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IDENTIFICATION AND CHARACTERIZATION OF A 9-*CIS*-HEXADECENOIC ACID *CIS-TRANS* ISOMERASE FROM A PSYCHROTROPHIC BACTERIUM, *PSEUDOMONAS* SP. STRAIN E-3

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Abstract: A cell-free extract of *Pseudomonas* sp. strain E-3 (*Pseudomonas* E-3) had activities that catalyzed the conversion of 9-*cis*-hexadecenoic acid [16:1(9c)] to 9-*trans*-hexadecenoic acid [16:1(9t)] in the free acid form, and when 16:1(9c) was esterified to phosphatidylethanolamine (PE). A soluble 16:1(9c) *cis-trans* isomerase (9-Iase) was purified to complete homogeneity from the extract of *Pseudomonas* E-3 and characterized. Electrophoresis on both denaturing and incompletely-denaturing polyacrylamide gels of the purified enzyme preparation showed the single band of a protein with a molecular mass of 80 kDa, suggesting that the 9-Iase is a monomeric protein of 80 kDa. The 9-Iase, assayed with 16:1(9c) as a substrate, had a specific activity of 22.8 μmol per h per mg of protein and a K_m of 118 μM. The enzyme had the optimum temperature for catalysis at 30°C and catalyzed the *cis* to *trans* conversion of a double bond of 16:1(9c) in the free acid form, but it was able to isomerize 16:1(9c) esterified to PE in the presence of the cell membrane fraction.

Irrespective of the temperature at which cells of *Pseudomonas* E-3 were grown, the level of 16:1(9t) was around 2–4% of the total cellular fatty acids. However, when cells grown at 4°C were warmed up to 30°C at a rate of about 20°C/min, the level of 16:1(9t) was increased from 3% to 14%. Since the level *in situ* of free fatty acids in this bacterium is negligible, it is suggested that the 9-Iase is operative *in vivo* as the *cis* to *trans* isomerase of 16:1(9c) that is esterified to PE together with the membranous factor, and that the 9-Iase might work as a stringent modulator of membrane fluidity under abrupt alteration in growth temperature.

1. Introduction

Some bacterial strains contain substantial levels of mono-unsaturated fatty acids with a *trans* double bond (OKUYAMA *et al.*, 1990; HEIPIEPER *et al.*, 1992). When the psychrophilic bacterium *Vibrio* sp. strain ABE-l (*Vibrio* ABE-l) is grown at 20°C, the upper limiting temperature for growth of this bacterium, 16:1(9t) accounts for up to 12% of the total fatty acids in the membrane phospholipids (OKUYAMA *et al.*, 1990). By contrast, only a trace amount of 16:1(9t) is found in cells grown below 5°C. When cells of *Vibrio* ABE-l grown at 5°C were transferred to 20°C in the presence of cerulenin, an inhibitor of the synthesis *de novo* of fatty acid, the level of 16:1(9t)

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increased with a concomitant decrease in that of 16:1(9c). From these results we suggested that 16:1(9t) is synthesized by the *cis* to *trans* isomerization of 16:1(9c) in this bacterium (OKUYAMA *et al.*, 1991). In a previous paper (MORITA *et al.*, 1993) we presented evidence for the direct interconversions of 16:1(9c) and 16:1(9t) in cells of *Vibrio* ABE-1 that were supplied exogenously with stable tracers, namely, [2,2-$^2H_2$]16:1(9c) and [2,2-$^2H_2$]16:1(9t). DIEFENBACH *et al.* (1992) suggested the *cis* to *trans* conversion of double bonds in mono-unsaturated fatty acids in the phenol-degrading bacterium *Pseudomonas putida*. However, no biochemical information is available about *cis-trans* isomerases, except for reports on the isomerases that catalyze the *cis* to *trans* isomerization of double bonds with the shifting of double bond positions (MÜLLER-NEWEN and STOFFEL, 1993; CHEN *et al.*, 1994; KEPLER and TOVE, 1967). These isomerases are characteristic of enzymes that catalyze the *cis* to *trans* isomerization of double bonds with the shifting of double bond positions.

Significantly high rates of *cis* to *trans* isomerization were associated with the cell-free extract of the psychrotrophic bacterium *Pseudomonas* sp. strain E-3 (*Pseudomonas* E-3; see below), which is another organism that contains 16:1(9t) (OKUYAMA *et al.*, 1990). In this report, the identification and intracellular distribution of *cis-trans* isomerases in *Pseudomonas* E-3 and the purification of the enzyme are described.

2. Materials and Methods

2.1. Growth and processing of cells

Pseudomonas E-3 was grown with shaking at 20°C in a synthetic medium free of fatty acids (OKUYAMA *et al.*, 1990). Cells in the late-exponential phase of growth were harvested by centrifugation at 9000× g for 10 min and washed three times with 50 mM NaCl by centrifugation. Then 30 g of wet cells from 3000 m*l* of culture were suspended at 2 g/m*l* in 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM KCl, 5.0 mM $MgCl_2$ and 100 mM glycerol (referred to hereafter as medium G) and cells were ruptured in a French pressure cell at 2000 psi. Cell debris was removed by centrifugation at 10000× g for 20 min and a portion of the supernatant was used as the cell-free extract, and the remaining portion was centrifuged at 105000× g for 90 min. The supernatant was used as the cytosolic fraction. The pellet was suspended in medium G and homogenized with a Teflon homogenizer and the resultant suspension was centrifuged again at 105000× g for 90 min. The pellet was resuspended at 10 mg protein/m*l* in medium G and homogenized. This homogenate was used as the membrane fraction.

2.2. Shifting in growth temperatures

A fifty-milliliter culture of *Pseudomonas* E-3 grown at 4°C was tranferred to a 60°C-water bath and warmed to 30°C with vigorous mixing. It took about 1.3 min for the temperature of the culture to reach 30°C; then the culture was incubated at 30°C in a 30°C-water bath. One-milliliter portions of the culture were withdrawn at suitable time intervals and then subjected to lipid extraction.

2.3. Isomerase purification

The isomerase, 9-*cis*-hexadecenoic acid *cis-trans* isomerase (9-Iase), was purified by precipitation of the cytosolic fraction with ammonium sulfate and column chromatography. The protein with isomerase activity recovered between 30% and 40% saturation with ammonium sulfate was dialyzed against 50 mM potassium phosphate buffer (pH 8.0) and then applied to a high-performance liquid chromatograph (model CCPM; Tosoh, Tokyo, Japan) equipped with an ultraviolet detector (model UV-8010; Tosoh) and a column of DEAE-Toyopearl (type 650 M; 1.5 cm i.d.×15 cm, Tosoh) which had been equilibrated with the same phosphate buffer. The eluate was monitored by absorption of UV light at 280 nm. The isomerase activity which had passed through the DEAE-Toyopearl column was loaded on a hydroxyapatite column (1.5 cm i.d.×15 cm; Gygapite, Seikagaku Kogyo, Tokyo, Japan) by connecting the two columns. The protein was eluted from the hydroxyapatite column with a linear gradient of potassium phosphate buffer (pH 7.0) from 50 to 500 mM at a flow rate of 2.0 m*l*/min. The protein with isomerase activity was applied to an ether-Toyopearl column (1.5 cm i.d.×15 cm; Tosoh) which had been equilibrated with 50 mM potassium phosphate buffer (pH 8.0) containing 1.0 M ammonium sulfate, and then the column was eluted with a linear gradient of ammonium sulfate (1.0 to 0 M) at a flow rate of 1.0 m*l*/min. The eluted enzyme fraction was dialyzed against 50 mM potassium phosphate buffer (pH 6.0). The resultant protein with isomerase activity was loaded onto a column of SP-Toyopearl (1.5 cm i.d.×15 cm; Tosoh) which had been equilibrated with 50 mM potassium phosphate buffer (pH 6.0) and eluted with the same buffer at a flow rate of 1.0 m*l*/min. Since the isomerase activity passed through the SP-Toyopearl column, it was applied to the prepacked butyl-G column (0.82 cm i.d.×7.5 cm; Waters, Milford, MA, USA) which had been equilibrated with 50 mM potassium phosphate buffer (pH 7.0) containing 1.2 M ammonium sulfate, and then the column was eluted with a linear gradient of ammonium sulfate (1.2 to 0 M) at a flow rate of 1.0 m*l*/min. In the final step of the purification the isomerase fraction was applied to a gel filtration column (1.6 cm i.d.×60 cm; HiLoad, Superdex 200 16/60, Pharmacia, Uppsala, Sweden) which had been equilibrated with 50 mM potassium phosphate buffer (pH 8.0). The isomerase was eluted with the same buffer at a flow rate of 1.0 m*l*/min.

Protein content was measured by the method of BRADFORD (1976).

Protein samples, along with commercially obtained molecular mass standards (Bio-Rad, Tokyo, Japan), were electrophoresed on gels with a linear gradient of 5–20% acrylamide, according to LAEMMLI (1970). When indicated, an incompletely denaturing SDS-PAGE system was used, in which non-heat-denatured protein samples in Laemmli sample buffer containing no 2-mercaptoethanol were electrophoresed at room temperature. Following electrophoresis, gels were silver-stained.

2.4. Assay of isomerase activity

For the assay of the isomerase activity, 16:1(9c) and its derivatives were used as substrates. A solution that contained 1.0 μmol of lipid in chloroform in a screw-capped test tube was evaporated to dryness under a stream of nitrogen gas.

Then 0.9 m*l* of medium G and 0.1 m*l* of the preparation of enzyme, containing approximately 0.1 to 1 mg of crude sample of protein or 1 to 2 μg of purified protein, were added, and the reaction mixture was subjected to sonic vibration over ice-cold water for 30 s. When the isomerization activity of 16:1(9c) esterified to phosphatidylethanolamine (PE) was measured, the membrane fraction was added. The reaction was allowed to proceed at 30°C for 1 or 2 h and then stopped by the addition of 5.0 m*l* of a mixture of chloroform and methanol (1:1, v/v). After vigorous mixing with a vortex mixer and centrifigation at 3000× g at room temperature, the chloroform layer was recovered as the lipid fraction. The lipid fraction was evaporated to dryness and was subsequently heated with 10% (v/v) acetyl chloride in methanol, together with pentadecanoic acid (15:0) as an internal standard, at 90°C for 3 h. The resultant fatty acid methyl esters were analyzed by gas-liquid chromatography (GLC) as described previously (OKUYAMA *et al.*, 1990). The isomerase activity was expressed as μmoles of 16:1(9t) synthesized per hour per mg of protein.

2.5. Chemicals

Authentic 16:1(9c), 16:1(9t), 15:0, sodium salt of 16:1(9c), and CoA and methyl esters of 16:1(9c) were purchased from Sigma (St. Louis, MO, USA). 16:1(9c)/16:1(9c)-PE was prepared from *sn*-1,2-9-*cis*-dihexadecenoyl phosphatidylcholine (Avanti Polar Lipids Inc., Alabaster, AL, USA) by transphosphatidylation with ethanolamine by phospholipase D from *Streptomyces lyticus* (SHIMBO *et al.*, 1990). Synthesized 16:1(9c)/16:1(9c)-PE was used after purification by TLC.

3. Results and Discussion

3.1. Isomerization

Figure 1 shows the profiles after GLC of fatty acid methyl esters recovered after the isomerization of free 16:1(9c) to 16:1(9t) by a cell-free extract. More than half of the 16:1(9c) added as a substrate was converted to 16:1(9t) (Fig. lC). When the cell-free extract was omitted from the reaction mixture, the isomerization of 16:1(9c) did not occur (Fig. 1B). With respect to the isomerization of endogenous 16:1(9c), we calculated that 0.02 μmol 16:1(9t)/h/mg protein was synthesized. When 16:1(9c)/16:1(9c)-PE was used as a substrate, a value of 0.122 μmol/h/mg protein was obtained.

3.2. Distribution of isomerases

The cell-free extract was separated into soluble and membrane fractions by ultracentrifugation. The membrane fraction that had been washed once with medium G had little capacity for the *cis-trans* isomerization of 16:1(9c): neither free 16:1(9c) nor 16:1(9c) esterified to PE was isomerized to 16:1(9t) by the membrane fraction. However, the cytosolic fraction by itself isomerized 16:1(9c) to 16:1(9t) in the free acid form at a rate of 0.015 to 0.02 μmol/h/mg protein, but it did not isomerize 16:1(9c) in the esterified form. By contrast, 16:1(9c) esterified to PE was isomerized to 16:l(9t) by the cytosolic fraction in the presence of the membrane fraction. Thus, it is suggested that *Pseudomonas* E-3 has two types of activities that catalyze the *cis* to

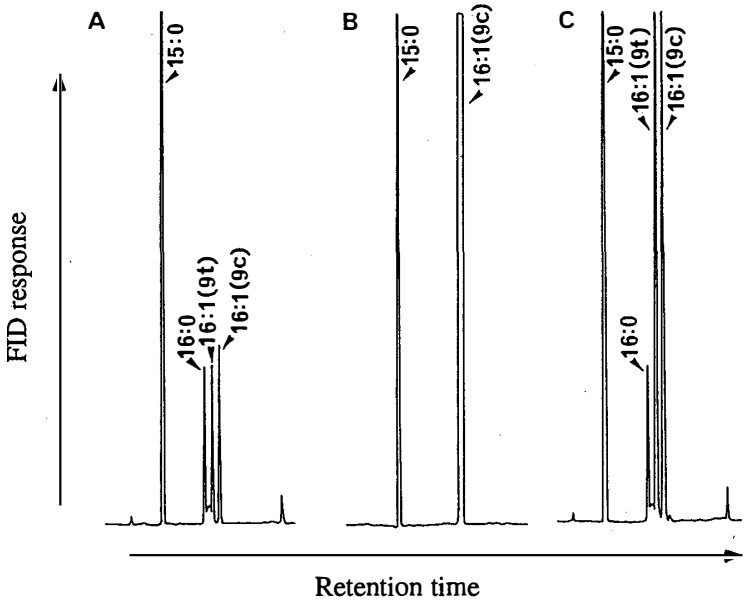

Fig. 1. Gas-chromatographic profiles of fatty acid methyl esters prepared from products of the isomerase assay. A cell-free extract (1 mg of protein) was incubated without (A) and with (C) 1 μmol of 16:1(9c) for 2 h at 30°C. In (B), 1 μmol of 16:1(9c) were treated as described above but without addition of the cell-free extract. Fatty acid methyl esters were prepared as described in the text. 15:0 was an internal standard. FID, Flame ionizing detector.

trans isomerization of 16:1(9c). The *cis-trans* isomerase of 16:1(9c), namely, 16:1(9c) *cis-trans* isomerase (9-Iase), which was located in the cytosolic fraction, was purified and characterized (see below).

3.3. Purification of 9-Iase

Since the 9-Iase activity did not bind at all to anion-exchange (DEAE or QE) resins under our conditions, we applied the proteins that had passed through the DEAE-Toyopearl column directly to the hydoroxyapatite column by connecting the two columns. Proteins on the hydroxyapatite column were eluted with a linear gradient of 50 to 500 mM potassium phosphate buffer (pH 7.0). The protein fraction with 9-Iase activity which had been eluted at 250 mM potassium phosphate after hydroxyapatite chromatography was applied to a column of ether-Toyopearl. A peak of protein with 9-Iase activity appeared at 0 to 0.9 M ammonium sulfate. The resultant 9-Iase fraction was applied to an SP-Toyopearl column. Subsequently the active fraction which had not been bound to the SP-Toyopearl gels was then applied to a butyl-Toyopearl column and the activity was eluted at 0 M ammonium sulfate. Finally the homogenous 9-Iase preparation was obtained by gel-filtration column chromatography. The purification procedure is summarized in Table 1. The 9-Iase was purified 5340-fold at the final step with a recovery of 5%.

The enzyme preparation after gel-filtration chromatography yielded a single band on incompletely-denaturing and denaturing PAGE, and the molecular mass was calculated to be 80 kDa (Fig. 2). The same molecular mass of the protein was also obtained by comparing its elution time with those of authentic standards in

Table 1. Purification of 9-Iase from Pseudomonas *E-3.*

Fraction	Total protein (mg)	Total activity (μmol/h)	Specific activity (μmol/h/mg protein)	Purification (fold)	Yield (%)
Crude extract	40274	284	0.0071	1	100
Ammonium sulfate	10670	144	0.0138	2	51
DEAE-Toyopearl & hydroxyapatite	172.3	120	0.6965	99	42
Ether-Toyopearl	12.11	37.6	3.1073	441	13
SP-Toyopearl	4.061	33.1	8.1606	1157	12
G-Butyl	1.617	18.1	11.169	1584	6
Gel filtration	0.390	14.7	37.641	5339	5

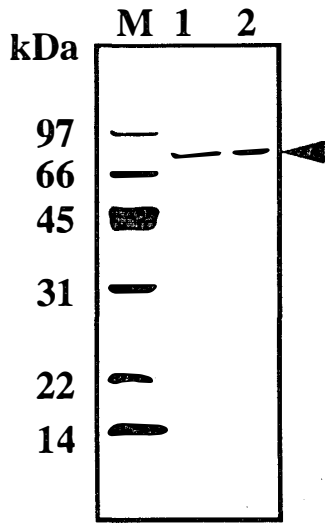

Fig. 2. Purified 9-Iase after SDS-PAGE. Lane 1, molecular mass standards; lane 2, 1 μg of purified 9-Iase (denatured sample); lane 3, 1 μg of purified 9-Iase (incompletely-denatured sample). The arrow indicates the enzyme protein.

gel-filtration chromatography (not shown). All these results suggest that the 9-Iase is a monomeric protein with a molecular mass of 80 kDa. The molecular form of the 9-Iase is very different from those of other unsaturated fatty acid *cis-trans* isomerases that have been reported, namely, the 200 kDa dienoyl-CoA 3,5-*cis*,2,4-*trans*-isomerase (EC 5.3.3.–) from rat liver, which consists of four identical subunits (CHEN *et al.*, 1994), and the 60 kDa dodecenoyl-CoA 3-*cis*,2-*trans*-isomerase (EC 5.3.3.3) from rat liver mitochondria, which consists of identical two subunits (STOFFEL and GROL, 1978). According to KEPLER and TOVE (1967) the linoleate 12-*cis*,11-*trans*-isomerase (EC 5.2.1.5) is an isomerase using a fatty acid in the free acid form as its substrate. However, that enzyme had not yet been purified, and thus structural information about it is not available.

When using a puried enzyme preparation, the apparent activity of 9-Iase depended on the amount of the enzyme protein and the rate of the reaction remained constant for about 2 h (not shown). The K_m for 16:1(9c) and the V_{max} of the 9-Iase were 118 μM and 22.8 μmol/h/mg protein, respectively. The K_m was about 10 times larger than the K_m of a linoleate 12-*cis*,11-*trans*-isomerase from *Butyrivibrio fibrisolvens* for linolenic acid (KEPLER and TOVE, 1967).

3.4. Effect of temperature on the 9-Iase activity

The 9-Iase activity was determined at various temperatures in the range from 0°C to 60°C. As shown in Fig. 3, the enzyme activity exhibited a maximum at 30°C and about 30% of the maximum was retained at 0°C and 60°C.

3.5. Substrate specificity of 9-Iase

As shown in Table 2, free 16:1(9c) was used as an effective substrate for this enzyme. However, CoA and methyl esters of 16:1(9c) and 16:1(9c) esterified to PE were not available for substrates and sodium salt of 16:1(9c) was poorly isomerized. When 16:1(9c)/16:1(9c)-PE was served as a substrate, it was isomerized by the 9-Iase in the presence of the membrane fraction. The combined fraction of the 9-Iase and

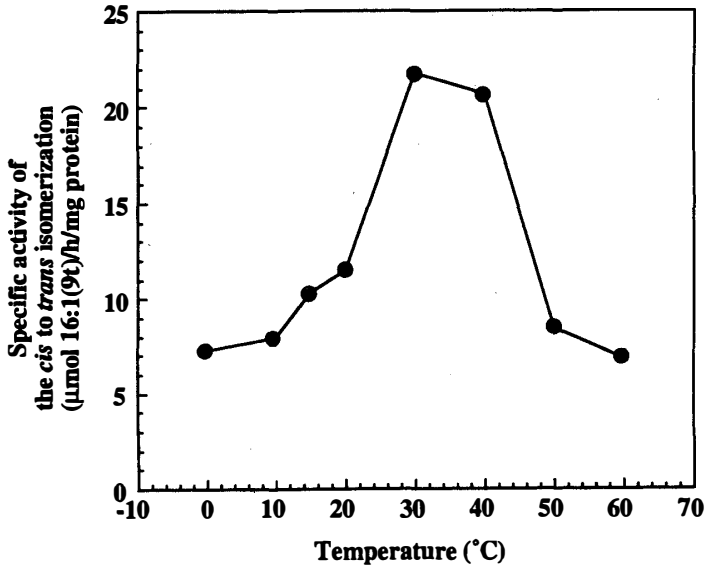

Fig. 3. Effect of temperature on the 9-Iase activity. To 1.0 μmol of 16:1(9c), 0.9 ml of medium G and 0.1 ml of the preparation of enzyme containing approximately 1 μg of purified protein were added as described in Section 2. The reaction was allowed to proceed at indicated temperature for 1 h.

Table 2. Substrate specificity of 9-Iase.

Substrate	Relative activity (%)
16:1(9c)	100
16:1(9c)-CoA	0
16:1(9c)-ME*	0
16:1(9c)-Na	5
16:1(9c)/16:1(9c)-PE	0
16:1(9c)/16:1(9c)-PE**	48
16:1(9c)/16:1(9c)-PC**	0

*Methyl ester of 16:1(9c).

**The reaction was carried out in the presence of the membrane fraction (5 μg of protein) prepared from *Pseudomonas* E-3.

membrane fraction did not isomerize 16:1(9c) esterified to phosphatidylcholine, suggesting that this activity has a specificity to the polar head group of phospholipid.

To the present time several kinds of *cis-trans* isomerase catalyzing a geometric isomerization of the double bond of unsaturated fatty acids have been reported. An enoyl-CoA 3,2-*trans*-isomerase (EC 5.3.3.8; MÜLLER-NEWEN and STOFFEL, 1993) and an enoyl-CoA 3-*cis*,2-*trans*-isomerase (STOFFEL and ECKER, 1969) have been purified from the soluble fraction of rat liver mitochondria. CHEN *et al.* (1994) and LUO *et al.* (1994) purified a dienoyl-CoA 3,5-*cis*,2,4-*trans*-isomerase from rat liver homogenates. All these enzymes use an acyl-CoA as their substrate and catalyze the geometric isomerization of the double bond with its positional shifting. A linoleate 12-*cis*,11-*trans* isomerase which was found in the soluble fraction from *Brevibacterium fibrisolvens*, uses a free fatty acid. However, it catalyzes the geometric and positional isomerization of a double bond simultaneously (KEPLER and TOVE, 1967). By contrast, the 9-Iase by itself uses a free unsaturated fatty acid as substrate and the isomerization is not accompanied by positional isomerization of the double bond. According to DIEFENBACH and KEWELOCH (1994), the phenol-degrading bacterium *Pseudomonas putida* has an activity isomerizing 9-*cis*-octadecenoic acid to 9-*trans*-octadecenoic acid, suggesting that this bacterium might have an isomerase of the same type as the 9-Iase. However, no biochemical information about the enzyme protein is available at present.

3.6. *Effect of a temperature shift on fatty acid composition*

When *Pseudomonas* E-3 cells were grown at 30°C, the level of 16:1(9t) was from 2% to 3%, which was almost the same as that of cells grown at 4°C (Table 3). However, when the culture of *Pseudomonas* E-3 grown at 4°C for 24 h was warmed up to 30°C at a high rate (about 20°C/min), the level of 16:1(9t) significantly increased from 2% to 14% at the expense of 16:1(9c) (from 46% to 30%, see Table 3) 2 h after the transfer. Levels of 16:0 and 18:1(11c) were slightly changed in the cells. Since the addition of cerulenin to the culture of *Pseudomonas* E-3 grown at 4°C did not inhibit the increase in the level of 16:1(9t) after the transfer to 30°C (data not shown), it is suggested that the abrupt changes in growth temperature might induce activation of the 9-Iase. Although the optimum temperature for activity of the 9-Iase is 30°C (Fig. 3), the 9-Iase seems not to be operative in cells constantly kept at 30°C (see Table 3).

Table 3. Effect of growth conditions on fatty acid composition of Pseudomonas *E-3.*

Growth conditions	16:0	16:1(9c)	16:1(9t)	18:1(11c)	Others
		% of total			
4°C, 24 h	24	46	2	26	2
30°C, 24 h	57	18	2	13	10
30°C, 48 h	56	16	3	11	14
2 h after shift to 30°C*	25	30	14	21	10
24 h after shift to 30°C*	43	24	5	15	13

*A culture of *Pseudomonas* E-3 grown at 4°C was shifted to 30°C.

The biological significance of *trans*-unsaturated fatty acids is not well understood. However, as shown in Table 3, the level of 16:1(9t) in *Pseudomonas* E-3 significantly increased in the cells of which had been rapidly warmed. And as indicated previously (SILVIUS, 1982; OKUYAMA *et al.*, 1991), the temperature for the phase transition from the gel to the liquid-crystal phase of phospholipids with *trans*-unsaturated fatty acids is seen to be intermediate between those of the phospholipids with saturated and *cis*-unsaturated fatty acids, when each has the same number of carbon atoms. As shown in Table 3, the level of 16:1(9t) in continuously grown cells at 30°C for 24h after the transfer to 30°C was decreased to 5% and the level of 16:0 was significantly increased. Thus it is suggested that 16:1(9t) might be urgently synthesized so as to adjust the hyper-fluid conditions of the cytoplasmic membrane, which was abruptly induced in this bacterium. Since *Pseudomonas* E-3 is rich in PE (WADA *et al.*, 1987) in its cytoplasmic membrane and the level *in situ* of free fatty acid is negligible, it is suggested that the 9-Iase could be operative *in vivo* as the *cis* to *trans* isomerase for 16:1(9c) that is esterified to PE, together with the membranous factor.

Acknowledgments

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