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Growth Temperature-Dependent Conversion of *De novo*-Synthesized Unsaturated Fatty Acids into Polyhydroxyalkanoic Acid and Membrane Cyclopropane Fatty Acids in the Psychrotrophic Bacterium *Pseudomonas fluorescens* BM07

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Abstract A psychrotrophic bacterial strain, *Pseudomonas fluorescens* BM07, synthesized unsaturated fatty acids (UFA) from fructose in response to lowering of growth temperature, and incorporated them into both polyhydroxyalkanoic acid (PHA) and membrane lipid. The blocking of PHA synthesis by adding 5 mM 2-bromooctanoic acid to the growth medium, containing 70 mM fructose, was found to be a useful means to profile the composition of membrane lipid by gas chromatography. As the growth temperature changed from 35 to 5°C, the total content of two UFA, 3-hydroxy-*cis*-5-dodecenoic acid ($C_{12:1}$) and 3-hydroxy-*cis*-7-tetradecenoic acid ($C_{14:1}$), in PHA increased from 31 to 44 mol%. The growth at lower temperatures also led to an increase in the level of two major UFA, palmitoleic acid (C16:1 *cis*9) and *cis*-vaccenic acid (C18:1 *cis*11), in membrane lipid. A fraction of these membrane-lipid UFA was converted to their corresponding cyclopropane fatty acids (CFA). The CFA conversion was a function of culture time, exhibiting biphasic increase before and after entering the stationary phase. However, pH changes in growth media had no effect on the CFA conversion, which is contrary to the case of *E. coli* reported. The cells grown at 30°C responded to a cold shock (lowering the medium temperature down to 10°C) by increasing the level of C16:1 *cis*9 and C18:1 *cis*11 up to that of 10°C-grown control cells and concomitantly decreasing the relative level of *cis*-9,10-methylenehexadecanoic acid (the CFA converted from C16:1 *cis*9) from 14 to 8 mol%, whereas the 10-grown cells exhibited little change in the lipid composition when exposed to a warmer environment of 30°C for 12 h. Based on this one-way response, we suggest that this psychrotrophic strain responds more efficiently and sensitively to a cold shock than to a hot shock. It is also suggested that BM07 strain is a good producer of two unsaturated 3-hydroxyacids, $C_{12:1}$ and $C_{14:1}$.

Key words: Polyhydroxyalkanoic acids (PHA), cyclopropane fatty acids (CFA), *Pseudomonas fluorescens* BM07, unsaturated PHA, psychrotrophic bacterium

Unsaturated fatty acids (UFA) are principally derived via the fatty acid *de novo* synthesis pathway [1, 2, 15]. A fraction of the UFA derivatives [e.g., palmitoleic acid (C16:1 *cis*9) and *cis*-vaccenic acid (C18:1 *cis*11)] are incorporated into the constituents of membrane lipids to control the fluidity of membrane. In bacteria, UFA shortened by the β-oxidation pathway are converted to polyhydroxyalkanoic acid (PHA) via the PHA synthesis pathway [6, 8, 11, 14]. Usually, two unsaturated monomers, 3-hydroxy-*cis*-5-dodecenoic acid ($C_{12:1}$) and 3-hydroxy-*cis*-7-tetradecenoic acid ($C_{14:1}$), at significant levels are detected in PHA, when bacteria are grown with unrelated carbon sources [11, 14], and the level of their incorporation depends on cultivation temperature. For example, the mesophilic bacterium *Pseudomonas putida* KT2442 grown with glucose exhibits an increased level of the unsaturated monomer units in PHA (totally less than 10 mol%), when the growth temperature is lowered from 30 to 15°C [11]. Analysis of relative positions of the double bond in the structure demonstrates that the two unsaturated coenzyme A (CoA) monomer precursors used for the PHA synthesis are derived from the acyl carrier protein derivatives of C16:1 and C18:1. The enzyme transacylase PhaG is known to link the two pathways; the fatty acid *de novo* synthesis and PHA synthesis by converting the (R)-3-hydroxyacyl-acyl

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carrier protein to the corresponding derivatives [17]. We also confirmed the linkage by using 2-bromooctanoic acid, an inhibitor which specifically inhibits PHA synthesis when *Pseudomonas fluorescens* BM07 is grown with fructose [14]. The psychrotrophic bacterium *P. fluorescens* BM07 can increase the level of $C_{12:1}$ and $C_{14:1}$ up to 30 and 5 mol% at 30°C, respectively.

The unsaturated acyl moieties in membrane lipid play important roles in the physiology of organisms [2, 15]. Low temperature increases the proportion of UFA in the membrane lipid, and the increase in the level of unsaturated acyl groups is known to increase the membrane fluidity, thus resulting in the viability at low temperatures.

In many bacteria, however, the UFA such as C16:1 and C18:1 in the membrane lipid are converted to cyclopropane fatty acids (CFAs) by CFA synthases [9]. These acids are formed by the addition of a methylene group, derived from the methyl group of *S*-adenosyl-L-methionine, across the carbon-carbon double bond of the UFA. The methylene group is transferred to mature phospholipid molecules already present in the membrane bilayers, but not to free fatty acids or intermediates in phospholipid biosynthesis. CFA formation occurs via post-synthetic modification of bacterial membrane lipid bilayers, however, the exact physiological function of CFA has not been known. Recently, it was suggested that the membrane CFA content is the major factor in acid resistance of *Escherichia coli* [3].

Psychrotrophic bacteria are expected to exhibit much more efficiently growth-temperature dependent PHA synthesis than mesophilic bacteria, since the average growth temperature of psychrotrophs ranges from 0 to +40°C [10]. The high level of UFA in PHA synthesized by the psychrotrophic *P. fluorescens* BM07 is expected to allow us to easily and clearly trace the metabolic flow of unsaturated acyl groups, using gas chromatography. To the best of our knowledge, however, there has been no detailed report on the PHA synthesis of any psychrotrophic strain. Therefore, we undertook to characterize the PHA synthesis of *P. fluorescens* BM07 together with growth temperatures. To find any relationship existing between the level of UFA in the membrane lipid and that of unsaturated 3-hydroxyacids in PHA, the growth-temperature dependent composition of fatty acids in the membrane lipid of *P. fluorescens* BM07 was also investigated. To understand in detail the fate of the UFA at physiological level, we also investigated CFA formation under conditions such as temperature shock, acid stress, and different type of carbon sources. Two CFAs, *cis*-9,10-methylenehexadecanoic acid (cyclo-C17) and *cis*-11,12-methylene-octadecanoic acid (cyclo-C19), were detected in the membrane lipids of *P. fluorescens* BM07. Furthermore, the CFA formation was dependent on culture time. The treatment of cells with 2-bromooctanoic acid [14, 16] was found to be a useful means to characterize the composition of membrane lipid by gas chromatography, eliminating a probable complexity due to the presence of PHA in cells.

Materials and Methods

Bacterial Strain, Culture Media, and PHA Accumulation in Cells

The strain isolated in our lab, *Pseudomonas fluorescens* BM07 [14], was used in the following experiments. Nutrient-rich (NR) medium was used in the seeding, maintenance, and storage of the strain, and contained 1% yeast extract, 1.5% nutrient broth, and 1% ammonium sulfate [14]. The modified M1 mineral-salts medium was used as the PHA synthesis medium, and its mineral composition was similar to that reported earlier [5]. The culture grown in NR medium at 30°C and 180 rpm for 12 h was transferred to 500 ml mineral-salts medium containing an appropriate amount of a carbon source in a 2-l flask. When the cells were grown to a steady state at a desired temperature, the cells were then harvested, washed with methanol, and dried under vacuum at room temperature.

Concentrations of ammonium ion remaining in the medium were measured by the Nesslers reagent method [5], consumption of fructose remaining in the medium was determined by the DNS method [5, 14], and the content of PHA in the cells was determined by methanolysis reaction of the dried cells with a mixture of sulfuric acid/methanol, followed by GC determination of the resulting 3-hydroxy-methylesters. Gas chromatograms were obtained by a Hewlett Packard 5890A gas chromatograph equipped with a HP-1 column and a flame ionization detector.

Polyesters were extracted from an appropriate amount of cells, which had been dried under vacuum overnight at 50°C, with hot chloroform in a Pyrex Soxhlet apparatus for 6 h. The concentrated solvent extract was precipitated by cold methanol while rapidly stirring. Isolated polymers were dried overnight under vacuum at ambient temperature, and then weighed. Quantitative determination of the monomer units in the polyesters was performed by gas chromatography as described above. The standardization of the GC chromatograms was made against the PHA of known structure characterized by a quantitative NMR experiment.

Transmission Electron Microscopy

The washed cells were fixed doubly with 2% glutaraldehyde and 1% osmium tetroxide. Ultrathin sectioning was performed using an LKB-Ultratome with a diamond knife. These sections were then collected on a copper grid coated with a Formvar-carbon film and were poststained with lead citrate and uranyl acetate [14]. Electron micrographs were taken with a HITACHI H-600 electron microscope (Tokyo, Japan) under an acceleration voltage of 75 kV.

Characterization of Membrane Lipid

Cultures were centrifuged (10,000 ×*g*, 15 min), the supernatant was decanted, and approximately 0.1 g of the cell pellet was weighed into duplicate Pyrex screw-cap tubes. Fatty acids of cell membrane were saponified by heating (100°C) for 10 min in the presence of 1.0 ml of 0.5 N NaOH in methanol and 1.0 ml of ethylether. Tubes were immediately cooled (0°C), and sufficient amount of Na_2SO_4 was added to remove water. Saponified fatty acids were transferred to another test tube and methylated by heating (80°C) in the presence of 1.0 ml of 1.5 M methanolic HCl for 10 min [7]. Tubes were immediately cooled (0°C), and 1.0 ml each of hexane and distilled water were added to extract methylated fatty acids. The hexane layer was transferred to a test tube, and sufficient Na_2SO_4 was added to remove water. Each extract was analyzed by a Hewlett Packard 5890A gas chromatograph equipped with a HP-1 column and flame ionization detector. Peak areas were normalized. At least, three batches of different cultures were analyzed and statistically averaged.

RESULTS

Temperature Dependence of PHA Synthesis from Fructose

Table 1 shows the temperature-dependent PHA synthesis in *P. fluorescens* BM07 grown on 70 mM fructose. The initial concentration of ammonium sulfate was 1.0 g/l in PHA synthesis M1 mineral medium where the approximate molar C/N ratio was ~10. The cultivation at 5°C continued for 7 days to reach a steady-state growth. In a 500-ml flask culture, the maximal dry biomass of 4 g/l at 30°C and the minimal dry biomass of 1.6 g/l at 5°C were obtained. The maximum PHA yield was 25(wt/wt)% at 30°C and the minimum yield was 5(wt/wt)% at 35°C. Overall, the PHA yield was rather low compared to other *Pseudomonas* species reported [5, 6, 8, 11]. Cell growth and PHA yield were parallel to each other with culture temperature [14]. Six monomers at the level higher than 1 mol% were detected, which included 3-hydroxyoctanoic acid (C_8), 3-hydroxydecanoic acid (C_{10}), 3-hydroxydodecanoic acid (C_{12}), 3-hydroxy-*cis*-5-dodecenoic acid ($C_{12:1}$), 3-hydroxytetradecanoic acid (C_{14}), and 3-hydroxy-*cis*-7-tetradecenoic acid ($C_{14:1}$). Generally, *P. fluorescens* BM07 incorporates relatively larger amounts of long monomers, such as C_{12} and C_{14}, into the polymer chain than other *Pseudomonas* species. The contents of the two major monomers, C_{12} and $C_{12:1}$, and the two minor monomers, C_{14} and $C_{14:1}$, in PHA were most temperature sensitive. The level of C_{12} in PHA decreased from 24 mol% at 35°C to 9 mol% at 5°C, whereas the level of $C_{12:1}$ increased from 26 mol% at 35°C to 41 mol% at 5°C. On the other hand, the relative amount of the major saturated monomer C_{10} increased slightly with decreasing the cultivation temperature. In general, the production of PHA decreased with decreasing the culture temperature.

Temperature Dependence of PHA Synthesis from Mono- and Dicarboxylic Acids

Similar to other Pseudomonas such as *P. putida* [11], *P. oleovorans* [8], and *P. citronellolis* [5], *P. fluorescens* BM07 utilizes low mono-carboxylic acids (e.g., from acetic acid to capric acid) mostly for cell growth, but not for PHA accumulation; however, the strain produces PHA from several low dicarboxylic acids. As seen in Table 2, the PHA had a similar ratio of monomer compositions to that produced from fructose. A long cultivation time was needed at low temperatures to reach a steady-state growth, similar to the growth on fructose. When the cells were grown on succinic acid, a significant amount of $C_{12:1}$ was increasingly incorporated into PHA with decrease of cultivation temperature. In octanoic acid grown cells, no remarkable change in monomer composition was observed with growth temperature. On the contrary, in the longer acid such as tetradecanoic acid, the content of $C_{12:1}$ in PHA changed significantly from 0.2 mol% at 30°C to 10 mol% at 10°C. In addition, the cultivation on tetradecanoic acid led to 7 mol% incorporation of the C_{14} monomer with the

Table 1. Temperature-dependent biosynthesis of PHAs in *Pseudomonas fluorescens* BM07 grown on 70 mM fructose.

Culture temp. (°C)	Culture time (h)	Dry cell wt (g/l)	Acetone precipitated dry exopolysaccharide wt (g/l)	Polyester content (wt%)	Polyester composition (mol%)[a]					
					C_8[b]	C_{10}	C_{12}	$C_{12:1}$	C_{14}	$C_{14:1}$
35	58	3.61±0.323	0.09±0.034	5.1±0.91	5.8±0.09	30.4±1.63	24.3±1.42	26.0±1.63	5.9±0.65	5.6±0.53
30	44	4.00±0.264	0.39±0.102	25.2±2.64	5.2±0.12	35.5±1.02	17.2±1.74	29.1±2.05	3.3±0.76	4.3±0.37
25	52	3.90±0.211	0.77±0.138	25.0±3.51	4.7±0.31	37.1±1.82	15.2±1.26	32.7±2.42	2.6±0.46	4.1±0.65
20	62	2.84±0.325	1.42±0.245	23.2±1.75	5.0±0.14	40.1±2.03	13.0±1.64	36.5±2.16	1.7±0.74	3.7±0.26
15	77	2.61±0.438	2.05±0.437	21.8±2.83	4.9±0.25	40.6±1.04	11.9±1.09	37.2±1.62	1.8±0.52	3.7±0.32
10	102	2.46±0.520	2.32±0.513	19.0±2.41	4.6±0.28	40.0±0.09	9.9±0.94	40.5±1.27	1.4±0.85	3.3±0.46
5	152	1.63±0.354		14.7±3.04	4.7±0.21	41.1±2.01	9.1±0.91	40.8±1.98	1.3±0.38	2.8±0.25

[a] Calculated from GC data.

[b] C_8, 3-hydroxyoctanoic acid; C_{10}, 3-hydroxydecanoic acid; C_{12}, 3-hydroxydodecanoic acid; $C_{12:1}$, 3-hydroxydodecenoic acid; C_{14}, 3-hydroxytetradecanoic acid; $C_{14:1}$, 3-hydroxytetradecenoic acid.

Table 2. Temperature dependence of PHA production by *Pseudomonas fluorescens* BM07 from various carboxylic acids.

Carbon source (mM)	Culture temp. (°C)	Culture time (h)	Dry cell wt (g/l)	Polyester content (wt%)	Polyester composition (mol%)[a]									
					C_4[b]	C_6	C_8	C_{10}	C_{12}	$C_{12:1}$	C_{14}	$C_{14:1}$	C_{16}[a]	$C_{16:1}$
Succinic acid (70 mM)	30	44	2.13±0.321	21.2±3.32	0.1±0.03	1.5±0.06	8.4±0.56	35.7±1.54	16.9±2.01	29.8±1.67	2.5±0.03	4.2±0.53	0.6±0.09	0.3±0.03
	20	60	3.49±0.254	15.3±2.75	0.2±0.05	0.7±0.08	5.5±0.31	38.1±1.98	14.3±1.93	34.5±2.31	2.0±0.01	3.9±0.13	0.4±0.02	0.4±0.06
	10	76	2.59±0.316	7.2±2.46	0.2±0.06	0.6±0.05	4.9±0.09	36.6±1.52	12.5±1.42	38.5±1.43	2.2±0.03	4.5±0.32		
Octanoic acid (40 mM)	30	44	2.47±0.321	23.3±2.64	0.4±0.03	11.8±1.03	84.4±2.05	1.8±1.76	0.4±0.02	0.4±0.06	0.4±0.01	0.1±0.03	0.2±0.02	0.2±0.01
	20	60	3.15±0.215	18.8±2.16		14.1±0.98	78.0±2.34	2.9±0.09	0.8±0.03	3.1±0.03	0.2±0.01	0.5±0.01	0.2±0.01	0.2±0.02
	10	120	2.42±0.186	12.2±1.95	0.3±0.05	13.9±1.76	78.2±1.87	1.5±0.01	0.3±0.01	0.2±0.01	0.4±0.02	0.6±0.01	2.5±0.05	2.1±0.01
Tetradecanoic acid (20 mM)	30	50	2.70±0.232	35.3±3.56			38.4±1.04	35.6±1.34	17.0±1.34	0.2±0.02	7.0±1.32			
	10	120	2.04±0.321	14.1±3.79			34.6±2.01	31.4±1.86	17.0±2.76	10.0±1.99	7.0±2.09			

[a]Calculated from GC data.

[b]C_8, 3-hydroxyoctanoic acid; C_{10}, 3-hydroxydecanoic acid; C_{12}, 3-hydroxydodecanoic acid; $C_{12:1}$, 3-hydroxydodecenoic acid; C_{14}, 3-hydroxytetradecanoic acid; $C_{14:1}$, 3-hydroxytetradecenoic acid; C_{16}, 3-hydroxyhexadecanoic acid; $C_{16:1}$, 3-hydroxyhexadecenoic acid.

same number of carbon as the fed substrate. For all carboxylic acids tested, a decrease of cultivation temperature resulted in a reduction of PHA yield, which may be due to splitting of carbon flow into PHA and exopolysaccharide, as observed in the cultivation with saccharides (manuscript in preparation).

Efficient Fatty Acid Profiling for Membrane Lipid by Blocking PHA Formation with 2-Bromooctanoic Acid

It is troublesome to analyze the fatty acid composition of membrane lipid by methyl-ester-formation reaction, because the fatty acids incorporated into PHA are co-esterified with those in the lipid and detected in the region of similar retention time. 2-Bromooctanoic acid at concentrations 2 to 5 mM is known to completely inhibit PHA synthesis [14]. Thus, the cells treated with 2-bromooctanoic acid demonstrated GC chromatograms totally different from those of non-treated fructose grown cells (Fig. 1): The treatment with 5 mM 2-bromooctanoic acid almost completely abolished the peaks associated with PHA monomers, shown for the untreated cells. The disappearance of PHA in the 2-bromooctanoic acid treated cells was confirmed also in the transmission electron microscopic picture (Fig. 1). Thus, the peaks that appeared at longer than 12 min of retention time were considered to be methyl esters of the membrane fatty acids. The PHA-related fatty acid methyl esters eluted earlier than the membrane fatty acid esters in the GC column. The membrane fatty acid esters were identified by GC/MS analysis as C16, C16:1, cyclo-C17, C18, C18:1, and cyclo-C19, using a standard mixture of the methyl esters. Cyclopropane fatty acid (cyclo(9,10)-C17) was one

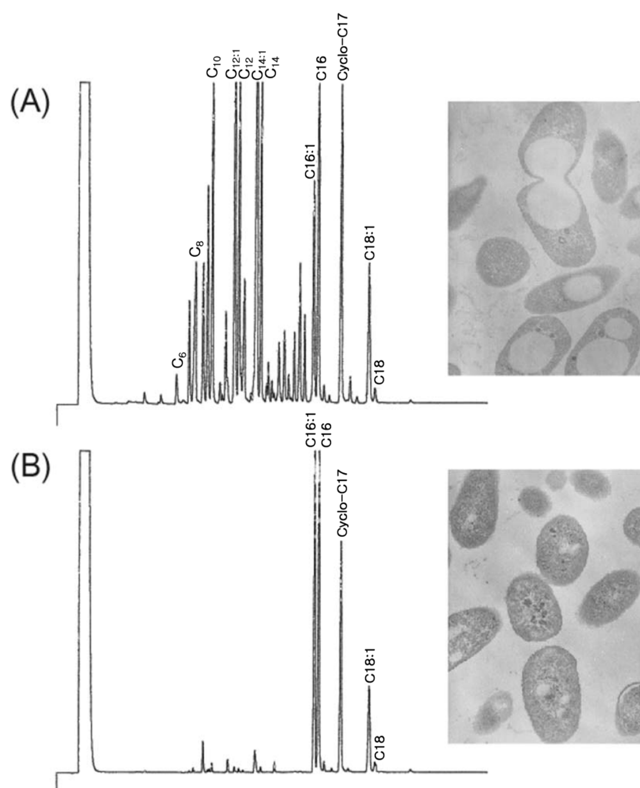

Fig. 1. Fatty acid profiling for the membrane lipids of *P. fluorescens* BM07 cells grown in the absence or presence of 2-bromooctanoic acid.

The transmission electron micrograph shown on the right of each chromatogram represents *P. fluorescens* cells grown with 70 mM fructose in the absence (the upper) or presence (the lower) of 5 mM 2-bromooctanoic acid.

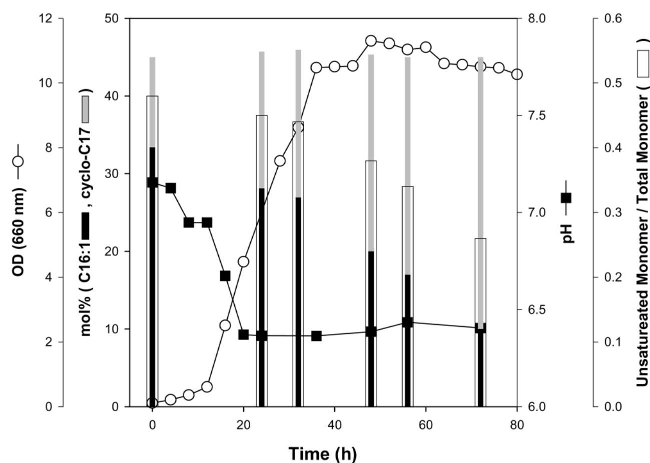

Fig. 2. Culture-time dependent fatty acid composition profiling of the membrane lipid of *P. fluorescens* BM07 cells grown with fructose at 30°C.

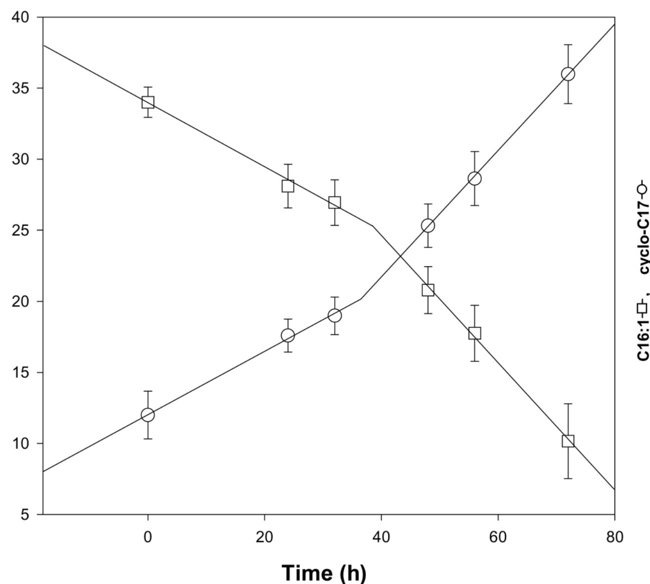

Fig. 3. Biphasic occurrence of palmitoleate (C16:1) and *cis*-9,10-methylenehexadecanoic acid (cyclo-C17 CFA) in the membrane lipid of *P. fluorescens* BM07 grown with fructose at 30°C.

of the major components. Therefore, the cyclo(9,10)-C17 was considered to have been derived from *cis*-9-hexadecenoic acid (C16:1) through the addition of methylene to C16:1. The complete and clear resolution of the PHA and membrane lipid peaks would be greatly helpful for characterization of the fatty acid composition of membrane lipid.

Dependence of Membrane Lipid Composition on Temperature and Carbon Source

Figure 2 shows the fatty acid composition profiling for the cells grown with 70 mM fructose at 30°C. It is of interest to note a gradual decrease in the ratios of C16:1 to cyclo-C17 and unsaturated to saturated fatty acid with culture time. Since it has been reported that the formation of cyclopropane fatty acids is related with acid resistance of *E. coli* [3], the pH of the culture medium was monitored. The pH of the fructose-containing medium decreased initially until mid-exponential growth period, and remained constant at around 6.4. The total pH change was 1. A careful analysis and re-plotting of the ratio of C16:1 to cyclo-C17 at 30°C revealed a biphasic feature with culture time (Fig. 3), and the breaking point was at around 40 h of cultivation when the exponential growth ended. The initial slower increase of cyclo-C17 might be attributable to the pH change of the medium during cultivation (Fig. 2). However, our detailed investigation described in the next subsection showed that the medium pH had no effect on the CFA formation in BM07 cells. In the case of PHA synthesis, no variation of monomer composition was observed with culture time.

Different types of carbon source, such as 70 mM fructose and 40 mM octanoic acid, did not significantly affect the composition of the membrane lipid (Table 3), when the cells were grown at the same temperatures. The final pH of octanoate grown medium was 7.8, slightly higher than the initial pH (7.2). As shown in Table 3, decrease of culture temperature generally induced significant increase in the level of unsaturated fatty acids, C16:1 and C18:1, in the membrane lipid. At 30°C, the steady-state maximum cell growth occurred at 48 h in the fructose medium and at 72 h in the octanoate medium. Lowering of culture temperature to 10°C slowed the growth rate. However, for both carbon sources, the steady-state plateau growth occurred after 160 h at the lowered temperature. At 10 and 30°C, the levels of C16, C18, and C18:1 were constant throughout the exponential and extended growth, whereas those of the other two components, C16:1 and cyclo-C17, reciprocally changed each other with time: That is, the sum of the C16:1 and cyclo-C17 contributions was invariant throughout the cultivation for both fructose and octanoate grown cells. Thus, the invariance of the sum strongly suggests that cyclo-C17 was derived from C16:1. For all cultures at 10 and 30°C, a decrease in the C16:1 level proportionately induced an equivalent increase in the cyclo-C17 level. However, the extent of variation for the two components was more significant for 30°C grown cells than for 10°C grown cells. This suggests that the regulation of membrane fluidity in this psychrotrophic bacterium occurs through interconversion between the two fatty acids, C16:1 and cyclo-C17.

Effect of Buffer Strength on Membrane Lipid Composition

In the cultivation in 37 mM phosphate buffered PHA synthesis medium containing 70 mM fructose (control) at 30°C, the pH of the medium was found to drop from 7.2

Table 3. Temperature and carbon source dependence of membrane lipid composition in *Pseudomonas fluorescens* BM07.

Carbon source (mM)	Culture temp. (°C)	Culture time (h)	Membrane lipid composition (mol%, avg±SE)[a]					C16:1+ cyclo-C17 (mol%)	Unsaturated monomer/ total monomer
			C16:1[b]	C16	cylclo-C17	C18:1	C18		
NR[c]	30	12	33.4±0.40	39.5±0.30	11.6±0.31	14.3±0.19	1.2±0.03	45	0.48
Fructose (70 mM)	30	24	28.1±1.53	34.9±0.63	17.6±1.16	17.0±0.96	2.4±0.09	46	0.45
		30	27.0±1.61	35.0±0.47	19.0±1.32	16.8±1.32	2.4±0.32	46	0.44
		48	20.8±1.65	35.0±0.70	25.3±1.53	17.0±1.33	1.9±0.57	46	0.38
		56	17.8±1.97	35.5±1.06	28.6±1.90	16.4±1.65	1.7±1.02	46	0.34
		72	10.2±2.63	36.0±0.79	36.0±2.07	15.4±1.06	2.5±1.41	46	0.26
	10	120	46.2±1.03	19.3±0.98	3.8±2.13	29.0±0.70	1.6±0.31	50	0.75
		160	43.5±1.64	19.6±0.65	7.1±1.27	28.7±0.88	1.1±0.22	51	0.72
		192	34.1±2.13	19.7±0.64	16.3±0.96	28.0±1.65	1.9±0.65	50	0.62
Octanoic acid (40 mM)	30	52	27.7±1.37	36.0±0.95	21.7±0.62	12.4±1.03	2.3±0.63	49	0.40
		72	24.0±2.04	37.0±0.91	24.6±2.03	12.7±0.64	1.8±0.72	49	0.37
		96	19.3±1.70	35.8±1.32	30.0±1.07	13.0±0.46	2.1±0.43	49	0.32
	10	120	49.1±1.76	15.7±1.03	3.0±0.70	30.0±0.95	2.4±0.43	52	0.79
		160	46.9±1.13	16.1±0.65	4.6±1.07	31.1±1.63	1.4±0.32	51	0.78
		192	42.6±2.31	15.8±0.92	9.5±1.95	30.5±0.65	2.0±0.55	52	0.73

[a]Calculated from the GC data.
[b]C16:1, palmitoleic acid; C16, palmitic acid; cylco-C17, *cis*-9,10-methylenehexadecanoic acid; C18:1, *cis*-vaccenic acid; C18, stearic acid.
[c]NR; nutrient-rich medium.

to 6.4. Therefore, to examine the effect of pH on the cyclopropane fatty acid formation, we first modulated the buffer strength of the medium by changing the amount of phosphate (Table 4). In the medium with five-fold buffer strength (185 mM phosphate), no pH change was observed during growth. Within the range of ionic strength studied (12 to 185 mM phosphate), the time-dependent growth profiles for 70 mM fructose grown cultures were unchanged. However, cells did not grow in the 185 mM phosphate buffered medium containing 40 mM octanoic acid. For fructose grown cells, five-fold increase in the buffer capacity did not change the fatty acid composition of the membrane lipid at each culture time, compared with that in the control experiment (37 mM phosphate medium). This means that the CFA formation may not be related with the pH drop. Furthermore, in a poorly buffered medium

Table 4. Effect of buffer strength on the composition of membrane lipid in *Pseudomonas fluorescens* BM07 grown on 70 mM fructose.

Carbon source (mM)	Culture temp. (°C)	Buffer strength (mM)	Culture time (h)	Membrane lipid composition (mol%, avg±SE)[a]					C16:1+ cyclo-C17 (mol%)	Unsaturated monomer/ total monomer	Final pH
				C16:1[b]	C16	cylclo-C17	C18:1	C18			
NR[c]	30		12	33.4±0.40	39.5±0.30	11.6±0.31	14.3±0.19	1.2±0.03	45	0.48	
Fructose (70 mM)	30	12[d]	24	36.0±2.23	34.0±1.33	10.8±1.32	17.0±1.33	2.2±0.65	47	0.53	4.0
			48	35.1±2.00	33.9±0.95	12.1±1.03	16.5±1.32	2.4±1.04	47	0.52	4.3
		37	24	28.1±1.53	34.9±0.63	17.6±1.16	17.0±0.96	2.4±0.09	46	0.45	6.4
			48	20.8±1.65	35.0±0.70	25.3±1.53	17.0±1.33	1.9±0.57	46	0.38	6.6
		185	24	28.0±2.00	35.6±1.04	18.5±0.80	16.0±0.87	2.0±0.68	47	0.44	7.2
			48	19.3±1.32	36.0±1.10	26.8±0.89	15.7±1.65	2.3±0.79	46	0.35	7.2
	10	12	72	50.0±2.03	20.7±1.30	0.2±0.12	28.0±1.69	1.1±0.69	50	0.78	5.9
			160	42.3±1.03	20.1±0.65	8.0±2.13	27.6±1.65	2.0±0.35	50	0.70	4.3
		37	120	46.2±1.03	19.3±0.98	3.8±2.13	29.0±0.70	1.6±0.31	50	0.75	6.8
			160	43.5±1.64	19.6±0.65	7.1±1.27	28.7±0.88	1.1±0.22	51	0.72	6.5
		185	72	49.5±2.32	20.4±1.36	1.7±1.33	27.0±1.66	1.5±0.35	51	0.76	7.2
			160	37.6±2.13	20.0±1.33	13.3±1.36	27.3±0.91	1.8±0.35	51	0.65	7.2

[a]Calculated from GC data.
[b]C16:1, palmitoleic acid; C16, palmitic acid; cylco-C17, *cis*-9,10-methylenehexadecanoic acid; C18:1, *cis*-vaccenic acid; C18, stearic acid.
[c]NR; Nutrient-rich medium.
[d]Concentration (mM) of phosphate added to the culture medium.

Table 5. Effect of temperature shock on the composition of membrane lipid in *Pseudomonas fluorescens* BM07 grown on NR medium.

Initial culture temp. (°C)	Initial culture time (h)	Shocked temp. (°C)[c]	Dry cell wt. (g/l)	Membrane lipid composition (mol%, avg±SE)[a]					C16:1+ cyclo-C17 (mol%)	Unsaturated monomer/total monomer
				C16:1[b]	C16	cylclo-C17	C18:1	C18		
30	12		2.99±0.271	32.4±0.67	37.5±0.60	13.6±0.81	15.3±0.69	1.2±0.06	46	0.48
	12 & transfer	30	3.12±0.398	15.9±0.25	39.1±0.18	29.3±0.59	14.7±1.67	1.0±0.09	45	0.31
		10	3.20±0.339	41.1±0.87	22.4±1.10	7.9±2.32	27.6±1.57	1.0±0.07	49	0.69
		35	2.75±0.346	15.9±0.75	43.1±1.55	28.8±1.22	11.3±1.32	0.9±0.013	45	0.27
10	28		2.47±0.231	47.3±0.24	18.3±0.35	3.0±0.23	30.5±0.42	0.9±0.09	50	0.78
	28 & transfer	30	2.34±0.153	42.6±0.32	20.6±0.32	5.6±0.15	30.6±0.32	0.6±0.08	48	0.73
		10	2.60±0.315	45.3±0.16	18.1±0.32	5.0±0.20	31.0±0.43	0.6±0.22	50	0.76
		35	2.17±0.351	43.1±0.51	21.4±0.65	4.6±0.20	30.1±0.21	0.8±0.09	48	0.73

[a]Calculated from GC data.

[b]C16:1, palmitoleic acid; C16, palmitic acid; cylco-C17, *cis*-9,10-methylenehexadecanoic acid; C18:1, *cis*-vaccenic acid; C18, stearic acid.

[c]All transferred cells were incubated at the indicated shock temperature for 12 h and harvested for analysis.

(12 mM phosphate), despite the significant pH drop down to ~4.0, the CFA formation was suppressed by as low as half of the highly buffered system at 30°C for 70 mM fructose grown cells. In this weak-buffered medium, many cells were found to undergo lysis in which only about 1 g of cell mass was recovered, compared to the usual 3.5 g for the control cells. In this buffer-strength experiment, the levels of three fatty acids, C16, C18, and C18:1, were constant irrespective of final pH and ionic strength, however, only the difference was a lower conversion of C16:1 to cyclo-C17 for 12 mM phosphate grown cells than for the more highly buffered grown cells. This again shows that the CFA formation in *P. fluorescens* BM07 was not related with the acid resistance, as was suggested for *E. coli* cells.

When the cells grown in NR medium at 30°C for 12 h were further grown in a fructose medium at 10°C, the CFA formation was highly suppressed (Table 4). Instead, the level of two unsaturated fatty acids, C16:1 and C18:1, increased significantly up to ~70 mol%, compared with 30 to 50 mol% at 30°C. During growth at 10°C, the increase in the level of cyclo-C17 with growth time was observed in all three buffered media. The highest conversion of C16:1 to cyclo-C17 was observed in 185 mM phosphate buffer medium. Similar to that at 30°C, the sum of the C16:1 and cyclo-C17 contents in the membrane lipid was always conserved in all media, irrespective of ionic-strength and final pH. In addition, the conversion was at least independent of final pH, thus again suggesting that the conversion is not related with acid resistance in this psychrotrophic strain.

Effect of Temperature Shock on Membrane Lipid Composition

It has been reported that the addition of methylene across the double bond is irreversible in *E. coli* [9]. *P. fluorescens* BM07 is a psychrotrophic strain, which may have a means to react flexibly against any heat or cold shock to survive the thermal shock. To investigate the possible role of CFA formation in the heat-shock response, temperature-shock experiments were performed by growing the 30°C-steady-state NR-grown cells (grown for 12 h) further at lowered temperature of 10°C without changing media or by growing the 10°C-steady-state NR-grown cells (for 28 h) further at elevated temperature of 30°C. As shown in Table 5, all steady-state NR-grown cells did not accumulate PHA, irrespective of culture temperature.

The maximal cell growth in NR medium at 10°C occurred at 24 h. When the cells (2.47±0.23 g/l) grown at 10°C for 28 h were further grown at 10, 30, and 35°C for 12 h, the dry cell weights were 2.60±0.32, 2.34±0.15, and 2.17±0.35 g/l, respectively, indicating no further significant cell growth at each temperature. The extended cell growth at the same or elevated temperatures induced little change in the composition of the membrane lipid. It is of interest to note still the high level of two unsaturated fatty acids, C16:1 and C18:1, and the low level of cyclo-C17 in the membrane lipid at the elevated temperatures of 30 and 35°C. This indicates that the enzymes related with CFA synthesis may not be wholly expressed at 10°C, and this remains to be identified.

Further growth of the 30°C-steady-state NR-grown cells (2.99±0.27 g/l) for 12 h yielded the cell masses, 3.20±0.34 g/l at 10°C, 3.12±0.40 g/l at 30°C, and 2.75±0.35 g/l at 35°C. Under these conditions of no more significant cell growth, therefore, a change in the membrane lipid composition is considered to occur mostly through *in situ* modification of membrane lipids. For the cold-shocked cells grown at 10°C, the level of UFA increased as expected, whereas the level of the saturated fatty acid C16 decreased down to as much as half of the control cells grown in NR at 30°C. The extended cultivation at 10°C also reduced the level of cyclo-C17 from 13.6 to 7.9 mol%. The reduction of cyclo-C17 level, as the growth temperature was lowered, may indicate a probable reversible conversion of cyclo-C17 to

C16:1. However, probable dilution by the *de novo* synthesized lipids that lack CFAs cannot be ruled out. In the extended growth at 30 and 35°C, the change only in the levels of C16:1 and cyclo-C17 occurred, while the level of other components remained rather unchanged. The extended growth caused a decrease in the level of C16:1 and an increase in the level of cyclo-C17, and the sum of the two components (mol%) was constant at 45 mol%. This again clearly demonstrates that cyclopropanation reaction occurs at the post-synthetic level.

DISCUSSION

Temperature-Dependent Factors on the PHA Monomer Composition and Synthesis Yield

Growth temperature is one of the important parameters to alter the monomer composition ratio in PHA, especially when the unrelated carbon sources, such as saccharide or dicarboxylic acid, are used as carbon source (Tables 1 and 2). However, the monomer compositions of octanoic acid grown cells are independent of the growth temperature. The monomer composition depends on the specificity of the PHA synthase and the supply rate of acyl-CoA monomers [19]. Thus, the *in-situ* concentrations of the acyl-CoA monomers available for polymerization may be extremely critical in determining the composition. Even at 30°C, the PHA from octanoate grown cells contained very low levels (less than ~2 mol%) of chain-elongated side monomers and 12 mol% of C_6 monomer produced by one round of β-oxidation of the substrate molecule. This intrinsically low availability of chain-elongated side monomers seems to result in no significant variation in PHA composition with temperature. This means that C_8 monomer is the central metabolic intermediate pool. In contrast, in the unrelated carbon-source grown cells, a wide spectrum of monomer distribution was seen even at 30°C, which is considered to result from the rather long metabolic pathway for the carbon source, via fatty acid synthesis and then PhaG to convert acyl-ACP to acyl-CoA [17]. Thus, the resulting temperature-dependent monomer distribution could be controlled by the related enzymes in the pathways which lead to PHA synthesis. In addition, such temperature-independent and rather constant monomer composition for the octanoate grown cells may indicate that the substrate specificities of the *P. fluorescens* BM07 PHA synthase(s) for various CoA substrates are linear with respect to temperature. Their specific activities may decrease linearly with decreasing temperature, which may result in the low PHA yield at low temperatures. In contrast, the yield of PHA synthesis in psychrophilic bacteria increases with decreasing the culture temperature [1].

3-Hydroxydecanoyl-CoA and 3-hydroxy-*cis*-5-dodecenoyl-CoA, the major constituents of the PHA synthesized from the unrelated carbon sources, are structurally very different. The structural difference is discernible from their CPK molecular models: The former is linear, but the latter is relatively spherical. However, the increase in the level of both monomers in PHA with decrease of culture temperature may also indicate that their relative composition depends strongly on the supplying rate of the corresponding monomers. Thus, the monomer composition of PHA is not strictly regulated at the level of specificity of the synthase enzyme, if only a single enzyme is assumed to be involved in the polymerization. The significant increase of $C_{12:1}$ level at 10°C, compared to that at 30°C, for the tetradecanoic acid grown cells additionally supports the view.

For the fructose grown cells, lowering the growth temperature induced an increase of $C_{12:1}$ level in PHA as well as C16:1 level in the membrane lipid. In the case of octanoic acid grown cells, however, we found an increase only in the level of C16:1 in the membrane lipid as the temperature decreased. This means that the fructose grown cells produced a large amount of C12:1-ACP, a part of which was converted to the corresponding acyl-CoA used for storage in the form of PHA. Part of the C12:1-ACP was chain-elongated to C16:1 which was incorporated into the acyl moiety of the membrane lipid. The storage of the excess C12:1 in the form of PHA may be a means for easy supply of the unsaturated precursors for survival of the bacterium under colder environments.

Biphasic Regulation of CFA Formation

Despite various efforts, the physiological significance of CFAs in bacteria has not yet been convincingly elucidated [9]. Recently, however, it was suggested that membrane CFA level might be an important factor in acid resistance of *Escherichia coli* [3]. As shown in Fig. 3, the initial pH decrease might increase the CFA levels over the entire cultivation period. However, other data on *P. fluorescens* BM07 culture are in disagreement with the above suggestion. For example, the CFA levels of octanoate grown cells steadily increased during cultivation, while the pH of the media increased from 7.4 to 7.8. Furthermore, in the buffer strength dependence experiments, the CFA levels were not correlated with the pH changes. Only culture-time dependent increase of CFA levels was observed. This suggests that the CFA conversion may be related with factors other than pH [18]. It has been reported that native *E. coli* CFA synthase is a short-lived protein *in vivo* whose half-life is in the range of 40 to 60 min [4]. However, the drastic increase in the CFA level even after the stationary phase indicates that the half-life of the psychrotropic *P. fluorescens* BM07 CFA synthase may be long enough to cause CFA formation over the entire culture period. As shown in heat-shock experiments, little increase in the CFA level of the 10°C-NR-grown cells was observed, even when the culture temperature was elevated to 30°C. This supports the view

that CFA synthase is expressed at the early growth phase, but the expression must be temperature dependent.

The biphasic CFA conversion for the 30°C-grown cells suggests that CFA formation is under different regulations, depending on the stage of growth of *P. fluorescens* BM07. On the contrary to the case of *E. coli*, the rate of CFA formation at the initial log-phase was slower than at the steady state, suggesting that the initial CFA formation at least was not related with a pH stress. The faster rate of CFA formation after entering the stationary phase may indicate an additional expression of the CFA synthase. CFA synthesis is an energy-requiring process, since the formation of each *S*-adenosyl-L-methionine molecule, the methylene group carrier, costs the cell three ATPs [9]. If the CFA in *P. fluorescens* BM07 would be reversibly converted to unsaturated fatty acids via a hither-to-unidentified pathway, the CFA formation at the stationary growth phase could be regarded as an additional energy storage means.

Gas chromatographic analysis of cell membrane fatty acid methyl esters was suggested to be useful for rapidly distinguishing closely related bacterial species from each other [12, 13, 20]. However, in this study, membrane fatty acid profiling was dependent on the growth time. Therefore, the growth temperature-dependent variation of membrane fatty acid profiling should be considered as a means for differentiating organisms.

Response to Temperature Shifts

P. fluorescens BM07 is a psychrotrophic bacterium, therefore, the bacterium is believed to have genes to respond to any temperature shock. The regulation of membrane fluidity is necessary for the survival in colder environments. The 30°C-grown cells well adapted to colder environment (10°C) by increasing the level of unsaturated fatty acid in the membrane lipid, but the 10°C-grown cells did not exhibit any change in the lipid composition in spite of the increase in the culture temperature by 20°C. The lowered CFA level for the cold-shocked cells may suggest possible presence of reversibly converting system. However, the increase of CFA at higher temperature may render the cell to resist any oxidative reaction of the unsaturated fatty acids at the elevated temperature. Thus, *P. fluorescens* BM07 responds well to the down-shift of growth temperature by increasing the unsaturated fatty acid level, but poorly to the up-shift of temperature. This one-way response may suggest that the psychrotrophic organism has evolved and adapted progressively to environmental temperature shift from hot to cold.

In conclusion, in *P. fluorescens* BM07, the incorporation of unsaturated fatty acid into lipids and PHA shares the original precursors synthesized via the fatty acid synthesis pathway, depending on the type of carbon sources used. Further conversion of the unsaturated fatty acids to CFA occurs in *P. fluorescens* BM07 even after entering the steady-state growth phase. This fact is different from that reported for the so-far well-studied *E. coli*. The biphasic and pH independent CFA formation in *P. fluorescens* BM07 indicates that the CFA formation in the psychrotrophs occurs via a mechanism different from that used by the mesophilic bacterium. The CFA formation in the psychrotrophic bacterium may be a means to flexibly respond to thermal shocks.

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