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THE RELATIONSHIP BETWEEN ENVIRONMENTAL TEMPERATURE, CELL GROWTH AND THE FLUIDITY AND PHYSICAL STATE OF THE MEMBRANE LIPIDS IN *BACILLUS STEAROTHERMOPHILUS*

RONALD N. McELHANEY[a] and KENNETH A. SOUZA[b]

[a]*Department of Biochemistry, The University of Alberta, Edmonton, Alberta, T6G 2H7 (Canada) and* [b]*Biological Adaptation Branch, NASA-Ames Research Center, Moffett Field, Calif. 94035 (U.S.A.)*

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SUMMARY

A definite and characteristic relationship exists between growth temperature, fatty acid composition and the fluidity and physical state of the membrane lipids in wild type *Bacillus stearothermophilus*. As the environmental temperature is increased, the proportion of saturated fatty acids found in the membrane lipids is also markedly increased with a concomitant decrease in the proportion of unsaturated and branched chain fatty acids. The temperature range over which the gel to liquid-crystalline membrane lipid phase transition occurs is thereby shifted such that the upper boundary of this transition always lies near (and usually below) the temperature of growth. This organism thus possesses an effective and sensitive homeoviscous adaptation mechanism which maintains a relatively constant degree of membrane lipid fluidity over a wide range of environmental temperatures. A mutant of *B. stearothermophilus* which has lost the ability to increase the proportion of relatively high melting fatty acids in the membrane lipids, and thereby increase the phase transition temperature in response to increases in environmental temperature, is also unable to grow at higher temperatures. An effective homeoviscous regulatory mechanism thus appears to extend the growth temperature range of the wild type organism and may be an essential feature of adaptation to temperature extremes.

Over most of their growth temperature ranges the membrane lipids of wild type and temperature-sensitive *B. stearothermophilus* cells exist entirely or nearly entirely in the liquid-crystalline state. Also, the temperature-sensitive mutant is capable of growth at temperatures well above those at which the membrane lipid gel to liquid-crystalline phase transition is completed. Therefore, although other evidence suggests the existence of an upper limit on the degree of membrane fluidity compatible with cell growth, the phase transition upper boundary itself does not directly determine the maximum growth temperature of this organism. Similarly, the lower boundary does not determine the minimum growth temperature, since cell growth ceases at a temperature at which most of the membrane lipid still exists in a fluid state. These observations do not support the suggestion made in an earlier study, which utilized electron spin resonance spectroscopy to monitor membrane lipid lateral phase separations, that the minimum and maximum growth temperatures of this organism might be

directly determined by the solid-fluid membrane lipid phase transition boundaries. Evidence is presented here that the electron spin resonance techniques used previously did not in fact detect the gel to liquid-crystalline phase transition of the bulk membrane lipids, which, however, can be reliably measured by differential thermal analysis.

INTRODUCTION

Almost all organisms thus far examined possess the ability to vary the fatty acid composition of their cellular lipids in a characteristic fashion in response to alterations in the environmental temperature. Changes in membrane lipid fatty acid composition as a function of the temperature of growth are particularly evident in the prokaryotic microorganisms, some of which have become adapted to temperature extremes ranging from −10 to nearly 100 °C [1–3]. An increase in environmental temperature typically results in the production of membrane lipids containing a relatively higher proportion of saturated fatty acids and a lower proportion of unsaturated and/or anteisobranched fatty acids. In addition, an increase in the growth temperature often results in the production of membrane lipids whose hydrocarbon chains have a greater average length (for reviews see refs. 4 and 5). These characteristic changes in fatty acid composition would be expected to progressively increase the temperature of the gel to liquid-crystalline phase transition, which arises from a cooperative melting of the hydrocarbon chains in the interior of a lipid bilayer, as the growth temperature is increased, and indeed this effect has been confirmed experimentally in several microorganisms [6–8]. These alterations in the phase transition temperatures would have the effect of minimizing changes in the fluidity and phase state of the membrane lipids which would otherwise accompany alterations in the environmental temperature. *Escherichia coli*, for example, is able to maintain the fluidity of its membrane lipids nearly constant over its entire growth temperature range by appropriate alterations in the fatty acid composition of its membrane lipids, a process termed "homeoviscous adaptation" [8]. Since the permeability properties of the cellular membrane [9] and the activity of certain membrane-bound enzymes [10–12] and transport systems [13–15] are markedly dependent on the fluidity and physical state of the membrane lipids, it seems likely that homeoviscous adaptation represents an important mechanism for maintaining optimal levels of cell growth over a wide range of environmental temperatures [5, 16].

Esser and Souza [17] have recently published an interesting study relating membrane lipid fluidity and phase state to maximum and minimum growth temperatures of wild type (WT) *Bacillus stearothermophilus* and of a temperature-sensitive (TS-13) mutant of this organism which is unable to alter its fatty acid composition in response to changes in growth temperature in the normal fashion. These investigators monitored changes in the fluidity and physical state of the lipids in spheroplast membranes and isolated lipid-water dispersions by electron spin resonance (ESR) spectroscopy, using both a fatty acid spin label and TEMPO, a spin probe which partitions differentially between water and lipids in the fluid or solid state. Changes of slope in Arrhenius plots of ESR spectral parameters were interpreted as "lateral lipid phase separations". Wild type cells were found to increase the temperatures at which the lateral phase separations were detected in response to increases in

growth temperature, so that the same physical state of the membrane was maintained at all temperatures. Somewhat surprisingly, the TS-13 mutant also exhibited a similar increase in the lateral phase separation temperature boundaries, despite the fact that its fatty acid composition was not markedly altered with increasing growth temperatures. These data were interpreted to indicate that the maximum and minimum growth temperatures of this organism are determined by the onset and conclusion of the lateral lipid phase separations and that the simultaneous presence of isolated solid and fluid lipid domains are required for functional membrane assembly. However, the nature of the "lateral phase separations" detected in *B. stearothermophilus* membranes by ESR spectroscopy were not specified and the conclusions reached in that study are at variance with previous work on the relationship between membrane lipid physical state and cell growth in certain other microorganisms [6–8, 18–23]. In order to clarify the nature of the previously observed lateral phase separations and to test the validity of the conclusions reached, we decided to reinvestigate the relationship between membrane lipid fluidity and phase state and cell growth in *B. stearothermophilus* using differential thermal analysis to detect membrane lipid phase transitions in spheroplast membranes and isolated membrane lipid. Our results indicate that the ESR spectroscopy techniques previously utilized did not in fact measure the gel to liquid-crystalline phase transition of the bulk lipid in *B. stearothermophilus* membranes.

MATERIALS AND METHODS

B. stearothermophilus, strain YTG-2, and a temperature-sensitive mutant (TS-13) derived from nitrosoguanidine-treated YTG-2, were used throughout this study. The taxonomic characteristics of strain YTG-2, growth conditions, mutant isolation procedure and the extraction and fatty acid analysis of the membrane lipids have been described in detail elsewhere [24]. Spheroplast membranes were prepared by the procedure of Wisdom and Welker [25].

The differential thermal analysis of both the spheroplast membranes and distilled water dispersions of the total membrane lipids were carried out with a Dupont 900 Thermal Analyzer essentially as previously described [9]. The macro heating block was utilized with glass beads in the control well and distilled water as the reference material in order to minimize initial baseline drift. Heating and cooling rates of 5–15 °C per min were used with an instrumental sensitivity setting of 0.1 °C per inch (membranes) or 0.2 °C per inch (lipid dispersions). The lower and upper boundaries of the membrane lipid phase transition were taken as the temperatures at which the differential thermal analysis thermogram starts to depart from and returns to the baseline, respectively. The midpoint of the phase transition was defined as the temperature at which one-half of the membrane lipid had been converted from the gel to the liquid-crystalline state, as determined from a planimetric measurement of the thermogram peak area.

The enthalpies of the membrane lipid phase transitions were also measured on a DuPont 900 Thermal Analyzer equipped with a differential scanning calorimetry cell. The enthalpy was determined from a planimetric measurement of the transition peak area after establishing the calibration coefficient using synthetic phospholipids and the total membrane lipids from *Acholeplasma laidlawii* B as standards.

RESULTS

Relation of cell growth to environmental temperature

The rates of growth of both WT and TS-13 cells of *B. stearothermophilus*, expressed as generations per h, as a function of environmental temperature are presented in Fig. 1. WT cells exhibit a growth temperature profile characteristic of thermophilic prokaryotic microorganisms, with minimum, optimum and maximum growth temperatures of 37, 65 and 72 °C, respectively. In contrast, TS-13 cells are unable to grow at the upper end of the WT growth temperature range but appear to grow normally at temperatures below about 50 °C. The minimum growth temperatures of TS-13 and WT cells are the same, indicating that the entire growth range of TS-13 cells has not shifted to lower temperatures. However, the optimum growth temperature of the TS-13 cells is depressed to about 58 °C. The decline of cell growth with increasing temperature above about 50 °C is very marked in the TS-13 cells, which are unable to grow and eventually lyse at temperatures of 60 °C or higher.

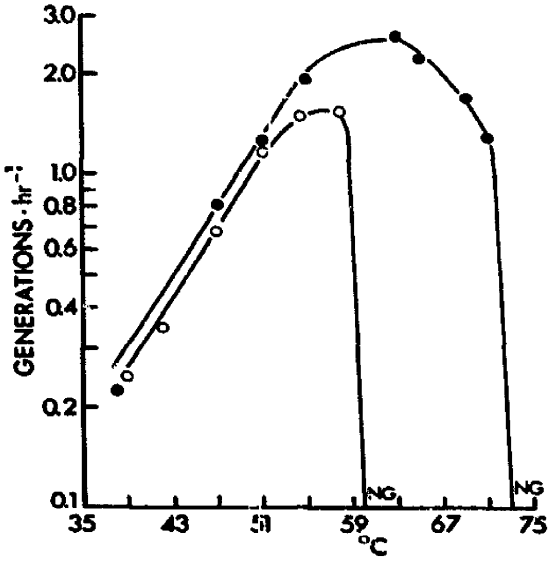

Fig. 1. Growth rate, expressed as generations per h, as a function of temperature for (●), WT; and (○), TS-13 cells grown aerobically in 1 % tryptic Soy Broth.

Relation of fatty acid composition to growth temperature

The fatty acid compositions of the membrane lipids of WT and TS-13 cells of *B. stearothermophilus* grown at various temperatures are shown in Table 1. WT cells exhibit the expected shifts in fatty acid composition in response to changes in environmental temperature over the entire growth temperature range studied. As the growth temperature is increased the proportion of the relatively high melting point saturated fatty acids, particularly palmitic acid, increases markedly while the proportion of the lower melting unsaturated and anteisobranched fatty acids also decreases markedly. The proportion of the isobranched fatty acids, which have intermediate melting points, tends to decrease slightly with increasing growth temperature. The net result of these

TABLE I

THE FATTY ACID COMPOSITION OF WT AND TS-13 CELLS GROWN AT VARIOUS TEMPERATURES

Values are expressed as percent of total fatty acids. Averages of at least four determinations representing two different cell batches and two analyses of each are reported.

Fatty acid methyl esters*	TS-13			WT			
	42 °C	52 °C	58 °C	42 °C	52 °C	58 °C	65 °C
Unidentified	tr	tr	tr	tr	tr	tr	tr
14 : 0*i*	2.2	2.4	2.5	1.5	1.7	1.7	1.2
14 : 0	3.6	4.2	4.5	2.5	4.4	5.7	6.6
15 : 0*i*	14.2	13.2	17.4	15.4	10.3	9.7	10.1
15 : 0*a*	3.5	3.9	4.0	4.0	3.4	2.5	2.3
15 : 0	3.4	4.3	3.8	3.4	2.9	2.2	2.2
16 : 0*i*	23.3	22.6	19.7	21.0	21.6	21.0	16.7
16 : 0*a*	1.9	tr	tr	1.8	tr	tr	tr
16 : 0	12.1	20.0	20.8	8.9	28.4	34.3	44.3
16 : 1	11.2	6.6	2.6	9.3	5.9	3.3	tr
17 : 0*i*	5.2	6.9	8.6	6.0	6.9	5.1	4.9
17 : 0*a*	9.3	10.8	12.8	14.0	9.0	5.8	4.5
17 : 0	1.6	1.9	1.8	1.8	1.8	1.2	tr
Unidentified	tr	tr	tr	tr	tr	tr	tr
18 : 0	4.4	2.1	2.0	4.1	2.1	2.5	2.7
18 : 1	2.4	tr	tr	2.1	2.2	2.5	1.5
Unidentified	tr	tr	tr	tr	tr	tr	tr

* Number of carbon atoms: number of double bonds; *i*, iso; *a*, anteiso.
tr, trace, < 1.0 %.

characteristic alterations in fatty acid composition is to produce membrane lipids which are progressively enriched in the relatively higher-melting fatty acyl species at higher environmental temperatures.

At a growth temperature of 42 °C, the fatty acid composition of the TS-13 cells is rather similar to that of the WT. However, increasing the growth temperature to 52 °C does not produce as great a shift in the fatty acid spectrum as noted in WT cells. Although the proportion of saturated fatty acid in TS-13 cells increases somewhat, this change is not as pronounced as normally observed. Also, although the proportion of unsaturated fatty acid falls markedly, the proportion of iso- and anteisobranched fatty acids actually increases with temperature, in contrast to the WT strain. Increasing the growth temperature of TS-13 cells to 58 °C results in little additional change in fatty acid composition. Thus, the TS-13 mutant is capable only of producing membrane lipids which are slightly enriched in the higher melting fatty acids as the temperature is increased over the lower temperature portion of the growth temperature range, and loses the ability to regulate its fatty acid composition entirely in the upper portion of the growth temperature range.

Relation of the physical state of the membrane lipids to the growth temperature

Temperature-base thermograms of aqueous dispersions of the total membrane lipids, extracted from isolated spheroplast membranes of WT and TS-13 *B. stearo-*

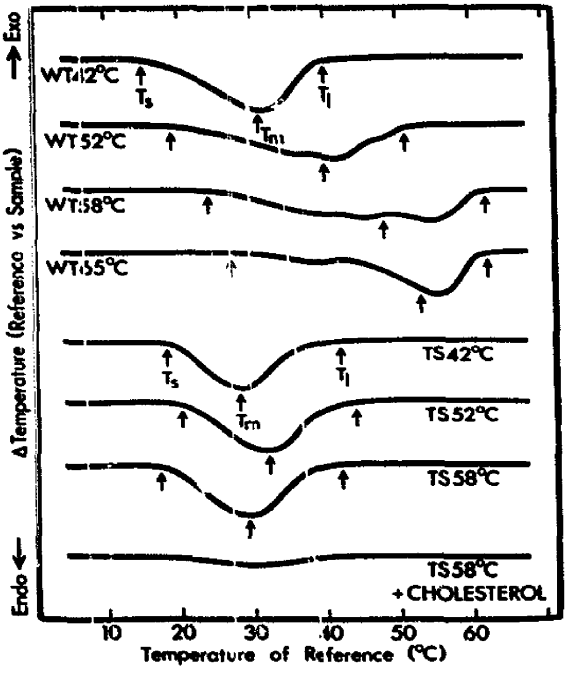

Fig. 2. Temperature-base thermograms of aqueous dispersions of the total membrane lipids extracted from isolated spheroplast membranes of WT and TS-13 *B. stearothermophilus* cells grown at several temperatures. The temperature differentials between the lipid-water dispersions and the inert reference material are plotted as a function of the temperature of the reference, using a heating rate of 5 °C/min. The lower boundary, transition midpoint and upper boundary of each phase transition are denoted by arrows and the symbols T_s, T_m and T_l, respectively. Cooling the samples at 5 °C/min results in essentially identical (but exothermic) curves, except that the entire transition is shifted by 2–3 °C to lower temperatures. The thermogram at the bottom of the figure is of an aqueous dispersion of the total membrane lipids of TS-13 cells mixed with an equal weight of cholesterol.

thermophilus cells grown at several temperatures, are presented in Fig. 2. These thermograms depict the gel to liquid-crystalline phase transitions of the membrane lipids, which under these conditions are known to exist as multilamellar bilayers separated by water-filled spaces [26]. These lipid phase transitions have the following properties: (i) the transitions are thermotropic and (ii) they are completely reversible upon sample cooling; (iii) the transition temperature shows the expected dependence on the fatty acid compositions of the membrane lipids; (iv) the phase transition enthalpies are of the order of 4 cal/g, similar to those noted for other biological lipid-water dispersions [6, 7, 27, 28]; and (v) the phase transition can be broadened and eventually abolished by adding cholesterol to the membrane lipid-water dispersion. These phase transitions or lateral phase separations are all rather broad, extending over a range of roughly 25–35 °C. This broadness is due to the heterogeneous nature of the polar headgroups and fatty acids of the *B. stearothermophilus* membrane lipids. At temperatures below the lower boundary of the phase transition (T_s), the lipids exist entirely in the gel state; at temperatures above the upper boundary of the transition (T_l), all the lipids are in the liquid-crystalline state. At temperatures within the phase transition temperature range, various proportions of gel and liquid-crystalline

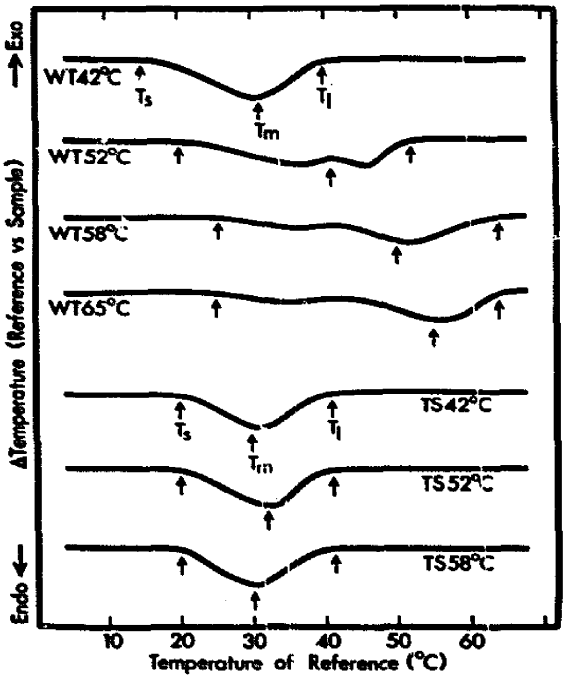

Fig. 3. Temperature-base thermograms of isolated spheroplast membranes derived from WT and TS-13 *B. stearothermophilus* cells grown at several different temperatures. The temperature differentials between the lipid-water dispersions and the inert reference material are plotted as a function of the temperature of the reference, using a heating rate of 7.5 °C/min. The lower boundary, transition midpoint and upper boundary of each phase transition are denoted by arrows and the symbols T_s, T_m and T_l, respectively. Cooling the samples at 7.5 °C/min results in essentially identical (but exothermic) curves, except that the entire transition is shifted by 2–3 °C to lower temperatures.

phases exist simultaneously, so that the lipid bilayers are heterogeneous with respect to their physical state [29]. In WT membrane lipid-water dispersions, the lower and upper boundaries of the phase transition and the phase transition midpoints (T_m) are progressively shifted to higher temperatures as the temperature of growth is increased. In contrast, the position of the phase transitions of the TS-13 membrane lipid preparations changes little with increases in the growth temperature.

Temperature-base thermograms of the isolated spheroplast membranes, derived from WT and TS-13 *B. stearothermophilus* cells grown at several temperatures, are shown in Fig. 3. Since the transitions detected by differential thermal analysis in the intact membranes are very similar in all respects to those observed for the corresponding membrane lipid-water dispersions, we believe that these thermograms are accurately depicting the gel to liquid-crystalline phase transition of the lipids in the isolated membranes. In particular, the similar heats of transition noted in both the membranes and lipid-water samples indicate that the bulk of the lipids in the spheroplast membranes are participating in the gel to liquid-crystalline phase transition. As can be noted in Table II, the lower and upper boundaries and the midpoints of the phase transitions are essentially the same, within experimental error, for the intact membranes and the corresponding membrane lipid-water dispersions, indicating that

TABLE II

A summary of the membrane lipid phase transition temperatures, as detected by differential thermal analysis and electron spin resonance spectroscopy, of spheroplast membranes and isolated lipid-water dispersions from WT and TS-13 *B. stearothermophilus* cells grown at various temperatures. The results are expressed in °C.

Cell type	Growth temperature	Differential thermal analysis				Electron spin resonance spectroscopy*	
		Membranes		Lipids			
		Ts-Tl	Tm	Ts-Tl	Tm	Membranes Ts-Tl	Lipids Ts-Tl
WT	42	15–40	31	15–40	31	37–45	—
	52	20–52	41	19–51	40	40–52	—
	58	25–64	50	24–62	48	50–58	—
	65	24–65	55	27–62	53	58–65	30–63
TS-13	42	20–41	30	18–42	27	42–45	—
	52	20–40	32	20–44	31	52–58	—
	58	20–40	30	17–42	28	52–58	—

* Data taken from Esser and Souza [17].

the membrane proteins do not significantly alter the thermal properties of the bulk of the membrane lipids. As noted in the lipid-water dispersions, lipid phase transitions occurring in the spheroplast membranes are broad, and the entire phase transition is shifted to higher temperatures as the growth temperature is increased only in the case of the WT cells.

A definite and consistent relationship exists between the fluidity and phase transition temperatures of the membrane lipids and the growth temperature in WT *B. stearothermophilus* cells. As the growth temperature is increased a corresponding increase in the temperatures of the phase transition midpoint and upper boundary is noted (see Table II). The lower boundary of the phase transition is also shifted upward, although to a lesser extent, so that the phase transitions tend to become broader at higher growth temperatures. The proportional increase in the phase transition midpoint and upper boundary temperatures with increases in the temperature of growth is particularly striking. As the growth temperature is raised from 42 to 65 °C (an increase of 23 °C) the phase transition midpoint temperature increases from 31 to 53–55 °C (an increase of 22–24 °C) and the upper boundary temperature increases from 40 to 62–64 °C (also an increase of 22–24 °C). This excellent correlation between the growth temperature and the position of the gel to liquid-crystalline membrane lipid phase transition indicates that WT *B. stearothermophilus* cells possess a very efficient mechanism for homeoviscous adaptation through variation in the fatty acid composition of the membrane lipids. It is also clear that over most of the growth temperature range WT cells prefer to grow near but slightly above the upper boundary of the membrane lipid phase transition, so that essentially all of the membrane lipids exist in the liquid-crystalline state at the growth temperature. WT cells maintained at 58 °C, however, are growing slightly below the upper boundary of the phase transition, but even in this case the great majority of the membrane lipid is in a fluid state. It should be noted that the minimum growth temperature of this organism

of 37 °C falls well above the lower boundary of the gel to liquid-crystalline phase transition. Also, the maximum growth temperature of WT cells usually falls slightly above the upper boundary of the lipid phase transition, although the organism does shift the position of this transition such that the upper boundary falls near the temperature of growth.

In contrast to WT cells, the membrane lipid phase transition midpoints and boundaries are not appreciably altered as the growth temperature of the TS-13 cells is increased (see Table II). This observation is not surprising in view of the inability of these cells to alter their fatty acid composition in the characteristic manner as a function of the temperature of growth. It is of interest to note that from 42 to 52 °C, the TS-13 mutant grows nearly as well as the WT cells despite the fact that at 52 °C the midpoint and upper boundaries of the TS-13 membrane lipid phase transition occur about 10 °C below that of the WT cells. This result strongly suggests that *B. stearothermophilus* cell membranes are capable of normal function at temperatures completely above the phase transition temperature range and demonstrates that the upper boundary of the gel to liquid-crystalline membrane lipid phase transition per se does not directly determine the maximum growth temperature, at least in TS-13 cells. However, if the environmental temperature is raised still farther above the static position of the TS-13 membrane lipid phase transition, the rate of increase of cell growth with environmental temperature slows and cell growth ceases abruptly when the environmental temperature exceeds the upper boundary and midpoint of the phase transition by about 18–19 °C and 28–30 °C, respectively. This latter result implies that an upper limit exists on the degree of membrane lipid fluidity which is compatible with cell growth and suggests that the process of homeoviscous adaptation serves to extend the growth temperature range in WT cells.

Comparison of the phase transition detected by differential thermal analysis and electron spin resonance spectroscopy

The membrane lipid phase transitions monitored by differential thermal analysis in the present study are compared with the lateral phase separations detected by ESR spectroscopy in the previous study by Esser and Souza [17] in Table II. For the WT spheroplast membrane preparations, the ESR technique indicates a rather narrow phase separation encompassing a range of 7–12 °C, which is centered at a higher temperature than the gel to liquid-crystalline phase transition detected by differential thermal analysis. The onset of the lateral phase separation detected by ESR occurs between the transition midpoint and upper boundary temperature of the thermograms. In the case of the TS-13 spheroplast membranes, the ESR transition is even narrower, encompassing a range of only 3–6 °C, and occurs at temperatures completely above the membrane lipid phase transitions monitored by differential thermal analysis. However, the ESR technique reports a much broader lateral phase separation centered at lower temperatures in the isolated membrane lipids from WT cells grown at 65 °C, in excellent agreement with the phase transition detected by differential thermal analysis. The marked difference between the lateral phase separations detected by ESR in membranes and lipid dispersions was ascribed by Esser and Souza [17] to the ordering influence of the membrane proteins. However, this explanation appears unlikely since studies on model systems indicate that hydrophobic lipid-protein interactions generally decrease the cooperativity of the lipid-lipid fatty acyl

chain interactions and thus tend to lower and broaden the gel to liquid-crystalline membrane lipid phase transitions, not to raise and sharpen them [6, 7, 28, 29]. In view of the evidence presented earlier that differential thermal analysis does accurately reflect the gel to liquid-crystalline phase transition of the bulk membrane lipids of this and other organisms, the most likely explanation for all of the above results is that the ESR technique is in fact not monitoring a solid-fluid lateral phase separation of the bulk of the membrane lipids. This conclusion is strengthened by the observation that the fatty acid spin label probe indicates that the phase transition temperature increases to a similar extent in both WT and TS-13 cells, whereas the fatty acid and thermal analytical phase transition data indicates that this should not be the case. These results suggest that the ESR technique is sensing some process which is dependent on the growth temperature but not on the fatty acid composition or "melting" properties of the membrane lipids.

DISCUSSION

The relations observed between the fluidity and physical state of the membrane lipids and the ability of *B. stearothermophilus* to grow at various temperatures are generally similar to those previously reported for the mesophilic microorganisms *A. laidlawii* B [19, 20] and *E. coli* [13, 30]. The absolute minimum growth temperatures of *A. laidlawii* and *E. coli* are not defined by the fatty acid compositions of the membrane lipids when cells are enriched in fatty acids giving rise to gel to liquid-crystalline membrane lipid phase transitions whose lower boundaries fall below this temperature. The cold denaturation of one or more enzymes or regulatory proteins probably determines the absolute minimum growth temperature of these organisms, as may also be the case with *B. stearothermophilus*. However, in *A. laidlawii* and unsaturated fatty acid auxotrophs of *E. coli*, the elevated minimum growth temperatures of cells enriched in relatively high-melting fatty acids is clearly limited by the lower boundary of the membrane lipid phase transition. Thus, although it is highly probably that *B. stearothermophilus* would also not be able to grow if its membrane lipids existed entirely or nearly entirely in the gel state, the growth of this organism has already ceased at temperatures well above those needed to completely solidify the membrane lipid. The optimum and maximum growth temperatures of *A. laidlawii* and *E. coli* are also influenced indirectly by the physical state of the membrane lipids, being significantly reduced for cells having a membrane lipid phase transition centered at low temperatures. This apparent upper limit to the fluidity of the membrane lipid may arise from an excessively "leaky" membrane, a structurally unstable lipid bilayer, or an inability of excessively fluid lipid to stabilize certain membrane proteins in a functional configuration (see ref. 5). Again, a similar situation seems to obtain for TS-13 *B. stearothermophilus* cells. However, both *A. laidlawii* and *E. coli* unsaturated fatty acid auxotrophs are capable of normal growth at temperatures above the upper boundaries of the membrane lipid phase transitions, again as noted here with *B. stearothermophilus* TS-13 cells. Wild type *E. coli* and *Micrococcus lysodeikticus* cells in fact adjust the physical state of their membrane lipids so that they exist entirely in the liquid-crystalline state at the temperature of growth [7, 8, 28].

The marked differences noted between the membrane lipid phase transition detected by differential thermal analysis in the present study and the lateral phase

separations measured with a fatty acid spin label probe underlines the need for caution in the interpretation of phase transition data gathered by secondary techniques such as ESR spectroscopy. A substantial amount of evidence exists that lipid spin probes can perturb and "fluidize" local environments in various lipid-water model systems [31]. Nitroxide derivatives of stearic acid and cholesterol occupy a much greater area per molecule when spread as monolayers at the air-water interface than do stearic acid or cholesterol under similar conditions [32]. Thus 5- and 12-nitroxide stearic acids in phospholipid monomolecular films under membrane-like conditions should read too high a fluidity [33]. This prediction is borne out by the recent experimental demonstration that various spin probes tumble freely in lipid multilayers at temperatures where the bulk lipid is solid [34]. The fact that the nitroxide group imports additional polarity as well as bulk to fatty acid molecules may also limit the usefulness of these spin label probes, particularly in studies on biological membranes. The ability of 5- and 12-nitroxide stearic acid to adopt bent configurations in lipid monomolecular films, with the nitroxide as well as the carboxyl group being located in the aqueous phase, could produce misleading results [33, 35]. Finally, the tendency of fatty acid spin probes to become localized in non-representative local domains introduces still another complicating factor which must be considered in interpreting ESR studies of model and natural membranes [29, 35].

Esser and Souza [16] have recently attempted to reconcile the ESR and differential thermal analysis data on *B. stearothermophilus* spheroplast membranes and membrane lipid-water dispersions by suggesting that the fatty acid spin probe may be reporting a "demixing" of different lipid classes within the same phase and not the conversion of a solid to a liquid phase. Evidence for fluid-fluid immiscibility in a binary mixture of dipalmitoylphosphatidylethanolamine and dielaidoylphosphatidylcholine in specified temperature and composition ranges has in fact recently been reported [36]. However, since only one of a number of binary mixtures exhibited this behavior and since phospholipids in biological membranes have very different distributions of fatty acids from the synthetic phospholipids utilized in that study, the biological relevance of this finding is unclear. Although a similar phenomenon could well occur in *B. stearothermophilus* membranes, one would have to make the additional postulate that this immiscibility is conferred upon the lipid phase by the membrane proteins, since such a fluid-fluid phase separation is not detected in membrane lipid-water dispersions. It is also possible that the fatty acid spin probe is preferentially localized in the boundary or halo lipid region [37, 38] and is reporting changes in lipid-protein interactions. Alternatively, the apparent phase separations noted in the intact membranes could be due to the anomalous behavior of the probe molecule and may not reflect the behavior of the native membrane lipid. Until the nature of the phenomenon being reported by the fatty acid spin probe is clarified, interpretation of this data remains difficult. The results of the present study again emphasize the need to interpret ESR experiments on biological membranes with caution and underlines the need to apply ESR in conjunction with other physical methods, even if the spin probe appears to behave in the expected manner in lipid-water model systems.

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