Channeling of Intermediates Derived from Medium-Chain Fatty Acids and *De novo*-Synthesized Fatty Acids to Polyhydroxyalkanoic Acid by 2-Bromooctanoic Acid in *Pseudomonas fluorescens* BM07

LEE, HO-JOO[1†], JONG KOOK RHO[1], KAMBIZ AKBARI NOGHABI[1], SEUNG EUN LEE[1], MUN HWAN CHOI[3], AND SUNG CHUL YOON[1,2,3,4]*

[1]*Division of Applied Life Sciences (BK21), Graduate School,* [2]*Division of Life Science, College of Natural Sciences,* [3]*PMBBRC,* [4]*Environmental Biotechnology National Core Research Center,* *Gyeongsang National University, Chinju 660-701, Korea*

Abstract 2-Bromooctanoic acid (2-BrOA) is known to block the formation of polyhydroxyalkanoic acid (PHA) in *Pseudomonas fluorescens* BM07 without any influence on the cell growth when grown on fructose, but it inhibits the cell growth when grown on octanoate (OA) (Lee *et al.*, *Appl. Environ. Microbiol.* **67**: 4963–4974, 2001). We investigated the role of 2-BrOA in the PHA synthesis of the bacterium grown with mixtures of fructose and fatty acids. OA, 11-phenoxyundecanoic acid (11-POU), and 5-phenylvaleric acid (5-PV) were selected as model substrates. When supplemented with 50 mM fructose, all these carboxylic acids suppressed the formation of PHA from fructose, however, the β-oxidation coenzyme A monomers derived from the carboxylic acids were efficiently polymerized, but the conversion yield [(mol of carboxylate substrate converted into PHA)/(mol of carboxylate substrate in the feed)] was low (e.g., maximally ~53% for 5 mM 11-POU). Addition of 2-BrOA (up to 5 mM) to the mixed carbon sources raised the conversion yield sensitively and effectively only at low levels of the acid substrates (e.g., 2 mM 11-POU or 5 mM OA): For instance, 100% of 2 mM 11-POU were converted into PHA in the presence of 5 mM 2-BrOA, whereas only ~10% of the 11-POU were converted in the absence of 2-BrOA. However, at highly saturated suppressing levels (e.g., 5 mM 11-POU), 2-BrOA inhibitor showed no significant additional effect on the conversion (60–70% conversion irrespective of 2-BrOA level). The existence of competitive and compensative relationship between 2-BrOA and all the carboxylic acid substrates used may indicate

that all the acid substrate-derived inhibiting species bind to the same site as the 2-BrOA inhibiting species does. We, therefore, suggest that 2-BrOA can be used for efficiently increasing the yield of conversion of expensive substituted fatty acids into PHA and then substituted 3-hydroxyacids by hydrolyzing it.

Key words: Polyhydroxyalkanoic acid, 2-bromooctanoate, *Pseudomonas fluorescens* BM07, monomer channeling, *de novo* fatty acids

*Corresponding author
Phone: 82-55-751-5942; Fax: 82-55-753-0765;
E-mail: scyoon@nongae.gsnu.ac.kr

†Present address: Section on Brain Physiology and Metabolism, Bldg. 10, Rm. 6N202, National Institute on Aging, National Institute of Health, Bethesda, MD 20892, U.S.A.

Polyhydroxyalkanoic acids (PHAs) are accumulated in the form of granular inclusion bodies in a wide range of microorganisms [1, 12]. These bipolymers have attracted much interest because of their potential applications for bioplastics and biomedical materials. The composition of the PHA is dependent mainly on the substrate specificity of the PHA synthase and on the metabolic routes which provide precursors from various carbon sources. There are two types of hydroxyalkanoic acid coenzyme A thioester monomers; short-chain-length (SCL) (3–5 carbon atoms) and medium-chain-length (MCL) (6–14 carbon atoms). It is possible to produce PHA by using many techniques such as PHA synthesis-related gene insertion [12], a combination of different precursor carbon sources [8, 12], multistep cultures [2], and pathway routing by inhibitors [5, 13]. *Pseudomonas* spp. can incorporate saturated and unsaturated MCL monomers into PHA from unrelated carbon sources, however, the incorporation level of unsaturation depends on the type of species. *Pseudomonas fluorescens* BM07 was reported to be able to incorporate up to 35 mol% of 3-hydroxy-*cis*-5-dodecenoate-unit into the PHA when grown on fructose [9]. The MCL monomers are derived from the
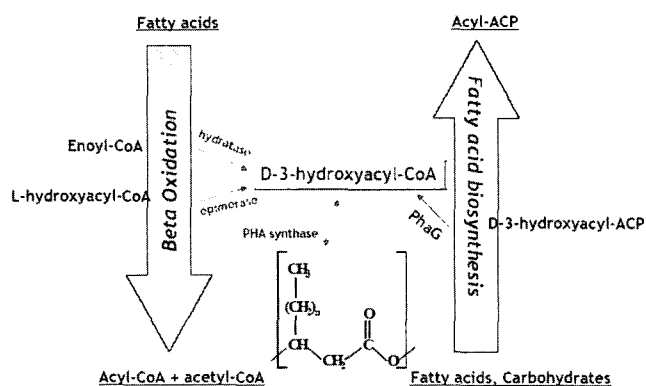
Fig. 1. Role of 3-hydroxyacyl-ACP:CoA transacylase PhaG in linking the two pathways, *de novo* fatty acid synthesis and PHA synthesis, in *Pseudomonas* spp.

A coenzyme-A thioester derivative of 2-BrOA (2-bromo-3-ketooctanoyl-CoA or 2-bromooctanoyl-CoA) is suggested to specifically inhibit the enzyme PhaG.

acyl carrier protein (ACP) intermediates of the fatty acid *de novo* synthesis pathway [4, 6, 12, 14] and the substrate of MCL-PHA synthase is (R)-3-hydroxyacyl-coenzyme A (CoA). Thus, to serve as a substrate for the PHA synthase, (R)-3-hydroxyacyl-ACP must be converted to the corresponding CoA derivative. Recently, it was found that 3-hydroxyacyl-ACP:CoA transacylase PhaG plays the role to link the two pathways of fatty acid synthesis and PHA synthesis [4, 6, 14] (Fig. 1). Thus, it is highly likely that *P. fluorescens* BM07 has high PhaG activity against the unsaturated 3-hydroxyacyl-ACP, when grown on unrelated carbon sources.

PHA synthesis-related inhibitors can be used to elucidate the metabolic pathway through which precursors for PHA synthesis are supplied as well as to channel intermediates of a specific pathway to PHA synthesis [5, 13]. Previously, we observed that 2-bromooctanoic acid (2-BrOA) highly specifically (K_i=60 μM) inhibited PHA synthesis in *P. fluorescens* BM07 when grown with fructose [9]. In the experiment, no cell growth inhibition was observed. It was suggested that a CoA thioester derivative of 2-BrOA (2-bromo-3-ketooctanoyl-CoA or 2-bromooctanoyl-CoA) might specifically inhibit the enzyme PhaG. *Ralstonia eutropha* has been known to incorporate only SCL monomers into PHA [12]. However, acrylate, known as a β-oxidation inhibitor for the bacterium, induced the synthesis of PHA, containing MCL monomers as comonomers, when the wild-type cells were cultivated with octanoic acid (OA) as the carbon source [5].

Preparation of MCL-PHA from ω-functional-group substituted MCL fatty acids is an important tool to obtain modified 3-hydroxyacids and PHA, which may be used as starting intermediates in pharmacological [10, 11] and other related chemical sciences. However, the conversion yield is relatively low, having 10 to 30% at most, when aromatic

PHA is synthesized from 11-phenoxyundecanoic acid (11-POU) and 5-phenylvaleric acid (5-PV) by *Pseudomonas putida* BM01 [15, 16]. The poor yield results from their consumption for energy even under the condition of cofeeding with non-functional carbon sources, such as saccharides or low carboxylic acids [7]. Therefore, it is necessary to develop a means to increase the production yield of such functional PHA from expensive substituted fatty acids.

In this study, we investigated the role of 2-BrOA in the PHA synthesis of *P. fluorescens* BM07 grown with mixtures of fructose and fatty acids. OA, 5-PV, and 11-POU were selected as model substrates: OA (structurally similar to 2-BrOA) for an unsubstituted fatty acid, and 11-POU and 5-PV (with a bulky substituent at ω-position) for substituted fatty acids. The substituted fatty acids were chosen for two reasons: one, for their bulkiness which may be a structural analogy to 2-BrOA at least in terms of molecular volume because it has a big bromine atom at the position 2; and the other, to find parameters to increase the conversion yield to PHA. Surprisingly, all three carboxylic acids tested were found to suppress PHA synthesis in *P. fluorescens* BM07 from fructose and were preferentially utilized for PHA production. Furthermore, as expected from their structural similarity, an apparent competitive relationship between 2-BrOA and OA or 11-POU was found. The inhibitor 2-BrOA raised their conversion yield most sensitively and effectively at relatively low concentrations (similar to 2-BrOA level) of the co-added carboxylic acid. This study shows 2-BrOA can be used to increase the yield of conversion of expensive substituted fatty acids into PHA, up to 100% in the case of 11-POU.

MATERIALS AND METHODS

Microorganism and Culture Media

Pseudomonas fluorescens BM07 isolated in the authors' laboratory [9] was used throughout the experiments. Nutrient rich (NR) medium, containing 1% yeast extract, 1.5% nutrient broth and 1% ammonium sulfate, was used in the seeding, maintenance, and storage of the strain. The modified M1 mineral-salts medium of the same composition as that reported earlier [3] was used as PHA synthesis medium. The culture (5 ml) grown in NR medium at 30°C and 180 rpm for 12 h was transferred to 500 ml M1 mineral-salts medium, containing an appropriate amount of a carbon source and 1.0 g/l ammonium sulfate, in a 2-l flask and cultivated to maximal growth. The cells were then harvested, washed with methanol, and dried under a vacuum at room temperature. The carbon sources including OA, 11-POU, 5-PV, and fructose and the inhibitor 2-BrOA were purchased from Sigma Co. and used without further purification.

Table 1. Effect of various medium-chain carboxylic acids on the formation of PHA in *P. fluorescens* BM07, grown on mixtures of 50 mM fructose and the co-added carboxylic acid with or without 2-BrOA inhibitor.

Co-added compounds	(mM)	Culture time (h)	Dry cell weight (g/l)	Polyester content (wt%)	C_6^a	C_8	C_{10}	C_{12}	$C_{12:1}$	3HPV	5POHV	7POHH	9POHN	Conversion yield[e]
Control (fructose only)	50	48	2.7±0.32	24.8±3.54		5.9±0.12	36.4±2.42	19.5±1.03	29.8±1.20[d]					
11-POU[b]	0.2	72	2.5±0.25	27.9±4.52		6.1±0.14	37.4±1.66	30.3±2.00	19.9±1.32		0.6±0.01			0.08
	0.5	72	2.4±0.37	26.4±3.97		6.2±0.02	36.5±1.62	30.8±2.01	20.2±1.85		1.7±0.01			0.09
	1	72	2.2±0.26	30.1±3.15		6.2±0.03	34.3±1.89	30.0±1.26	20.5±0.63		4.5±0.02			0.12
	5	72	2.3±0.35	34.7±2.64		1.5±0.01	3.9±0.35	5.3±0.02	3.9±0.02		56.7±1.62	25.7±1.67	3.0±0.02	0.53
	10	72	2.7±0.31	39.5±4.05		1.8±0.01	4.2±0.12	5.0±0.03	3.5±0.02		57.6±3.02	24.7±1.45	3.2±0.03	0.35
2-BrOA (+2 mM 11-POU)	0.1	72	2.5±0.21	29.6±2.36		5.6±0.10	36.1±1.23	18.9±1.09	29.3±1.96		6.8±0.16	3.2±0.23	0.1±0.01	0.15
	0.5	72	2.4±0.31	30.5±3.85		3.9±0.08	24.9±1.65	12.9±1.35	19.8±1.65		25.9±1.35	12.4±0.95	0.2±0.01	0.55
	1	72	2.3±0.28	30.6±3.35		3.4±0.06	20.5±1.75	10.5±0.92	15.6±0.95		33.2±2.03	16.4±1.65	0.4±0.01	0.68
	3	72	2.3±0.35	32.5±3.74		2.5±0.07	14.6±0.97	8.5±0.75	12.1±0.78		41.8±2.84	19.9±2.03	0.6±0.02	0.90
	5	72	2.2±0.20	34.6±3.61		1.9±0.06	11.6±0.64	7.1±0.95	9.2±1.65		47.1±1.97	22.2±1.65	0.9±0.01	0.99
2-BrOA (+5 mM 11-POU)	0.1	72	2.2±0.16	46.2±2.63		2.0±0.02	3.2±0.05	4.0±0.01	2.0±0.03		64.1±2.09	21.4±2.32	3.3±0.01	0.70
	0.5	72	2.1±0.43	45.7±3.53		1.8±0.01	2.4±0.02	3.8±0.01	1.9±0.01		64.2±1.76	22.6±2.02	3.3±0.01	0.67
	1	72	2.2±0.32	46.3±3.86		1.3±0.01	2.8±0.01	4.6±0.01	2.2±0.01		64.1±1.58	20.7±1.93	4.3±0.03	0.71
	3	72	1.9±0.29	46.7±2.43		2.3±0.02	3.0±0.03	2.7±0.02	1.9±0.01		63.4±1.27	21.6±2.40	5.1±0.01	0.62
	5	72	2.0±0.28	48.1±1.98		3.4±0.01	3.2±0.02	3.2±0.01	2.1±0.02		63.5±1.64	19.6±1.07	5.0±0.02	0.66
5-PV	0.1	96	2.6±0.23	16.4±2.42		5.4±0.05	35.2±1.56	22.4±1.23	30.6±1.96	1.5±0.01				0.33
	0.5	96	2.3±0.28	13.8±1.42		5.5±0.02	34.1±2.84	20.3±1.03	29.3±2.03	5.0±0.02				0.16
	1	96	2.2±0.21	11.9±1.75		5.2±0.08	29.1±2.36	18.6±0.85	27.9±1.08	14.4±0.95				0.20
	5	96	2.1±0.26	23.2±1.46		1.8±0.01	6.9±0.52	10.1±0.75	15.6±2.00	64.1±1.09				0.32
	10	96	2.3±0.18	29.1±2.54		1.5±0.02	2.6±0.06	3.8±0.01	5.8±0.35	85.7±2.01				0.30
	20	96	2.3±0.24	39.0±2.11						100.0±0.01				0.23
OA	5	72	2.8±0.12	24.3±1.53	0.2±0.01	4.4±0.01	35.0±1.57	18.9±0.85	30.2±2.85					0.04
	10	72	2.7±0.22	26.2±2.53	3.7±0.02	29.3±1.43	28.2±1.78	23.5±0.63	15.3±1.36					0.13
	20	72	2.6±0.26	27.2±3.53	6.0±0.42	50.0±2.13	17.5±1.48	14.2±0.85	7.5±0.75					0.11
	30	72	2.6±0.15	26.8±3.12	8.4±0.12	60.9±2.99	15.5±1.02	10.3±1.02	4.9±0.08					0.09
	40	72	2.4±0.32	26.1±2.89	10.6±0.08	68.8±2.42	9.4±0.85	7.6±0.21	3.6±0.02					0.07

Table 1. Continued.

Co-added compounds (mM)		Culture time (h)	Dry cell weight (g/l)	Polyester content (wt%)	Monomer composition, mol%									Conversion yield[e]
					C_6[a]	C_8	C_{10}	C_{12}	$C_{12:1}$	3HPV	5POHV	7POHH	9POHN	
OA	5	72	2.8±0.12	24.3±1.53	0.2±0.01	4.4±0.01	35.0±1.57	18.9±0.85	30.2±2.85					0.04
	10	72	2.7±0.22	26.2±2.53	3.7±0.02	29.3±1.43	28.2±1.78	23.5±0.63	15.3±1.36					0.13
	20	72	2.6±0.26	27.2±3.53	6.0±0.42	50.0±2.13	17.5±1.48	14.2±0.85	7.5±0.75					0.11
	30	72	2.6±0.15	26.8±3.12	8.4±0.12	60.9±2.99	15.5±1.02	10.3±1.02	4.9±0.08					0.09
	40	72	2.4±0.32	26.1±2.89	10.6±0.08	68.8±2.42	9.4±0.85	7.6±0.21	3.6±0.02					0.07
2-BrOA (+5 mM OA)	0	72	2.8±0.12	24.3±1.53	0.2±0.01	4.4±0.01	35.0±1.57	18.9±0.85	30.2±2.85					0.04
	0.1	72	2.7±0.12	24.1±2.55	2.0±0.12	20.0±3.24	30.2±1.45	13.8±2.49	26.4±3.56					0.16
	0.5	72	2.6±0.09	23.5±2.73	2.5±0.20	21.4±3.95	29.1±2.18	12.4±2.36	26.2±2.84					0.16
	1	72	2.5±0.14	22.6±1.84	2.8±0.17	23.8±5.54	29.0±3.36	12.2±3.63	25.3±3.59					0.17
	3	72	2.4±0.12	19.3±1.60	5.0±0.25	45.6±4.26	18.7±3.51	8.4±3.95	16.7±3268					0.27
	5	72	2.3±0.18	13.5±3.27	7.1±1.06	62.2±5.62	13.1±4.21	3.4±1.62	10.7±3.62					0.24
2-BrOA (+20 mM OA)	0	72	2.6±0.26	27.2±3.53	6.0±0.42	50.0±2.13	17.5±1.48	14.2±0.85	7.5±0.75					0.11
	0.1	72	2.4±0.09	27.8±4.25	6.2±1.00	52.8±3.85	17.4±3.13	13.9±2.26	7.2±2.08					0.11
	0.5	72	2.3±0.09	26.3±5.12	6.7±0.52	54.1±3.21	17.1±2.82	13.6±2.19	7.0±3.05					0.10
	1	72	2.1±0.06	27.5±4.85	6.9±0.96	58.2±4.15	16.0±1.23	11.8±2.42	5.9±1.89					0.11
	3	72	2.1±0.05	25.5±3.88	7.6±1.36	66.5±5.62	15.2±2.52	6.3±2.23	3.4±1.36					0.11
	5	72	2.0±0.11	26.5±4.97	8.1±1.42	70.1±5.12	14.2±2.68	4.5±2.78	2.4±1.38					0.12
OA[c]	40	72	2.5±0.16	23.3±2.54	11.8±0.14	84.4±2.86	1.8±0.12	0.4±0.01	0.4±0.01					0.08

[a]C_6, 3-hydroxyhexanoate; C_8, 3-hydroxyoctanoate; C_{10}, 3-hydroxydecanoate; C_{12}, 3-hydroxydodecanoate; $C_{12:1}$, 3-hydroxydodecenoate; 3HPV, 3-hydroxy-5-phenylvalerate; 5POHV, 3-hydroxyphenoxyvalerate; 7POHH, 3-hydroxyphenoxyheptanoate; 9POHN, 3-hydroxyphenoxynonanoate.

[b]11-POU, 11-phenoxyundecanoate; 2-BrOA, 2-bromooctanoate.

[c]The cells were grown with OA as single carbon source.

[d]The minor monomers principally composed of C_{14} and $C1_{4:1}$ were omitted. Therefore, the summation may not lead to 100%.

[e]The conversion yield is defined as the ratio: [mol of carboxylate substrate converted into PHA]/[mol of carboxylate substrate in the feed].

Quantitation of Remaining Nitrogen and Carbon Sources and PHA in Cells

The cell growth was monitored by turbidity measurements at 660 nm in a Spectronic 20 spectrophotometer. The concentrations of ammonium ion remaining in media were measured using the Nessler reagent method [3], the concentration of fructose remaining by using DNS method [3] and the concentration of remaining organic acid, including both carbon sources and inhibitors, by reacting the chloroform extract of NaCl saturated medium with a sulfuric acid/methanol mixture followed by gas chromatographic (GC) determination of the resultant methyl esters [9]. For the analysis of PHA in cells, 10 mg of dried cells were reacted with a mixture containing 1 ml of chloroform, 0.85 ml of methanol, and 0.15 ml of conc. sulfuric acid: The reaction mixture in a closed screw-capped tube was incubated at 100°C for 3 h and the organic layer containing the reaction products was separated, dried over Na_2SO_4, and analyzed by gas chromatography. Each peak was standardized against standard 3-hydroxy-methylesters which were obtained by methanolysis of purified PHA with known compositions, determined by quantitative NMR analysis [9, 15, 16]. Gas chromatograms were obtained on a Hewlett Packard 5890A gas chromatograph equipped with a HP-1 column and a flame ionization detector. Minimum triplicate experiments were carried out and statistically averaged for all cultivations.

Isolation of PHA and Characterization of Monomer Composition

PHA was extracted from an appropriate amount of cells, which had been dried overnight under a vacuum at 50°C, with hot chloroform in a Pyrex Soxhlet apparatus for 6 h. After concentration, the solvent extract was precipitated with cold methanol while rapidly stirring. The isolated PHA was dried overnight under a vacuum at ambient temperature and then weighed. Quantitative determination of the monomer units in the PHA was performed by GC, as described above.

Thermal Characterization of PHA Blend Formed in *P. fluorescens* BM07

The dried PHA recovered from the cells, which had grown with 50 mM fructose and 30 mM OA, was aged at room temperature for at least one month or more to obtain fully crystallized structures [15, 16]. Thermal characterization of the PHA was carried out under a dry nitrogen purge using a Setaram differential scanning calorimeter (micro-DSC, Setaram Co., Caluire, France) equipped with a data station. The room-temperature annealed PHA samples were heated at a rate of 1°C/min from 10 to 80°C. The sample size was approximately 25 mg. The enthalpy value for each sample was calculated using the standard software supplied with the instrument. For comparison, several PHAs obtained from the cells grown with fructose or OA as the only carbon source were also characterized.
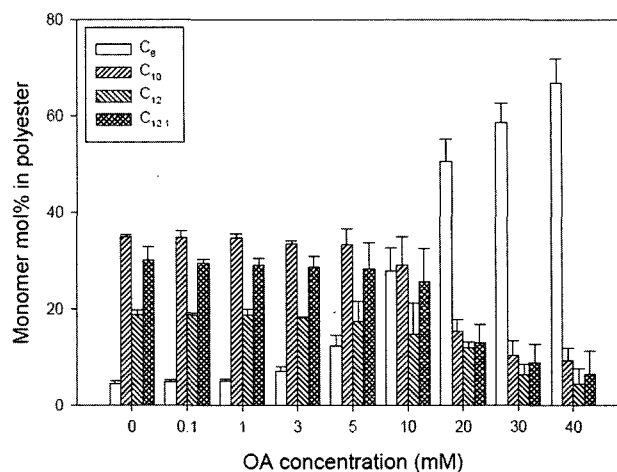
Fig. 2. Effect of octanoate (OA) level in the feed mixture of OA and 50 mM fructose on the monomer composition of PHA produced by *P. fluorescens* BM07, grown aerobically at 30°C for 72 h.

Display of the minor monomers such as C_6, C_{14}, and $C_{14:1}$ is omitted for simplicity.

RESULTS

Suppression of Fructose-Metabolizing PHA Synthesis by Unsubstituted Medium-Chain Fatty Acids

Compared to the monomer composition of the PHA in cells grown with fructose (50 mM) as sole carbon source (the control), a gradual increase of octanoic acid (OA) level in the mixture of OA and 50 mM fructose gradually lowered the levels of the monomers 3-hydroxydecanoic acid (C_{10}), 3-hydroxydodecanoic acid (C_{12}), and 3-hydroxy-*cis*-5-dodecenoic acid ($C_{12:1}$), all of which were the principal monomers of the control fructose-metabolized PHA (Table 1 and Fig. 2). The increase of the OA level to 40 mM in the mixed feed led to the production of a PHA with the monomer composition similar to that for the cells grown with OA only. The shift of the major monomer-unit occurred from C_{10} at 0 mM OA to 3-hydroxyoctanoic acid (C_8) at 10 mM or higher OA. In this fructose/OA system, C_{10} is derived mostly from fructose, and C_8 mostly from OA. The C_8 monomer was incorporated exponentially at around 10 to 20 mM OA.

To understand how the level of fructose-derived monomers was suppressed by the addition of OA, the time-course profiles for the consumption of the two carbon sources and the cell growth were investigated for the 50 mM fructose and 30 mM OA system (Fig. 3A). The consumption of fructose and ammonium sulfate occurred in a biphasic manner; an initial slower consumption and then a faster consumption after depletion of OA. When OA was used as a sole carbon source, it took BM07 strain at least 72 h to consume it, including 24 h of induction time (Fig. 3B). The PHA monomer composition profiling showed that C_8 was
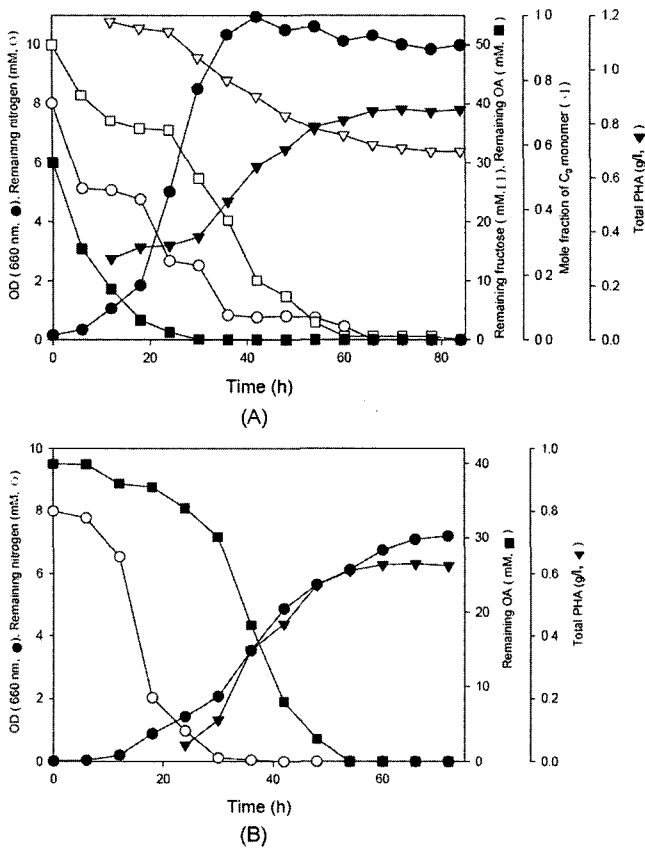
Fig. 3. Time-course profiles for PHA synthesis in *Pseudomonas fluorescens* BM07 grown in PHA synthesis mineral salts medium containing (A) 50 mM fructose plus 30 mM octanoate (OA) or (B) 40 mM OA as carbon source at 30°C.

Each medium contained 1.0 g/l ammonium sulfate. PHA formation was followed by a sulfuric acid/methanol reaction and then GC determination of the resulting 3-hydroxy-methylesters. The remaining fructose and OA was determined by the DNS and GC methods, respectively.

Fig. 4. Culture-time dependent (A) variation of monomer composition of the PHA produced by *P. fluorescens* BM07, grown with 50 mM fructose and 30 mM octanoate, and (B) comparative differential scanning calorimetric characterization of the PHA recovered at (c) 24 h and (b) 72 h.

All samples for DSC run were 25 mg. The DSC run was performed after all samples were fully crystallized by aging them under room temperature. The other samples in (B) were designated: (a), PHA from fructose grown cells exhibiting no melt endothermic peak; (e), PHA from octanoate grown cells exhibiting a melt endothermic peak at 52°C; (d), a mixture of 40% of the PHA in (a) and 60% of the PHA in (e).

incorporated principally into the polymer during the initial 30 h, its mol% ranging 95 to 80 (Figs. 3A and 4A). The initially disappeared fructose must have been utilized mostly for cell growth. In the 2nd period of fructose consumption, the monomers C_{10}, C_{12}, and $C_{12:1}$ derived from fructose started to incorporate into the polymer. Thus, the resulting PHA was a blend-type PHA, not a copolymer. The parallel biphasic consumption of fructose and ammonium sulfate also suggests that the carbon source for cell growth was supplied mostly from fructose, and the OD profile pattern was in agreement with the above suggestion. Fructose is used up by BM07 strain cells within 24 h, when it is fed as the sole carbon source [9]. Thus, the addition of OA significantly retarded the consumption of fructose and also prevented its derivatives from flowing into PHA.

The blend-like nature of the PHA synthesized with the mixtures of OA and fructose was confirmed by the differential scanning calorimetric (DSC) traces of the PHA (Fig. 4B), in which all purified samples were fully crystallized by

storing them at ambient temperature for at least 1 month. The DSC traces in Fig. 4B for the crystallized polyesters were obtained from the medium used in Fig. 4A. The melting-enthalpy value of 4.5 cal/g for the 24 h culture in (c) of Fig. 4B is close to the value of 4.9 cal/g for P(3HO) polymer recovered from OA only grown cells in (e), whereas the value of 1.3 cal/g for the 72 h culture in (b) of Fig. 4B is comparable to the value of 2.9 cal/g for the blended mixture in (d), which is composed of 60% P(3HO) and
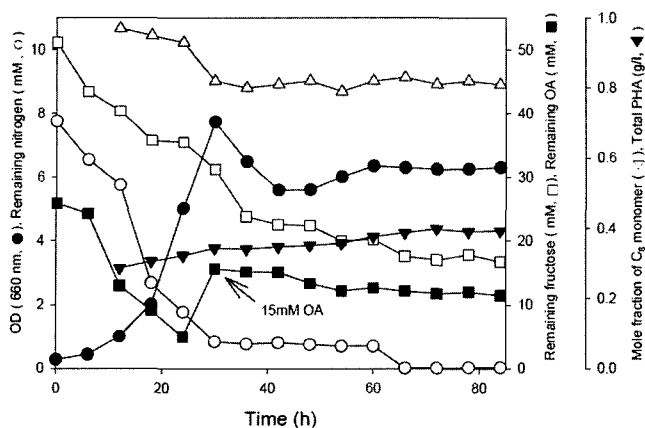
Fig. 5. Effect of an interim addition of 15 mM octanoate on the fructose consumption of *P. fluorescens* BM07, grown on 50 mM fructose and 30 mM octanoate.

After the addition of OA at 30 h of cultivation, no significant consumption of fructose occurred, and the mole fraction of C_8 (3-hydroxyoctanoate) remained constant at 80 mol%.

40% PHA from cells grown on fructose only. The comparably lower enthalpy value clearly demonstrates that the PHA from the mixed carbon source was not a homogeneous random copolymer.

An interim addition of 15 mM OA to the 50 mM fructose plus 30 mM OA system clearly revealed the effect of OA on the PHA synthesis from fructose. The OA was added at 30 h of cultivation, when the ammonium sulfate was almost depleted (Fig. 5), therefore, cell growth was halted; however, the fructose consumption seemed to still continue, but less vigorously, than in the case of no interim OA addition shown in Fig. 3A. Thus, 35% of the fructose remained unconsumed by the addition of 15 mM OA. The level of PHA content increased slightly as the OA was utilized, even though the rate of OA consumption was rather slower. However, the levels of both the fructose-derived monomers and C_8 were relatively constant throughout the cultivation after the interim addition. This result shows that the additional OA may partially block the pathway leading to PHA synthesis via PhaG, and that the resulting C_8-CoA was continuously and predominately polymerized while the supply of 3-hydroxyacyl-coenzyme A available through PhaG was significantly suppressed.

To find out whether there is any competitive relationship between OA and 2-BrOA, 2-BrOA was added to the mixture of 50 mM fructose plus 20 mM OA. In the 50 mM fructose/20 mM OA system, as the concentration of 2-BrOA was increased from 0 mM to 5 mM, only a slight increase in the mole% of C_8 was observed (Fig. 6A). However, lowering of OA co-feeding to 5 mM resulted in a significant proportionate increase in the relative C_8 level, as the 2-BrOA level increased (the inserted figure in Fig. 6B). The linear increase may suggest one-to-one substitution of 2-BrOA-derived species for OA-derived species bound to a common binding site. In
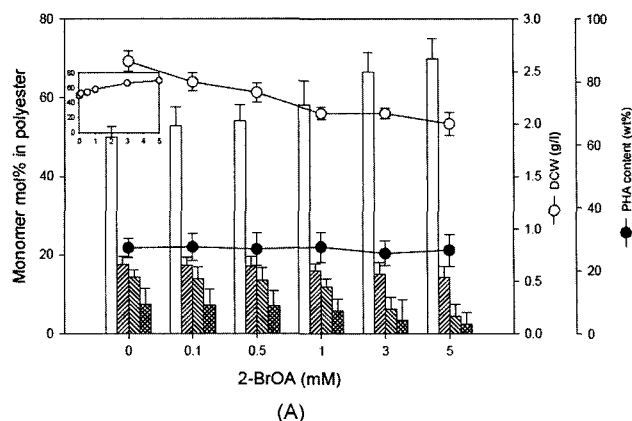
(A)
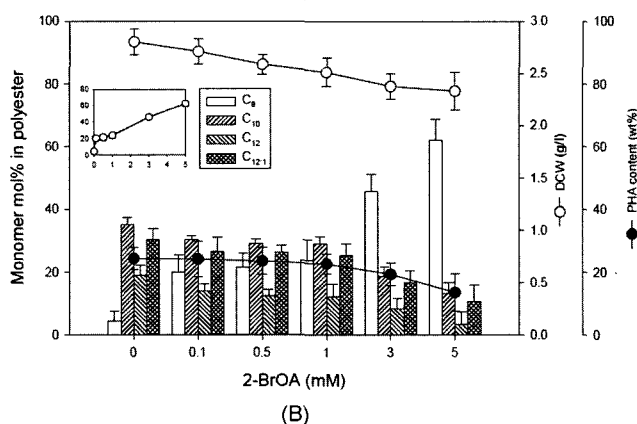
(B)

Fig. 6. Effect of 2-BrOA on the incorporation of C_8 monomer into PHA produced by *P. fluorescens* BM07, grown on (A) 50 mM fructose and 20 mM octanoate and (B) 50 mM fructose and 5 mM octanoate.

The minor monomers such as C_6, C_{14}, and $C_{14:1}$ were omitted for simplicity. The cells were cultivated using one-step cultivation technique in a manner similar to that in Fig. 1. The mole percent of C_8 monomer was replotted against the linear concentration of 2-BrOA, and the plots were inserted into each figure.

the 50 mM fructose/20 mM OA medium, 11% of the fed OA was converted into PHA, irrespective of the concentration of 2-BrOA, whereas at 3 to 5 mM 2-BrOA, 25% of the fed OA was converted in the 50 mM fructose/5 mM OA medium (Table 1). A more significant effect of 2-BrOA concentration on the conversion at lower OA concentration indicates a competitive nature existing between OA and 2-BrOA. Thus, the increase of OA concentration to 20 mM abolished the effect of 0 to 5 mM 2-BrOA addition.

Suppression by Phenyl-Group Substituted Medium-Chain Fatty Acids

Similar to that in OA/fructose media, both 11-POU and 5-PV exhibited concentration dependency on the PHA composition, when the bacterium was cultivated on the mixtures of phenyl-group substituted acid and 50 mM fructose. In the 11-POU/fructose system, 11-POU at only a few mM concentration suppressed the incorporation of the
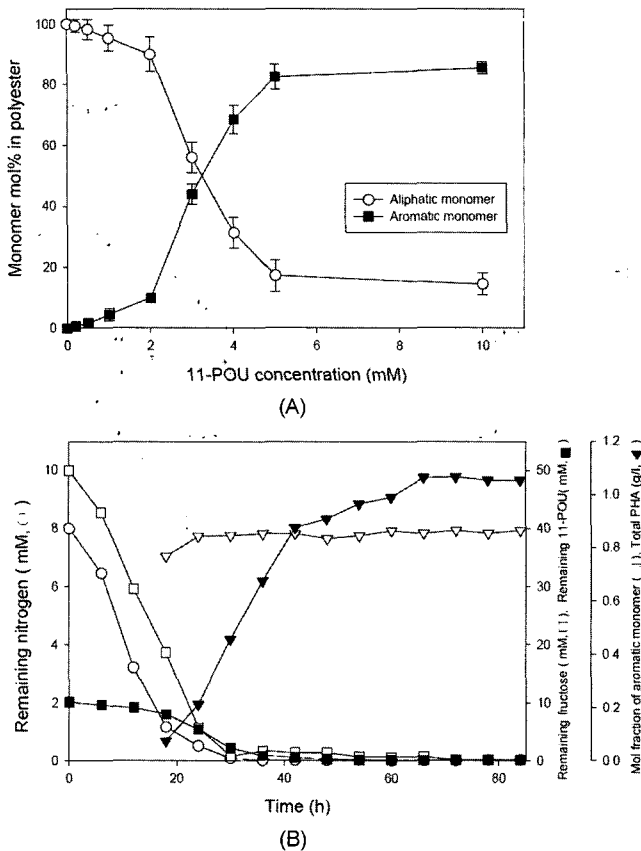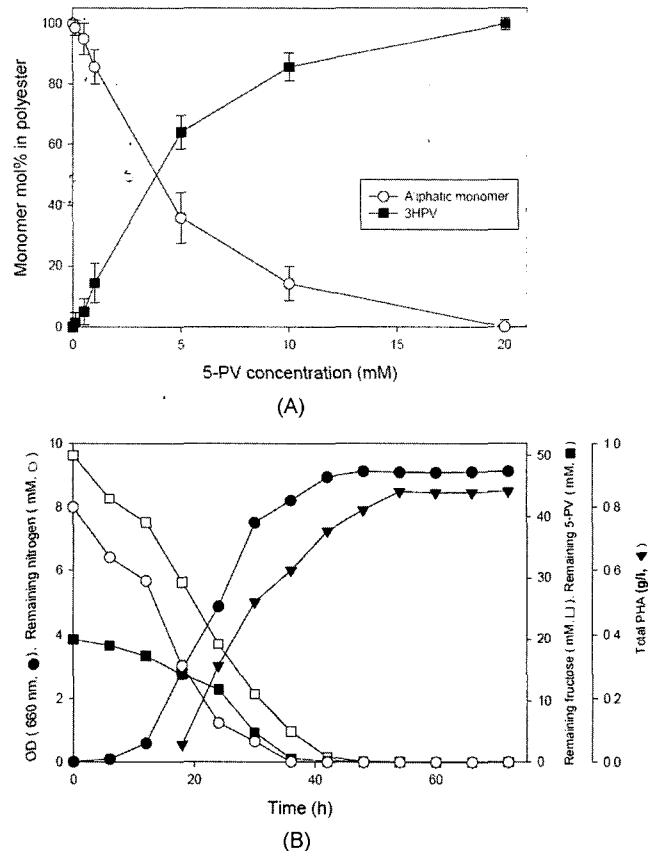
Fig. 7. Suppression of fructose-metabolized aliphatic monomer incorporation into PHA in *P. fluorescens* BM07 by phenyl-group substituted medium-chain fatty acids.

(A) Concentration-dependent effect of 11-POU. The cells were co-fed with 50 mM fructose and cultivated aerobically at 30°C for 72 h. (B) Time-course profiles for PHA synthesis in *P. fluorescens* BM07, grown in PHA synthesis mineral salts medium containing 50 mM fructose and 10 mM 11-POU.

Fig. 8. Suppression of fructose-metabolized aliphatic monomer incorporation into PHA in *P. fluorescens* BM07 by phenyl-group substituted medium-chain fatty acids.

(A) Concentration-dependent effect of 5-PV. The cells were co-fed with 50 mM fructose and cultivated aerobically at 30°C for 96 h. (B) Time-course profiles for PHA synthesis in *P. fluorescens* BM07, grown in PHA synthesis mineral salts medium containing 50 mM fructose and 20 mM 5-PV.

PhaG-mediated CoA monomers, significantly lower fed concentration than in the OA/fructose system (Fig. 7A and Table 1). Three to four mM 11-POU were enough to suppress the incorporation of the aliphatic monomers down to 20 mol% or less. At the concentration of 0.2 to 1 mM 11-POU, a significant reduction of $C_{12:1}$ and elevation of C_{12} level in PHA was observed, compared to the fructose control system (Table 1). Only 3-hydroxy-5-phenoxyvalerate (5POHV) was detected in PHA at these low 11-POU concentrations, and the longer monomers, such as 3-hydroxy-7-phenoxyheptanoate (7POHH) and 3-hydroxy-9-phenoxynonanoate (9POHN), were not found. However, an increase of 11-POU concentration induced the incorporation of the longer monomers such as 7POHH up to 25 mol% and 9POHN to 3 mol%, in which 5POHV was the dominant monomer (57 mol%).

In the 5-PV/fructose system, the level of 3-hydroxy-5-phenylvalerate (3HPV) was a function of concentration of the co-added substrate in a manner similar to that in the 11-

POU/fructose system (Fig. 8A). When the concentration of 5-PV was increased to 20 mM, the resulting PHA was found to be homopolymer poly(3-hydroxy-5-phenylvalerate), P(3HPV). At this 5-PV concentration, supply of the fructose-metabolized CoA monomers is believed to be completely blocked (Table 1). As shown in the time-course profiles for the 50 mM fructose/20 mM 5-PV system, the consumption rates of fructose and ammonium sulfate were parallel to each other, but the consumption rate of 5-PV was initially rather sluggish, later showing a faster pattern (Fig. 8B). *P. fluorescens* BM07 does not grow on 5-PV as the sole carbon source: Compared to the OA/fructose system, 5-PV at 20 mM did not affect the cell growth when co-fed with 50 mM fructose. Throughout the cultivation, no aliphatic monomers were incorporated into PHA, resulting in P(3HPV) homopolymer. Thus, a coenzyme A derivative of 5-PV inhibited PhaG.

The time-course profile of PHA synthesis in the 11-POU/fructose system (Fig. 7B) was quite different from that in

the OA/fructose system. First of all, biphasic consumptions of fructose and nitrogen found in the co-added OA system was not observed: The consumption of fructose was not affected by the presence of 11-POU, therefore, the fructose was preferentially consumed within 30 h (1.9 mmole/l·h), and 70 mM fructose as a sole carbon source was also consumed by a similar rate (2.3 mmole/l·h) [9]. Furthermore, the monomer composition of the PHA in the 10 mM 11-POU/50 mM fructose system was relatively constant throughout the cultivation, in which the fructose-derived monomers constituted only minor components, four monomers totally amounting to 15 mol%, whereas the phenoxy-monomers were dominant (totally 85 mol%). The parallel consumption of fructose and ammonium sulfate indicates that the fructose was utilized principally for the cell growth. Thus,
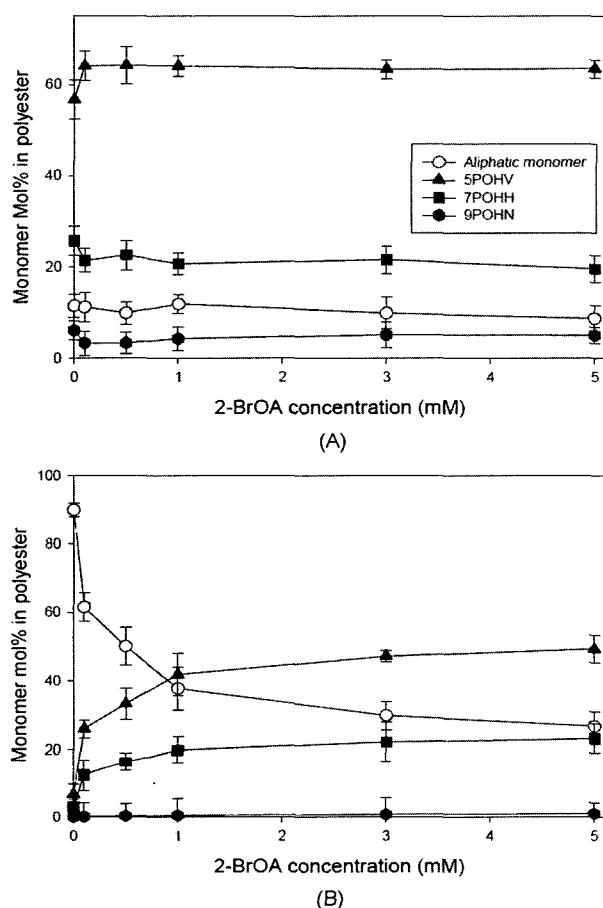
Fig. 9. Effect of 2-BrOA on the incorporation of fructose-metabolized aliphatic monomers into PHA in *P. fluorescens* BM07, grown on (A) 50 mM fructose and 5 mM 11-POU and (B) 50 mM fructose and 2 mM 11-POU.

The mol% of all aliphatic monomers was added and expressed as a single value for simplicity. The cells were cultivated using one-step cultivation technique for 72 h. A leveling effect of 5 mM 11-POU appeared to override the addition of 2-BrOA, however, at a concentration down to 2 mM, the ineffective suppression of 2 mM 11-POU was compensated by an increasing addition of 2-BrOA. See text for details.

the presence of 11-POU-derived CoA derivatives prevented the fructose-derived monomers from being incorporated into PHA via PhaG.

To ascertain whether 11-POU and 2-BrOA competitively blocked the carbon flow into PHA from fructose, the effect of 2-BrOA at various concentrations (0 to 5 mM) on the incorporation of aliphatic monomers into PHA was investigated in 2 mM 11-POU/50 mM fructose and 5 mM 11-POU/50 mM fructose media (Fig. 9). At 5 mM 11-POU, a similar amount of aliphatic monomers (~10 mol%) was incorporated into PHA irrespective of the 2-BrOA concentration (Fig. 9A), suggesting that 5 mM 11-POU was enough to block the flow of the aliphatic monomers through PhaG. However, in the 2 mM 11-POU/50 mM fructose system, a compensatory effect of 2-BrOA on the PHA composition was observed (Fig. 9B): An increase of the 2-BrOA concentration suppressed the incorporation of fructose-derived aliphatic monomers and enhanced the incorporation of the aromatic monomers derived from 11-POU.

The yield of conversion of 11-POU into PHA is also a function of concentration of 11-POU and 2-BrOA. At 5 mM 11-POU, the addition of 0.1 to 5 mM 2-BrOA resulted in a slight increase in the conversion (60–70%) compared to the control value (53%), irrespective of the 2-BrOA concentration. However, at the lowered 2 mM 11-POU system, the conversion yield strongly depended on the concentration of 2-BrOA: The addition of 5 mM 2-BrOA to 2 mM 11-POU/50 mM fructose medium completely converted 2 mM 11-POU into PHA. Thus, 2-BrOA can be used as an "effector" to convert a substituted MCL fatty acid into the corresponding 3-hydroxyacid in a high yield.

DISCUSSION

As long as the co-added OA remained in the OA/fructose medium, the incorporation of the fructose-derived comonomers in the bacterium was almost completely blocked. However, after the OA was exhausted, the PhaG-mediated CoA monomers began to incorporate into PHA. At high OA concentrations (e.g., 20 mM or higher), even 5 mM 2-BrOA had no significant additional effect on the comonomer flow into PHA from fructose. However, at lower OA concentrations (e.g., 5 mM or less), the blocking effect of 2-BrOA was highly dependent on its concentration. This concentration dependency of 2-BrOA blockage clearly indicates that the two structural analogues (the CoA thioester derived from OA and the CoA derived from 2-BrOA) may compete for the same enzyme which links fatty acid synthesis and PHA synthesis (probably PhaG). However, since 2-BrOA (at ≥1.5 mM) inhibits the growth of *P. fluorescens* BM07 fed on OA as a sole carbon source, but less significantly PHA

synthesis [9], it is likely that a CoA thioester derivative of 2-BrOA also acts as a weak inhibitor for the β-oxidation enzyme(s). Therefore, 2-BrOA appears to affect the enzymes on the two pathways, strongly inhibiting PhaG and weakly inhibiting the β-oxidation enzyme (Fig. 1).

In contrast to the OA/fructose system, no such significant lag time in consumption between the carboxylic acid and fructose was observed for 11-POU/fructose and 5-PV/fructose systems (Figs. 7 and 8). Thus, the inhibitory mechanism of OA appears to be different from that of 11-POU and 5-PV, however, the common competitive effect of 2-BrOA against the two different types of substrates may exclude the above possibility. Thus, the fructose-derived PHA-synthesis inhibitory species which are derived from 2-BrOA, OA, and 11-POU compete for the same target site, implying a broad specificity of the common target enzyme.

One hundred μM 2-BrOA was enough to substantially block the carbon flow into the PHA synthesis pathway in BM07 strain [9]. Therefore, the inhibitory power (estimated from % of suppressed PHA synthesis at 5 mM acid substrate) of the acids tested in this study may decrease in the order of 2-BrOA>11-POU>5-PV>OA. 2-BrOA is not catabolized in the BM07 strain [9], but the other three acids are metabolized. Thus, the ω-phenyl or phenoxy group-substituted medium-chain fatty acids appear to be more potent in the inhibition of fructose metabolizing PHA synthesis than the unsubstituted acids. The high inhibitory potency may arise from rather longer half-lives of the inhibiting species, because of their slower degradation in cells and/or stronger binding strengths, resulting from higher hydrophobicity of the phenyl group. Thus, in order to prepare specially designed PHA composed of both β-oxidation and fatty acid *de novo* synthesis pathway intermediates (Fig. 1), 2-BrOA may be used to efficiently control the supplying rate of MCL-comonomers, which are produced via the fatty acid *de novo* synthesis pathway.

11-POU and 5-PV have the higher PHA conversion yield than the unsubstituted OA, which could be attributed to slower degradation of the aromatic species in the cell, thereby maintaining their higher intracellular concentrations available for PHA synthesis. However, 2-BrOA can further increase the conversion yield of medium-chain fatty acids into PHA more effectively at their low feed concentrations. The further increase of the conversion yield by 2-BrOA may be due to an additional role of 2-BrOA (probably caused by a species different from the species involved in PhaG blocking) to affect the β-oxidation enzyme(s), thereby slowing down the degradation rate of β-oxidation intermediates (Fig. 1). In the case of 11-POU, the slower degradation may contribute to the increase of the conversion yield up to 100%. In addition, this high yield may also result from more efficient supply of acetyl-CoA from fructose to cell growth than from the co-added fatty acids.

Based on the results, we suggest that the inhibition of fructose metabolizing PHA synthesis occurs at the enzymatic reaction level. However, especially in the case of OA, a question also arises about the possibility of blocking at the transcriptional level, where the *phaG* expression may be regulated. However, this possibility may be eliminated by the fact that fructose-derived monomers were immediately incorporated, followed by the depletion of OA (Figs. 3 and 4). A detailed enzymatic study for PhaG is underway.

Acknowledgments

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