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Lipid and Protein Composition of Membranes of *Bacillus megaterium* Variants in the Temperature Range 5 to 70°C

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Membranes were prepared from four temperature range variants of *Bacillus megaterium*: one obligate thermophile, one facultative thermophile, one mesophile, and one facultative psychrophile, covering the temperature interval between 5 and 70°C. The following changes in membrane composition were apparent with increasing growth temperatures: (i) the relative amount of iso fatty acids increased and that of anteiso acids decreased, the ratio of iso acids to anteiso acids being 0.34 at 5°C and 3.95 at 70°C, and the pair iso/anteiso acids thus seemed to parallel the pair saturated/unsaturated acids in their ability to regulate membrane fluidity; (ii) the relative amount of long-chain acids (C_{16} to C_{18}) increased fivefold over that of short-chain acids (C_{14} and C_{15}) between 5 and 70°C; (iii) the relative amount of phosphatidylethanolamine increased, and this phospholipid accordingly dominated in the thermophilic strains, whereas diphosphatidylglycerol was predominant in the two other strains; and (iv) the ratio of micromoles of phospholipid to milligrams of membrane protein increased threefold between 5 and 70°C. Moreover, a quantitative variation in membrane proteins was evident between the different strains. Briefly, membrane phospholipids with higher melting points and packing densities appeared to be synthesized at elevated growth temperatures.

Most microorganisms alter their membrane composition in response to changes in the environmental temperature. The physical properties of the membrane lipid bilayer are believed to be important for this temperature adaptation. *Escherichia coli* thus maintains a nearly constant fluidity of its membrane lipids over its entire temperature range of growth, a process called "homeoviscous adaptation" (45). The mechanism, in this case, involves the production of membrane lipids containing a higher proportion of saturated fatty acids relative to unsaturated ones at higher temperatures.

Generally, the highest growth temperature for a microorganism is considered to be determined by the thermal destruction of the most heat-labile essential cell component. Membrane integrity has been put forward in one of the theories attempting to explain the phenomenon of thermophily (44). Esser and Souza (13) concluded that for a temperature-sensitive mutant of the obligate thermophile *Bacillus stearothermophilus*, the maximum and minimum growth temperatures were determined by the onset and cessation of the membrane lipid phase separation. However, McElhaney and Souza (30), in a reexamination of this correlation, stated that the upper boundary of the phase transition interval in itself did not directly determine the maximum growth temperature of this thermophile; nor did the lower boundary of the phase transition interval, which occurred well below the growth temperature range, determine the minimum growth temperature of *B. stearothermophilus*, although the minimum growth temperatures of *Acholeplasma laidlawii* and *E. coli* (29, 39, 50) can be determined by this lower boundary. Thus, the thermostability of microorganisms probably involves many factors, and the specific composition of the membrane is but one requirement. McElhaney (29) states that changes in the physical state of the membrane lipids alone will not convert mesophiles into psychrophiles or thermophiles.

The fatty acid compositions of lipids from *Bacillus* species grown at different temperatures have been investigated (4, 8, 22, 44, 47, 52, 56). The species most commonly used was *B. stearothermophilus*. The temperature intervals studied were rather narrow, about 20°C, and the results are conflicting with respect to some fatty acids. Thus, for iso-C_{15}, anteiso-C_{15}, iso-C_{16}, iso-C_{17}, and anteiso-C_{17} fatty acids, increasing as well as decreasing relative amounts with increasing growth temperature were reported. Shen et al. (44) compared six different *Bacillus* species,

and the observed fatty acid composition might have been affected by inherent genetic differences between these species as well as by temperature.

The present investigation used temperature range variants of *B. megaterium* (48). Such variants have extended the capability of temperature adaptation outside the normal limits of their parent bacteria. The temperature-dependent changes in membrane composition could be determined with greater correctness in a genetically homogenous system of strains spanning a growth temperature range of 65°C. Changes in the relative amounts of phospholipid classes, in the average chain length of the fatty acids, in the relative proportions of iso and anteiso acids, and in membrane protein composition were observed in these *B. megaterium* strains when growth temperature was altered.

MATERIALS AND METHODS

Organisms. Four *B. megaterium* strains, designated Fp 1, M 1, Ft R32, and Ot 32, were used throughout this work. Strain Fp 1 is facultatively psychrophilic, strain Ft R32 is facultatively thermophilic, and strain Ot 32 is obligately thermophilic. These strains originate from the same mesophilic wild-type strain, M 1, of *B. megaterium*. Their isolation and characteristics have been described (48). Characteristics of relevance to the present investigation are given in Table 1.

Culture conditions. Stock cultures of the strains were kept on slants of tryptone-starch agar (tryptone [Difco Laboratories, Detroit, Mich.], 10 g; soluble starch [E. Merck AG, Darmstadt, Germany], 2 g; agar, 30 g; distilled water, 1,000 ml) at 4°C. Temperature range stability (Table 1) was tested at different temperatures on this medium before the start of an experiment.

Cultures for inoculum were grown in tryptone-starch broth and transferred at least twice at the temperature intended for the main experiment. Erlenmeyer flasks (1 liter) containing 250 ml of broth were inoculated with 2% (vol/vol) of the preculture and incubated in thermostatically controlled water baths (±0.1°C) with reciprocal shaking. The flasks were equipped with side tubes for turbidimetric measurements, which were made periodically. In all experiments, the cultures were harvested in the mid-exponential growth phase at the same turbidity reading.

The facultative psychrophile was grown at 5, 10, 25, and 35°C; the mesophile was grown at 10, 15, 25, 35, and 44°C; the facultative thermophile was grown at 17, 25, 35, 45, and 55°C; and the obligate thermophile was grown at 40, 50, 55, 60, 65, and 70°C.

Membrane preparation. Cell membranes were prepared by the relatively rapid procedure of Konings et al. (25), which circumvents the usual protoplast formation step. According to this method, the cells are treated with lysozyme in a hypotonic medium. The cell wall is thereby partially hydrolyzed, which results in an immediate lysis of the cells and formation of membrane vesicles. Added deoxyribonuclease and ribonuclease hydrolyze the liberated DNA and RNA. Cell wall remnants are further hydrolyzed by continued incubation in the presence of lysozyme. The membranes were collected by centrifugation for 30 min at 34,000 × *g* (at 4°C). No intact cells were present in the membrane preparations when examined by phase-contrast microscopy. The protein content of membranes was estimated by the method of Lowry et al. (27) modified according to Hartree (16). Bovine serum albumin (Cohn V) was used as a standard.

Polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed as described by Neville (35) with the modifications of Jergil and Ohlsson (18). The gels were cast in 9.5- by 7.5- by 0.3-cm slab gel forms. Membrane fractions containing 120 μg of protein were brought to a volume of 25 μl by addition of deionized water. An equal volume was then added of double-strength upper reservoir buffer containing sodium dodecyl sulfate, β-mercaptoethanol, ethylenediaminetetraacetate, sucrose, and phenol red. The samples were placed in a boiling-water bath for 3 min. After cooling to room temperature, the entire sample was placed in a sample well of the slab gel. Electrophoresis was performed at a constant current of 20 mA per slab gel and was terminated just before the phenol red tracking dye migrated out of the gel. The gels were stained for 12 h with 0.05% (wt/vol) Coomassie brilliant blue R 250 in methanol-acetic acid-water (50:7:43, vol/vol) and destained for 48 h in methanol-acetic acid-water (25:7:68, vol/vol). Reference proteins used for molecular weight determinations were: bovine serum albumin (68,000), ovalbumin (43,000), deoxyribonuclease I (31,400), trypsin (23,300), and lysozyme (14,300).

An Eppendorf photometer equipped with a gel and photo scanner was used for scanning the negatives of

TABLE 1. *Genealogy, cardinal temperatures, and important characteristics of the B. megaterium variants*[a]

Strain	Derivation	Cardinal temp (°C)			Characteristic	
		Maximum	Optimum	Minimum	Megacin producer	Phage sensitive
Ot 32	Spontaneous from M 1 at 60°C	73	64	38	−	+
Ft R32	Acridine revertant from Ot 32	58	50	16	+	+
M 1	Mesophilic wild type of *B. megaterium*	45	36	8	−	+
Fp 1	Spontaneous from M 1 at 5°C	42	35	0	−	+

[a] Data taken from reference 48.

gel photos. The qualitative transmission was recorded, and areas under peaks were estimated from the weights of the cut out paper recordings.

Lipid extraction. Lipids were extracted from membrane suspensions according to a modification of the method of Bligh and Dyer (3). Each 1 ml of membrane suspension, containing 1 to 3 mg of protein, was mixed with 3.75 ml of methanol-chloroform (2:1, vol/vol) and extracted for 2.5 h at room temperature with intermittent shaking. The suspension was then subjected to sonic treatment in a Bransonic bath for 0.5 h. The membrane residue was removed by centrifugation at 10,000 × *g* for 10 min, and the supernatant was stored at 4°C. The pellet was extracted once more for 1 h after suspension in 4.75 ml of methanol-chloroform-water (2:1:0.8, vol/vol). The suspension was subjected to sonic treatment and centrifuged as described above. The two supernatants were then combined, and 2.5 ml each of chloroform and water were added. The mixture was centrifuged at 2,000 × *g* for 5 min, and the lower, chloroform phase was recovered and then diluted with a small amount of benzene to remove traces of water. The chloroform was removed by evaporation under a stream of nitrogen at 35 to 40°C. The extracted lipids were finally suspended in chloroform-methanol (2:1, vol/vol) and stored at −20°C.

Separation and identification of phospholipids. Phospholipids were separated and analyzed by thin-layer chromatography with Merck Silica Gel H (0.5 mm thick) buffered with 0.5 g of sodium acetate per 100 g of gel (33). Chromatograms were developed in chloroform-methanol-water (65:25:4, vol/vol) or chloroform-acetic acid-methanol-water (80:18:12:5, vol/vol).

The lipid components were characterized by comparing their R_f values with those of known phospholipids and by their reactions with specific spray reagents. Reference lipids were phosphatidylglycerol and diphosphatidylglycerol from *A. laidlawii* A strain EF 22, previously identified by acid and mild alkaline hydrolysis (53). A lipid extract from *E. coli* strain K-12 containing phosphatidylglycerol, phosphatidylethanolamine, and diphosphatidylglycerol (5) was also used. The spray reagents used were: rhodamine 6 G, molybdate reagent for phosphate (10), diphenylamine reagent for lipid sugars (46), ninhydrin (Sigma Chemical Co., St. Louis, Mo.) for amino groups, and periodate-Schiff reagents for α-glycols (43).

Quantitative determination of phospholipids. Phospholipids were separated by thin-layer chromatography as described above, the spots were visualized with iodine vapor, and the individual lipid spots were scraped off into glass tubes. The organic phosphorus assay of McClare (28) was then used. A calibration curve was constructed by applying known amounts of L-α-lysophosphatidyl choline (Sigma) to thin-layer chromatography plates and treating these spots in the same way as the samples.

Isolation of fatty acids. Fatty acids were converted to their methyl esters by treating the lipid extract (0.5 to 1.5 μmol of lipid) for 2 h at 70°C in tubes containing 4 ml of 5% (vol/vol) H_2SO_4 in water-free methanol. The methyl esters were extracted with *n*-hexane (Merck, spectroscopic grade) and analyzed by gas-liquid chromatography.

Identification and quantitative determination of fatty acids. The fatty acids were identified on a Perkin-Elmer model F apparatus equipped with two different columns: (i) a 1.83-m-long glass column, 0.635 cm in diameter, packed with 5% (wt/wt) free fatty acid phase on Chromosorb G AW-DMCS, 80 to 100 mesh, and (ii) a 3.05-m-long stainless steel column, 0.318 cm in diameter, packed with 10% (wt/wt) diethylene glycol succinate on Chromosorb W AW, 80 to 100 mesh. In the former case, the column and injector temperatures were 175 and 300°C, respectively, and the nitrogen flow rate was 25 ml/min. In the latter case, the corresponding parameters were 160 and 330°C and 7 ml/min, respectively. The individual fatty acids were characterized by comparison of retention times with standard mixtures containing methyl esters of straight- and branched-chain C_{13} to C_{20} acids (Larodan Lipids, Malmö, Sweden) run under identical conditions.

Peak areas were calculated with an analog electronic integrator. A known amount of methyl elaidate (Sigma) was added to each sample as an internal standard to obtain absolute values.

RESULTS

Membrane protein composition. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of membrane proteins gave about 35 to 40 well-resolved polypeptide bands (Fig. 1). Most of the bands were found in all strains and over the whole temperature interval investigated, but the relative amounts, as judged from staining intensities, differed for certain proteins. The greatest differences were evident in comparisons between strains (Fig. 1), but small alterations were also noted within each strain when grown at different temperatures (not shown). The two high-molecular-weight proteins (top bands, Fig. 1c) present in the facultatively thermophilic strain Ft R32 were particularly prominent. In the other strains, the 130,000-dalton protein was totally absent, and the 110,000-dalton protein was present in very small amounts. A spectrophotometric scanning of the gel pattern for strain Ft R32 showed that the 130,000-dalton polypeptide band made up 11% and that the 110,000-dalton band made up 28% of the total stain. Thus, roughly 40% of the membrane proteins in strain Ft R32 consisted of these high-molecular-weight proteins. This strain is unique in producing megacin(s) (48, Table 1).

A comparison of the protein patterns between strains Fp 1 and Ot 32 would indicate the magnitude of the difference between the psychrophilic and thermophilic states. About 20 protein bands out of 43 differed in staining intensities when strain Fp 1, grown at 5°C, was compared with strain Ot 32, grown at 70°C (not shown).

Phospholipid composition. Based on comparisons of R_f values and staining behavior with reference lipids on thin-layer chromatography

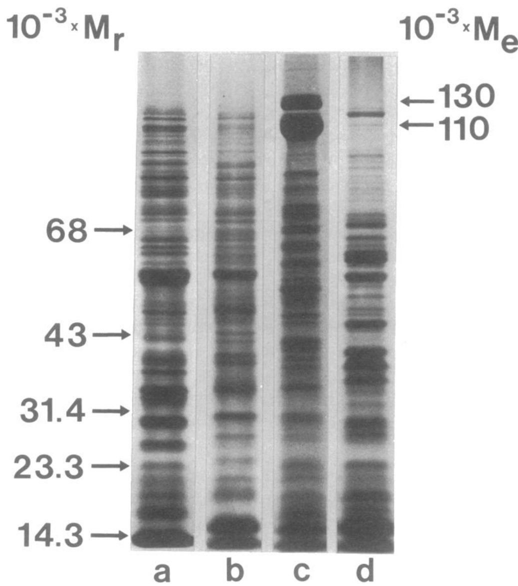

FIG. 1. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of B. megaterium membranes, prepared and solubilized as described in the text. Comparison of protein patterns for strains Fp1, facultative psychrophile (a); M 1, mesophile (b); Ft R32, facultative thermophile (c); and Ot 32, obligate thermophile (d). Each strain was grown close to its optimum temperature (Table 1). The positions of five molecular weight marker proteins are indicated by arrows on the left, and the estimated molecular weights of membrane proteins are indicated on the right.*

plates (24, 53; Materials and Methods), three phospholipids were found in the membranes of all strains and at all growth temperatures. These lipids were phosphatidylethanolamine, phosphatidylglycerol, and diphosphatidylglycerol. Neutral lipids were also present, but these were not further characterized.

Relative changes in phospholipid composition. In the obligate thermophile Ot 32 and the facultative thermophile Ft R32, the dominating phospholipid was phosphatidylethanolamine (Figure 2a and b). The fraction of this lipid increased slightly when the strains were grown at higher temperatures. In both strains, phosphatidylglycerol decreased and diphosphatidylglycerol increased with temperature, although these trends were more pronounced for strain Ft R32.

Diphosphatidylglycerol was the minor phospholipid component in the thermophilic strains with the exception of strain Ft R32 grown at 55°C (Fig. 2a and b). In the mesophile M 1 and the facultative psychrophile Fp 1, however, this phospholipid was the predominant one (Fig. 2c). For the latter strains, changes in phospholipid composition with temperature were not statistically significant. The diphosphatidylglycerol content fluctuated around a mean value of 57% (mol/mol), which was three to six times more than that in the thermophilic strains. The average contents of phosphatidylglycerol and phosphatidylethanolamine were 19 and 23% (mol/mol), respectively.

The lipid-to-protein ratio has been reported to decrease with increasing temperature in membranes of *B. stearothermophilus* (55). This trend can be considered logical, since a higher protein content may render the membrane more rigid and thus help the organism to maintain a functional membrane at higher growth temperatures

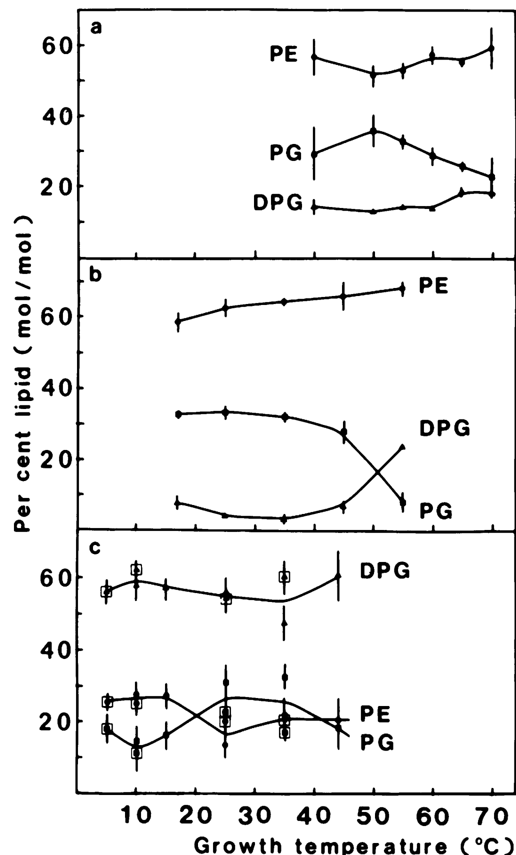

FIG. 2. *Effect of growth temperature on the relative distribution of membrane phospholipids from B. megaterium strains Ot 32, obligate thermophile (a); Ft R32, facultative thermophile (b); and M 1, mesophile/Fp 1 (framed symbols), facultative psychrophile (c). Abbreviations: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol. Values reported are the means of four determinations, representing two different cell batches and two analyses of each. Vertical bars indicate standard error.*

(42). However, the phospholipid-to-protein ratio increased over threefold for *B. megaterium* in the presently studied temperature interval (Fig. 3). Similar results have been reported also for the extreme thermophile *Thermus aquaticus*, which is gram-negative but has mainly branched fatty acids (41).

Distribution of fatty acids. The fatty acid methyl esters were analyzed qualitatively by gas-liquid chromatography on two columns with different stationary phases (Materials and Methods). The column containing diethylene glycol succinate gave the best resolution of individual peaks and was further used for the quantitative determinations.

The dominating fatty acids in the membranes of the four *B. megaterium* strains were six branched-chain acids—12-methyltridecanoic (iso-C_{14}), 13-methyltetradecanoic (iso-C_{15}), 14-methylpentadecanoic (iso-C_{16}), 15-methylhexadecanoic (iso-C_{17}), 12-methyltetradecanoic (anteiso-C_{15}), and 14-methylhexadecanoic (anteiso-C_{17}) acids—representing between 60 and 90% (mol/mol) of the total fatty acids, and four straight-chain acids—tetradecanoic (n-C_{14}), pentadecanoic (n-C_{15}), hexadecanoic (n-C_{16}), and octadecanoic (n-C_{18}) acids. In addition, a hexadecenoic acid (n-$C_{\overline{16}}$) was produced in significant amounts by the obligate thermophile Ot 32 when cultivated at temperatures close to the minimum. At 40°C this hexadecenoic acid made up 13% (mol/mol) of the fatty acid total in this strain.

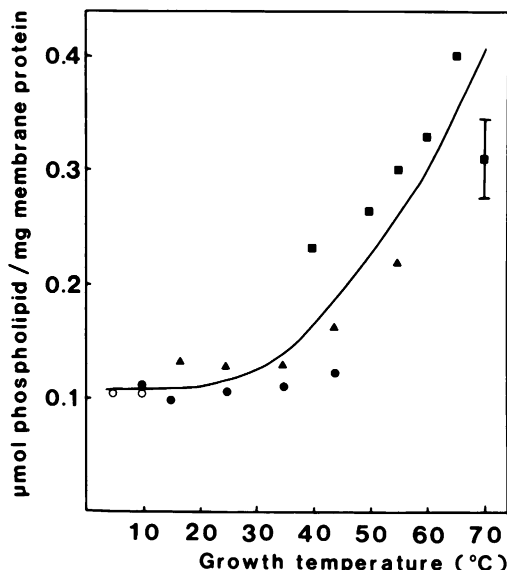

FIG. 3. *Effect of growth temperature on the ratio of micromoles of phospholipid to milligrams of membrane protein in membranes from B. megaterium. Values are shown for strains Fp 1 (○), facultative psychrophile; M 1 (●), mesophile; Ft R32 (▲), facultative thermophile; and Ot 32 (■), obligate thermophile. The curve represents the average trend. Standard error was 8 to 12% of the values (vertical bar).*

Since the odd-numbered (C_{15} and C_{17}) iso and anteiso acids were the qualitatively predominant ones, only results concerning these acids have been included in the graphs. Figure 4 shows the relative amounts of the branched C_{15}-acids at different growth temperatures. A comparison between strains reveals profound changes in the relative distribution of these acids. The total amount of the acids was approximately constant over the entire temperature interval investigated, but the proportion of iso-C_{15} rose markedly with increased growth temperature. A difference of 41% (mol/mol) was apparent between strain Fp 1 at 5°C and strain Ot 32 at 50°C. In the latter strain, iso-C_{15} then decreased by 15%

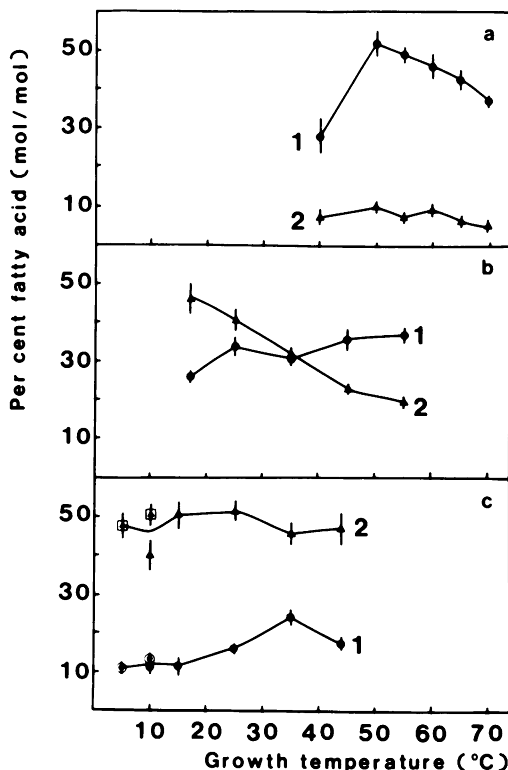

FIG. 4. *Effect of growth temperature on the relative distribution of branched-chain C_{15}-fatty acids (iso [1] and anteiso [2]) in membranes from B. megaterium strains Ot 32, obligate thermophile (a); Ft R 32, facultative thermophile (b); and M 1, mesophile/Fp 1 (framed symbols), facultative psychrophile (c). Values reported are the means of four determinations, representing two different cell batches and two analyses of each. Vertical bars indicate standard error.*

(mol/mol) when the temperature of growth was further raised from 50 to 70°C. Anteiso-C_{15} revealed a 43% (mol/mol) decrease when going from the lower to the higher end point of the temperature interval. The ratios of iso-C_{15} to anteiso-C_{15} increased from 0.23 to 0.40, from 0.56 to 1.87, and from 4.1 to 7.2 for strains Fp 1/M 1, Ft R32, and Ot 32, respectively. Thus, the total rise in this ratio function between 5 and 70°C was over 30-fold. The increase in growth temperature was responsible for a 10-fold rise, whereas the remainder was due to discontinuities between strains.

The trend for iso-C_{17} was similar to that noted for iso-C_{15} (Fig. 4), the increase being 20% (mol/mol) between the end points of the temperature interval (Fig. 5). The relative amount of anteiso-C_{17} also increased with growth temperature when the entire interval was considered, but it decreased within the growth ranges of strains M 1 and Ft R32. In strain Ot 32, the amount of anteiso-C_{17} reached the highest levels. The amount of the branched C_{17}-acids thus increased markedly in this strain, constituting 32% (mol/mol) of the fatty acid total at 70°C. In spite of the same general trend exhibited by these acids, an inversion in the relative amounts was apparent. The ratios of iso-C_{17} to anteiso-C_{17} increased from 0.11 to 0.27, from 0.26 to 3.8, and from 0.86 to 1.81 for strains Fp 1/M 1, Ft R32, and Ot 32, respectively, which corresponds to an over 15-fold rise in this parameter between the end points of the temperature interval.

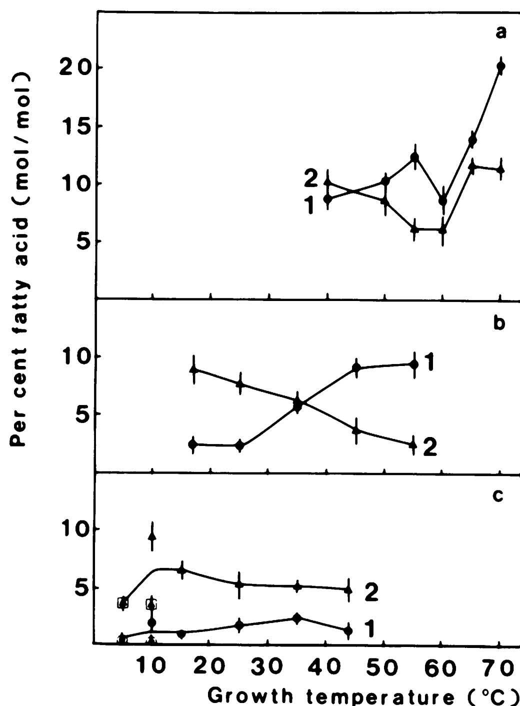

FIG. 5. *Effect of growth temperature on the relative distribution of branched-chain C_{17}-fatty acids (iso [1] and anteiso [2]) in membranes from B. megaterium strains Ot 32, obligate thermophile (a); Ft R32, facultative thermophile (b); and M 1, mesophile/Fp 1 (framed symbols), facultative psychrophile (c). Values reported are the means of four determinations, representing two different cell batches and two analyses of each. Vertical bars indicate standard error.*

The two remaining branched fatty acids, iso-C_{14} and iso-C_{16}, showed minor but consistent trends. The relative amounts of iso-C_{14} decreased from 7% (mol/mol) at 5°C to 1% at 70°C, and iso-C_{16} correspondingly increased from 1% (mol/mol) to 8% between the same temperatures (data not shown).

Evidently, iso acids were synthesized in relatively larger amounts at elevated temperatures (Fig. 4 and 5) than were their anteiso counterparts. Figure 6 shows how the ratio of all iso acids to all anteiso acids depends on growth temperature. A more than 10-fold difference in the value of this parameter between the end points of the investigated temperature interval is evident. This relation thus clearly indicates the important influence of melting points and the packing properties of the respective fatty acids in the functional adaptation of the membrane composition to environmental temperature.

Elongation of the acyl chains increases the fatty acid melting point (49). The fatty acids of the present *B. megaterium* strains may be classified into long- and short-chain groups. The former group includes all saturated C_{16}-, C_{17}-, and C_{18}-acids, and the latter includes all saturated C_{14}- and C_{15}-acids. There was about a fivefold increase of long-chain fatty acids over short-chain ones in the present temperature interval (Fig. 7). The ratios of iso-C_{17}/iso-C_{15}, anteiso-C_{17}/anteiso-C_{15}, and iso-C_{16}/iso-C_{14} increased from 0.04 to 0.55, from 0.08 to 2.2, and from 0.14 to 8.5, respectively, between 5 and 70°C.

DISCUSSION

The results presented have centered on only one aspect of temperature adaptation in these *B. megaterium* variants. Apart from changes in membrane composition, there is a genetic aspect to temperature adaptation. It was suggested (48) from data on the origin and reversion of these variants that plasmids could be involved in the phenomenon. The two high-molecular-weight proteins in strain Ft R32 (Fig. 1c) could be

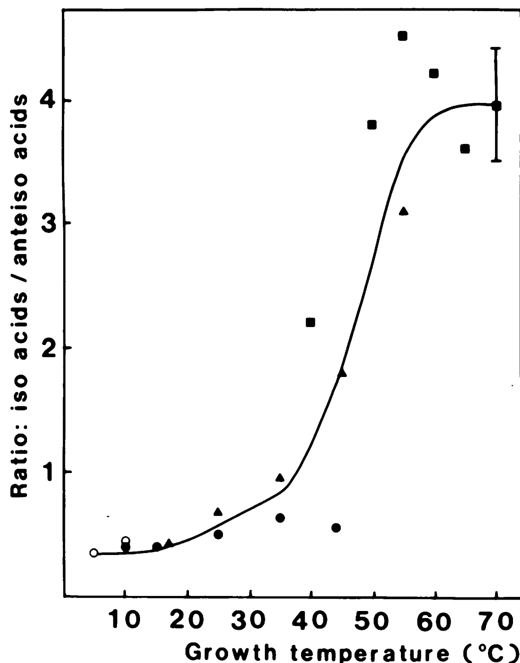

FIG. 6. *Effect of growth temperature on the ratio between relative amounts of iso and anteiso fatty acids in membranes from B. megaterium. Iso fatty acids represent the sum of iso-C_{14}, iso-C_{15}, iso-C_{16}, and iso-C_{17}, and anteiso fatty acids represent the sum of anteiso-C_{15} and anteiso-C_{17}. Values are shown for strains Fp 1 (○), facultative psychrophile; M 1 (●), mesophile; Ft R32 (▲), facultative thermophile; and Ot 32 (■), obligate thermophile. The curve represents the average trend. Standard error, calculated by propagation of error, was 7 to 10% of the values (vertical bar).*

plasmid determined. Iyer (17) reported substantial variations in the amounts of two proteins (34,000 and 36,500 daltons) located in the outer membrane of *E. coli* B/r when the organism harbored different antibiotic resistance plasmids. Similarly, a 24,000-dalton protein was found in membrane preparations from strains of *E. coli* K-12 carrying the F-factor and the derepressed plasmids R100-1 and R1-19 (32). More detailed comparisons are premature, since, to our knowledge, no information is available concerning membrane protein patterns in thermophilic bacteria.

The distribution of fatty acids in the mesophilic wild type M 1 corresponds to that reported for *B. megaterium* (19). The mesophilic *Bacillus* species may be divided into two groups based on their respective fatty acid compositions: one producing a high proportion of anteiso acids, with anteiso-C_{15} dominating, and the other producing odd-numbered iso acids, especially iso-C_{15}, in the largest amount (20). *B. megaterium* is usually included in the former group (23), which is even clearly possible for strain M 1 (Fig. 4c and 5c). However, in the obligate thermophile Ot 32, the odd-numbered iso acids are predominant (Fig. 4a and 5a), relating this strain to the second group. The transition in group relations occurs within the growth range of the facultative thermophile Ft R32. When grown below 35°C, it belongs to group one, but at temperatures above 35°C, it belongs to group two (Fig. 4b and 5b). The validity of such a classification may therefore be questioned. The thermophilic strains at high temperatures resemble *B. stearothermophilus* (8, 47, 56), *B. caldolyticus*, and *B. caldotenax* (52) in producing mainly odd-numbered iso acids.

The same increasing trend from the lower to the higher end point of the temperature interval was apparent for the ratios of both iso-C_{15}/anteiso-C_{15} and iso-C_{17}/anteiso-C_{17} (Fig. 4, 5). This trend appears logical when viewed in light of the different physical properties of the

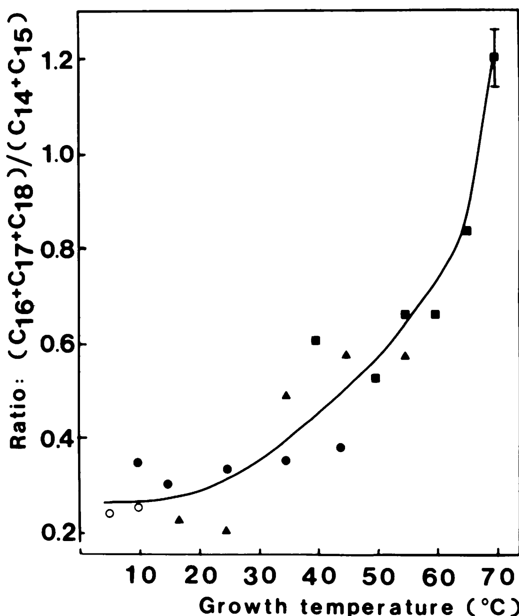

FIG. 7. *Effect of growth temperature on the ratio between relative amounts of long-chain and short-chain fatty acids in membranes from B. megaterium. Long-chain acids are represented by the sum of iso-C_{16}, n-C_{16}, iso-C_{17}, anteiso-C_{17}, and n-C_{18}, and short chain acids are represented by the sum of iso-C_{14}, n-C_{14}, iso-C_{15}, anteiso-C_{15}, and n-C_{15}. Values are shown for strains Fp 1 (○), facultative psychrophile; M 1 (●), mesophile; Ft R32 (▲), facultative thermophile; and Ot 32 (■), obligate thermophile. The curve represents the average trend. Standard error, calculated by propagation of error, was 5 to 8% of the values (vertical bar).*

fatty acids involved. The iso-C_{15}- and iso-C_{17}-acids have melting points similar to those of their straight-chain isomers, but their lateral packing areas are at least 1.5 times larger (54). The anteiso acids, on the other hand, have significantly lower melting points (about 25°C) than the corresponding straight-chain acids and occupy still larger lateral packing areas (54). At higher growth temperatures, the incorporation of the iso acids would be preferred to that of the anteiso acids, thus preventing the membrane from becoming too fluid and leaky at the higher temperatures. A deviation from the general tendency of increased amounts of iso-C_{15}-acids at higher growth temperatures was noted in strain Ot 32 between 50 and 70°C. Iso-C_{15} was, however, replaced primarily by iso-C_{17} and, to a smaller extent, by *n*-C_{16} and *n*-C_{18} in this interval, all acids having higher melting points than iso-C_{15}.

A decrease in the total amount of branched fatty acids with increasing growth temperature has been reported in several investigations of *Bacillus* species (4, 8, 44, 47, 52, 56). This trend was most obvious in *B. stearothermophilus* (8, 47, 56) and was, in this species, accompanied by a drastic increase in the amount of *n*-C_{16}. In strain Ot 32 grown between 50 and 70°C and in strain Ft R32, the total amounts of branched fatty acids decreased 8 and 15% (mol/mol), respectively, with concomitant increases in the amounts of *n*-C_{16} and *n*-C_{18}. However, the changes between branched-chain and straight-chain acids were neither the dominant ones nor the ones solely responsible for the observed alterations in membrane fatty acid composition with growth temperature. Instead, the changes between the two groups of branched-chain fatty acids, iso and anteiso, were of much greater magnitude in all strains. The high-melting iso acids seem to play the role of saturated acids and the low-melting anteiso acids seem to play that of unsaturated acids in their abilities to regulate membrane fluidity, when *B. megaterium* is compared to an organism synthesizing exclusively straight-chain acids. As seen in Fig. 4 through 6, each strain has the capacity to alter the ratio of iso acids to anteiso acids within certain limits, but this alteration is carried out at different ratios in the different strains. At a growth temperature common to all strains, for instance 40°C, the iso-C_{15}/anteiso-C_{15} ratios were 0.46, 1.26, and 4.1 for strains M 1, Ft R32, and Ot 32, respectively.

The synthesis of unsaturated acids by the genus *Bacillus* has been reported for *B. stearothermophilus* (8, 15, 56) and for a number of mesophilic and psychrophilic species (15, 21, 22). In strain Ot 32, the hexadecenoic acid together with *n*-C_{16} and iso-C_{16} replaced iso-C_{15} at 40°C.

When the hydrocarbon chain is elongated within a homologous series of fatty acids, the acids acquire higher melting points because of the additive London-van der Waals forces (49). The elongation process is thus another way to solidify the membrane lipids in *B. megaterium* at higher growth temperatures (Fig. 7). The phenomenon has been observed in other investigations as well (38, 44, 52).

The finding of phosphatidylethanolamine, phosphatidylglycerol, and diphosphatidylglycerol in the *B. megaterium* membranes is in agreement with earlier observations (2, 36, 37). Neither glycosaminyl nor lysyl derivatives of phosphatidylglycerol were synthesized under the presently used growth conditions. The pH of the culture during growth was between 6.5 and 7.5, and the glycosaminyl derivative is formed mainly at a pH around 5 (2, 36, 37).

The biosyntheses of phosphatidylethanolamine, on one hand, and phosphatidylglycerol and diphosphatidylglycerol, on the other, proceed along different pathways from the common precursor, CDP-diglyceride. These pathways have been established for *E. coli* as well as for some *Bacillus* species (14, 26, 40). In *E. coli*, the ratio between the relative amounts of phosphatidylethanolamine and phosphatidylglycerol/diphosphatidylglycerol is fairly constant in the temperature range of the organisms (5, 9, 45). We found small variations in this ratio within each *B. megaterium* strain and a drastic inversion when going from the mesophilic to the thermophilic state (Fig. 2). The different chemical and physical properties of the phospholipids may partly explain these results. There were no differences in fatty acid content in the individual phospholipids (unpublished results). The biophysical behavior of the lipids as determined by the polar headgroup is therefore of main interest. Phosphatidylethanolamine is known to have its phase transition temperature well above that of phosphatidylglycerol when the fatty acid content is identical in both lipids (6, 51). Phosphatidylethanolamine can accordingly be more densely packed, and an increased relative amount of this lipid in the membranes at higher growth temperatures would be favorable in maintaining membrane integrity. A similar trend has been noted in *Tetrahymena pyriformis* (31). Moreover, a sixfold increase in the ratio of phosphatidylethanolamine to phosphatidylcholine in membranes from mouse LM cells was accompanied by a markedly raised viscosity (12).

An additional explanation of the reversed phospholipid pattern in strains Fp 1 and M 1 as compared with the thermophilic strains is possible. Several respiratory chain enzymes require

primarily diphosphatidylglycerol to attain full activity (7, 11). The thermophilic strains are more anaerobic than the mesophilic ones (48). When grown under the same conditions of aeration, the latter strains probably synthesize greater amounts of the respiratory chain components and accordingly need more diphosphatidylglycerol. Op den Kamp et al. (37) cultivated *B. megaterium* MK 10D under strong aeration and found that this lipid made up only 5% (wt/wt) of the phospholipid total. It has been suggested that diphosphatidylglycerol contributes to the thermal stability of *B. stearothermophilus* membranes (34). However, in our *B. megaterium* strains, this function of the lipid is less probable, since it was the minor membrane phospholipid component in the thermophilic strains (Fig. 2).

It should be pointed out that profound changes in the investigated parameters occurred in the facultatively thermophilic strain Ft R32 between 35 and 50°C (Fig. 3 through 7). This temperature range corresponds to the boundary between bacterial mesophily and thermophily established for *B. licheniformis* (1).

The regulation of membrane lipid properties occurring in *B. stearothermophilus*, *B. caldolyticus*, and *B. caldotenax* (8, 47, 52, 56) cannot be considered as specific for thermophilic species, since a similar regulation mechanism is found in a thermophilic variant of an otherwise mesophilic *B. megaterium*. The genetic information for adaptation of the cell membrane composition to more or less extended thermophilic growth may thus be present in the *B. megaterium* wild-type population but is not expressed in this population under mesophilic growth conditions.

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LITERATURE CITED

1. **Bausum, H. T., and T. S. Matney.** 1965. Boundary between bacterial mesophilism and thermophilism. J. Bacteriol. **90**:50–53.
2. **Bertsch, L. L., P. P. M. Bonsen, and A. Kornberg.** 1969. Biochemical studies of bacterial sporulation and germination. XIV. Phospholipids in *Bacillus megaterium*. J. Bacteriol. **98**:75–81.
3. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**:911–917.
4. **Chan, M., Y. P. Virmani, R. H. Himes, and J. M. Akagi.** 1973. Spin-labeling studies on the membrane of a facultative thermophilic bacillus. J. Bacteriol. **113**:322–328.
5. **Cronan, J. E., Jr., and P. R. Vagelos.** 1972. Metabolism and function of the membrane phospholipids of *Escherichia coli*. Biochim. Biophys. Acta **265**:25–60.
6. **Cullis, P. R., and B. de Kruyff.** 1976. ^{31}P NMR studies of unsonicated aqueous dispersions of neutral and acidic phospholipids. Effects of phase transitions, p^{2}H and divalent cations on the motion in the phosphate region of the polar headgroup. Biochim. Biophys. Acta **436**:523–540.
7. **Dancey, G. F., and B. M. Shapiro.** 1977. Specific phospholipid requirement for activity of the purified respiratory chain NADH dehydrogenase of *Escherichia coli*. Biochim. Biophys. Acta **487**:368–377.
8. **Daron, H. H.** 1970. Fatty acid composition of lipid extracts of a thermophilic *Bacillus* species. J. Bacteriol. **101**:145–151.
9. **De Siervo, A. J.** 1969. Alterations in the phospholipid composition of *Escherichia coli* B during growth at different temperatures. J. Bacteriol. **100**:1342–1349.
10. **Dittmer, J. C., and R. L. Lester.** 1964. A simple, specific spray for the detection of phospholipids on thin-layer chromatograms. J. Lipid Res. **5**:126–127.
11. **Esfahani, M., B. B. Rudkin, C. J. Cutler, and P. E. Waldron.** 1977. Lipid-protein interactions in membranes. Interaction of phospholipids with respiratory enzymes of *Escherichia coli* membrane. J. Biol. Chem. **252**:3194–3198.
12. **Esko, J. D., J. R. Gilmore, and M. Glaser.** 1977. Use of a fluorescent probe to determine the viscosity of LM cell membranes with altered phospholipid compositions. Biochemistry **16**:1881–1890.
13. **Esser, A. F., and K. A. Souza.** 1974. Correlation between thermal death and membrane fluidity in *Bacillus stearothermophilus*. Proc. Natl. Acad. Sci. U.S.A. **71**:4111–4115.
14. **Finnerty, W. R., and R. A. Makula.** 1975. Microbial lipid metabolism. Crit. Rev. Microbiol. **4**:1–40.
15. **Fulco, A. J.** 1967. The effect of temperature on the formation of Δ^5-unsaturated fatty acids by bacilli. Biochim. Biophys. Acta **144**:701–703.
16. **Hartree, E. F.** 1972. Determination of protein: a modification of the Lowry method that gives a linear photometric response. Anal. Biochem. **48**:422–427.
17. **Iyer, R.** 1977. Plasmid mediated alterations in composition and structure of envelopes of *Escherichia coli* B/r. Biochim. Biophys. Acta **470**:258–272.
18. **Jergil, B., and R. Ohlsson.** 1974. Phosphorylation of proteins in rat liver. Endogenous phosphorylation and dephosphorylation of proteins from smooth and rough endoplasmatic reticulum and free ribosomes. Eur. J. Biochem. **46**:13–25.
19. **Kaneda, T.** 1967. Fatty acids in the genus *Bacillus*. I. Iso- and anteiso-fatty acids as characteristic constituents of lipids in 10 species. J. Bacteriol. **93**:894–903.
20. **Kaneda, T.** 1971. Factors affecting the relative ratio of fatty acids in *Bacillus cereus*. Can. J. Microbiol. **17**:269–275.
21. **Kaneda, T.** 1971. Major occurrence of cis-Δ^5 fatty acids in three psychrophilic species of *Bacillus*. Biochem. Biophys. Res. Commun. **43**:298–302.
22. **Kaneda, T.** 1972. Positional preference of fatty acids in phospholipids of *Bacillus cereus* and its relation to growth temperature. Biochim. Biophys. Acta **280**:297–305.
23. **Kaneda, T.** 1977. Fatty acids in the genus *Bacillus*: an example of branched-chain preference. Bacteriol. Rev. **41**:391–418.
24. **Kates, M.** 1972. Techniques of lipidology: isolation, analysis and identification of lipids, p. 269–610. *In* T. S. Work and E. Work (ed.), Laboratory techniques in biochemistry and molecular biology, vol. 3, part II. North-Holland Publishing Co., Amsterdam.
25. **Konings, W. N., A. Bisschop, M. Veenhuis, and C. A. Vermeulen.** 1973. New procedure for the isolation of membrane vesicles of *Bacillus subtilis* and an electron microscopy study of their ultrastructure. J. Bacteriol.

116:1456–1465.

26. **Larson, T. J., T. Hirabayashi, and W. Dowhan.** 1976. Phosphatidylglycerol biosynthesis in *Bacillus licheniformis*. Resolution of membrane-bound enzymes by affinity chromatography on cytidine diphospho-*sn*-1,2-diacylglycerol sepharose. Biochemistry 15:974–979.
27. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
28. **McClare, C. W. F.** 1970. An accurate and convenient organic phosphorus assay. Anal. Biochem. 39:527–530.
29. **McElhaney, R. N.** 1976. The biological significance of alterations in the fatty acid composition of microbial membrane lipids in response to changes in environmental temperature, p. 255–281. *In* M. R. Heinrich (ed.), Extreme environments: mechanisms of microbial adaptation. Academic Press Inc., New York.
30. **McElhaney, R. N., and K. A. Souza.** 1976. The relationship between environmental temperature, cell growth and the fluidity and physical state of the membrane lipids in *Bacillus stearothermophilus*. Biochim. Biophys. Acta 443:348–359.
31. **Martin, C. E., K. Hiramitsu, Y. Kitajima, Y. Nozawa, L. Skriver, and G. A. Thompson, Jr.** 1976. Molecular control of membrane properties during temperature acclimation. Fatty acid desaturase regulation of membrane fluidity in acclimating *Tetrahymena* cells. Biochemistry 15:5218–5227.
32. **Minkley, E. G., Jr., and K. Ippen-Ihler.** 1977. Identification of a membrane protein associated with expression of the surface exclusion region of the F transfer operon. J. Bacteriol. 129:1613–1622.
33. **Minnikin, D. E., and H. Abdolrahimzadeh.** 1971. Thin-layer chromatography of bacterial lipids on sodium acetate-impregnated silica gel. J. Chromatogr. 63:452–454.
34. **Mosley, G. A., G. L. Card, and W. L. Koostra.** 1976. Effect of calcium and anaerobiosis on the thermostability of *Bacillus stearothermophilus*. Can. J. Microbiol. 22:468–474.
35. **Neville, D. M., Jr.** 1971. Molecular weight determination of protein-dodecyl sulphate complexes by gel electrophoresis in a discontinuous buffer system. J. Biol. Chem. 246:6328–6334.
36. **Op den Kamp, J. A. F., U. M. T. Houtsmuller, and L. L. M. van Deenen.** 1965. On the phospholipids of *Bacillus megaterium*. Biochim. Biophys. Acta 106:438–441.
37. **Op den Kamp, J. A. F., W. van Iterson, and L. L. M. van Deenen.** 1967. Studies on the phospholipids and morphology of protoplasts of *Bacillus megaterium*. Biochim. Biophys. Acta 135:862–884.
38. **Oshima, M., and A. Miyagawa.** 1974. Comparative studies on the fatty acid composition of moderately and extremely thermophilic bacteria. Lipids 9:476–480.
39. **Overath, P., H. U. Schairer, and W. Stoffel.** 1970. Correlation of *in vivo* and *in vitro* phase transitions of membrane lipids in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. 67:606–612.
40. **Patterson, P. H., and W. J. Lennarz.** 1971. Studies on the membranes of bacilli. I. Phospholipid biosynthesis. J. Biol. Chem. 246:1062–1072.
41. **Ray, P. H., D. C. White, and T. D. Brock.** 1971. Effect of temperature on the fatty acid composition of *Thermus aquaticus*. J. Bacteriol. 106:25–30.
42. **Rottem, S., and A. Samuni.** 1973. Effects of proteins on the motion of spin-labeled fatty acids in *Mycoplasma* membranes. Biochim. Biophys. Acta 298:32–38.
43. **Shaw, N.** 1968. The detection of lipids on thin-layer chromatograms with the periodate-Schiff reagents. Biochim. Biophys. Acta 164:435–436.
44. **Shen, P. Y., E. Coles, J. L. Foote, and J. Stenesh.** 1970. Fatty acid distribution in mesophilic and thermophilic strains of the genus *Bacillus*. J. Bacteriol. 103:479–481.
45. **Sinensky, M.** 1974. Homeoviscous adaptation—a homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. 71:522–525.
46. **Skipski, V. P., and M. Barclay.** 1969. Thin-layer chromatography of lipids. Methods Enzymol. 14:530–598.
47. **Souza, K. A., L. L. Kostiw, and B. J. Tyson.** 1974. Alterations in normal fatty acid composition in a temperature-sensitive mutant of a thermophilic bacillus. Arch. Microbiol. 97:89–102.
48. **Ståhl, S., and O. Olsson.** 1977. Temperature range variants of *Bacillus megaterium*. Arch. Microbiol. 113:221–229.
49. **Tourtellotte, M. E.** 1972. Mycoplasma membranes: structure and function, p. 439–470. *In* C. F. Fox and A. D. Keith (ed.), Membrane molecular biology. Sinauer Associates, Inc., Stamford, Conn.
50. **Tsukagoshi, N., and C. F. Fox.** 1973. Abortive assembly of the lactose transport system in *Escherichia coli*. Biochemistry 12:2816–2822.
51. **Van Dijck, P. W. M., P. H. J. Th. Ververgaert, A. J. Verkleij, L. L. M. van Deenen, and J. de Gier.** 1975. Influence of Ca^{2+} and Mg^{2+} on the thermotropic behaviour and permeability properties of liposomes prepared from dimyristoyl phosphatidylglycerol and mixtures of dimyristoyl phosphatidylglycerol and dimyristoyl phosphatidylcholine. Biochim. Biophys. Acta 406:465–478.
52. **Weerkamp, A., and W. Heinen.** 1972. Effect of temperature on the fatty acid composition of the extreme thermophiles *Bacillus caldolyticus* and *Bacillus caldotenax*. J. Bacteriol. 109:443–446.
53. **Wieslander, Å., and L. Rilfors.** 1977. Qualitative and quantitative variations of membrane lipid species in *Acholeplasma laidlawii* A. Biochim. Biophys. Acta 466:336–346.
54. **Willecke, K., and A. B. Pardee.** 1971. Fatty acid-requiring mutant of *Bacillus subtilis* defective in branched-chain α-keto acid dehydrogenase. J. Biol. Chem. 246:5264–5272.
55. **Wisdom, C., and N. E. Welker.** 1973. Membranes of *Bacillus stearothermophilus*: factors affecting protoplast stability and thermostability of alkaline phosphatase and reduced nicotinamide adenine dinucleotide oxidase. J. Bacteriol. 114:1336–1345.
56. **Yao, M., H. W. Walker, and D. A. Lillard.** 1970. Fatty acids from vegetative cells and spores of *Bacillus stearothermophilus*. J. Bacteriol. 102:877–878.