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THE EFFECT OF ETHANOL ON THE PHASE BEHAVIOR OF MEMBRANE LIPIDS EXTRACTED FROM *CLOSTRIDIUM THERMOCELLUM* STRAINS

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The phase behavior of aqueous dispersions of extracted lipids from *Clostridium thermocellum* wild-type and ethanol-tolerant C919 cells has been examined by DSC. The optimum growth temperature of this anaerobe is 60°C. The wild-type lipids exhibit a broad phase transition centered at 30°C; the C919 mutant lipids show a 10°C lower T_m. The direct addition of growth inhibiting concentrations of ethanol has no significant effect on T_m or headgroup mobility (monitored by ^{2}H-NMR) of either set of lipids. In contrast, wild-type cells adapted to growth in ethanol exhibit a broadened and lower T_m (15–25°C plateau); C919 membrane lipids do not exhibit significantly altered phase behavior when adapted to growth in ethanol. Both wild-type and mutant membranes have fatty acid composition changes upon growth in ethanol, which increases lower-melting components. It is concluded that fatty acid changes which occur upon adaptation of the organism to growth in ethanol are secondary responses and not necessarily direct responses to alter membrane fluidity.

Introduction

Clostridium thermocellum is an anaerobic, thermophilic bacterium that ferments cellulose to ethanol, acetate, and lactate. Interest in this organism for fuel production has stemmed from its possession of a cellulase enzyme [1,2]. However, wild-type *Cl. thermocellum* is relatively sensitive to ethanol: 50% growth inhibition is reached with 5 g/l ethanol [3]. When exponentially growing cells are exposed to ethanol there is an initial period of uninhibited growth (I), followed by a period of growth arrest (L), and then inhibited but sustained exponential growth (II) [3]. During phase-L changes in the lipid composition occur so that the membrane in phase-II has a different fatty acid profile than phase-I [4]. For a series of *n*-alkanols, the amount of compound required for 50% inhibition correlates with the concentration of alcohol partitioned in the lipid bilayer [3]. This suggests that a hydrophobic target, potentially the cell membrane, is involved.

Alcohols can partition into a lipid bilayer [5–7] and modulate a variety of cellular functions which depend on membrane fluidity, specific lipid/protein interactions, permeability, etc. If the membrane of *Cl. thermocellum* has a characteristic phase transition around the optimum growth temperature (60°C), then ethanol could alter membrane fluidity and hence disrupt membrane protein-mediated phenomena. The ethanol-tolerant mutant, C919, would be expected to show a different thermal profile and response to ethanol.

We have measured the thermotropic phase behavior of dispersions of lipids extracted from *Cl. thermocellum* ATCC 27405 (wild-type), ethanol-

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Abbreviation: $N(C^2H_3)_3$-POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-N-$(C^2H_3)_3$-choline.

adapted or phase-II wild-type cells, and C919 organisms grown in the absence and presence of ethanol. DSC profiles exhibit broad but defineable transitions 30°C below optimum growth temperatures. Growth-inhibiting concentrations of ethanol do not significantly affect DSC profiles. In addition, the physical behavior of the lipid headgroup region has been monitored by ^{2}H-NMR of membrane lipid liposomes doped with 2–5% $N(C^2H_3)_3$-POPC. These results confirm that the changes in fatty acid composition that occur in phase-L do produce lower melting membranes, but they clearly show that growth-inhibiting concentrations of ethanol do not directly affect *Cl. thermocellum* lipid phase behavior. The relationship of this to the mechanism of ethanol-inhibition of cell growth is discussed.

Methods

Lipid extraction. Total lipids of *Clostridium thermocellum* ATCC 27405 and the mutant strain C919 grown in CM4-Cb medium in the presence and absence of ethanol were extracted as described previously [4]. The pooled organic extract was washed with 200 ml saturated KCl containing 0.01 M EDTA, pH 7.2, to remove paramagnetic contaminants. The organic phase was filtered, dried, and stored at −20°C under a nitrogen atmosphere.

Differential scanning calorimetry. Lipid extracts in $CHCl_3/CH_3OH$ (1 : 1) were dried under N_2 in Perkin-Elmer DSC sample pans (50 μl capacity), desiccated under a vacuum overnight, hydrated with 50% ethylene glycol in water or 0.6–1.2 M ethanol in water and sealed. The lipid concentration was approx. 9 wt.%. Scanning calorimetry was carried out using a Perkin-Elmer DSC-2 scanning calorimeter at a scan rate of 5°C/min.

Liposome preparations. Liposomes doped with $N(C^2H_3)_3$-POPC (synthesized as described previously [8]) for ^{2}H-NMR were prepared as follows. The lipid extract (ca. 30 mg) and $N(C^2H_3)_3$-POPC were mixed in organic solvent, then dried under vacuum. The lipids were dispersed in 1 ml of ^{2}H-depleted water (Aldrich), vortexed vigorously at 30°C, and then lyophilized. (This exchange was introduced to reduce the intensity of natural abundance H^2HO.) The lyophilized sample was resuspended in ^{2}H-depleted water and used for ^{2}H-NMR.

^{2}H-NMR spectroscopy. ^{2}H (41.4 MHz) NMR spectra were obtained on a Bruker 270 spectrometer equipped with a Nicolet 1080 computer. ^{2}H spectra were run without sample spinning. Each quadrupolar coupling constant is the average of two measurements. The sample temperature was controlled ±1°C.

Results

Thermal properties of extracted lipids

DSC profiles of aqueous dispersions of lipids extracted from *Cl. thermocellum* are shown in Fig. 1. Lipids were hydrated with ethylene glycol in water to allow observation at temperatures below 0°C. Average T_m values from duplicate runs on different batches of extracted lipids are shown in Table I. Lipids from the wild-type organism show a broad transition with a maximum around 30°C (Fig. 1a). No transition is seen in the vicinity of the growth temperature (60°C). Addition of 0.6 M ethanol (five times the amount of ethanol causing 50% growth inhibition) has no significant effect on

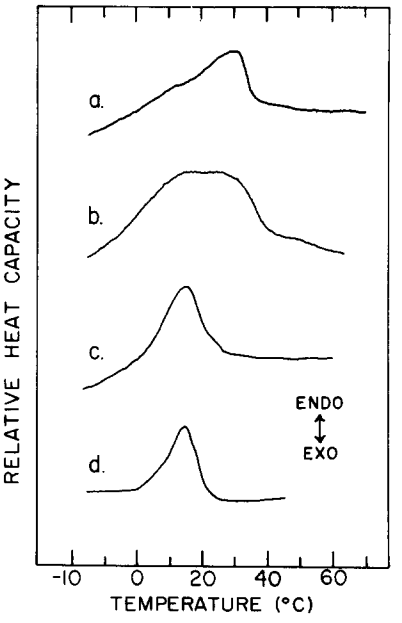
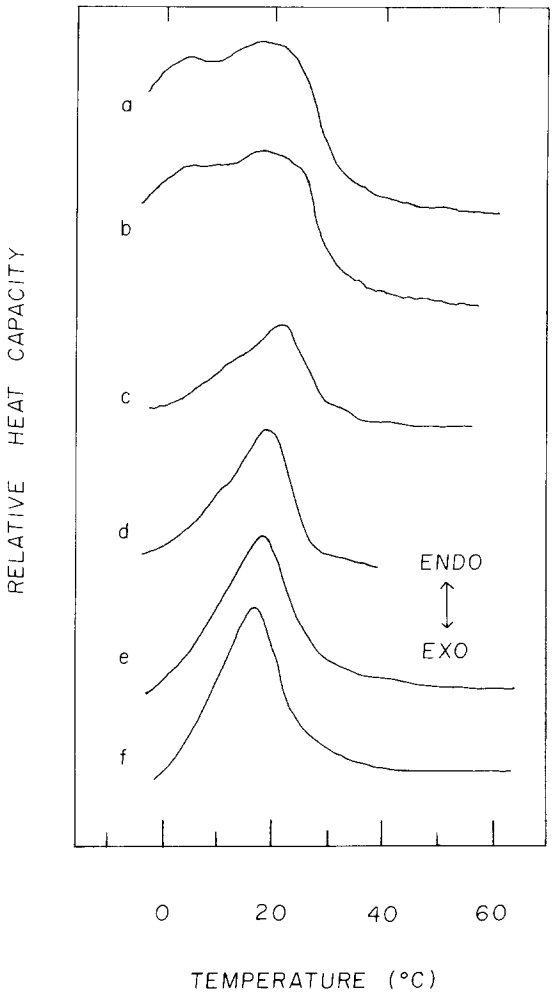


Fig. 1. Differential scanning calorimetry traces (heating runs) of dispersions of lipid extracts (hydrated with 50% ethylene glycol in water) from (a) ATCC 27405 cells, (b) ethanol (5 g/l) adapted ATCC cells, (c) C919 cells, and (d) C919 cells grown in the presence of 15 g/l ethanol.



the DSC behavior. Lipids from wild-type phase-II cells grown in the presence of 5 g/l ethanol (the concentration which causes 50% growth inhibition) show an altered profile (Fig. 1b). The ethanol-adapted ATCC extract has a broader transition range which is lower than that of the ATCC wild-type extract, although the exact shape and maximum transition temperatures vary somewhat with each batch of adapted cells (compare Figs. 1b and 2a). There is a broad double peak which indicates the presence of lower-melting components in the ethanol-adapted ATCC wild-type cell lipids. Addition of 0.6 M ethanol has no significant effect on the thermal behavior of these extracted lipids (Fig. 2b).

The lipids from the mutant strain C919 exhibit a lower T_m than wild-type (Fig. 1c), but the thermal profile is not as broad as that of the phase-II ethanol-grown wild-type cells. The absolute maximum T_m varies slightly from batch to batch of extracted lipids (from 16°C, Fig. 1c, to 22°C, Fig. 2c). The difference between the T_m of wild-type and mutant C919 lipids is approx. 10°C, indicating a more 'fluid' mutant membrane. This ob-

Fig. 2. Differential scanning calorimetry traces (heating runs) of dispersions of membrane lipids hydrated with water (a, c, e) or aqueous ethanol (b, d, f). The ethanol concentration was 0.6 M in (b) and 1.2 M in (d) and (f). The lipid extracts are from (a, b) ATCC 27405 wild-type cells adapted to growth in 5 g/l ethanol, (c, d) C919 mutant cells, and (e, f) C919 cells adapted to growth in 15 g/l ethanol.

TABLE I

T_m (°C) VALUES FOR LIPIDS EXTRACTED FROM *CLOSTRIDIUM THERMOCELLUM* STRAINS

Organism	Range for lipid extracts	Individual batch of extracted lipids	+ Ethanol
ATCC 27405	29±2	29	28 [a]
ATCC 27405 grown in ethanol [b]	15–25 [c]	19	20 [a]
		5	6
C919	19±3	22	19 [d]
C919 grown in ethanol [e]	17±2	19	17.5 [d]

[a] 0.6 M ethanol was added to extracted lipids (no ethylene glycol present).
[b] Ethanol concentration for growth was 5 g/l (50% growth inhibition).
[c] Broad plateau observed in most DSC traces; a lower melting distinct peak can often be discerned around 5°C in some adapted batches.
[d] 1.2 M ethanol was added to extracted lipids.
[e] Ethanol concentration for growth was 15 g/l (40% growth inhibition).

servation is consistent with trends in fatty acid composition of these organisms when adapted to growth in ethanol [4]. Addition of 1.2 M ethanol (about three times the concentration which decreases cell growth 50%) to C919 lipids causes a small decrease of about 2–3°C in T_m (Fig. 2d). When C919 is grown in 15 g/l ethanol (ca. 40% growth inhibition), the extracted lipids show a temperature profile nearly equivalent to C919 alone (Fig. 2e). The membrane fluidity of the mutant strain is much less altered by growth in ethanol than that of the wild-type, even at three times higher alcohol concentration. Addition of 1.2 M ethanol to the lipids of ethanol-adapted C919 cells produces no significant effect (Fig. 2f).

^{2}H-NMR studies of lipid headgroups and the response to ethanol

A series of NMR experiments were undertaken to see if growth inhibiting concentrations of ethanol perturbed the headgroup region of extracted lipid bilayers. ^{2}H-NMR spectra of wild-type and C919 liposomes doped with $N(C^2H_3)_3$-POPC exhibit similar quadrupolar coupling constants at a given temperature. $\Delta\nu_q$ observed for the extracted lipids is considerably less than for pure POPC liposomes. At 17°C, which is 20°C above the phase transition of POPC (−3°C), $\Delta\nu_q$ for pure $N(C^2H_3)_3$-POPC bilayers is 1.4 kHz. For POPC doped in extracted *Cl. thermocellum* lipids at 50°C (20°C above T_m), $\Delta\nu_q$ is 0.62 kHz for wild-type liposomes and 0.71 kHz for C919 liposomes. Lipids from ethanol-adapted wild-type cells and C919 cells exhibit similar $\Delta\nu_q$ values. This suggests similar headgroup environments for the deuterated probe in all four extracted lipids (i.e. no large variation in phosphatidylcholine headgroup order among these lipids). The temperature dependence of $\Delta\nu_q$ in extracted lipids is similar to that of pure POPC liposomes; there is a monotonic increase with decreasing temperature (Fig. 3). Bulk lipid chain ordering (which shows a transition around 20–30°C) is not sensed by this probe. The addition of 0.1–1.0 M ethanol does not alter $\Delta\nu_q$ for POPC in any of the extracted lipid bilayers. Thus ethanol has no significant effect on the phosphatidylcholine headgroup of *Cl. thermocellum* extracted lipids.

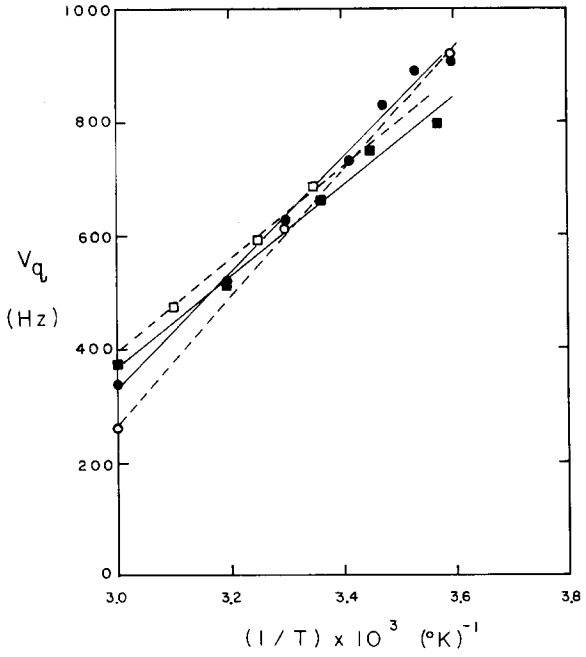


Fig. 3. The quadrupolar coupling constant, ν_q (Hz), of N-$(C^2H_3)_3$-POPC doped at 5 wt.% in liposomes formed from extracted lipids of wild type *Cl. thermocellum* grown without (●) and with (○) 5 g/l ethanol, and C919 mutant grown without (■) and with (□) 15 g/l ethanol.

Discussion

Lipids in biomembranes can undergo reversible thermotropic phase changes from liquid crystalline to gel state [9,10]. Each organism has characteristic phase behavior (usually much broader in temperature range than for single lipid systems) and, in general, the bacterial membrane needs to be in the liquid crystalline state to function in transport and for optimal activity of membrane-associated enzymes. Lipid fatty acid composition affects the transition temperature, and is often used as an indicator of changes in membrane fluidity. Most mesophilic organisms that have been studied show an optimum growth temperature 20–40°C higher than T_m [11,12]. Although few thermophiles have been examined in depth, the general trend is for their membranes to be composed of fatty acids with higher melting points than mesophiles [13–15]. They also grow optimally at temperatures above T_m. For example, *Thermus T* 351 grows optimally at 75°C and ESR probes detect phase transitions at 19, 39 and 66°C [16].

The anaerobic thermophile *Cl. thermocellum* follows these basic patterns in the phase behavior of its lipids. The optimum growth temperature of this microorganism is 60°C while the constituent lipid T_m is around 30°C. The difference between optimum growth temperature and T_m is even more pronounced for the ethanol-tolerant mutant C919 (around a 40°C difference). The fatty acid composition of *Cl. thermocellum* shows a large proportion of palmitate and saturated branched chain fatty acids; unsaturated fatty acids are minor components by comparison [4]. This fatty acid distribution contributes to the higher T_m of the thermophile compared to mesophiles (-20 to -10°C for organisms that grow at approx. 37°C).

The fatty acid composition of biomembranes, and by inference T_m, is often modified by a microorganism as a response to external factors, for example, alterations in growth temperature [19–20] or upon addition of small organic molecules such as ethanol [4,21–24]. The growth of *Cl. thermocellum* is particularly sensitive to *n*-alkanols. Alcohol inhibition potency increases as the chain length increases, i.e., a hydrophobic target is implicated. An obvious hydrophobic site in the cell is the plasma membrane.

We have observed that addition of growth inhibiting concentrations (0.6–1.2 M) of ethanol has no significant effect on the thermal behavior of wild-type or ethanol-adapted lipids; lipids from the ethanol tolerant mutant C919 show slight (ca. 2°C) decreases in T_m in the presence of 1.2 M ethanol. While these results indicate no effect of ethanol on acyl chain melting behavior, ethanol could modify the hydration state of polar headgroups in membranes and hence change 'interfacial regulation' [25]. For ethanol concentrations that inhibit growth by 90%, ^{2}H-NMR experiments failed to detect any such changes in the lipid headgroup region.

While ethanol has little direct effect on membrane phase behavior, both wild-type and C919 cells adjust their fatty acid composition in phase-L to produce a phase-II membrane with lower melting fatty acids [4]. Wild-type *Cl. thermocellum* membranes show a broader and *lower* T_m when grown in ethanol (T_m plateau 15–30°C). Thus, physical studies of extracted lipids provide direct confirmation of fatty acid composition analyses which suggest that the membrane has altered phase behavior when adapted to growth in ethanol. The mutant, whose T_m is already 7–10°C lower than wild-type, does not dramatically alter T_m when adapted to growth in ethanol even though comparable fatty acid changes occur [4]. Therefore, the mutant is resistant to the fluidity changes which occur when wild-type cells are grown in ethanol. C919 could alter lipid headgroups to counteract the fatty acid changes observed.

Several questions about *Cl. thermocellum* phase behavior must now be addressed: (i) how relevant are T_m values of 20–30°C to growth temperatures of 60°C?; and (ii) is the change in T_m for wild-type in phase-II a primary response to counter ethanol effects or is it a secondary, non-adaptive response (for example, ethanol affecting specificity in fatty acid synthesizing complexes)? Because growth occurs 30–40°C above T_m, the membrane is clearly liquid crystalline and will not undergo phase changes when ethanol is added. Therefore, we can rule out the explanation that ethanol inhibition of *Cl. thermocellum* growth is caused by changing lipid phase behavior. Furthermore, because the ethanol-tolerant mutant C919 does not lower T_m significantly when adapted to growth in ethanol, we can suggest that the adaptation effects of T_m in wild-type are not necessary for cell growth and performance.

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