Part 1: transition from log to long-term-survival phase. Growth curves at 35°C demonstrated that once cells of *L. monocytogenes* ATCC 19115 were inoculated into fresh TSBYE, there was a log phase (Fig. 1A) lasting for ~ 14 h until the population reached $\sim 3 \times 10^9$ CFU/ml (stationary phase; Fig. 1B). After an ~ 2 -h stationary phase (data not shown) the cells rapidly entered the death phase (Fig. 1C), where ca. 90% of the cells died within 24 h. However, after this rapid death the viable cell concentration remained at $\sim 10^8$ CFU/ml for at least 1 month without any addition of nutrients, indicating that the cells had entered a long-term-survival phase (Fig. 1D). Pairwise comparisons revealed that there was no significant difference ($P > 0.05$) in CFU/ml within the long-term-survival phase at 70, 214, 502, and 718 h (Fig. 1D).

ANOVA revealed that log reductions after high pressure or thermal processing were significantly ($P < 0.001$) affected by growth phase (Fig. 2). Log reductions after pressure treatment

FIG. 1. Growth of *L. monocytogenes* ATCC 19115 in TSBYE at 35°C for different times to yield log-phase (A), stationary-phase (B), death-phase (C), or long-term-survival-phase (D) cells. The data points and error bars represent means and standard deviations based on three replications of the experiment.

at 400 MPa for 180 s decreased significantly ($P < 0.001$) from 5.1 in the late-log and stationary phases to 2.1 in the death phase to 0.3 in the long-term-survival phase. Log reductions after pressure treatment of late-death-phase cells at 41 h and long-term-survival-phase cells at 70, 214, 502, and 718 h were significantly ($P < 0.05$) lower than at previous sampling times. Log reductions after heat treatment at 62.8°C for 30 s decreased significantly ($P < 0.001$) from 5.7 in the stationary phase to 3.8 in the death phase to 1.6 in long-term-survival phase. Log reductions after heat treatment of long-term-survival-phase cells at 70, 214, 502, and 718 h were significantly ($P < 0.05$) lower than at previous sampling times. Similar patterns were observed using *L. monocytogenes* strain F5069 and strain Scott A (data not shown).

Both barotolerance and thermotolerance significantly ($P < 0.001$) increased as cells of *L. monocytogenes* ATCC 19115 progressed from the late-log phase to the long-term-survival phase (Fig. 3). Inactivation of cells in the late-log and late-death phases by HPP followed log-linear kinetics, and thus

FIG. 2. Growth of *L. monocytogenes* at different phases of the life cycle and subsequent survival after HPP and heat treatments. Cells of *L. monocytogenes* ATCC 19115 were grown in TSBYE at 35°C for different times to yield log-phase (A), stationary-phase (B), death-phase (C), or long-term-survival-phase (D) cells. Milk inoculated with *L. monocytogenes* was pressure-treated at 400 MPa for 180 s or heat treated at 62.8°C for 30 s. The data points and error bars represent means and standard deviations based on three replications of the experiment.

FIG. 3. Barotolerance (A) and thermotolerance (B) of *L. monocytogenes* at different growth phases. Cells of *L. monocytogenes* ATCC 19115 were grown in TSBYE at 35°C for 12 h, 16 h, 22 h, 41 h, or 30 days to yield late-log-phase, stationary-phase, early-death-phase, late-death-phase, or long-term-survival-phase cells. Milk inoculated with *L. monocytogenes* was pressure treated at 400 MPa for different times or heat treated at 62.8°C for different times. The data points and error bars represent means and standard deviations based on three replications of the experiment.

linear regression was used to calculate $D_{400 \text{ MPa}}$; however, inactivation of cells in the stationary, early-death, or long-term-survival phase revealed nonlinear inactivation kinetics (Fig. 3A). The inactivation curve of stationary-phase cells exhibited a short shoulder and a small tail (Fig. 3A). The inactivation curve of early-death-phase cells also exhibited a tail, and that of long-term-survival-phase cells showed both a shoulder and a tail (Fig. 3A). Therefore, to calculate $D_{400 \text{ MPa}}$, linear regression was only applied to the linear portion (the part without a shoulder or a tail) of the inactivation curves for the stationary, early-death, and long-term-survival phases. ANOVA revealed that $D_{400 \text{ MPa}}$ was significantly ($P < 0.001$) affected by growth phase. Pairwise comparisons showed that the $D_{400 \text{ MPa}}$ values of cells were as follows: late-log phase (23.2 s) < early-death phase (64.5 s) < late-death phase (242.0 s) = long-term-survival phase (230.1 s) ($P < 0.05$). The $D_{400 \text{ MPa}}$ value of cells in stationary phase (30.2 s) was not significantly ($P > 0.05$) different from the late-log or early-death phase. In the thermotolerance study (Fig. 3B), all curves exhibited log-linear kinetics; thus, linear regression of all data points was used to calculate D values at 62.8°C. ANOVA revealed that $D_{62.8^\circ\text{C}}$ was significantly ($P < 0.001$) affected by the growth phase. Pairwise comparisons of the $D_{62.8^\circ\text{C}}$ values of cells were as follows: late-log phase (3.3 s) < late-death phase cells (19.6 s) < long-term-survival cells (61.4 s) ($P < 0.05$). The $D_{62.8^\circ\text{C}}$

FIG. 4. LM, SEM, and TEM images of *L. monocytogenes* at different growth phases. Cells of *L. monocytogenes* ATCC 19115 were grown in TSBYE at 35°C for various times to yield cells at different phases and then observed using LM (Gram stain), SEM, and TEM. Bars: 10 μm (LM), 1 μm (SEM), and 0.5 μm (TEM).

values of cells in the stationary (9.4 s) and early-death (10.8 s) phases were not significantly ($P > 0.05$) different from the late-log or late-death phase.

Representative photomicrographs of cells of *L. monocytogenes* ATCC 19115 in different phases are shown in Fig. 4. Gram stains and SEM photomicrographs showed that cells decreased in size as they transitioned from the log phase to the long-term-survival phase (Fig. 4, LM and SEM). SEM results

showed that all cells in the late-log, stationary, and early-death phases were rod-shaped, and cocci started to appear in the late-death phase. Pairwise comparisons revealed that the percentage of cocci measured by Gram stain and SEM significantly ($P < 0.05$) increased from 41 h to 30 days. Similar results were observed using *L. monocytogenes* strains F5069 and Scott A (data not shown).

ANOVA of the SEM data revealed that cell length was also

FIG. 5. Growth of long-term-survival-phase cells of *L. monocytogenes* after inoculation into fresh TSBYE at 35°C and subsequent survival after HPP and heat treatments. Cells of *L. monocytogenes* ATCC 19115 were grown in TSBYE at 35°C for 30 days to yield long-term-survival-phase cells, and 10-ml portions of cultures were centrifuged. Pellets were suspended in 10 ml of fresh TSBYE and then incubated at 35°C for various times before being diluted 1:100 in UHT whole milk. Milk inoculated with *L. monocytogenes* was pressure treated at 400 MPa for 180 s or heat treated at 62.8°C for 30 s. The data points and error bars represent means and standard deviations based on three replications of the experiment.

significantly ($P < 0.001$) affected by the growth phase. Pairwise comparisons revealed cell lengths as follows: late-log phase (1.25 μm) > stationary phase (1.00 μm) = early-death phase (0.97 μm) = late-death phase (0.92 μm) > long-term-survival phase at 9 days (0.81 μm) > long-term-survival phase at 30 days (0.58 μm) ($P < 0.05$) (Fig. 4, SEM). As cells transitioned from log phase to long-term-survival phase, a weak correlation was observed between cell length before treatment and log reductions after pressure treatment ($R^2 = 0.69$) or after heat treatment ($R^2 = 0.76$). Some cocci exhibited wrinkled surfaces (Fig. 4F, SEM). TEM revealed cells in the long-term-survival phase at 30 days with a dark thin cell wall and a network in the cytoplasm, which appeared condensed and attached to the cell wall (Fig. 4F, TEM).

Part 2: transition from the long-term-survival phase back to the log and stationary phases in TSBYE at 35°C. When cells of *L. monocytogenes* ATCC 19115 in the long-term-survival phase after 30 days of incubation were inoculated into fresh, sterile TSBYE and incubated at 35°C, cells transitioned from lag to log and then to stationary phase (Fig. 5). ANOVA revealed that log reductions after pressure or thermal processing were both significantly ($P < 0.001$) affected by growth phase (Fig. 5). Pairwise comparisons demonstrated mean log reductions as follows: long-term-survival-phase cells after pressure treatment (0.4) = lag phase (0.2) < log phase (1.2) < stationary phase (2.2) ($P < 0.001$). Pairwise comparisons revealed mean log reductions as follows: lag-phase cells after heat treatment (1.4) < long-term-survival-phase cells (1.8) < log-phase cells (2.8) < stationary-phase cells (4.9) ($P < 0.001$).

After inoculation of long-term-survival cells into fresh, sterile TSBYE, the mean length of cells and the proportion of rod-shaped cells significantly ($P < 0.05$) increased, as cells rapidly transitioned from the lag phase to the stationary phase (data not shown). Slide culture results showed cocci converting to rod-shaped cells and then forming microcolonies (Fig. 6). During the transition from long-term-survival back to the log and stationary phases a strong correlation was observed be-

FIG. 6. Phase-contrast photomicrographs of long-term-survival-phase cells of *L. monocytogenes* after inoculation into fresh TSBYE. Photomicrographs were taken at 0 h (A), 1.5 h (B), 2.5 h (C), 3.5 h (D), 5 h (E), and 15 h (F) of incubation. Arrows indicate the transition from a coccoid cell to a rod-shaped cell that formed a microcolony. Bars, 10 μm .

tween cell length before treatment and log reductions after pressure ($R^2 = 0.96$) or heat treatment ($R^2 = 0.93$).

Part 3: transition from the long-term-survival phase back to the log and stationary phases in milk at 4°C. After inoculation

FIG. 7. Growth of long-term-survival-phase cells of *L. monocytogenes* after inoculation into UHT whole milk at 4°C and subsequent survival after HPP and heat treatments. Cells of *L. monocytogenes* ATCC 19115 were grown in TSBYE at 35°C for 30 days to yield long-term-survival-phase cells, and 10-ml portions of cultures were centrifuged. Pellets were suspended in 10 ml of UHT whole milk and then incubated at 4°C for various times before being diluted 1:100 in UHT whole milk. Milk inoculated with *L. monocytogenes* was pressure treated at 400 MPa for 180 s or heat treated at 62.8°C for 30 s. The data points and error bars represent means and standard deviations based on three replications of the experiment.

into UHT whole milk and incubation at 4°C, cells of *L. monocytogenes* ATCC 19115 in the long-term-survival phase transitioned to the log phase and remained there for 6 days. After 6 days of incubation, cells transitioned into the stationary phase and remained there for at least 29 days (Fig. 7). ANOVA again revealed that log reductions after pressure or heat treatment were significantly ($P < 0.001$) affected by growth phase. Pairwise comparisons revealed the following log reductions: long-term-survival-phase cells after pressure treatment (0.4) < log-phase cells (0.9) < stationary-phase cells (1.3) ($P < 0.001$). Pairwise comparisons showed the following log reductions: long-term-survival-phase cells after heat treatment (1.7) < log-phase cells (2.0) < stationary-phase cells (3.2) ($P < 0.001$).

Pairwise comparisons revealed that log reductions after pressure treatment of log-phase cells in part 1 > part 2 = part 3 and that log reductions of stationary-phase cells were as follows: part 2 > part 2 > part 3 ($P < 0.001$). Similarly, log reductions after heat treatment of log-phase cells in part 1 > part 2 > part 3 ($P < 0.001$), and log reductions after heat treatment of stationary-phase cells in part 1 > part 2 > part 3 ($P < 0.05$).

DISCUSSION

In the present study, the stationary phase (Fig. 1B) was relatively short, followed by the death phase (Fig. 1C); however, the mechanism(s) of the transition from stationary phase to death phase is not clear. It is possible that entry to death phase is a random (or stochastic) event. In this “passive” model, when cells enter the death phase depends only on the level of available nutrients (29). On the other hand, cell death could be a programmed event (apoptosis) regulated by quorum sensing (1). In this latter “active” model, bacteria sense that the population is too high for the limited nutrients, and they then communicate a “suicide” command to other cells via signaling molecules (9). By dying and lysing, dead bacteria release nutrients into the medium, which can serve as food for

survivors. This might partially explain the long-term survival (Fig. 1D) of cells that survive the death phase. Lysed dead cells are known to release another signal to let survivors exit apoptosis (9), but this signal might not be effective until it reaches a threshold concentration when 90 to 99% of cells are dead. Survivors can then respond to this signal and live on the debris of dead cells. This phenomenon has been termed cryptic growth (9, 29). Such surviving cells might exhibit long-term survival (29). Long-term starvation-survival has been characterized in *L. monocytogenes* (22, 31, 36) and many other microorganisms (6, 17, 29, 45, 47).

In the long-term-survival phase, bacteria may be in a dormant state. Dormancy induced by starvation has been reported in soil and rock microorganisms (30) and marine bacteria (38, 41). Additional research is needed to determine whether or not the coccoid form of *L. monocytogenes* in the long-term-survival phase (Fig. 1D) is in a dormant state. Another possibility is that the long-term-survival phase is metabolically dynamic with populations changing over time (9, 33). In this scenario newly created mutants that are more competitive than the parent culture finally take over the whole population. This phenomenon occurs reproducibly in the long-term-survival phase of *E. coli* (9, 29, 51).

The present study demonstrated that barotolerance significantly ($P < 0.05$) increased from log to long-term-survival phase (Fig. 2 and 3A). Novitsky and Morita (40) found that starvation for 1 week enhanced the barotolerance of a marine vibrio, which could also change cellular morphology from rods to cocci (37). We speculate that the enhanced barotolerance in the long-term-survival phase seen in the present study may be due to cytoplasmic condensation and subsequent lowered water activity in coccoid cells. Lowered water activity was previously shown to dramatically enhance the barotolerance of *L. monocytogenes* (19).

In the present study, tailing was evident in early-death or long-term-survival-phase cells, but not in late-log or late-death-phase cells (Fig. 3A). Nonlinear inactivation, or tailing, of microorganisms during HPP has been reported by other researchers (20, 49). The causative mechanism of tailing is still unclear but may be due to the presence of pressure-resistant cocci. In contrast, the linear part of the inactivation curve might be due to the inactivation of pressure-sensitive rods (Fig. 3A).

Cells in the stationary phase were not significantly ($P > 0.05$) more thermotolerant than cells in the log phase (Fig. 3B), a finding that disagrees with previous studies (25, 32). Perhaps stationary-phase cells in the present study still had not transitioned to resistant cocci (Fig. 4). The finding that cells in long-term-survival phase were the most thermotolerant is consistent with other reports that starvation enhances heat resistance in many microorganisms, including *L. monocytogenes* (22, 32), *E. coli* (24), *Arthrobacter globiformis* (6), and *Lactococcus lactis* (18). It is possible that the high thermotolerance in the long-term-survival phase in *L. monocytogenes* results from a lowered water activity in the coccus-shaped cells, similar to what is thought to occur in the core of bacterial spores (2, 3, 35). This is reasonable, since non-spore-forming cells would also benefit from a fitness strategy that produces long-term survival in natural environments. It is also possible that starvation stress prior to or during the long-term-survival phase

induces the synthesis of stress proteins, which are also known to protect cells against both heat (24) and pressure (21).

L. monocytogenes is generally rod-shaped; however, an early report indicated that it could form coccoid cells in broth cultures (16). Starvation is known to induce a change in cell shape from rods to cocci in *Arthrobacter crystallopoietes* (17), *A. globiformis* (6), a marine vibrio (37), and *Rhizobium leguminosarum* (45). Starvation also induced the formation of smaller coccoid cells of *Staphylococcus aureus* (47). After glucose starvation, cells of *L. monocytogenes* were reportedly shorter and wider than log-phase cells (22). The formation of cocci may be due to cell division without an increase in total biomass (29, 39, 45). Cocci might also be formed by cell shrinkage and cytoplasmic condensation (29), which might have produced the textured surface of cocci seen in the present study (Fig. 4F, SEM). Cytoplasmic condensation might lead to lowered water activity by decreasing cell volume and water content, and/or by increasing solute concentration. The percentage of cocci in the long-term-survival phase at 30 days (85%) as determined by SEM was significantly ($P < 0.05$) higher than by Gram stain (26%) (Fig. 4F), which might be due to the serial dehydration utilized in the SEM preparation. The occurrence of cocci (41 to 718 h) coincided with the occurrence of maximum barotolerance (41 to 718 h) and with the occurrence of maximum thermotolerance (70 to 718 h) (Fig. 2 and 3). Smaller cocci have a larger surface-to-volume ratio than rods, which can enhance simple or facilitated diffusion for nutrient uptake, which reduces the need for energy in nutrient transportation during starvation (8).

In part 2, after being inoculated into fresh TSBYE, the barotolerance and thermotolerance of cells in the long-term-survival phase significantly ($P < 0.05$) decreased as they reentered the log phase (Fig. 5). This rapid decrease in resistance could be largely explained by the increase in cell length, which is consistent with the hypothesis that resistance is due to cytoplasmic condensation. A weak correlation between cell length and resistance was observed when cells transitioned from rods to cocci (part 1), possibly because resistance was due to both synthesis of stress proteins and cytoplasmic condensation. In contrast, a strong correlation was observed when cocci transitioned to rods (part 2), which may have been due to the loss of resistance after rehydration of the cytoplasm without synthesis of stress proteins. Also, log reductions following either pressure or heat treatments of cells in either log or stationary phase were significantly ($P < 0.05$) lower in part 2 compared to part 1 (Fig. 2 and 5). Perhaps this higher resistance is due to the presence of highly resistant cocci in the log and stationary phases in part 2 (data not shown), which may be due to failure of some cocci to "germinate." This phenomenon has also been observed in *Bacillus cereus* spores (23). Once "germinated," cocci of *L. monocytogenes* were able to quickly enlarge to regain the rod shape and thereafter start cell division in fresh TSBYE (Fig. 6). Similar observations were reported in *A. crystallopoietes* (17) and marine microorganisms (26). Therefore, the rod-coccus-rod life cycle of *L. monocytogenes* is similar to that of other microorganisms that inhabit natural environments.

In part 3, after inoculation of long-term-survival-phase cells into milk, barotolerance and thermotolerance decreased significantly ($P < 0.05$) as cells reentered the log phase (Fig. 7).

Both log- and stationary-phase cells in part 3 showed significantly ($P < 0.05$) higher barotolerance and thermotolerance, compared to cells in the same phases in parts 1 and 2. This might be due to the protective effect of milk and/or induction of protective cold shock proteins at 4°C (13).

In conclusion, the present study revealed that cells of *L. monocytogenes* formed cocci in the long-term-survival phase, where they also became significantly ($P < 0.05$) more barotolerant and thermotolerant. These coccoid cells lost their resistance when they transitioned back to rods when placed in fresh media. Additional strains of *L. monocytogenes* and possibly other species of *Listeria* should be tested in the future to confirm that concomitant changes of barotolerance, thermotolerance, and cellular morphology throughout the life cycle are a general phenomenon in *L. monocytogenes* and possibly in other species of *Listeria*. Food safety studies on the resistance of microorganisms are usually based on the use of stationary-phase cells, which might not be the most resistant. Therefore, the food processing or preservation methods based on these processes might be inadequate, and thus their effectiveness may be overestimated. Also, cells of *L. monocytogenes* may persist in food processing plants in a resistant long-term-survival state, especially inside hard-to-clean harborage sites (46) or biofilms (12). They might then transition to infectious rod-shaped vegetative cells when they contaminate RTE foods after pasteurization. Further research is needed to address these important issues.

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