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RELATION BETWEEN GROWTH TEMPERATURE OF *E. COLI* AND PHASE TRANSITION TEMPERATURES OF ITS CYTOPLASMIC AND OUTER MEMBRANES

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Summary

Cells of wild-type *E. coli* B were grown at 17, 27 and 37°C, and their cell membranes were fractionated into the cytoplasmic and the outer membranes. Chemical assay proved that the molar ratio of saturated to unsaturated fatty acids increases in phospholipids extracted from each membrane as the growth temperature increases.

The transition temperature at which the solid phase disappears was determined by X-ray diffraction in these biomembranes and also membranes of extracted phospholipids and of extracted lipopolysaccharide. The transition temperatures of the cytoplasmic membrane and of the membranes of phospholipids extracted from the cytoplasmic and the outer membranes increased with the growth temperature in good parallelism to the molar ratio of saturated to unsaturated fatty acids. The transition temperature of the outer membrane was less sensitive to the growth temperature, presumably due to the presence of lipopolysaccharide. The transition temperature of the membranes of lipopolysaccharide extracted from the outer membrane was 25°C, for the cells grown at 27 and 37°C. For the cells grown at 17°C, the extracted lipopolysaccharide gave a broad diffraction peak and did not exhibit a solid-fluid phase transition between —5 and 40°C.

Introduction

It is well known that *E. coli* performs temperature adaptation in its cell envelope, as demonstrated by studies of the effects of different growth tem-

peratures on the fluid properties of the total cell envelope [1], and as surveyed by Melchior and Steim [2]. The cell envelope of *E. coli*, like other Gram negative bacteria, is a complex composed of three morphologically distinct layers [3,4]: the cytoplasmic membrane, a rigid peptidoglycan layer external to the cytoplasmic membrane, and a second membraneous structure at the outermost surface of the cell called the outer membrane. Recently, several workers studied the effects of different growth temperatures on the fatty acid composition [5,6] and on the fluid properties [7] of the cytoplasmic and the outer membrane separately. These data are, however, not fully in accordance with each other. For instance, the phase transition temperature of the cytoplasmic membrane as determined by the ESR method [7] does not shift significantly with growth temperature, whereas the determined fatty acid composition [5,6] seems to suggest more drastic change of the transition temperature. Therefore, it seemed worthwhile to study the effects of different growth temperatures on each membrane by simultaneous determination of lipid composition and the phase transition temperature, in order to establish a basis for discussion on the mechanism of the temperature adaptation.

We fractionated the cytoplasmic and the outer membranes from wild-type *E. coli* B grown at 17, 27 and 37°C. Chemical assay of the phospholipid molecular species was made. X-ray diffraction was used to study phase transitions of the cytoplasmic and the outer membranes and also membranes of phospholipids extracted from each membrane and lipopolysaccharide extracted from the outer membrane. Results revealed a close correlation between growth temperature, phase transition temperature and phospholipid molecular species.

Materials and Methods

Bacterial growth

Wild-type *E. coli* B was grown up to an absorbance of 0.8—1.0 at 660 nm, i.e., in the mid-logarithmic phase, with vigorous aeration and agitation at 17, 27 and 37°C. The medium used was similar to medium A of Osborn et al. [8]: 1% (w/v) proteose peptone (Difco Lab.), 0.1% meat extract (Kyokuto Seiyaku Co.) and 0.5% NaCl (pH 7.5). Cells were harvested by centrifugation at 10 000 × *g* for 5 min at 0—4°C.

Membrane preparation

Following the procedure of Osborn et al. [8], membranes were prepared at 0—4°C (except when checking the effects of preparation temperature as described later). Cells were lysed by pouring a suspension of spheroplasts into the same volume of cold distilled water, followed by sonication five times for 20-s durations at 30-s intervals. Membranes were fractionated by continuous sucrose gradient centrifugation from 35% (w/w) (density 1.15 g/cm^3) to 50% (w/w) (density 1.23 g/cm^3) on a cushion of sucrose solution of 55% (w/w) (density 1.26 g/cm^3), all containing 5 mM EDTA (pH 7.5). Two distinct fractions were obtained: a light fraction at the density of 1.18 g/cm^3 and a heavy fraction at the density of 1.22 g/cm^3. During the work, we noticed as in the report by Letellier et al. [9] that several bands appeared

in the sucrose gradient when membranes of an *E. coli* fatty acid auxotroph were fractionated at low temperature. They concluded that phase separation resulted in membrane fractions with different density. Therefore, we examined whether membrane preparation at each growth temperature (17, 27 and 37°C) yielded results different from those at low temperature (0—4°C). Band patterns obtained were the same, independent of the preparation temperature. This disagreement with the results of Letellier et al. [9] may be attributed to the difference in bacterial strains. Since spheroplast formation and lysis at low temperature gave better yields, our data reported below were obtained from fractions prepared at 0—4°C.

Lipid extraction and chemical assay

Total lipids were extracted according to the method of Bligh and Dyer [10]. Phosphorus content was determined by using the method of Bartlett [11] assuming the average molecular weight of phospholipids to be 750. Protein concentration was determined by using the method of Lowry et al. [12] using bovine serum albumin as a standard. Lipopolysaccharide was extracted by using the method of Westphal and Jann [13]. Lipopolysaccharide content was estimated by the 2-keto-3-deoxyl octonose assay using the thiobarbituric acid test [14]. A standardization by dry weight of extracted lipopolysaccharide was performed. The distribution of cytochrome was estimated by difference spectroscopy at 427 nm using hydrosulfite-reduced and air-oxidized samples and a correction for base-line absorption. Extracted phospholipids were subjected to the silica gel thin-layer chromatography and gas-liquid chromatography as described by Kito et al. [15].

X-ray diffraction measurements

Each fraction was washed in 10 mM NaCl, 5 mM Tris-HCl (pH 7.5) twice and centrifuged at 200 000 × *g* for more than 12 h. The extracted lipids and lipopolysaccharide were dried and an appropriate amount of buffer solution was added. The resultant pellet was sealed into a plastic holder of 1 mm thickness with a thin aluminium window. Water content of the samples was 50—80% (w/w), measured by drying in vacuo over P_2O_5 for more than 3 days. X-rays were obtained from a Rigaku rotating-anode microfocus generator RU-3HM and nickel-filtered CuKα radiation (wavelength 1.542 Å) was used. The X-rays were focused with Elliot toroidal optics [16]. Scattered X-rays were detected using a position-sensitive proportional counter system of the Gabriel-Dupont type [17] with a gas-flow chamber made by Rigaku Denki Co. The counter chamber was set at an angle of 70° to the incident beam to prevent the high angle reflections from being broadened due to the inclined incidence. The X-ray path from specimen to the detector was evacuated to avoid air scattering. Diffraction spacings were calibrated with $Pb(NO_3)_2$ and sodium myristate powder patterns. Temperature of the specimens was controlled to within ±0.1°C by a flow of nitrogen gas which was produced by evaporation of liquid nitrogen and passage through a heater. Each X-ray measurement was carried out within 15 min. The background scattering was determined with the buffer solution mentioned above at each temperature. All the X-ray experiments were repeated at least twice starting from independent cultures.

Results

Chemical assay

As described above, we obtained two distinct membrane fractions for wild-type *E. coli* B: a light fraction of density 1.18 g/cm^3 and a heavy one of density 1.22 g/cm^3. They were subjected to chemical assays. As reported by Osborn et al. [8], cytochrome was found almost exclusively in the light fraction and much more lipopolysaccharide was present in the heavy fraction than in the light one, indicating that the light fraction corresponds to the cytoplasmic membrane and the heavy one to the outer membrane. Relative amounts of phospholipids, lipopolysaccharide and cytochrome in the two membranes are shown in Table I, which may be compared with Table II of Ref. 8. Fatty acid compositions of the two membranes are shown for three growth temperatures in Table II. They are quite similar to the case of *E. coli* K12 [5]. The bottom four lines give the sums of lipids which have the same degree of unsaturation or cyclopropane. 1,2-disaturated lipids increase and 1,2-diunsaturated lipids decrease with increasing growth temperature in both the cytoplasmic and the outer membranes, in accordance with the results reported on *E. coli* K12 [5,6]. The four bottom lines also show that the outer membrane has more 1,2-disaturated lipids and less 1,2-diunsaturated lipids than the cytoplasmic membrane at each growth temperature.

Lipid phase transition

Fig. 1 shows X-ray diffraction patterns by the outer membranes from 37°C-grown cells. The abscissa is an inverse of the spacing: $1/d = 2\sin\theta/\lambda$, where 2θ is the diffraction angle and λ is the wavelength (1.542 Å). A sharp reflection peak of d of 4.2 Å appeared at low temperatures but disappeared at about 31°C, above which only a broad peak of d of about 4.5 Å was present. This broad peak was present also below 31°C together with the sharp peak, giving a shoulder to the sharp peak in Fig. 1. This fact indicates coexistence of the solid (or ordered) and the fluid (or disordered) phases, as generally observed in membranes or multicomponent lipids (e.g., Ref. 18). We shall call the temperature at which the 4.2 Å peak disappears the transition temperature and denote it as T_f. This is the temperature at which all the lipid

TABLE I

COMPOSITION OF MEMBRANE FRACTIONS

Components	17°C-grown		27°C-grown		37°C-grown	
	Cytoplasmic	Outer	Cytoplasmic	Outer	Cytoplasmic	Outer
Phospholipids (mg/mg protein)	0.89	0.53	1.00	0.71	0.84	0.43
Lipopoly-saccharide (mg/mg protein)	0.20	0.93	0.25	1.01	0.20	0.98
Cytochrome (A_{427}/mg protein)	0.26	—	0.23	—	0.21	0.01

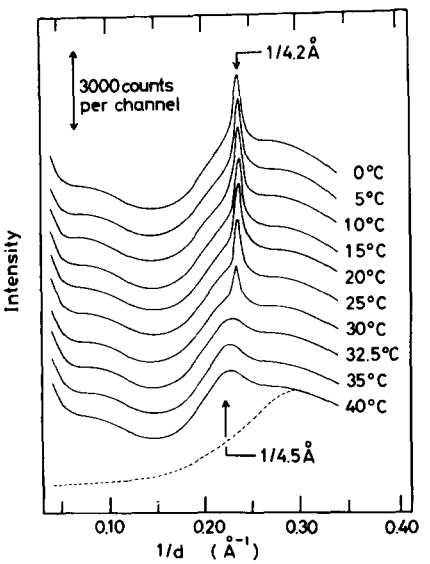
TABLE II

FATTY ACID MOLECULAR SPECIES DISTRIBUTION IN PHOSPHOLIPIDS

Molecular species C-1 C-2 *	17° C-grown		27° C-grown		37° C-grown	
	Cytoplasmic	Outer	Cytoplasmic	Outer	Cytoplasmic	Outer
16 : 1-16 : 1	3	3	1	1	1	0
18 : 1-16 : 1	20	14	22	12	9	2
18 : 1-18 : 1	20	14	20	12	5	3
16 : 0-16 : 1	23	24	28	21	22	8
16 : 0-18 : 1	34	39	22	39	34	28
16 : 0-14 : 0	—	2	2	6	6	14
16 : 0-16 : 0	—	1	1	3	1	—
16 : 0-17 : 0∇**	—	2	4	6	21	36
16 : 0-19 : 0∇	—	1	0	0	1	9
1.2-disaturated 1-saturated, 2-unsaturated	—	3	3	9	7	14
	57	63	50	60	56	36
Cyclopropane ***	—	3	4	6	22	45
1,2-diunsaturated	43	31	43	25	15	5

* C-1, position 1 of *sn*-glycerol-3-phosphate; C-2, position 2 of *sn*-glycerol-3-phosphate.
** ∇, cyclopropane.
*** 1-saturated, 2-cyclopropane fatty acyl species.

bilayer in the membrane turns into the fluid state, and thus it is different from the transition temperature defined as the center of the transition ranges in Refs. 2 and 7. Shechter et al. [19] observed that the fluid phase persists at low temperatures for a fatty acid auxotroph supplemented with unsaturated



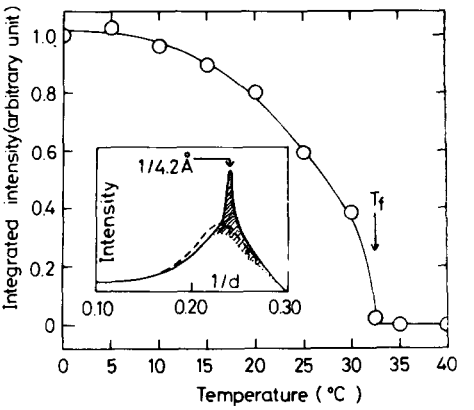


Fig. 1. X-ray diffraction patterns from outer membranes of 37° C-grown *E. coli* cells observed at various temperatures. Recorded with a position-sensitive detector. d, spacing ($1/d = 2\sin\theta/\lambda$). The dotted curve indicates background scattering by buffer solution at 40° C.

Fig. 2. Temperature dependence of integrated intensity of the 4.2 Å peak in Fig. 1. T_f, transition temperature. The inset shows the definition of the integrated intensity (see text).

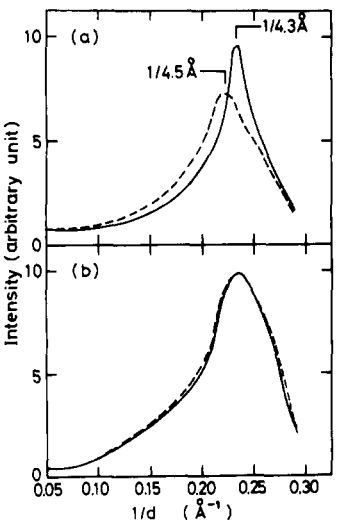
TABLE III

TRANSITION TEMPERATURE OF VARIOUS MEMBRANES

Growth temperature (°C)	Transition temperature (°C)				
	Membrane fraction		Phospholipids		Lipopoly-saccharide
	Cytoplasmic	Outer	Cytoplasmic	Outer	
17	10	23	10	23	—
27	15	23	12.5	27.5	25
37	28.5	31.5	27.5	45	25

fatty acids. Taking this into account, we define the integrated intensity as the shaded area in the inset of Fig. 2, where the background (the dotted line) was drawn by appropriately scaling the 4.5 Å peak at 40° C (the broken line) and assuming that the tail of the 4.2 Å peak becomes zero at $1/d = 0.20$ and 0.30 $Å^{-1}$. Fig. 2 shows how the integrated intensity of the 4.2 Å peak depends upon temperature. The temperature where integrated intensity becomes zero corresponds to T_f. This temperature itself could be determined also by visual inspection of disappearance of the 4.2 Å peak on such a set of diffraction curves as shown in Fig. 1. The curves were recorded at 2.5 Å intervals in the vicinity of T_f, so that T_f could be determined to within 2.5° C in general. The difference of T_f for the different experiments starting from independent cultures was less than 5° C. The average values are given in Table III.

Formation of bilayers by lipopolysaccharide in water was observed by Burge and Draper [20]. When the lipopolysaccharide extracted from the



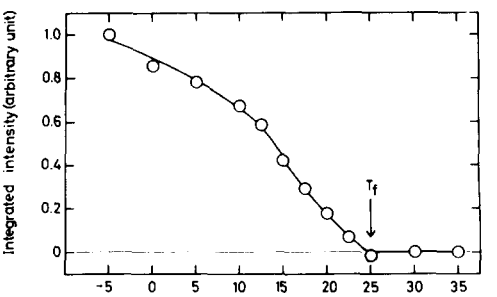


Fig. 3. Diffraction patterns by lipopolysaccharide in buffer solution. The lipopolysaccharide was extracted from the outer membranes of 27° C-grown cells (a) and of 17° C-grown cells (b). Solid curve was obtained at 30° C, and the broken curve at —5° C (a) and 0° C (b). d, spacing ($1/d = 2 \sin\theta/\lambda$).

Fig. 4. Temperature dependence of the integrated intensity of the 4.3 Å peak in Fig. 3a.

outer membranes of the 27°C- and 37°C-grown cells was dispersed into the buffer solution, a sharp peak appeared having a d value of 4.3 Å at low temperatures (Fig. 3a) as observed by Emmerling et al. [21]. The sharp peak, however, had a slightly larger width than that of lipid bilayers suggesting that arrangement of hydrocarbon chains is less ordered in the lipopolysaccharide bilayers than in phospholipid bilayers. Comparison of the diffraction peaks revealed that the peak of the outer membrane had more similarity to that of the extracted phospholipids than that of the extracted lipopolysaccharide. Fig. 4 shows the integrated intensity of the 4.3 Å peak as a function of temperature for the lipopolysaccharide extracted from the outer membranes of the 27°C-grown cells. The transition temperature, T_f, determined by such plots (with the help of the visual inspection of diffraction curves) was 25°C for both the 27°C- and 37°C-grown cells. Lipopolysaccharide extracted from the 17°C-grown cells gave a peak in the range of d of 4.3—4.4 Å (Fig. 3b). Its profile was similar to the 4.5 Å peak in other cases, as shown in Fig. 3b. Its position and profile were, however, practically independent of temperature in the examined range of —5 to 40°C.

Discussion

Fig. 5 shows molar ratios of saturated, unsaturated and cyclopropane hydrocarbon chain calculated from Table II for the cytoplasmic and the outer membranes. The molar ratios of saturated chains increase with the growth temperature (denoted below as T_g) in both membranes, implying that the sum of the unsaturated and cyclopropane chains decreases. The molar ratios

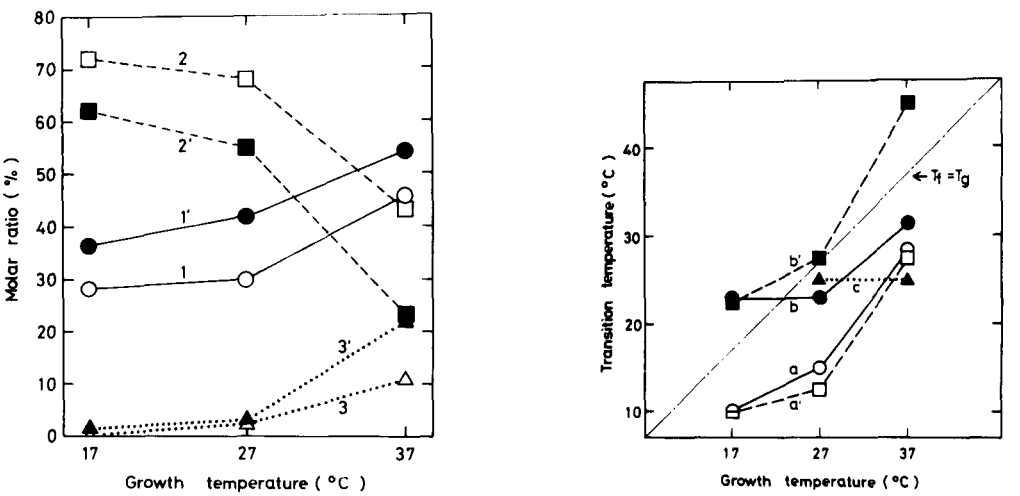


Fig. 5. Molar ratios of saturated, unsaturated and cyclopropane hydrocarbon chains in the cytoplasmic and the outer membranes as functions of growth temperature. ○, saturated, cytoplasmic; ●, saturated, outer; □, unsaturated, cytoplasmic; ■, unsaturated, outer; △, cyclopropane, cytoplasmic; ▲, cyclopropane, outer.

Fig. 6. Transition temperatures of membranes as functions of growth temperature. ○, cytoplasmic membrane; ●, outer membrane; □, phospholipids extracted from the cytoplasmic membrane; ■, phospholipids extracted from the outer membrane; ▲, lipopolysaccharide extracted from the outer membrane.

of unsaturated chains markedly decrease in both membranes with increasing T_g. It was reported [22] that the cyclopropane is derived from the double bond. Therefore, the decrease in unsaturated chains seems to be caused partly by reduction of their biosynthesis, and partly by an increase in cyclopropane chains. Fig. 6 illustrates how the transition temperatures, T_f, listed in Table III depend upon T_g in the five membranes. Unsaturated hydrocarbon chains of lipids have a tendency to disturb the ordered arrangement of saturated hydrocarbon chains in lipid membranes and to lower T_f, as demonstrated in synthetic lipid membranes [18,23]. Cyclopropane hydrocarbon chains are expected to have a similar tendency due to their steric heterogeniety. Taking these into account, the conclusions of our present study may be summarized as follows.

(1) T_f increased with T_g in the membranes of lipids extracted from the cytoplasmic and the outer membranes (curves a′ and b′ in Fig. 6) in accordance with increase in the molar ratio of saturated hydrocarbon chains as shown by curves 1 and 1′ in Fig. 5. These increases were larger between 27 and 37°C than between 17 and 27°C in each these four curves. One possible explanation for this might be that the ability of temperature adaption of *E. coli* decreases as the temperature approaches the lower temperature limit of growth (8°C [24]).

(2) T_f of the membrane of lipids extracted from the outer membrane is higher than that from the cytoplasmic membrane (curves a′ and b′ in Fig. 6) in accordance with the fact that the molar ratio of the saturated hydrocarbon chain is larger in the outer membrane than in the cytoplasmic membrane (curves 1 and 1′ in Fig. 5).

(3) The differences $T_g - T_f$ are all positive (7, 12 and 8.5°C for T_g of 17, 27 and 37°C, respectively) in the cytoplasmic membrane (curves a and $T_f = T_g$ in Fig. 6). Naturally, this means that the whole part of the cytoplasmic membrane is in the fluid state at the growth temperatures. One of the aims of regulation of molecular species of lipid might be to control fluidity of the bilayer part of the cytoplasmic membrane rather than the outer membrane.

(4) Fig. 6 shows that there is fairly good parallelism between T_f of the cytoplasmic membrane and extracted lipid membrane (curves a and a′), whereas the parallelism is not so good in the case of the outer membrane (curves b and b′). Lipopolysaccharide may suppress the change of T_f, because extracted lipopolysaccharide has a constant T_f in the growth temperature range of 27—37°C, and the discussion in the next item (5) suggests some intermixing occurred between hydrocarbon chains of lipopolysaccharide and of phospholipids in the outer membrane.

(5) The extracted lipopolysaccharide gave a characteristic 4.3 Å peak (Fig. 3a) with wider peak-width than in the case of phospholipid. The outer membrane itself did not give such a peak (Fig. 1), and profile of its 4.2 Å peak was quite similar to that of extracted phospholipids, suggesting that there is some intermixing between hydrocarbon chains of lipopolysaccharide and of phospholipids in the outer membrane.

(6) Several authors [25,26] reported that lipopolysaccharide molecules obtained from *E. coli* have substantially no unsaturated hydrocarbon chains. Therefore, it is understandable that we observed the same transition temper-

ature (25°C) in the 27°C- and 37°C-grown cells (curve c in Fig. 6). Lipopolysaccharide from the outer membrane of the 17°C-grown cells gave peculiar peaks as shown in Fig. 3b and exhibited no phase transition. One possibility is that lipopolysaccharide formed bilayers which were in a fluid-like state in the examined temperature range of −5 to 40°C. Another is that it formed some structure other than a bilayer with prevailing spacing of about 4.3—4.4 Å.

As cited in Introduction, Lugtenberg and Peters [6] and Ishinaga et al. [5] determined the fatty acid composition of phospholipids in the cytoplasmic and the outer membranes of *E. coli* K12 grown at different temperatures. Their results show the same tendency as ours on the strain B in the following respects: saturated hydrocarbon chains in phospholipids increase with growth temperature in both membranes, and more saturated chains are present in the outer membrane than in the cytoplasmic membrane at each growth temperature.

Melchior and Steim [2] studied the phase transition in the cytoplasmic and the outer membranes of *E. coli* W945 with differential scanning calorimetry. They defined the transition temperature by the peak position on the differential heat flow vs. temperature curve. Peaks were observed at 5—10°C and 25°C for 20°C-grown cells. They could be compared with T_f of the 17°C-grown cells in our case: 10°C for the cytoplasmic membrane and 23°C for the outer membrane. More data, however, will be needed for more detailed discussion.

Recently Janoff et al. [7] have studied how the transition temperature T_f (T_U by their notation) changes with the growth temperature in the outer and the cytoplasmic membranes of *E. coli* K12 using an ESR probe. Their results, however, are quite different from ours: T_f of the outer membrane is higher than ours by 10—15°C and T_f of the cytoplasmic membrane is almost constant (about 24°C), irrespective of the growth temperatures (12, 20, 37 and 43°C), compared to our results shown in Fig. 6. It is difficult to see what caused this discrepancy. Our results were reproducible: all the X-ray measurements were repeated twice starting from independent cultures, resulting in similar results. The molar ratio of the saturated fatty acid depended upon the growth temperature in good parallelism with the X-ray-determined transition temperature as shown in Figs. 5 and 6. Also our results of fatty acid composition are in accordance with those by Ishinaga et al. [5] and by Lugtenberg and Peters [6].

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