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A Novel Strictly Anaerobic Recovery and Enrichment System Incorporating Lithium for Detection of Heat-Injured *Listeria monocytogenes* in Pasteurized Milk Containing Background Microflora

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Heat-injured cells of *Listeria monocytogenes* were recovered from heated raw milk containing noninjured *Enterococcus faecium* by combining a simple method for obtaining strict anaerobiosis with a novel enrichment broth, Penn State University broth (PSU broth). Strictly anaerobic conditions were rapidly achieved by adding 0.5 g of filter-sterilized cysteine per liter to PSU broth and then purging the preparation with N_2 gas. Little resuscitation or growth occurred in strictly anaerobic PSU broth without lithium chloride because of overgrowth by *E. faecium*. The growth of *E. faecium* decreased dramatically with increasing LiCl concentration; LiCl concentrations of 8 and 10 g/liter were completely bacteriostatic. The mechanism of inhibition by LiCl appeared to involve competition with the divalent cations Ca^{2+} and Mg^{2+}. Heat-injured *L. monocytogenes* consistently recovered and grew rapidly in strictly anaerobic PSU broth containing 4, 6, or 7 g of LiCl per liter. The use of strictly anaerobic PSU broth containing 7 g of LiCl per liter permitted detection of severely heat-injured *L. monocytogenes* in one simple recovery-enrichment step by eliminating oxygen toxicity and inhibiting the growth of background microflora, without preventing the resuscitation and subsequent growth of heat-injured *L. monocytogenes*. *L. monocytogenes* heated in raw milk at 62.8°C for 10, 15, and 20 min could be consistently recovered from strictly anaerobic PSU broth enrichment cultures at 30°C after 48, 96, and 144 h, respectively, and hence, use of PSU broth may result in better recovery of both injured and noninjured cells from foods than currently used U.S. Department of Agriculture and Food and Drug Administration preenrichment procedures.

Pathogens are often present in foods in an injured state because of various food-processing operations that produce physical and/or chemical stresses (40). Many pathogenic microorganisms, including *Listeria monocytogenes*, suffer reversible injury (30, 40) and can remain virulent upon recovery (1, 32). Various conditions in foods may permit the resuscitation and growth of injured pathogens, including refrigeration (33), extended shelf life (36), and lack of oxygen (28, 30). This is especially a concern with *L. monocytogenes*, because this potentially lethal food-borne pathogen can grow at refrigeration temperatures. Therefore, to ensure the microbiological safety of a food product, any detection system used must be capable of detecting injured cells (1, 7, 40).

Superoxide dismutase is an essential enzyme in most aerobic organisms, where it converts toxic $O_2^{-}\cdot$ radicals to H_2O_2, another toxic species. Catalase detoxifies H_2O_2 to H_2O and O_2 (35). Reactive oxygen species can react with critical intracellular molecules to cause lethal effects (14, 46). In *L. monocytogenes* both catalase and superoxide dismutase activities decrease rapidly upon heating at 60°C (11); however, neither the addition of pyruvate or catalase nor the use of conventional anaerobic procedures increases the detection of injured cells (11, 43). By using strictly anaerobic Hungate techniques, Knabel et al. (30) demonstrated that severely heat-injured *L. monocytogenes* could be recovered in a nonselective medium if the cells were immediately placed in a medium devoid of O_2. The use of a novel, strictly anaerobic method dramatically enhanced the resuscitation of heat-injured *L. monocytogenes* compared with aerobic methods (29). In contrast to the exogenous addition of antioxidants, complete removal of O_2 prevents the intracellular formation of superoxide (27) and the extremely toxic hydroxyl radical ·OH (26).

Historically, food microbiologists have faced a dilemma in trying to rapidly detect injured pathogens in foods containing background microflora; namely, nonselective media permit overgrowth by background microflora, while selective media inhibit the resuscitation of injured cells. The cold enrichment method (21) for detecting *L. monocytogenes* in foods involves inhibiting growth of background microflora by using low temperature. While this method inhibits the growth of most background microfloras and permits resuscitation (32) and subsequent growth (21) of *L. monocytogenes*, it is too time-consuming for practical use in detecting food-borne pathogens. Injured pathogens can resuscitate and grow faster at higher incubation temperatures; however, rapid overgrowth by background microflora must then be inhibited by selective agents, which in turn inhibit resuscitation (17, 19, 43). As a result, it has become almost a dogma in food microbiology that injured microorganisms can be detected only if they are first allowed to repair themselves in a nonselective environment before they are exposed to selective media (5, 23, 40, 48, 49).

Growth of background microflora, especially enterococci, often inhibits the detection of heat-injured *L. monocytogenes* (4; unpublished data). The currently used Food and Drug Administration (FDA) and U.S. Department of Agriculture (USDA) primary enrichment media for *L. monocytogenes* both contain selective agents to inhibit the growth of background

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microflora. Unfortunately, these media also inhibit the resuscitation of heat-injured *L. monocytogenes* (10, 23, 43). The selective agent LiCl was thought to prevent the recovery of injured *L. monocytogenes* in both selective enrichment broth (48) and selective agar (41). Thus, LiCl is not used in either the FDA (31) or USDA (34) primary enrichment media but is used at a concentration of 3 g/liter in the USDA's secondary enrichment medium, Fraser broth (18).

Numerous techniques have been used to detect heat-injured *L. monocytogenes*, with varied success. Heat-injured *L. monocytogenes* in milk could not be detected by direct plating immediately after pasteurization (12, 15), but viable cells were detected after a period of liquid enrichment. Interestingly, Doyle et al. (12) did not achieve consistent detection with any of the three aerobic, liquid enrichment procedures which they used. Bunning et al. (6) detected more *L. monocytogenes* at higher heating temperatures by using a sterile heating menstruum, a nonselective medium, and a suboptimal incubation temperature (25°C). While the FDA's *Listeria* enrichment broth (LEB) and the USDA's University of Vermont medium (UVM) can detect low levels of noninjured *L. monocytogenes*, both of these media were deemed inadequate for detecting heat-injured cells that might survive high-temperature short-time (HTST) pasteurization; however, LEB, but not UVM, often gave levels of recovery similar to the levels obtained with Trypticase soy broth containing yeast extract (TSBYE) after extended incubation (10). Recently, Bailey et al. (2) reported that UVM was more efficient than LEB for recovering heat-injured *L. monocytogenes* in the presence of a competitive microflora from chicken and Brie cheese. They attributed this difference to a decrease in the pH of LEB because it contains a fermentable carbohydrate (glucose) but lacks adequate buffering capacity.

The purpose of this study was to determine the critical parameters needed to detect heat-injured *L. monocytogenes* in the presence of background microflora, including noninjured *Enterococcus faecium*. The information which we obtained was then used to develop a simple and efficient system for detection of heat-injured *L. monocytogenes* in pasteurized milk.

(Some of the results were presented at the Annual Meeting of the American Society for Microbiology in Atlanta, Ga., on 19 May 1993.)

MATERIALS AND METHODS

Bacterial cultures and culture conditions. *L. monocytogenes* F5069 (serotype 4b) was obtained from Robert Weaver, Centers for Disease Control and Prevention, Atlanta, Ga. This strain was isolated from raw milk obtained from a farm that supplied dairy products which were incriminated in an outbreak of listeriosis (16). *L. monocytogenes* Scott A was obtained from Elmer Marth, University of Wisconsin, Madison. A strain of *Enterococcus faecium* that had caused problems in detecting heat-injured *L. monocytogenes* in pasteurized milk was isolated at The Pennsylvania State University. Isolates were identified as *L. monocytogenes* by using the following characteristics: Gram stain reaction, presence of catalase, hemolysis on horse blood Columbia agar bilayer plates (Remel, Lenexa, Kans.), tumbling motility at 25°C, formation of umbrella-shaped growth in motility test medium (Difco Laboratories, Detroit, Mich.), appearance on modified Oxford medium (MOX) (Oxoid/Unipath, Columbia, Md.), fermentation of xylose and rhamnose, and growth at 4 and 45°C (31). The identity of *Enterococcus faecium* was determined by its Gram stain reaction, by its growth at 10 and 45°C, at pH 9.6, and in the presence of 6.5% NaCl, and by its carbohydrate fermentation profile (API Rapid CH; API Analytab Products, Plainview, N.Y.). All stock cultures were grown on Trypticase soy agar containing 0.6% yeast extract (TSAYE) (BBL Microbiology Systems, Cockeysville, Md.) at 35°C for 18 h and were maintained at 4°C with monthly transfers.

Modification of LEB. LEB was prepared as described by Lovett (31), except that acriflavine was not added because preliminary studies by workers in our laboratory revealed that 15 mg of acriflavine per liter did not inhibit enterococci or enhance the detection of heat-injured *L. monocytogenes* in reduced LEB containing LiCl. DNA intercalating agents, such as acriflavine, are known to inhibit RNA synthesis, which is necessary for the resuscitation of injured cells (25). Resazurin (2.5 mg/liter; Fisher Scientific Co., Pittsburgh, Pa.) was added as an E_h indicator. Isolation Roll Streak Tubes (Bellco, Vineland, N.J.) containing LEB (30 ml per tube) were sterilized by autoclaving them at 121°C for 15 min and then were cooled to 30°C in a water bath. The tubes containing LEB were reduced by adding a 50% (wt/vol) solution of filter-sterilized L-cysteine hydrochloride (Sigma Chemical Co., St. Louis, Mo.) to give a final concentration of 0.5 g of L-cysteine hydrochloride per liter. Other supplements were added, and other modifications were made to LEB as described below. The headspace of each tube was rapidly purged of oxygen by using a 14-gauge, 4-in (10.16-cm) cannula attached to a 2-ml Luer-Lok, sterile-cotton-plugged glass syringe (Becton Dickinson, Cockeysville, Md.). The barrel of the syringe was connected to a tank of N_2 gas with amber latex tubing (Fisher Scientific). The commercial N_2 gas used in this study was always free of O_2; however, to achieve the results reported below, we tested the N_2 for O_2, and if O_2 was found, the N_2 was passed over a heated copper column to remove the traces of O_2. During purging, the flame-sterilized cannula was inserted between the inner side of the vessel and the butyl rubber stopper, which created a small opening for oxygen gas to escape. After approximately 5 s, purging was complete and the cannula was removed while the stopper was simultaneously pushed and then twisted firmly in place to form an airtight seal. When this procedure was used, the contents of the vessels remained strictly anaerobic throughout the incubation period (30).

Preparation of bacteria. *L. monocytogenes* F5069 or Scott A was grown in TSBYE at 43°C for 18 h and inoculated into sterile blender jars containing 100 ml of raw milk to yield approximately 10^7 *L. monocytogenes* cells per ml of raw milk. Cells of *L. monocytogenes* were grown at 43°C because cows infected with and shedding *L. monocytogenes* can have temperatures as high as 42.8°C (12). In addition, cells grown at 43°C are more thermotolerant (13, 30) and maintain this thermotolerance when they are stored at 4°C for 2 days prior to pasteurization (13). The aerobic plate counts (after incubation on TSAYE at 30°C for 48 h) of the raw milk were approximately 10^4 CFU/ml before heating and 10^2 CFU/ml after thermal injury. Raw milk was used as the heating menstruum in the first experiment (see Fig. 2) to simulate actual prepasteurization conditions and because sterile milk slightly inhibited resuscitation of heat-injured *L. monocytogenes* (unpublished data). Sterile milk, however, had to be used in a later experiment (see Fig. 3), because overgrowth by background microflora would have prevented enumeration of *L. monocytogenes* on TSAYE. Milk and *L. monocytogenes* were mixed by using five 1-s high-speed bursts of a Waring blender, and the resulting preparation contained approximately 10^7 *L. monocytogenes* cells per ml of milk; 2.5-ml portions of this mixture were dispensed into Pyrex thermal death time (TDT) tubes (outside diameter, 9 mm; inside diameter, 7 mm; length 150 mm). The TDT tubes were sealed by using a type 3A

blowpipe (Veriflo Corp., Richmond, Calif.) and then were placed in a 5°C refrigerator for 10 to 15 min to allow temperature equilibration before thermal injury. Noninjured *Enterococcus faecium* cells were added to enrichment broth cultures (final concentration, 10^3 cells per ml of broth) to further challenge the ability of the detection systems to inhibit growth of large numbers of problematic background organisms. *Enterococcus faecium* was not added in one of the experiments (see Fig. 3) to permit enumeration of *L. monocytogenes* on TSAYE.

Thermal injury. Thermal injury was accomplished by completely submerging the TDT tubes containing milk inoculated with *L. monocytogenes* in a Lauda model MS 20 circulating water bath set at 62.8 ± 0.1°C. The TDT tubes were heated for 6 min (1 min come-up time plus 5 min at 62.8°C). This heat treatment resulted in an approximately 5 to 6 $\log_{10}$ reduction in the numbers of *L. monocytogenes* cells, as determined by colony formation on aerobic TSAYE incubated for 96 h at 30°C. Additional experiments were performed with heating times of 10, 15, 20, 25, and 30 min at 62.8°C. After heating, the TDT tubes were immediately cooled in an ice water bath at 0°C and the contents were then used to inoculate enrichment broth media. The total number of injured cells per milliliter of enrichment broth was determined by spreading 1.0 ml of broth onto anaerobic TSAYE plates inside a Bactron model 2 anaerobic chamber (Anaerobe Systems, Inc., San Jose, Calif.). Before inoculation the plates had been held in the anaerobic chamber for 24 h to completely reduce the medium (to change the resazurin indicator from pink to clear). Enrichment broth was also spread onto aerobic TSAYE plates. Both aerobic and anaerobic TSAYE plates were incubated at 30°C for 96 h before colonies were counted. All plating experiments were performed in duplicate. All surviving cells were determined to be initially injured, as they failed to form colonies on aerobic MOX agar plates after incubation for 96 h at 30°C.

MIC of lithium chloride. The MICs of lithium chloride for *L. monocytogenes* (heat-injured cells and non-heat-injured cells) and noninjured *Enterococcus faecium* were determined by using a tube dilution test. Twofold dilutions of LiCl in LEB were prepared to give the following concentrations of LiCl: 2.5, 3.75, 5.0, 7.5, 10, 15, and 20 g/liter. Three sets of Isolation Roll Streak Tubes containing LEB and LiCl were prepared, and in each set one tube containing LEB and no LiCl was used as a control. All of the LEB tubes were sterilized by autoclaving them at 121°C for 15 min, cooled to 30°C in a water bath, and reduced by first adding filter-sterilized cysteine and then purging with N_2 gas.

The members of two sets of strictly anaerobic LEB tubes were each inoculated with 0.2 ml of a diluted culture of either noninjured *L. monocytogenes* or noninjured *Enterococcus faecium*. Each organism was grown in TSBYE at 35°C for 18 h to a final concentration of approximately 10^5 CFU/ml (when we determined MICs in Fraser broth, final concentrations of 10^4 CFU/ml were used). The members of a separate set of LEB tubes were inoculated with 0.2-ml portions of *L. monocytogenes* that had been grown in TSBYE at 35°C for 18 h and then heat injured at 62.8°C for 5 min in TSBYE. An extra tube containing sterile LEB was added as an uninoculated control. After inoculation all LEB tubes were purged with nitrogen gas, stoppered, and incubated at 30°C for 48 h. The lowest concentration of LiCl that resulted in no visible turbidity in LEB after 48 h was considered the MIC. The experiment was replicated three times, and the data obtained were evaluated by using chi-square analysis (24).

Effects of metal cations on inhibition of *Enterococcus faecium* by LiCl. To determine how LiCl might inhibit enterococci, a well-plate technique was used to determine whether selected metal ions could counteract the inhibitory effect of LiCl on the growth of *Enterococcus faecium*. *Enterococcus faecium* cells were grown at 35°C for 18 h in TSBYE. Sterile cotton swabs were used to spread the resulting culture onto the surfaces of MOX agar plates. A sterile, size 11 cork borer was then used to cut a well in the center of each inoculated plate, and each well was filled with a sterile solution of 1.0 M $MgCl_2$, 1.0 M $FeCl_3$, 1.0 M $MnCl_2$, 1.0 M KCl, and 1.0 M $CaCl_2$, or a solution containing both 1.0 M $MgCl_2$ and 1.0 M $CaCl_2$. The well of a separate MOX agar plate inoculated with *Enterococcus faecium* was filled with sterile water and was used as a control. All plates were incubated at 35°C for 48 h and checked for growth and blackening (esculin hydrolysis) around the wells after 24 and 48 h.

Effect of LiCl on resuscitation and growth of heat-injured *L. monocytogenes* in PSU broth. *L. monocytogenes* grown at 43°C for 18 h was heat injured in raw milk as described above. Isolation Roll Streak Tubes containing 30 ml of reduced Penn State University (PSU broth) or 250-ml volumetric flasks (Fisher Scientific) containing 225 ml of reduced PSU broth were prepared as described above. The concentration of LiCl in each vessel was then adjusted to 4, 5, 6, 7, 8, or 10 g/liter. The Isolation Roll Streak Tubes and volumetric flasks were then inoculated with 0.3- and 25-ml portions of a heat-injured *L. monocytogenes* culture, respectively. Enough noninjured *Enterococcus faecium* was added to achieve a final concentration of 10^3 cells per ml. All of the vessels were nitrogen purged, stoppered, and incubated at 30°C for 60 h. At 12-h intervals, aliquots of broth were removed from each vessel and plated in duplicate on anaerobic and/or aerobic MOX agar and TSAYE. The aliquots were removed with a 1-ml pipette attached to a pipette aid (Drummond Scientific Co., Broomall, Pa.); the exhaust line was connected to an N_2 gas source to allow purging of the pipette prior to sampling. The plates were incubated at 30°C, and bacterial colonies were counted after 48 h. Colonies on TSAYE were confirmed to be *Enterococcus faecium* or *L. monocytogenes* colonies by using the tests described above. The experiment was replicated three times, and standard errors of all means were calculated as previously described (24).

Fraser broth was prepared according to the formula of Fraser and Sperber (18), and modified Fraser broth was prepared by increasing the LiCl concentration in Fraser broth to 10 g/liter. At 12-h intervals, 0.1 ml of the primary enrichment broth from each Isolation Roll Streak Tube was transferred to each of three corresponding Fraser broth and modified Fraser broth tubes. The contents of all tubes were mixed by vortexing, and the tubes were incubated aerobically at 35°C for 24 h. After 24 h, broth samples from the Fraser broth and modified Fraser broth tubes that were black were streaked in duplicate on MOX agar plates. The plates were incubated at 35°C for 48 h and then checked for presumptive *L. monocytogenes* colonies (34).

PSU broth. PSU broth was prepared by using medium ingredients that collectively supported the resuscitation and growth of heat-injured *L. monocytogenes* under strictly anaerobic conditions, while suppressing the growth of enterococci and other background organisms. PSU broth contained (per liter) 17.0 g of Casitone, 3.0 g of soytone, 5.0 g of sodium chloride, 2.5 g of glucose, 1.0 g of esculin, 6.0 g of yeast extract, 9.6 g of Na_2HPO_4, 1.35 g of KH_2PO_4, 7.0 g of lithium chloride, 0.05 g of cycloheximide, 0.04 g of nalidixic acid, and 2.5 mg of resazurin. All of the ingredients except LiCl were sterilized by autoclaving at 121°C. To avoid formation of insoluble lithium salts during autoclaving, a filter-sterilized LiCl solution (50%,

wt/vol) was added immediately after the medium was autoclaved and cooled. Oxygen was removed from PSU broth by using cysteine hydrochloride and N_2 purging as described above. The final concentration of cysteine hydrochloride in PSU broth was 0.5 g/liter, and the final pH was 7.1.

Resuscitation of cells with different degrees of injury in PSU broth. *L. monocytogenes* cells were heat treated as described above, except that sterile milk was used as the heating menstruum to eliminate background microflora and thus permit enumeration of *L. monocytogenes* on TSAYE. The experiment was replicated three times, and standard errors of all means were calculated as described previously (24). In this study the different degrees of injury were defined as follows. Severely injured cells could recover and form colonies on strictly anaerobic TSAYE (TSAYE/anaerobic), but not on aerobic TSAYE (TSAYE/aerobic). Therefore, the number of severely injured cells equaled the difference between the counts on TSAYE/anaerobic and the counts on TSAYE/aerobic. Moderately injured cells could recover and form colonies on TSAYE/aerobic, but not on aerobic MOX agar (MOX/aerobic). Therefore, the number of moderately injured cells equaled the difference between the counts on TSAYE/aerobic and the counts on MOX/aerobic (see Fig. 3). Uninjured cells were defined as cells that formed colonies on MOX/aerobic. The percentage of total survivors that represented each category of cells was calculated by using the following relationships: percent severely injured, [(TSAYE/anaerobic − TSAYE/aerobic) × 100]/(TSAYE/anaerobic); percent moderately injured, [(TSAYE/aerobic − MOX/aerobic) × 100]/(TSAYE/anaerobic); and percent uninjured, (MOX/aerobic × 100)/(TSAYE/anaerobic).

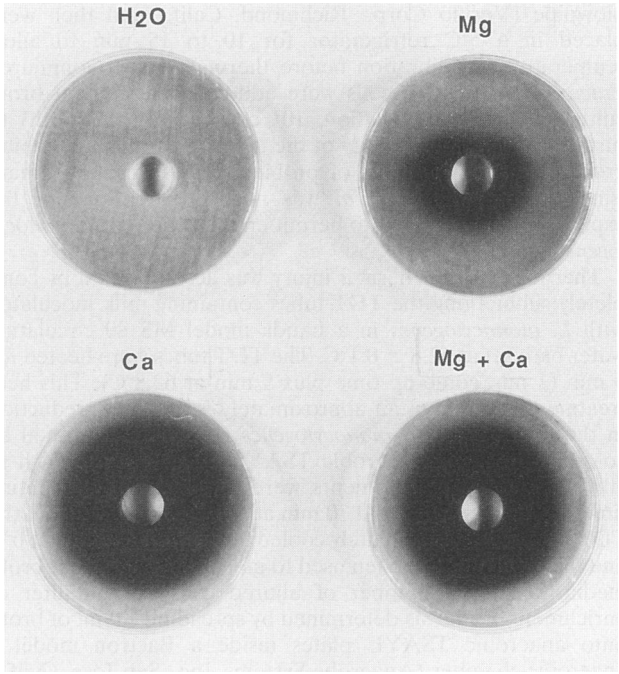

FIG. 1. Effects of $CaCl_2$, $MgCl_2$, and $MgCl_2$ plus $CaCl_2$ on the growth of *Enterococcus faecium* on MOX agar. The black zones around the wells represent esculin hydrolysis due to growth of *Enterococcus faecium* for 48 h at 35°C.

RESULTS

MICs of lithium chloride. In order to determine a concentration of LiCl that would inhibit *Enterococcus faecium* but not *L. monocytogenes*, the MICs of LiCl for both *Enterococcus faecium* and *L. monocytogenes* F5069 were evaluated in LEB at 30°C under strictly anaerobic conditions. The MICs of LiCl for noninjured *L. monocytogenes* (40 g/liter), heat-injured *L. monocytogenes* (15 g/liter), and noninjured *Enterococcus faecium* (10 g/liter) were significantly different ($P < 0.005$). Surprisingly, heat-injured *L. monocytogenes* resuscitated and grew in strictly anaerobic LEB that contained up to 10 g of LiCl per liter, whereas *Enterococcus faecium* failed to grow in LEB containing that same concentration of LiCl. The MICs of LiCl in aerobic Fraser broth at 35°C for noninjured *L. monocytogenes* F5069 and *Enterococcus faecium* were 12 and 7 g/liter, respectively (data not shown).

Effects of metal ions on inhibition of *Enterococcus faecium* by LiCl. *Enterococcus faecium* did not grow on MOX agar containing 15 g of LiCl per liter; however, it did grow well and hydrolyze esculin on MOX agar without LiCl. After 24 and 48 h *Enterococcus faecium* grew around wells on MOX plates containing Ca^{2+} alone or Ca^{2+} plus Mg^{2+}; smaller zones of growth were observed around wells containing Mg^{2+} alone (Fig. 1). *Enterococcus faecium* did not grow on MOX agar containing 15 g of LiCl per liter when only deionized water was added to the wells (Fig. 1). Manganese, iron, and potassium salts failed to relieve growth inhibition by LiCl (data not shown).

Effect of LiCl on resuscitation and growth of heat-injured *L. monocytogenes*. The average population size of the background microflora in LEB without LiCl (which initially contained approximately 10^4 heat-injured *L. monocytogenes* cells per ml and 10^3 noninjured *Enterococcus faecium* cells per ml) was 10^9 CFU/ml after 24 h, and there was no further increase after incubation for up to 60 h. The vast majority of the background microorganisms isolated were confirmed to be *Enterococcus faecium*. The background microflora counts decreased dramatically as the LiCl concentration increased. After 48 h, the total background microflora population sizes in LEB containing 4 and 6 g of LiCl per liter were 4.6×10^7 and 1.3×10^5 CFU/ml, respectively. Higher concentrations of LiCl (8 and 10 g/liter) had a bacteriostatic effect on the background microflora up to 48 h, after which the counts increased slowly to about 1.0×10^4 CFU/ml at 60 h. In the presence of noninjured *Enterococcus faecium*, *L. monocytogenes* resuscitated and grew in LEB containing 4 or 6 g of LiCl per liter but not in LEB that lacked LiCl. Detection of heat-injured *L. monocytogenes* in LEB containing 8 or 10 g of LiCl per liter was inconsistent (data not shown).

In a separate set of experiments, LiCl concentrations of 0, 4, 5, 6, 7, 8, and 10 g/liter were evaluated for their effects on the resuscitation and growth of heat-injured *L. monocytogenes* F5069 in reduced LEB that contained *Enterococcus faecium* at a final concentration of 10^3 CFU/ml. *L. monocytogenes* F5069 was heat injured in raw milk at 62.8°C for 5 min. Heat-injured *L. monocytogenes* resuscitated and grew in the presence of all LiCl concentrations tested; however, an LiCl concentration of 10 g/liter consistently delayed detection. Heat-injured *L. monocytogenes* F5069 was rarely detected in reduced LEB lacking LiCl. Very similar results were obtained with *L. monocytogenes* Scott A (data not shown).

In subsequent experiments an LiCl concentration of 7 g/liter was found to be optimal for both suppressing growth of the background microflora, including *Enterococcus faecium*, and permitting resuscitation and growth of heat-injured *L. monocytogenes*. While phosphate buffer decreased the rate of resuscitation, both the growth rate and the final cell numbers of *L.*

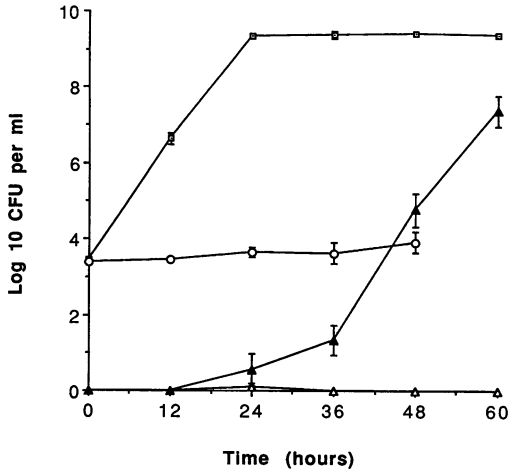

FIG. 2. Effect of growth of *Enterococcus faecium* on the resuscitation and growth of heat-injured *L. monocytogenes* F5069 in PSU broth containing 0 and 7 g of lithium chloride per liter. Raw milk containing approximately 10^7 *L. monocytogenes* cells per ml was heated for 5 min at 62.8°C, yielding approximately 10^4 viable, but heat-injured *L. monocytogenes* cells per ml; the resulting preparation was then inoculated into PSU broth containing approximately 10^3 noninjured *Enterococcus faecium* cells per ml, and the culture was incubated at 30°C. Symbols: ⊡, *Enterococcus faecium*, no LiCl, aerobic TSAYE; △, *L. monocytogenes*, no LiCl, aerobic MOX agar; ○, *Enterococcus faecium*, 7 g of LiCl per liter, aerobic TSAYE; ▲, *L. monocytogenes*, 7 g of LiCl per liter, aerobic MOX agar. The values are the means of the values obtained from three replicates of the same experiment. The bars indicate ±1 standard error.

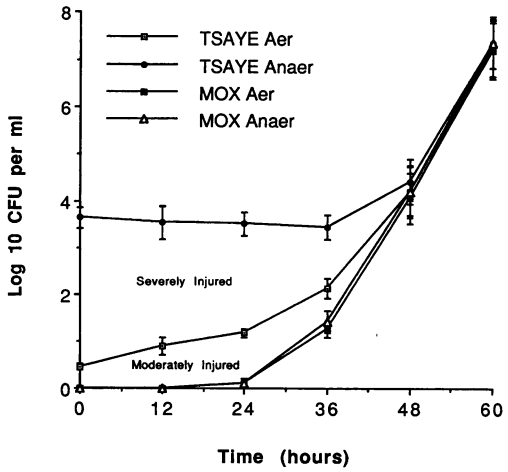

FIG. 3. Resuscitation of *L. monocytogenes* cells with different degrees of heat injury in PSU broth. Sterile milk containing approximately 10^7 *L. monocytogenes* cells per ml was heated for 5 min at 62.8°C, yielding approximately 10^4 viable but heat-injured *L. monocytogenes* cells per ml of PSU broth; the resulting preparation was incubated at 30°C. No *Enterococcus faecium* cells were added to the PSU broth in this experiment. The values are the means of the values obtained from three replicates of the same experiment. The bars indicate ±1 standard error. Aer, aerobic; Anaer, anaerobic.

monocytogenes F5069 or Scott A were higher when phosphates were incorporated into LEB (data not shown).

PSU broth. The population size of the background microflora in PSU broth (which initially contained approximately 10^4 heat-injured *L. monocytogenes* cells per ml and 10^3 noninjured *Enterococcus faecium* cells per ml) was approximately 2.7×10^3 CFU/ml after 12 h and increased slowly to 8.3×10^3 CFU/ml after 48 h. Enumeration of the background microflora on TSAYE after 48 h was not possible because of overgrowth by *L. monocytogenes*. The growth rate of the background microflora remained drastically reduced through 48 h; this allowed rapid growth of resuscitated *L. monocytogenes* cells from 36 to 60 h (Fig. 2). The sizes of the heat-injured *L. monocytogenes* F5069 populations, which recovered in PSU broth containing 7 g of LiCl per liter, were approximately 6.0×10^4 and 2.4×10^7 CFU/ml after 48 and 60 h, respectively. *L. monocytogenes* remained undetectable in PSU broth lacking LiCl, but the size of the *L. monocytogenes* population was approximately 10^7 CFU/ml in PSU broth containing 7 g of LiCl per liter (Fig. 2). Very similar results were obtained with *L. monocytogenes* Scott A and ATCC 984 (data not shown). In the absence of background microflora, the rate of resuscitation of heat-injured *L. monocytogenes* was slightly less in PSU broth than in LEB (data not shown). At 24 h, false-positive esculin hydrolysis reactions were detected in PSU broth lacking LiCl, whereas PSU broth containing 7 g of LiCl per liter yielded only true *Listeria*-positive reactions at 48 and 60 h (data not shown).

Resuscitation of cells with different degrees of injury in PSU broth. When initial populations (1.0×10^7 CFU/ml) of *L. monocytogenes* F5069 were heated at 62.8°C for 5 min in sterile milk, 100% of the surviving cells were injured (no colonies appeared on aerobic MOX agar after 0 and 12 h of incubation in PSU broth) (Fig. 3); identical results were obtained with *L. monocytogenes* Scott A (data not shown). When we used our definitions for the different degrees of injury (see Materials and Methods), we found that at time zero severely injured cells (TSAYE/anaerobic − TSAYE/aerobic) accounted for 99.9 and 99.6% of the remaining viable *L. monocytogenes* F5069 (Fig. 3) and Scott A (data not shown) cells, respectively. Moderately injured cells (TSAYE/aerobic − MOX/aerobic) accounted for 0.06 and 0.4% of the remaining viable *L. monocytogenes* F5069 (Fig. 3) and Scott A (data not shown) cells, respectively. These moderately injured cells took 48 h to form visible colonies on aerobic TSAYE at 30°C. After *L. monocytogenes* F5069 was heated for 8 min at 62.8°C, all cells were determined to be severely heat injured, because at time zero colonies of *L. monocytogenes* developed on strictly anaerobic TSAYE but not on aerobic TSAYE (data not shown). *L. monocytogenes* F5069 heated in raw milk at 62.8°C for 10, 15, and 20 min was consistently recovered after 48, 96, and 144 h of incubation in strictly anaerobic PSU broth, respectively, as determined by the formation of *L. monocytogenes* colonies on aerobic MOX agar.

DISCUSSION

Many authors have stated that salts in general inhibit the resuscitation of injured food-borne pathogens. Indeed, the inability of injured cells to grow in media containing 4 to 7.5% NaCl has traditionally been used to define what is meant by an injured cell (7, 8, 17, 25, 40). While low concentrations of NaCl do not inhibit either injured pathogens or background microflora, we found that a low concentration (0.7%) of a different monovalent salt, LiCl, totally inhibits the growth of uninjured *Enterococcus faecium* but has little or no effect on the resuscitation of heat-injured *L. monocytogenes* (Fig. 2). Cox et al. (9) determined that 3 g of LiCl per liter severely inhibited the growth of uninjured *Enterococcus faecalis*. It is doubtful that in our study the low concentration of LiCl in PSU broth (0.7%)

directly stimulated recovery, as severely heat-injured *L. monocytogenes* can recover just as rapidly in the absence of LiCl (30). Heat-injured cells were more sensitive to higher concentrations of LiCl (1.5%), which is consistent with other findings that heat-injured cells are more sensitive to higher concentrations of salts and cannot recover in their presence (17, 40, 43).

Enterococcus faecium exhibited different sensitivities to NaCl and LiCl probably because these salts have different antimicrobial mechanisms of action. High concentrations of NaCl inhibit bacteria by increasing osmotic stress and cellular plasmolysis (42). In contrast, lower concentrations of LiCl appear to inhibit bacterial growth by competing with essential divalent cations, such as calcium and magnesium (3) (Fig. 1). It is possible that critical metalloenzymes are inactivated when the monovalent cation Li^+ replaces divalent cations on these enzymes. Umeda et al. (47) demonstrated that pyruvate kinase from *Escherichia coli* K-12 was strongly inhibited by 50 mM LiCl and suggested that lithium inhibited this enzyme by substituting for potassium on the enzyme. The concentration of LiCl in PSU broth (7 g/liter or 165 mM) may be high enough to have a similar inhibitory effect on metalloenzymes in *Enterococcus faecium*. The hydrated ionic radius of calcium (0.321 nm) is closer to that of lithium (0.340 nm) than to that of magnesium (0.467 nm) (3). This may explain the better growth of *Enterococcus faecium* around the well containing $CaCl_2$ than around the well containing $MgCl_2$ (Fig. 1).

Lithium chloride has been used in solid media to detect *Staphylococcus aureus* in foods (9). Fraser and Sperber (18) were the first workers to report the use of LiCl (3 g/liter) in a secondary enrichment broth for *Listeria* spp. Later, van Netten et al. (48) used 10 g of LiCl per liter of L-PALCAMY selective enrichment broth to isolate *Listeria* spp. from foods. The latter authors concluded that it was not possible to use selective agents, such as LiCl, to inhibit background microflora and still detect injured cells. In contrast, we present evidence in this paper that heat-injured *L. monocytogenes* can be detected in the presence of a significant level of LiCl (7 g/liter), because even severely heat-injured *L. monocytogenes* cells are more resistant to LiCl than noninjured *Enterococcus faecium* cells are (Fig. 2). This resistance of heat-injured *L. monocytogenes* to LiCl does not appear to be a physiological effect of growth temperature (43°C) because *L. monocytogenes* cells that were grown at 35°C and then heat injured at 62.8°C for 5 min resuscitated and grew in LEB containing 10 g of LiCl per liter. It is important to realize that the MIC of LiCl for heat-injured *L. monocytogenes* is the concentration that prevents resuscitation, which is much lower than the concentration which prevents growth.

To gain a better understanding of the relationship between degree of injury and speed of recovery, injured cells were divided into two categories (severely injured and moderately injured) on the basis of their ability to resuscitate in different combinations of media and oxygen conditions (Fig. 3). Note that all cells heated at 62.8°C for 5 min were initially injured, as no colonies of *L. monocytogenes* appeared on MOX agar plates until after 12 h of preenrichment in PSU broth. Interestingly, counts on anaerobic TSAYE did not increase significantly during the first 36 h, while counts on aerobic TSAYE did increase significantly, indicating that severely injured cells were recovering. The recovery of severely injured cells continued after 36 h until there was no significant difference between anaerobic TSAYE and aerobic TSAYE cultures (Fig. 3). The data described above, along with our finding that some cells in cultures heated at 62.8°C for 10 min (a heat treatment that severely heat injures all cells) could be recovered from PSU broth after 48 h of incubation at 30°C (data not shown), support the hypothesis that some severely heat-injured cells from cultures that had been heated for 5 min at 62.8°C were recovered from PSU broth after 48 h. It is very likely that the few moderately injured cells recovered and initiated growth first, while the very severely injured cells took longer than 48 h to recover. This hypothesis is consistent with previous findings that very severely injured cells take much longer to recover both in selective enrichment broth (10) and on nonselective agar (30). Further research with solid PSU agar in place of PSU broth will be needed to enumerate the organisms and thus accurately determine the relationship between degree of heat injury and time course of recovery in this new recovery-enrichment system.

Severely injured cells need a much longer time for resuscitation than cells with slight injuries (40). In this study *L. monocytogenes* heated in raw milk at 62.8°C for 5, 10, 15, and 20 min could be consistently recovered only after 36, 48, 96, and 144 h, respectively. Therefore, the use of a 6-h preenrichment in aerobic, nonselective broth, as currently recommended by the FDA for detection of injured *Listeria* spp. (23), may not permit resuscitation of moderately or severely heat-injured cells. Incubation in nonselective broth for longer periods of time would not be effective either, because overgrowth by background microflora would inhibit resuscitation and growth (Fig. 2). This was the reason why heat-injured *L. monocytogenes* could not be recovered in media lacking LiCl. Consistent detection was achieved in strictly anaerobic PSU broth because both oxygen toxicity and background microflora were inhibited, while resuscitation of severely heat-injured cells was not. Severely heat-injured cells cannot respond rapidly to various stresses because protein synthesis is inactive (44), and thus these cells are subject to both a buildup of toxic oxygen intermediates (30) and overgrowth by background microflora (12, 43) during extended incubation in enrichment media.

Some of the most problematic members of the background microflora in procedures to detect *L. monocytogenes* in foods are the enterococci (4), because they can be present as both pre- and postpasteurization contaminants and because they hydrolyze esculin to produce false-positive reactions in secondary enrichment broth (18, 51). We demonstrated in this study that *Enterococcus faecium* can also cause false-negative results (Fig. 2). A few of the moderately heat-injured cells were able to resuscitate in the presence of large numbers of background microflora but were unable to replicate (Fig. 2). Therefore, these cells would probably have remained undetected with currently used methods. In this study, increasing the LiCl concentration to 7 and 10 g/liter totally eliminated false-positive and false-negative results in PSU broth and Fraser broth, respectively.

Growth of the background microflora can affect the resuscitation of injured cells in various ways. Bailey et al. (2) attributed the lack of resuscitation of heat-injured *L. monocytogenes* in LEB to the low pH (pH <5.2) of this medium when background microflora was present. Enterococci can survive heating at 60°C for 30 min and can subsequently ferment a variety of carbohydrates to rapidly lower the pH of their growth medium to pH 4.2 to 4.6 or less (37). However, in this study, *L. monocytogenes* cells that were heat injured for 5 min at 62.8°C could not be detected in the absence of LiCl even when the pH of phosphate-buffered LEB was 6.45 after 48 h. This might be explained in part by nutrient depletion due to the growth of the background microflora (7). In this study, addition of nutrients (Casitone, soytone, glucose, and yeast extract) to buffered, spent LEB (broth in which *Enterococcus faecium* was allowed to grow for 48 h at 30°C before the medium was filter sterilized) only slightly increased detection

of heat-injured *L. monocytogenes* (data not shown). Therefore, factors other than low pH and lack of nutrients, such as production of antagonistic metabolites by the background microflora (20, 45), may also be responsible for inhibiting the resuscitation and growth of heat-injured *L. monocytogenes* in nonselective laboratory media.

Detection of injured *L. monocytogenes* in foods by classical or modern methods has been limited by the ineffectiveness of the currently used enrichment systems (4). Hitchins (23) stated that no well-characterized preenrichment culture procedure for repairing stress-damaged *Listeria* spp. has been developed. Recently, the three most popular methods for detecting *L. monocytogenes* were found to yield more than 25% false-negative results (22). Various combinations of oxygen-mediated toxicity, overgrowth by background microflora, and inhibition by selective agents may explain why injured cells of *L. monocytogenes* are sometimes not detected with currently used methods. Preliminary data from workers in our laboratory indicate that even moderately injured cells (cells that recover on aerobic nonselective media) are not detected by the USDA and FDA primary enrichment media now in use. A negative laboratory result obtained with the currently used methods may provide a false sense of security, as *L. monocytogenes* cells that are only moderately injured may recover and grow in extended-shelf life, refrigerated foods that are typically nonselective in nature (33). More severely heat-injured cells may recover in those food systems that provide a low-O_2 environment because of a vacuum, modified atmospheres, and/or growth of background microflora that deplete O_2. Research is currently under way in our laboratory to compare the effectiveness of the PSU broth system with the effectiveness of the UVM, LEB, and L-PALCAMY broth systems for detection of heat-injured *L. monocytogenes* in various foods.

The PSU broth system appears to overcome all previously mentioned obstacles and has other advantages over currently used systems. The PSU broth system allows both resuscitation and growth to occur in one vessel, resulting in a one-step, rather than two-step, recovery-enrichment procedure. This results in significant savings of time and money, since two different detection methods may not be required, as previously recommended (22), and no preparation and use of secondary enrichment media are necessary (34). The high *Listeria*-to-background organism ratio in PSU broth (Fig. 2) guarantees a strong, clear signal for detection of low numbers of injured *L. monocytogenes* cells. Combining the PSU broth system with modern molecular methods (38, 39, 50) should result in a hybrid system that is simple, inexpensive, and highly specific for the detection of heat-injured *L. monocytogenes* in foods containing background microflora. The strategy outlined in this paper might also be applied to the detection of numerous other facultatively anaerobic, food-borne pathogens, including *Salmonella* spp., *Escherichia coli*, *Staphylococcus aureus*, *Shigella* spp., *Yersinia* spp., *Vibrio* spp., etc.

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