Effects of Above-Optimum Growth Temperature and Cell Morphology on Thermotolerance of *Listeria monocytogenes* Cells Suspended in Bovine Milk

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The thermotolerances of two different cell forms of *Listeria monocytogenes* (serotype 4b) grown at 37 and 42.8°C in commercially pasteurized and laboratory-tyndallized whole milk (WM) were investigated. Test strains, after growth at 37 or 42.8°C, were suspended in WM at concentrations of approximately 1.5 × 10^8 to 3.0 × 10^8 cells/ml and were then heated at 56, 60, and 63°C for various exposure times. Survival was determined by enumeration on tryptone-soya-yeast extract agar and *Listeria* selective agar, and D values (decimal reduction times) and Z values (numbers of degrees Celsius required to cause a 10-fold change in the D value) were calculated. Higher average recovery and higher D values (i.e., seen as a 2.5- to 3-fold increase in thermotolerance) were obtained when cells were grown at 42.8°C prior to heat treatment. A relationship was observed between thermotolerance and cell morphology of *L. monocytogenes*. Atypical *L. monocytogenes* cell types (consisting predominantly of long cell chains measuring up to 60 μm in length) associated with rough (R) culture variants were shown to be 1.2-fold more thermotolerant than the typical dispersed cell form associated with normal smooth (S) cultures (*P* < 0.001). The thermal death-time (TDT) curves of R-cell forms contained a tail section in addition to the shoulder section characteristic of TDT curves of normal single to paired cells (i.e., S form).

The factors shown to influence the thermoresistance of suspended *Listeria* cells (*P* < 0.001) were as follows: growth and heating temperatures, type of plating medium, recovery method, and cell morphology. Regression analysis of nonlinear data can underestimate survival of *L. monocytogenes*; the end point recovery method was shown to be a better method for determining thermotolerance because it takes both shoulders and tails into consideration. Despite their enhanced heat resistance, atypical R-cell forms of *L. monocytogenes* were unable to survive the low-temperature, long-time pasteurization process when freely suspended and heated in WM.

*Listeria monocytogenes* is a facultative anaerobic bacterium that is distributed ubiquitously in the environment (19) and has a higher thermotolerance than many other nonsporeforming food-borne pathogens (14, 21). Because this potentially lethal pathogen is found occasionally in raw milk (2, 10, 11, 14, 36) and in other nonprocessed foods (2, 19, 25, 26) and can grow in foods under refrigerated storage (22), considerable emphasis has been placed on its complete destruction during pasteurization and during other minimal thermal food processes (3, 4, 6–8, 12–14, 31, 32).

Many factors are known to influence microbial thermotolerance in foods such as the composition of the food and the physiological condition of vegetative cells and spores (15, 24, 52). Bacterial thermotolerance can also increase after exposure to a variety of environmental stress conditions including heating at sublethal temperatures, presence of deleterious chemicals in the growth medium (e.g., hydrogen peroxide, dyes, and antibiotics, etc.), viral infections, and osmotic and acidic shocks (18, 19, 29).

Any temperature above the optimum growth temperature will exert a stress effect (24); while the optimum temperature for growth of *L. monocytogenes* lies between 30 and 37°C, it can grow between 1 and 45°C (19, 48). For most microbial species growth at or short-term exposure to temperatures above optimum induces higher thermotolerances (1, 37–40). It is believed that these temperatures trigger physiological responses that lead to the synthesis of special proteins known as heat shock proteins (HSPs) (31, 33). In *Escherichia coli* K-12, when cells were shifted to 42°C from an incubation temperature of 30°C, the rate of HSP formation increased 5 to 20-fold (cited in reference 48). Gram-positive and -negative bacteria appear to behave similarly: *Streptococcus faecalis* grown at 45°C were more heat resistant than when grown at 27°C (53), while the D<sub>55°C</sub> value for *Salmonella senftenberg* 775W increased as growth temperature was raised from 15 to 44°C (43).

Many authors have reported an increase in the thermotolerance of *L. monocytogenes* as a result of heat shock prior to heating (20, 28, 31, 34, 35, 44). Results from Linton et al. (35) and Pagán et al. (44) are particularly interesting as they suggest that the magnitude of the effect of heat shock treatments is highly dependent on the temperature and duration of the treatment, with higher heating temperatures and longer treatments favoring an increase in heat resistance. Whereas the former researchers observed that *L. monocytogenes* attained its greatest thermotolerance after 20 min of heat shocking at 48°C, Pagán et al. (44) reported a sevenfold increase in thermotolerance achieved by extending the duration of heat shock to 180 min at 45°C. While most thermotolerance studies have utilized *L. monocytogenes* cells at or below 37°C (3, 4, 6–8, 10, 12, 13, 23, 51), the body temperature of a cow suffering from listeriosis can reach as high as 42.8°C (10, 14). Doyle et al. (14) reported the low-level survival of *L. monocytogenes* in high-temperature short-time (HTST)-pasteurized milk from a cow that had been artificially infected with the organism. Also, Knabel et al. (31) showed that growth of *Listeria* at temperatures above 37°C for 18 h (39 to 43°C) resulted in cells that were sixfold more thermotolerant than cells grown at 37°C.
Interestingly, both research groups used serotype 4b strains, a serotype implicated in a number of fatal food-borne outbreaks (19, 21, 25, 26) and shown recently by Sörgqvist to be the second-most heat resistant of seven serotypes examined (49). While production of HSPs are typically a response to temperature upshifts, extended growth at above-optimum temperatures has been shown to result in the expression of HSPs (45). Microbial cell morphology has been linked with increased thermotolerance (41, 46). The acquisition of thermotolerance in heat-shocked *Aquaspirillum arcticum* was shown to be directly related to the formation of long cells (40). Jørgensen et al. (29) showed that *L. monocytogenes* cells grown in medium containing 1.5 mol of NaCl liter⁻¹ prior to heating were 22-fold more heat tolerant than similarly treated cells grown with 0.09 mol of NaCl liter⁻¹. Cells grown in medium containing 1.5 mol of NaCl liter⁻¹ became 50 times longer, but no link to thermotolerance could be made. The change from short rods to long cell chains also occurs under nonstress conditions; Kuhn and Goebel (32) showed that spontaneous mutants of *L. monocytogenes* that form long cell chains occur at a relatively high frequency (about 1 in 10,000 colonies). These long cell chain forms exhibited thermal death-time (TDT) curves that were characterized by both shoulder and tail sections. Other researchers have reported similar TDT curves in *L. monocytogenes* as a result of heat shocking (20, 31, 34, 35); none, however, have mentioned whether the cell morphology of this organism had altered as a result of heating.

The present study was undertaken to investigate the effect of growth at the above-optimum temperature of 42.8°C (i.e., similar to the body temperature of a cow infected with *L. monocytogenes*) on the heat resistance of different cell forms of *L. monocytogenes* (serotype 4b). Thermotolerance was calculated by determining both D values (decimal reduction times) and Z values (numbers of degrees Celsius required to cause a 10-fold change in the D value) and by using the end point recovery method.

### MATERIALS AND METHODS

**Bacterial culture and media.** Two different morphological culture forms of *L. monocytogenes* were used, the normal smooth or S type, consisting of short single and/or paired rods (0.4 to 0.5 μm in diameter and 0.5 to 2 μm in length), and the atypical rough or R type, predominantly consisting of long cell chains of up to 60 μm in length (Fig. 1). The two S-type strains used were NCTC 9863 and 11994 (both of these serotype 4b strains were originally isolated from patients with meningitis and are referred to as S₁ and S₂, respectively, throughout the text) and were obtained from the National Collection of Type Cultures, Public Health Laboratories, Colindale, London, United Kingdom. The two R-form culture variants, R₁ and R₂, were derived previously from the S₁ and S₂ strains, respectively (46), via heating studies as follows: the parent S₁ and S₂ cultures were grown in tyndallized whole milk (WM) at 42.8°C for 24 h without shaking prior to being heated at 60°C for 7 min and at 63°C for 3 min. The R₁ and R₂ culture variants were obtained on tryptone-soya agar plates supplemented with 0.6% yeast extract (TSYE). The cultures were plated immediately after the 7- and 3-min intervals described above and were incubated for 48 h at 37°C. The purity of the strains was confirmed by Gram, catalase, and oxidase reactions; tumbling motility at 25°C; CAMP test reaction; and biochemical profiling with the API Listeria gallery (Biomerieux Ltd.). Stock cultures were grown on TSYE at 37°C for 18 to 20 h and were maintained at 4°C with monthly transfer.

Fifty milliliters of tryptone-soya broth supplemented with 0.6% yeast extract (TSYE) contained in a 250-ml flask was inoculated with the test strain and incubated at 30°C with shaking at 150 rpm on a rotary incubator (model RFI-125; INFORS AG, Bottmingen, Switzerland). Growth was monitored by measuring the optical density at 625 nm (OD₆₂₅) of the culture with a spectrophotometer (model UV-120-02; Shimadzu Corp., Kyoto, Japan). Cells from the late-exponential phase (an absorbance of 0.2 at OD₆₂₅, yielding approximately 10⁵ *Listeria* cells/ml) were harvested by centrifugation at 3,000 × g in a refrigerated (4°C) centrifuge, washed twice, and resuspended in 5 ml of precooled phosphate-buffered water (0.01 M, pH 7 at 4°C). Two duplicate 250-ml flasks each containing 50 ml of commercially pasteurized WM were tyndallized to sterility and inoculated to give initial cell densities of approximately 10⁵ cells/ml. Cultures were grown without shaking for 24 h at 37 or 42.8°C to the respective maximum stationary stage.

**Thermal resistance studies and enumeration.** Heat treatments were performed using screw-cap 28-ml dilution bottles containing 10 ml of WM. The bottles were equilibrated at 56, 60, or 63°C utilizing a circulating constant tem-
temperature water bath (model HE30; Grant Instruments Ltd., Cambridge, United Kingdom) equipped with a thermoregulator capable of maintaining temperature to within ±0.05°C (model TE-SA; Techne Ltd., Cambridge, United Kingdom); the level of the water in the water bath was maintained ca. 5 cm cm from the submerged bottles. A mercury thermometer was inserted into an uninoculated bottle and was checked periodically during the experimental runs to ascertain that the heating temperatures were maintained. One milliliter of the overnight-grown cells was then added to give 1.5 × 10⁸ to 3 × 10⁹ CFU/ml of WM. At predetermined heating intervals (over 2 h at 56°C, 1 h at 60°C, and 30 min at 63°C), a 1-ml sample was removed from each of the bottles and added to 9 ml of WM. The bottles containing heat-treated samples were transferred quickly into a beaker containing tap water at 22°C and held for 1 min (which ensured near-instantaneous cooling of the samples) and then placed into a beaker containing an ice-water mixture. Preliminary experiments showed no growth during this period of time (data not shown) (46). Heat treatments at each temperature were repeated at least twice.

Recovery of surviving cell populations was determined at each heating interval in the heat-treated suspensions, and dilutions thereof, by spread, pour, and spiral plating samples (model B; Spiral Systems Inc., Shipley, United Kingdom) onto TSYEA and Listeria selective agar (LSA) (Oxford formulation; Oxoid). Susceptive dilutions were performed with 9 ml of WM, and all counts were done with triplicate plates. WM was used as the diluent to provide greater protection against the deleterious effects of heat treatment when L. monocytogenes cells were grown, heat treated, and enriched in the same nutrient medium compared with that provided by changing the nutritional composition of the heating medium and/or the diluent (data not shown) (46). Plates were incubated aerobically at 37°C for 48 h, and colonies were counted. The cell morphologies of three randomly selected colonies were examined per plate. All bottles containing treated cultures from each sample heating interval were then incubated and/or resuscitated without shaking at 30°C for 2 days, and total aerobic counts were obtained after a 2-day enrichment period in WM (Table 1). 2.5- to 3-fold increase in thermotolerance (P ≤ 0.001) compared to that for growth at 37°C (Table 1). This enhanced thermotolerance was seen at each heating temperature (Table 1). Table 2 lists the factors that significantly influenced the heat resistance of Listeria cells shown in Table 1. While treatment temperature was shown to have the most significant effect on thermotolerance (i.e., the higher heating temperatures resulting in greater reductions in cell numbers), other factors which provided greater levels of cell protection were growth at 42.8°C prior to heat treatment and recovery of thermally injured cells on the nonselective TSYEA medium (Tables 1 and 2). Greater recovery of heat-injured Listeria cells was obtained after a 2-day enrichment period in WM (Table

### Table 1. Heat resistance of S- and R-culture forms of L. monocytogenes grown in WM at 37 or 42.8°C prior to heating at 56, 60, and 63°C as determined by the interval sample plating method

<table>
<thead>
<tr>
<th>Heating Temp (°C)</th>
<th>Growth Temp (°C)</th>
<th>D or Z value (min)</th>
<th>culture forms plated on indicated media</th>
</tr>
</thead>
<tbody>
<tr>
<td>56</td>
<td>37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>63</td>
<td>37</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Results and Discussion

#### Effects of growth temperature and other factors on the thermotolerance of L. monocytogenes

Growth and heating temperatures and types of plating media and recovery methods had significant effects (P < 0.001) on the survival of heated L. monocytogenes (Tables 1 and 2). Growth of Listeria cells in bovine WM at the above-optimum temperature of 42.8°C resulted in a 2.5- to 3-fold increase in thermotolerance (P ≤ 0.001) compared to that for growth at 37°C (Table 1). This enhanced thermotolerance was seen at each heating temperature (Table 1). Table 2 lists the factors that significantly influenced the heat resistance of Listeria cells shown in Table 1. While treatment temperature was shown to have the most significant effect on thermotolerance (i.e., the higher heating temperatures resulting in greater reductions in cell numbers), other factors which provided greater levels of cell protection were growth at 42.8°C prior to heat treatment and recovery of thermally injured cells on the nonselective TSYEA medium (Tables 1 and 2). Greater recovery of heat-injured Listeria cells was obtained after a 2-day enrichment period in WM (Table 1).
3. Results from total viable counts (data not shown) showed that growth occurred in the enriched bottles at each sample interval up to and including the end point.

The results of the present study are in agreement with the findings of other researchers (14, 31), who showed that growth of L. monocytogenes cells at an above-optimum temperature (i.e., 39 to 43°C) resulted in an increase in thermotolerance. Growth of L. monocytogenes F5069 under anaerobic conditions at 43°C prior to heat treatment and enumeration of survivors resulted in $D_{62.5}$ values that were at least sixfold greater than those previously obtained by using cells grown at 37°C and with aerobic plating (31). Whereas Knabel et al. (31) reported that under their test conditions, high levels of L. monocytogenes survived the minimum low-temperature, long-time (LTLT) pasteurization process, we showed that L. monocytogenes cells grown at 42.8°C were less heat tolerant, surviving approximately 27 min of heating at 63°C when enumerated after a 2-day enrichment period. Knabel and coworkers attributed the greater recovery of severely heat-injured L. monocytogenes cells to the absence of O$_2$ in the enrichment medium (i.e., the O$_2$ sensitivity of heat-injured Listeria cells has been attributed in part to the inactivation of catalase and superoxide dismutase). Dallmier and Martin (9) reported that catalase and superoxide dismutase were rapidly inactivated when cells were heated at temperatures of 55 to 60°C. Inactivation of these two enzymes was thought to result in the accumulation of H$_2$O$_2$. The production and action of a specific set of HSPs, synthesized during the growth of L. monocytogenes cells at 42.8°C, may also account for the acquired thermotolerance in the present study (27, 31, 45). While the precise role of HSPs in acquired heat resistance remains controversial, HSPs might help cells cope with stress-induced damage by promoting the degradation of abnormal proteins (e.g., lon and Clp proteases) and/or the reactivation of stress-damaged proteins by functioning as molecular chaperones, preventing the aggregation and promoting the proper refolding of denatured proteins (45). Rapid degradation of damaged proteins reduces the possibility of deleterious interactions between polypeptides and functional proteins, prevents accumulation of insoluble aggregates, and releases the amino acids contained in nonfunctional polypeptides for synthesis of new proteins (45). As is the case for all organisms studied so far, L. monocytogenes responds to sudden increases in temperature by synthesizing a particular set of HSPs (28). Some stress-induced proteins are also produced in organisms in order to sustain long-term survival at above-optimum temperatures (45). For instance, in Saccharomyces cerevisiae, some HSPs are required for growth at temperatures near the upper end of the normal growth range (e.g., HSP70), others are required for long-term survival at moderately high temperatures (e.g., ubiquitin), and still others are required for tolerance to extreme temperatures (e.g., HSP104) (45). In some cases, expression of genes encoding for the production of proteins associated with one stimulus (e.g., heat shock) can be induced during other stresses; for example, various HSPs in E. coli cells are also synthesized when the cells are exposed to hydrogen peroxide, ethanol, UV, puromycin, and nutrient or amino acid deprivation (27). Therefore, the large increase in heat resistance observed when L. monocytogenes cells were grown at 42.8°C, compared with that of cells grown at 37°C, may have been due to the accumulation of large amounts of postexponential HSPs that were induced by elevated growth temperature, nutrient deprivation, and/or other stresses (27, 45). Jenkins et al. (27) concluded that the increased heat resistance of stationary-phase E. coli cells could have been a result of synthesis of postexponential HSPs that were induced during glucose starvation. The induction of HSPs, therefore, may prepare cells for growth at elevated sublethal temperatures while playing only a minor role in acquired thermotolerance at lethal temperatures.

Under the present test conditions, heat-injured Listeria cells suspended in the heating menstruum were subjected to minimal mixing before enumeration and then enriched for 2 days without shaking. Due to the differences in cell recovery between immediate aerobic plating and 2-day enrichment methods (Table 3), it is probable that the enhanced thermostolerance seen with the latter method was due in part to the provision of a reduced O$_2$ environment in the enrichment bottles. Nonetheless, R-form cells cultivated at 42.8°C prior to heating at 63°C were recovered from the 27-min sample interval, i.e., an additional heat tolerance of 16 min compared with that of the immediate plating method resulted (Table 3). As heat-injured cells grew in WM over the 2-day enrichment period, it was not possible to convert end point survivor data to D values (Table 3). The enhanced recovery of cells grown at 42.8°C seen after enrichment in WM at 30°C is important in terms of milk safety, because HTST pasteurization (which is used commercially) has been shown to result in approximately 10-fold fewer log reductions of L. monocytogenes than the LTLT pasteurization process used in this study (6). Therefore, under similar growth and recovery conditions (enrichment in WM at 30°C), HTST pasteurization might yield survivors that can grow in milk.

The significance of L. monocytogenes in relation to food safety is mainly due to its ability to grow in foods under refrigerated storage (2, 10, 11, 14, 28). Many authors have reported that in non-heat-shocked cells of different bacterial species, higher growth temperatures lead to higher thermostolerance (31, 37–39). There is little knowledge, however, about the influence of growth at above-optimal temperatures in relation to the acquisition and maintenance of thermostolerance in foods during refrigeration. Whereas the thermostolerance de-

### Table 3. Effects of growth temperature on the thermotolerances of S- and R-cell forms of L. monocytogenes using the end point recovery method

<table>
<thead>
<tr>
<th>Growth temp (°C)</th>
<th>Plating medium</th>
<th>Immediate plating of heated samples</th>
<th>Plating after 2-day enrichment period at 30°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>D$_{62.5}$ for indicated cell form with:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>S$_1$</td>
<td>S$_2$</td>
</tr>
<tr>
<td>42.8</td>
<td>TSYEA</td>
<td>7.3 (0.5)$^{a,b}$</td>
<td>7.6 (0.6)$^{a,b}$</td>
</tr>
<tr>
<td>37</td>
<td>TSYEA</td>
<td>3.0$^{a,b}$</td>
<td>3.1 (0.1)$^{a,b}$</td>
</tr>
<tr>
<td>42.8</td>
<td>LSA</td>
<td>0.7 (0.7)$^{a,b}$</td>
<td>1.0$^{a,b}$</td>
</tr>
<tr>
<td>37</td>
<td>LSA</td>
<td>0.7 (0.7)$^{a,b}$</td>
<td>1.0$^{a,b}$</td>
</tr>
</tbody>
</table>

* Values are means (± standard deviations). Values in the same column within each heating temperature with different superscript uppercase letters are significantly different ($P < 0.05$). Values in the same row with different superscript lowercase letters are significantly different ($P < 0.05$).
developed by Salmonella typhimurium after heat shocking was lost during storage in just 1 h (37). Farber and Brown (17) reported that L. monocytogenes still maintained, after a 24-h period at 6°C, the heat resistance developed after a 2-h heat shock at 48°C. Smith and Marmer (47) showed that L. monocytogenes grown at 10°C did not attain the heat resistance of non-heat-shocked cells grown at 37°C. Pagan and coworkers (44), however, showed that storage of heat-shocked L. monocytogenes (42.5°C for 180 min) for 24 h at 4°C before heating did not affect the D values seen for heat-shocked cells. These authors also reported that for non-heat-shocked Listeria cells stored for 7 and 14 days at 4°C, the proportion of cells responding to heat shock not only depended on the duration of heat shock but also on the duration of previous storage. The longer the duration of heat shock and the shorter the duration of storage, the greater the proportion of cells responding to heat shock. For instance, after a 14-day storage at 4°C, Pagan et al. (44) reported that only 10% of the cells given the longest heat shock of 120 min responded, while only 1.5% of those given the mildest heat shock of 15 min did so.

Factors affecting the thermotolerance of L. monocytogenes R- and S-cell forms. The thermotolerance of the R- and S-cell forms varied as a result of the various growth, heating, and recovery conditions used in the present study. Atypical R-type culture forms (predominantly consisting of long cell chains) exhibited higher D and Z values (Table 1) than those of typical S forms (consisting of single and paired cells). R-cell forms were 1.2-fold more heat tolerant as measured by D value ratios at each heating temperature (Table 1). R cultures obtained from the two L. monocytogenes strains (NCTC 11994 and 9863) did not differ in thermotolerance nor did the parent S forms (P ≤ 0.001). Both cell forms of L. monocytogenes grown at 37 and 42.8°C showed nonlinear TDT curves (Fig. 2). Whereas the R-form TDT curves contained both a shoulder and tail section, S-form survivor curves exhibited a shoulder section only (Fig. 2).

Atypical R-form cells consistently survived longer at 62.8°C than S-form cells as shown by both recovery methods (Table 3). Both cell forms were recovered after a longer duration of heating when enumerated on TSYEA immediately after heat-
ing and after a 2-day enrichment period (Table 3). Other researchers also reported inferior recovery of heat-injured *L. monocytogenes* as a result of enumeration on selective plating media (31, 34, 35, 47, 48). Despite their enhanced thermotolerance, atypical cell forms of *L. monocytogenes* were unable to survive the LTTLT pasteurization process (Table 3). Jørgensen et al. (29) also reported the existence of long cell chains in *L. monocytogenes* which exhibited TDT curves that contained both a shoulder and tail section post heating. These atypical *Listeria* cells, however, were obtained by severe osmotic shock due to growth in medium containing 1.5 mol of NaCl/liter, and these cells became up to 50 times longer than cells grown in medium containing 0.09 mol of NaCl/liter. Cells which had adapted to a high salinity before heat treatment showed a 10-fold increase in thermotolerance in minced beef compared to a 22-fold increase in tryptic phosphate broth. However, no link between the acquisition of thermotolerance and cell morphology was made. While TDT curves obtained during the present study, as a result of growth at an above-optimum temperature, were similar to the type of survivor curves obtained by Jørgensen et al. (29), atypical *Listeria* cells were shown to be 1.2-fold more heat tolerant than single-celled cultures at each heating temperature (Table 1). McCallum and Innis (41) reported a direct link between thermotolerance and cell morphology, where the acquisition of thermotolerance in *A. arcticum* was related to the formation of filamentous cells.

It is particularly interesting that the majority of researchers reporting greater thermotolerance in *L. monocytogenes*, as a result of growth at above-optimal temperatures and/or heat shock, showed nonlinear TDT curves that contained both a shoulder and tail section (16, 20, 37, 44) or just an initial shoulder section (30, 35, 50). In addition, the majority of researchers reporting survivor curves in *L. monocytogenes* used the same serotype, 4b (16, 20, 29, 31, 35, 37, 50); this serotype has been isolated from patients with meningitis and from foods implicated in a number of food-related illnesses (19, 21, 25, 26).

Irrespective of the shape of the thermal death kinetic data, calculations on the level of thermotolerance in *Listeria* cells have been based on logarithmic death kinetics (i.e., D values that were calculated as the absolute value of the inverse slope of the least square regression line fitted to log reduction in viable cell numbers versus heating time) (16, 20, 31, 35, 37, 50). Very often no allowance has been made in these calculations for the shoulder and/or tail section in survivor curves. King et al. (30) showed that the function (log log \(N_r/a\) - log N) = \(kt + c\) could be successfully used to linearize the survivor curves obtained from *B. fulva*, a mold that produced heat-resistant ascospores. \(N_0\) and \(N\) are the initial and surviving number of organisms, respectively, at time \(t\), the death rate constant is given by \(k\), and \(c\) is a constant for a set of data. The \(a\) value is derived from the least squares slope of a plot of log(log \(N_r/a\) - log N) versus log time.

King and coworkers showed that the function does not change significantly as the severity of the lethal treatment is increased. When comparing the survival of microbial species under different thermal processes in foods, a large number of log units of kill should be used so the final calculations will incorporate the shoulder and the rapid death phase of the curve. For instance, a 1 log unit destruction of *L. monocytogenes* NCTC 11994 (i.e., \(S_1\) in Fig. 2) takes 6.1 min at 60°C, but the second log unit takes 3.4 min, the third takes 3.3 min, the fourth takes 3.1 min, and the fifth takes 2.6 min. Because of the shoulder of the curve, a third of the total time for a 5 log unit reduction is required for the first log unit of destruction. While the \(D_{1/2}\) for \(S_1\) was 3.7 min, the analogous value for a 1-log unit destruction (using the linearizing function) increased to 3.9 min. Application of this function also showed that the thermotolerance of other bacterial pathogens was underestimated. The time taken for a 6-log unit reduction in *Salmonella serftenberg* 750W was observed to be 119 min but was calculated to be 132 min using the function (43). However, use of this formula did not apply to TDT curves containing both shoulder and tail sections (data not shown) (46). Shoulders of TDT curves have been postulated to be due to spore activation, repair of heat injury, cell disaggregation, or even methodological problems (24, 42). Moats et al. (42) reported extensive tailing in the survivor curves for *E. coli, S. faecalis, Salmonella serftenberg* 775W, and *S. antum* and attributed these deviations from the exponential death rates to differences in heat resistances in a single bacterial culture and/or clumping (i.e., where clumps of two or more cells produce a colony as long as one cell in the clump is viable). Therefore, while measurement of thermotolerance by the TDT curve method (i.e., D value determination) provides detailed data on thermal death rate kinetics that cannot be obtained by the end point recovery method (24, 44, 52), regression analysis of nonlinear data can underestimate survival of *L. monocytogenes* (Table 3). The end point recovery method appears to be a better method for determining microbial thermotolerance because it takes both shoulders and tails into consideration.

Little knowledge is available on the putative virulence capability of R-transformed *L. monocytogenes*. Kuhn and Goebel (32) reported that long cell chains of this organism result from an impairment in the synthesis of a major extracellular protein, p60 (considered an important housekeeping protein for virulent strains of *L. monocytogenes*). It was suggested that p60 protein may be a murcin hydrolase and that its synthesis is not under the control of the transcriptional activator, PrfA (which regulates the synthesis of many virulence factors in the gene cluster). The p60 mutants form long cell chains (also designated R forms), with unseparated septae between the individual bacterial cells, which disaggregate to normal-sized single bacteria upon treatment with partially purified p60. R-mutant forms were reported to be avirulent as they were unable to invade phagocytic 3T6 mouse fibroblast cells (5, 32). These researchers showed, however, that these R-mutant forms were still capable of adhering to and invading epithelial human colon carcinoma cells (CaCo-2), albeit at a reduced level of invasiveness (5). As spontaneously occurring mutants of *L. monocytogenes* with R-form cell characteristics were isolated previously at a relatively high frequency (1 in 10,000 colonies) (32), and were also shown to emerge under conditions of severe osmotic (29) and heat stress (46), the potential pathogenicity of ingested R-form *L. monocytogenes* in vulnerable groups remains unanswered. In the present study, the rever-sion rate from R form to normal-sized single bacteria was shown to be approximately 3 in 500 colonies (data not shown) (46).

In view of the fact that many foods are subject to mild heat treatments followed by lengthy periods of refrigerated storage, e.g., sous-vide (19), greater research is needed to determine the pathogenicity of long cell chain forms of *L. monocytogenes*, their frequency of occurrence in foods, and the relevance of survivor curves (particularly TDT curves with tail sections) in food systems. The D value concept, which assumes a linear relationship from R form to normal-sized single bacteria upon treatment with partially purified p60. R-mutant forms were reported to be avirulent as they were unable to invade phagocytic 3T6 mouse fibroblast cells (5, 32). These researchers showed, however, that these R-mutant forms were still capable of adhering to and invading epithelial human colon carcinoma cells (CaCo-2), albeit at a reduced level of invasiveness (5). As spontaneously occurring mutants of *L. monocytogenes* with R-form cell characteristics were isolated previously at a relatively high frequency (1 in 10,000 colonies) (32), and were also shown to emerge under conditions of severe osmotic (29) and heat stress (46), the potential pathogenicity of ingested R-form *L. monocytogenes* in vulnerable groups remains unanswered. In the present study, the reversion rate from R form to normal-sized single bacteria was shown to be approximately 3 in 500 colonies (data not shown) (46).

In view of the fact that many foods are subject to mild heat treatments followed by lengthy periods of refrigerated storage, e.g., sous-vide (19), greater research is needed to determine the pathogenicity of long cell chain forms of *L. monocytogenes*, their frequency of occurrence in foods, and the relevance of survivor curves (particularly TDT curves with tail sections) in food systems. The D value concept, which assumes a linear relationship between the log number of cell survivors and heating time, accurately describes only some of the data presented. More importantly, D values calculated from linear sections of the TDT curve, and not from the entire curve, could lead to an underestimation of the time and temperature required to achieve the desired level of cell destruction. Therefore, the end point method may be the best approach for determining mi-
crobial thermotolerance as it takes both shoulders and tails into account.

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REFERENCES


