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Electron paramagnetic resonance studies of the membrane fluidity of the foodborne pathogenic psychrotroph *Listeria monocytogenes*

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Abstract

Listeria monocytogenes is a foodborne psychrotrophic pathogen that grows at refrigeration temperatures. Previous studies of fatty acid profiles of wild-type and cold-sensitive, branched-chain fatty acid deficient mutants of *L. monocytogenes* suggest that the fatty acid 12-methyltetradecanoic (*anteiso*-$C_{15:0}$) plays a critical role in low-temperature growth of *L. monocytogenes*, presumably by maintaining membrane fluidity. The fluidity of isolated cytoplasmic membranes of wild-type (SLCC53 and 10403S), and a cold-sensitive mutant (*cld-1*) of *L. monocytogenes*, grown with and without the supplementation of 2-methylbutyric acid, has been studied using a panel of hydrocarbon-based nitroxides (2N10, 3N10, 4N10, and 5N10) and spectral deconvolution and simulation methods to obtain directly the Lorentzian line widths and hence rotational correlation times (τ_c) and motional anisotropies of the nitroxides in the fast motional region. τ_c values over the temperature range of −7°C to 50°C were similar for the membranes of strains SLCC53 and 10403S grown at 10°C and 30°C, and for strain *cld-1* grown with 2-methylbutyric acid supplementation (which restores branched-chain fatty acids) at 30°C. However, strain *cld-1* exhibited a threefold higher τ_c when grown without 2-methylbutyric acid supplementation (deficient in branched-chain fatty acids) compared to strains SLCC53, 10403S, and supplemented *cld-1*. No evidence was seen for a clear lipid phase transition in any sample. We conclude that the fatty acid *anteiso*-$C_{15:0}$ imparts an essential fluidity to the *L. monocytogenes* membrane that permits growth at refrigeration temperatures.

Keywords: Electron paramagnetic resonance; Membrane fluidity; Simulation; Spectral deconvolution; Rotational correlation time; Branched-chain fatty acid; (*Listeria monocytogenes*)

1. Introduction

Psychrophilic and psychrotrophic bacteria can often grow at temperatures as low as 0°C, and are of considerable importance as agents of food spoilage and foodborne disease. The ability to grow at low temperature has profound effects on microbial cell structure and function, involving the structural integrity of macromolecules, macromolecular assemblies, protein synthesis and nutrient uptake [1,2]. Major themes in studies of growth at low temperature include cold-induced gene expression and protein synthesis, ribosome function, and membrane fluidity at low temperatures.

Listeria monocytogenes is a psychrotrophic bacterium that is the causative agent of listeriosis, a dis-

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ease which encompasses meningoencephalitis, meningitis, focal infections in the immunocompromised, and stillbirths and neonatal sepsis from infection of pregnant women [3]. *L. monocytogenes* was a newly recognized foodborne pathogen in the 1980s causing both common source and sporadic disease often associated with the consumption of refrigerated food. The fatality rate from listeriosis is about 30%. Over the last several months in the United States there has been a widespread outbreak of listeriosis resulting in several fatalities, associated with hot dogs and other meat products, as well as several product recalls due to *L. monocytogenes* contamination [4].

Psychrophiles have optimum growth temperatures around 15°C and cannot grow above 25°C, whereas psychrotrophs have higher optimum and maximum growth temperatures. It has been reported that *L. monocytogenes* can grow at just less than 0°C in chicken broth [5]. This ability of the organism to grow at low temperatures, where many other bacteria cannot grow, means that refrigeration can result in the enrichment of *L. monocytogenes* in foods. Indeed, foods that pose the highest risk as a source of *L. monocytogenes* infection include those that are ready to eat and stored at refrigeration temperatures for long periods [6].

In order for growth to occur at low temperatures, the cytoplasmic membrane must retain sufficient fluidity to ensure the necessary physical state required for membrane structure and function [7]. Membrane lipid fatty acid composition plays a major role in determining membrane fluidity, especially in bacteria, which typically lack sterols in their membranes [8,9]. We have recently shown that the fatty acid *anteiso*-$C_{15:0}$ plays an important role in the ability of *L. monocytogenes* to grow at low temperatures [10]. The fatty acid composition of *L. monocytogenes* is dominated to an unusual extent by branched-chain fatty acids, and at low temperatures *anteiso*-$C_{15:0}$ becomes the major fatty acid. Furthermore, two cold-sensitive mutants that are deficient in branched-chain fatty acids are severely impaired in their abilities to grow at low temperature [10]. Jones et al. [11] reported that *L. monocytogenes* had a lower proportion of *anteiso*-$C_{17:0}$ and a higher proportion of *anteiso*-$C_{15:0}$ and short chain fatty acids when grown in continuous culture at 10°C compared to 30°C. Mastronicolis et al. [12] reported that *anteiso*-$C_{15:0}$ increased in all lipid classes in *L. monocytogenes* grown at 6°C. However, although the proportion of *anteiso*-$C_{15:0}$ increases with decreasing growth temperature, we found that *L. monocytogenes* grown at 37°C could reinitiate growth at 5°C before major alterations in the proportions of fatty acids had occurred [10]. This implies that the cytoplasmic membrane of *L. monocytogenes* grown at 37°C is sufficiently fluid to allow growth at lower temperatures. To date, no study has correlated fatty acid composition with determinations of membrane fluidity in *L. monocytogenes*.

Given the above, it is clear that verification of the relationship between membrane fluidity and fatty acid composition of the *L. monocytogenes* membrane is called for. In this study, four nitroxide spin labels (Fig. 1) were used to obtain rotational correlation times, τ_c, and motional anisotropies of the nitroxides at different depths of the *L. monocytogenes* cytoplasmic membrane. These parameters were measured as a function of temperature in two wild-type strains (SLCC53 and 10403S) and *cld-1*, a 10403S mutant strain deficient in branched-chain fatty acid synthesis, grown at 10°C and 30°C.

2. Materials and methods

2.1. Chemicals

All chemicals were reagent grade. Ethanol was obtained from Aldrich. Water (18 MΩ resistance or greater) was obtained from a Barnstead Nanopure water purification system.

2.2. Nitroxides

The nitroxides 2N10, 3N10, 4N10, and 5N10 were kindly provided by Dr. Wayne Zeller of Illinois State University and were synthesized by the method of Keana et al. [13].

2.3. Bacterial strains and growth conditions

L. monocytogenes strains SLCC53, 10403S, and the cold-sensitive mutant *cld-1* [10,14] were grown in brain heart infusion broth (Difco) with shaking (200 rpm) at various temperatures. When required,

2-methylbutyric acid was filter-sterilized and added to cultures to a final concentration of 100 μM.

2.4. Membrane preparation

Three 1-l cultures of *L. monocytogenes* in 2-l Erlenmeyer flasks were grown at 10°C or 30°C to early stationary phase (OD_{580} of about 2.5). The cells were harvested by centrifugation (15 344 × *g*, 5 min, 4°C) and washed by resuspension and centrifugation (15 344 × *g*, 5 min, 4°C) in 50 mM Tris–HCl, pH 7.2. The cells were broken by passage through a French pressure cell at 16 000 lb/inch^2. After breakage, the suspension was placed on ice and 1 mg DNase (Sigma) was added to the broken suspension for 5–10 min to reduce viscosity. Unbroken cells and cell walls were removed by centrifugation (15 344 × *g*, 10 min, 4°C). Membranes were recovered from the supernatant by ultracentrifugation (66 226 × *g* average, 30 min, 4°C) which yielded a yellowish translucent pellet. The membranes were washed twice by resuspension in ice-cold 50 mM Tris–HCl (pH 7.2) containing 5 mM $MgCl_2$ followed by centrifugation as above (two washes were necessary to remove residual respiratory activity which reduced the nitroxide spin labels). The membranes were resuspended in 500 μl of Tris–HCl–$MgCl_2$ buffer and kept at 4°C. Membrane protein concentration was estimated using the BioRad DC Protein Assay (BioRad). Membrane suspensions were adjusted to 50 mg protein/ml for EPR spectroscopy.

2.5. Fatty acid analysis

Total fatty acids were analyzed by gas–liquid chromatography according to the method of Mayberry and Lane [15] as described by Annous et al. [10].

2.6. Instrumentation

All EPR experiments were performed on a JEOL JES-TE200 spectrometer equipped with a Platform-Independent Data Acquisition Module and EWWIN 4.0 (Scientific Software Services, Bloomington, IL). Standard spectrometer settings were: microwave power of 1.0 mW, center field of 322.0 mTesla, sweep width of 10.0 mTesla, modulation amplitude of 50×10^{-3} mTesla, frequency 8.9934 GHz and time constant of 0.003 s. Spectra were taken as 1024 data points. Instrument gain ranged from 125 to 1000 with scan times ranging from 4 to 15 min.

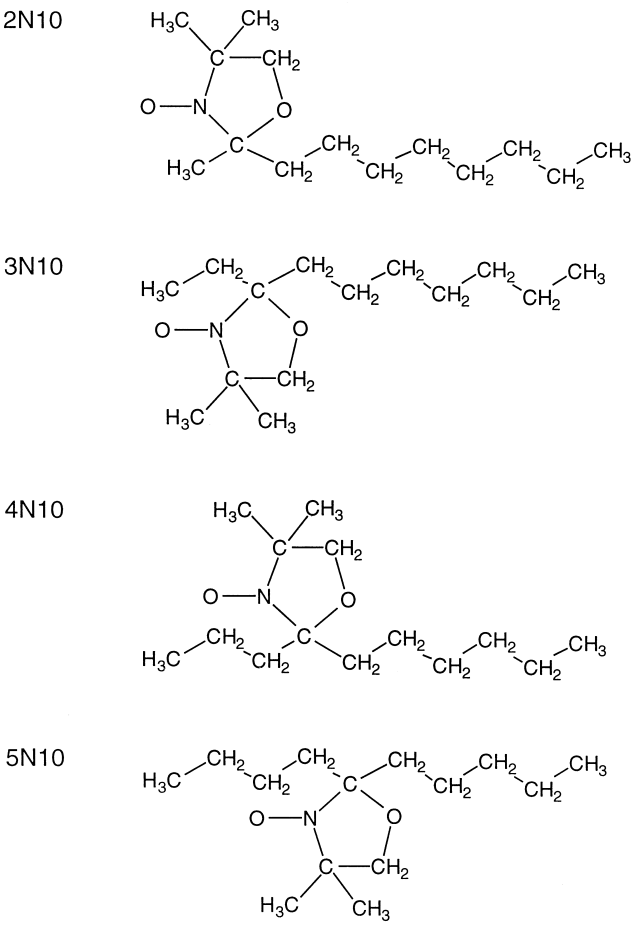

Fig. 1. Structures of the nitroxides used in this study.

Sample temperature was maintained using a Varian variable temperature controller modified by Research Specialties (Chicago, IL). In a typical experiment, the cavity Dewar was equilibrated to the lowest expected temperature prior to insertion of the sample. Temperature was increased in approximately five degrees increments throughout the course of experimentation. Temperature stability of ±0.1°C was typical.

2.7. Nitroxide preparation

Nitroxides were stored in ethanol. All nitroxides were standardized against a 1.01 mM 2,2,6,6-tetra-

methylpiperidine-*N*-oxyl-4-ol (TEMPOL) solution in ethanol. Stock solutions were analyzed periodically to check for loss of signal (no loss of signal was observed). Concentrations of the nitroxides stock solutions used for EPR measurements were 0.94 mM (2N10), 5.72 mM (3N10), 3.98 mM (4N10), and 0.81 mM (5N10). These concentrations were chosen so that partitioning of each nitroxide into the membrane preparations was approximately the same at room temperature. The molar ratio of nitroxide/lipid was never greater than 1:100.

2.8. EPR sample preparation

An EPR sample consisting of isolated *L. monocytogenes* membranes and the appropriate spin label was made as follows. Ten microliters of 2N10, 3N10, 4N10, or 5N10 were placed in a microcentrifuge tube and ethanol was removed under a stream of dry nitrogen. The appropriate isolated membrane preparation (100 μl) was added directly to the microcentrifuge tube and the sample was mixed using a pulse vortexing procedure where the vortex was applied for 2 s, then stopped for 1 s continuously over a period of 2 min. The sample was then drawn into a capillary tube that was flame-sealed at both ends.

Samples were placed in an EPR tube along with a copper–constantan thermocouple that was positioned just below the top of the capillary tube. The thermocouple was present to measure the sample temperature inside the cavity only; it was not linked to the actual temperature controller.

Each membrane preparation was analyzed using the four different nitroxides.

2.9. Spectral analysis

Spectral analysis included the simulation of both the aqueous and hydrocarbon contributions to the spectra with the simulation/fitting program EW-VoigtN (Scientific Software Services, Bloomington, IL, [16]). The program simulated the entire two-component EPR spectra assuming a Voigt line shape under conditions of relatively rapid nitroxide motion. Lorentzian and Gaussian line widths, hyperfine splitting constants, amplitude, and position of the spectral lines were obtained from the simulation. Aqueous and hydrocarbon spectra and the residual were saved as separate files. Fig. 2 shows examples of fits obtained at temperature extremes.

At low temperatures a spectral contribution was present which was incapable of being simulated by

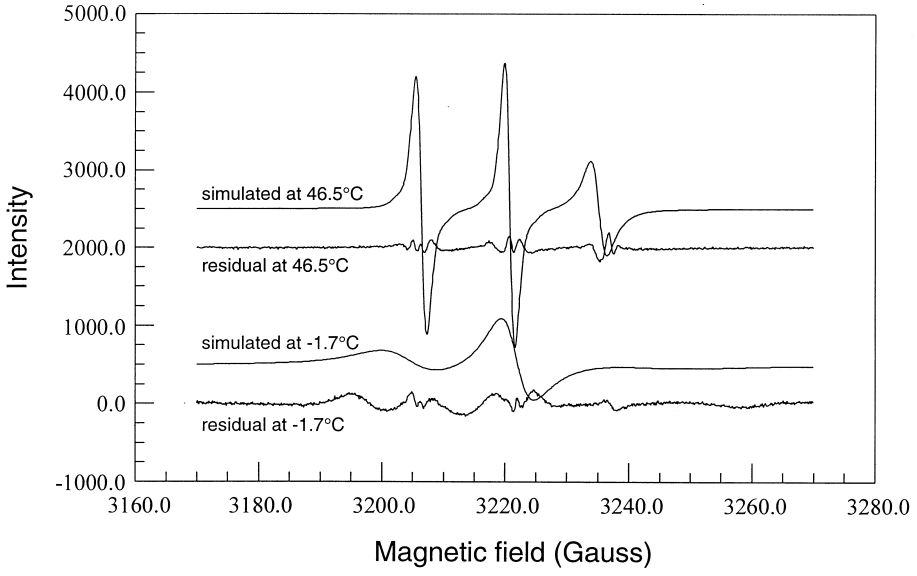

Fig. 2. Membranes and residual spectra at −1.7°C and 46.5°C obtained from simulation. Membranes are from strain *cld-1* supplemented with 2-methylbutyric acid and the nitroxide used is 5N10. The spectrometer settings are as described in the text.

EWVoigtN (Fig. 2, spectrum at −1.7°C). This portion of the spectrum was measured as a peak-to-peak distance in magnetic field units on the residual spectrum obtained from EWVoigtN after a 51-point smooth (smoothing had no effect on the position or shape of these broad lines).

Assuming rapid reorientation of the nitroxide, rotational correlation times were calculated from the equation:

$$T_2(M)^{-1} = A + BM + CM^2 \quad (1)$$

where T_2^{-1} was the Lorentzian line width of the Mth line. A, B, and C were parameters arising from the magnetic anisotropy of the unpaired electron [17–20].

Hyperfine parameters and g values were taken from Jost et al. [21] for a representative oxazolidine nitroxide ($g_{xx} = 2.0088$; $g_{yy} = 2.0058$; $g_{zz} = 2.0022$; $A_{xx} = 0.63$ mTesla; $A_{yy} = 0.58$ mTesla; $A_{zz} = 3.36$ mTesla). Parameters B and C were calculated from the line widths as follows:

$$B = (T_{2(-1)} - T_{2(+1)})/2 \quad (2)$$

$$C = ((T_{2(-1)} + T_{2(+1)})/2) - T_{2(0)} \quad (3)$$

Comparison of rotational correlation times calculated from the B and C parameters were used to estimate the anisotropy of nitroxide motion [22].

3. Results

3.1. Fatty acid composition

The fatty acid composition of the various strains grown at 10°C and 30°C are shown in Table 1. The major fatty acids of both strains SLCC53 and 10403S are *anteiso*-$C_{15:0}$, *anteiso*-$C_{17:0}$, and *iso*-$C_{15:0}$ when grown at 30°C. In both strains, the levels of *anteiso*-$C_{17:0}$ decreased and *anteiso*-$C_{15:0}$ increased in cells grown at 10°C.

There were low levels of *anteiso*-$C_{17:0}$ and *anteiso*-$C_{15:0}$ and increased levels of *iso*-$C_{14:0}$, $C_{14:0}$, *iso*-$C_{16:0}$, and $C_{16:0}$ in strain *cld-1* grown at 30°C in the absence of 2-methylbutyric acid. The levels of *anteiso*-$C_{15:0}$ and *anteiso*-$C_{17:0}$ were restored by adding their metabolic precursor, 2-methylbutyric acid, to the growth medium. Under supplementation, the levels of even-numbered branched and straight-chain fatty acids decreased to very low levels. These observations are entirely consistent with our previous studies [10]. The fatty acid composition of membranes isolated from strain 10403S cells grown at 30°C is very similar to that of whole cells (Table 1).

3.2. Qualitative analysis of EPR spectra

The spectral line widths of all four nitroxides decreased with increasing sample temperature. The de-

Table 1
Fatty acid composition of wild-type and cold sensitive mutant of *Listeria monocytogenes*

Fatty acid	Strains						
	SLCC53 grown at 30°C	SLCC53 grown at 10°C	10403S grown at 30°C	Isolated membrane of strain 10403S grown at 30°C	10403S grown at 10°C	*cld-1* mutant grown at 30°C	*cld-1* mutant supplemented with 2-MBA grown at 30°C
iso-$C_{13:0}$	ND	1.7	ND	ND	<1	ND	ND
anteiso-$C_{13:0}$	ND	3.1	ND	ND	1.7	ND	<1
iso-$C_{14:0}$	<1	2.4	<1	<1	2.1	7.5	<1
$C_{14:0}$	ND	ND	<1	<1	0.8	19.5	1.7
iso-$C_{15:0}$	18.6	18.8	11.7	10.4	16.6	2.3	<1
anteiso-$C_{15:0}$	56.0	68.6	48.8	44.7	65.4	6.3	50.4
$C_{15:0}$	ND	ND	ND	ND	ND	6.9	ND
iso-$C_{16:0}$	1.1	<1	2.2	2.0	1.5	14.9	1.1
$C_{16:0}$	<1	<1	1.4	1.2	<1	34.5	4.8
iso-$C_{17:0}$	1.5	ND	3.1	3.9	<1	1.0	<1
anteiso-$C_{17:0}$	21.4	3.3	31.8	36.2	8.9	4.2	40.0
$C_{18:0}$	<1	<1	ND	<1	<1	1.0	<1

ND, not detected.

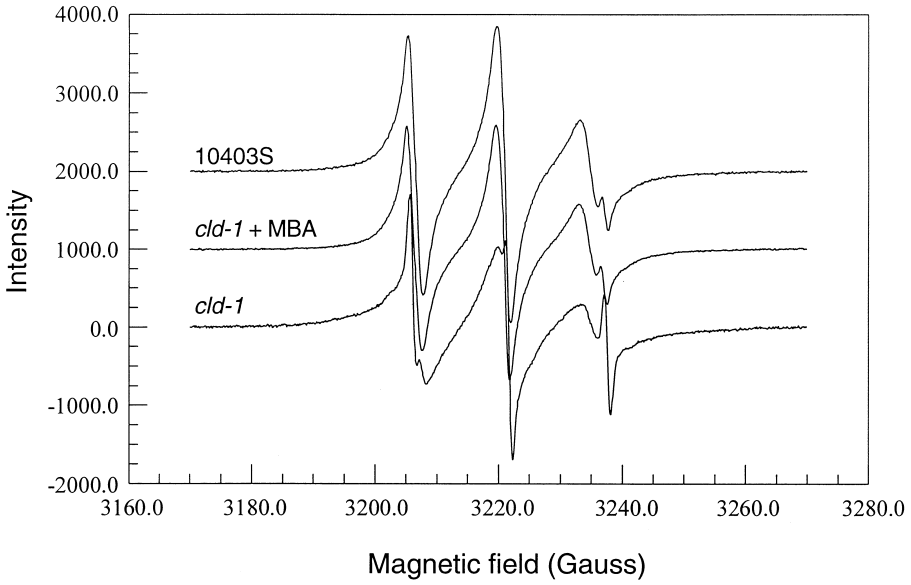

Fig. 3. EPR spectra of the nitroxide 3N10 in the cytoplasmic membrane of *L. monocytogenes* strains 10403S, *cld-1*, and *cld-1* supplemented with 2-methylbutyric acid, at 30°C.

gree of narrowing at a given temperature was similar for all membrane preparations except for *cld-1* grown without 2-methylbutyric acid supplementation. At any given temperature the line widths from the unsupplemented *cld-1* membranes were broader than those from either the wild-type membranes or the *cld-1* supplemented with 2-methylbutyric acid membrane. Also, the aqueous signal was larger in the unsupplemented *cld-1* strain membrane (Fig. 3).

All four nitroxides showed a discernible water signal at low temperatures in all of the membranes. As the measurement temperature increased, the intensity of the aqueous signal decreased (Fig. 4). This indicates increased partitioning of the nitroxides into the

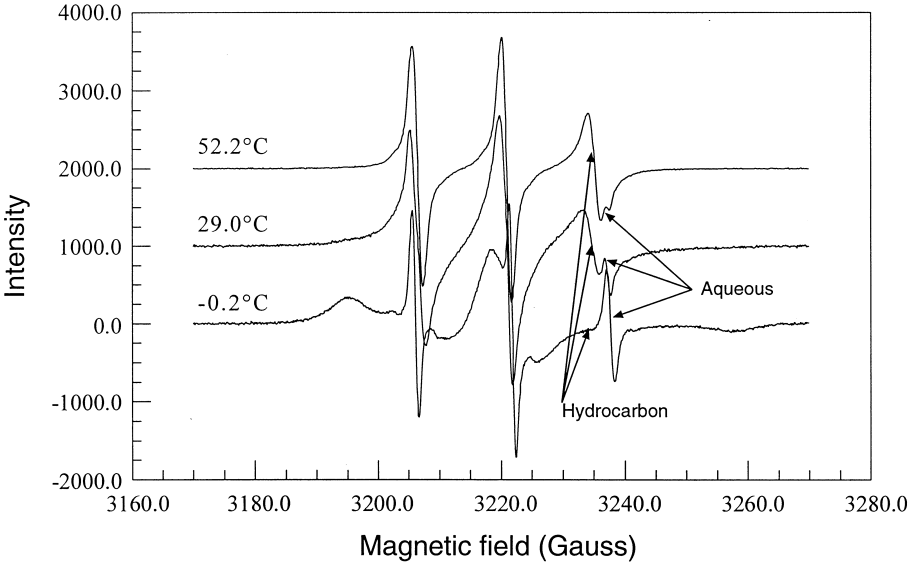

Fig. 4. Decrease in intensity of aqueous signal with increasing temperature for 4N10 in membranes from strain 10403S grown at 30°C.

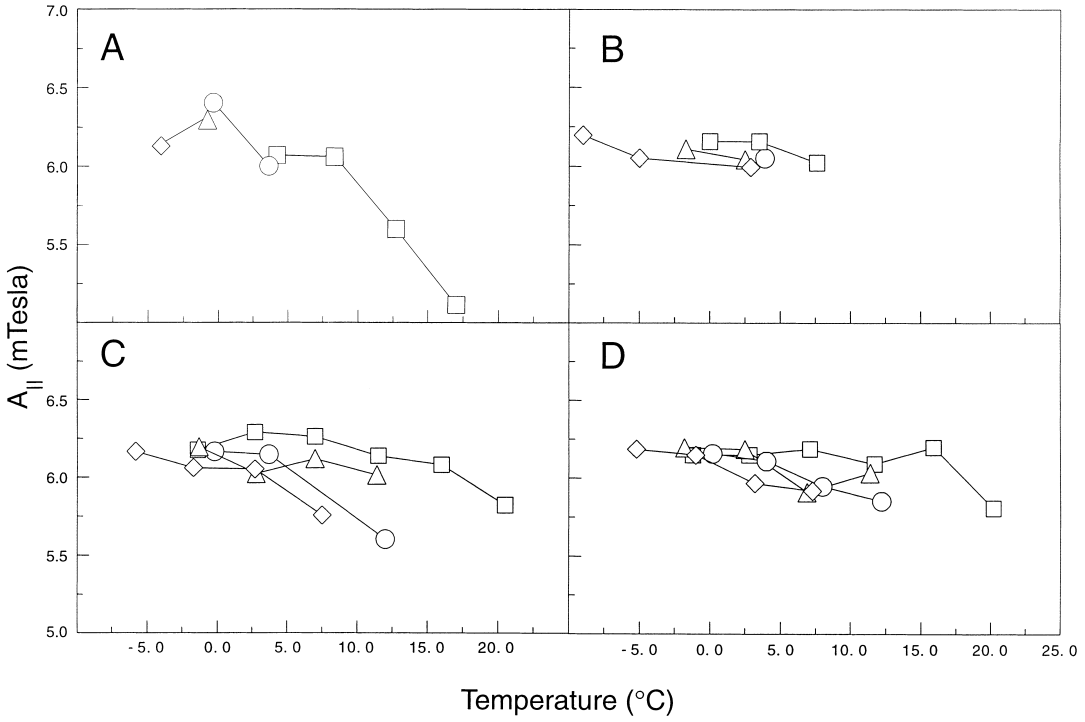

Fig. 5. Values of $A_{||}$ for (A) 2N10, (B) 3N10, (C) 4N10, (D) 5N10 of (□) *cld-1* grown at 30°C; (△) *cld-1* supplemented with 2-methylbutyric acid grown at 30°C; (○) 10403S grown at 30°C; (◇) 10403S grown at 10°C.

membrane with increasing temperature. Typical hyperfine couplings for spectra arising from the hydrocarbon region of the membranes are: 2N10, 1.454 ± 0.001 mTesla; 3N10, 1.439 ± 0.001 mTesla; 4N10, 1.436 ± 0.001 mTesla; 5N10, 1.432 ± 0.001 mTesla. This shows that the nitroxides partition deeper into the membrane hydrocarbon region as the oxazolidine group is positioned further toward the middle of the decane chain.

Spectra of all four nitroxides in each of the membranes taken at 0°C showed a hyperfine splitting consistent with the contribution to the spectrum of nitroxides oriented along the nitroxide *z*-axis ($A_{||}$). As temperature increased, there was a decrease in intensity of the peaks defining $A_{||}$ and a decrease in hyperfine splitting. This spectral component was present in all six membranes labeled with all four nitroxides (Fig. 5A–D) and was observed in the membranes of unsupplemented *cld-1* over the largest temperature range for all nitroxides.

3.3. Motional anisotropy

Graphs of *C*/*B* versus temperature were used to determine motional anisotropy of the nitroxides in the membrane. A *C*/*B* value of 1 indicates completely isotropic motion whereas values greater than 1 indicate a preferred axis of rotation, presumed, for these nitroxides, to be along the long axis of the hydrocarbon chain.

For clarity, only data for strain 10403S and unsupplemented *cld-1* are shown (all other samples were similar to the strain 10403S). Anisotropy decreased with increasing temperature for all nitroxides in all samples except for unsupplemented *cld-1* with nitroxides 3N10 and 5N10 (Fig. 6A–D).

3.4. Rotational correlation times

Calculating τ_c time from the true Lorentzian line widths gives a quantitative measure of spin label motion. By comparing the rate of probe motion in different membranes at different temperatures, the relative membrane fluidity can be determined. Obtaining τ_c of membrane probes as a function of both temperature and membrane type, as well as a function of depth, provides a detailed description of the physical properties of the membrane.

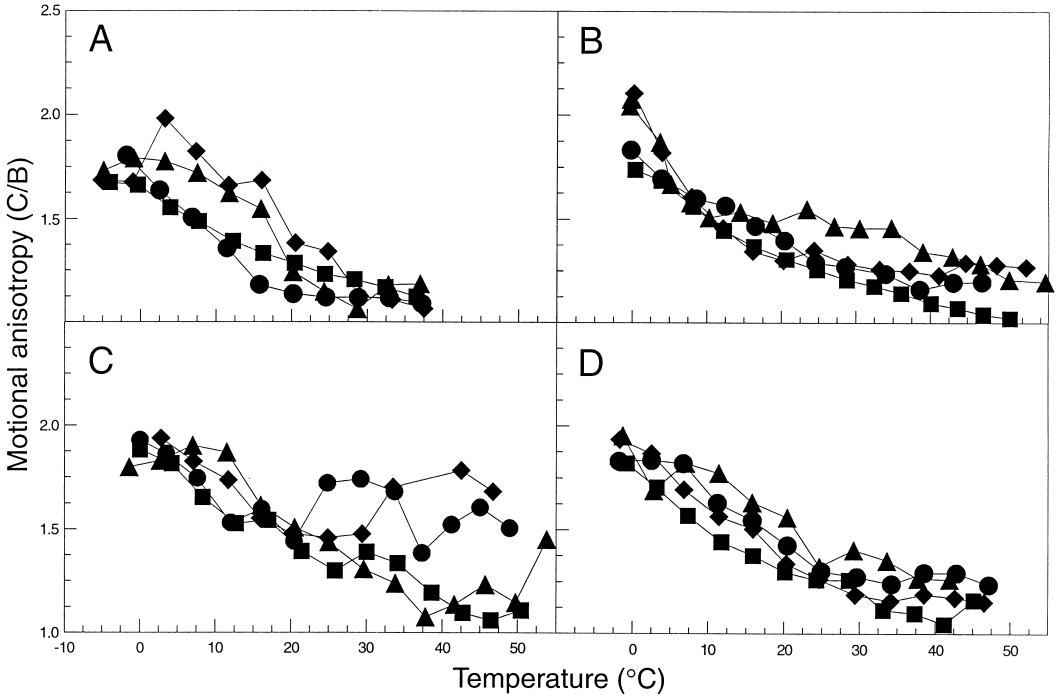

Fig. 6. Motional anisotropy of all four nitroxide labels in membranes of (A) 10403S grown at 10°C, (B) 10403S grown at 30°C, (C) unsupplemented *cld-1* grown at 30°C, and (D) *cld-1* supplemented with 2-methylbutyric acid grown at 30°C. (■) 2N10; (●) 3N10; (▲) 4N10; (◆) 5N10.

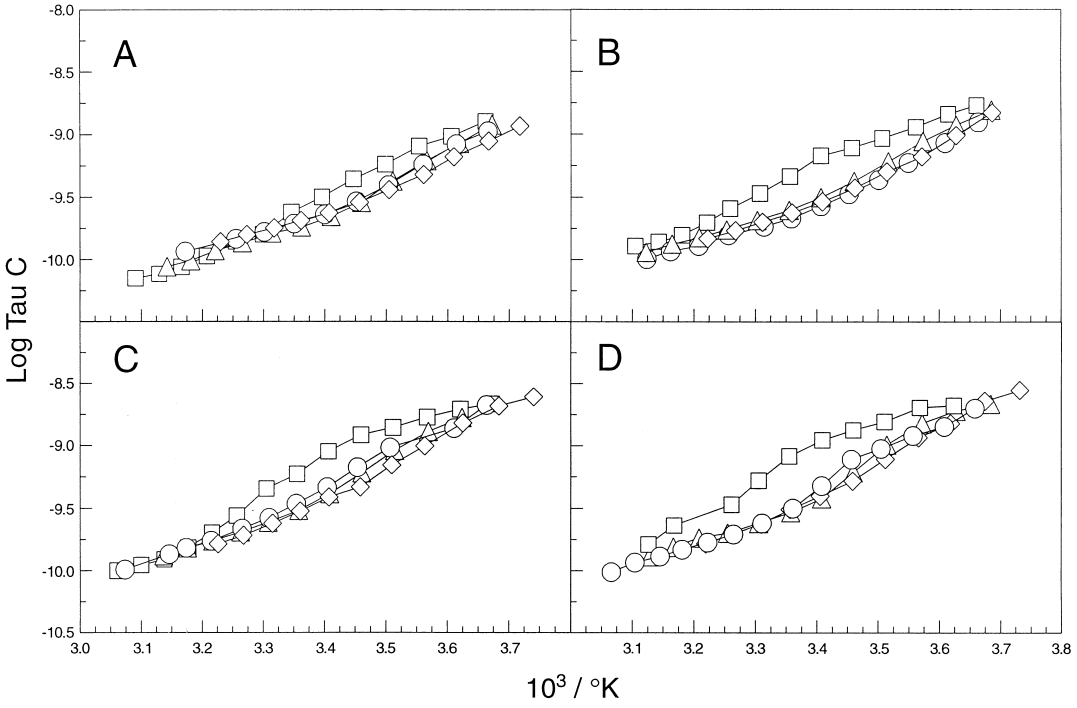

Fig. 7. Rotational correlation times (τ_c) calculated for each nitroxide as a function of temperature. Data are presented as Arrhenius plots (log τ_c vs. $1/K \times 1000$). (A) 2N10; (B) 3N10; (C) 4N10; (D) 5N10. (□) *cld-1* grown at 30°C; (△) *cld-1* supplemented with 2-methylbutyric acid grown at 30°C; (○) 10403S grown at 30°C; (◇) 10403S grown at 10°C.

Fig. 7A–D show that τ_c decreased by approximately a factor of ten over the temperature range of −7°C to 50°C. Only the membranes isolated from the unsupplemented *cld-1* mutants showed a measurable difference in rotational motion (increase in τ_c) when compared to all the other membrane types. This difference was most prominent with the nitroxides 3N10, 4N10, and 5N10.

4. Discussion

Overall, our results show that the membrane of the unsupplemented *cld-1* mutant is markedly less fluid than the membranes of the other strains or the supplemented *cld-1* mutant. These results correlate with the fatty acid composition of the membranes; *anteiso*-$C_{15:0}$ is virtually absent only in the unsupplemented *cld-1* mutant. *Anteiso*-$C_{15:0}$ is a branched-chain fatty acid and, from these results, it is clear that it exerts its influence on membrane fluidity deeper in the membrane. These changes are discussed in detail below.

4.1. EPR spectra at low temperature (0–20°C)

The spectral component corresponding to $A_{\|}$ indicates that the nitroxides are rotating slowly and that averaging of the hyperfine tensors are incomplete during the lifetime of the excited state. $A_{\|}$ persisted to higher temperature in the membranes from the unsupplemented *cld-1* mutant where it disappeared at 15°C for 2N10, at 5°C for 3N10 and at 20°C for 4N10 and 5N10. These results suggest that the head-group region of the membrane does not participate strongly in interactions that restrict the motion of the nitroxides at these temperatures. Another interpretation is that 2N10 and 3N10 are physically excluded from some portion of the membrane where slower motion occurs. We cannot currently distinguish between these possibilities although exclusion of 2N10 and 3N10 from such a phase would lead to enrichment of 2N10 and 3N10 in the more mobile phase that would in turn lead to line broadening. We did not observe line broadening for these nitroxides although we cannot rule out very small increases in line widths less than 0.1 μTesla. $A_{\|}$ disappeared at slightly lower temperatures for the SLCC53 and 10403S strains grown at 10°C compared to the same bacterial strain at 30°C, which indicates a subtle increase in membrane fluidity at lower growth temperature for both strains. This is consistent with the increase in *anteiso*-$C_{15:0}$ fatty acid content in cells grown at 10°C compared to 30°C.

4.2. Motional anisotropy

As shown in Fig. 6A–D, the nitroxides tumble anisotropically in all membrane preparations and the increase of the *C*/*B* ratio at low temperatures indicates that the degree of motional anisotropy of the nitroxides increased with decreasing temperature. The increase in anisotropy implies a more motionally restrictive (more ordered) environment at low temperatures.

The plots of *C*/*B* versus temperature were smooth, with no sudden changes in *C*/*B* value and all four nitroxides give similarly shaped graphs. This argues against the presence of abrupt phase transitions in the membrane preparations over the temperature ranges studied. The nearly identical *C*/*B* values, regardless of the probe used, shows that the location of the nitroxide in the membrane has very little effect on its motional anisotropy; the long hydrocarbon chain of the nitroxide governs the tumbling.

The *C*/*B* values for the unsupplemented *cld-1* mutant are not easily explainable (Fig. 6). The initial decrease in anisotropy characteristic of all the membrane preparations was seen as temperatures increased from −7°C to 20°C, but above this temperature the isotropy of motion, as described by *C*/*B*, varied widely with no pattern noted. The *C*/*B* value for each nitroxide began to diverge as well. The changes in *C*/*B* did not indicate either a smooth decrease in anisotropy with increasing temperature, or a phase transition in the membranes. Difficulty in simulating the unsupplemented *cld-1* membrane spectra could contribute to the erratic *C*/*B* values, although problem simulations were generally noted at low, not high, temperature. EWVoigtN gives error analyses of each simulated parameter, and the error of each *C*/*B* value is less than the size of the symbol on the graph. We cannot at this time attach any physiological significance to the variations in the

C/B values seen in the unsupplemented *cld-1* membranes.

4.3. Rotational correlation times (τ_c)

Changes in the shape of the Arrhenius plots of τ_c were observed as a function of temperature, but no sharp breaks in the slope were present (Fig. 7A–D). Thus, changes in membrane fluidity are gradual, with no apparent liquid-crystalline to gel transition.

The data revealed very little difference in τ_c as a function of cell growth temperature (as an example, τ_c of the nitroxides changed by about a factor of 10 over the range of temperature of 0°C to 45°C while the viscosity of water changes by a factor of 3 over the same range [23]). The τ_c of probes in the membranes of SLCC53, 10403S, and the supplemented *cld-1* mutant grown at 30°C were nearly indistinguishable from those grown at 10°C. These results imply that the fatty acid composition of the cell membranes produced under optimal growth conditions provides a sufficiently fluid environment for bacterial growth over a very wide range of temperatures. The differences in membrane fluidity exhibited by the wild-type bacteria grown at 30°C and 10°C were similar, even in the presence of changes in the fatty acid composition on the order of 10–15% of *anteiso*-$C_{15:0}$ (Table 1).

The only truly noticeable difference was observed with membranes from the unsupplemented *cld-1* strain. The τ_c of probes in the unsupplemented *cld-1* membranes at low temperatures varied considerably from those exhibited by the wild-type bacteria and the *cld-1* mutant supplemented with 2-methylbutyric acid. Changes in τ_c correlate with large (50%) changes in levels of *anteiso*-$C_{15:0}$. Membranes of the *cld-1* mutant contain almost no branched-chain fatty acids.

While differences in τ_c, and therefore membrane fluidity, were noted between the wild-type and unsupplemented *cld-1* membranes at low temperatures, very little difference was seen at elevated temperatures. At temperatures above 30°C, both wild-type *L. monocytogenes* and the unsupplemented *cld-1* strain are capable of growth and the membranes exhibit similar physical properties as measured with the nitroxides. At higher temperatures, both the branched-chain and the straight-chain components of the cell membranes are apparently in a fluid state.

4.4. Lack of a phase transition

The phase transition range depends on the ability of the lipids to pack. For example, the transition temperature in *Acholeoplasm laidlawii* membranes can be shifted from about −20°C to 60°C depending upon whether the organism is supplemented with oleic or stearic acid respectively, which are incorporated into the membrane lipids [24].

We did not observe a clearly defined phase transition in our studies of the fluidity of *L. monocytogenes* membranes. Wild-type *L. monocytogenes* membranes contain almost exclusively odd-numbered branched-chain fatty acids, predominantly *anteiso* fatty acids. *Anteiso* fatty acids occupy significantly larger cross-sectional areas than either normal straight chain or *iso* fatty acids [25], thus disrupting close packing. Fatty acid *anteiso*-$C_{15:0}$ has a low melting temperature (25.8°C), and phosphatidylcholine containing *anteiso*-$C_{15:0}$ has a transition temperature of −13.9°C [9]. X-Ray diffraction studies have shown that fatty acids in membranes rich in branched-chain fatty acids are more loosely packed in the crystalline state than in membranes lacking branched-chain fatty acids [26,27]. We suspect that the phase transition temperature of the *L. monocytogenes* membrane lipids is below our lowest EPR measurement temperature.

4.5. Homeoviscous and homeophasic adaptation of membrane fluidity

Sinensky [7] reported that the fluidity of *Escherichia coli* membrane lipids grown at 15°C and 43°C were nearly equivalent when measured at their respective growth temperature. Maintenance of constant membrane fluidity at different growth temperatures was termed homoviscous adaptation. However, microorganisms are able to grow and function normally with membranes of widely different fluidities [28]. It is estimated that growth of *E. coli* ceases when more than 55% of the lipid is in the ordered state [29]. McElhaney [29] has proposed that microorganisms regulate their lipid composition to maintain the bilayer in a fluid state at lower tem-

peratures and he termed this 'homeophasic adaptation'. Homeoviscous adaptation would represent a fine-tuning of fluidity. We found previously that wild-type *L. monocytogenes* grown at 37°C, when transferred to 5°C, would initiate growth before any significant alterations in fatty acid composition had occurred [10]. However, in long-term cultures of *L. monocytogenes* grown at low temperatures, *anteiso*-$C_{15:0}$ levels increase so that it becomes the predominant fatty acid in the membrane. This alone suggests that *anteiso*-$C_{15:0}$ provides a more fluid environment for growth in the cold. However, in strains SLCC53 and 10403S, we found no obvious differences in membrane fluidity in cultures grown at 10°C and 30°C, which suggests that the physiological role of *anteiso*-$C_{15:0}$ of is more complex than previously thought.

4.6. Influence of membrane fluidity on minimum growth temperature

McElhaney [29] found that the minimum growth temperature of 8°C of *A. laidlawii* was not determined by the fatty acid composition of membrane lipids providing that the lipids had phase transition temperatures below this temperature. However, when lipids with higher phase transition temperatures were present, the minimum growth temperature was clearly defined by the fatty acid composition of the membrane lipids. We suspect that the fatty acid composition of *L. monocytogenes* does not determine the minimum growth temperature of the organism but rather permits growth of the organism at low temperatures. The cold-sensitive mutant *cld-1* has low amounts of branched-chain fatty acids, lower membrane fluidity, and a higher minimum growth temperature than the wild type.

4.7. Conclusions

We conclude that in *L. monocytogenes*, branched-chain fatty acids impart essential membrane fluidity that remains relatively unchanged over a wide range of growth and measurement temperatures. Absence of these branched-chain fatty acids, as occurs for a cold-sensitive mutant, decreases fluidity and impairs growth at low temperatures.

Our results suggest that antibacterial agents that affect branched-chain fatty acid metabolism may be a potentially fruitful approach to controlling the growth of *L. monocytogenes* at low temperatures.

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