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The effect of growth temperature on the thermotropic behavior of the membranes of a thermophilic *Bacillus*. Composition-structure-function relationships

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The following study was carried out with the aim of widening our understanding of the thermoadaptive mechanisms of the membrane of thermophiles, using *Bacillus stearothermophilus* var. *nondiastaticus* as test-organism. The phospholipids and their acyl chain composition of this *Bacillus* studied in relation to the physical properties of its membrane from bacteria grown at various temperatures. Phospholipids account for 68–75 weight% of the total lipid in cells grown at 45, 55 or 65°C. Phosphatidylglycerol and diphosphatidylglycerol constitute up to 90% of the total phospholipids; no amino phospholipids were found. Increasing the growth temperatures from 45° to 65°C caused an approximately 4-fold decrease in the proportion of the branched-chain fatty acids and a 2-fold increase in the amount of the saturated acyl chains. The reduced proportion of the branched fatty acids was mainly due to a decrease in their anteiso forms. Unsaturated fatty acids were not produced by cells grown at 65°C. In accordance with the fatty acid composition, the molecular packing of phospholipids in monolayers was more expanded with phospholipids from 45°C grown cells as compared with cultures grown at 55°C. The thermotropic gel to liquid-crystalline phase transition of the membrane lipids was monitored by differential scanning calorimetry and fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene. With increase of the growth temperature the phase transition was progressively shifted to higher but narrower range of temperatures. Completion of the lipid melting occurred always at temperatures below those employed for growth. A constructed phase diagram enabled to relate the growth temperature, the fatty acid composition and the lipid apparent microviscosity at temperatures not used in the present study for growth of the thermophile. The minimum temperature for growth and the upper boundary temperature of the least saturated lipid crystallization were extrapolated in this manner; they correspond to the experimentally determined minimal growth temperature. The apparent microviscosity, a measure of membrane order, decreased gradually and conspicuously as the growth temperature was elevated. The delimiting apparent microviscosity values, at the maximal (65°C) and minimal (41°C) growth temperatures were 0.8 and 1.8 poise, respectively. This lack of rigorous homeostatic control of the bulk lipid viscosity prompted reevaluation of the physiological significance of 'homeoviscous adaptation' in *Bacillus stearothermophilus*.

Introduction

Recent advances in understanding the structure and function of biological membranes have focused

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interest on thermotropic (temperature-induced) changes of their lipid constituents. The ability of poikilothermic organisms to survive at adverse temperatures suggests that they may serve as a powerful model to understand the relationship between composition, structure and function of the membrane [1–6]. Generally, thermotropic changes in the physical state of the membrane lipids, displayed by perturbed molecular mobility and change of degree of order of the hydrocarbon chains, may deleteriously affect functional properties of the membrane. A thermoadaptive compensation response in the lipid composition and in particular in the fatty acid composition serves to maintain the membrane dynamics and order in the requisite state [1–6].

Much of the progress in understanding the interrelationship between the environmental temperature, the physical state or the chemical composition of the lipids and the physiological functions of membranes has been achieved through studies of mesophilic microorganisms [2,4–8]. However, thermophilic microorganisms, by their ability to grow and multiply at a much wider range of temperatures, seem to present a better model for understanding the thermoadaptive processes. This is apparently due to their thermoadaptive response in the fatty acid composition of their polar lipids [3,9,10]. Still, until now these organisms received only limited attention mainly by the extensive studies of Souza and co-workers [10–12].

The following study was carried out with the aim of extending our knowledge of the thermoadaptive mechanisms operating in membranes of thermophiles, using as a test organism *Bacillus stearothermophilus* var. *nondiastaticus*.

Materials and Methods

Bacteria and growth conditions. The tyrosine and leucine auxtrophic mutant of *Bacillus stearothermophilus* var. *nondiastaticus,* used in this study, has been previously described [13].

Cells were grown at 45, 55 or 65°C in minimal salt medium supplemented with tyrosine and leucine [13]. For large scale growth of cells, fermentors (New Brunswick) containing 10 or 100 liters of the minimal salt medium were prepared. The medium was inoculated with 5 to 7% (v/v) of a starter found in the logarithmic growth phase. Aeration was achieved by an air flow at a rate of 0.5 v/v per min which was increased during the early log phase to a rate of 1 v/v per min; agitation speed was 250–350 rpm. The pH of the culture was maintained at pH 7.2 by a pH controller (Modcon Ltd.). Growth was measured with a Klett-Summerson colorimeter with filter 42. Mid-logarithmically grown cells were cooled (4°C) and collected in a Sharpless Centrifuge. Packed cells were kept at −70°C until used.

Membrane isolation. Membranes were isolated according to Konings et al. [14] washed and resuspended in 0.1 M phosphate buffer (pH 6.6) and stored at −70°C until used.

Analysis of lipids. Total lipids were extracted according to the method of Bligh and Dyer [15]. Fractionation of the neutral and polar lipids was performed by column chromatography on activated (3 h, 110°C) silicic acid (HR, Merck). Elution was carried out stepwise with chloroform containing increasing proportions of methanol [16]. The lipid components were separated and identified by thin-layer chromatography on plates of Silica gel HR (0.25 mm) or on commercial Silica gel plates (Type Q. Quantum Ind., Fairfield, NJ). The plates were developed with petroleum ether (b.p. 40–60°C)/ethyl ether/acetic acid (60 : 40 : 1, v/v) for determination of the neutral lipids composition and with chloroform/methanol/acetic acid/water (100 : 20 : 12 : 5, v/v) for phospholipids. Two-dimensional thin-layer chromatography was also used to confirm phospholipids composition; chloroform/methanol/28% ammonia (65 : 25 : 5, v/v) was used in the first direction followed by chloroform/methanol/water (65 : 25 : 4, v/v) in the second direction. Iodine vapors or charring with potassium dichromate in sulfuric acid [16] was employed for use for lipid detection. Individual lipids were detected and identified using specific spray reagents (ninhydrin, for free amino groups; molybdate, for organic phosphorus; and naphtol, for sugars [16]). Authentic standards (Sigma, St. Louis, MO) were used as identification markers. The identity of the phospholipids was finally confirmed by chromatography of the deacylated products, obtained by mild alkaline hydrolysis, and comparison to similarly deacylated authentic lipid standards [17].

For quantitative determination the phospholipid spots on the chromatogram were first visualized by iodine vapors, then the iodine was removed by hot fan and the silica gel was scrapped off and assayed for lipid phosphorus content (see Refs. 16–18). Controls of scrapped areas without phospholipids were simultaneously assayed. Approx. 93% (average of eight determinations) of the total phosphate initially loaded was recovered by this procedure. Lipids containing sugars were hydrolyzed in methanolic solution of 1 M HCl (3.5 h at 100°C) in sealed ampoules; the hydrolysate was extracted twice with heptane and the aqueous layer was assayed for total sugars [44] and glucose (see Sigma Technical Bull. No. 510).

Fatty acids were isolated after saponification (0.4 M KOH in methanol, 3 h at 37°C) and esterified with BF_3-methanol (Sigma). The methyl esters of the fatty acids were separated by gas-liquid chromatography using a column of 15% diethylene glycol succinate (DEGS) on Chromosorb W (Applied Sciences Lab. Inc., PA) or a column packed with 10% SP 2340 on Chromosorb W (Supelco Inc., Bellfonte, PA). Both columns were operated isothermally at 150°C. Helium or nitrogen was used as the carrier gas at a flow rate of 40 ml/min. The fatty acids were identified by comparing their retention times with commercially available standards (Applied Science) or by the linear relationship between the logarithm of retention time and the number of carbon atoms in homologous fatty acids. The weight percentage composition was calculated from the areas of each component on the chromatogram. Identification of individual esters was confirmed by combined gas chromatography-mass spectrometry and comparison with known fatty acid methyl esters or reference spectra. A mass spectrometer (Varian CH5DF) equipped with a gas chromatograph inlet system was employed. The identity of unsaturated fatty acids was confirmed by analyzing the products obtained following oxidation with periodate-permanganate [16].

Analytical methods. Total protein, DNA and RNA were determined as described in Refs. 45, 46 and 47, respectively.

Preparation of liposomes. Multilamellar large vesicles in final lipid concentration of 1 mM were prepared as described elsewhere [19,20] in 0.1 M potassium phosphate buffer (pH 6.6) containing 15% w/v sucrose. The latter was included to prevent precipitation of vesicles during the fluorescence depolarization measurements [20].

Differential scanning calorimetry. Differential scanning calorimetry of intact bacterial membranes suspended in 0.1 M potassium phosphate buffer (pH 6.6) was performed using a Dupont 990 differential scanning calorimeter equipped with a cell base II and especially constructed cooling device. The calibration mode was employed using cooling and heating rates of 2 and 5 deg.C/min and sensitivity of 0.02 and 0.1 $mcal \cdot s^{-1} \cdot inch^{-1}$, respectively. t_s and t_l describe the lower (solidus) and upper (liquidus) boundaries (respectively) of the phase transition [21]. While t_m is defined as the temperature at which the excess specific heat reaches maximum, it does not necessarily represent the midpoint of the transition (see the asymmetry index below; for review, see Ref. 21). $t_{1/2}$ describes the temperature width at half height of the endotherm and it serves as a measure of phase transition cooperativity [21]. The degree of asymmetry of the phase transition is evaluated by an asymmetry index [22]. To obtain the asymmetry index value a horizontal line is drawn on the thermogram at the half-maximal excess heat capacity. This line ($t_{1/2}$) intersects the thermogram above and below the t_m. The asymmetry index is obtained by dividing the temperature difference between t_m and lower intersection difference by the difference between the upper intersection temperature and t_m. All measurements were repeated at least three times for each sample in order to distinguish between phase transition, involving membrane lipids (which are reversible), from those involving protein denaturation (which are not reversible [21]).

Fluorescence depolarization measurements. The thermotropic behaviour of both intact bacterial membranes and multilamellar vesicles made of their lipids extracts was determined from steady-state fluorescence depolarization measurements of the fluorophore 1,6-diphenyl-1,3,5-hexatriene (DPH, Aldrich) embedded in the membranes (for reviews, see Refs. 23–25). All measurements were performed using the membranes made of (for multilamellar vesicles) or containing (for intact membrane) 0.5 mM lipids and a diphenylhexatriene to

lipid mole ratio of 1 : 1000–1 : 2000. The experimental procedure is described elsewhere [25] using a modified Perkin-Elmer MPF 44 spectrofluorimeter [25] or the T system described by Shinitzky and Barenholz [19]. Steady-state fluorescence anisotropy (r). The apparent microviscosity ($\bar{\eta}$) and the microviscosity activation energy were determined after correcting for the G (grating) factor and light scattering as described elsewhere [23,25]. The data were calculated and plotted on a CDC-6400 computer equipped with a Gerber Plotter. It should be stressed that the apparent microviscosity as determined by diphenylhexatriene is not a trivial expression [23,24,26]. The apparent microviscosity involves two components: one is related to the order of the acyl chain (order parameter) and the second is a real motion parameter. In most cases studied the order parameter is dominant (for reviews, see Refs. 24 and 26). Therefore the term 'apparent microviscosity' ($\bar{\eta}$) is used here as a membrane order parameter. It has the convenient property of linear Arrhenius plots (describing (ln $\bar{\eta}$) as function of $1/T$) in the absence of membrane phase transition, while 'breaks' due to dramatic increases in the slope of Arrhenius curve occur at membrane phase transition. The range of transition is defined by t'_s and t'_1 (which are equivalent to t_s and t_1 values obtained by differential scanning calorimetry). t'_{sf} and t'_{1f} are obtained by extrapolation of the temperature range in the phase transition region in which the slope of the Arrhenius curve or the temperature-dependent anisotropy curve are maximal, with the slope in the solidus and liquidus regions, respectively [27]. These values are obtained from either one of the three curves: (1) Temperature-dependent steady-state fluorescence anisotropy (Fig. 2). (2) Arrhenius plots describing the natural logarithm of the apparent microviscosity as function of the reciprocal of the absolute temperature ($1/T$) (data not shown). (3) The temperature-dependent 'microviscosity activation energy' obtained by numerical differentiation of the logarithm of apparent microviscosity with respect to the reciprocal of the absolute temperature (Fig. 3) [23,27]. The latter is also used to obtain t'_m values (equivalent to t_m of differential scanning calorimetry) which are the temperature at which maximal change in the apparent microviscosity occurs. Phase transition profiles obtained by fluorescence measurement are somewhat different from those obtained by differential scanning calorimetry probably due to the fact that the two methods are measuring different parameters and differ in their sensitivity. The main advantage of the diphenylhexatriene fluorescence depolarization measurement is that in addition to the thermodynamic characterization of the phase transition, information on the bilayer acyl chain order is also obtained.

Results

Effect of growth temperature on the gross composition of the bacterial membranes

B. stearothermophilus was grown at 45, 55, 55 and 65°C and the membranes obtained were analyzed for protein, lipid, nucleic acids and hexosamines content. The analysis accounted for 88 and 98% of the membranal dry weight. The protein and lipid contents represent 54 to 59% and 25 to 29%, respectively, of the dry weight of the isolated membranes and were not significantly affected by variations in growth temperature. By contrast, the minor membrane components like RNA, DNA and hexosamine decreased considerably at higher growth temperatures. Thus membranes from cells grown at 45, 55 and 65°C contained 11.7, 1.1 and 0.95% RNA, respectively; 2.5-fold more DNA was found in membranes from cells grown at 45°C than from 65°C grown cultures. The minute amounts of hexosamines found in the above membrane preparations (0.2–0.89%) speak for their relative purity and lack of contamination with residual cell-wall components.

Three classes of polar lipids were detected in the membrane preparations which also contain neutral lipids. The main group of the polar lipids are the phospholipids, which comprised 68 to 75% (by weight) of the total lipid fraction, the aminolipids which are phosphorus free, of much lower content (5.2 to 3.3% by weight), which decreased slightly with the increase in the growth temperature (see Table I). The rest, 20–30% of the lipids, were neutral lipids and glycolipids. No detailed analysis was performed on these two groups. However, presence of acylglycerols and minute amounts of free fatty acids was qualitatively indicated by thin-layer chromatography of the neutral

lipids. Regarding the glycolipids, their acidic hydrolysis released carbohydrates of which 36 to 58% reacted with glucose oxidase, suggesting the presence of glucolipids in the thermophile membrane. Table I shows the phospholipid composition of the bacterial membranes. The diphosphatidylglycerol (cardiolipin) and phosphatidylglycerol (PG) together comprised about 90% of the total phospholipids in cells grown at various indicated temperatures. The proportion of these phospholipids was affected by the growth temperature; the diphosphatidylglycerol content decreased while the phosphatidylglycerol increased at elevated growth temperatures. These changes were much more pronounced when the growth temperature was elevated from 55 to 65°C rather than from 45 to 55°C. At the lower temperature range (45 to 55°C) only minor changes of these phospholipids occurred (see Table I).

Three other minor and distinct phospholipid fractions comprised about 10% of the total phospholipids and their further identification was not attempted. Nevertheless, none of the phospholipids, resolved by thin-layer chromatography, was ninhydrin positive, indicating the absence of amino phospholipids in this strain of *B. stearothermophilus*.

TABLE I

EFFECTS OF GROWTH TEMPERATURE ON THE PHOSPHOLIPID COMPOSITION [a]

Lipid composition [a]	Growth temperature (0°C)		
	45	55	65
Total amino lipids [a,b]	5.2	4.6	3.3
Neutral and glycolipids [a]	26.5	21.0	21.0
Total phospholipids [a]	68.3	74.4	75.7
Phospholipid composition [c]			
Diphosphatidyl-glycerol [c]	78.0	76.5	48.7
Phosphatidyl-glycerol [c]	12.0	16.6	40.1
Phospholipid-$X_1^{c,d}$	4.7	4.7	5.2
Phospholipid-$X_2^{c,d}$	0.8	1.6	4.8
Phospholipid-$X_3^{c,d}$	3.6	0.4	0.8

[a] Values are given in weight% of total lipid weight (average of eight experiments with standard deviation not exceeding ±3%).

[b] Amino lipids devoid of phosphorus, no amino phospholipids were detected in the bacterial lipids (see Results).

[c] Values are given as mol% phosphorus equivalent, total phospholipid phosphorus equals 100 mol%.

[d] Unidentified phospholipids (see text).

Effect of growth temperature on fatty acid composition

Mild alkaline hydrolysis of the phospholipids (see Methods) caused their complete hydrolysis to fatty acids and water soluble phosphorus containing molecules. This led to the conclusion that all phospholipids were esterified glycerophospholipids. Table II lists the fatty acid composition of lipids extracted from membranes of bacteria grown at 45°C, 55°C and 65°C and their melting points. The fatty acid composition of the individual phospholipid was almost identical to this of the total lipids.

TABLE II

EFFECT OF GROWTH TEMPERATURE ON FATTY ACID COMPOSITION OF THE MEMBRANE LIPIDS [a]

Fatty acid [b]	m.p. [d] (°C)	Growth temperature (°C) [a]		
		45	55	65
n-14:0	54.4	8.3	5.9	2.2
n-15:0	52.3	3.0	2.0	1.5
n-16:0	62.8	34.4	54.6	79.0
n-17:0	61.3	0.7	0.8	0.6
n-18:0	69.6	0.1	1.8	5.7
i-14:0	50–53	2.5	1.0	0.9
i-15:0	52.2	9.3	6.4	1.5
i-16:0	62.0	14.6	11.0	4.5
i-17:0	60.5	3.8	4.2	2.5
a-15:0	25.8	8.9	4.0	0.6
a-17:0	38.0	7.6	6.2	1.4
n-16:1	0±0.5	6.8	2.1	0
Subgroups of fatty acids				
m.p. ≤ 45		23.3	12.	2.0
m.p. ≥ 62.5		34.5	56.4	84.7
62.5 ≥ m.p. ≥ 45		42.2	31.3	13.3

[a] Values are in weight% of total fatty acids (average of four determinations).

[b] *n*-, i-, and a- represent normal-, iso-, and anteiso- fatty acids, respectively. Fatty acids are designated $x:y$, where x is the number of carbon atoms and y is the number of double bonds per molecule.

[c] Percentage of fatty acids having melting point (m.p.) higher than 62.5°C, lower than 45°C, or in range of 45°C–62.5°C.

[d] All melting point values were taken from 'Handbook of Biochemistry', 2nd Edn. (1970) published by Chemical Rubber Co., Cleveland, OH, U.S.A.

It is evident from Table II that there are major thermoadaptive alterations in the lipid acyl chain composition. In general one can conclude that elevation of growth temperature causes a large increase in the content of fatty acids whose melting point is equal or above 63°C (m.p. of palmitic acid) at the expense of decrease in content of most fatty acids of melting point lower than 63°C. The detailed analysis (Table II) reveals the following fine picture: Major increases in *n*-16 : 0 and *n*-18 : 0 at higher growth temperatures (2.4-fold and 57-fold, respectively, comparing bacteria grown at 45°C and 65°C). This was accompanied by a decrease of the unsaturated monenoic acid, Δ^5-hexadecenoix acid, till its complete disappearance from membranes of 65°C grown cells. This correlates well with the generally observed inverse relationship between growth temperature and the proportion of unsaturated fatty acids [2,6]. Likewise, growth at higher temperatures resulted in lower proportions of saturated normal fatty acids with chains shorter than 16 carbon atoms.

The pattern of the alterations among the branched-chain fatty acids was also inversely related to the growth temperature: increasing the temperature by 20 deg. C (from 45 to 65°C) resulted in 3.2-fold reduction of the total iso-forms and 8.2-fold decrease of the anteiso acids. The latter decrease was mainly due to the considerable decline (15-fold) in anteiso 15 : 0 with the lowest melting point (25.8°C) of the saturated acids in this thermophile. Moreover, although the anteiso-pairs (a-15 : 0 + a-17 : 0) and the corresponding iso-pairs (i-15 : 0 + i-17 : 0) were relatively abundant, their values decreased progressively as the growth temperature increase; the reduction of the anteiso-pair, with lower melting temperature, exceeded that of their iso-counterparts. Thus, the ratio of (i-15 : 0 + i-17 : 0) to (a-15 : 0 + a-17 : 0) increased from 0.8 to 2.0 in cultures shifted from growth at 45°C to growth at 65°C. The same trend in the temperature-dependent alterations of the fatty acid composition is demonstrated by the higher ratio (2.7-fold) of a-17 : 0 to a-15 : 0 observed upon increasing the temperature from 45 to 65°C.

Thermotropic behaviour of bacterial membranes

The thermotropic behaviour of intact bacterial membranes isolated from bacteria grown at three different temperatures (45°C, 55°C and 65°C) was studied using two independent methods. Differential scanning calorimetry and fluorescence depolarization of the fluorophore 1,6-diphenylhexatriene embeded in the membranes. Excellent agreement between these two methods was obtained (Table III). The thermotropic behaviour of multilamellar large vesicles prepared of phospholipids extracted of membranes of bacteria grown at the above three temperatures was also studied.

(a) Differential scanning calorimetry. The thermograms obtained from membranes isolated from cells grown at 45, 55 and 65°C are shown in Fig. 1. All three thermograms show one endotherm. The estimated temperature ranges of these endotherms (defined as t_s–t_l) are from 22.5 to 46.0, from 31 to 52°C and from 46 to 62°C for membranes obtained from cells grown at the above respective temperatures. The thermotropic behaviour was reversible and rescanning of the same samples resulted in identical thermograms. Table III shows the t_m and the width of the phase transitions (t_l–t_s) in comparison with the same parameters as obtained by fluorescence depolari-

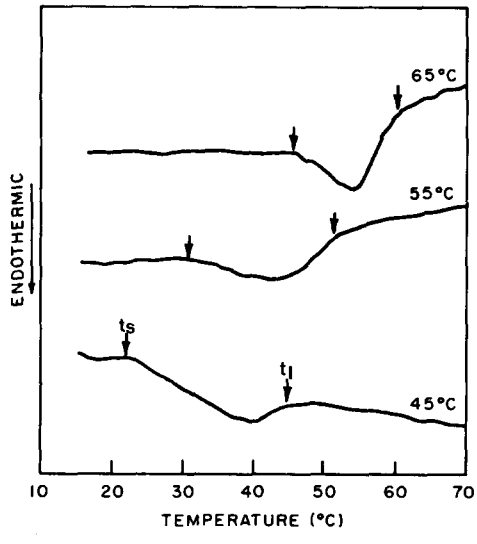

Fig. 1. Thermograms obtained by differential scanning calorimetry of membranes isolated from cultures grown at 45°C, 55°C or 65°C. Growth temperatures are indicated on the thermograms. Arrows denote the phase transition boundaries (t_s and t_l).

TABLE III

CHARACTERIZATION OF PHASE TRANSITION IN MEMBRANES AND MULTILAMELLAR VESICLES OBTAINED FROM CELLS GROWN AT 45, 55 and 65°C

The table describes features of the thermotropic behaviour of bacterial membranes and multilamellar vesicles prepared of lipids derived of these membranes, as obtained by differential scanning calorimetry and by diphenylhexatriene fluorescence depolarization.

Growth temp. (°C)	Bacterial membranes									Multilamelar vesicles Diphenylhexatriene fluorescence depolarization			
	Differential Scanning Calorimetry				Dephenylhexatriene fluorescence depolarization								
	t_m	t_s	t_l	Asymmetry index	t'_m	t'_s	t'_l	t'_{sf}	t'_{lf}	t'_s	t'_l	t'_{sf}	t'_{lf}
45	39.0	22.5	46.0	3.40	36.0	22.0	50.0	25.0	41.0	22.0	48.0	24.0	41.0
55	43.0	31.0	52.0	1.39	42.0	29.0	47.0	33.0	44.0	32.0	47.0	34.0	46.0
65	52.5	46.0	62.0	0.71	51.5	38.0	56.0	46.0	52.0	39.0	53.0	44.0	52.0

zation of diphenylhexatriene (t'_m, t'_l–t'_s) also the range in which most of the phospholipids molecules undergo their phase transition (t'_{lf}–t'_{sf}). These parameters were essentially similar to those obtained with the corresponding multilamellar vesicles prepared of extracts of phospholipids of bacterial membranes (Table III). This finding, together with the reversibility of the endothermic transitions suggests that they depict the gel-to-liquid-crystalline phase transition of the membrane lipids, with the membrane proteins having much smaller effect (for review see Ref. 21).

The endotherms exhibit the expected dependence on growth temperature and on fatty acid composition of the membrane lipids; cells grown at elevated temperatures and possessing increased proportions of fatty acids with higher melting points also exhibited progressively higher t_m and higher temperatures of the whole range of the gel-to-liquid-crystalline phase transition. As expected the acyl chain homogeneity had a reciprocal relationship to the phase transition width (Tables II and III). This is better observed from t'_{lf}–t'_{sf} which defines the temperature range in which the majority of phospholipid molecules undergo the phase transition (see fluorescence depolarization data).

Another important parameter is the asymmetry index of the phase transition.This shows that for bacteria grown at 45°C the melting was much less cooperative at the lower temperature range of the phase transition (for $t < t_m$). At growth temperature of 55°C the phase transition was more symmetric with slightly less cooperative phospholipids melting at $t < t_m$. While for bacteria grown at 65°C their membrane phospholipids melting was faster (more cooperative) at $t < t_m$ than for the range of the transition in which $t > t_m$. This asymmetry phenomenon suggests micro-phase separation in the bacterial membrane. It may be a result of overlapping phase transitions which are not well resolved. Possibly it is related to the quantity of minor components of the membrane, phospholipids containing *n*-16 : 1 and a-15 : 0 acyl chains or *n*-18 : 0 for bacteria grown at 45°C and 65°C, respectively.

(b) Fluorescence depolarization. Fig. 2 shows the temperature-dependent profiles of the steady-state fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (r) in membranes and vesicles prepared of membrane lipids of cells grown at 45°C, 55°C and 65°C. For all preparations used, the r was inversely proportional to the temperature of measurement. Comparing the three membrane preparations and the three respective vesicle dispersions at any given temperature the r was found to be inversely related to the growth temperature, thus e.g., membranes from cells grown at 65°C exhibited the highest r while those from cells grown at 45°C showed the lowest anisotropy.

It is worth noting that similar maximal r values (0.31–0.35) were obtained for membranes at the three different temperatures. However, the temperature at which these values were attained was considerably higher for membranes or lipid dispersions of cells grown at 65°C as compared with

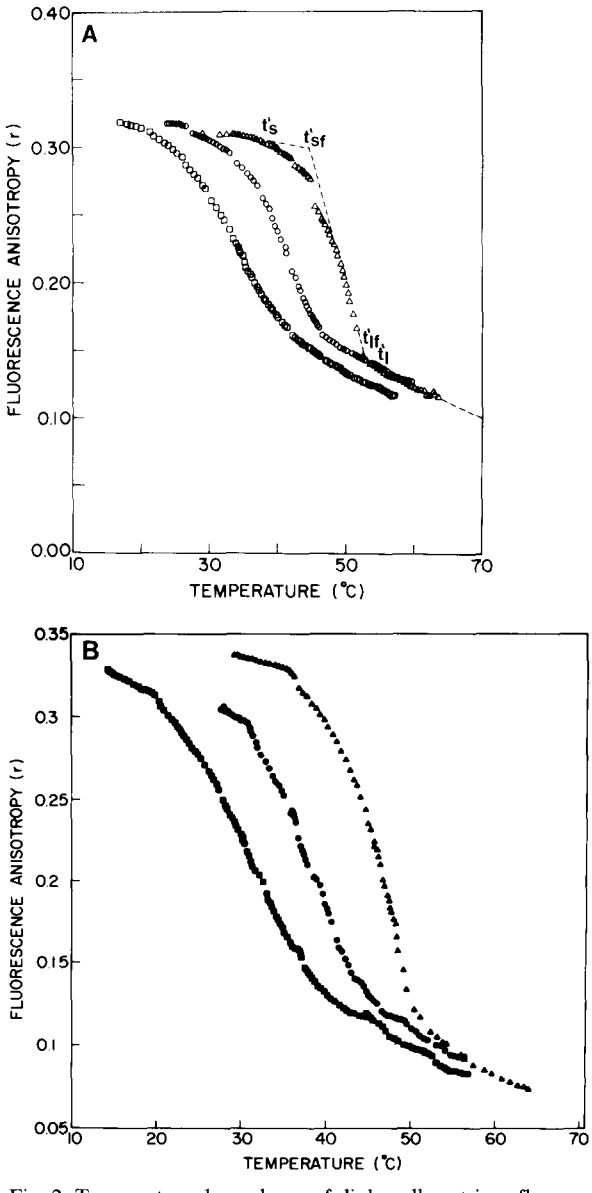

Fig. 2. Temperature dependence of diphenylhexatriene fluorescence anisotropy in bacterial membranes (A) and in multilamellar prepared of total phospholipid extracted from these membranes (B), using bacteria grown at 45°C (□, ■); 55°C (○, ●) and 65°C (△, ▲), for A and B, respectively. Arrows denote the boundaries of the phase transition, starting from t'_s (where the melting process of the gel phase lipids started through t'_{sf} t'_{1f} and t'_1 (the temperature in which the melting was terminated and all the lipids are in a liquid-crystalline state).

those of 45°C or 55°C grown cultures. The effect of membrane proteins was demonstrated by the slightly higher r values obtained for the membrane preparations when compared with the corresponding liposomes at identical recorded temperatures. The gel-to-liquid-crystalline phase transition of the bulk lipids, represented by the steep changes of diphenylhexatriene anisotropy, is delimited by lower (t'_{sf}) and upper (t'_{1f}) boundaries (see Fig. 2A) which were shifted to higher temperatures as the growth temperature was elevated. Table III summarizes the phase transition parameters obtained by differential scanning calorimetry and by fluorescence depolarization of diphenylhexatriene.

The slopes in the Arrhenius plots describing the natural log of the apparent microviscosity as function of the reciprocal of the absolute temperature ($1/T$) represent the energetic changes taking place during the transition process. These changes are proportional to the flow activation energy (see Refs. 23 and 26 for review) and are shown in Fig. 3, where $d(\ln \bar{\eta})/d(^1/T)$ is plotted as function of temperature. The temperature ranges which characterize the gel-to-liquid-crystalline phase transitions according to Fig. 3 also verify that growth temperatures are always higher than the corresponding t_1 of the lipid phase transition.

When the apparent microviscosity was compared at the corresponding growth temperature (Table IV) a conspicuously progressive increase of the membrane or of the liposomal apparent microviscosity, was found as growth temperature was

TABLE IV

APPARENT MICROVISCOSITY OF THE MEMBRANES OR THE CORRESPONDING MULTILAMELLAR VESICLES AS DETERMINED AT THE GROWTH TEMPERATURES

Temperature [a] (°C)	Apparent microviscosity (poise)	
	Membranes	Lipid dispersions
40	1.78 [b]	–
45	1.58	1.07
55	1.18	0.73
65	0.83 [c]	0.47 [c]

[a] The same temperatures have been employed for growth and for apparent microviscosity determination.
[b] Value obtained from Fig. 4.
[c] These are maximal values since the microviscosity was determined at 64°C.

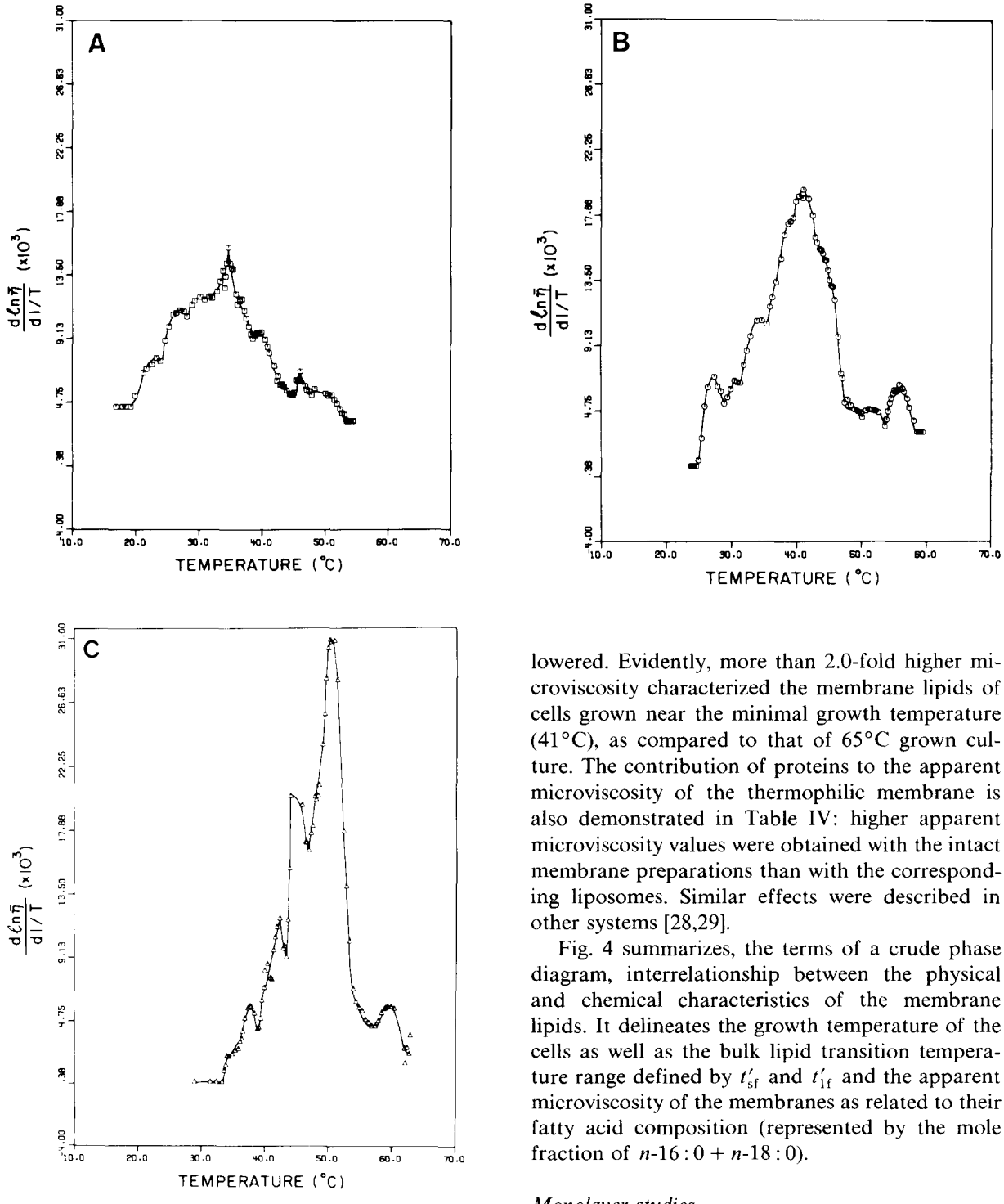

Fig. 3. Temperature dependence of diphenylhexatriene-derived microviscosity activation energy $d(\ln \bar{\eta})/d(^1/T)$ of membranes isolated from 45°C (A); 55°C (B) and 65°C (C) grown bacteria.

lowered. Evidently, more than 2.0-fold higher microviscosity characterized the membrane lipids of cells grown near the minimal growth temperature (41°C), as compared to that of 65°C grown culture. The contribution of proteins to the apparent microviscosity of the thermophilic membrane is also demonstrated in Table IV: higher apparent microviscosity values were obtained with the intact membrane preparations than with the corresponding liposomes. Similar effects were described in other systems [28,29].

Fig. 4 summarizes, the terms of a crude phase diagram, interrelationship between the physical and chemical characteristics of the membrane lipids. It delineates the growth temperature of the cells as well as the bulk lipid transition temperature range defined by t'_{sf} and t'_{lf} and the apparent microviscosity of the membranes as related to their fatty acid composition (represented by the mole fraction of *n*-16 : 0 + *n*-18 : 0).

Monolayer studies

Fig. 5 depicts the pressure-area curves for monolayers of the total phospholipids extracted

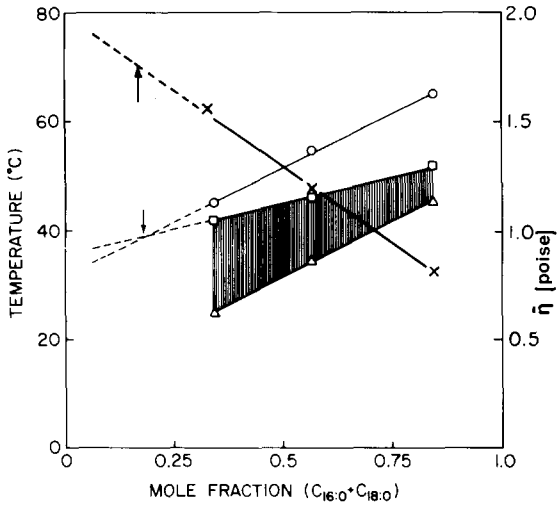

Fig. 4. The correlation between temperature range of lipid bulk phase transition ($t'_{sf}-t'_{lf}$), growth temperature and apparent microviscosity of the bacterial membranes as related to their fatty acid composition. Fatty acid composition is represented by the mole fraction of palmitic plus stearic acids ($C_{16:0}+C_{18:0}$). The gel-to-liquid-crystalline phase transition of the bulk lipids represented by the steep changes in diphenylhexatriene anisotropy as delimited by lower (t'_{sf}, Δ) and upper (t'_{lf}, □) boundaries (see Fig. 2A). The stripped area denotes this temperature range of the phase transition. Symbols (○———○) and (×———×) describe the growth temperature and the apparent microviscosity, respectively. The values of apparent microviscosity (poise) are described by the left ordinate. The lower arrow denotes the intersection point of the extrapolated growth temperature curve (□- - - - -□). The upper arrow denotes the apparent microviscosity extrapolated (×- - - - - -×) to maximal growth temperature of this bacteria.

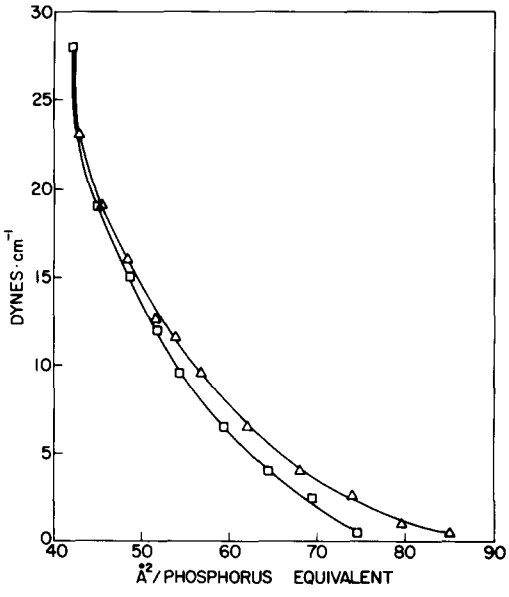

Fig. 5. Force-area curve of monomolecular layers of bacterial phospholipids at the air water interface. Total phospholipids were extracted of membranes of bacteria grown at 45°C (△) and 55°C (□). The measurements were performed at 23°C. For more details see Results and Ref. 43.

from 45°C and 55°C grown cells. This was measured exactly as described by Quinn and Barenholz [43]. The mean minimal molecular area (calculated per phospholipid phosphorus) appears to be similar for both extracts 42 Å^2 at the higher range of the pressure imposed; however, more expanded molecular packing is apparent for the phospholipids from 45°C grown cells at pressures lower than 15 dyn/cm. The difference in the molecular packing of the phospholipids of the 45°C and 55°C grown bacteria is most likely due to their different fatty acid composition (Table II) since no significant changes were observed in their composition as based on hydrophilic head groups (Table I).

Discussion

The phospholipid and fatty acid composition of the membranes of *B. stearothermophilus*, a thermophile growing between 40°C and 70°C, were studied with the aim of finding the mechanisms which allow its adaptation and growth at such a wide range of temperatures. This was done by growing the bacteria at different temperatures in identical medium and enabling the bacteria to make their own lipids having the desired fatty acyl composition. The ratio of the lipid to protein is not affected by the growth temperature. On the other hand the following two variables are affected. (a) The reciprocal relationship between the increase in phosphatidylglycerol and the parallel decrease in diphosphatidylglycerol, which together comprise about 90% of the cellular phospholipids. (b) The change in acyl chain composition of all membrane lipids. However, while the acyl chain composition changes continuously with the increase in temperature, the changes in diphosphatidylglycerol : phosphatidylglycerol ratio are

observed mainly upon shifting the temperature between 55°C and 65°C. The change in the ratio of these two lipids is not fully understood. It is possible that the high phosphatidylglycerol content accumulated at 65°C is a result of a decrease in the enzymatic activities in the pathway leading from phosphatidylglycerol to diphosphatidylglycerol. One has to be aware that the diphosphatidylglycerol has a smaller packing parameter than phosphatidylglycerol [30], therefore in the liquid-crystalline state diphosphatidylglycerol may undergo a transformation from a lamellar to a nonlamellar structure especially in the presence of calcium ions (for reviews, see Refs. 8 and 31), while phosphatidylglycerol in water forms only stable bilayers. The relevancy of this behaviour is not clear as yet. Possibly certain proportions of bilayer and hexagonal phase preferring molecules must be maintained in order to optimize membrane structure and function (for reviews, see Ref. 8 and references listed therein).

A better defined process is the thermoadaptive response of the fatty acid composition. The increase in growth temperature is manifested by the following phenomena: (a) A marked increase in the abundance of the longer homologues of saturated-linear fatty acids. (b) A gradual depletion of the unsaturated fatty acids and (c) a prominent increase in the ratio between iso to anteiso fatty acids. Nevertheless, increasing the growth temperature did not significantly affect the average chain length of the fatty aids produced. A similar finding was also described with another thermophilic bacillus [6,32]. The abundance of branched-chain fatty acids, particularly at lower growth temperature, corresponds to the preponderance of these acids in mesophilic [33] or thermophilic [12,32,34] species of the genus *Bacillus*. It further demonstrates the ability of these acids, mainly of the anteiso series, to replace the unsaturated acids [35] in 'fluidizing' the membrane lipids at lower temperatures.

The apparent paucity of fatty acids with melting points higher than the maximal growth temperature is characteristic of this thermophile as well as of other thermophilic [12,32,36] or caldoactive [34,37] bacteria. For example, stearic acid (m.p. 69.5°C) accounts for less than 6% of the total fatty acids even at the maximal growth temperature of 65°C. This property is further demonstrated in the presently studied thermophile by the calculated average melting temperature of the total fatty acids: 50.8, 57.1 and 62.4°C for cells grown at 45, 55 and 65°C, respectively.

All the above phenomena can be explained in terms of the range at which the free volume and/or the ratio between the hydrophobic and hydrophilic mass enable the preservation of the non-leaky lamellar structure in which the membranal functions are retained.

The effect of growth temperature on the thermotropic behaviour of membrane lipids in situ can be interpreted in terms of homeoviscous adaptation such as maintenance of similar apparent microviscosity. Such is the case of *Escherichia coli*, which presumably maintains similar apparent microviscosity at all temperatures examined [5]. This is, however, not the case of the thermophile studied by us (Table IV). Decreasing the growth temperature resulting in progressive increase of its membrane apparent microviscosity, the *B. stearothermophilus* 'fails' to adjust continuously the fatty acid composition to counterbalance the thermally induced alterations in the lipid organization. Our findings also disagree with a previous study [12] which suggests the presence of sensitive and efficient mechanisms for homeoviscous adaptation in another strain of *B. stearothermophilus*. This discrepancy presumably stems from the use of different strains or rather, from the methods employed. While the other study [12] is based on DSC data only, in the present one a combination of differential scanning calorimetry and fluorescence depolarization of diphenylhexatriene has been employed. The differential scanning calorimetry data monitor mainly information regarding the gel-to-crystalline phase transition and the fluorescence depolarization monitors in addition the organization of the membrane lipids and their degree of order [24,26]. The inability to maintain a constant microviscosity at a wide range of growth temperatures was also observed in other poikilothermic organisms [6,37–41]. It should be stressed that the microviscosity represents mainly an order parameter which is determined by the packing and free volume in the membrane [24,26].

Throughout the wide range of growth temperatures tested, the physical state of the *B.*

stearothermophilus membrane is such that the majority of its lipid molecules are in the liquid-crystalline state. The growth temperature is always above the liquidus line in the phase diagram (Fig. 4). However, the temperature difference between the growth temperature and the liquidus line is increasing with elevating growth temperature (from 4 deg. at 45°C to 15 deg. at 65°C). This can be explained by wider fluctuation in growth temperature occurring at higher temperature (65°) than at lower temperature (45°C). Such fluctuation may bring the bacterial membranes below the liquidus line into the phase transition range which will prevent bacterial growth (see Fig. 4).

The inability of the cells to grow at temperatures below the liquidus line which elicit crystallization of the membrane lipids enables us to predict, with the aid of Fig. 4 and Table III, the range of temperature which is incompatible with optimal growth unless prerequisite changes will take place in the fatty acid composition or in the apparent microviscosity. Although no direct examination has been performed toward this end, a previous study [42[provides us with some preliminary support. Accordingly, cells transferred from 55°C to 65°C assume growth immediately (without apparent lag) and with a rate characteristic to cultures growing at 65°C. Similar findings were obtained with temperatures shifts in the opposite direction (65°C → 55°C). In contrast, a long lag phase, which was frequently accompanied with partial lysis, preceded growth when the culture was transferred from 45°C to 65°C, or vice versa. These extreme shifts result in solid membrane lipids (65°C → 45°C) or in low apparent microviscosity (low degree of order) (45°C → 65°C) which are most likely incompatible with growth.

It seems that keeping membrane apparent microviscosity at certain range rather than at a fixed value is the key for the thermal adaptation in *B. stearothermophilus* var. *nondiastaticus*. This range has to be slightly above the liquidus line. The temperature gap between the growth temperature and this line is increasing with the elevation of growth temperature to prevent disturbance by downward fluctuation in growth temperature. This parameter is important for the determination of the viable microviscosity range. This concept differs from the classical homeviscous adaptation [5] and applies also to eucaryotic uni- and multicellular organisms. Such thermal adaptation is achieved by the thermophile by manipulating its membrane lipids acyl chain composition and possibly its membrane phosphatidylglycerol to diphosphatidylglycerol mole ratio. Again one has to be aware that the modified concept of hemoviscons adaptation does not deal with a purely dynamic aspect of motion but with an order parameter affected by the packing and free volume in the membrane. This structural parameter is affecting also the membrane dynamics.

The biochemical mechanism of the thermal adaptation occurring in the membranes of *B. stearothermophilus* var. *nondiastaticus* is not yet clear. Data available from other bacterial systems (see Refs. 2 and 6 for review) suggest the direct involvement of few enzymes which are responsible for the synthesis of unsaturated and branched fatty acids. Elevation of growth temperature causes deactivation of these enzymes thereby increasing the incorporation of saturated fatty acids (palmitic and stearic in our system) to the bacterial phospholipids, resulting in an increased level of the fully saturated phospholipids [2,6]. However, the exact details have to await further studies.

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