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Critical Role of Anteiso-$C_{15:0}$ Fatty Acid in the Growth of *Listeria monocytogenes* at Low Temperatures

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Listeria monocytogenes* is a food-borne pathogen capable of growth at refrigeration temperatures. Membrane lipid fatty acids are major determinants of a sufficiently fluid membrane state to allow growth at low temperatures. *L. monocytogenes* was characterized by a fatty acid profile dominated to an unusual extent (>95%) by branched-chain fatty acids, with the major fatty acids being anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, and iso-$C_{15:0}$ in cultures grown in complex or defined media at 37°C. Determination of the fatty acid composition of *L. monocytogenes* 10403S and SLCC 53 grown over the temperature range 45 to 5°C revealed two modes of adaptation of fatty acid composition to lower growth temperatures: (i) shortening of fatty acid chain length and (ii) alteration of branching from iso to anteiso. Two transposon Tn*917*-induced cold-sensitive mutants incapable of growth at low temperatures had dramatically altered fatty acid compositions with low levels of i-$C_{15:0}$, a-$C_{15:0}$, and a-$C_{17:0}$ and high levels of i-$C_{14:0}$, $C_{14:0}$, i-$C_{16:0}$, and $C_{16:0}$. The levels of a-$C_{15:0}$ and a-$C_{17:0}$ and the ability to grow at low temperatures were restored by supplementing media with 2-methylbutyric acid, presumably because it acted as a precursor of methylbutyryl coenzyme A, the primer for synthesis of anteiso odd-numbered fatty acids. When mid-exponential-phase 10403S cells grown at 37°C were temperature downshocked to 5°C they were able, for the most part, to reinitiate growth before the membrane fatty acid composition had reset to a composition more typical for low-temperature growth. No obvious evidence was found for a role for fatty acid unsaturation in adaptation of *L. monocytogenes* to cold temperature. The switch to a fatty acid profile dominated by a-$C_{15:0}$ at low temperatures and the association of cold sensitivity with deficiency of a-$C_{15:0}$ focus attention on the critical role of this fatty acid in growth of *L. monocytogenes* in the cold, presumably through its physical properties and their effects, in maintaining a fluid, liquid-crystalline state of the membrane lipids.

Lipids in biological membranes are usually maintained in the fluid, liquid-crystalline state, so that the main gel-to-liquid-crystalline phase transition temperature is below the environmental temperature (30). The correct physical state of membrane lipids is required for optimal membrane structure and function. Temperature markedly affects membrane lipid composition, and changes in lipid composition are thought to occur in order to maintain an appropriate amount of the liquid-crystalline state. The major way in which bacteria, which generally lack cholesterol, maintain this ideal membrane physical state is by changing their fatty acid composition (4, 23, 30). As the growth temperature decreases, lower-melting-point fatty acids are incorporated into lipids, which have lower phase transition temperatures. This has been described as a homeoviscous adaptation by Sinensky (27), where the ideal membrane lipid physical state is maintained through lipid changes in response to changes in growth temperature.

The low G+C gram-positive bacterium *Listeria monocytogenes* is a food-borne pathogen, and its ability to grow at refrigeration temperatures is an important aspect of its role as a food-borne pathogen. *L. monocytogenes* can grow at up to 45°C, giving it an unusually wide temperature range for a pathogen of warm-blooded animals (26). Different investigators have reported different minimum growth temperatures for *L. monocytogenes*, including just less than 0°C in chicken broth (31).

The fatty acids of *L. monocytogenes* are characterized by a high proportion of iso and anteiso, odd-numbered, branched-chain fatty acids (11, 22). Fatty acid changes in response to lowering of growth temperatures have been studied to a limited extent in *L. monocytogenes* (12, 21). Püttman et al. (21) studied the fatty acids of cells grown at 37, 20, and 4°C on solid medium. The proportion of a-$C_{17:0}$ was lower and that of a-$C_{15:0}$ was higher in cells grown at 4°C than at 37°C. Juneja and Davidson (12) did not differentiate between iso and anteiso fatty acids and reported that the proportion of branched $C_{17:0}$ was lower and that of branched $C_{15:0}$ was higher in cells grown on solid medium at 7°C than at 37°C.

Detailed analyses of the fatty acids of bacteria containing iso and anteiso branched fatty acids have revealed subtleties and complexities in the changes in fatty acids in response to lowering of the growth temperatures (30). Suutari and Laakso (30) have described three modes of adaptation in fatty acid composition in response to temperature: (i) the type of branching in the methyl end of the fatty acid, i.e., anteiso versus iso, (ii) fatty acid chain length, and (iii) the degree of fatty acid unsaturation. Anteiso fatty acids have lower melting points than iso fatty acids, and shortening fatty acid chain length or increasing unsaturation also results in lower-melting-point fatty acids. In

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TABLE 1. Effect of growth temperature on total fatty acid composition of *L. monocytogenes* 10403S and SLCC 53

Strain, medium, and growth temperature	% Total fatty acid													
	n12:0	i13:0	a13:0	i14:0	n14:0	i15:0	a15:0	i16:0	n16:1	n16:0	i17:0	a17:0	n18:1	n18:0
10403S														
Tryptic soy broth														
37°C	0.1	<0.1	0.1	0.7	0.4	13.0	40.7	3.4	0.1	2.7	5.6	31.7	0.5	1.0
5°C	1.3	0.8	1.9	2.6	1.4	17.2	65.7	1.1	ND[a]	0.5	0.7	6.2	ND	0.4
Defined medium														
Alone, 37°C	ND	ND	ND	ND	0.2	2.0	43.8	0.8	0.2	0.9	1.3	50.0	ND	0.5
Plus 1 mM glycine betaine, 37°C	ND	ND	ND	ND	0.3	2.1	45.7	0.8	ND	1.1	1.1	48.1	ND	0.6
Alone, 5°C	0.1	ND	1.4	0.2	0.3	1.8	73.0	0.3	ND	0.7	0.3	15.9	ND	0.6
Plus 1 mM glycine betaine, 5°C	0.1	ND	1.1	0.2	0.4	2.9	78.0	0.3	0.17	0.8	0.3	14.8	ND	0.5
SLCC 53														
Tryptic soy broth														
37°C	0.1	0.1	0.3	0.3	0.3	17.7	52.4	0.8	0.1	1.0	1.9	23.7	0.4	0.9
5°C	0.6	1.8	5.5	0.6	0.4	12.0	72.1	ND	ND	1.1	0.2	3.8	0.6	1.3
Defined medium														
Alone, 37°C	ND	ND	ND	ND	ND	2.4	44.3	0.4	ND	0.7	0.8	50.9	ND	0.6
Plus 1 mM glycine betaine, 37°C	ND	ND	ND	ND	ND	2.5	46.2	0.5	ND	0.6	0.7	49.0	ND	0.6
Alone, 5°C	ND	ND	1.1	ND	ND	1.6	78.9	ND	ND	0.5	ND	16.7	ND	0.5
Plus 1 mM glycine betaine, 5°C	ND	ND	1.8	ND	ND	2.1	85.4	ND	ND	0.5	ND	9.1	ND	0.5

[a] ND, not detected.

studies of *Bacillus subtilis* and *Bacillus megaterium* Suutari and Laakso (29) observed notable species-specific differences in the modes of regulation of fatty acid compositions in temperature adaptation.

We are interested in understanding the underlying mechanisms that allow growth of *L. monocytogenes* in the cold (2). In this study we demonstrated the occurrence of two modes of regulation of fatty acid composition in *L. monocytogenes* in response to decreases in the growth temperature. We have also studied the fatty acid composition of two transposon Tn*917*-induced cold-sensitive mutants. Our results focus attention on the critical role of a-$C_{15:0}$ in growth of *L. monocytogenes* in the cold.

MATERIALS AND METHODS

Strains, culture maintenance, and media. *L. monocytogenes* SLCC 53 was supplied by T. Chakraborty (16), and strains 10403S and DP-L910 (10403S carrying the Tn*917* plasmid pLTV3) were supplied by D. A. Portnoy (3). *L. monocytogenes* transposon mutants were generated by the method of Camilli et al. (3). Potential mutants were selected by plating onto brain heart infusion agar with erythromycin (1 μg/ml) and lincomycin (25 μg/ml). Mutants were scored as previously described (2). Southern hybridization according to the method of Sambrook et al. (25) was used to confirm that *L. monocytogenes cld-1* and *cdl-2* each contained a single insertion of Tn*917*. All cultures were maintained and routinely transferred on tryptic soy agar (Difco Laboratories, Detroit, Mich.). Complex media used were tryptic soy broth and brain heart infusion broth (Difco). Defined medium was that described by Pine et al. (20).

Inoculum development and general growth procedures. A 2% (vol/vol) aliquot of an overnight culture grown in the appropriate medium at 37°C was used to inoculate 25 ml of medium in 300-ml nephelometer flasks (Bellco). Growth was monitored by A_{580} measurements with a Bausch and Lomb Spectronic 20 spectrophotometer. Cultures were grown to mid-logarithmic phase (A_{580} = 0.5), harvested by centrifugation at 18,000 × *g* for 15 min at 4°C, and washed once with cold H_2O. Glycine betaine and 2-methylbutyric acid were added to the medium as filter-sterilized solutions at final concentrations of 1 mM and 100 μM, respectively.

Fatty acid analysis. The total fatty acids were extracted and methyl esterified from 30 to 40 mg (wet weight) of cells as described by Mayberry and Lane (18), using 1 M KOH at 55°C for 1 h followed by hydrolysis with 2 M HCl at 100°C. The MIDI microbial identification system (Microbial ID, Inc., Newark, N.J.) was used for separation, detection, and identification of the fatty acids. The system consisted of a 5890A gas-liquid chromatograph (Hewlett-Packard, Avondale, Pa.) equipped with a split/splitless injector, a flame ionization detector, and a 25-m by 0.2-mm Ultra 2 capillary column (Hewlett-Packard); an automatic sampler; an integrator; and a program which identifies the fatty acids (Microbial ID). The carrier gas (hydrogen) flow was adjusted to 80 ml/min, and the injector and detector temperatures were maintained at 250 and 300°C, respectively. Sample (2 μl) was injected in the split mode (ratio, 5:3) and the column temperature was raised from 170 to 270°C at a rate of 5°C/min.

All chemicals were analytical-grade reagents. Hydrogen (>99.9% pure) was purchased from Matheson Gas Co. (Joliet, Ill.). Glassware used in the fatty acid determinations was cleaned with Nochromix solution (Godax Laboratories, Inc., New York, N.Y.) and rinsed repeatedly with distilled water before use.

RESULTS

Effect of temperature on fatty acid composition of steady-state cultures in tryptic soy broth. Cells were grown to mid-exponential phase (A_{580} = 0.5) in tryptic soy broth at different temperatures, and their fatty acid compositions were analyzed. Cells were grown at 45, 42, 37, 30, 20, 10, and 5°C, and the culture mean generation times of strain 10403S at these temperatures were 0.8, 0.6, 0.6, 0.8, 1.8, 10.2, and 23.1 h, respectively. At 45°C growth ceased after 3 h. Lag phases of 24 h or more were noted in the cultures at 5 and 10°C. The fatty acid compositions of strains 10403S and SLCC 53 at 37 and 5°C are shown in Table 1. At 37°C, the major fatty acids in both strains were, in order, a-$C_{15:0}$, a-$C_{17:0}$, and i-$C_{15:0}$. Branched fatty acids represented 95 and 97% of the total fatty acids in strains 10403S and SLCC 53, respectively. At 5°C the major changes were a large decrease in a-$C_{17:0}$ content and a significant increase in a-$C_{15:0}$. Also, there was a general shortening of the fatty acids in the profile: C_{12} to C_{15} fatty acids increased and C_{16} to C_{18} fatty acids decreased at 5°C. Similar changes in fatty acid composition were seen in cells grown to late exponential phase (data not shown).

The changes in the three major fatty acids with temperature are shown in Fig. 1a and b. In both strains, a-$C_{17:0}$ levels declined over the entire temperature range studied. From 45 to 20°C, levels of i-$C_{15:0}$ increased and those of a-$C_{15:0}$ did not change much. However, below 20°C the percentage of a-$C_{15:0}$ increased significantly and that of i-$C_{15:0}$ decreased slightly. Thus, two modes of fatty acid change were seen over the

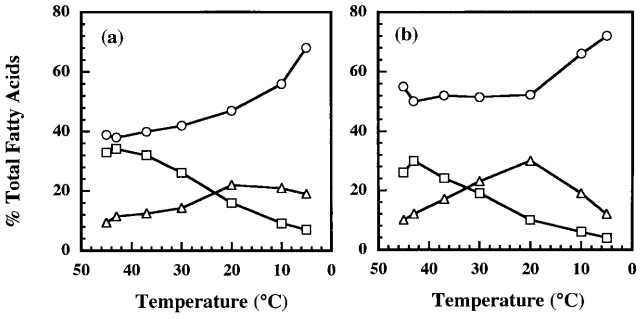

FIG. 1. Effect of growth temperature on the composition of the major fatty acids of *L. monocytogenes* 10403S (a) and SLCC 53 (b). Cells were grown to mid-exponential phase at the indicated temperatures in tryptic soy broth. Symbols: ○, a-$C_{15:0}$; □, a-$C_{17:0}$; △, i-$C_{15:0}$.

temperature range 45 to 5°C. From 45 to 20°C, there was a shortening of fatty acids, but from 20 to 5°C, fatty acid shortening and continued the branching switched from iso to anteiso branching.

Effect of temperature and glycine betaine on fatty acid composition of steady-state cultures in defined medium. The fatty acid compositions of mid-exponential-phase cells of both strains grown in defined medium at 37 and 5°C are shown in Table 1. In both strains 10403S and SLCC 53, branched-chain fatty acids represented 98 and 99%, respectively, of the total fatty acids. Strain 10403S grown in defined medium at 37°C contained 44% a-$C_{15:0}$, 50% a-$C_{17:0}$, and 2% i-$C_{15:0}$, compared to 41% a-$C_{15:0}$, 32% a-$C_{17:0}$, and 13% i-$C_{15:0}$ in tryptic soy broth-grown cells. Hence, cells had less iso odd-numbered fatty acid and more anteiso odd-numbered fatty acid in defined medium than in tryptic soy broth. At low temperatures, a-$C_{15:0}$ was an extremely prominent (70% or more) component of the fatty acid profile of both strains. Inclusion of the cryoprotectant glycine betaine (14) in the medium appeared to lead to a further small decrease in a-$C_{17:0}$ levels and an increase in a-$C_{15:0}$ levels (Table 1).

Two cold-sensitive mutants are deficient in odd-numbered branched-chain fatty acids. (i) Brain heart infusion broth-grown cells. In a previous report from this laboratory two cold-sensitive *L. monocytogenes* mutants that do not appear to be impaired for cold shock protein induction were described (2). The fatty acid compositions of *cld-1* and *cld-2* mutant strains were compared with those of the parent 10403S grown in brain heart infusion broth at 37°C. Brain heart infusion broth was used for these studies because somewhat better growth of the mutants was obtained in this medium than in tryptic soy broth. The mutants had strikingly different fatty acid compositions from the parent. At 37°C, the major fatty acids in the mutants were the even-numbered straight-chain and iso branched-chain fatty acids (i-$C_{14:0}$, $C_{14:0}$, i-$C_{18:0}$, and $C_{16:0}$), whereas odd-numbered branched-chain fatty acids were the predominant fatty acids in the parent (97% of the total) (Fig. 2a). Even-numbered straight-chain and branched-chain fatty acids made up 83 and 91% of the total fatty acids in *cld-1* and *cld-2* mutants, respectively, versus about 6% in the parent strain.

The *cld* mutants appear to be deficient in odd-numbered branched-chain fatty acids, in particular a-$C_{15:0}$ and a-$C_{17:0}$. Anteiso fatty acids are biosynthesized from isoleucine (Fig. 3). After uptake, the branched-chain amino acids participate in transamination reactions, producing 2-keto-3-methylvaleric acid in the case of isoleucine. 2-Keto-3-methylvaleric acid is then decarboxylated by branched-chain keto acid dehydrogenase to produce 2-methylbutyryl coenzyme A (CoA), which acts as a primer molecule for anteiso fatty acids (13). It appears that *cld-1* and *cld-2* strains are defective in production of anteiso odd-numbered fatty acid primer 2-methylbutyryl-CoA. Consequently, the ability of 2-methylbutyric acid to restore levels of a-$C_{15:0}$ and a-$C_{17:0}$ was tested in the *cld* mutants, and the results of this experiment are shown in Fig. 2b. Inclusion of 100 μM 2-methylbutyric acid in the medium restored the levels

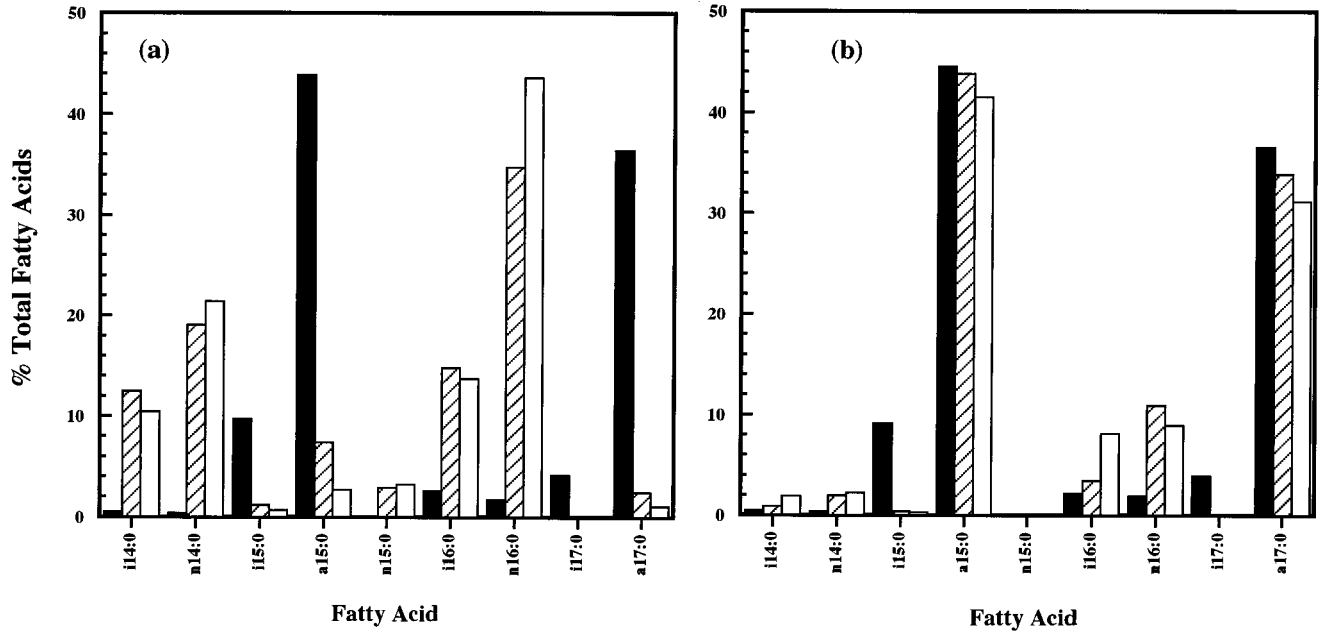

FIG. 2. Fatty acid composition of *L. monocytogenes* 10403S (■) and cold-sensitive *cld-1* (▨) and *cld-2* (□) mutants and the effect of 2-methylbutyric acid on fatty acid composition. Cells were grown to mid-exponential phase in brain heart infusion broth in the absence (a) and presence (b) of 2-methylbutyric acid at 37°C.

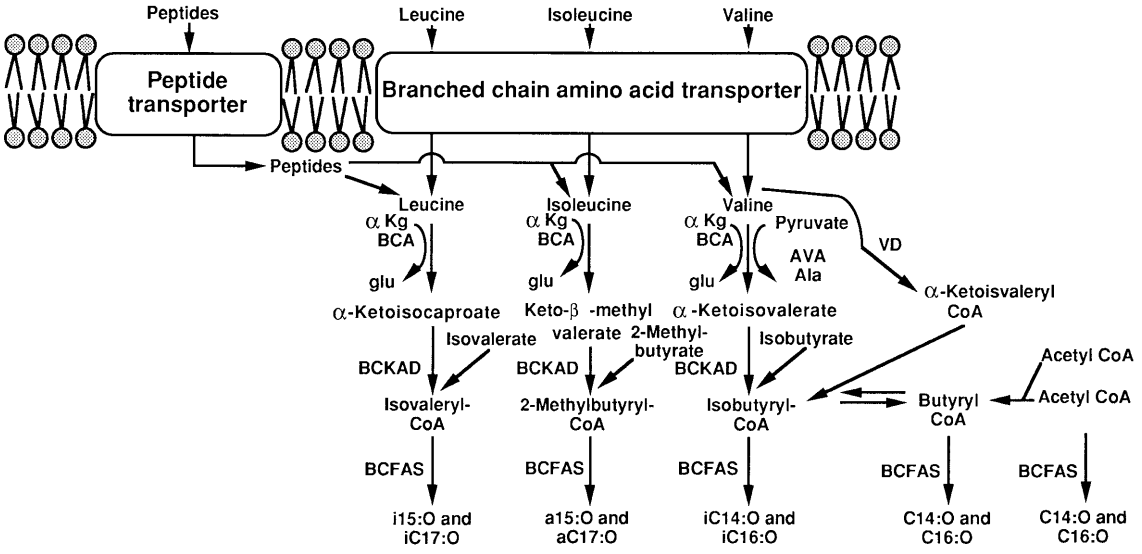

FIG. 3. Hypothetical scheme of transport and metabolism related to branched- and straight-chain fatty acid biosynthesis. This figure was based on data from references 1, 6, 13, 14, and 32. BCA, branched-chain amino acid aminotransferase; AVA, alanine-valine aminotransferase; VD, valine dehydrogenase; BCKAD, branched-chain α-keto-acid dehydrogenase; BCFAS, branched-chain fatty acid synthetase; αKg, α-ketoglutarate.

of a-$C_{15:0}$ and a-$C_{17:0}$ in *cld-1* and *cld-2* mutants to levels similar to those present in the parent strain 10403S. 2-Methylbutyric acid had no impact on the fatty acid composition of strain 10403S.

(ii) Defined-medium-grown cells. The fatty acid compositions of 10403S and *cld-1* and *cld-2* mutants grown in defined medium in the presence and absence of 100 μM 2-methylbutyric acid are shown in Table 2. Defined-medium-grown *cld-1* and *cld-2* cells were less deficient in a-$C_{15:0}$ and a-$C_{17:0}$ fatty acids than cells grown in brain heart infusion broth. Nevertheless, they had lower levels of these fatty acids than strain 10403S and elevated levels of $C_{14:0}$ and $C_{16:0}$. Under these conditions, the cold-sensitive mutants showed less increase in i-$C_{14:0}$ and i-$C_{16:0}$ levels than when they were grown in brain heart infusion broth. Inclusion of 2-methylbutyric acid in defined medium restored the levels of a-$C_{15:0}$ and a-$C_{17:0}$ to approximately those of the wild type (Table 2).

2-Methylbutyric acid restores the ability of the cold-sensitive mutants to grow at low temperatures. The *cld* mutants grew poorly at 20°C in brain heart infusion broth, reaching A_{580} values of 0.25 (*cld-1*) and 0.18 (*cld-2*) only after 120 h. Supplementation of cultures with 100 μM 2-methylbutyric acid not only increased the content of a-$C_{15:0}$ and a-$C_{17:0}$ at 20°C but also improved the growth of the cultures, restoring normal growth rates and resulting in final culture turbidities similar to those of the parent strain (data not shown). The influence of 100 μM 2-methylbutyric acid on growth at 10°C was analyzed in detail (Fig. 4). Starter inocula were grown overnight in brain heart infusion broth without 2-methylbutyric acid at 37°C and used to inoculate medium with and without 2-methylbutyric acid at 10°C. The parent strain 10403S initiated growth after a lag phase and grew normally (Fig. 4a). The lengths of lag phase, growth rates, and final culture turbidities were essentially identical in the presence and absence of 2-methylbutyric acid. Growth of *cld-1* and *cld-2* mutants in the absence of 2-methylbutyric acid was exceedingly slow (Fig. 4a). However, inclusion of 2-methylbutyric acid in the medium allowed both strains to grow (Fig. 4a). The lag phases of the mutants were

TABLE 2. Fatty acid composition of *L. monocytogenes* 10403S and cold-sensitive *cld-1* and *cld-2* mutants grown in defined medium

Strain and growth conditions	% Total fatty acid							
	i14:0	n14:0	i15:0	a15:0	i16:0	n16:0	i17:0	a17:0
10403S								
37°C	ND	ND	2.4	47.7	1.1	1.1	1.1	46.6
37°C + 100 μM 2-methylbutyrate	ND	ND	2.3	45.1	0.8	1.2	1.2	49.4
20°C	ND	0.2	3.9	56.9	ND	0.7	0.7	37.6
cld-1 mutant								
37°C	1.3	12.4	2.3	32.1	2.4	34.5	<1.0	15.0
37°C + 100 μM 2-methylbutyrate	<1.0	<1.0	<1.0	46.9	<1.0	4.7	ND	48.4
20°C	1	15.8	4.5	48.5	ND	15.8	0.5	13.9
cld-2 mutant								
37°C	1.0	3.0	6.9	38.6	3.4	16.9	3.3	26.9
37°C + 100 μM 2-methylbutyrate	<1.0	<1.0	3.9	46.2	<1.0	3.3	1.6	45.0
20°C	0.3	4.7	8.3	48.8	ND	11.3	1.9	24.7

[a] ND, not detected.

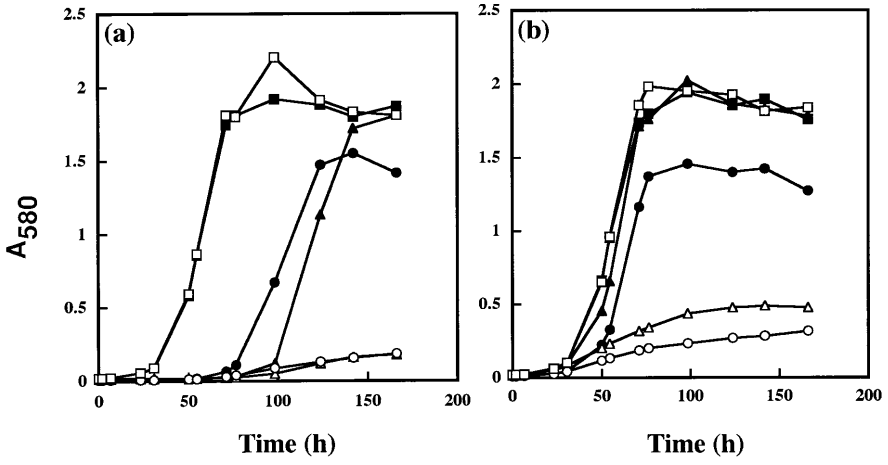

FIG. 4. Influence of 2-methylbutyric acid on growth of the cold-sensitive mutants at low temperatures. The starter inoculum was grown overnight in brain heart infusion broth at 37°C in the absence (a) and presence (b) of 2-methylbutyric acid and used to inoculate brain heart infusion broth with (filled symbols) and without (open symbols) 2-methylbutyric acid, incubated at 10°C. ○ and ●, *cld-1* mutant; □ and ■, 10403S; △ and ▲, *cld-2* mutant.

longer than that of the parent strain, but the growth rates and final turbidities of the cultures were not a great deal less than those of the parent strain under these conditions. Starter cultures also were grown with 100 μM 2-methylbutyric acid to investigate subsequent effects on strain growth at 10°C in the presence and absence of 100 μM 2-methylbutyric acid (Fig. 4b). Growth of *cld-1* and *cld-2* starter cultures with 2-methylbutyric acid, which corrects the fatty acid composition (Fig. 2), dramatically reduced the lag phase of the strains in medium containing 2-methylbutyric acid at 10°C (Fig. 4b) compared to non-2-methylbutyric acid-grown starter cultures (Fig. 4a). Starter cultures of *cld-1* and *cld-2* mutants grown with 2-methylbutyric acid and used to inoculate medium lacking 2-methylbutyric acid at 10°C were able to grow slowly, until growth appeared to cease at low culture density.

The *cld* mutants grew well in defined medium at 20°C, and their fatty acid compositions under these conditions are shown in Table 2. Good growth at this temperature is consistent with the larger amounts of odd-numbered anteiso fatty acids in defined-medium-grown cells than in those grown in brain heart infusion broth.

Effect of cold shock in tryptic soy broth on fatty acid composition. Several proteins known as cold shock proteins are induced when *L. monocytogenes* is cold shocked from 37 to 10 or 5°C, and there is a lag before growth is reinitiated (2). It was of interest to study changes in fatty acid composition in response to cold shock. Mid-exponential-phase cultures were chilled rapidly from 37 to 5°C, and samples were taken at intervals for determination of fatty acid composition.

The growth of strain SLCC 53 is shown in Fig. 5. The A_{580} of the culture increased from 0.44 to 0.48 from 0 to 4 h and then entered a lag phase where A_{580} increased from 0.48 to only 0.52 from 4 to 24 h before a new exponential growth rate was established. The fatty acid composition changed slowly over time (Fig. 5). By 48 h, when growth was well under way, a-$C_{17:0}$ had decreased from 22% at time zero to 10%, a-$C_{15:0}$ had increased from 51 to 63%, and i-$C_{15:0}$ had decreased from 19 to 14%.

However, when the same experiment was performed with strain 10403S the culture did not establish growth upon temperature downshock in tryptic soy broth. The fatty acid profile showed essentially no change over the course of the experiment. This strain has a lower ratio of i+a-$C_{15:0}$ to i+a-$C_{17:0}$ than strain SLCC 53 when grown at 37°C (1.63 versus 2.5), and whereas this ratio increased about fivefold in strain SLCC 53 over 4 days, there was only a minor increase in this ratio in strain 10403S. Additionally, these results indicate that high cell density has a negative impact on cold adaptation in strain 10403S, because cultures initiated from a small inoculum,

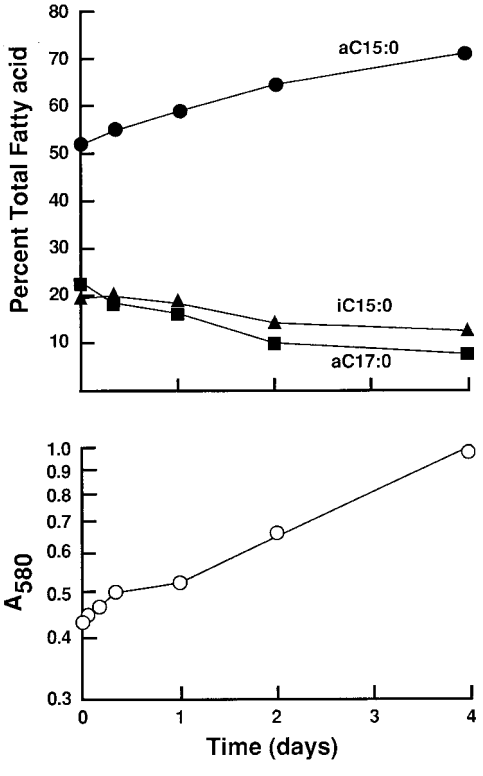

FIG. 5. Effect of cold shock on fatty acid composition. Mid-exponential-phase cultures of strain SLCC 53 grown in tryptic soy broth at 37°C were downshocked to 5°C, and samples were taken for A_{580} and fatty acid determinations at intervals.

TABLE 3. Effect of cold shock on fatty acid composition of *cld-1* strain

Conditions[a]	% Total fatty acid								
	i14:0	n14:0	i15:0	a15:0	n15:0	i16:0	n16:0	a17:0	n18:0
Exponential phase, 37°C	11.8	19.0	0.6	2.7	3.2	15.6	44.5	0.9	2.1
Cold shocked at 20°C for 1 h	11.8	17.2	1.3	6.5	2.9	15.7	40.0	3.0	1.6

[a] Cells were grown in brain heart infusion broth at 37°C.

which are thus also downshocked, grew and established steady-state growth (Table 1).

When mid-exponential-phase cultures of either strain grown in defined medium were cold shocked to 5°C, the cultures were able to reestablish growth after a lag phase, before there was much alteration in fatty acid composition (data not shown). This indicates that the fatty acid composition of cells grown at 37°C in defined medium (Table 1) is adequate for growth to occur before the fatty acid composition adjusts to one more ideal for a particular temperature in long-term steady-state cultures.

Failure to demonstrate a role for desaturation of *L. monocytogenes* fatty acids in cold adaptation. A fatty acid desaturating system is induced in *Bacillus* species by growth at low temperatures, particularly in response to temperature downshock (5, 8). We found no evidence for increased production of unsaturated fatty acids in steady-state or cold-shocked cultures of *L. monocytogenes* in complex medium (Fig. 5) or defined medium (data not shown). Straight-chain fatty acids ($C_{16:0}$ and $C_{14:0}$) are major components of the fatty acid profile of the *cld-1* strain grown in brain heart infusion broth. This strain appeared to offer an increased possibility for detecting any desaturase activity resulting in production of $C_{16:1}$ and $C_{14:1}$ that might be induced by cold shock. However, we were unable to detect any evidence of unsaturation of fatty acids in *cld-1* or *cld-2* cultures cold shocked at 20 or 5°C (Table 3).

Southern analysis of *L. monocytogenes* 10403S and *cld-1* and *cld-2* mutants. DNA isolated from *L. monocytogenes* cold-sensitive *cld-1* and *cld-2* mutants was analyzed by Southern hybridization to determine if each mutant carried a single insertion of the transposon. Digesting the chromosomal DNA with *Xba*I restricts within the transposon and in the DNA region flanking the transposon. Probe DNA hybridized to a single band of approximately 16 kb in each of the *L. monocytogenes* cold-sensitive mutants but did not hybridize to the *L. monocytogenes* 10403S DNA (data not shown). In each of the cold-sensitive mutants, the probe hybridized to a slightly different-size band. This indicates that in the two mutants, the insertion is in a different location.

DISCUSSION

Determination of the fatty acid composition of exponential-phase *L. monocytogenes* cultures grown at different temperatures in tryptic soy broth revealed two modes of change in fatty acid composition as temperature decreased. These modes of change were as follows: (i) fatty acid shortening over the entire range of temperatures studied (45 to 5°C), i.e., a decrease in a-$C_{17:0}$, and (ii) switch in branching from iso to anteiso, i.e., i-$C_{15:0}$ to a-$C_{15:0}$, from 20 to 5°C. These results confirm and extend those of others (12, 21). No mechanism whereby an existing a-$C_{17:0}$ fatty acid can be shortened by 2 carbon units is known. Fatty acid synthetase elongates fatty acids by 2 carbon units, and its activity of adding 2 carbon units to the a-$C_{15:0}$ intermediate is possibly inhibited at low temperatures. In *Micrococcus cryophilus*, a fatty acid elongase is temperature regulated and plays a role in determining fatty acid chain length in response to temperature (23).

When cells were grown in defined medium at low temperatures, a-$C_{15:0}$ became an even more prominent component of the fatty acid profile than in cells grown in tryptic soy broth at the same temperature. Switching from i-$C_{15:0}$ to a-$C_{15:0}$ fatty acid requires a switch in utilization of fatty acyl primers. The primer used for a-$C_{15:0}$ synthesis is the CoA ester of 2-methylbutyric acid, which is derived from isoleucine, and that for i-$C_{15:0}$ synthesis is the CoA ester of isovaleric acid derived from leucine (Fig. 3). Temperature in some way appears to alter the primer selection specificity of the fatty acid synthetase. Nichols and Russell (19) have described changes in utilization of fatty acid primer molecules in an Antarctic psychrophilic bacterium as a response to environmental stress. Alternatively, it is possible that alterations in primer pools may occur in response to lower temperature.

The fatty acid compositions of the two cold-sensitive mutants were strikingly different from that of the parent strain. The major fatty acids of the *cld* mutants were even-numbered iso and straight-chain fatty acids, in contrast to the odd-numbered branched-chain fatty acids of the parent strain. It appears that the *cld* mutants are deficient in the production of the primer of anteiso odd-numbered fatty acids, i.e., 2-methylbutyryl-CoA. Addition of 2-methylbutyric acid to culture medium restored the levels of the anteiso fatty acids to those of the wild type and restored the ability of the mutants to grow at low temperatures. Preadaptation of the *cld* mutants by culturing them in the presence of 2-methylbutyric acid allowed them to initiate growth more rapidly when shifted to low temperatures. This is presumably because the anteiso fatty acids in the membrane allow a better initial adaptation to low temperatures. The production of even-numbered iso branched fatty acids and straight-chain unsaturated fatty acids was increased to compensate for the deficiency in odd-numbered branched fatty acids. It seems possible that the mutants are impaired in branched-chain aminotransferase activity which converts the branched-chain amino acids to the corresponding α-keto branched-chain carboxylic acids (Fig. 3). Such mutants would be able to synthesize the valine-derived fatty acids, and alternative pathways to isobutyryl-CoA not involving branched-chain aminotransferase via alanine-valine aminotransferase or valine dehydrogenase may operate (Fig. 3). Alternatively, the mutants could be impaired in branched-chain keto acid dehydrogenase activity. The valine-derived fatty acids could be produced via α-ketoisovaleryl-CoA produced by valine dehydrogenase activity. Pyruvate dehydrogenase has some activity in converting branched-chain keto acids to their CoA derivatives (13), and it may function as an alternative pathway if branched-chain keto acid dehydrogenase activity is impaired. Another alternative would be the formation of isobutyryl-CoA by isomerization of butyryl-CoA.

The precursor for straight-chain even-numbered fatty acids in bacteria with a preponderance of branched-chain fatty acids is not entirely clear (13). Acetyl-CoA is a poorly used primer

for branched-chain fatty acid synthetase, but the acetyl-acyl carrier protein derivative serves as an excellent primer for straight-chain fatty acids. Butyryl-CoA is also an excellent primer of straight-chain fatty acids for branched-chain fatty acid synthetase.

The deficiency in odd-numbered branched-chain fatty acids is more pronounced in the *cld* mutants grown in brain heart infusion broth than defined medium. It is possible that this might be related to the pool levels of amino acids. Strains of *L. monocytogenes* typically require several amino acids for growth, including leucine, isoleucine, and valine (26). In defined medium, the amino acids are supplied as individual amino acids and presumably enter the cell via branched-chain amino acid carrier activity (28) (Fig. 3). In brain heart infusion broth, peptone supplies significant amounts of amino acids in the form of peptides which are believed to be transported in by a separate transporter and then hydrolyzed to individual amino acids in the cytoplasm (1).

Cloning and sequencing of the DNA flanking the transposon in either of the mutants would be expected to provide insight into the affected gene. However, repeated attempts to clone directly the transposon-flanking DNA (3) have been unsuccessful. Possibly genes that are toxic to *Escherichia coli* are present.

We were unable to detect a role for fatty acid desaturase activity in production of unsaturated fatty acids in temperature downshocked wild type or *cld* mutants in defined or complex media. This is in contrast to the situation in species of *Bacillus* (5, 8). Previous studies have generally reported low levels of unsaturated fatty acids in the lipids of *L. monocytogenes*. However, Mastronicolis et al. (17) recently reported that a *L. monocytogenes* phosphoamino lipid contained 46% unsaturated fatty acid.

In the cold shock experiments, it was noted that high cell density seemed to have a negative impact on the ability of strain 10403S to reestablish growth in tryptic soy broth. The cells clearly initiated growth at cold temperatures at the low cell densities typical of inoculation into culture medium (2% [vol/vol] inoculum). Possibly some sort of quorum sensing (24) occurs, with a negative effect on growth at low temperatures at high cell densities. Interestingly, the signaling molecules in quorum sensing in gram-negative bacteria contain a fatty acid as part of their structure (24). In *Myxococcus xanthus*, *esg* mutants are defective in production of a signal in cell-cell communication (7). The *esg* locus encodes components of branched-chain keto acid dehydrogenase involved in branched-chain fatty acid production in this organism. It is proposed that branched-chain fatty acids constitute the E-signal transmitted among *M. xanthus* cells to complete development.

The experiments described herein focus attention on a-$C_{15:0}$ as playing a critical role in the growth of *L. monocytogenes* at low temperatures. This fatty acid becomes the major fatty acid in cold-grown organisms in both complex and defined media. The *cld* mutants which are deficient in odd-numbered branched-chain fatty acids have significantly higher minimum growth temperatures than the wild type. The phase transition temperature of phosphatidylcholine containing a-$C_{15:0}$ (−13.9°C) is significantly lower than those of phosphatidylcholine containing a-$C_{17:0}$ (9.2°C), i-$C_{15:0}$ (−7.0°C), or $C_{16:0}$ (40°C) (30). Anteiso fatty acids occupy significantly larger cross-sectional areas than either normal straight-chain saturated fatty acids or iso fatty acids (34), thus disrupting close packing of fatty acyl chains and imparting greater fluidity to the membrane. Haest et al. (9), using wide-angle X-ray diffraction spectra of *B. subtilis* and *Staphylococcus aureus* membranes, observed a wide (4.3-Å) reflection at low temperatures, indicating perturbation of lipid order due to the large amounts of branched-chain fatty acids in these membranes. An *E. coli* unsaturated fatty acid auxotroph can be grown on branched-chain fatty acids (15). Freeze-fracture electron microscopy of *E. coli* cells grown on a mixture of *B. subtilis* fatty acids revealed no segregation of proteins at low temperatures due to perturbation of the lipid order by the branched-chain fatty acids. This was in contrast to the segregation of proteins observed in *E. coli* grown with a-$C_{19:0}$ fatty acid, which does not perturb lipid order at low temperatures.

The physical properties of a-$C_{15:0}$ probably impart a membrane fluidity over a wide temperature range. Defined-medium-grown cells, with their high content of anteiso fatty acids, were able to resume growth after temperature downshock before the membrane fatty acid composition had reset to a composition ideal for the particular temperature, as attained in steady-state cultures grown at low temperatures. Ko et al. (14) have described a cold-activated transport system for the osmoprotectant and cryoprotectant glycine betaine. Evidently the membrane fatty acid composition established by growth of cells at 37°C provides a physical environment within which the glycine betaine transport system can function, even at low temperatures.

The *cld* mutants compensate for their deficiency of odd-numbered branched-chain fatty acids by incorporating increased proportions of even-numbered normal and iso branched fatty acids. At low temperatures the membrane probably assumes a gel-like state which detrimentally affects cell function, thus preventing growth. In this connection, the *cld* mutants incorporated L-[^{35}S]methionine into proteins upon cold shock at about half the rate of the parent strain (2). The mutants provide strong evidence for a critical role for a-$C_{15:0}$ in growth of *L. monocytogenes* in the cold.

Willecke and Pardee (34) described a mutant of *B. subtilis* lacking branched-chain keto acid dehydrogenase activity, created in a mutant already lacking pyruvate dehydrogenase activity. The mutant required short branched-chain fatty acids for growth, for conversion to primers of fatty acid biosynthesis. The fatty acid composition of the mutants could be manipulated by feeding them different fatty acid precursors. However, no results of the effect of lower growth temperature on the mutant were reported.

It is suggested that in *L. monocytogenes*, a-$C_{15:0}$ coupled with unusually low amounts of straight-chain saturated fatty acids plays a critical role in providing an appropriate degree of membrane fluidity for growth at low temperatures. *B. subtilis* and *S. aureus* are closely related to *L. monocytogenes*, and both these organisms contain large amounts of iso and anteiso fatty acids (4, 33). However, both organisms contain much larger amounts of straight-chain fatty acids than *L. monocytogenes* and have higher minimum growth temperatures. The data of Jackson and Cronan (10) indicate that *E. coli* can grow with a substantial amount of its lipids in the ordered state, although growth ceases if more than 55% of the lipids are ordered. Clearly, studies of the order/disorder status and of the gel-to-liquid-crystalline transition temperatures of *L. monocytogenes* membrane lipids would be of great interest. These studies also suggest that an agent interfering with the biosynthesis of a-$C_{15:0}$ may be useful in controlling the growth of *L. monocytogenes* in refrigerated foods.

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