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Temperature-Dependent Morphological Changes in Membranes of Bacillus stearothermophilus

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Bacillus stearothermophilus cells vary the lipid fatty acid composition of cytoplasmic membranes with growth temperature. Spin label studies of such membranes have been interpreted to indicate lateral lipid phase separations at the growth temperature. We have now used freeze-fracture electron microscopy to confirm the spin label studies. Freeze-fracture faces of protoplasts indicate slight but distinct protein aggregation at the growth temperature. Aggregation increases rapidly with decreasing quench temperature in wild-type cells. In contrast we were unable to demonstrate extended protein segregation in membranes of a temperature-sensitive mutant that contains more than 58% branched fatty acids.

Storage of protoplasts for prolonged times below the lipid phase transition results in the appearance of corrugated fracture faces with 300- to 500-Å repeat patterns, although this organism does not synthesize lecithins.

Key words: **freeze-fracturing, membranes, lipid phase separations, B stearothermophilus, temperature adaptation**

Half a century ago, Heilbrunn [1] postulated that poikilothermic organisms adapt to changes in temperature by altering their lipid composition and that heat resistance, therefore, was related to the melting temperature of lipids. Since then this proposal has been confirmed in a vast number of experiments. With a few exceptions, practically all organisms were shown to be capable of such adaptive changes (for an early review see

Abbreviations: DTA, differential thermal analysis; EPR, electron paramagnetic resonance; TSB, 1% (w/v) Tryptic Soy Broth; doxyl, 4,4-dimethyloxazolidine-N-oxyl; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxyl.

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Chapman [2] and for more recent ones see Fulco [3] and Cronan and Gelman [4]). For microorganisms one of the reasons for such changes in lipid composition apparently is the need to keep the "viscosity" of membrane lipids at a constant level – a process for which Sinensky [5] has coined the term "homeoviscous adaptation."

Our earlier studies on membranes of Bacillus stearothermophilus, aimed to provide further insight into the mechanisms that enable thermophilic bacteria to adapt to extremely high temperatures, established a close correlation between membrane fluidity and growth temperature in this organism [6]. Furthermore, we suggested that the maximal growth temperature at which these cells grow is determined by lateral lipid phase separations in the plane of the plasma membrane [7]. Our hypothesis was based on EPR spin label experiments with spheroplast membranes obtained from wild-type cells and a temperature-sensitive mutant. However, a subsequent DTA study [8], while in partial agreement with the spin label results obtained on wild-type cells, did not correlate with spin label data obtained on the mutant. This mutant does not alter its lipid composition in a normal manner with increases in growth temperature and lyses when placed at temperatures above 60° while the parent strain grows well to approximately 70°. In the present study we have used freeze-fracture electron microscopy in order to understand the reason for this apparent discrepancy.

METHODS

Bacteria

The source and growth characteristics of the thermophilic baccillus (YTG-2), identified as a strain of B stearothermophilus has been reported previously [9]. A temperature-sensitive mutant derived from the wild-type and designated TS-13 was also studied. The growth characteristics and lipid analysis of this mutant have been described [9, 10].

Media and Growth Conditions

The cells were grown aerobically at different temperatures in 1% (w/v) Tryptic Soy Broth (TSB) (Difco), supplemented with Fe^{++}, Mg^{++}, and trace elements [9], to late log-phase ($OD_{450} \cong 0.9–1.0$).

Preparation of Protoplasts

Cells were harvested at 20° by centrifugation for 5 min at 12,000 g_{max}. Pellets were immediately resuspended in 50 ml TSB containing 20% sucrose, 10 mM $MgSO_4$, and lysozyme (5 mg/ml) which was prewarmed to the growth temperature. The suspension was incubated at the growth temperature in a shaking water bath for 10–15 min. Light microscopic evaluation indicated a better than 85% conversion of rods to spherical forms. Since electron microscopic observations (see below) revealed the absence of cell wall material these preparations are referred to as protoplasts [11]. Protoplast suspensions were then centrifuged at 4°C for 10 min at 14,500 g_{max}, washed twice with 50 mM phosphate–5 mM $MgSO_4$ buffer (pH 7.0), resuspended in 3–5 ml of the same buffer and stored at –20° until used. As published earlier [6] repeated freezing and thawing did not affect the integrity of the protoplasts nor their lipid phase transitions. For crosslinking experiments protoplasts were suspended in phosphate–Mg buffer, equilibrated at the growth temperature and mixed with an equal volume of prewarmed 5% glutaraldehyde, and incubated for 30 min. After three washings with 100 mM ammonium acetate fixed protoplasts were resuspended in a small volume and stored at –20°.

Freeze-Fracture Electron Microscopy

Unfixed protoplasts were heated to the quench temperature and equilibrated at that temperature for 15 min. Small droplets (approx 2 μl) were pipetted into tall, hollow-stem gold planchets (Balzers) resting on a copper block maintained at the same temperature by a recirculating, constant-temperature water bath (B. Braun). The temperature of a droplet in a reference planchet was monitored with a fine-wire thermocouple. The test planchets were plunged rapidly into partially solidified Freon 22 (duPont), then transferred to liquid nitrogen, in which they were stored until fractured in a Balzers BA360M freeze-etching device. Fracturing was carried out at −110°C and etching at −100°C. Replicas were floated off and cleaned by standard methods. After mounting on 400 mesh uncoated grids the replicas were then examined and photographed in Hitachi HU7 or HU11A electron microscopes at a direct magnification of 30,000 ×. Glutaraldehyde-fixed protoplasts were frozen from room temperature. For a few experiments, protoplasts were also suspended in 20% glycerol as a cryoprotectant and frozen from the desired temperatures. The nomenclature of Branton et al [12] is used to identify the different fracture and etch surfaces.

RESULTS

To aid the reader in recognizing the different temperatures at which lipid transitions in B stearothermophilus membranes were recorded by DTA or by spin labels, we have summarized these transition temperatures in Table I. Although both techniques recorded similar values for high-temperature transitions (T_h) that were very close to or identical with the cells' growth temperature, considerable differences are apparent for the low-temperature (T_l) transition.

In order to understand the reason for the observed differences in T_l we have performed freeze-fracture studies on membranes quick-frozen or crosslinked at these various temperatures. Replicas of the wild-type strain (WT) protoplasts quenched from several degrees above the growth temperature show fracture faces that are covered with densely packed particles (Fig 1a). If quenching occurs at or near the growth temperature (± 1.5°C) a slight but distinct aggregation of intramembranous particles becomes apparent (Fig 1b).

TABLE I. Summary of Lipid Transition Temperatures in Membranes of B stearothermophilus WT and TS-13 Grown at Different Temperatures as Detected by EPR Spin Label Experiments* and by DTA†

Cell type	Growth temperature (°C)	High-temperature transition (T_h)		Midpoint-transition temperature (T_m)	Low-temperature transition (T_l)	
		EPR	DTA	DTA	EPR (T_{l-1})	DTA (T_{l-2})
WT	42	42	40	31	36	15
	52	52	52	41	40	20
	58	58	58	50	50	25
	65	65	65	55	57	24
TS-13	42	47	41	30	42	20
	52	58	40	32	52	20
	58	58	40	30	52	20

*From Esser and Souza [6].
†From McElhaney and Souza [8].

This aggregation is more pronounced about 7–10°C below the growth temperature (Fig 1c), the temperature at which, according to the results of spin label tests, the lateral phase separation is completed (T_{l-1}). After further decrease in freezing temperature to the mid-point temperature (T_m), as measured by DTA, fracture faces appear that display smooth domains separated by very densely packed network-like regions (Fig 1d). The total areas covered by smooth regions and particulate regions are approximately equal. When freezing is performed at a temperature at which all lipids are in the solid state (below T_{l-2}) large smooth areas are visible (Fig 1e) within the membrane. Although most of the particles are squeezed into one hemisphere, the smooth areas still contain a significant number in a dispersed state.

Particle segregation can also be observed on replicas of freeze-fractured whole cells (Fig 2), which proves that this phenomenon is not simply an artifact of protoplast preparation. Furthermore, quick-freezing at the desired temperature, or crosslinking of protoplasts with glutaraldehyde at the same temperature followed by freezing, yields similar pictures. Although the photographs shown here were taken on replicas of protoplasts prepared from WT cells grown at 65°, similar results were obtained on WT cells grown at 58° and 52°.

The membrane views shown in Figures 1e and 2 are in general agreement with the ones published earlier by Sleytr [13] from a different strain of B stearothermophilus. We observed numerous cross fractures of whole cells exhibiting vesicular structures that were interpreted by him to be the remainders of incompletely extruded mesosomes. But we did not find the rectangular, paracrystalline patterns of intramembraneous particles that were observed by him. Similar arrays were found by Weiss [14] in magnesium-starved Escherichia coli membranes. The absence of such arrays in our preparations could perhaps be due to differences in magnesium concentrations or due to strain differences. Our preparations, however, reveal an interesting band pattern that was not mentioned by Sleytr [13]. Protoplast samples that have been stored for prolonged periods of time below T_{l-2} prior to freezing and fracturing show banded structures with repeat distances of 300–500 Å (Fig 1f).

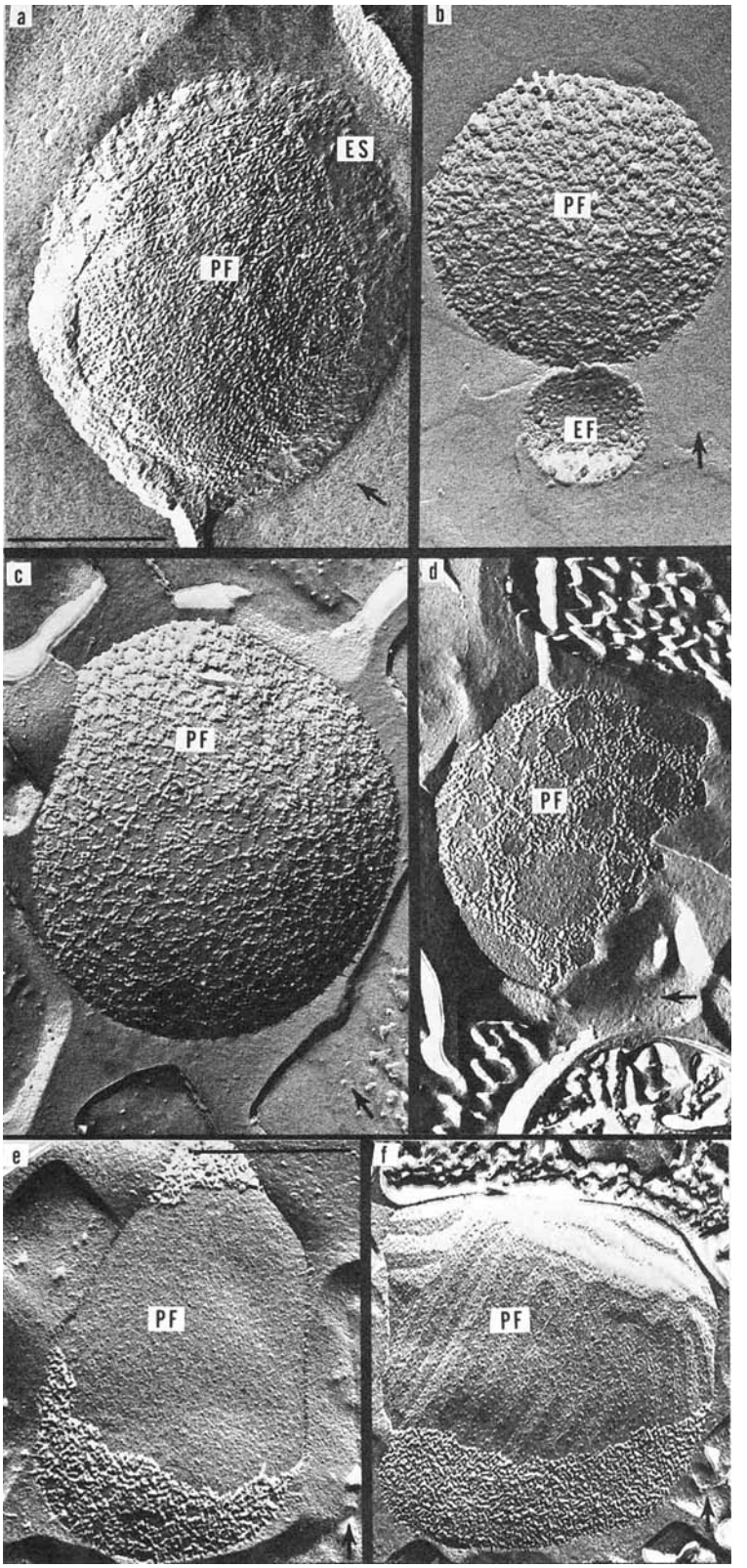
In contrast to the wild-type the mutant TS-13 did not reveal such extended segregations as those shown in Figs 1d and e. TS-13 protoplast membranes show only slight aggregation with very little dependence on quench temperature and no dependence on growth temperature. The membrane views shown in Fig 3 were not influenced much by freezing of the protoplasts at the growth temperature or at T_{l-2} or any temperature in between. The aggregation state in the PF plane shown in Fig 3b was the strongest we have ever observed for the mutant. Usually, particle segregation remained intermediate between the states shown in Fig 3a and 3b. In addition we noticed that these membranes fractured poorly along the middle of the bilayer; most preparations were cross-fractured. For this reason we have only a limited number of views of fracture planes in mutant membranes to base evaluations on. A similar situation was encountered with protoplasts prepared from WT cells that were grown at 42°, approximately 5° above the minimal growth temperature for this organism.

DISCUSSION

The studies described here clearly demonstrate that integral plasma membrane proteins in B stearothermophilus protoplasts are not dispersed randomly at the growth

Fig 1. Freeze fracture faces of membranes from B stearothermophilus WT grown at 65°C. Protoplast samples were quick-frozen from a) 70°, b) 65°, c) 58°, d) 54°, e) 20°, and f) 20° after holding the protoplasts for several days at 4°. Bar corresponds to 500 mμ; arrow indicates direction of shadowing.

Abbreviations: PF = protoplasmic face, EF = exoplasmic face, ES = exoplasmic surface. (See Branton et al [12]).



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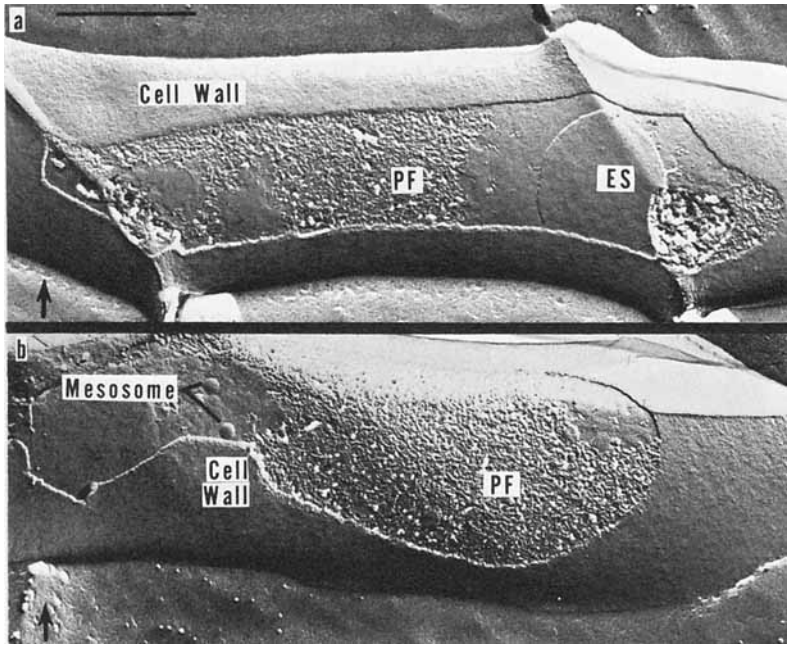

Fig 2. Freeze fracture faces of whole cells of B stearothermophilus WT grown at 65°C. Samples were quick-frozen from room temperature. Bar corresponds to 500 mμ; arrow indicates direction of shadowing.

temperature. Although DTA results had indicated that in WT membranes lipid hydrocarbon chains are almost completely in the disordered ("fluid") state, membrane views obtained by freeze-fracturing demonstrate a slight but distinct protein aggregation. This observation is even more striking in the case of the mutant where the lipid phase transition was shown to be 18° below the growth temperature [8]. It could be argued that protein aggregation is not present at the growth temperature but is induced during the freezing process, which might be too slow to lock proteins in place. However, similar membrane views were obtained after fixation of protoplasts at the different quench temperatures. Furthermore, if particle aggregation was determined by the time required to freeze the membranes, then we should have obtained identical pictures for membranes quenched at the same temperature but derived from cells grown at different temperatures. This was not the case. Protoplast membranes isolated from cells grown at 65° or 58° but quenched from 58° displayed quite different fracture faces.

A comparison of the freeze-fracture patterns shown in Fig 1 and the lipid transitions tabulated in Table I reveals some interesting correlations. When protoplast membranes from cells grown at 65° are lowered in temperature to 58°, only about 10% of their lipids are in the solid state as determined by DTA (see also Fig 3 in McElhaney and Souza [8]). Yet the fracture faces of membranes quenched or crosslinked at that temperature show smooth areas comprising far more than 10% of the total membrane area. What are the forces that keep the particles segregated even when lipids are 90% fluid?

Several investigators have suggested that intercalated proteins aggregate upon solidification or separation of lipids (15–18). As lipids solidify, proteins are squeezed out, since

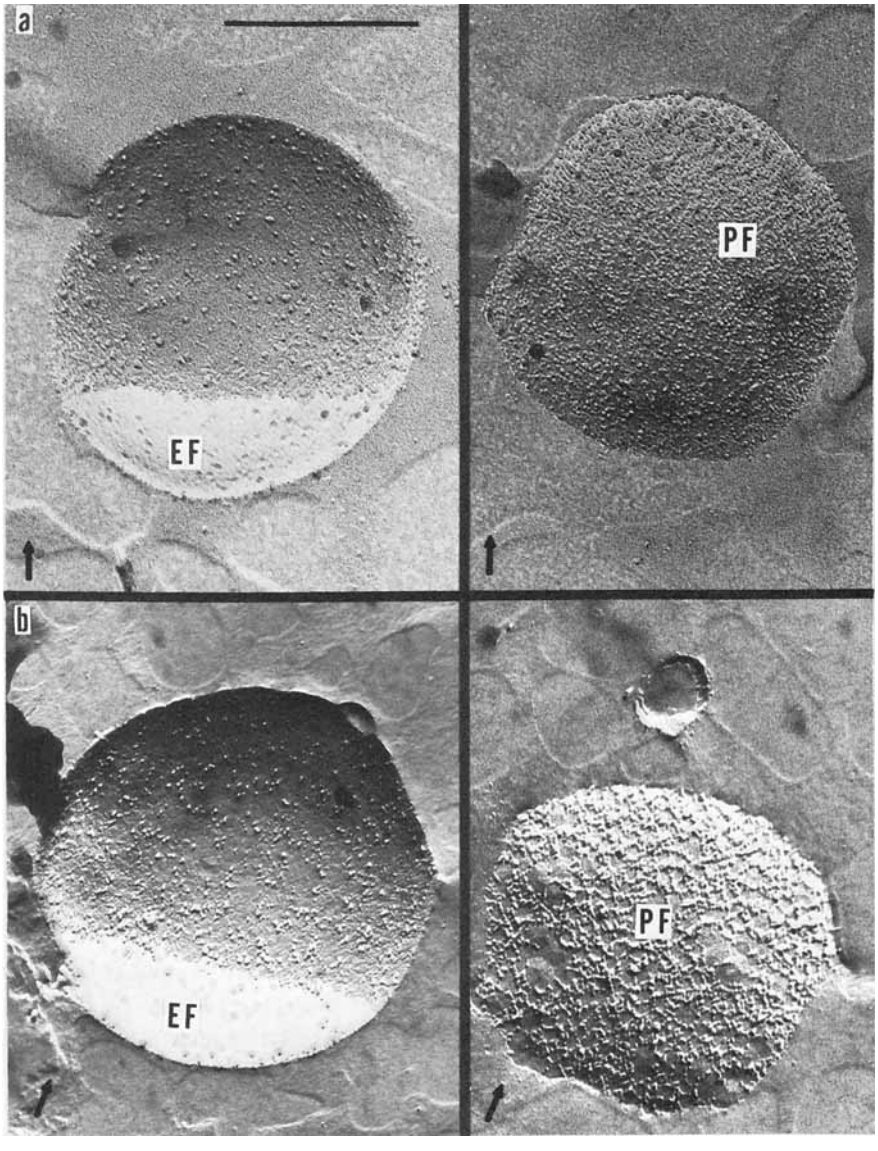

Fig 3. Freeze fracture faces of protoplasts from B stearothermophilus TS-13 grown at 58°C. Protoplast samples were quick-frozen from a) 58° and b) 20°. Bar corresponds to 500 mμ, arrow indicates direction of shadowing.

they would interfere with close hexagonal packing of ordered hydrocarbon chains (ie, crystal formation). When lipids separate, proteins concentrate in a lipid region for which they show preferential affinity, thereby depleting other lipid domains. The behavior of ATPase from sarcoplasmic reticulum in liposomes exemplifies the solidification events [16], whereas the human myelin basic protein with its preference for acidic phospholipids is an example of the separation sequence [19]. In both cases the total energy change for

the final state must outweigh the entropy that would be gained by random mixing. With respect to B stearothermophilus membranes it can be seen that a comparatively small temperature incrase of about 10° results in complete randomization, making protein-protein interactions an unlikely explanation. Consideration of the fact that spin label results agree well with DTA data in determining transitions in isolated, protein-free lipids [8] (but not in membranes) suggests that lipid-lipid interactions should also be dismissed as the impetus for particle separation, leaving lipid-protein interactions as the most likely cause. Apparently, then, proteins cause some form of lipid clustering even when almost all lipids are fluid. These clusters are quite large, strongly suggesting that the interactions must be of long range order. Spin labels such as 5-doxyl stearate and Tempo have the potential to sense such clustering [20, 21], which when associated with little or no change in enthalpy, could remain unnoticed by DTA.

In contrast to the results with WT cells grown at 65°, 58°, or 52° we were unable to demonstrate pronounced particle aggregation in membranes of protoplasts from WT cells grown at 42° or from TS-13 cells grown at any temperature. Verkleij and Ververgaert [17] have listed three possible explanations for the absence of extended particle aggregation: 1) protein-protein interactions are strong enough to prevent concentration by lipids; 2) protein-lipid interactions prevent the formation of large lipid phases; and 3) some lipids do not fuse into crystals because they remain loosely packed below the transition point. These authors postulated that branched lipids belong to the latter class of lipids, since incorporation of about 60% of branched fatty acids into Acholeplasma laidlawii membranes prevented aggregation of particles. Our results are in excellent agreement with this proposal. As shown in Table II, in wild-type cells the percentage of branched fatty acids increases with decreasing growth temperature, reaching 62% at 42°, while in the mutant the trend is reversed but the total amount never falls below 58%. The inhibition of protein aggregation by branched fatty acids prevents us from drawing conclusions on the relationship between protein segregation and the temperature-sensitive lesion in the mutant. But recent studies by Mulks [22] clearly point out that at the restrictive temperature the mutant is unable to transport cell wall monomer units through the membrane thereby stopping cell wall synthesis.

An interesting pattern of banding is observed on protoplast samples from wild-type cells that have been stored for prolonged peridos at temperatures below T_{l-2} prior to freezing and fracturing (Fig 1f). The repeat period in these corrugated structures is in the range of 300–500 Å. Repeat distances of 120–500 Å, associated with the $P_{\beta'}$ lipid phase (nomenclature of Tardieu, Luzzati, and Reman [23]), were observed earlier by Luna and McConnell [24] in freeze-fracture electron micrographs of saturated lecithins. This partic-

TABLE II. Comparison of Branched, Straight-Chain, and Unsaturated Fatty Acids of B stearothermophilus WT and TS-13 by Growth Temperature*

	TS-13			WT			
	42°	52°	58°	42°	52°	58°	65°
Fatty acid group							
Branched	58	59	65	62	53	46	40
Straight	25	33	33	21	40	46	57
Unsaturated	17	8	2	17	7	8	3

*Values are given as percentages of total fatty acids (from Kostiw and Souza [10]).

ular phase was only observed within a narrow temperature range and these authors suggested that the corrugated structure was associated with the well-known "pretransition" that occurs prior to the main transition in lecithins [25]. The nature of the pretransition remains disputed. Rand, Chapman, and Larsson [26] interpret their X-ray diffraction data as indicating a transition from the $L_{\beta'}$ to the L_{β} phase while Janiak, Small, and Shipley [27], on the basis of similar X-ray data, favor a transition from the $L_{\beta'}$ to the $P_{\beta'}$ state. Both groups, however, agree that admixture of more than 10 mole % of other lipids such as phosphatidylethanolamine (PE) or cholesterol abolishes the pretransition of the lecithins. Accordingly, Janiak, Small, and Shipley [27] postulated that only pure, saturated lecithins would form the $P_{\beta'}$ phase. B stearothermophilus does not synthesize choline-containing lipid but has about 20% PE, the remainder being cardiolipin (CL) and phosphatidylglycerol (PG) [10]. To make the aforementioned hypothesis compatible with the result shown in Fig 1f, one has to assume that in its banded phase the membrane is free of PE and that CL and/or PG are also capable of forming $P_{\beta'}$-like structures.

Summarizing these results, we can say that B stearothermophilus WT, and especially the mutant TS-13, are capable of growing at a temperature at which most or all of the lipid hydrocarbon chains are in a fluid state. This raises the question: If all the lipids are fluid, can "excess fluidity" hamper growth and function? It should be remembered that even in fluid lipids, as defined by DTA results, interactive forces can prevent membrane constituents from moving in a completely unrestricted fashion. The nature of the forces that keep some lipids in clusters or domains has been described [18, 20]. Further increase in temperature above the lipid melting temperature weakens these forces to a point at which randomization sets in, resulting in loss of function. For this reason we proposed in answer to the last question that microorganisms can adapt their membranes to higher temperatures as long as they can synthesize a lipid mixture that does not form an unrestrictive, randomizing two-dimensional fluid in the presence of all other membrane constituents such as water, proteins, and ions. Obviously, there are several means by which such restrictions can be created, as Chapman, Cornell, and Quinn [18] have discussed, and such processes can operate in a fluid or partially solid membrane; however, the particular mechanisms that operate in B stearothermophilus cells remain to be elucidated.

Although some authors have cast doubt on the validity of the spin label technique when applied to biological membranes [4, 8], our freeze-fracture electron microscopic observations clearly validate our earlier conclusions [7] based on spin label experiments on B stearothermophilus membranes.

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