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Correlation between Thermal Death and Membrane Fluidity in *Bacillus stearothermophilus*

(thermophiles/spin labels/lateral lipid phase separation/membrane biogenesis)

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ABSTRACT **Paramagnetic resonance spectra of spin labels partitioned into spheroplast membranes of *Bacillus stearothermophilus* indicate lateral lipid phase separations. Cells adjust their lipid composition in response to temperature changes so that the same change of state in membrane phospholipids is achieved at the respective growth temperature. A temperature-sensitive mutant that fails to change its lipid composition above a certain temperature can survive only up to the higher temperature boundary for lateral phase separation. These data are interpreted to indicate that the maximal and minimal growth temperatures of thermophiles are regulated by the onset and conclusion of phase separations of the particular lipid composition they synthesize. It is suggested that isolated lipid domains are required for functional membrane assembly.**

Prokaryotic microorganisms have the remarkable ability to adapt to temperature extremes that are usually considered lethal for other forms of life. This so-called biokinetic temperature zone for microorganisms extends from −10° to nearly 100° (1, 2). The capacity of thermophilic organisms to grow at very high temperatures resides in their ability to synthesize cell components that have a greater heat stability than the corresponding components synthesized by mesophiles and psychrophiles. It is well documented (for review, see ref. 3) that proteins from thermophiles are not readily heat-denatured. Likewise, T_m values, which characterize the melting behavior of DNA and RNA, are higher for thermophilic strains of a given genus (2, 4). However, these observations do not give an indication about the mechanisms that lead to the creation of thermostable macromolecules. In addition, there is no ready explanation for the observed differences in *maximal* growth temperature (T_{max}), *optimal* growth temperature (T_{opt}), and *minimal* growth temperature (T_{min}) among different species.

The idea that the maximal growth temperature any organism can achieve is determined by its membrane stability has also been entertained by many investigators. In early work, Heilbrunn (5) and Bělehrádek (6) postulated that organisms adapt to changes in temperature by altering their plasma lipid composition and that heat resistance was, therefore, related to the melting temperature of the lipids. At the time of their work, the presence of lipids in membranes was not yet known. More recently, Johnston and Roots (7) argued that cells must maintain lipids near the critical point of phase transition in order to achieve an appropriate degree of expansibility and solid–liquid ratio. They argued further that varying the degree of fatty acid unsaturation could provide an effective way to preserve this particular state. Only in the past few years were these ideas incorporated into proposals for membrane structure in general (for review, see ref. 8).

The importance of lipid phase transitions to the thermal stability of thermophilic microorganisms has been supported by the work of De Siervo (9) and Daron (10), who found an inverse relationship between the degree of unsaturation and growth temperature. Babel *et al.* (2), however, interpreted these data differently and dismissed lipids as likely candidates which could confer thermostability. In order to investigate this apparent controversy we studied in more detail the "melting" behavior *in vivo* of lipids in membranes from a thermophilic bacillus and a temperature-sensitive mutant. To determine these phase changes we chose the spin label technique, which has been proven to be a powerful tool for such studies in microorgansims (11–13). Our results indicate a close relationship between growth temperature and lipid phase changes *in vivo*.

MATERIALS AND METHODS

Bacterial Strains. Bacillus stearothermophilus, strain YTG-2, and a temperature-sensitive mutant (TS-13) derived from YTG-2 were used throughout this work. Taxonomic characteristics, growth conditions, isolation of the mutant, and lipid analysis are given elsewhere (14). Spheroplast membranes were prepared following a procedure of Wisdom and Welker (15).

Spin Labels. Spheroplasts were spin-labeled with either 4′,4′-dimethyloxazolidine-*N*-oxyl derivative of 5-keto stearate (5-doxyl stearate; Syva, Palo Alto, Calif.) or 2,2,6,6-tetramethylpiperidine-1-oxy (TEMPO), and the electron paramagnetic resonance (EPR) spectra were recorded and averaged as described (16). The total lipid-to-spin-label ratio in the membranes was kept above 200.

RESULTS

EPR spectra for 5-doxyl stearate incorporated into spheroplast membranes of *B. stearothermophilus* and recorded at different temperatures are presented in Fig. 1. The spectra at low temperatures are characteristic of an immobilized fatty acid spin label in a viscous environment (17). At higher

Abbreviations: 5-doxyl stearate, 4′,4′-dimethyloxazolidine-*N*-oxyl derivative of 5-keto stearate; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; EPR, electron paramagnetic resonance.

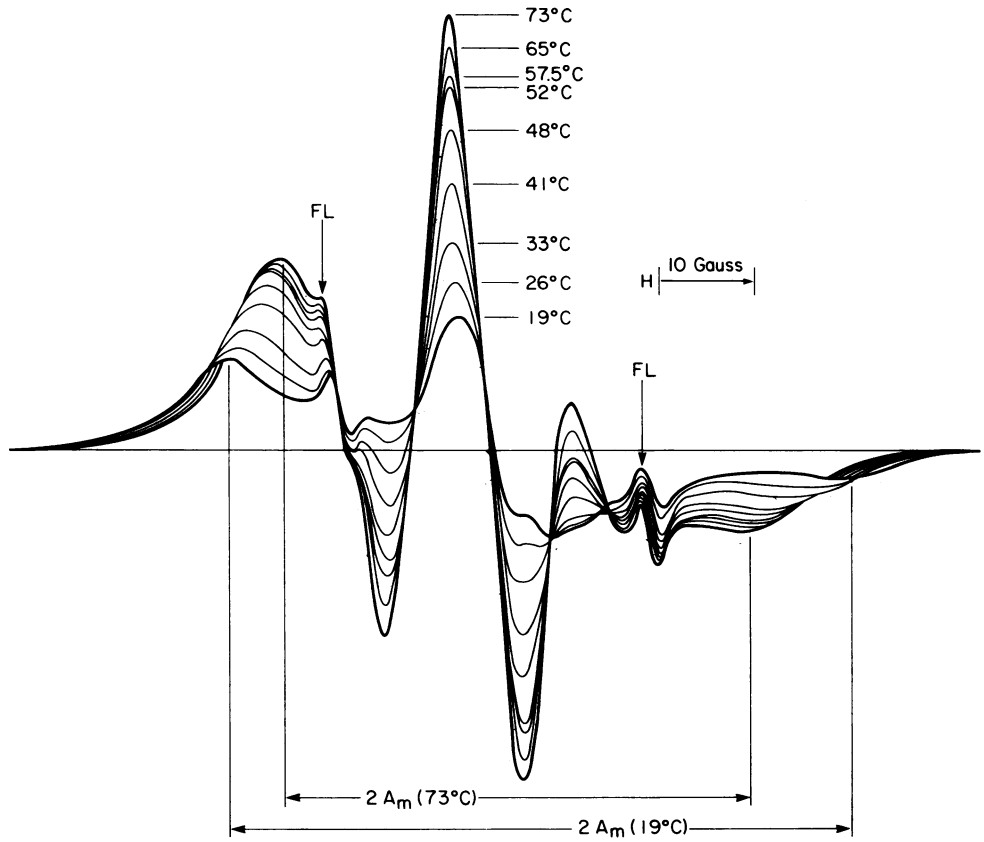

FIG. 1. EPR spectra of 5-doxyl stearate partitioned into spheroplasts of *B. stearothermophilus* YTG-2 grown at 58°. The spectra were recorded at the indicated temperatures, with the Varian variable-temperature-accessory and a small flat cell for liquid samples. Microwave power: 5 mW; modulation amplitude: 0.9 gauss; average of 16 scans.

temperatures, especially above the growth temperature, the spectra indicate a less restricted spin label. Similar spectra, but characteristic of an increase in mobility of the spin label group, can be obtained with 12-doxyl and 16-doxyl stearate (data not shown), indicating that the spheroplast membrane is a typical lipid bilayer membrane (17). The small peaks labeled FL are due to a more mobile spin label and correspond to about 2% of the total label. The failure of ascorbate to reduce these peaks indicates that these spin label molecules are not free in the outer medium but are most probably in the membrane, or, less likely, in the interior of the spheroplast.

The main spectra probably represent only one type of spin label population. Sometimes it is difficult to determine whether or not a spectrum is a composite one, i.e., one that is due to two different populations. Griffith *et al.* (18) have recently shown that, in spin-labeled cytochrome oxidase vesicles, the stearate label samples two different lipid regions. The presence of at least three well-defined isosbestic points in Fig. 1, however, is taken as evidence that the majority of the label (98%) is present in a single lipid environment only. If two populations were present, one would expect (*a*) that the partition coefficient for the label in two environments would change with temperature and (*b*) that the spectral response to changes in temperature would be different for the label in either environment. Both effects combined should not allow the appearance of isosbestic points.

The distance ($2A_m$) between the two outer hyperfine maxima is sensitive to anisotropic rotation of the spin-carrying nitroxyl group. It can be used to assess the freedom of motion of this group and thus characterize the rigidity of the lipid environment. Plots for the temperature dependence of this spectral parameter are shown in Fig. 2. At each of the growth temperatures tested, characteristic discontinuities are apparent. For the wild-type organism, the second break (i.e., the one at the higher temperature) corresponds very closely to the growth temperature. It is important to note that the same $2A_m$ values (51 ± 1 gauss), indicating conservation of spin label mobility, are always recorded at the different growth temperatures.

Such breaks in temperature dependence for spin label parameters have been successfully correlated with either lipid *transitions* (8, 12, 17) or with lipid phase *separations* (19). The latter results were obtained with the partitioning properties of the spin label TEMPO. TEMPO is a water- and lipid-soluble molecule. When membranes undergo a transition from a gel to a more solid state, TEMPO is excluded from the hydrophobic environment and appears in the aqueous phase. The spectral difference exhibited by TEMPO in the two phases can be used to calculate a partitioning parameter *f* (for further procedural details, see ref. 19). We attempted to measure phase separations in *B. stearothermophilus* membranes using this label, but this proved to be very difficult. The spheroplasts reduced the label very effectively, especially near or at the growth temperature, so that only two or three temperatures could be measured on a single sample before the signal disappeared. These spheroplasts are very stable against physical stress (15). They did not lose their spherical shape and structural integrity, and considerable

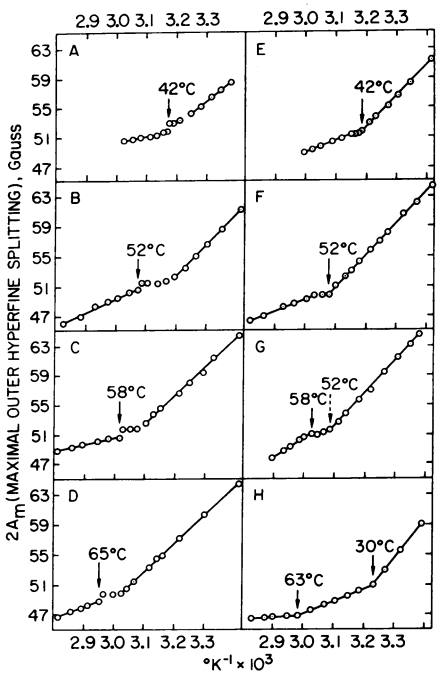

FIG. 2. Temperature dependence of spin label parameter $2A_m$ obtained from spectra similar to Fig. 1. (A, B, C, and D) Spheroplasts from wild-type cells grown at 42°, 52°, 58°, and 65°, respectively; (E, F, and G) spheroplasts from mutant cells grown at 42°, 52°, and 58°, respectively; (H) lipid vesicles prepared from total lipids extracted from wild-type cells grown at 65°. Instrumental parameters as in Fig. 1.

activity for TEMPO reduction was still present even after six freeze-thaw cycles and after several washings with ferricyanide. Nevertheless, by using multiple samples we were able to perform one set of experiments on spheroplasts prepared from cells grown at 58°. As can be seen in Fig. 3, a break in the data at the growth temperature is clearly indicated despite the large error limit caused by the reduction of the label. This transition and the one at lower temperatures correspond reasonably well with the ones observed for 5-doxyl stearate (see Fig. 2). Because the reduction problem was less severe with the stearate label, we did not continue the use of TEMPO on cells grown at other temperatures.

Our studies were then extended to a temperature-sensitive mutant, TS-13, derived from the wild-type strain. This mutant is unable to grow and will lyse at temperatures above 58°, while the parent strain grows optimally at 65° and maximally at 72°. Earlier studies (14) indicated that the defect in the mutant may involve lipid metabolism. Results for the mutant are also shown in Fig. 2. Again, two transitions were detected; however, only for cells grown at 58° did the growth temperature correspond to the point of the higher temperature change in slope. For cells grown at 42° and 52°, the growth temperature is identical with the lower temperature transition. These differences in results between wild-type and mutant spheroplasts may reflect differences in their lipid composition, as will be discussed later.

Since the pioneering studies of Steim *et al.* (20), it has been known that lipids, when extracted from membranes, exhibit the same phase changes *in vitro*. Thus, we extracted total lipids from *B. stearothermophilus* cells grown at different temperatures and redispersed them in buffer to form vesicles.

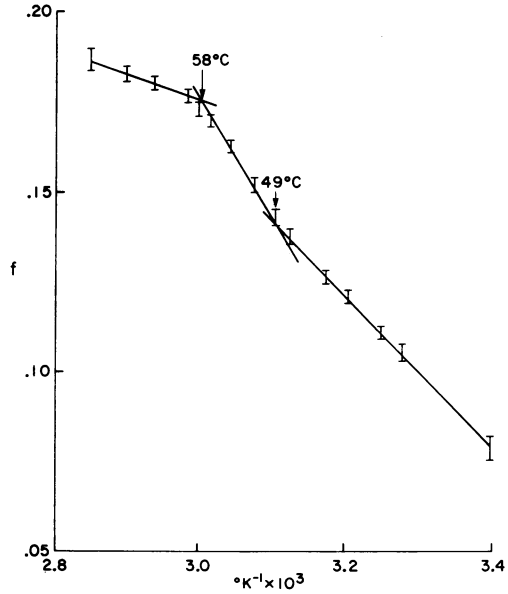

FIG. 3. Temperature dependence of TEMPO parameter *f*, measured on spheroplasts from wild-type cells grown at 58°. The TEMPO parameter *f* was measured and calculated as given by Shimshick and McConnell (19).

These preparations of multilamellar lipid vesicles show transitions similar to the ones observed with spheroplasts. An example is given in Fig. 2H. As was noticed in many earlier studies, the transitions in these lipid dispersions are always at lower temperatures than the corresponding ones in membrane preparations. This shift is due most probably to the influence proteins exert on lipids *in vivo*.

DISCUSSION

Many earlier investigations had amply demonstrated that the lipid composition in plasma membranes of microorganisms is strongly influenced by the growth temperature (for review, see ref. 21). But there seemed to be less agreement on the necessity and the reason for such changes. Our spin label data show that the $2A_m$ values and, thus, the dynamics of the lipid phase are unchanged at the different growth temperatures. These data indicate strongly that the preservation of the physical state of the lipids in the plasma membrane of *B. stearothermophilus* is one of the reasons for temperature-induced changes in lipid composition. Earlier spin label studies on thermophilic bacteria by Chan *et al.* (22) were interpreted to indicate that the membrane of cells grown at higher temperatures is more rigid than the one from cells grown at lower temperatures. In our hands this is only true if the comparison is made at temperatures below the growth temperature; there are no differences detectable *at* the growth temperature. A more detailed comparison of our data with those of Chan *et al.* (22), however, is not warranted since these authors used lyophilized cells while we studied intact spheroplasts.

In pure, one-component systems, transitions such as those seen in Fig. 2 can best be explained as phase transitions. However, in multicomponent systems it is more likely that the observed breaks indicate phase separations, as has been discussed by Shimshick and McConnell (19). Following their lead, we likewise interpret the data obtained with TEMPO

(Fig. 3) as phase separations whereby the lower transition signals the onset and the higher one the conclusion of the separation. Since the transitions observed with TEMPO correspond reasonably well to those observed with 5-doxyl stearate, we conclude that the latter label reports the same phase separations.

Thus, it seems that the plasma membrane of *B. stearothermophilus* is another system that requires lateral lipid phase separations for function. Studies by Linden *et al.* (13) had shown a similar requirement for transport functions in *Escherichia coli* membranes. Freeze-fracture electron microscopy combined with spin label studies by James and Branton (23) also indicated the presence of separate phases in *Acholeplasma laidlawii* cells.

Equivalent phase separations can be observed in the mutant membrane. We have no explanation at the moment for the finding that in the mutant the two lower growth temperatures correspond to the onset of the phase separation. Usually one observes that the growth temperature is closer to the end of the heterogeneous phase (13), as was found for the wild-type strain. However, too few organisms have been tested to permit a general conclusion. In addition it should be remembered that there are marked differences in lipid composition between the wild-type and the mutant when grown at 42°, 52°, and 58°.

Two main questions can now be asked with respect to thermophilism. (*1*) What advantage to the cell is a mechanism to alter the composition of its lipids in response to temperature? (*2*) Is there a relationship between lipid phase separations and T_{max}? The answers to both questions are obviously not independent. One can rephrase question *1* somewhat and ask, why do *B. stearothermophilus* cells grow only "within the phase transition"? Overath *et al.* (24) have suggested that there exists an upper limit to lipid phase fluidity that is compatible with growth. This is reminiscent of the older theories of Heilbrunn (5) and Bělehrádek (6), which state that the maximal growth temperature is limited by the melting points of the lipids. Babel *et al.* (2), however, pointed out that an exact relationship between T_{max} and melting points could not be demonstrated. We propose that the temperature limit for thermophilic growth may be determined by the boundary conditions of the phase diagram for the total lipid mixture in the membrane. The latter conditions are obviously dependent on, but not equivalent to, the melting points of the individual lipids.

The results we have obtained with the mutant support our proposal. We reported previously (14) that in wild-type cells, a shift in growth temperature from 42° to 65° results in an increase from 42% to 69% of fatty acids with melting points above 55°. In the mutant the ability to make such changes is severely limited above 52°; Fig. 4 summarizes these results. While the wild-type strain is able to make the drastic adjustments in fatty acid composition that are necessary to stay within the boundaries for phase separations, such as decreasing the concentration of low-melting-point, branched fatty acids and increasing the high-melting-point, straight-chain composition, the mutant must attempt to survive with a more or less stagnant fatty acid composition. The boundary temperatures are 52° and 58° for the mutant grown at 52° (Fig. 2F); thus, if our proposal is correct, then T_{max} should be at 58°. As was published earlier (see Fig. 2 of ref. 14), the growth limit for the mutant is indeed 58°.

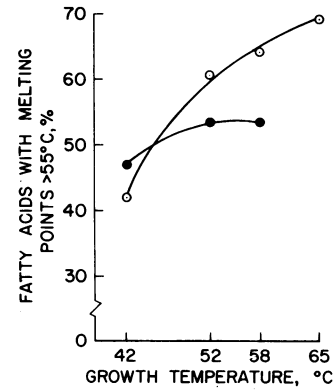

FIG. 4. Alteration in the percentage of fatty acids with melting points greater than 55° as a function of growth temperature in wild-type (○) and mutant (●) cells.

The detailed studies by Overath *et al.* (24) and Fox (13, 25) and their coworkers have documented the fact that membrane assembly in *E. coli* does not proceed correctly when the lipids are in an "all frozen" state. This state is present below the temperature at which the phase separation begins. At the conclusion of the phase separation the lipids are in an "all fluid" state, and Overath *et al.* (24) have postulated that this is a necessary requirement for successful insertion of proteins and lipids into membranes. The fluid state assures a fast and complete randomization of lipids in the membrane. However, it is known that the function of several enzymes is dependent upon the presence of specific lipids (for review, see refs. 21 and 26). The presence of distinct, *physically* different lipid halos around enzymes was convincingly demonstrated for cytochrome oxidase (18) and cytochrome *P*-450 (27). Whether these boundary lipids consist of *chemically* different lipids or whether all lipids present in the membrane participate in the halo formation, of course, remains to be elucidated. Mechanistically, a newly synthesized, lipid-requiring enzyme either (*a*) may be inserted directly into portions of the membrane having a specific lipid domain, or (*b*) may form a complex with specific lipids prior to being inserted into the membrane (25, 26). Case *a* would require the presence of isolated lipid domains, a condition that is possible within the temperature boundaries for phase separation. In thermophilic organisms, the lowest and highest boundary temperature of any possible lipid mixture synthesized by the organism may determine T_{min} and T_{max}.

We wish to emphasize that we do not consider our interpretation of membrane assembly, i.e., case *a*, to be generally applicable to all bacterial membranes. Esser and Lanyi (16) have presented evidence for the presence of only bound, nonfluid lipids in the membrane of *Halobacterium cutirubrum*. The assembly process in that bacterium most likely will be different from that proposed for *E. coli* (24, 25) and *B. stearothermophilus*.

While our hypothesis can predict successfully correlations between T_{min} and T_{max} and temperature boundaries for lipid phase separations, we cannot predict T_{opt} from lipid phase studies. We stated earlier that thermophilic organisms must be able to synthesize thermostable macromolecules such as proteins and nucleic acids in order to survive higher temperatures. The synthesis of these molecules might very well determine T_{opt}. Recent preliminary data by Kruyssen and MacElroy (personal communication) indicate that the fidelity

of the protein-synthesizing machinery in thermophilic bacteria is less accurate on either side of T_{opt}.

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