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RELATIONSHIP OF GROWTH TEMPERATURE AND THERMOTROPIC LIPID PHASE CHANGES IN CYTOPLASMIC AND OUTER MEMBRANES FROM *ESCHERICHIA COLI* K12

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Summary

Purified cytoplasmic and outer membranes isolated from cells of wild type *Escherichia coli* grown at 12, 20, 37 and 43°C were labelled with the fatty acid spin probe 5-doxyl stearate. Electron spin resonance spectroscopy revealed broad thermotropic phase changes. The inherent viscosity of both membranes was found to increase as a function of elevated growth temperature. The lipid order to disorder transition in the outer membrane but not the cytoplasmic membrane was dramatically affected by the temperature of growth. As a result, the cytoplasmic membrane presumably existed in a gel + liquid crystalline state during cellular growth at 12 and 20°C, but in a liquid crystalline state when cells were grown at 37 and 43°C. In contrast, the outer membrane apparently existed in a gel + liquid crystalline state at all incubation temperatures. Data presented here indicate that the temperature range over which the cell can maintain the outer membrane phospholipids in a mixed (presumedly gel + liquid crystalline) state correlates with the temperature range over which growth occurs.

Introduction

The cell envelope of Gram-negative bacteria is a complex multilayered structure comprised of two membranes with a peptidoglycan matrix sandwiched in between [1]. The cytoplasmic membrane has been shown to be a typical bilayer

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; 5-DS, 5-doxyl stearate.

containing protein and phospholipid [2]. The outer membrane, which lies distal to the peptidoglycan matrix, contains, in addition, substantial amounts of lipopolysaccharide [3]. The relative fluidity of both of these membranes has been described [4—7]. Moreover, the effect of different growth temperatures on the fluidity and composition of the unseparated envelope has been studied [8,9]. Surprisingly, however, the extent to which the fluid properties of each membrane are altered with changes in growth temperature has not been fully investigated. Furthermore, the lipid state of each membrane at the growth temperature is not known. These parameters may be critical with respect to the cell's ability to survive environmental stress. It has been suggested that the freezing out of membrane lipids may define the lower temperature limit for cell growth [10].

In this study, purified cytoplasmic and outer membranes isolated from cells of wild type *Escherichia coli* K12 grown at various temperatures were labelled with the fatty acid spin probe 5-doxyl stearate. Electron spin resonance (ESR) spectroscopy revealed broad thermotropic lipid phase changes. The temperature of the phase change in the outer but not the cytoplasmic membrane was shifted dramatically with growth temperature. Data presented here indicate that the temperature range over which the cell can maintain the outer membrane phospholipids in a mixed (presumably gel + liquid crystalline) state correlates with the temperature range over which growth occurs.

Materials and Methods

Growth of cells. E. coli strain W1485F$^-$ was grown for 15—20 generations at 12, 20, 37 and 43°C in M9 minimal medium supplemented with 0.4% glucose (final concentration). The absorbance of the cultures was monitored at 560 nm using a Beckman DB spectrophotometer. Growth was stopped late in logarithmic phase by the addition of chloramphenicol (20 μg/ml, final concentration).

Preparation of membranes. Cells were washed twice in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.5, and then lysed with a French pressure cell. Membranes were isolated according to the procedure of Schnaitman [11] except that the discontinuous sucrose gradient contained 12 ml of 0.77 M, 16.8 ml of 1.44 M, and 4.8 ml of 2.12 M sucrose in 10 mM Hepes. The gradient was centrifuged at 25 000 rev./min in a Spinco SW27 rotor for 18 h. Following fractionation the outer and cytoplasmic membrane bands were washed twice in 10 mM Hepes, pH 7.5.

Assays. Succinate dehydrogenase was determined by the method of Osborn et al. [12]. Ketodeoxyoctanoic acid was assayed using the method of Dröge et al. [13]. Protein concentrations were determined using the procedure of Lowry et al. [14]. Membrane proteins were characterized on sodium dodecyl sulfate (SDS)-polyacrylamide slab gels [15]. Prior to dry weight determination, membrane isolates were washed twice in 0.1 mM Hepes, pH 7.5.

Spin labelling. The fatty acid spin probe 5-doxyl stearate (5-DS, Synvar Corp., Palo Alto, CA) was dissolved at a 15 mM solution in absolute ethanol. Depending on protein concentration, between 3 and 10-μl aliquots of this stock solution were added to between 0.4 ml and 0.7 ml of fresh, refrigerated mem-

branes in 10 mM Hepes. The mixture was then placed in a bath sonicator (125 watts) for 10 min at room temperature. The concentration of spin label approximated 0.1% of the lipid weight of the membranes. All electron spin resonance (ESR) experiments were carried out with a Varian Century Line ESR spectrometer, model E-112, equipped with a variable temperature controller. An external calibrated thermistor probe (Omega Engineering, Inc., Stamford, CT) was used to monitor the temperature of the sample. Control experiments (not shown) indicated that the ethanol added during labelling did not alter the ESR spectra.

The order parameter (S) was calculated as described elsewhere [16]. The experimental curves (hyperfine splitting parameter vs. temperature and S vs. temperature) were analyzed in terms of linear components by fitting regression lines to appropriate sections using the method of least squares. This method allows the determination of break points [17,18]. For each growth temperature membranes were isolated 2—4 times, and each isolate was fully characterized.

Results

Membrane isolation

The degree of purity of cytoplasmic and outer membrane isolates derived from cells grown at 12, 20, 37 and 43°C is shown in Table I. Succinate dehydrogenase activity was between seven and 20 times greater in cytoplasmic membrane fractions while ketodeoxyoctanoic acid, a component of lipopolysaccharide, was between five and eight times more concentrated in outer membrane fractions. The percentage of protein in either membrane fraction was not appreciably influenced by the growth temperature. Outer membrane protein electrophoretic patterns on SDS-polyacrylamide gels indicated that outer membrane protein 1a (Schnaitman's nomenclature) decreased and protein 3b increased with increasing temperatures (data not shown) as has been previously reported [19,20]. Growth temperature also affected the concentrations of several cytoplasmic membrane proteins (not shown).

Membrane spin labelling

Typical ESR spectra of 5-DS-labelled cytoplasmic membranes are shown in

TABLE I

BIOCHEMICAL CHARACTERIZATION OF ISOLATED CYTOPLASMIC AND OUTER MEMBRANE FRACTIONS

	Cytoplasmic membrane				Outer membrane			
Growth temperature (°C):	12	20	37	43	12	20	37	43
mg protein/mg membrane	0.49	0.57	0.49	0.52	0.52	0.50	0.49	0.52
μM ketodeoxyoctanoic acid/mg membrane	0.03	0.03	0.02	0.03	0.17	0.14	0.16	0.14
Relative succinate dehydrogenase activity/mg membrane	1.8	3.2	4.9	3.6	0.12	0.16	0.32	0.50

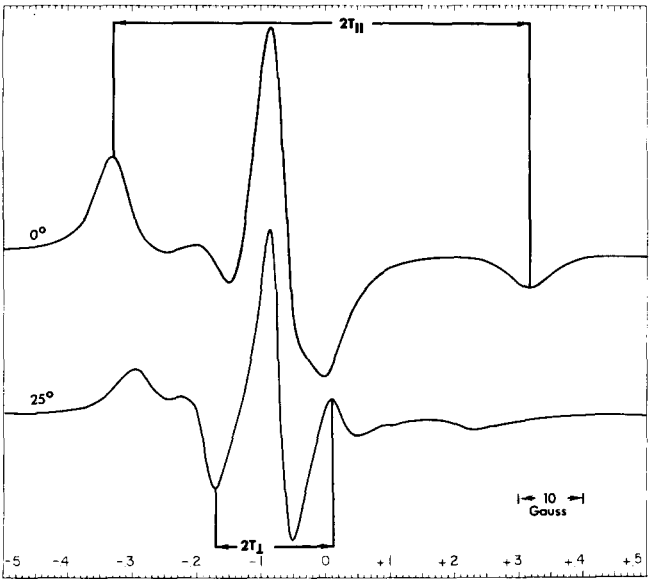
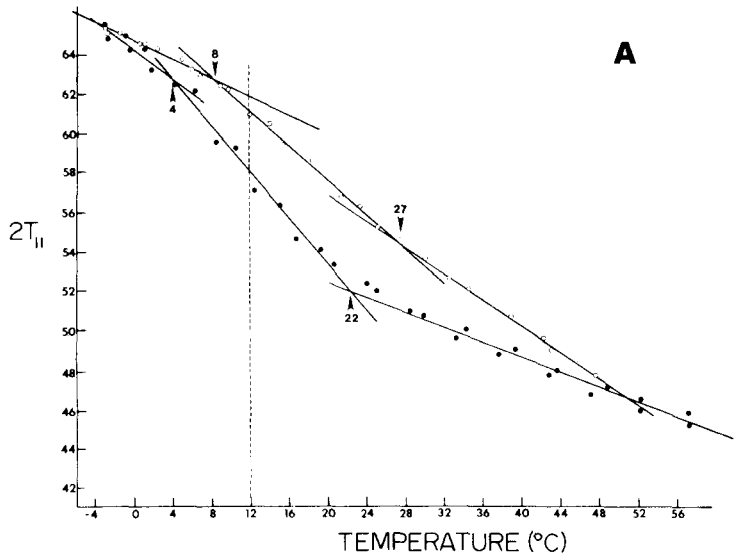


Fig. 1. Electron paramagnetic resonance spectra of *E. coli* cytoplasmic membranes isolated from cells grown at 37° C and labelled with 5-doxyl stearate. The spectra were taken at the temperatures indicated. Scan range was 100 gauss.

Fig. 1. Little or no unbound probe was present. The single symmetric low field peak was taken as evidence that the majority of label was present in a single lipid environment. The low field peak remained symmetric over the temperature interval examined. Similar data were obtained with 5-DS-labelled outer membrane samples. The hyperfine splitting parameter, $2T_{\parallel}$ (Fig. 1), is related to the rotational mobility of the spin label and therefore reports the local fluidity of the membrane lipids [21]. High values of $2T_{\parallel}$ reflect low fluidity.

The temperature dependence of $2T_{\parallel}$ in cytoplasmic and outer membranes isolated from cells of *E. coli* grown at the temperatures indicated is illustrated in Fig. 2. Cytoplasmic membranes exhibited lower $2T_{\parallel}$ values at midrange temperatures than did outer membrane isolates. This suggests a greater overall fluidity in the cytoplasmic membrane fraction. In all fractions $2T_{\parallel}$ decreased with increasing temperatures and discontinuities in slope were inferred (Fig. 2). Such breaks in the temperature dependence of spin label parameters have been correlated with lipid phase separations or lipid phase transitions from gel → gel + liquid crystalline, and from gel + liquid crystalline → liquid crystalline lipid states [17,18,22–27]. The data presented in Fig. 2 suggest that at all growth temperatures the outer membrane phospholipids existed in a mixed (presumably gel + liquid crystalline) lipid state. Cytoplasmic membranes, however, appeared to exist in a liquid crystalline state at the growth temperature when derived from cells grown at 37 and 43° C, but in a mixed lipid state at the growth temperature when derived from cells grown at 20 and 12° C.

From the electron spin resonance spectra which were recorded at temperatures higher than about 12° C the order parameter, S, could also be determined. The order parameter, which is dependent on $2T_{\perp}$ (Fig. 1) as well as $2T_{\parallel}$, mea-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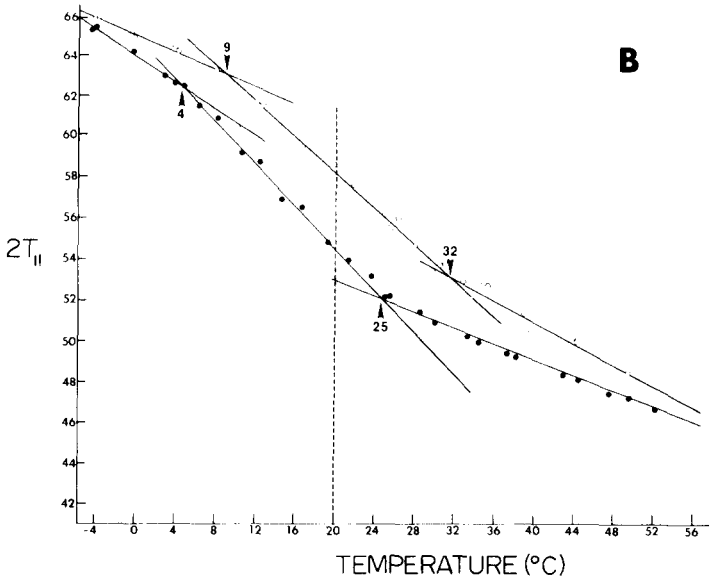
TEMPERATURE (°C)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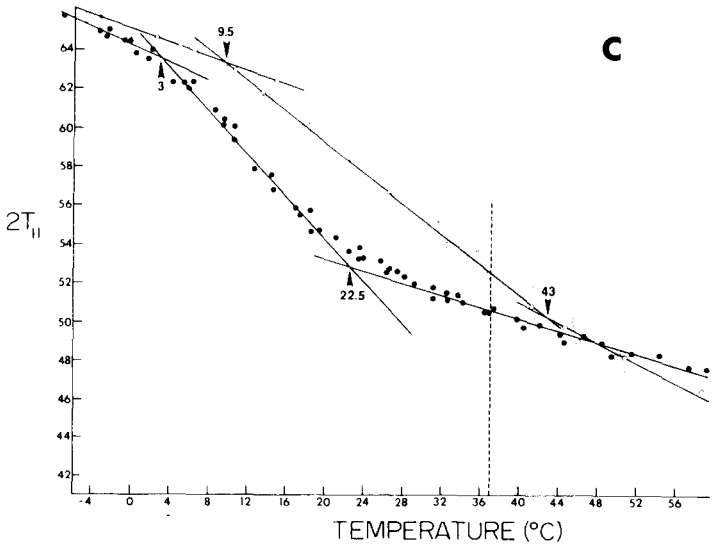
C
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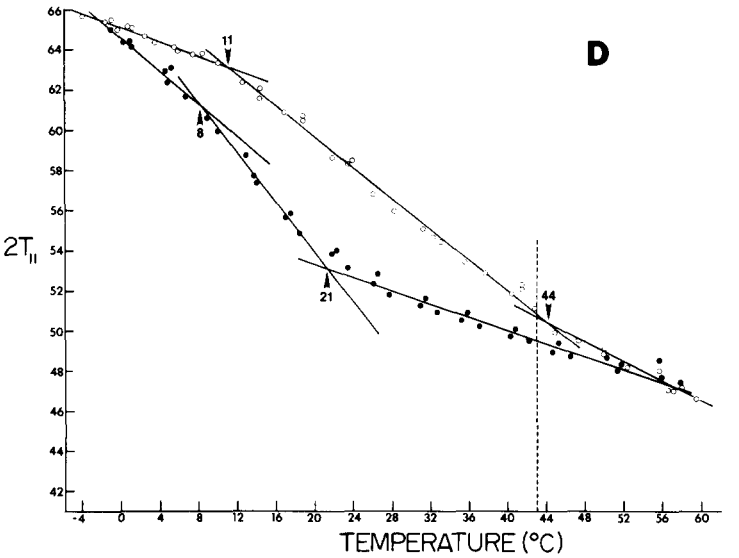

Fig. 2. Hyperfine splitting parameter, $2T_{\parallel}$ (gauss), as a function of temperature in *E. coli* outer (○) and cytoplasmic (●) membranes labelled with 5-doxyl stearate. Membranes were isolated from cells grown at (A) 12°; (B) 20°; (C) 37° and (D) 43°C. The vertical broken line indicates the growth temperature. The arrows indicate phase changes.

sures the deviation of the observed ESR signal from the case of a completely uniform orientation of the probe. For a uniformly oriented sample, $S = 1$; for a random sample, $S = 0$.

When the order parameter was plotted as a function of sample temperature, changes in slope occurred corresponding to presumed lipid phase change temperatures. These break points agreed quite well with those determined by the hyperfine splitting parameter. Fig. 3 shows, for example, the temperature dependence of the order parameter in cytoplasmic membranes isolated from cells grown at 37°C.

Table II summarizes the lower (T_L) and upper (T_U) transition temperatures

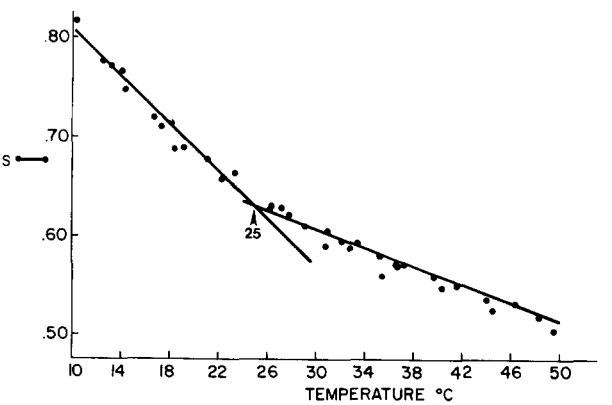

Fig. 3. The temperature dependence of the order parameter (S) in *E. coli* cytoplasmic membranes labelled with 5-doxyl stearate. Cells were grown at 37°C. The arrow indicates the phase change.

TABLE II

LIPID PHASE TRANSITION TEMPERATURES (°C) OF ISOLATED CYTOPLASMIC AND OUTER MEMBRANE FRACTIONS AS DETERMINED BY THE HYPERFINE SPLITTING PARAMETER AND THE ORDER PARAMETER

T_L, lower transition; T_U, upper transition; T_M, transition midpoint.

	Cytoplasmic membrane				Outer membrane			
Growth temperature (°C):	12	20	37	43	12	20	37	43
T_L	4.0	4.3	3.0	7.8	8.3	8.8	9.5	10.7
T_U	24.5	25.4	23.7	21.2	28.0	31.8	43.0	44.5
T_M	14.2	14.8	13.4	14.5	18.2	20.3	26.3	27.6

TABLE III

ORDER PARAMETER, S, AS A FUNCTION OF GROWTH TEMPERATURE IN CYTOPLASMIC AND OUTER MEMBRANE ISOLATES

	Cytoplasmic membrane				Outer membrane			
Growth temperature (°C):	12	20	37	43	12	20	37	43
Cuvette temperature (°C)								
12	0.76	0.79	0.79	0.80	0.87	0.89	0.89	0.89
20	0.67	0.69	0.69	0.67	0.77	0.79	0.81	0.82
37	0.54	0.55	0.57	0.58	0.59	0.61	0.63	0.65
43	0.50	0.51	0.55	0.55	0.54	0.56	0.56	0.59

and transition midpoint temperatures (T_M) found in cytoplasmic and outer membrane isolates. The lower transition temperatures were determined using the hyperfine splitting parameter. The upper transition temperatures represent an average of values determined using the hyperfine splitting parameter and the order parameter. These data show that T_U in outer membrane isolates changed markedly such that T_U was always above the cell growth temperature. Indeed the T_M values found in outer membranes increased with increasing incubation temperatures. In contrast, T_U and T_M in cytoplasmic membrane isolates did not change appreciably in response to changes in the growth temperature. Data presented in Table III indicate, however, that both cytoplasmic and outer membrane isolates exhibited increased order when isolated from cells grown at increasing incubation temperatures.

Discussion

The evidence presented here indicates that of the two membrane components in the intact cellular envelope of *E. coli*, the outer membrane represents an area of greater order and microviscosity at physiological temperatures. In addition, the inherent order of both cytoplasmic and outer membrane fractions was found to increase as a function of elevated growth temperatures. These findings are consistent with those of Lugtenberg and Peters [28] who have shown that the outer membrane is enriched in saturated fatty acids, and the saturated fatty acid content of both membranes increases with increasing

growth temperatures. Saturated fatty acids are known to increase membrane lipid order [29,30]. Moreover, while doxyl stearate spin probes have been shown to situate only in phospholipid domains in the outer membrane [5,6] their mobility is reportedly significantly reduced by the presence of lipopolysaccharide in this membrane fraction [31].

In multicomponent systems, such as biological membranes, it is more likely that inflections in the temperature dependence of ESR spectral parameters reflect phase separations rather than phase transitions [18]. It is possible, however, that spin probes could report changes in miscibilities rather than solid to fluid transitions or separations. Nevertheless at temperatures between the upper and lower inflections, lipids can be assumed to exist in two phases (a mixed state) either because of gel and liquid crystalline separation or partial lipid immiscibilities.

In these studies, over a wide range of growth temperatures (12—43°C), the phospholipids in isolated outer membranes were found to exist within their broad phase change in a mixed (presumably gel + liquid crystalline) state, at the temperature of growth. This occurred because T_U in this membrane fraction changed dramatically so that it always existed above the temperature of growth. In contrast, T_U in the cytoplasmic membrane did not change appreciably with growth temperature. As a consequence, the lipids in this inner membrane apparently existed in a mixed state during growth at low temperatures, but in a disordered state during growth at elevated temperatures.

The temperatures of the phase changes which we observe in isolated outer membranes are similar to those reported by Emmerling et al. [32]. Using fluorescence techniques, they reported phase transition midpoints at between 25 and 29°C in outer membrane-murein complexes isolated from cells grown at 30°C. Further, Melchior and Steim [10] reported that endothermic transitions attributed to the outer membrane occurred at higher temperatures than those attributed to the cytoplasmic membrane. In contrast, Overath and coworkers [7] have reported that the two membranes from an unsaturated fatty acid auxotrophic mutant of *E. coli* have similar transition temperatures. The discrepancy between their data and that presented here may be due to differences in their experimental procedure: cells aberrant in lipid synthesis were cultured in the presence of fatty acids and a non-ionic detergent. During the membrane purification the murein was degraded and the membranes were treated with ethylenediaminetetraacetic acid, sonicated and frozen. Such manipulations could have altered or rearranged membrane components.

Esser and Souza [33] and McElhaney [34] have proposed that the existence of two lipid phases in bacterial membranes at the growth temperature may be necessary for survival and division. We suggest that in *E. coli* the cell must keep the outer but not the cytoplasmic membrane in a mixed lipid state to be able to grow.

Fig. 4 shows that as the growth temperature was raised, the difference between T_U in the outer membrane and the temperature of growth diminished in a linear fashion. Thus, at a hypothetical growth temperature of 47°C, T_U would be expected to occur at 47°C and the membrane would exist essentially in a liquid crystalline lipid state. Similarly, it can be predicted that at a growth temperature of 8°C, T_L in the outer membrane would occur at 8°C and the

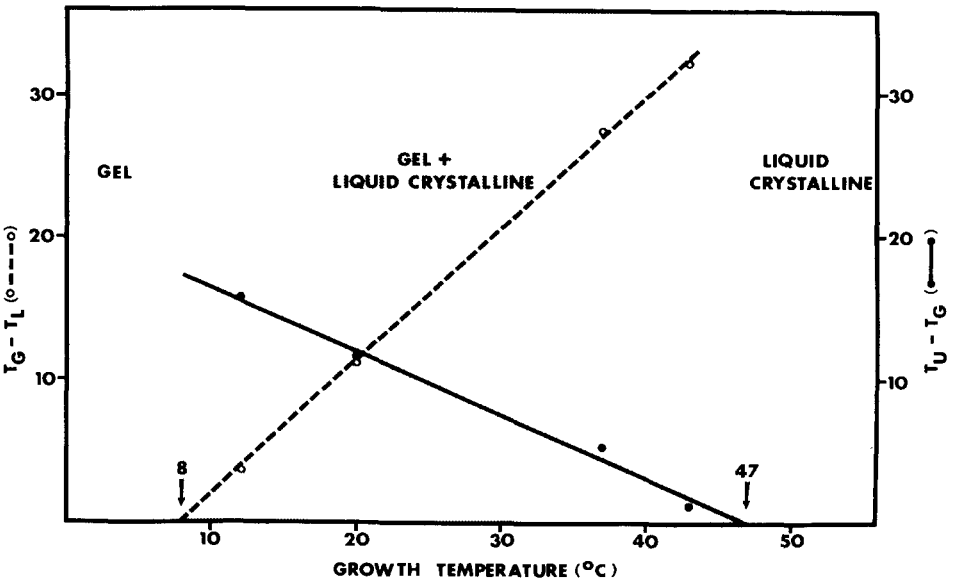

Fig. 4. The difference between the growth temperature, T_G, and the upper transition temperature, T_U, (———, $T_U - T_G$) or between T_G and the lower transition temperature, T_L, (- - - - -, $T_G - T_L$) in outer membrane isolates as a function of growth temperature. The area between 8 and 47°C indicates the temperature range over which the outer membrane can exist in a heterogenous (presumably gel + liquid crystalline) lipid state.

membrane would exist essentially in a gel state (Fig. 4). The temperature range over which the outer membrane lipids can exist in a mixed state, therefore, is assumed to be from about 8 to 47°C. Shaw and coworkers [35] have shown that the lower temperature limit for *E. coli* cell growth in a glucose minimal medium is 7.8°C, and we have found that the upper temperature limit in glucose minimal media is about 45°C. We therefore suggest that the temperature range over which the outer membrane can exist in a heterogeneous lipid state may define the temperature range over which cell growth can occur.

It is understandable that the adaptability of the outer membrane may be crucial for the cell's ability to grow over a broad range of temperatures. The outer membrane appears to act as a molecular sieve allowing small hydrophilic molecules to enter into the periplasm of the cell. This is thought to occur because outer membrane proteins are associated in a specific manner to form pores or transmembrane channels [36,37]. The maintenance of a pore structure may require a specific bulk lipid state, specific phospholipid domains or a specific physical state of the boundary lipids. Thus at high temperatures where a mixed lipid state can no longer be maintained, the resulting state might be too disordered to allow proper association of the pore-forming proteins or proper lipid-protein interactions. The mediation of strong protein-protein interactions by the lipid matrix is consistent with theoretical models [38]. Mutants defective in outer membrane synthesis are known to be temperature sensitive. Yet growth at elevated temperatures has been demonstrated in at least some of these mutants, when divalent cations, which rigidify membranes, were added to the medium [39,40].

Several investigators have suggested that the inability of *E. coli* to grow at temperatures below 8°C is linked to an inhibition of protein synthesis [41—43]. Low temperatures, however, have been shown to inhibit in vitro protein synthesis to a lesser extent than whole cell protein synthesis and growth in *E. coli* [44]. This implies that the primary cause of growth inhibition at low temperatures could be a membrane-mediated phenomenon. It seems likely that at low temperatures the highly ordered state found in the outer membrane restricts the incorporation of membrane components and in turn restricts growth.

Acknowledgements

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