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Change in Chemical Composition of Membrane of *Bacillus caldotenax* After Shifting the Growth Temperature

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Abstract. Membranes from *Bacillus caldotenax* contain neutral lipids and phospholipids such as phosphatidylethanolamine, phosphatidyl glycerol and cardiolipin. Each of the lipids has almost the same fatty acid composition. When the growth temperature decreases, not only the fatty acid composition but also the lipid composition changes such that the membrane fluidity increases, and the composition of membrane-bound proteins also changes. On shifting the growth temperature from 65° to 45° C, the bacterium grows immediately with a doubling time at 45° C, but the compositions of proteins and lipids in membranes gradually change and reach the compositions typical of cells growing at 45° C one doubling time after the temperature shift, respectively. It is concluded that the change in chemical composition of membrane of the bacterium on the temperature shift from 65° to 45° C is not prerequisite for growth at 45° C.

Key words: Thermophilic bacterium – Fatty acids – Lipids – Memebrane-bound proteins – Membrane fluidity – Shift of growth temperature.

Thermophilic bacteria as well as mesophilic bacteria have been shown to alter the fatty acid composition of membrane lipids on changing the growth temperature (Souza et al., 1974; Oshima and Miyagawa, 1974; McElhaney and Souza, 1976; McElhaney, 1976). Such a change in fatty acid composition has been considered to be prerequisite for maintaining the fluidity of membrane lipids nearly constant over entire growth temperature range, a process termed "Homeoviscous adaptation" (Sinensky, 1974). Shaw and Ingraham (1965) reported that *Escherichia coli* can grow with a doubling time at 10° C even when the bacterium possesses the fatty acid composition of cells growing at 37° C. This may indicate that the change in fatty acid composition in membrane lipids of *E. coli*, when transferred from 37° to 10° C, was not indispensable for the growth of the bacterium at 10° C. In order to examine whether the change in fatty acid composition of membrane lipids of a bacterium, when transferred from one growth temperature to another, is prerequisite for the growth at the new temperature, the change in chemical composition of lipids of a thermophilic bacterium, *Bacillus caldotenax*, was examined after shifting the growth temperature from 65° to 45° C, and compared the change with that in the growth rate after the temperature shift.

Materials and Methods

Microorganism and Culture. The thermophilic bacterium used in the present study was *Bacillus caldotenax*, strain YT-G, obtained from Dr. H. Zuber, Zürich. Solid (i) and liquid (ii) media of the following compositions were used (g/l of deionized water). (i) Trypticase, 10; Polypeptone, 5; Yeast extract, 3; Glucose, 2; and Agar, 20. (ii) Polypeptone, 10; Trypticase, 5; Yeast extract, 1; Glucose, 4; NaCl, 5; KH_2PO_4, 2; $MgSO_4 \cdot 7H_2O$, 1; and KNO_3, 1. The pH values of the media were 7.5.

The organisms from a fresh culture on solid medium (65° C, 14–16 h) were transferred in 1 l of the culture medium and incubated at 65° C under vigorous aeration, the pH being kept at 7.5 with 1 N NaOH. When the value of the optical density at 650 nm (OD_{650}) of the culture reached about 0.8, the cells were collected by centrifugation and used as the bacterium grown at 65° C. The organisms grown to a middle logarithmic phase at 65° C were transferred into a fresh medium at a cell concentration of $OD_{650} = 0.1$ and aerobically grown at 45° C. The organisms in a logarithmic phase ($OD_{650} = 0.8$) were used as the bacterium grown at 45° C.

When the analyses for chemical compositions of membranes were carried out after shifting the growth temperature from 65° to 45° C, the culture in early logarithmic phase ($OD_{650} = 0.3$) was rapidly cooled from 65° to 45° C within 2 or 3 min and aerobic incubation was continued at 45° C.

Preparation of Membranes. The organisms (wet weight, 1 g) were suspended in 80 ml of 20 mM phosphate buffer (pH 6.0) containing 0.4 M sucrose, 5 mM $MgCl_2$ and 0.9% NaCl, and 4 mg lysozyme

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(EC 3.2.1.17) was added. The mixture, after stirring at 37° C for 15 min, was centrifuged at 7,000 × *g* for 10 min. The precipitates were suspended in 80 ml of deionized water. After stirring the suspension with 25 µg deoxyribonuclease I (EC 3.1.4.5) for 5 min at room temperature, undisrupted cells and cell debris were removed by centrifugation and the resultant supernatant was centrifuged at 35,000 × *g* for 20 min. Membranes thus prepared were washed twice with 20 mM Tris-HCl buffer (pH 7.5) and suspended in the same buffer.

Analyses of Lipids. Total lipids were extracted with chloroform-methanol from freezed and dried cells accroding to Bligh and Dyer (1959). More than 95% of lipids were extracted as judged from the comparison of the amount of fatty acids in the extracted lipids with that in whole cells determined by the direct methanolysis of cells. The extracted lipids were subjected to thin layer chromatography on 0.5 mm Silica Gel (Merck, Type 60), using a solvent system of chloroform:methanol:water (70:30:5, v/v) according to Blank et al. (1964), and color was developed with I_2 vapor (Sims and Larose, 1962). Four I_2-positive spots were observed on the chromatogram, and one of the spots migrated almost at the solvent front. When the chromatogram was treated with α-naphthol specific for glycolipids (Siakotos and Rouser, 1965), only one small, faint blue spot was found at the solvent front. As compared the sizes and shades of color of the I_2- and α-naphthol-positive spots, it may be considered that neutral lipids and glycolipids co-chromatographed in the present developing solvent system, and the content of the former was higher than that of the latter. In the present paper, therefore, the designation of neutral lipids (NL) was used for the sum of neutral lipids and glycolipids.

The phospholipids of the thermophilic bacterium was identified by comparing their R_f values on the thin layer chromatogram, as described above, to those of the phospholipids from an alkalophilic *Bacillus* (Koyama and Nosoh, 1976). To confirm the identities, the chloroform-methanol soluble lipid fraction, after a mild deacylation according to Sawson (1960), was subjected to paper chromatography on Toyo Nr. 53 filter paper, using the two different solvent systems of phenol:water:acetic acid:ethanol (80:20:10:12, v/v) and phenol:water (80:20, v/v), according to Dawson (1960). The color was developed with a Hanes and Isherwood reagent (Hanes and Isherwood, 1949). The standard samples of deacylated phospholipids were prepared by the deacylated procedure as described above from caldiolipin (CL) from bovine heart, phosphatidylethanolamine (PE) from *Escherichia coli* and phosphatidyl glycerol (PG) from egg yolk.

The spots corresponding to PE and PG on the thin layer chromatogram were combined, extracted with the developing solvent (chloroform:methanol:water = 70:30:5, v/v), and subjected to rechromatography. NL and CL on the first chromatogram and PE and PG on the second chromatogram were recovered and quantitated as follows. Recovery of lipids was 100%, as judged from the comparison of the amounts of the fatty acids in total lipids before and after chromatography.

Fatty acid compositions of lipids were determined by gas chromatography with a Hitachi 103 gas chromatograph according to Oshima and Miyagawa (1974). The standard fatty acids were obtained from Dr. M. Oshima, Kanagawa. The relative amounts of neutral lipids and phospholipids (weight percent) were estimated by measuring the fatty acid contents of the lipids. Molar ratios of phospholipids were calculated by measuring the Pi contents of phospholipids according to Bartlett (1959).

Analysis of Membrane-Bound Proteins. The proteins were solubilized from membranes by treating with 1% sodium dodecyl sulfate (SDS) and 1% β-mercaptoethanol for 3 min in boiling water, and subjected to electrophoresis on polyacrylamide gel (5.6%) containing 0.1% SDS. Proteins were stained with Coomasie brilliant blue (Fairbanks et al., 1971). The gels were scanned at 560 nm with an Atago KEMIC densitometer.

Analyses for Membrane-Bound Enzymes. NADH, malate and cytochrome *c* oxidases were assayed by determining oxygen consumption of membranes in 20 mM Tris-HCl buffer (pH 7.5) containing 5 mM $MgCl_2$ and 0.9% NaCl with 1 mM NADH (Esfahani et al., 1977), 5 mM malate (Dawson and Chappell, 1978) and 2.5 mM ascorbate plus 0.25 mM tetramethyl-*p*-phenylenediamine (Dawson and Chappell, 1978) as substrates, respectively. Oxygen consumption was measured with a Bioxygraph (Kyusuikagaku Co., Tokyo), and activities were expressed as µl O_2 consumed/min · mg protein of membranes. Protein concentration was determined according to the method of Lowry et al. (1951).

Measurement of Polarization. To 3 ml of membrane suspension in 20 ml Tris-HCl buffer (pH 7.5) (final concentration, 0.24 mg protein/ml) was added 10 µl of 1,6-diphenyl-1,3,5-hexatriene (final concentration, 2 µM) in tetrahydrofuran. Fluorescence of the dye in membranes, when excited at 366 nm, was measured at 428 nm with a Shimadzu RF-502 recording spectrofluorimeter, and polarization (P) was determined according to Chen and Bowman (1965).

Chemicals. Tripticase was purchased from Difco Co.; polypeptone from Wako Pure Chemical Industries (Tokyo); yeast extract from Kyokuto Seiyaku Co. (Tokyo). Deoxyribonuclease, NADH, bovine heart CL, *E. coli* PE and egg yolk PG were purchased from Sigma and lysozyme from Seikagaku Co. (Tokyo). 1,6-Diphenyl-1,3,5-hexatriene was from Tokyo Kasei Co. (Tokyo). Other chemicals were reagent grade. Methanol, chloroform and phenol of reagent grade were redistilled before use.

Results and Discussion

Bacillus caldotenax exhibited a maximum growth at 65° C and no growth below 42° C under the present culture condition. Analyses of chemical compositions of membranes of the bacterium were then carried out with the organisms grown at 65° and 45° C.

Membranes of *B. caldotenax* grown at 65° and 45° C contained 10 and 17% of neutral lipids and 90 and 83% of phospholipids, respectively.

When the chloroform-methanol soluble fraction of the bacterium was subjected to thin layer chromatography in the solvent system of chloroform:methanol:water (70:30:5, v/v), four lipid components were separated. One of the components which migrated at the solvent front was found to be neutral lipids including glycolipids (NL, see Materials and Methods). The other iodine-positive spots having the R_f values of 0.78, 0.62 and 0.53 were shown to coincide with those of CL, PE and PG prepared from an alkalophilic *Bacillus* (Koyama and Nosoh, 1976). The three spots on the paper chromatograms of the phospholipids, after deacylation, had the R_f values of 0.60, 0.41 and 0.23 in the solvent system of phenol:water:acetic acid:ethanol (80:20:10:12, v/v) anf those of 0.52, 0.33 and 0.12 in the solvent system of phenol:water (80:20, v/v) and coincided with those of the standard deacylated phospholipids, PE, PG and CL, respectively. The head groups of phospholipids from the bacteria grown at 65° and 45° C were thus identified to be PE, PG and CL.

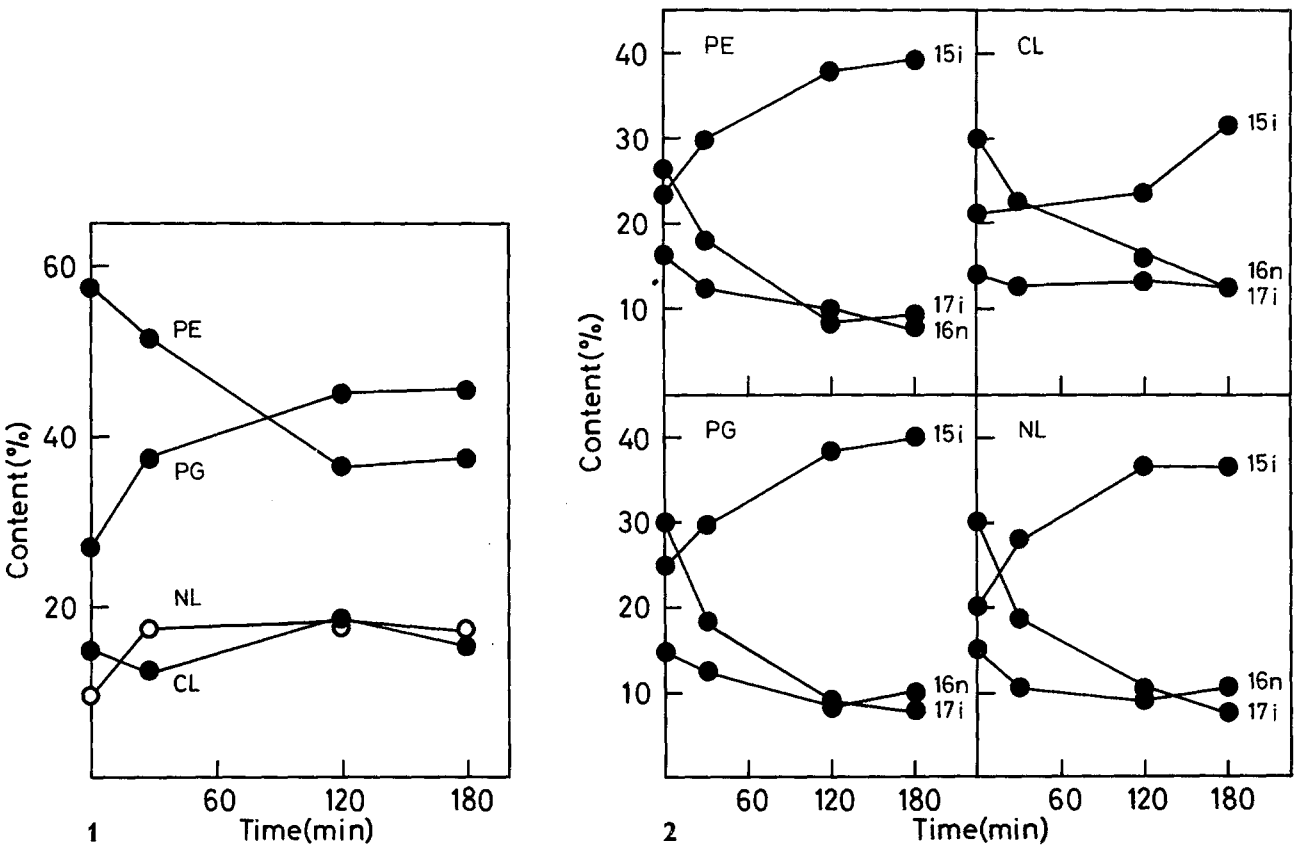

Fig. 1. Change in lipid composition of *Bacillus caldotenax* after transferring from 65° to 45° C. The NL content was expressed as weight percent of total fatty acids in total lipids. The contents of phospholipids (*PE*, *PG* and *CL*) were expressed as mol percent of total phospholipids.

Fig. 2. Change in fatty acid composition in each of the lipids of *B. caldotenax* after transferring from 65° to 45° C. The contents were expressed as mol percent of total fatty acids in each lipid

Molar ratios of PE, PG and CL in the bacteria grown at 65° and 45° C were estimated to be 58, 27 and 14% and 37, 46 and 17%, respectively, when expressed as percent of total phospholipids. Souza et al. (1974) determined the phospholipid composition of a thermophilic *Bacillus*, which was later identified as *B. stearothermophilus*, strain YTG-2 (Esser and Souza, 1974), at different temperatures. The contents of PE, PG and CL of *B. stearothermophilus* grown at 65° and 42° C were 27.6, 17.7 and 37.4% and 43.9, 44.4 and 7.6%, respectively, indicating an increase in both of the PE and PG contents accompanied by a decrease in the CL content on decreasing the growth temperature. In *B. caldotenax* , on the other hand, the PG content increased and the PE content decreased, although the CL content remained constant, when the growth temperature decreased. The melting points of PE and PG with the same fatty acid chain have been reported to be different (Oldfield and Chapman, 1972; Kimelberg and Papahadjopoulos, 1974), and the melting point of the former is 25° C higher than that of the latter. Such a change in phospholipid composition as observed with *B. caldotenax* may contribute to a decrease in the phase transition temperature of the membranes of the bacterium, when the growth temperature decreases.

The fatty acid composition of *B. caldotenax* grown at 65° and 45° C are shown in Table 1. Unsaturated fatty acids were not observed. Major fatty acids were palmitic acid (C_{16n}), 13-methyltetradecanoic acid (C_{15i}), 15-methylhexadecanoic acid (C_{17i}) and 14-methylhexadecanoic acid (C_{17a}). As in the cases of other thermophilic bacteria (Oshima and Miyagawa, 1974; Souza et al., 1974; McElhaney and Souza, 1976), the proportion of the relatively high melting point fatty acids (C_{16n} and C_{17i} in the present bacterium) decreased and that of the lower melting point fatty acids (C_{17a}) increased on decreasing the growth temperature. As shown in Table 1, the lipids (NL, PE, PG and CL) of *B. caldotenax* had essentially the same fatty acid composition, and the fatty acid composition in the lipids changed almost similarly on decreasing the growth temperature to 45° C.

Table 1. Fatty acid composition of each of the membrane lipids of *Bacillus caldotenax* grown at 65° and 45° C[a]

Fatty acid[b]	PE		PG		CL		NL	
	65°	45°	65°	45°	65°	45°	65°	45°
14n	4.1	2.5	3.6	3.2	3.9	4.2	4.6	2.9
15n	1.0	T[c]	1.1	T	2.0	1.7	3.1	1.8
16n	26.7	8.8	29.2	7.5	29.8	11.9	29.8	6.3
18n	3.7	6.0	2.1	4.7	3.9	6.0	T	2.5
14i	T	1.2	T	1.4	T	1.0	T	1.4
15i	23.6	39.1	24.4	39.3	21.9	31.8	20.3	36.3
16i	7.8	8.6	8.2	7.7	8.2	7.7	9.4	11.1
17i	17.3	8.2	15.9	10.0	14.5	11.8	15.7	11.3
15a	4.4	9.1	4.1	9.1	4.5	7.8	3.8	7.6
17a	11.7	15.1	10.3	16.3	10.6	14.8	13.3	15.5

[a] Averages of three determinations are given.

[b] Number of carbon atoms; N, normal; i, iso; a, anteiso.

[c] T = Trace, < 0.1%.

On shifting the growth temperature of *B. caldotenax* from 65° to 45° C, the composition of head groups and fatty acids (C_{16n}, C_{15i} and C_{17i}) in each lipid gradually changed and reached constant level typical of cells grown at 45° C about 120 min after the temperature shift, respectively (Figs. 1 and 2).

Bacterial membranes consists of lipids and proteins. As in the case of membrane lipids, the composition of membrane-bound proteins, especially of higher molecular weights, considerably changed on decreasing the growth temperature (Fig. 3). When the bacterium was transferred from 65° to 45° C, the composition of the membrane-bound proteins gradually changed, and attained the composition typical of the 45° C grown cells about 120 min after the temperature shift.

The value of the reciprocal of polarization (1/P) for a fluorescence dye in membranes has been considered to reflect the membrane fluidity (DeKruff et al., 1974; Cossins, 1977; Cossins and Prosser, 1978). The results

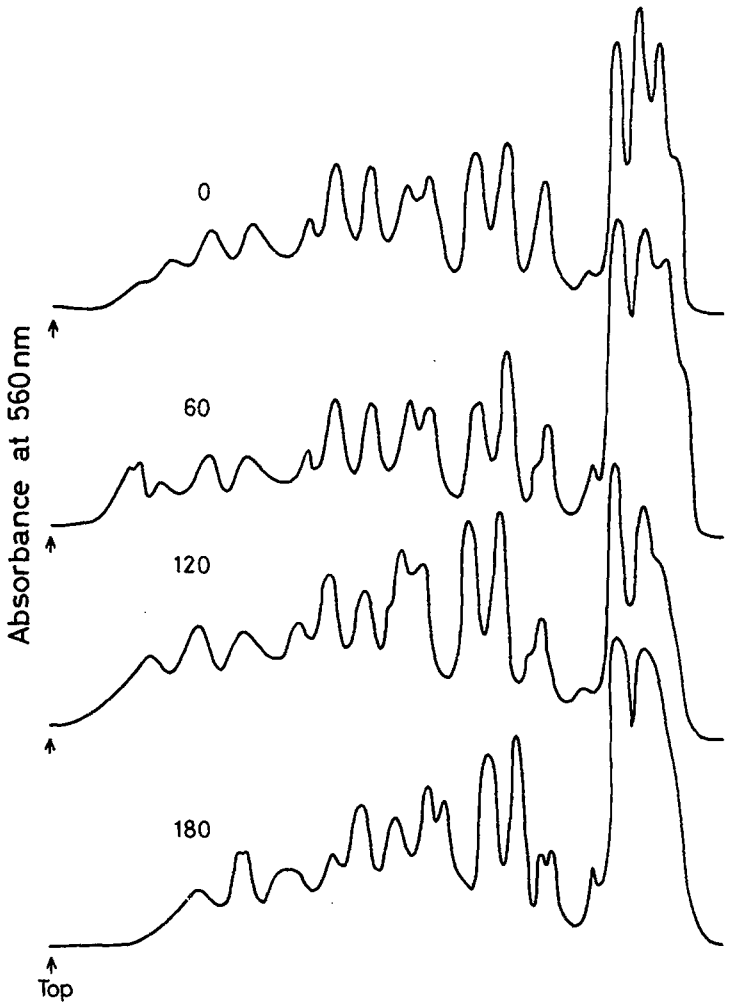

Fig. 3
Change in composition of membrane-bound proteins of *B. caldotenax* after transferring from 65° to 45° C. Electrophoresis was carried out after transferring from 65° to 45° C. Numeral on each curve indicates the time (min) after transferring from 65° to 45° C

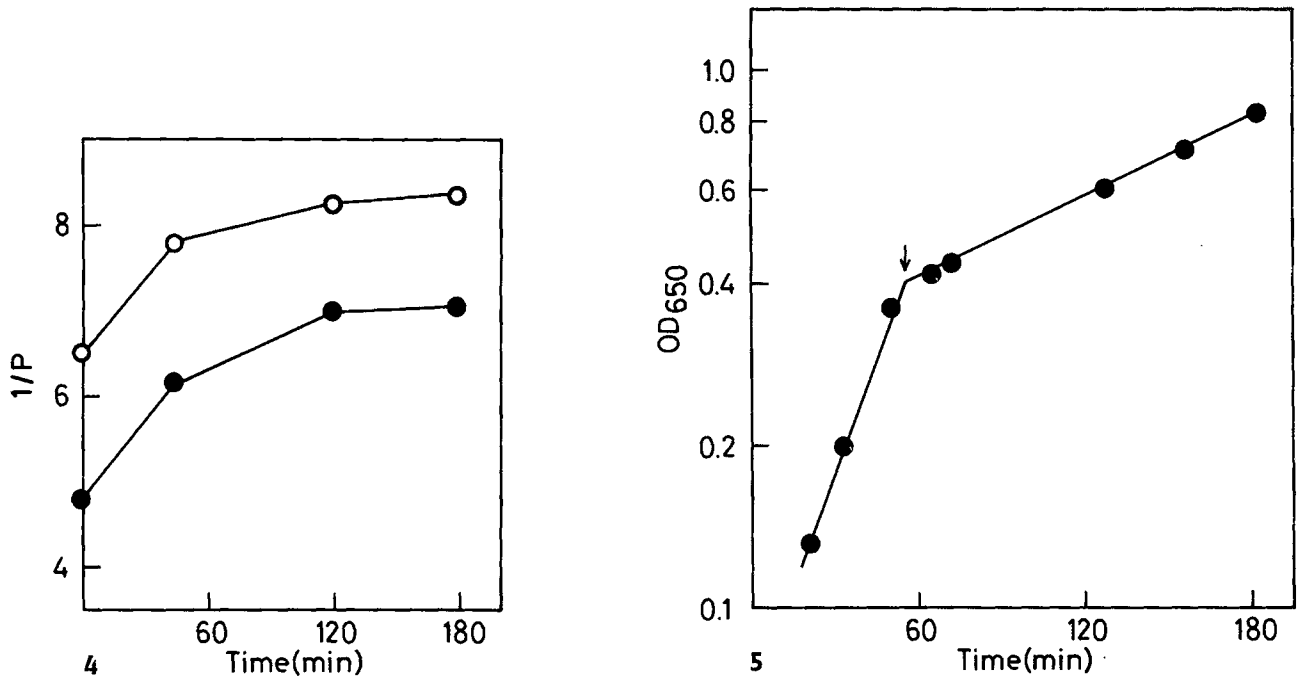

Fig. 4. Change in 1/P for 1,6-diphenyl-1,3,5-hexatriene in membranes from *B. caldotenax* after transferring from 65° to 45° C. 1/P was measured at 45° (●) and 65° C (○)

Fig. 5. Change in growth rate of *B. caldotenax* after transferring from 65° to 45° C. The growth temperature was changed from 65° to 45° C at the time indicated by an arrow

shown in Fig. 4 may indicate that the membrane fluidity of *B. caldotenax*, on shifting the growth temperature from 65° to 45° C, changed proportionally to the change in liquid composition and attained almost a constant level typical of the 45° C grown cells about 120 min after the temperature shift.

The membrane fluidity of thermophilic bacteria has been shown to be kept constant over growth temperature range by changing the fatty acid composition of membrane lipids (Souza et al., 1974; Oshima and Miyagawa, 1974; Esser und Souza, 1974; McElhaney and Souza, 1976). This indicates, as Esser and Souza (1974) demonstrated with the membrane lipids of *B. stearothermophilus* using the spin-label method, that the membrane fluidity of thermophilic bacteria is almost the same over the growth temperature range. The value of 1/P for 1,6-diphenyl-1,3,5-hexatriene in the membranes from the 65° grown cells (65° C-membrane) at 65° C was almost the same as that with the 45° C-membrane at 45° C (Fig. 4). This may indicate that the membrane fluidity of *B. caldotenax* was kept nearly constant over the growth temperature range by changing the head group composition (PE and PG) and fatty acid composition in each of the lipids (NL, PE, PG and CL).

As shown in Fig. 4, the 65° C-membrane was more rigid than the 45° C-membrane, when compared at the same temperature, for instance, at 45° C. If the homeoviscous adaptation is indispensable for the membrane function such as permeability and membrane-bound enzymes to exhibit a maximum activity, membrane function of the 65° C-membrane may be different from that of the 45° C-membrane at 45° C. As shown in Table 2, however, the activities of certain membrane-bound enzymes, NADH, malate and cytochrome *c* oxidases, of the 65° C-membrane at 45° (or 65° C) were almost the same as those of the 45° C-membrane at

Table 2. Activities of NADH, malate and cytochrome *c* oxidases in membranes from *B. caldotenax* grown at 65° (65°-membrane) and at 45° C (45°-membrane)

Enzyme		Activity[a] (O_2 µl/min · mg protein)	
		65°	45°
NADH oxidase	65°-membrane	380	132
	45°-membrane	325	129
Malate oxidase	65°-membrane	28	11
	45°-membrane	27	12
Cytochrome *c* oxidase	65°-membrane	209	136
	45°-membrane	233	141

[a] Activities were measured at 65° and 45° C

45° C (or 65° C), respectively. The membrane fluidity appears not affect the activities of the membrane-bound enzymes over the growth temperature range.

Escherichia coli, when transferred from 37° to 10° C in a glucose-containing medium, commenced to grow after a 4.5 h lag period (Shaw and Ingraham, 1965). During the lag, the fatty acid composition gradually changed and attained the composition typical of cells grown at 10° C at the end of the lag period. When the temperature shift was made after starvation for glucose at 37° C, no change in fatty acid composition occurred. Addition of glucose to the medium 4.5 h after the temperature shift resulted in an immediate growth at 10° C, although the fatty acid composition was still typical of cells grown at 37° C. The results indicate that the change in fatty acid composition was not prerequisite for the growth of *E. coli*, when transferred from 37° to 10° C. As shown in Fig. 5, *B. caldotenax* grew with a doubling time at 45° C immediately after transferring from 65° to 45° C. The composition of head groups and fatty acids of membrane lipids and also of the membrane-bound proteins of the bacterium, when transferring from 65° to 45° C, gradually changed and attained the levels of cells grown at 45° only 120 min (about one doubling time at 45° C) after the temperature shift, respectively. Although the physiological meaning of the change of the composition of membrane-bound proteins of *B. caldotenax* on transferring from 65° to 45° C is quite obscure at present, the change in lipid composition which is considered to determine the membrane fluidity is not prerequisite for the growth and the activities of certain membrane-bound enzymes of the thermophilic bacterium at 45° C.

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