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Development of a Repair-Enrichment Broth for Resuscitation of Heat-Injured *Listeria monocytogenes* and *Listeria innocua*

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The ability of the divalent cations magnesium, iron, calcium and manganese; yeast extract; pyruvate; catalase; and the carbohydrates glucose, lactose, sucrose, esculin, fructose, galactose, maltose, and mannose to facilitate repair of heat-injured *Listeria monocytogenes* and *Listeria innocua* was evaluated. *Listeria* populations were injured by heating at 56°C for 50 min. To determine the effects on repair, Trypticase soy broth (TSB) was supplemented with each medium component to be evaluated. Repair occurred to various degrees within 5 h in TSB supplemented with glucose, lactose, sucrose, yeast extract, pyruvate, or catalase. Chelex-exchanged TSB was supplemented with divalent cations; magnesium and iron cations were found to have a role in repair. *Listeria* repair broth (LRB) was formulated by utilizing the components that had the greatest impact upon repair. When incubated in LRB, heat-injured *Listeria* cells completed repair in 5 h. After the repair, acriflavin, nalidixic acid, and cycloheximide were added to LRB to yield final concentrations identical to those of the selective enrichment broths used in the procedures of the Food and Drug Administration and the U.S. Department of Agriculture. The efficacy of LRB in promoting repair and enrichment of heat-injured *Listeria* cells was compared with that of existing selective enrichment broths. Repair was not observed in the Food and Drug Administration enrichment broth, *Listeria* enrichment broth, or University of Vermont enrichment broth. The final *Listeria* populations after 24 h of incubation in selective enrichment media were 1.7×10^8 to 9.1×10^8 CFU/ml; populations in LRB consistently averaged 2.5×10^{11} to 8.2×10^{11} CFU/ml.

Listeria monocytogenes is a food-borne pathogen that causes severe and often fatal infections in susceptible human hosts. Food-borne illness outbreaks with high mortality rates involving *L. monocytogenes* serotype 4b have been documented in North America. An outbreak in the Maritime Provinces of Canada in 1981 was caused by the consumption of contaminated cole slaw. Two of 7 (29%) infected adults and 17 out of 34 (49%) infants died despite appropriate antibiotic therapy (35). Pasteurized milk was implicated as the vehicle of infection in an outbreak of listeriosis that occurred in Massachusetts in 1983. Seven cases involved fetuses or infants, and 42 cases involved immunosuppressed adults. The mortality rate recorded in this outbreak was 39% (14). A third listeriosis outbreak occurred in California in 1985; the mode of transmission involved the consumption of Mexican-style cheese. A total of 145 cases were reported, and 46 deaths occurred (32% mortality rate). Of the afflicted individuals, 67% were mother-infant pairs (26).

Detection of *L. monocytogenes* in suspect food products or food processing environments is accomplished by use of a variety of standard or rapid procedures. Among the most widely used are protocols devised by the U.S. Department of Agriculture-Food Safety Inspection Service for the detection of *L. monocytogenes* in meats (7, 25, 32) and the Food and Drug Administration for the detection of *L. monocytogenes* in dairy products (29). Also widely used are rapid commercial procedures that employ enzyme-linked immunosorbent assay or DNA probe technology for *L. monocytogenes* detection (23, 31). All of these procedures use highly selective enrichment media to facilitate growth of *Listeria* cells over competitive background flora. These highly selective enrichment procedures do not account for the recovery of sublethally injured *Listeria* cells that could exist within a variety of heated, frozen, or acidified foods.

It is well recognized that *L. monocytogenes* can be injured as a result of exposure to a variety of processing treatments, including sublethal heating, freezing, exposure to sanitizing compounds, and exposure to acids (4, 6, 8, 17, 18, 37). Injured *Listeria* cells can no longer tolerate selective agents to which they would be otherwise resistant. Therefore injured *Listeria* cells are not recoverable by procedures that employ selective media. The need for a procedure that is efficient in recovering sublethally injured *Listeria* cells has recently been expressed by numerous investigators (6, 10, 12, 17, 27, 37, 39). Detection of injured *Listeria* cells in food products is essential to ensure public health safety. Sublethally injured bacteria are capable of repair in food products and are able to regain pathogenic potential (40).

The objective of this study was to evaluate the ability of specific medium components, including carbohydrates, minerals, yeast extract, pyruvate, and catalase, to facilate repair of heat-injured *L. monocytogenes* and *Listeria innocua*. The components that promoted repair were then used to devise a repair-enrichment medium for repair of heat-injured listeriae. The efficacy of the devised repair-enrichment medium was then compared with those of existing selective enrichment procedures.

MATERIALS AND METHODS

Bacterial cultures. The strains of *L. monocytogenes* used in this study represented the serotypes most frequently isolated from food products (14). *L. monocytogenes* F5069 (serotype 4b) and *L. monocytogenes* F5027 (serotype 1a) were obtained from Robert Weaver, Centers for Disease Control, Atlanta, Ga. *L. innocua* CWD350, a dairy plant environmental isolate, was also used in this study. All cultures were maintained at −70°C in 10% glycerol.

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Media. The nonselective plating medium used in these studies to evaluate injury in bacterial populations was tryptose phosphate agar (TPA), which contained 20.0 g of tryptose (Difco Laboratories, Detroit, Mich.), 2.0 g of glucose, 5.0 g of NaCl, 2.5 g of Na_2HPO_4, and 15.0 g of agar per liter of distilled water. TPA supplemented with 4% NaCl (TPAN) was used as the selective plating medium. Tryptose phosphate broth (TPB) was used as the growth medium for obtaining 18-h cultures and also as the heating menstruum for injury studies. TPB is identical to TPA but without the agar.

To evaluate the effect of individual medium components on repair of heat-injured listeriae, Trypticase soy broth (TSB; BBL Microbiology Systems, Cockeysville, Md.) was prepared with 30.0 g of TSB per liter of distilled water. TSB was supplemented with each individual medium component under evaluation. The carbohydrates evaluated were glucose, lactose, sucrose, esculin, fructose, galactose, maltose, and mannose at concentrations of 0.5, 1.0, 2.0 and 3.0%. Tryptone base broth, a carbohydrate-free control, was prepared with 20.0 g of tryptone (Difco), 5.0 g of NaCl, and 2.5 g of Na_2HPO_4 per liter of distilled water.

To examine the effect of divalent cations on repair of heat-injured *Listeria*, TSB was first subjected to cation exchange with Chelex 100 cation-exchange resin (sodium form, 200-400 mesh; Bio-Rad Laboratories, Richmond, Calif.) to remove divalent cations (43). The medium was exchanged at 25°C in a Pyrex column (66 by 4.5 mm [inner diameter]) at a flow rate of 10 ml/min. After the medium exchange, the resin was regenerated with 2 bed volumes of 1 N HCl, 5 bed volumes of distilled water, 4 bed volumes of acetate buffer (pH 6.2), and 5 bed volumes of distilled water. Chelex-exchanged TSB (CE-TSB) was supplemented with magnesium sulfate, ferrous sulfate, calcium chloride, or manganese chloride to yield cation concentrations of 1.0, 5.0, 10.0, 20.0, and 30.0 mM. CE-TSB served as the control medium in mineral evaluation. Mineral analysis of unexchanged, exchanged, and mineral supplemented CE-TSB was performed by using an inductively coupled argon plasma spectrophotometer (Thermo Jarrel Ash 61, Franklin, Mass.) as recommended by the manufacturer.

TSB supplemented with 0.6% yeast extract, 1.0% pyruvate (Sigma Chemical Co., St. Louis, Mo.), or 0.04% filter-sterilized catalase (Sigma) was separately evaluated.

To compare repair rates of heat-injured listeriae in selective enrichment broths, the following broths were examined: University of Vermont modified *Listeria* enrichment broth (UVM broth) (Difco) (13), Food and Drug Administration enrichment broth (FDA broth) (28), and *Listeria* enrichment broth (LEB) (31). The pH of all recovery broths was 7.2 ± 0.1. All media were autoclaved for 15 min at 121°C.

Heat injury and repair procedure. An 18-h *Listeria* culture was grown at 37°C in 100 ml of TPB inoculated with a loopful of frozen stock culture. Eight milliliters of the 18-h culture was transferred into 800 ml of TPB in a 2.8-liter Fernbach flask that was preheated for 30 min in a 56°C water bath. The temperature of the broth (55.5°C) was monitored with a digital thermometer (VWR model 100). Immediately after inoculation, the broth was mixed and 1.0 ml was removed and placed on ice. This sample was diluted in cold Butterfield phosphate-buffered diluent (33) and plated on TPA and TPAN by using a spiral plater (model D; Spiral Systems, Inc., Cincinnati, Ohio) to determine the initial concentration (16). The flask was heated with gentle agitation for exactly 50 min. Aliquots of 200 ml were transferred to 250-ml centrifuge bottles, cooled on ice, and centrifuged at 10,000 × *g* for 10 min. Cell pellets were resuspended in 100 ml of the appropriate recovery broth. One milliliter was removed, diluted in phosphate-buffered saline, and plated on TPA and TPAN to determine the percent injury. Recovery broths were incubated at 37°C, and samples were removed at 1-h intervals and plated as described above. Plates were incubated at 30°C and counted at 72 h. All plating was performed in duplicate.

Determination of percent injury. The percent injury in each population of heat-stressed cells was determined by a comparison of counts on selective (TPAN) versus nonselective (TPA) agar. Nonselective media support growth of both injured and noninjured cells, whereas selective media only support growth of noninjured cells. The percent injury is calculated by using the equation [1 − (count on selective agar/count on nonselective agar)] × 100.

RESULTS

Heat injury of *L. monocytogenes* F5069. *L. monocytogenes* F5069 was chosen as the test organism for studies evaluating the effect of specific medium components on repair of heat injury. Initial populations of *L. monocytogenes* F5069 (2.8×10^7 cells per ml) heated at 56°C for 50 min consistently recorded injury in the range of 98.1 to 99.9%. Populations on nonselective media (TSA) were normally present at a level of 3.6×10^3 to 1.5×10^5 CFU/ml after heat treatment, whereas populations on selective media (TSAN) typically ranged from 2.0×10^1 to 5.3×10^3 CFU/ml. To study the impact on repair, TSB was chosen as the nonselective basal medium. TSB supported only modest growth of heat-injured *L. monocytogenes* F5069 during a 5-h period (Fig. 1), making it an ideal medium for this study. TSB did support repair of heat-injured *L. monocytogenes* F5069 (Fig. 1), yet an extensive lag period (2 h) was observed before the initiation of repair. In TSB alone, total repair was not accomplished within 5 h, and populations on selective media reached levels of only 3.0×10^2 CFU/ml. To determine whether the presence of supplements in TSB could accelerate repair, yeast extract (0.5%) and glucose (0.5%) were added to TSB. The rate and extent of repair were accelerated when these components were added to TSB (Fig. 1). In 5 h, cells incubated in TSB-glucose increased from 5.0×10^1 CFU/ml to 3.2×10^3 CFU/ml and those incubated in TSB-yeast extract increased from 5.0×10^1 CFU/ml to 5.0×10^2 CFU/ml when plated on TPAN. Tryptone broth, a basal medium devoid of a carbohydrate source, was also used as a control medium to evaluate the absence of carbohydrates on repair of heat-injured *L. monocytogenes* F5069. In tryptone medium, virtually no repair was recorded during a 5-h period. Colony counts were initially 2.0×10^1 CFU/ml and only reached 6.0×10^1 CFU/ml after 5 h of incubation in tryptone medium (Fig. 1). These results suggested a role for yeast extract and glucose in facilitating repair of heat-injured *L. monocytogenes*.

Effect of carbohydrates on repair of *L. monocytogenes* F5069. Carbohydrates other than glucose were evaluated for their effect on promoting repair of heat-injured *L. monocytogenes* F5069. The additional carbohydrates tested were lactose, sucrose, esculin, fructose, galactose, maltose, and mannose at concentrations of 0.5, 1.0, 2.0, and 3.0%. TSB supplemented with 0.5% glucose, lactose, or sucrose showed the greatest effect on repair in 5 h (Table 1). When 0.5% glucose, sucrose, or lactose was added to TSB, the colony counts on selective media increased 51.4, 51.4, and 48.6%, respectively. TSB supplemented with carbohydrates

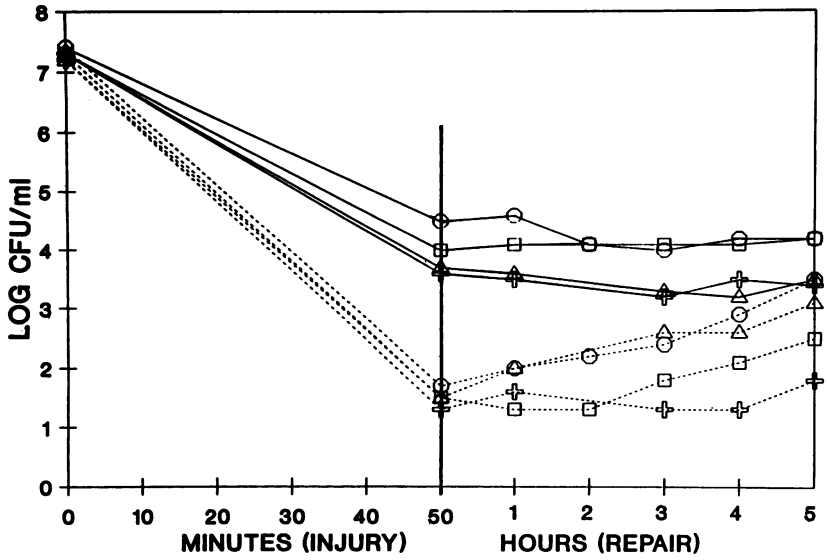

FIG. 1. Effect of TSB (□), TSB-yeast extract (△), TSB-glucose (○), or tryptone broth (✣) on the repair of heat-injured *L. monocytogenes* F5069 grown on TPA (——) or TPAN (– – –).

at concentrations higher than 0.5% facilitated repair to a lesser degree (data not shown). TSB supplemented with mannose, fructose, galactose, or esculin had less of an impact on repair than did TSB alone. Esculin, a carbohydrate frequently incorporated in *Listeria* enrichment media, had the least effect on repair of heat-injured *L. monocytogenes* F5069, allowing populations to increase by only 3.3% within 5 h (Table 1).

Effect of divalent cations on repair of *L. monocytogenes* F5069. The effect of divalent cations on repair of heat-injured *L. monocytogenes* F5069 was explored. Divalent cations were removed from TSB through cation exchange. Mineral analysis was performed to determine magnesium, iron, calcium, and manganese concentrations of unexchanged TSB and cation-exchanged TSB (Table 2). Magnesium and calcium were present at levels of 1,223.4 ± 78.9 μM/ml and 430.8 ± 14.3 μM/ml, respectively, in unexchanged TSB. Levels of these cations were reduced to 0.0 μM/ml for Mg^{2+} and 1.0 ± 0.4 μM/ml for Ca^{2+} after cation exchange. Iron was reduced from 10.4 ± 0.3 μM/ml to 2.4 ± 0.9 μM/ml, whereas manganese was reduced from 0.5 ± 0.2 μM/ml to 0.0 μM/ml. CE-TSB, used as the control medium in these studies, failed to support repair of heat-injured cells. Iron, magnesium, calcium, and manganese were added to CE-TSB at concentrations of 1.0 mM either alone (Fig. 2) or in combination with 30 mM magnesium (Fig. 3). Of the added cations, Fe^{2+} appeared to have the greatest impact upon repair. Calcium had a limited effect on repair, whereas with manganese as the sole cation injured and noninjured cells were killed. CE-TSB without mineral supplementation failed to support repair of injured cells.

Effect of sodium pyruvate and catalase on repair of *L. monocytogenes* F5069. The effect of the oxygen-scavenging compounds pyruvate and catalase on repair of heat-injured *L. monocytogenes* was studied. TSB supplemented with 1.0% sodium pyruvate or 0.04% catalase supported repair of injured cells within 5 h (Fig. 4). The initial colony counts were 2.9×10^2 and 1.2×10^2 CFU/ml, which increased to 1.5×10^4 and 7.4×10^3 CFU/ml in the presence of pyruvate and catalase, respectively.

LRB. By utilizing components that exhibited the greatest impact on repair, *Listeria* repair broth (LRB) was formulated. LRB consisted of 30.0 g of TSB, 5.0 g of glucose, 6.0 g of yeast extract, 4.94 g of magnesium sulfate, 0.3 g of ferrous sulfate, 10.0 g of pyruvic acid (sodium salt), 8.5 g of 3-*N*-morpholinepropanesulfonic acid (MOPS)-free acid (Sigma), and 13.7 g of MOPS sodium salt (Sigma) per liter of distilled water. LRB was evaluated for its ability to effect repair of heat-injured *L. monocytogenes* F5069 and F5027 and *L. innocua* CWD350. The repair curves were similar for all organisms, with repair initiating at 2 h and nearly com-

TABLE 1. Effect of carbohydrates on the repair of heat-injured[a] *L. monocytogenes* F5069

Carbohydrate added to TSB at 0.5%	Log_{10} CFU[b]		% Increase in log_{10} CFU
	0 h	5 h	
Glucose	1.7	3.5	51.4
Sucrose	1.7	3.5	51.4
Lactose	1.8	3.5	48.6
TSB control	1.6	2.9	44.8
Mannose	1.9	2.9	34.5
Fructose	2.0	2.8	28.6
Galactose	2.3	3.0	23.3
Esculin	2.9	3.0	3.3

[a] Percent injury in *Listeria* populations after heating ranged from 99.4 to 99.9%.

[b] Counts of *L. monocytogenes* F5069 on selective plating medium (TSAN).

TABLE 2. Concentrations of cations in unexchanged TSB and CE-TSB

Medium	μM of (mean ± SD)[a]			
	Mg^{2+}	Fe^{2+}	Ca^{2+}	Mn^{2+}
TSB	1,223.4 ± 78.9	10.4 ± 0.3	430.8 ± 14.3	0.5 ± 0.2
CE-TSB	0.0	2.4 ± 0.9	1.0 ± 0.4	0.0

[a] Values represent the means of four analyses.

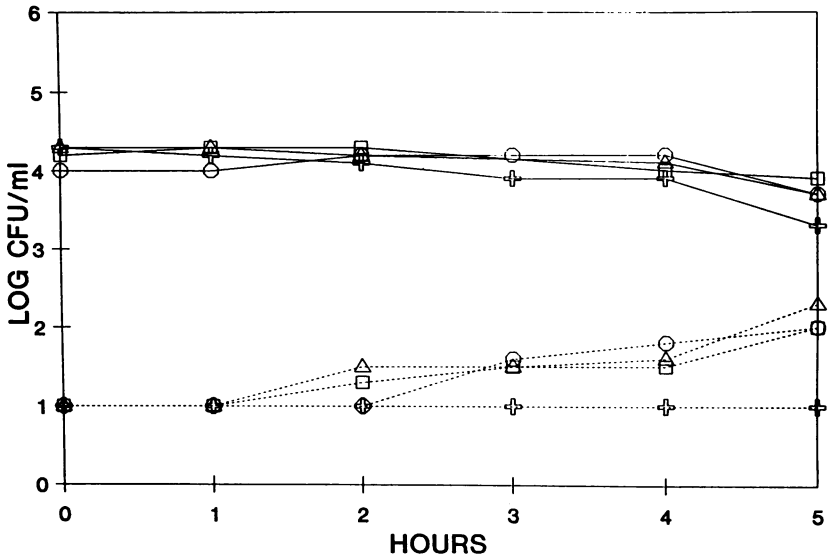

FIG. 2. Effect of Mg^{2+} (○), Fe^{2+} (△), or Ca^{2+} (□) at 1.0 mM or CE-TSB (✣) on the repair of heat-injured *L. monocytogenes* F5069 grown on TPA (——) or TPAN (– – –).

plete at 5 h of incubation. Filtered-sterilized acriflavine, nalidixic acid, and cycloheximide were then added to LRB to yield final concentrations identical to those in FDA and U.S. Department of Agriculture-Food Safety Inspection Service (USDA-FSIS) enrichment broths. LRB containing selective agents was incubated for 19 ± 1 h at 30°C. The plate counts at 24 h (total incubation time) averaged 2.5×10^{11} to 8.2×10^{11} CFU/ml (Fig. 5 and 6).

Repair of heat-injured *L. monocytogenes* F5069 and F5027 and *L. innocua* CWD350 in FDA enrichment broth, LEB, and UVM enrichment broth was also evaluated. Due to the highly selective nature of these media, injured cells could not repair themselves; the colony counts on selective agar increased only slightly after 5 h of incubation. The initial population levels of *L. monocytogenes* F5069 and F5027 and *L. innocua* CWD350 averaged 1.5×10^2 CFU/ml and increased to only 3.2×10^2 CFU/ml after 5 h of incubation on selective media. In contrast, cells increased from an average of 1.7×10^2 CFU/ml to 7.2×10^3 CFU/ml during 5 h of incubation in LRB. The final achievable populations (at 24 h) averaged 4.5×10^{11} CFU/ml in LRB and 4.4×10^8 CFU/ml in selective enrichment broths. The differences in these final achievable counts reflect the growth of injured cells that have repaired themselves in LRB.

The influence of pH on the repair of injured listeriae was evaluated. The initial pH readings of all repair broths were 7.2 ± 0.1. Readings taken at 5 h were essentially unchanged (7.1 ± 0.1) (data not shown). This observation eliminates the possibility of low pH preventing the repair of injured cells in these highly selective enrichment broths.

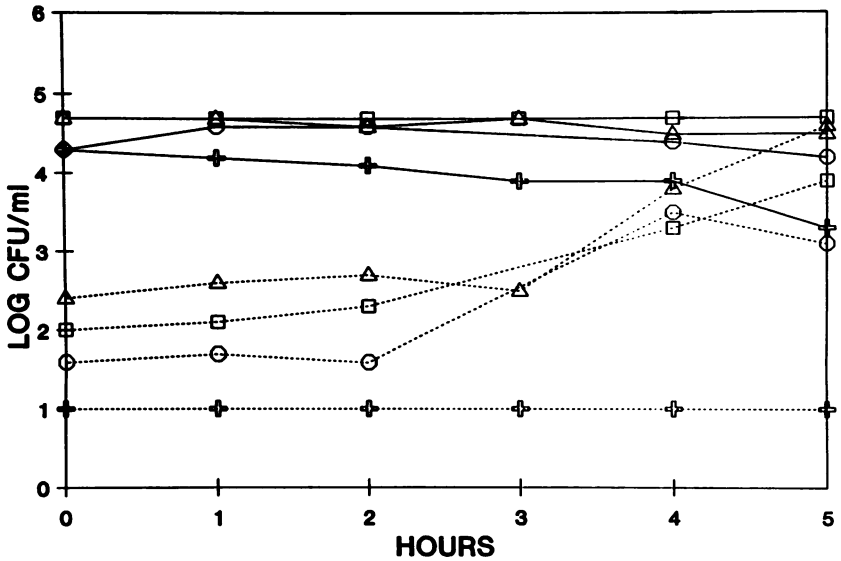

FIG. 3. Effect of Mg^{2+} at 30.0 mM plus Fe^{2+} (△), Ca^{2+} (□), or Mn^{2+} (○) at 1.0 mM or CE-TSB (✣) on the repair of heat-injured *L. monocytogenes* F5069 grown on TPA (——) or TPAN (– – –).

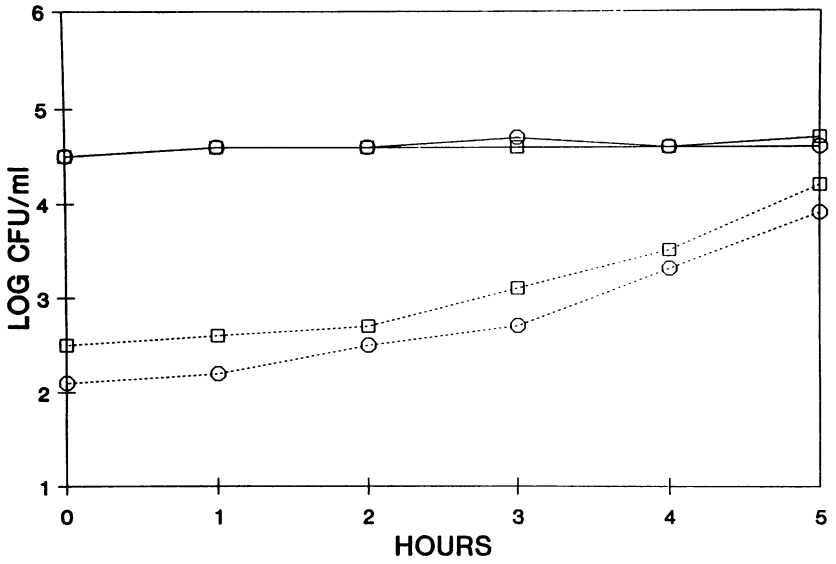

FIG. 4. Effect of pyruvate (□) or catalase (○) on the repair of heat-injured *L. monocytogenes* F5069 grown on TPA (——) or TPAN (– – –).

DISCUSSION

In a recent publication, Bailey et al. (1) reported that UVM enrichment broth consistently allowed for recovery of heat-injured *L. monocytogenes*, and these authors have recommended the use of UVM as a primary enrichment broth to effect recovery of heat-injured *L. monocytogenes*. In contrast to these results and recommendations, we failed to observe the ability of heat-injured *L. monocytogenes* to undergo repair in UVM enrichment broth. The discrepancy in these results may be due to the fact that Bailey et al. enumerated viable counts of *Listeria* that were capable of growth during a 24-h enrichment period in UVM broth and recorded higher counts in UVM enrichment broth than in FDA enrichment broth during 24 h of incubation. These authors attributed the differences in these counts to the recovery of injured organisms. These differences do not reflect repair of injured cells but in fact reflect higher growth rates of uninjured cells. Had Bailey et al. constructed repair curves, perhaps they would have observed differences in counts on selective plating medium versus nonselective plating medium over time. In their experimental design, populations of heat-injured cells were added to enrichment media, and differences in viable counts as a result of growth in selective media were compared. This experimental design does not allow for the examination of the fate of injured bacteria over time. Our results show a difference between LRB and UVM broth in the recovery of heat-injured listeriae. The differences in growth potential in UVM broth versus LRB reflect the ability of LRB to allow for repair of heat-injured cells.

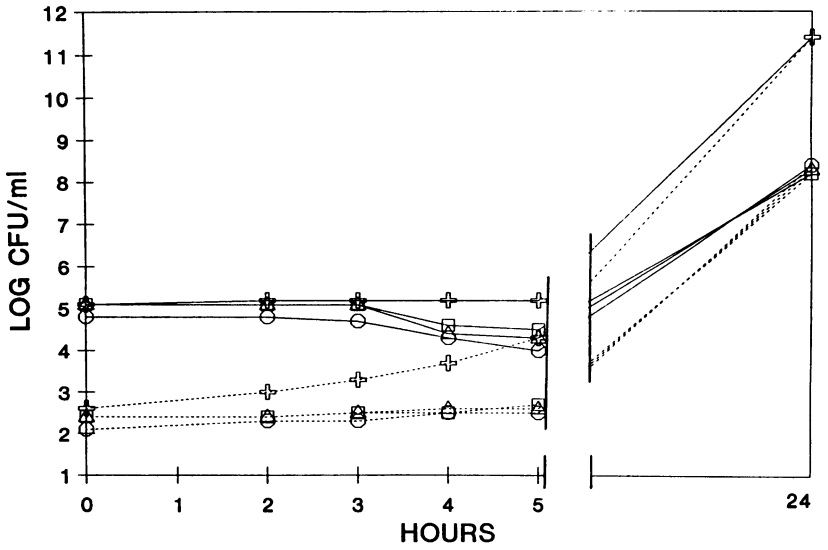

FIG. 5. Effect of FDA enrichment broth (□), LEB (△), UVM enrichment broth (○), or LRB (✣) on the repair of heat-injured *L. monocytogenes* F5027 grown on TPA (——) or TPAN (– – –).

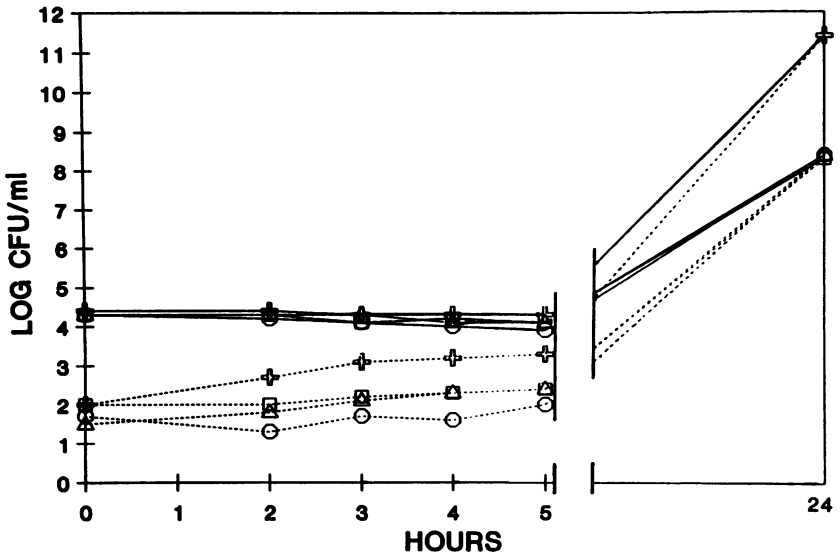

FIG. 6. Effect of FDA enrichment broth (□), LEB (△), UVM enrichment broth (○), and LRB (✣) on the repair of heat-injured *L. innocua* CWD 350 grown on TPA (——) or TPAN (– – –).

The role of specific medium components in the repair process for listeriae needs further investigation. Past research with heat-injured *Staphylococcus aureus* has revealed some information on repair mechanisms. Iandolo and Ordal (22) suggested that glucose serves as an energy source for recovering heat-injured *S. aureus*. They demonstrated that cell wall and protein syntheses were not necessary for the recovery of *S. aureus* through the addition of penicillin, cycloserine, and chloramphenicol to the recovery broth. The addition of actinomycin D prevented recovery, suggesting that RNA synthesis is required for repair. RNA synthesis is dependent on the concentration of amino acids that have leaked out through damaged membranes of heat-injured cells. Reconcentration of amino acids requires repair of the cell membrane, and both processes are mediated by energy supplied through glucose oxidation.

Hurst and Hughes (20, 21) demonstrated the magnesium requirement of *S. aureus* for repair of sublethal heat injury. The stability of ribosomes of sublethally heated *S. aureus* was also examined. When cells were heated in magnesium-chelating buffers, ribosome destruction occurred. The leakage of 260-nm-absorbing material was seen during heating; this material was shown to be RNA. These cells were not repaired in the presence of actinomycin D. Heating cells in buffers that did not cause the release of magnesium prevented ribosome destruction. These cells were capable of partial repair in the presence of actinomycin D. It was concluded that the effect on ribosomes was not due to heat but was a consequence of magnesium loss as a result of membrane damage caused by heating.

The addition of exogenous pyruvate to repair media has been shown to enhance recovery of heat-stressed *L. monocytogenes* (37) and *S. aureus* (2, 5, 38). Injured cells have decreased catalase and superoxide dismutase activities, rendering them sensitive to the lethal effects of hydrogen peroxide and the superoxide radical (3, 11, 30). Additionally, the anaerobic but not aerobic growth of heat-injured *L. monocytogenes* on the same medium has confirmed the hypothesis that the absence of oxygen is conducive to recovery (24).

The roles of yeast extract and iron in the repair of heat-injured listeriae are not yet clear. However, yeast extract is an excellent source of B-complex vitamins, for which listeriae have a requirement (36). Previous research has confirmed the requirement for thiamine and riboflavin by listeriae (15, 34, 42).

Sword (41) and Hao et al. (19) have found iron to be stimulatory for the growth of *L. monocytogenes*. A reduction in the 50% lethal dose of listeriae in the presence of additional iron was demonstrated by Sword (41). These findings prompted Cowart and Foster (9) to explore the relationship of virulence of listeriae to iron availability. Their work showed a stimulatory effect of iron on the growth of virulent strains. Iron is required for redox reactions, such as those catalyzed by catalase, peroxidase, and cytochromes. These essential compounds are possibly lost through cell membrane damage in heat injury so that they must be supplied in the recovery medium.

Failure to employ a nonselective repair-enrichment step when attempting to recover listeriae may allow heat-injured cells to escape detection. Repair of injured cells in foods presents a major public health risk. To account for these potentially deadly organisms, we recommend the use of LRB for recovery of heat-stressed *L. monocytogenes*.

ACKNOWLEDGMENT

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