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Effects of Growth Temperature and Strictly Anaerobic Recovery on the Survival of *Listeria monocytogenes* during Pasteurization†

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***Listeria monocytogenes* F5069 was suspended in either Trypticase soy broth–0.6% yeast extract (TSBYE) or sterile, whole milk and heated at 62.8°C in sealed thermal death time tubes. Severely heat-injured cells were recovered in TSBYE within sealed thermal death time tubes because of the formation of reduced conditions in the depths of the TSBYE. Also, the use of strictly anaerobic Hungate techniques significantly increased recovery in TSBYE containing 1.5% agar compared with aerobically incubated controls. The exogenous addition of catalase, but not superoxide dismutase, slightly increased the recovery of heat-injured cells in TSBYE containing 1.5% agar incubated aerobically. Growth of cells at 43°C caused a greater increase in heat resistance as compared with cells heat shocked at 43°C or cells grown at lower temperatures. Growth of *L. monocytogenes* at 43°C and enumeration by the use of strictly anaerobic Hungate techniques resulted in $D_{62.8°C}$ values that were at least sixfold greater than those previously obtained by using cells grown at 37°C and aerobic plating. Results indicate that, under the conditions of the present study, high levels of *L. monocytogenes* would survive the minimum low-temperature, long-time treatment required by the U.S. Food and Drug Administration for pasteurizing milk. The possible survival of low levels of *L. monocytogenes* during high-temperature, short-time pasteurization and enumeration of injured cells by recovery on selective media under strictly anaerobic conditions are discussed.**

Listeria monocytogenes is a gram-positive, catalase-positive, aerobic to facultatively anaerobic bacterium that has recently been implicated in several fatal foodborne disease outbreaks (23, 37, 54). Because the organism is present in raw milk (5, 23, 27, 43), can grow well in foods at refrigeration temperatures (7, 16, 53), and is a potentially lethal pathogen (43), considerable emphasis has been placed on ensuring its complete destruction during pasteurization.

Conflicting reports have appeared on the ability of *L. monocytogenes* to survive the minimum high-temperature short-time (HTST) pasteurization processing guidelines (71.7°C for 15 s) of the Food and Drug Administration (24). Bearns and Girard (4) were the first to report that *L. monocytogenes* was able to survive pasteurization; however, Donnelly et al. (17) determined that the method used by the previous authors could have resulted in an overestimation of the heat resistance of the organism. Numerous studies have indicated that *L. monocytogenes* would not survive the minimum HTST treatment given fluid milk (5, 9, 10, 12, 13, 16, 17, 29); however, a few workers have reported that *L. monocytogenes* in naturally and artificially contaminated raw milk can survive the minimum HTST process (18, 21, 22). Although *L. monocytogenes* could not be detected by direct plating immediately after pasteurization, the microorganism was detected after a period of liquid enrichment. Fleming et al. (23) and Doyle et al. (18) postulated that the ability of *L. monocytogenes* to survive pasteurization was due to a thermal protective effect provided by an intracellular location within polymorphonuclear leukocytes. Bunning et al. (12, 13) determined that intracellular location within bovine phagocytes did not significantly increase heat resistance. In addition, they did not obtain a significant difference between the direct plate and liquid enrichment methods in detecting positive samples (13). Incubation at 25°C resulted in the detection of more organisms at higher heating times, and a potentially unsafe HTST process of 3.7 D was indicated (13). Bunning et al. (13) concluded, however, that commercial pasteurization would completely inactivate the low levels of *L. monocytogenes* found in raw milk. Golden et al. (29) reported that different strains of *L. monocytogenes* differed in heat resistance. One strain, Brie-1, was significantly more heat resistant than previous strains, but the authors still believed that it was unlikely that this organism would survive pasteurization if freely suspended in milk. Most recently, in joint studies between the Food and Drug Administration and the U.S. Department of Agriculture, viable cells were not detected in milk that had been contaminated both in vitro and in vivo with *L. monocytogenes* and then subjected to the minimum HTST pasteurization process (W. H. Stroup, A. R. Prosser, J. T. Tierney, J. H. Bryner, and R. W. Dickerson, J. Food Prot. **51**:822, 1988; J. Lovett, J. G. Bradshaw, D. W. Francis, R. G. Crawford, C. W. Donnelly, G. K. Murthy, and I. V. Wesley, J. Food Prot. **51**:822, 1988).

Many factors influence the heat resistance of nonsporeforming microorganisms (32). Time and temperature of incubation dramatically affect the heat resistance of both gram-negative (20) and gram-positive bacteria (36, 58). Beuchat (6) speculated that growth at high temperatures results in the production of thermostable membranes, which results in increased thermotolerance. A factor that has received considerable attention recently is sublethal heat shock (45, 60), which induces the rapid synthesis of heat shock proteins (see reference 42 for a current review). Factors other than heat, such as hydrogen peroxide (49) and glucose starvation (38), also induce the synthesis of heat shock proteins. Evidence is accumulating that heat shock proteins are a major determinant of bacterial thermotolerance (38, 60).

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An important factor in thermotolerance may be the effect of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) on the recovery of bacteria injured by heat. Recoveries of heat-stressed facultative pathogens were greatly increased in media containing compounds such as catalase (CA) (46) and pyruvate (57) that degrade hydrogen peroxide; the addition of exogenous superoxide dismutase (SOD) had no effect on the recovery of heat-injured *Staphylococcus aureus* (11). Gregory and Fridovich (30) reported that the induction of SOD in *Escherichia coli* dramatically increased its resistance to hyperbaric oxygen, presumably by eliminating intracellular superoxide. Dallmier and Martin (15) observed no discernible correlation between intracellular levels of either CA or SOD and the resistance of *L. monocytogenes* to sublethal heat. However, a correlation was noted in their data between the rate of inactivation of SOD and resistance to sublethal heat injury.

The purpose of the present study was to determine whether the thermal resistance of *L. monocytogenes* could be enhanced by growth or sublethal heat shock at 43°C. In addition, an attempt was made to determine whether and why liquid medium results in superior recoveries of heat-injured *L. monocytogenes* compared with direct aerobic plating in a solid medium.

MATERIALS AND METHODS

Bacterial culture, culture conditions, and medium preparation. *L. monocytogenes* F5069, serotype 4b, was obtained from Robert Weaver, Centers for Disease Control, Atlanta, Ga. This strain was isolated from raw milk obtained from a farm that supplied dairy products incriminated in an outbreak of listeriosis (23). The identity of the isolate was established by Gram stain, presence of CA, hemolysis on horse blood agar, tumbling motility at 25°C, formation of umbrella-shaped growth in motility test medium (Difco Laboratories, Detroit, Mich.), and growth at 4 and 45°C (56). Stock cultures were grown on Trypticase soy agar–0.6% yeast extract (TSAYE) (BBL Microbiology Systems, Cockeysville, Md.) at 37°C for 18 h and were maintained at 4°C with monthly transfer.

Prereduced Trypticase soy broth–0.6% yeast extract (TS BYE) (BBL Microbiology Systems) and prereduced TSBYE containing 15 g of agar (Difco Laboratories) per liter (TSB YEA) were prepared as described previously (34, 35). Resazurin (Fisher Scientific Co., Pittsburgh, Pa.) (0.25 g/liter) was added as an E_h indicator, and cysteine hydrochloride · H_2O (Sigma Chemical Co., St. Louis, Mo.) (0.5 g/liter) was added after boiling to reduce the medium. Oxygen-free N_2 was obtained by passing N_2 gas through a hot copper column (34, 35). Extreme care was taken to avoid the introduction of even slight amounts of O_2 into any of the prereduced media. Any tubes containing oxidized medium (as detected by the presence of a pink color at the surface of the medium) were discarded.

Preparation of bacteria. *L. monocytogenes* F5069 was grown in TSBYE at 37, 39, 41, 43, and 45°C for 18 h. In addition, the organism was grown in TSBYE at 37°C and then subjected to a sublethal heat shock at 43°C for 5, 30, and 60 min. Cultures of *L. monocytogenes* subjected to the various incubation and incubation-heat shock treatments were inoculated into blender jars containing 200 ml of either TSBYE or sterile, whole, homogenized milk to yield approximately 10^6 cells per ml. The heating menstruum and inoculum were mixed by using five 1-s high-speed bursts on a Waring blender; 2.5-ml portions of this mixture were dispensed into Pyrex thermal death time (TDT) tubes (9 mm in outside diameter, 7 mm in inside diameter by 150 mm in length). The TDT tubes were sealed by using a type 3A blowpipe (Veriflo Corp., Richmond, Calif.) and then were placed in a water bath at 10°C for 3 min to allow temperature equilibration before thermal inactivation.

Thermal inactivation and enumeration. Thermal inactivation was accomplished by completely submerging TDT tubes in a model 11-V-8 circulating water bath (Precision Scientific, Chicago, Ill.) equipped with a thermoregulator capable of maintaining temperature to within ±0.05°C. TDT tubes were heated at 60°C (pull times of 0, 5, 10, 15, 30, and 60 min) or 62.8°C (pull times of 0, 2, 5, 10, 15, and 30 min). The time for the TDT tube contents to reach water bath temperature (zero time) was measured by using a TDT tube equipped with a thermal death time thermocouple (O. F. Ecklund Inc., Cape Coral, Fla.). Six TDT tubes containing inoculated milk from each treatment-time combination were removed at appropriate times. Four tubes were left sealed, one was plated in duplicate in TSBYEA and incubated aerobically, and one was enumerated in duplicate in prereduced TSB YEA by using the Hungate roll tube method (34, 35). In the latter procedure, 1:10 dilutions were made in tubes of prereduced TSBYE while the tubes were being flushed with oxygen-free N_2. Roll tubes containing 12 ml of melted, prereduced TSBYEA equilibrated to 45°C were inoculated with 0.1-ml portions of the appropriate dilutions. The roll tubes were purged of O_2 by using O_2-free N_2 gas and stoppered as described previously (34, 35). An anoxy tube roller model C (The McBee Laboratory, Bozeman, Mont.) containing ice water was used to solidify the agar evenly against the inside of the tubes. Any tubes showing signs of oxidation (pink color) were labeled as such, and the procedure was repeated, when necessary, to obtain roll tubes that were completely anaerobic (showing no sign of pink color). Sealed TDT tubes, aerobic plates, and roll tubes were incubated for 3 weeks at 25°C, a temperature found to be superior for the recovery of heat-injured *L. monocytogenes* (13). Isolates from the plates and roll tubes were then confirmed as *L. monocytogenes* as already described.

Effects of CA and SOD. Bovine liver CA (2,600 U/mg) and iron-SOD from *E. coli* (2,940 U/mg) were obtained from Sigma Chemical Co. The enzymes were rehydrated with distilled water and sterilized by filtration to obtain stock solutions with enzyme activities of 10,000 U/ml. *L. monocytogenes* F5069 was grown at 43°C for 18 h, diluted in TSBYE to approximately 10^6 cells per ml, and heated for 10 min at 62.8°C by using the sealed tube method. Enzyme(s) and heat-injured cells (0.1 ml each) were added to roll tubes containing 20 ml of melted, prereduced TSAYE that had been equilibrated to 45°C. Inactive enzymes (heated at 100°C for 5 min) were added to other roll tubes as controls. The contents of the roll tubes were inverted several times to distribute the cells and achieve a uniform enzyme concentration (50 U of enzyme activity per ml) and then poured into sterile petri plates. The plates were incubated aerobically at 25°C, and CFUs were counted after 14 days. Anaerobic roll tubes containing cells but no enzyme were prepared as described above.

RESULTS

Recovery in sealed TDT tubes. Growth temperature and type of recovery method had significant effects on the detection of heat-injured *L. monocytogenes*. Cells grown at elevated temperatures and both heated and recovered in

TABLE 1. Effects of growth temperature and recovery method on survival of *L. monocytogenes* F5069 heated at 60°C in TSBYE in sealed TDT tubes[a]

Time (min) at 60°C	Growth temp			
	37°C		45°C	
	APC (log_{10} CFU/ml)[b]	TDT tube (no.)[c]	APC (log_{10} CFU/ml)	TDT tube (no.)
0	6.7	3	6.1	3
5	6.1	3	5.8	3
10	2.2	3	5.5	3
15	<0	0	2.9	3
30	<0	0	<0	3
60	<0	0	<0	3

[a] Pour plates and TDT tubes were incubated at 25°C for 12 days.
[b] APC, Aerobic plate count (in TSAYE) immediately after heat treatment.
[c] Number of sealed TDT tubes, of three possible, showing growth after 12 days at 25°C.

TSBYE within sealed TDT tubes survived severe heat treatments (Tables 1 and 2), whereas cells plated aerobically in TSBYEA did not (Table 1). Elevated growth temperatures increased the organism's thermal resistance, as measured by its ability to recover in TSBYE within sealed TDT tubes (Table 2). Recovery was not observed in milk that had been heated at 62.8°C for 5 min or longer in sealed TDT tubes and then incubated at 25°C (data not shown). Reduction of resazurin occurred in the bottom of sealed TDT tubes containing TSBYE immediately after heating, but not in TDT tubes containing sterile whole milk treated similarly. The depth of reduction in TDT tubes containing TSBYE became progressively greater as heating time increased. The time required to detect growth in TSBYE within sealed TDT tubes increased as the heating time increased; TDT tubes heated at 62.8°C for 30 min required 12 days at 25°C before growth became visible.

Aerobic and anaerobic plate count method. Aerobic plating of heat-injured *L. monocytogenes* yielded nonlinear thermal destruction curves (Fig. 1). The rate of destruction increased dramatically after 5 min at 62.8°C with cells grown at 37°C and after 10 min with cells grown at 43°C (Fig. 1). The heat resistance of cells grown at 37°C increased with the time cells were subjected to sublethal heat shock (43°C) before thermal inactivation; however cells grown at 43°C were the most heat resistant (Fig. 1). Incubation of TSBYEA pour plates in anaerobic jars did not result in a significant increase in CFUs compared with aerobically incubated controls (data not shown).

TABLE 2. Effects of growth temperature on survival of *L. monocytogenes* F5069 heated at 62.8°C in TSBYE and recovered in sealed TDT tubes

Time (min) at 62.8°C	No. of TDT tubes showing growth at a temp (before heat treatment) of[a]:			
	37°C	39°C	42°C	43°C
0[b]	3[c]	3	3	3
10	3	3	3	3
15	3	3	3	3
20	0	1	3	3
25	0	0	1	3
30	0	0	0	1

[a] Number of sealed tubes, of three possible, showing growth after 12 days at 25°C. An 18-h culture in TSBYE was used.
[b] Approximately 10^6 *L. monocytogenes* cells per ml.

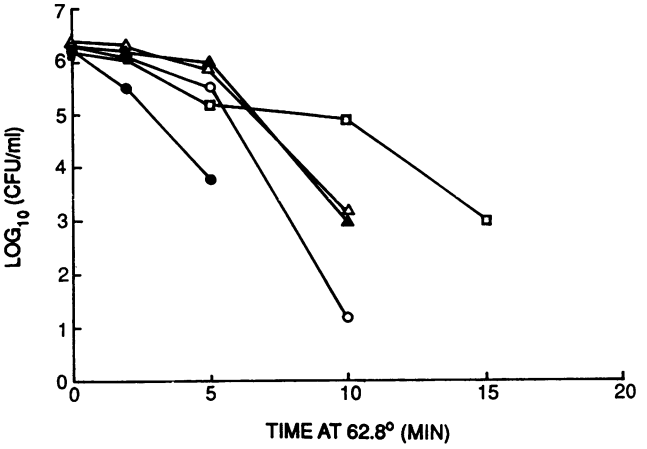

FIG. 1. Effects of heat shock time at 43°C versus growth at 43°C for 18 h on heat resistance of *L. monocytogenes* F5069 suspended in sterile TSBYE. Symbols: Cells grown at 37°C for 18 h and not heat shocked (●) or heat shocked for 5 (○), 30 (▲), and 60 (△) min; cells grown at 43°C for 18 h (□). All cells were plated and incubated aerobically.

Anaerobic roll tube method. Figure 2 depicts the effects of growth temperature and strictly anaerobic incubation on the enumeration of heat-injured *L. monocytogenes* F5069 (four replicate experiments were conducted and all four showed the same pattern as in Fig. 2). Strictly anaerobic incubation greatly increased the apparent heat resistance of *L. monocytogenes* compared with the aerobic plate count method (Fig. 2). *L. monocytogenes* grown at 43°C and inoculated into sterile, whole, homogenized milk before thermal inactivation resulted in a $D_{62.8°C}$ value of 243 s (Fig. 2). In contrast, cells grown at 37°C, which were treated the same but plated aerobically, yielded a $D_{62.8°C}$ value of 36 s (Fig. 2). Cells grown at 43°C and enumerated by using strictly anaerobic Hungate techniques resulted in thermal inactivation data with little deviation from a straight line (Fig. 2).

Effects of CA and SOD. The addition of both CA and SOD

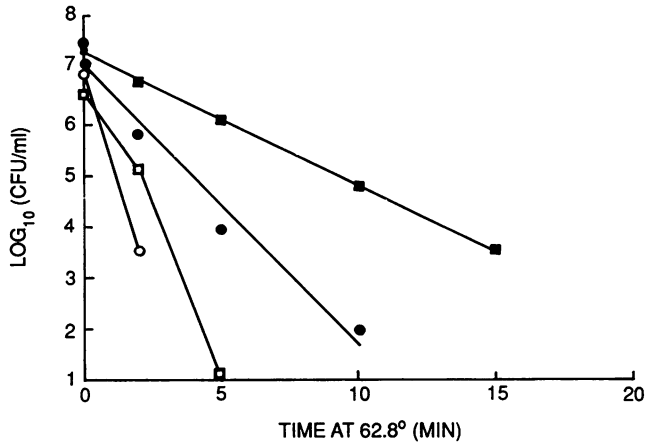

FIG. 2. Effects of growth temperature and strictly anaerobic recovery on heat resistance of *L. monocytogenes* F5069 suspended in sterile, whole, homogenized milk. Symbols: Cells grown at 37°C for 18 h—aerobic plate count (○), anaerobic roll tubes (●); cells grown at 43°C for 18 h—aerobic plate count (□), anaerobic roll tubes (■).

TABLE 3. Effects of CA, SOD, and strictly anaerobic recovery on enumeration of heat-injured *L. monocytogenes* F5069

Treatment[a]	Log_{10} cells per ml[b]
APC[c]	2.0
APC + active CA[d]	2.8
APC + active SOD[e]	2.2
APC + active SOD + active CA	2.8
Strictly anaerobic roll tube	4.0
APC + inactive CA[f] + inactive SOD[f]	2.1
APC + active CA + active SOD − cells	<1.0

[a] *L. monocytogenes* was grown at 43°C for 18 h, diluted to approximately 10^6 cells per ml in TSBYE, and then heated in sealed TDT tubes at 62.8°C for 10 min before enumeration.
[b] Average of two replicate experiments.
[c] APC, Aerobic plate count; plates were poured with TSAYE and then incubated aerobically for 14 days at 25°C.
[d] CA at 50 U/ml of TSAYE.
[e] SOD at 50 U/ml of TSAYE.
[f] Heated at 100°C for 5 min.

to TSBYEA increased the recovery of heat-injured *L. monocytogenes* upon aerobic incubation; CA was more effective than SOD (Table 3). Neither of these enzymes alone or in combination increased the number of CFUs as much as the use of anaerobic Hungate techniques (Table 3). The addition of heat-inactivated enzymes had no effect on the number of CFUs compared with the aerobic control (Table 3).

DISCUSSION

The reduction of resazurin in TSBYE inside sealed TDT tubes after heating indicated that the recovery of heat-injured *L. monocytogenes* was due to the absence of molecular O_2. The reduction of resazurin was probably a result of the creation of a vacuum in the headspace of the TDT tube during sealing and a lowering of O_2 solubility during heating. Upon heating, the O_2 moved from the broth into the headspace, which left the bottom of the TDT tube in a reduced state suitable for the repair and multiplication of heat-injured *L. monocytogenes*. In contrast to resazurin in TDT tubes containing TSBYE, resazurin in TDT tubes containing milk did not become reduced. Viable *L. monocytogenes* could be recovered from milk heated for 15 min at 62.8°C when the milk was sampled immediately after heating by using strictly anaerobic techniques (Fig. 2), but could not be recovered from milk in TDT tubes that had been heated for 5 min or longer and then incubated for 2 weeks at 25°C before sampling (data not shown). The exposure of heat-injured *L. monocytogenes* to the oxidized environment of the milk probably led to the eventual cell death observed. Growth of heat-injured cells in anaerobic roll tubes, but not in aerobic plates containing the same medium (Table 3 and Fig. 2), confirmed the hypothesis that the absence of O_2 was responsible for the recovery of severely heat-injured *L. monocytogenes*.

The O_2 sensitivity of heat-injured *L. monocytogenes* observed in this study probably was a result of the inactivation of CA and SOD. Dallmier and Martin (15) reported that these two enzymes were rapidly inactivated when *L. monocytogenes* was heated at temperatures of 55 to 60°C. Inactivation of these enzymes would result in the buildup of toxic levels of O_2 products, such as $O_2^{\cdot-}$ and H_2O_2 (46). Hydrogen peroxide can undergo a secondary reaction with iron (52) or $O_2^{\cdot-}$ (31) to form the extremely toxic hydroxyl (·OH) radical. Complete inactivation of these enzymes would have converted *L. monocytogenes* into an obligate anaerobe, a microorganism rapidly killed in the presence of molecular O_2. This hypothesis agrees with the enzyme-based theory of obligate anaerobiosis proposed by McCord et al. (48) and accounts for the large differences in thermotolerance observed when heated cells were incubated under aerobic and strictly anaerobic conditions (Fig. 2).

In the present study, exogenously added CA and SOD failed to effectively protect heat-injured *L. monocytogenes* plated aerobically (Table 3). Martin et al. (46) determined that thermal inactivation of CA in *S. aureus* resulted in the accumulation of toxic levels of H_2O_2, which significantly decreased recovery of this microorganism. In a subsequent study (11), the addition of SOD to an $O_2^{\cdot-}$-generating system resulted in protection of thermally stressed cells of *S. aureus*; however, incorporation of $O_2^{\cdot-}$ scavengers into the medium did not increase enumeration upon aerobic incubation, which was explained by the failure of exogenously added SOD to penetrate the cell membrane and react with intracellular $O_2^{\cdot-}$. Bagley et al. (2) treated Chinese hamster cells with paraquat, a chemical that mediates the production of intracellular $O_2^{\cdot-}$, and observed that when added to the growth medium neither CA nor SOD enhanced the formation of colonies. When introduced into the cells, however, SOD but not CA inhibited the toxicity of paraquat, indicating that the cytocidal action of paraquat was caused by the intracellular production of $O_2^{\cdot-}$. The intracellular cytocidal action of $O_2^{\cdot-}$ was also thought to be involved in the thermal destruction of *E. coli*, because strains low in SOD activity were more susceptible to heat injury than cells with high SOD activity (47). Therefore, the thermal inactivation of SOD and subsequent intracellular accumulation of $O_2^{\cdot-}$ may play a large role in the thermal destruction of *L. monocytogenes*.

Failure to detect an increase in heat resistance in the present study by incubation of pour plates in anaerobic jars (data not shown) has been reported previously (15, 22) and can be attributed to the "strict anaerobe" nature (48) of severely heat-injured *L. monocytogenes* (Table 3 and Fig. 2). Toxic O_2 products would have accumulated inside cells during aerobic diluting and plating operations and until all molecular O_2 had been expended inside the anaerobic jars. This would explain why no significant differences were observed between the numbers of colonies formed in TSB YEA plates incubated aerobically and those plates prepared aerobically and then incubated in anaerobic jars.

Doyle et al. (18) and Fernandez-Garayzabal and coworkers (21, 22), using naturally and artificially contaminated raw milk, did not detect viable *L. monocytogenes* by direct aerobic plating of milk immediately after pasteurization, but did detect viable cells when liquid enrichment techniques were used. More recently, Smith and Archer (57) and Crawford et al. (14) determined that the selective media currently in use to detect *L. monocytogenes* in foods are not satisfactory for the recovery of heat-injured cells. Crawford et al. (14) suggested that the presence of viable *L. monocytogenes* in HTST pasteurized milk, as detected by using selective enrichment media (18, 21, 22), was the result of postpasteurization contamination with uninjured cells. Dubos (19) observed that only a few cells of various facultative pathogens were required to initiate growth in a plain meat broth if the broth had been recently boiled, but after cooling for 24 h that same broth would not allow the growth of large numbers of the same microorganisms unless it was first reduced by the addition of cysteine. The addition of thioglycolic acid to a liquid enrichment medium greatly increased the ability of the medium to support growth of heat-treated facultative bacteria (51). Dubos (19) and Nelson (51) both

concluded that low oxidation-reduction potential was the main factor in allowing growth of heat-injured, facultative microorganisms. Mackey and Derrick (44) discovered that the solid forms of selective and nonselective media were significantly more inhibitory than the corresponding liquid media to heat-injured *Salmonella typhimurium*. The authors hypothesized that aerobic incubation of solid media exposed the cells to higher levels of O_2 which resulted in the formation of toxic H_2O_2, because the addition of CA increased the counts on nutrient agar to the level obtained in nutrient broth. Therefore, the negative recovery of heat-injured *L. monocytogenes* by using direct aerobic plate counts (9, 10, 12, 13, 16, 18, 22) and the negative (14; Lovett et al., J. Food Prot. **51**:822, 1988) and inconsistent (18, 22) recovery in liquid media may have depended more on the concentration of O_2 in the individual media than on the composition of the media per se. In the present study, recovery of severely heat-injured *L. monocytogenes* occurred only in reduced TSBYE inside sealed, undisturbed TDT tubes (Tables 1 and 2) or in roll tubes containing prereduced TSBYEA (Fig. 2). The ability of cells recovered under strictly anaerobic conditions to grow aerobically (data not shown) was probably a result of the cells regaining protective levels of CA and SOD activity.

Resting phagocytes consume little oxygen but obtain most of their energy from glycolysis (41). Oxygen consumption occurs only during the respiratory burst, which results in the introduction of O_2^- into the phagosome (1). *L. monocytogenes* is an intracellular pathogen that can survive and grow inside phagocytes because it either fails to initiate the respiratory burst (28) or possesses significant CA and SOD activities (8) that remove toxic O_2 metabolites. SOD located within the cytoplasm of phagocytes (39) survives pasteurization because it is more thermostable than the corresponding enzyme in *L. monocytogenes* (cf. references 15 and 33). Therefore, location inside phagocytes might protect heat-injured *L. monocytogenes* from O_2 toxicity. The localization of *L. monocytogenes* inside polymorphonuclear leukocytes was originally thought to serve as a "heat shield" and provide direct thermal protection (18, 23), but Bunning et al. (13) and Crawford and co-workers (14) determined that localization inside bovine phagocytes did not provide significant protection against the direct effects of heat during pasteurization. In those studies (13, 14), heat-injured *L. monocytogenes* were released from the phagocytes by sonication immediately after heating, followed by aerobic plating on a solid medium. This would have subjected the heat-injured, oxygen-sensitive bacteria to an oxygenated (toxic) environment, which explains why Bunning et al. (13) were unable to recover heat-injured, intracellular *L. monocytogenes* from HTST pasteurized milk. In contrast, by inoculating liquid enrichment media with nonsonicated milk, Doyle et al (18) may have given heat-injured, intracellular *L. monocytogenes* the reduced conditions necessary for recovery after pasteurization.

The heat resistance of cells grown at 37°C increased with the time they were subjected to a sublethal heat shock at 43°C (Fig. 1). This has been reported previously for *Salmonella typhimurium* (45), and may reflect a quantitative increase in the amounts of heat shock proteins synthesized with time (38, 42). Crawford et al. (R. G. Crawford, J. T. Tierney, J. T. Peeler, and V. K. Bunning, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, Q112, p. 348) observed a general trend of increased thermotolerance with an increase in time and temperature of heat shock for *L. monocytogenes* F5069; however, they concluded that the increased thermotolerance was not statistically significant. Most thermotolerance studies of *L. monocytogenes* have utilized cells grown at, or below, 37°C (5, 9, 10, 12, 13, 16, 17, 29). The normal body temperature of a cow is approximately 39°C, but temperatures can reach as high as 42.8°C when the cow is infected with *L. monocytogenes* (18). The large increase in heat resistance observed when cells were grown at 43°C, compared with cells that were grown at 37°C and then heat shocked at 43°C (Fig. 1), may be due to the accumulation of large amounts of heat shock proteins during the stationary phase of growth (38). Jenkins et al. (38) concluded that the increased heat resistance of stationary-phase cells (20, 36, 58) could be a result of the synthesis of postexponential heat shock proteins that are induced during glucose starvation. The presence of large amounts of postexponential heat shock proteins in stationary-phase cells grown at 43°C would explain why these cells were more heat resistant than either those grown at lower temperatures (Tables 1 and 2) or those grown at 37°C and then heat shocked at 43°C (Fig. 1). Heat resistance can also be induced by exposure to H_2O_2 (49), a condition known to occur within the phagosome (39). Therefore, cells of *L. monocytogenes* growing inside bovine phagocytes within an infected udder could be in a heat-resistant state that might persist during refrigerated storage prior to pasteurization. Heat-shocked cells of *L. monocytogenes* can remain heat resistant after being held for 24 h at 4°C (J. M. Farber and B. E. Brown, Dairy Food Environ. Sanit. **9**:274, 1989).

Recently, Bunning et al. (13) used cells grown at 37°C, aerobic plating, and sterile, homogenized, whole milk to obtain a $D_{62.8°C}$ value of 38.3 s for freely suspended *L. monocytogenes*. Based on their results and the use of a detailed risk analysis model (D. A. A. Mossel, Proceedings of the World Health Organization Conference on Listeriosis, agenda item 8, 1986), Bunning et al. (13) concluded that commercial HTST pasteurization of milk was not a problem. Earlier, however, Mossel and Van Netten (50) cautioned that, if careful attention is not paid to the recovery of stressed microorganisms, substantial overestimation of the microbial kill attained in processing may result. By combining growth at 43°C with strictly anaerobic recovery, the $D_{62.8°C}$ values were approximately 6.3 times greater in the present study than the value of 38.3 s just mentioned (13). Extrapolation of our results to the HTST range is scientifically inappropriate; however, given the conditions of the present study and the z_D value of 5.6°C calculated recently (13), low levels of *L. monocytogenes* (i.e., 10 per ml of raw milk) might survive the minimum HTST treatment. Therefore, unlike others (59), we believe further research is still needed on the thermal destruction of *L. monocytogenes*, especially during HTST pasteurization of milk.

The heat resistance of *L. monocytogenes* in foods other than dairy products is also of current interest (7, 25). The development of anaerobic environments in foods after thermal processing might permit the growth of heat-injured *L. monocytogenes*. Such conditions might exist in cooked meats (40) and other pasteurized foods that are packaged in containers having low levels of O_2. Potentially inadequate thermal processes currently exist for hot dogs (25), the consumption of which has been associated with sporadic listeriosis (55). Recently, a fatal case of listeriosis was associated with the consumption of vacuum-packaged turkey franks (3). Although it has not been shown that *L. monocytogenes* can survive the thermal process for hot dogs, the results of the present study should give cause for concern.

Many attempts have been made to develop an improved medium for the enumeration of injured *L. monocytogenes*, but such a medium is still to be realized (14, 57). The lethal effects of selective agents on injured cells is the main impediment to developing a selective, yet efficient, recovery method. Selective agents might increase lethality by enhancing the oxidative stresses that injured cells experience under aerobic incubation. Removal of this oxidative stress might allow direct enumeration of *Listeria* spp. in selective media. This hypothesis is supported by previous work with *L. monocytogenes* (57), *S. aureus* (46), and *Salmonella typhimurium* (44), in which the toxic effects of various selective agents were partially overcome by the addition of CA. A strictly anaerobic recovery period would eliminate both internal and external oxidation stress by excluding O_2, the necessary precursor of toxic O_2 radicals. Therefore, the use of strictly anaerobic recovery with solid selective media might permit rapid and accurate enumeration of injured, facultatively anaerobic, foodborne pathogens.

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