Thermotolerance of Heat-Shocked *Listeria monocytogenes* in Milk Exposed to High-Temperature, Short-Time Pasteurization

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The effect of prior heat shock (48°C for 15 min) on the thermotolerance of *Listeria monocytogenes* at the minimal high-temperature, short-time (71.7°C for 15 s) parameters required by the Pasteurized Milk Ordinance was examined. The mean *D* value for heat-shocked *L. monocytogenes* was 4.6 ± 0.5 s (control *D* = 3.0 ± 1.0 s); the ratio of *D* to control *D* was 1.5. The increased thermotolerance of heat-shocked *Listeria* cells was not significant and appeared unlikely to have practical implications, in terms of risk assessment, for the safety of pasteurized milk.

Acquired thermotolerance in *Listeria monocytogenes* (4, 13, 18, 19) and other bacteria (22, 23, 26) is induced by exposing cells to a brief heat shock at a temperature within or slightly above the range for normal cell growth. Heat shock results in increased resistance to the lethal effect of a higher temperature. A direct cause-and-effect relationship between the development of this acquired thermotolerance and the synthesis of global stress proteins, however, remains controversial (1, 8, 30, 33, 35).

Bacterial heat shock response is a major issue in food safety because certain foods are thermally processed to limit public exposure to pathogens (17). Initial studies by Mackey and Derrick (22, 23) with *Salmonella* spp. in broth, liquid whole egg, and reconstituted dried milk suggested that marginal heat treatments may be inadequate in certain instances. Recent outbreaks of listeriosis were epidemiologically linked to the consumption of dairy products and meats; hence, the thermal processing parameters of these foods are being reexamined (5, 10, 13, 16). Prevention of underprocessing is necessary because uninjured *Listeria* cells can grow at refrigeration temperatures (6).

Since the 1983 outbreak of listeriosis in Massachusetts, which was epidemiologically linked to high-temperature, short-time (HTST) pasteurized milk (16), we have examined the thermotolerance of *L. monocytogenes* relative to the following experimental variables: heat shock, intracellular location (within bovine milk phagocytes), suspending men- struum (raw versus sterile milk, milk versus broth), recovery method (enrichment versus direct plating, selective versus nonselective medium), and inactivation method (immersed sealed tube versus slug flow heat exchanger) (3–6). The issue of intracellular protection from thermal inactivation was raised when no evidence of faulty pasteurization was found at the dairy plants where milk from the above-mentioned outbreak was processed (16). Intracellular shielding from heat was dismissed simply on the known thermodynamics of the pasteurization equipment design (9). An alternative hypothesis is that intracellular *Listeria* cells are in a thermotolerant (acquired) state, which is mediated by induced stress proteins that overlap with specific heat shock proteins (5). The induction of potential virulence proteins, neoantigens, and a heat shock protein(s) by bacteria after either invasion into or phagocytosis by eucaryotic cells has been

determined (2, 32). Moreover, listeriolysin, a major virulence factor of *L. monocytogenes* that mediates intracellular survival, appears to be a heat shock protein (32).

This study was undertaken to determine the degree of acquired thermotolerance conferred by prior heat shock on *L. monocytogenes* under HTST minimal pasteurization guidelines (17). Cumulative data for HTST inactivation of *L. monocytogenes* under various conditions will be summarized and interpreted.

(Results of this study were presented at the 90th Annual Meeting of the American Society for Microbiology in Anaheim, Calif., 13 to 17 May 1990.)

*L. monocytogenes* F5069 was obtained from Robert Weaver, Centers for Disease Control, Atlanta, Ga. This strain belongs to serotype 4b and was isolated from raw bovine milk (4).

Bacteria were grown at 35°C in Trypticase soy broth (TSB; BBL Microbiology Systems, Cockeysville, Md.) containing 0.6% yeast extract (YE) (Difco Laboratories, Detroit, Mich.). The shock and inactivation medium was sterile whole bovine milk, prepared as previously described (5). After heating, bacterial cells were recovered by dilution in phosphate buffer, comparative plating on the nonselective TSBYE agar and the selective MMA, LPM, and Oxford (Oxoid, Basingstoke, England) agars, and incubation for 7 days at 25°C. The composition of these agar media was previously described (6). The selective and nonselective enrichment techniques used for recovery were also previously described (6).

Bacteria were thermally inactivated in a slug flow heat exchanger (5) at the minimal HTST pasteurization standard temperature of 71.7°C. For heat shock studies, the intake supply line was adapted with a stainless steel reservoir, with a residence time of 15 min, immersed in a 48°C water bath. These heat shock conditions have been shown to induce thermotolerance (4). The holding time after heat shock was 15 s. *D* values, the time required to reduce a given viable bacterial population by 90%, were calculated as previously described (4). The tests of significance were performed on the slopes of the linear regression (1/D). The slopes were compared by analysis of variance (the slopes were normally distributed under the assumptions of the algorithms), and the differences observed were noted by using Duncan's test (4).

The effect of prior heat shock on the thermotolerance of *L. monocytogenes* at the minimal HTST holding parameters of 71.7°C for 15 s, as required by the Pasteurized Milk Ord-
nance, is shown in Table 1 (conditions 1, 2, and 10). The mean $D_{71.7^\circ C}$ value for heat-shocked *Listeria* cells was 4.6 ± 0.5 s (condition 2) (control $D_{71.7^\circ C} = 3.0 ± 1.0$ s; condition 1); the ratio of $D$ to control $D$ was 1.5. This ratio is similar to the insignificant-to-slightly significant ratios (1.2 to 1.5) determined for *L. monocytogenes* at lower inactivation temperatures (4). As stated previously (5, 21), data variation may reflect differences in the two heating methods, i.e., immersed sealed tube and slug flow heat exchanger. The data also supported the toxic effect of selective media on heat-injured cells (6, 31), although heat shock (condition 10) provided an almost twofold degree of protection compared with control values determined earlier (condition 9) (6).

Corresponding enrichment techniques did not enhance recovery in either this study (data not shown) or previous experiments (6). Although anaerobic incubation may have increased recovery (18), the applicability of this risk factor to fluid milk pasteurization, as opposed to vacuum-packaged foods (4), is limited because of the failure of milk to achieve oxygen-reduced conditions after heating (18). Moreover, survivors, if any, would be heat injured (6) and unable to compete with the large number of thermoduric milk flora for glucose, the limiting nutrient for *Listeria* growth in milk (29). Finally, pasteurization studies (5, 6, 14, 20, 27) that used extensive enrichment protocols to recover *L. monocytogenes* after processing were unable to confirm previous survival studies (12, 15).

Stress mechanisms similar to heat shock may affect facultative intracellular pathogens within host cells (2, 5, 32). Our results on acquired thermotolerance in *L. monocytogenes* correlate with our previously observed intracellular and freely suspended mean $D_{71.7^\circ C}$ values of 5.0 and 3.1 s, respectively (5); the mean observed ratio of intracellular $D$ to freely suspended $D$ was 1.6.

An overall analysis of mean $D$ values suggested a clear trend for an increase in thermotolerance by either the heat shock or intracellular location stimuli, with the results near the point of statistical significance (Table 1, conditions 1, 2, and 3) (3–5). Because the Massachusetts epidemiological study indicated a potential problem with HTST pasteurization (16), we summarized our $D$ values, which were determined at the HTST minimal guidelines under various conditions (Table 1). Both the predicted (if calculable) and the highest observed mean $D$ values are stated for a given set of conditions to illustrate the statistical interpretation. The trend in these data supports the theory that intracellularly treated *L. monocytogenes* cells may be induced to a level of thermotolerance similar to that of heat-shocked cells. This comprehensive analysis also indicates the significant inhibitory effect of raw milk flora and selective media on recovery of heat-injured *Listeria* cells. For the latter situation, the condition 9 $D_{71.7^\circ C}$ value of 1.4 ± 0.3 s was the highest mean $D$ value determined for the given experimental conditions and was obtained by the Food and Drug Administration enrichment method (6). Other selective methods produced much lower $D$ values (6), which would have significantly increased the ratio.

Clearly, to define regulatory guidelines with an adequate safety margin, the heating of either intracellular or heat-shocked *L. monocytogenes* in sterile milk, with subsequent recovery on nonselective medium, has provided upper-limit $D$ values (Table 1). Assessment of these data within an adequate risk analysis model for HTST milk pasteurization (5, 6, 25, 34) has not given convincing reasons to raise the minimum temperature or holding time (34). Critical factors have included the following: low incidence and contamination levels in farm bulk tank surveys (6, 34); various levels of *Listeria* shedding by subclinically mastitic cows, with the highest number from naturally or artificially infected animals estimated at 10^6/ml (6, 34); inhibition of *Listeria* growth and/or thermal injury recovery by raw milk flora (3, 6); unexceptional laboratory thermal inactivation kinetics for various strains, as determined by valid heating techniques (3–6, 10, 14, 21, 24); unexceptional pilot plant pasteurization

### Table 1. Comparative D values determined at HTST minimal conditions

<table>
<thead>
<tr>
<th>Condition no.</th>
<th>Expit conditions</th>
<th>Mean observed $D_{71.7^\circ C}$ (s)</th>
<th>Predicted$^d$ $D_{71.7^\circ C}$ (s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Media$^a$ -</td>
<td>3.0 ± 1.0$^b$ (6)</td>
<td>2.7</td>
<td>5; this report</td>
</tr>
<tr>
<td>2</td>
<td>Milk$^b$ -</td>
<td>4.6 ± 0.5$^b$ (3)</td>
<td>4.1</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>Intracellular$^c$ -</td>
<td>5.0 ± 0.4$^b$ (6)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Heat shock$^d$ -</td>
<td>1.3 ± 0.1 (2)</td>
<td>1.6</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>ND</td>
<td>1.9</td>
<td>3</td>
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<tr>
<td>7</td>
<td></td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>8</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>1.4 ± 0.3 (2)</td>
<td>ND</td>
<td>6</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>2.6 ± 0.1$^b$ (3)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>1.5 ± 0.1$^b$ (3)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>&lt;0.3$^b$ (2)</td>
<td>ND</td>
<td>6</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>&lt;0.3$^b$ (2)</td>
<td>ND</td>
<td>6</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ +, selective; -, nonselective.

$^b$ +, raw; -, sterile.

$^c$ +, intracellular; -, freely suspended.

$^d$ +, heat shock; -, no heat shock.

$^e$ Highest mean $D$ value obtained at conditions given. The number of observations is shown in parentheses. ND, not determined.

$^f$ Predicted values from references 3 and 5 were obtained by linear regression analysis of thermal death plots.

$^{a,b,c,d}$ Indicates negative recovery at the shortest heating interval; calculated by dividing total time interval by log initial count.
studies for inactivation of *L. monocytogenes* (20); an approximate twofold increase in thermostolerance by heat shock or intracellular stimuli, as proposed here from data presented in this study and others (4, 5, 13, 18, 27); and the additional killing of *L. monocytogenes* cells by bovine mastitic milk phagocytes (7), which may contribute to the overall inactivation of the pathogen (11). A comprehensive bovine milk pasteurization risk analysis report, using *L. monocytogenes* as the prototype pathogen, is in progress (28).

REFERENCES


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