Growth Kinetics and Cell Morphology of 
Listeria monocytogenes Scott A as Affected by Temperature, NaCl, and EDTA†‡

LAURA L. ZAIKA* AND JOSEPH S. FANELLI

Microbial Food Safety Research Unit, Eastern Regional Research Center, U.S. Department of Agriculture, Agricultural Research Service, 600 East Mermaid Lane, Wyndmoor, Pennsylvania 19038 USA

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ABSTRACT

Growth kinetics and morphological characteristics of Listeria monocytogenes Scott A grown under stress conditions induced by increasing levels of NaCl and EDTA were studied as a function of temperature. L. monocytogenes Scott A was inoculated into brain heart infusion broth (pH 6) at 19, 28, 37, and 42°C. Test cultures contained NaCl (at concentrations of 4.5, 6.0, and 7.5%) or EDTA (at concentrations of 0.1, 0.2, and 0.3 mM); control cultures contained 0.5% NaCl. Growth curves were fitted from plate count data by the Gompertz equation, and growth kinetics parameters were derived. Stationary-phase cells were examined by scanning and transmission electron microscopy. Generation times (GTs) and lag phase duration times (LPDs) increased as additive levels were increased. The bacterium grew at all NaCl levels. At 37 and 42°C, growth was slow in media containing 7.5% NaCl, and no growth occurred in media containing 0.3 mM EDTA. Temperature was a major factor in certain stress conditions that led to cell elongation and loss of flagella. Cells in control media at 28°C grew as short rods (0.5 by 1.0 to 2.0 μm), while at 42°C most cells were 4 to 10 times as long. Higher levels of NaCl at higher temperatures resulted in longer and thicker cells. At 28°C, 0.1 mM EDTA had little effect on growth kinetics and morphology; however, 0.3 mM EDTA caused a sixfold increase in GT and LPD and loss of flagellae, with most cells being two to six times as long as normal. Cell length did not correlate with growth kinetics. The results of this study suggest that the effect of altered morphological characteristics of L. monocytogenes cells grown under stress on the virulence and subsequent survival of these cells should be investigated.

Listeria monocytogenes is a facultative intracellular pathogen that is widely distributed in the environment and frequently isolated from a variety of foods and food ingredients (8). Outbreaks of listeriosis, some resulting in fatalities, have focused attention on the need to control this bacterium in foods. Important considerations are the organism’s high thermal stability and its ability to grow and survive at refrigeration temperatures and at elevated salt levels (17).

The environment in which bacteria grow can influence their ability to grow and survive when they are subsequently subjected to inhibitory conditions. Bacteria grown under stress conditions are better able to survive when subjected to increased stress or even a different type of stress. For example, L. monocytogenes cells grown at pH 5.0 were found to survive a subsequent acid shock (pH 3.0) significantly better than cells grown at pH 7.0 (23), and cells grown at 43°C, a temperature above the optimum temperature for growth, were found to be more heat resistant than cells grown at lower temperatures (21). These data have important implications for attempts to control this bacterium in foods. Understanding the effects of stress on the characteristics of the L. monocytogenes cell may aid in determining more efficient methods for inactivating this pathogen.

The growth and survival of a microorganism are dependent on a combination of factors, such as temperature, pH, water activity (aw), metal ions, nutrients, and various other constituents of the medium. The temperature range for the growth of L. monocytogenes is −0.4 to 45°C (17). The bacterium’s tolerance of high salt levels has been demonstrated by growth at 35°C in tryptic soy broth containing 10% NaCl and at 25 and 10°C in broth containing 12% NaCl (32). Furthermore, Farber et al. (10), in studying the ability of L. monocytogenes strains to initiate growth at 4, 10, 15, 30, and 40°C in brain heart infusion (BHI) broth adjusted to various aw values, found that the bacteria grew at 15 and 30°C in the presence of 13 to 14% NaCl at an aw value of 0.91.

EDTA is widely used in the food industry as a stabilizer and sequestrant (12). Experiments indicate that it may also possess antimicrobial activity. Wang and Shelef (33) noted that EDTA applied as a dip at a concentration of 15 to 25 mM had a bacteriostatic effect on L. monocytogenes inoculated into cod fish fillets. A combination of 5 mM EDTA and 100 mg of lysozyme per kg was bactericidal to L. monocytogenes in several types of fresh vegetables but...
was less effective in sausage or Camembert cheese (16). In ultrahigh-temperature milk, a combination of 200 μg of lysozyme per ml and 2.5 mg of EDTA per ml reduced the population of *L. monocytogenes* by ca. 6 log cycles after 18 h at 36°C, while lysozyme or EDTA added alone had no significant effect (27). EDTA (0.1%) combined with sucrose laurate and butylated hydroxyanisole was found to prevent the growth of *L. monocytogenes* in skim milk stored for 20 days at 25°C (31).

Although much research on the growth and survival of *L. monocytogenes* under adverse environmental conditions has been reported (29), relatively little attention has been devoted to examining the effects of these conditions on cellular morphological characteristics, which may reflect the physiological state of the bacterium. Brzin (3, 4) observed that the growth of *L. monocytogenes* on 5% human serum agar containing 8 to 9% NaCl induced the formation of irregularly shaped colonies and cell filamentation. Maximum cell elongation occurred at 30 to 37°C, while changes were less pronounced at lower temperatures. Filament formation by *L. monocytogenes* in cultures containing high levels of NaCl was also observed by Isom et al. (18). Cells of *L. monocytogenes* grown in the presence of 9% NaCl were found to be more thermostable than cells grown in low-salt medium subsequently subjected to the same concentration of NaCl for up to 4 h (20). Furthermore, environmental conditions may influence the expression of proteins involved in survival and virulence (7).

The objective of this investigation was to measure *L. monocytogenes* growth under various temperature, NaCl, and EDTA conditions and to examine the effects of these conditions on the morphological characteristics of the cells.

**MATERIALS AND METHODS**

**Preparation of inoculum.** *L. monocytogenes* Scott A (clinical isolate, U.S. Food and Drug Administration) was used for the study. The stock culture was stored at −70°C in BHI (Difco Laboratories, Detroit, Mich.) broth supplemented with 10% (vol/vol) glycerol. To prepare the inoculum, 0.1 ml of stock culture was added to 10 ml of BHI broth and incubated without shaking for 18 to 24 h at 37°C, and the culture was diluted with sterile 0.1% peptone water to the experimental concentration.

**Test chemicals.** Sodium chloride and EDTA, disodium salt dihydrate (99%, Sigma-Aldrich, St. Louis, Mo.) were used as test materials.

**Growth in liquid cultures.** BHI broth adjusted to pH 6.0 (±0.1) with HCl was used as the growth medium. Test materials were added to BHI broth prior to pH adjustment and autoclaving. Control media contained 0.5% (0.0855 M) NaCl (present as a constituent of BHI), and test media contained 4.5, 6.0, or 7.5% (0.770, 1.026, or 1.283 M, respectively) NaCl or 0.00372, 0.00744, or 0.0112% (0.1, 0.2, or 0.3 mM, respectively) EDTA. Portions (50 ml each) of sterile medium in 250-ml Erlenmeyer flasks (250 ml) were inoculated with 0.5 ml of diluted *L. monocytogenes* culture to produce an initial level of 10^8 CFU/ml and were then incubated on a rotary shaker (150 rpm) at 19, 28, 37, or 42°C. At appropriate time intervals, bacterial population sizes were determined by surface plating the cultures, or their dilutions in sterile 0.1% peptone water, on tryptose agar (Difco) with a Spiral Plater (Model D, Spiral System Instruments, Inc., Bethesda, Md.). The plates were incubated for 24 to 48 h at 37°C, and the colonies were counted with the aid of a Bacteria Colony Counter (Model 500A, Spiral System Instruments).

**Determination of growth kinetics parameters.** *L. monocytogenes* population data were used to generate bacterial growth curves with the Gompertz equation (14) in conjunction with ABA-CUS, a nonlinear regression program employing a Gauss-Newton iteration procedure (6). The values of the Gompertz parameters (A, B, C, and M) were used to calculate exponential growth rates ($\log_{10}$ CFU/ml/h), generation times (GTs) (h), lag phase durations (LPDs) (h), and maximum population densities (MPDs) ($\log_{10}$ CFU/ml) as described previously (5, 14).

**Scanning electron microscopy.** Stationary-phase cultures of *L. monocytogenes*, grown under the combinations of conditions described above, were adsorbed to 22-mm glass coverslips and chemically fixed by immersion in 1% glutaraldehyde in 0.1 M imidazole buffer (pH 6.8) for 30 min. After they were washed in the buffer to remove glutaraldehyde, the cells on coverslips were dehydrated with a graded series of ethanol and critical-point dried from liquid CO₂. Coverslips with dry cells were glued to specimen stubs and coated with a thin layer of gold by DC sputtering (Edwards Scan Coat 6, West Sussex, UK). Samples were observed and digital images were created on an Imix work station (Princeton Gamma-Tech, Princeton, NJ) integrated to a Model JSM840A scanning electron microscope (JEOL USA, Peabody, Mass.) operated in the secondary electron imaging mode to record topographical details.

**Transmission electron microscopy.** Stationary-phase cultures of *L. monocytogenes*, grown as described above, were chemically fixed through the addition of glutaraldehyde to a final concentration of 1% and centrifuged to produce compact cell pellets. Following washing in 0.1 M imidazole buffer (pH 6.8), the cell pellets were postfixed in 1% osmium tetroxide solution in imidazole buffer for 1 h and were then washed in distilled water and dehydrated in a graded series of ethanol solutions and propylene oxide. The cell pellets were embedded in epoxy resin through infiltration in a 1:1 mixture of epoxy resin and propylene oxide and cured at 55°C. Thin sections of the cured blocks were cut with diamond knives, mounted on copper specimen screens, stained with solutions of uranyl acetate and lead citrate, and examined in a Model CM 12 transmission electron microscope (FEI Co., Hillsboro, Ore.) in the bright field imaging mode to obtain ultrastructural details.

**RESULTS**

**Growth kinetics.** Growth kinetics data for *L. monocytogenes* cultured aerobically in BHI broth (pH 6.0) under various temperature, NaCl, and EDTA conditions are summarized in Table 1. The bacteria grew rapidly to a maximum population density of $\approx 9.3 \log_{10}$ CFU/ml at all four temperatures in control cultures (0.5% NaCl), but growth inhibition increased with increasing levels of NaCl and EDTA. *L. monocytogenes* grew in the presence of 0.5, 4.5, 6.0, and 7.5% NaCl at 19, 28, 37, and 42°C. With the exception of differences between LPDs at 19 and 42°C, differences between growth patterns at 0.5 and 4.5% NaCl were relatively minor at all temperatures. Growth at all temperatures was progressively slower in the presence of 6 and 7.5% NaCl. Significantly lower maximum population densities were obtained when 7.5% NaCl was present at 37 and 42°C.
TABLE 1. Aerobic growth kinetics parameters of Listeria monocytogenes Scott A cultured in BHI broth adjusted to pH 6.0 at various temperatures, NaCl concentrations, and EDTA concentrations

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>NaCl concn (%)</th>
<th>EDTA concn (mM)</th>
<th>n b</th>
<th>Exponential growth rate (log_{10} CFU/ml/h)</th>
<th>Generation time (h)</th>
<th>Lag phase duration (h)</th>
<th>Maximum population density (log_{10} CFU/ml)</th>
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<td>4</td>
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a Numbers in parentheses are standard deviations. NG, no growth.
b Number of replicate cultures.

Increasing growth inhibition was observed with increasing concentrations of EDTA in the cultures. At 0.1 mM EDTA, the organism did not grow, but the bacterial population remained essentially unchanged for >300 h at 37°C, while at 42°C the population decreased to undetectable levels (<1.3 log_{10} CFU/ml) in ca. 100 h. In preliminary experiments, we also tested the effects of higher concentrations of EDTA on L. monocytogenes at 28°C. In the presence of 0.5 mM EDTA, no change in bacterial population size occurred for at least 300 h, while concentrations of 1 and 2 mM led to a decrease of 0.5 to 1 log_{10} CFU/ml in 438 h (data not shown).

Morphology. The morphological characteristics of late-stationary-phase cells obtained under the growth conditions studied were examined by scanning electron microscopy (SEM). All factors studied (temperature, NaCl concentration, and EDTA concentration) affected cellular morphological characteristics. The spectrum of morphological characteristics observed is shown in Figures 1 through 3 for cells grown at 28, 37, and 42°C, respectively. The SEM data for cells grown at 19°C (not shown) were essentially similar to data for cells grown at 28°C (Fig. 1). Although growth at 19°C was slower than at 28°C in control cultures, no significant differences were noted in the appearances of cells. Short cells (0.5 by 1 to 2 μm) with flagellae were obtained in both cases (Fig. 1A). Growth in control cultures at 37°C (Fig. 2A) and at 42°C (Fig. 3A) resulted in many long cells (4 to 10 times as long as cells grown at the lower temperatures) and loss of flagella. Increasing EDTA levels resulted in cell elongation and loss of flagella. At 28°C, growth in the presence of 0.1 mM EDTA had little effect on cellular morphology, while 0.3 mM EDTA resulted in cell elongation and loss of flagella (Fig. 1). Increasing levels of NaCl resulted in increased cell elongation, fewer short cells, loss of flagella, curved cells, and cell aggregation. These changes were particularly evident in cultures grown at the two higher temperatures (Figs. 2B, 2D, 2E, 3C, and 3D). It is possible that the lower maximum population densities obtained at 37 and 42°C for media containing 7.5% NaCl (Table 1) were due to cell aggregation.

The morphological characteristics of the L. monocytogenes...
FIGURE 1. Scanning electron micrographs for L. monocytogenes cells grown in BHI broth (pH 6.0) at 28°C: control culture (A) and cultures containing (B) 6% NaCl, (C) 0.1 mM EDTA, and (D) 0.3 mM EDTA.

genes cells were further examined by transmission electron microscopy (TEM). Thin sections of late-stationary-phase cells grown under various conditions used in this study are shown in Figures 4 and 5. In general, results obtained with TEM support those obtained with SEM. Cells grown in the presence of \( \geq 0.2 \) mM EDTA (Figs. 1D and 4B) are longer and thinner than cells grown in control media (Fig. 4A). Cells grown in the presence of increased levels of NaCl, particularly at the higher temperatures, were wider, and many curved cells were present (Fig. 4C and 4D). Growth in the presence of \( \geq 6\% \) NaCl resulted in the formation of thicker sections in the cell envelope, either at the septum (arrow in Fig. 5C) or in other areas of the cell (arrows in Fig. 4D). Increased NaCl concentrations induced the collapse of nucleoid material and the formation of clear, irregular, poorly defined areas within the cell. This effect became more pronounced as the growth temperature increased (arrows in Fig. 5B and 5D).

DISCUSSION

The response of bacteria to short-term exposure to various stress factors, such as heat, cold, osmolarity, and acids, under conditions that do not result in bacterial growth has been well documented. However, the effect of growth under stress conditions on the morphological characteristics and composition of bacterial cells has received little attention. Bacterial growth, cell composition, and morphological characteristics may be profoundly affected by various physicochemical factors. Temperature alone can cause morphological changes. The formation of long cells of the psychrophile Bacillus insolitus was found to occur when this bacterium was grown at 30°C but not when it was grown at 20°C (11). Our SEM data indicate that in control media (without added NaCl or EDTA), the growth of the psychrotrophic L. monocytogenes at 19 and 28°C resulted in short, uniformly sized cells, while growth at 37 and 42°C resulted in cell elongation and other morphological changes.

Morphological changes in L. monocytogenes grown in the presence of high levels of NaCl and at 10 to 37°C were studied by Brzin (3, 4), who found that growth on agar media containing 8 to 9% NaCl was accompanied by cell elongation (filamentation) and some degree of spheroplasting. The degree and extent of elongation increased as the
growth temperature increased. Incubation at 37 and 30°C resulted in the longest cells (and these cells were slightly thicker), while the effects on morphological characteristics were much less pronounced after incubation at 10°C. Our findings are consistent with these observations. Isom et al. (18) observed morphological changes in L. monocytogenes grown at 37°C in tryptic soy broth containing various NaCl concentrations (up to 8.8%). Filament formation was apparent at an NaCl concentration of 5.8%, and filament length increased as the NaCl concentration increased. Our results indicate a similar trend, and the morphological changes were more apparent at the lower temperatures studied, 19 and 28°C. Although we studied L. monocytogenes Scott A, reports indicate that high levels of NaCl in the growth media can induce filamentation in other strains of L. monocytogenes, such as L. monocytogenes LO28 (1) and L. monocytogenes SLCC 5764 (18).

Our TEM analyses indicated that the growth of L. monocytogenes in media containing the higher NaCl levels resulted in the collapse of the nucleoid material (Fig. 5B and 5D). A similar effect on the nucleoid material was noted for Escherichia coli grown in the presence of increasing levels of KCl (15). Shrinkage of the cytoplasm in L. monocytogenes cells was also observed after long-term storage at 4°C (9). Since the NaCl levels in most foods are <4.5%, NaCl may not exert a major effect on the growth or cellular characteristics of L. monocytogenes in foods, particularly at moderate temperatures, below 28°C.

Bacterial growth in media deficient in Mg²⁺ can cause cell elongation (35). Previously, we reported that the growth of L. monocytogenes in the presence of long-chain (average chain lengths, 6, 13, and 21) sodium polyphosphate sequestrants was inhibited and resulted in cell elongation (36). However, normal growth was restored when the polyphosphate-containing media were supplemented with polyvalent metal ions (Ca²⁺, Mg²⁺, Fe³⁺, Mn²⁺, or Zn²⁺) (37). Kraniak and Shelef (22) suggested that EDTA inhibited the growth of Staphylococcus aureus by forming stable chelates in the media with polyvalent metal ions that are essential for cell growth. The addition of Fe³⁺, Zn²⁺, or Ca²⁺ to broth containing 1.7 mM EDTA eliminated inhibition at an EDTA/cation molar ratio of 1:1, whereas a
larger quantity of Mg$^{2+}$ was required to achieve this effect. According to Jen and Shelef (19), the concentration of metal ions (such as Mg, Ca, Fe, and Zn) important to microbial growth is low (0.43 mM) in BHI medium. Thus, the addition of 0.3 mM EDTA may result in levels of available essential metal ions that are insufficient for the normal growth and cell development of *L. monocytogenes*. Other factors, such as high growth temperature, may enhance the effect of EDTA. The maximum level of EDTA currently permitted for use as a direct additive to food depends on the food type and is in the range of 0.005 to 0.05% (0.134 to 1.343 mM, respectively, calculated as disodium EDTA dihydrate). However, the concentration of metals (Mg, Ca, Fe, and Zn) in foods is relatively high, generally ≥9 mM (34). Thus, these concentrations of EDTA may not exert any significant effect on *L. monocytogenes* if they are present in foods.

Our experiments used BHI adjusted with HCl to pH 6, a pH value representative of many foods, such as meat products. Isom et al. (18) reported that some filamentation, together with normal coccoid rods, was observed when *L. monocytogenes* was grown in tryptic soy broth adjusted to a pH of 5.0 to 6.0 with citric acid, but no morphological changes were observed for cells grown in untreated broth or in broth acidified with hydrochloric, acetic, or lactic acid. Apparently, the chelating properties of citric acid played a role in the morphological effect, as was the case with EDTA in our experiments. The application of additional stress conditions, such as acidification, may enhance cell elongation. Bereksi et al. (1) reported that cell elongation was observed when *L. monocytogenes* Scott A was grown at 22°C in the presence of 10% NaCl in Trypticase soy broth–yeast extract medium at pH 5 but not in medium at pH 7.

*L. monocytogenes* has a characteristic temperature-dependent motility attributable to its possession of peritrichous flagella. Peel et al. (28) investigated the temperature-dependent expression of flagella of *L. monocytogenes*. Many flagella were seen in cells grown at 20°C, whereas very few were present in cells grown at 37°C. Also, with the use of the Western blotting technique, these authors determined that flagellin (29-kDa protein) was abundant in...
organisms grown at 20°C but detectable only in trace amounts in organisms grown at 37°C. This finding is in agreement with our observations with regard to the effect of temperature on the morphological characteristics of cells. Flagella were observed in cells grown in control cultures at 19°C (data not shown) and 28°C (Fig. 1A); however, few if any flagella were evident in cells grown at 37°C (Fig. 2A) and 42°C (Fig. 3A). The morphological changes induced by growth under stress conditions persist only as long as the organism is cultured under stress conditions, and normal morphology is observed when the stress is removed (3, 4, 18, 20).

The relationship between growth characteristics and changes in L. monocytogenes cells induced by growth under stress conditions are not clear. However, the potential for such cells to withstand exposure to subsequent stresses, such as heat inactivation (20), should be examined more systematically. In recent years, much information concerning the virulence genes and proteins of L. monocytogenes has become available (2, 7). Much of this information has been obtained with the use of mutant strains grown under standardized conditions. The influence of environmental and nutritional conditions for cell growth on the resulting virulence characteristics of L. monocytogenes is still not well understood (7, 29). Leimeister-Wächter et al. (24) reported that the expression of virulence genes in L. monocytogenes is thermoregulated and that an essential virulence protein, listeriolysin, was expressed in cultures grown at 37°C but not in cultures grown at 20 or 30°C. However, little work on the relationship of the growth conditions for L. monocytogenes and its infectivity has been reported to date (13, 26). The production of catalase, listeriolysin, and superoxide dismutase was more extensive for bacteria grown at 37°C in tryptic soy broth containing 428 mM NaCl or KCl, while no significant differences were observed for bacteria grown in broth without these salts in mouse model virulence tests (25).

The morphological characteristics of L. monocytogenes have received relatively little attention, particularly in relation to the virulence characteristics of the bacterium. It is of interest to note that cell length per se may not be a major determinant of virulence. Rowan et al. (30) described atypical rough cell forms of L. monocytogenes isolated from clinical and food samples that showed wild-type levels of adherence to, invasion of, and cytotoxicity to HeLa, Hep-2, and Caco-2 cells. Morphological examination of these rough forms, designated FR variants, showed that they consisted of single or paired filaments up to 96 μm long. In our work, cells 10 to 20 μm long were commonly observed in cultures grown under in-

![Figure 4](image1.png)

**FIGURE 4.** Transmission electron micrographs for L. monocytogenes cells grown in BHI broth (pH 6.0) at 37°C: control culture (A) and cultures containing (B) 0.2 mM EDTA, (C) 4.5% NaCl, and (D) 7.5% NaCl. The formation of thicker sections in the cell envelope is indicated by arrows (D).

![Figure 5](image2.png)

**FIGURE 5.** Transmission electron micrographs for L. monocytogenes cells grown in BHI broth (pH 6.0) at 19°C: control culture (A) and cultures containing (B) 6% NaCl, (C) 7.5% NaCl (28°C), and (D) 6% NaCl (42°C). The thickening of the cell envelope at the septum is indicated by an arrow (C). The collapse of nucleoid material and the formation of clear, irregular areas within the cells are indicated by arrows (B, D).
creased stress conditions; however, longer cells were occasionally observed.

We conducted a systematic study on the effects of increases in temperature, NaCl concentration, and EDTA concentration on the growth characteristics of *L. monocytogenes* Scott A. Under stress conditions, major changes in growth kinetics were reflected in changes in cellular morphology. In particular, a combination of a high growth temperature (37 or 42°C) and a high concentration of test compound ($\geq 6\%$ NaCl or $\geq 0.2$ mM EDTA) resulted in major changes in cellular morphology, as determined by SEM and TEM. Additional research is needed to determine how bacterial growth under stress conditions influences subsequent growth, survival, attachment, and virulence characteristics of the pathogen.

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