Variation of Branched-Chain Fatty Acids Marks the Normal Physiological Range for Growth in *Listeria monocytogenes*

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**Listeria monocytogenes** is a food-borne pathogen responsible for numerous outbreaks of disease in North America, Europe, and Australia linked to the consumption of commercial food products (9, 15). The ability of *L. monocytogenes* to grow at refrigeration temperatures has led to concerns about infection from cold-stored foods, particularly those consumed without subsequent cooking (i.e., ready-to-eat foods) (11). While low levels of *L. monocytogenes* contamination are not uncommon in such foods, outbreaks of disease have occurred in cases where the bacterium has proliferated in foods stored at low temperatures over time (15).

A major adaptation of *L. monocytogenes* to growth at low temperature is the significant proportion of branched-chain fatty acids present in the cell membrane. In particular, anteiso-C15:0 (a15:0) was demonstrated to be essential for growth at low temperatures (3, 10). *L. monocytogenes* is also unusual in its temperature range for growth, which is toward the upper end of the range of temperatures reported for bacteria (−2 to 45°C [approximately 48°C]) (21).

Nichols et al. (17) reported that they found a correlation between branched-chain fatty acid content and the normal physiological range (NPR) for growth of the psychrophilic bacterium *Shewanella gelidimarina* by using fine-scale determinations over the entire biokinetic temperature range. The objective of this study was to investigate the phenotypic regulation of branched-chain fatty acids in *L. monocytogenes* by using the same methodology.

**MATERIALS AND METHODS**

**Bacterial strains.** *L. monocytogenes* Scott A was maintained on slopes of tryptone soya broth (catalog no. CM 129; Oxoid) with 0.6% yeast extract (catalog no. L21; Oxoid).

**Temperature gradient incubator experiments.** The effect of temperature on the rate of growth of *L. monocytogenes* was determined by using a temperature gradient incubator (Toyo Kagaku Sangyo, Tokyo, Japan). Four replicate experiments were conducted. The growth medium was tryptone soya broth with 0.6% yeast extract. Tubes were inoculated with an actively growing culture, and growth was monitored by measuring the percentage of transmittance at a wavelength of 540 nm until growth was complete. Cultures were shaken by the oscillatory motion of the incubator through an arc of 60° at a rate of 60 oscillations min⁻¹. The growth rate at each temperature was calculated by fitting a modified Gompertz function to the data (14). The growth temperature of each incubation tube was determined in triplicate with a Fluke 51 K/J thermometer and Fluke 80PK-1 thermocouple following cessation of growth. The calculated growth rate data sets were then fitted to the modified four-parameter-square root model of Ratkowsky et al. (20) by using UltraFit software. Growth rate data were also fitted to the model of Rosso et al. (22) in order to obtain optimal growth temperature (TOPT) estimates. This model contains T₉₀ as a parameter and thereby allows standard errors to be determined. The two models were found to give similar results for other parameters.

**NPR.** The NPR for growth was estimated by expressing the growth rate data as an Arrhenius plot. The midpoint of the data set was determined, individual data points were sequentially added in both directions, and a linear function was fitted. The temperature points whose inclusion resulted in the r² value of the fitted function falling below 0.990 were designated the boundaries of the NPR.

**Lipid extraction and analysis.** When individual incubation tubes reached a transmittance value of 27 to 30%, corresponding to the mid-exponential phase, the cultures were immediately harvested by filtration onto glass fiber filters (Whatman) which had been previously heated at 400°C for 24 h. Sample filters were then frozen at −20°C until lipid analysis. Filters were extracted by a direct transesterification procedure (7) demonstrated to yield fatty acid profiles comparable to those obtained by the Bligh-Dyer solvent extraction method (5, 24). Briefly, filters were added to screw-cap test tubes containing 3 ml of methanol-chloroform-hydrochloric acid (10:1:1, vol/vol/vol). The tubes were heated at 80°C for 1 h before they were cooled to room temperature. After addition of Milli-Q water (1 ml), the resultant fatty acid methyl esters were extracted three times with 1.5 ml of hexane-chloroform (4:1, vol/vol).

Fatty acid methyl esters were analyzed by using a Hewlett-Packard 5890 II gas chromatograph and 5970A mass selective detector equipped with a cross-linked methyl silicone (film thickness, 0.33 μm) fused-silica capillary column (length, 50 m; internal diameter, 0.22 mm). The operating conditions were similar to those detailed by Nichols et al. (17). Fatty acid methyl esters from all samples were identified by comparing the component spectra to the spectra of known standards. The double bond positions and geometry in monounsaturated isomers of selected samples were determined by producing and analyzing dimethyl disulfide adducts (18). The data presented below are the means and standard errors to be determined.
deviations for four complete replicate experiments in which the standard deviation of the temperature values was less than 0.5°C. Significant differences between adjacent mean values were assessed by a Student’s t test. The significance of outlying data points was assessed by analysis of standardized residuals for each data point compared to the fitted trends (see Fig. 3).

RESULTS

Growth rate. L. monocytogenes had a theoretical growth range of 46.5°C under the experimental conditions employed (Fig. 1). The fitted theoretical minimum growth temperature and maximum growth temperature were 1.5 ± 0.5 and 48.0 ± 0.2°C, respectively, and the estimated Topt was 36.6 ± 0.3°C. The variation in the growth rate between data sets (as assessed by standard deviation) increased immediately preceding and following the Topt (Fig. 1).

NPR. An estimate of the NPR was obtained by defining the linear portion of the Arrhenius plot (Fig. 2). Growth rate data, as an additive series, in the temperature range from 14.0 to 26.1°C were well described by a linear function (r² ≈ 0.996). Inclusion of data points outside this range resulted in a decrease in r² (r² < 0.990). The boundaries of the NPR were therefore defined as 12.7 to 14.0 and 26.1 to 27.7°C.

Fatty acid composition. There was a high degree of variation (as assessed by standard deviation) in the percentage of branched-chain components between replicate experiments at a given growth temperature (Fig. 3). The variation was particularly marked when the proportion of total branched-chain fatty acids was considered. While there was no trend discernible from the percentage of total branched-chain fatty acids, two significant alterations in composition were evident for three temperature regions, 11.2 to 12.5°C (P < 0.01), 12.5 to 13.9°C (P < 0.001), and 31.3 to 32.8°C (P < 0.01) (Fig. 3). These alterations were due primarily to the contributions of the two C15 branched-chain components. The proportion of a15:0 decreased as the growth temperature increased, and the trend was well described by a second-order polynomial curve. The percentage of i15:0 was maximal at 17°C and minimal at 6 and 40°C and was well described by a third-order polynomial curve (Fig. 3). One data point (the 12.5°C data point) differed

FIG. 1. Plot of growth rate (1/√generation time) versus temperature for L. monocytogenes grown in tryptone soya broth with yeast extract. The data points represent the means of four replicates, and the bars indicate standard errors.
The percentages of both i17:0 and a17:0 increased with growth temperature in a generally linear manner (Fig. 3), and i17:0 exhibited much less variation than the other branched-chain components. There was no significant \( (P > 0.05) \) deviation of data points from the values on the fitted curves for these data sets. However, for a17:0, the largest standardized residual of the data set occurred at 12.5°C.

DISCUSSION

When the fatty acid composition of \( L. \) monocytogenes Scott A was examined, there were specific temperature regions where the proportion of \( C_{15} \) branched-chain components of the fatty acids deviated significantly from the trend established over the entire biokinetic growth range. In the region from 12 to 13°C there were significant deviations in both the percentage of i15:0 and the percentage of a15:0 together with a suggested deviation in a17:0, resulting in a significant change in the percentage of total branched-chain fatty acids. In the 31 to 33°C region the total percentage of branched-chain components changed significantly. These observed perturbations in fatty acid composition correlated with the estimated boundaries of the NPR as determined from the linear region of the Arrhenius growth rate plot. The goodness of fit of the fitted curves and the low standard deviation of the i17:0 data (Fig. 3) suggest that the variation observed in the proportions of other fatty acids was not a function of the experimental procedures.

To date, the most detailed study of \( L. \) monocytogenes fatty acid composition in relation to temperature was performed by Annous et al. (3). These workers reported fatty acid data for seven growth temperatures, two supraoptimal (42 and 45°C), one optimal (37°C), and four suboptimal (30, 20, 10, and 5°C). The reported trends in fatty acid composition were similar to those observed in the present study. In addition, the data of Annous et al. (3) illustrated that there were differences in the proportion of branched-chain fatty acids between strains and that medium composition had a marked effect on the proportion of a15:0 at low temperature for one strain but not for another strain. However, reliance on single culture determinations did not allow an assessment of reproducibility to be made. Some of the observations described by Annous et al. (3) may have been due to the inherent variation in fatty acid composition highlighted by this study. Future examinations of \( L. \) monocytogenes lipid composition will be required to consider this phenomenon.

The NPR has been described as the linear portion of the Arrhenius relationship for bacterial growth, and the boundaries of the NPR are defined at both high and low temperatures by deviation in the bacterial growth rate response from Arrhenius kinetics (16). However, the description of such a linear section may be subjective (14), and some authors argue
FIG. 3. Plots of *L. monocytogenes* fatty acid composition for cultures grown over the entire biokinetic temperature range in tryptone soya broth with yeast extract. (a) Total branched-chain fatty acids; (b) i15:0 fitted with a third-order polynomial curve ($r^2 = 0.93$); (c) a15:0 fitted with a second-order polynomial curve ($r^2 = 0.95$); (d) i17:0 fitted with a third-order polynomial curve ($r^2 = 0.99$); (e) a17:0 fitted with a third-order polynomial curve ($r^2 = 0.96$). The 12.5 and 31.3°C data points are highlighted (●). The data points represent the means of four replicates, and the bars indicate standard deviations.
that there are multiple linear regions in the growth rate response of L. monocytogenes (4). In this study we employed an independent method of linear regression to assess the NPR regions of the Arrhenius relationship for L. monocytogenes. The estimates of the resultant boundaries (12.7 and 27.7°C) may be considered surprising in relation to the broad cardinal temperatures of this bacterium.

Similar perturbations in the proportion of branched-chain fatty acids associated with the NPR boundaries of S. gelidimarina were revealed by close-interval sampling throughout the entire bio kinetic temperature range for growth (17). It was suggested that different primer specificities of β-ketoacyl-acyl carrier protein (β-ketoacyl-ACP) synthase enzymes may be responsible. In the case of Listeria it is also probable that the provision of branched-chain primer molecules plays a role. Branched-chain fatty acids are biosynthesized by the fatty acid synthase system with specific branched acyl primers. For example, a15:0 is synthesized from 2-methylbutyryl coenzyme A (2-methylbutyryl-CoA) as a chain primer (12). In Listeria 2-methylbutyryl-CoA is provided by branched-chain amino acid metabolism (2, 3) specifically, by diversion of the 2-keto-3-methylvalerate intermediate). Decarboxylation of 2-keto-3-methylvalerate by a branched-chain keto acid decarboxylase (19) yields the 2-methylbutyryl-CoA primer for a15:0 production by fatty acid synthase. However, Listeria is deficient in branched-chain amino acid anabolism and relies upon exogenous isoleucine for growth (3). In this case the 2-methylbutyryl-CoA used as a chain primer for the biosynthesis of a15:0 must be provided from the transamination of exogenous isoleucine by branched-chain amino acid aminotransferase (BcaT) (2, 3). Thus, two additional enzymes (BcaT and branched-chain keto acid decarboxylase) are critical for branched-chain fatty acid biosynthesis in Listeria and represent two other sites of temperature regulation which may provide a mechanism for the phenomena observed.

The important role of a15:0 in the low-temperature growth of L. monocytogenes has been highlighted previously (3, 8). This study revealed that the low-temperature variation observed in the percentage of a15:0 at 12.5°C resulted in a proportion equal to that found at a much higher growth temperature (ca. 23°C) (Fig. 3). While this phenomenon did not affect the growth rate (Fig. 1), it may point to the possibility of increased susceptibility to other membrane-perturbing effects at this temperature. Nisin, a polypeptide bacteriocin, is known to act by disruption of the cytoplasmic membrane (1, 6). While nisin resistance is described as a complex phenotype (6), resistant mutants have been reported to contain a lower proportion of branched-chain fatty acids and to differ in the proportion of phospholipid classes (6, 13, 23). Hence, the observed perturbations in L. monocytogenes fatty acid composition near the NPR boundaries are similar to those associated with nisin resistance.

Close-interval temperature sampling of L. monocytogenes fatty acid composition revealed significant patterns in the proportions of branched-chain components over the entire growth temperature range. The perturbations associated with the estimated boundaries of the NPR were similar to those described for the Antarctic psychrophile S. gelidimarina, which also contains an appreciable proportion of branched-chain fatty acids. This phenomenon may be related to the specific activity profile of enzymes associated with branched-chain fatty acid biosynthesis.

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