Headline Articles

Biosynthesis of Polyester Blends by *Pseudomonas* sp. 61-3 from Alkanoic Acids

Mikiya Kato,†† Toshiaki Fukui,† and Yoshiharu Doi*,†,††

†Polymer Chemistry Laboratory, The Institute of Physical and Chemical Research (RIKEN), Hirosawa, Wako, Saitama 351-01

††Department of Biological and Environmental Sciences, Saitama University, Shimo-Okubo, Urawa, Saitama 338

(Received September 25, 1995)

Pseudomonas sp. 61-3 accumulated poly(3-hydroxyalkanoates), P(3HA), in nitrogen-free media containing medium- and long-chain alkanoic acids or plant oils. The polyesters of 3HA units of even carbon numbers ranging from C4 to C12 were produced from the alkanoic acids of even carbon numbers, while those produced from the alkanoic acids of odd carbon numbers contained 3-hydroxybutyrate (3HB) and 3HA units of odd carbon numbers of C5 to C11. The strain produced simultaneously P(3HB) homopolymer and a random copolymer of 3HA units of C4 to C12 within cells from the alkanoic acids of even carbon numbers, while a copolymer of 3HB and 3-hydroxyvalerate was formed with a random copolymer of 3HA units from the alkanoic acids of odd carbon numbers. The results suggest that *Pseudomonas* sp. 61-3 has two types of PHA synthases with different substrate specificities.

Bacterial poly(hydroxyalkanoates)(PHAs) are a biodegradable thermoplastic with a wide range of physical properties.[1,2] A number of bacteria synthesize isotactic homopolymers and copolymers of (*R*)-3-hydroxyalkanoic acids (3HA) with four to fourteen carbon atoms as an intracellular storage material of carbon and energy.[3—5] Saturated,[6,7] unsaturated,[8,9] halogenated,[10—12] branched[13] and aromatic[14] side chains in 3HA monomeric units have been found as constituents in the sequence of bacterial polyesters. In addition, several bacteria have been found to produce copolymers containing hydroxyalkanoate monomeric units without side chains such as 3-hydroxypropionate,[15] 4-hydroxybutyrate,[16,17] and 5-hydroxyvalerate.[18] The PHA have been shown to occur in over 90 genera of bacteria, and sixty different constituents of PHA have been identified as various hydroxyalkanoic acids with three to fourteen carbon atoms.[4] The bacterial PHA can be broadly divided into two groups. One group of bacteria including *Alcaligenes eutrophus* produces short-chain PHAs with C3–C5 monomeric units, while the other group, including *Pseudomonas oleovorans*, produces medium-chain PHAs with C6–C14 monomer units. Recently, some bacteria have been found to produce polyesters consisting of 3HA units of C4 to C12.[19,20]

In general, bacterially produced copolyesters of hydroxyalkanoic acids have a narrow distribution of copolymer composition.[1,2] However, some bacterial copolyesters have been reported to be a mixture of several copolymers with different compositions. The copolymers of 3-hydroxybutyrate (3HB) and 4-hydroxybutyrate (4HB) produced by *Alcaligenes eutrophus*[21] and *Comamonas acidovorans*[22] were shown to be a mixture of random copolymers with two different 4HB contents. Mitomo et al.[23] fractionated the bacterial copolymers of 3HB and 3-hydroxyvalerate (3HV) into several fractions with different 3HV contents by a mixed solvent of acetone/water. In a previous paper,[19] we reported that a soil bacterium *Pseudomonas* sp. 61-3 produced a mixture of poly(3-hydroxybutyrate) homopolymer and a random copolymer of seven 3HA units of C4 to C12 when gluconate was fed as the sole carbon source. In this paper, we report that *Pseudomonas* sp. 61-3 strain produces a blend of two types of PHA from various alkanoic acids of C6 to C18 and plant oils under nitrogen-free conditions, and we discuss the biosynthetic pathway of this PHA blend.

Experimental

Bacterial Strain and Culture Conditions. *Pseudomonas* sp. 61-3 (FERM P-13108) was isolated from soil[19] and used in this study. The isolated bacterial strain 61-3 was a Gram-negative, aerobic, and motile rod, which was catalase and oxidase positive. Further tests revealed that the strain was non-fluorescent, capable of reducing nitrate, able to form acid on glucose, and able to utilize glucose, mannose and fructose. On the basis of these phenotypic properties, strain 61-3 was identified as a *Pseudomonas*

sp. Optical and electron microscopes clearly showed the presence of polar flagella. Although DNA–DNA hybridization of the strain 61-3 was respectively made with *Pseudomonas aeroginosa* (IFO 12689), *P. fluorescens* (IFO 14162), *P. chlororaphis* (IFO 3904), *P. putida* (IFO 14164), *P. stutzeri* (IFO 14165), and *P. mendocina* (IFO 14162), the species of this strain could not be identified due to a low homology intensities (13—27%). The taxonomic assignation of this strain was made in the Japan Food Research Laboratories.

Polyester synthesis was carried out by a two-stage fermentation of *Pseudomonas* sp. 61-3. *Pseudomonas* sp. 61-3 was first grown under aerobic conditions at 30 °C in a nutrient-rich medium (100 ml) containing 1 g yeast extract, 1 g peptone, 0.5 g meat extract, and 0.5 g $(NH_4)_2SO_4$. The cells were harvested by centrifugation after 10 h. Under these culture conditions, the cells accumulated P(3HB) homopolymer. Its content in dry cells was 2 wt%. To promote polyester synthesis, about 0.4 g (dry weight) quantity of the centrifuged cells was transferred into a 100 ml nitrogen-free mineral medium containing a different carbon substrate (0.5 g). The carbon substrates used were normal alkanoic acids of carbon numbers from C2 to C18 and plant oils. The pH value of media was adjusted at 7.0 by addition of NaOH. The composition of the mineral salts medium was as follows (per liter of distilled water): 3.8 g of Na_2HPO_4, 2.65 g of KH_2PO_4, 0.2 g of $MgSO_4$, and 1 ml of microelement solution (pH 7.0). The microelement solution contained 9.7 g of $FeCl_3$, 7.8 g of $CaCl_2$, 0.218 g of $CoCl_2 \cdot 6H_2O$, 0.156 g of $CuSo_4 \cdot 5H_2O$, 0.118 g of $NiCl_3 \cdot 6H_2O$ and 0.105 g of $CrCl_3 \cdot 6H_2O$ per liter of 0.1 M HCl (M = mol dm^{-3}). All cell growth and polyester synthesis experiments were performed under aerobic conditions in a temperature-controlled shaker (Taitec Bio-shaker BR-3000L) at 30 °C and 130 rpm. The cells were incubated in the nitrogen-free medium for 48 h at pH 7.0, harvested by centrifugation, washed with methanol or ethanol, and finally lyophilized. Polyesters were extracted from the lyophilized cells with hot chloroform in a Soxhlet apparatus and purified by reprecipitation with methanol.

Analytical Procedures. To determine the cellular polyester content and polymer composition, approximately 15 mg of dry cells was subjected to methanolysis with a solution consisting of 1.7 ml of methanol, 0.3 ml of 98% sulfuric acid and 2.0 ml of chloroform at 100 °C for 140 min to convert the constituents to their methyl esters. Addition of 1 ml of water to the reaction mixture induced phase separation. The lower chloroform layer was used for gas chromatography (GC) analysis on a Shimadzu GC-14A system equipped with a Neutra Bond-1 capillary column (30 m by 0.25 mm) and a flame ionization detector.

All molecular weight data were obtained by gel permeation chromatography (GPC) at 40 °C, using a Shimadzu 6A GPC system and a 6A refractive index detector with serial columns of Shodex K-80M and K-802 columns. Chloroform was used as eluent at a flow rate of 0.8 ml min^{-1}. Sample concentration was 1.0 mg ml^{-1}. Polystyrene standards with low polydispersity were used to construct a calibration curve.

The solution ^{13}C nuclear magnetic resonance (NMR) spectra of polyesters in chloroform were recorded on a JEOL α-400 spectrometer. The 100 MHz ^{13}C NMR spectra were recorded at 27 °C on a $CDCl_3$ solution of polyester sample (25 mg ml^{-1}) with 5 ms pulse width (45° pulse angle), 2.6 s pulse repetition, 27100 Hz spectral width, 16000 data points and 22000 accumulations. Tetramethylsilane (Me_4Si) was used as an internal shift standard.

Differential scanning calorimetry (DSC) data of polyesters were recorded in the temperature range of −150 to 200 °C on a Shimadzu DSC-50 equipped with a cooling accessory under a nitrogen flow of 30 ml min^{-1}. Samples were heated from 0 to 200 °C at a rate of 10 °C min^{-1}. The melting temperature (T_m) and the enthalpy of fusion (ΔH_m) were determined from the DSC endotherms. For measurement of the glass-transition temperature (T_g), the samples were maintained at 200 °C for 1 min, and then rapidly quenched at −150 °C. They were then heated from −150 to 200 °C at a heating rate of 20 °C min^{-1}. The T_g was taken as the midpoint of the heating capacity change.

Results and Discussion

Biosynthesis of Polyester from Various Alkanoic Acids. Polyester synthesis in *Pseudomonas* sp. 61-3 was carried out under aerobic conditions at 30 °C for 48 h in nitrogen-free media containing various alkanoic acids of C2 to C18 and plant oils (5 g dm^{-3}). When short-chain alkanoic acids of C2 to C5 were used as the sole carbon source, no polyester was produced within cells. Polyesters were produced from medium- and long-chain alkanoic acids of C6 to C18 and plant oils (see Table 1). The content of polyester in dry cells was as large as 26—33 wt% when alkanoic acids of C12 to C14 were fed to *Pseudomonas* sp. 61-3. When the alkanoic acids of even carbon numbers were used as the sole carbon source, the produced polyesters were composed of 3HA monomeric units of even carbon numbers ranging from C4 to C12. In contrast, the polyesters produced from the alkanoic acids of odd carbon numbers contained 3-hydroxybutyrate (3HB) and 3HA units of odd carbon numbers ranging from C5 to C11.

In the production of P(3HA) from C6–C12 alkanoic acids, the longest 3HA unit had the same chain length as the alkanoic acid fed, and other 3HA units had shorter chain lengths than that of carbon source. The result suggests that the pathway of P(3HA) synthesis is linked to the cyclic β-oxidation and to thiolytic cleavage of fatty acid. A close relationship between the pathways of both fatty acid oxidation and P(3HA) synthesis was first established by Lageveen et al.[8] who studied the biosynthesis of P(3HA) in *Pseudomonas oleovorans* from medium-chain alkanoic acids.

Formation of Polyester Blends. The produced polyesters may be a blend of P(3HB) homopolymer and P(3HA) copolymer, as reported in a previous paper.[19] Then, the fractionation of polyesters produced from alkanoic acids of C12 to C15 was carried out for 5 h with boiling acetone. The result is given in Table 2. The polyesters from the alkanoic acids of even carbon numbers were fractionated into P(3HB) homopolymer (acetone-insoluble fraction) and P(3HA) copolymer of C4–C12 monomeric units (acetone-soluble fraction). In contrast, the polyesters from those of odd carbon numbers were fractionated into a copolymer of short-chain 3HA units of C4 and C5 (acetone-insoluble fraction) and the copolymer consisting of both short- and medium-chain 3HA units ranging from C4 to C11 (acetone-soluble fraction).

Table 3 shows the molecular weights and thermal properties of the two fractions of polyesters. Each fraction had a single glass-transition temperature (T_g). The T_g values of acetone-soluble fractions were around −40 °C, and those of acetone-insoluble fractions were around 2 °C. The acetone-

Table 1. Production of Polyesters from Various Alkanoic Acids and Plant Oils by *Pseudomonas* sp. 61-3 for 48 h at 30 °C

Carbon source	Cell dry weight	Polyester yield	Polyester content[a]	PHA composition[b]/mol%								
5 g dm^{-3}	g dm^{-3}	g dm^{-3}	wt%	3HB	3HV	3HHx	3HHp	3HO	3HN	3HD	3HUD	3HDD
$CH_3(CH_2)_4COOH$	4.11	0.24	6	72	—	28	—	—	—	—	—	—
$CH_3(CH_2)_5COOH$	3.43	0.30	9	44	9	—	47	—	—	—	—	—
$CH_3(CH_2)_6COOH$	3.44	0.42	12	32	—	27	—	41	—	—	—	—
$CH_3(CH_2)_7COOH$	3.89	0.60	15	32	6	—	30	—	32	—	—	—
$CH_3(CH_2)_8COOH$	1.99	0.28	14	42	—	20	—	33	—	5	—	—
$CH_3(CH_2)_9COOH$	2.76	0.19	7	47	—	—	18	—	35	—	—	—
$CH_3(CH_2)_{10}COOH$	5.25	1.48	28	78	—	3	—	12	—	6	—	1
$CH_3(CH_2)_{11}COOH$	5.79	1.50	26	73	7	—	5	—	11	—	4	—
$CH_3(CH_2)_{12}COOH$	6.04	2.01	33	71	—	4	—	15	—	8	—	3
$CH_3(CH_2)_{13}COOH$	4.96	0.83	17	62	8	—	8	—	15	—	7	—
$CH_3(CH_2)_{14}COOH$	4.42	0.44	10	60	—	7	—	23	—	9	—	2
$CH_3(CH_2)_{15}COOH$	4.12	0.20	5	59	12	—	10	—	19	—	—	—
$CH_3(CH_2)_{16}COOH$	4.01	0.18	4	81	—	3	—	11	—	5	—	—
Corn oil	5.46	0.27	8	41	—	14	—	34	—	10	—	1
Olive oil	6.58	0.53	8	45	—	13	—	30	—	12	—	7
Palm oil	5.82	0.87	15	49	—	13	—	29	—	9	—	—

a) Polyester content in dry cells. b) Determined by GC analysis: 3HB; 3-hydroxybutyrate, 3HV; 3-hydroxyvalerate, 3HHx; 3-hydroxyhexanoate, 3HHp; 3-hydroxyheptanoate, 3HO; 3-hydroxyoctanoate, 3HN; 3-hydroxynonanoate, 3HD; 3-hydroxydecanoate, 3HUD; 3-hydroxyundecanoate, 3HDD; 3-hydroxydodecanoate.

Table 2. Fractionation of Produced Polyester with Boiling Acetone for 5 h

Carbon source	Polyester[a]	Fraction weight	PHA composition[b]/mol%								
		mg	3HB	3HV	3HHx	3HHp	3HO	3HN	3HD	3HUD	3HDD
$CH_3(CH_2)_{10}COOH$	Whole	177	78	—	3	—	12	—	6	—	1
	Soluble	64	11	—	12	—	48	—	24	—	5
	Insoluble	113	100	—	—	—	—	—	—	—	—
$CH_3(CH_3)_{11}COOH$	Whole	217	73	7	—	5	—	11	—	4	—
	Soluble	92	4	7	—	22	—	48	—	18	—
	Insoluble	125	95	5	—	—	—	—	—	—	—
$CH_3(CH_2)_{12}COOH$	Whole	240	71	—	4	—	15	—	8	—	3
	Soluble	95	10	—	11	—	43	—	26	—	10
	Insoluble	145	100	—	—	—	—	—	—	—	—
$CH_3(CH_2)_{13}COOH$	Whole	193	62	8	—	8	—	15	—	7	—
	Soluble	97	5	8	—	22	—	46	—	19	—
	Insoluble	96	95	5	—	—	—	—	—	—	—

a) Polyester was produced for 48 h at 30 °C from C12–C15 alkanoic acids (5 g dm^{-3}).

insoluble fractions were a partially crystalline polymer. The melting temperatures of P(3HB) homopolymer were around 175 °C, and those of copolymers of 3-hydroxybutyrate (95 mol%) and 3-hydroxyvalerate (5 mol%) were around 165 °C. In contrast, the acetone-soluble fractions were an amorphus polymer. This suggests that the fractions are random copolymers containing short- and medium-chain 3HA units.

The sequence distributions of 3HA units in copolymers were determined by analysis of the 100 MHz ^{13}C NMR spectra. The carbonyl resonances ($\delta = 167$—170) were clearly resolved into three peaks, arising from different diad sequences of connecting 3HB and other 3HA units of C5 to C12.[19,24] The diad sequence distribution data were compared with the Bernoullian statistics applicable to a statistically random copolymerization. In the Bernoullian model, the mole fraction F_{ij} of diad sequence ij can be expressed from the mole fractions F_i and F_j of i and j units as $F_{ij} = F_iF_j$. The diad fractions calculated from the mole fractions of 3HB and 3HA units are in good agreement with the observed values (see Table 3). Thus, ^{13}C NMR data revealed that the copolymers in both acetone-soluble and acetone-insoluble fractions have a statistically random distribution of 3HB and 3HA units of C5 to C12.

The time-dependent change in the formation of polyester blends in *Pseudomonas* sp. 61-3 cells was investigated in the nitrogen-free medium containing dodecanoic or tridecanoic acid (5 g dm^{-3}) as the sole carbon source. The results are shown in Figs. 1 and 2. When dodecanoic acid was used as the carbon source, both acetone-insoluble fraction of P(3HB) homopolymer and the soluble fraction of P(3HB-*co*-3HA) copolymer increased with time during the presence of a carbon source up to 20 h (see Fig. 1 (B)), which indi-

Table 3. Properties and Dyad Sequence Distributions of Fractionated Polyester Samples

Carbon source	Polyester	Molecular weight		Thermal properties			Relative intensities	Sequence[a]		
		M_n	M_w/M_n	T_g °C	T_m °C	ΔH_m J g^{-1}		F_{3HB^*-3HB}	$F_{3HB^*-3HA}+F_{3HA^*-3HB}$	F_{3HA^*-3HA}
$CH_3(CH_2)_{10}COOH$	Soluble	36000	5.7	−40	—	0	Obsd[b]	0	0.22	0.78
							Calcd[c]	0.01	0.20	0.79
	Insoluble	188000	4.3	3	174	83	Obsd[b]	1.00	0	0
$CH_3(CH_2)_{11}COOH$	Soluble	81000	3.7	−41	—	0	Obsd[b]	0	0.10	0.90
							Calcd[c]	0	0.08	0.92
	Insoluble	250000	3.8	2	162	75	Obsd[b]	0.90	0.10	0
							Calcd[c]	0.90	0.10	0
$CH_3(CH_2)_{12}COOH$	Soluble	109000	2.8	−44	—	0	Obsd[b]	0	0.20	0.80
							Calcd[c]	0.02	0.26	0.72
	Insoluble	292000	3.1	3	175	79	Obsd[b]	1.00	0	0
$CH_3(CH_2)_{13}COOH$	Soluble	82000	2.9	−39	—	0	Obsd[b]	0	0.16	0.84
							Calcd[c]	0	0.10	0.90
	Insoluble	387000	2.5	2	166	77	Obsd[b]	0.89	0.11	0
							Calcd[c]	0.90	0.10	0

a) 3HB; 3-hydroxybutyrate (C4), 3HA; 3-hydroxyalkanoate units of C5–C14. b) Determined from relative peak areas of carbonyl carbon resonances. c) Calculated by the Bernoullian statistics.

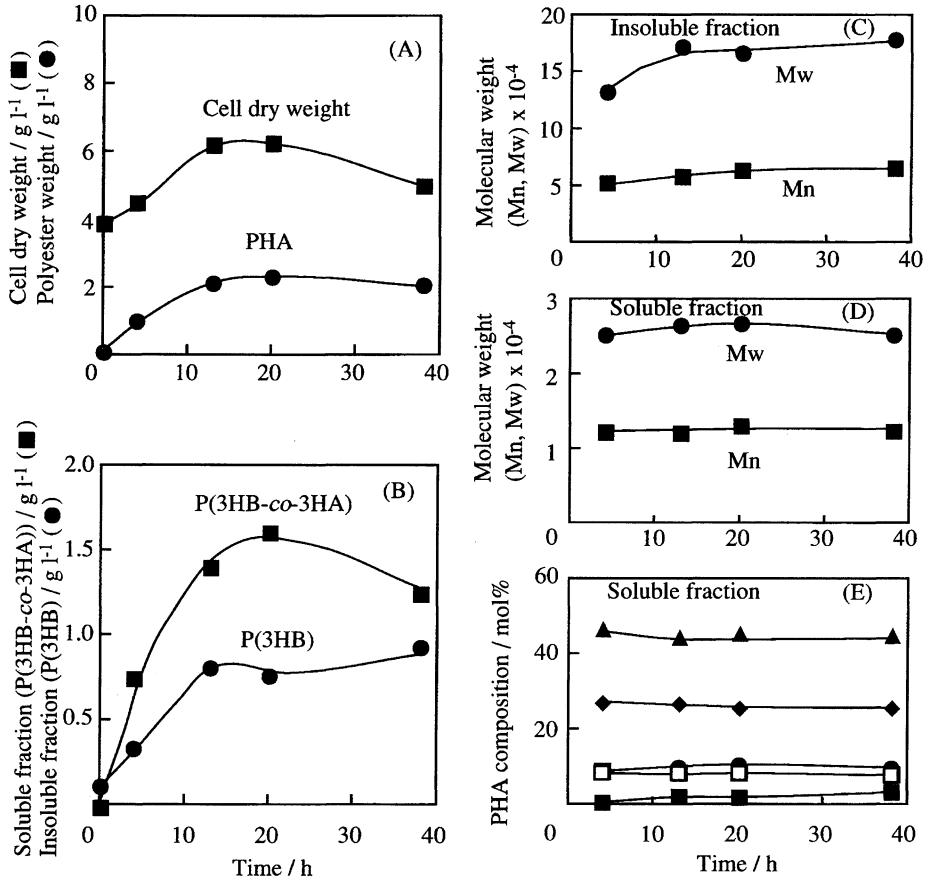

Fig. 1. Time courses of cell weight and PHA accumulation during the batch fermentation of *Pseudomonas* sp. 61-3 in a nitrogen-free medium containing dodecanoic acid (5 g dm^{-3}) at 30 °C. (E) Copolymer composition in the acetone-soluble fraction; 3HB (■), 3HHx (●), 3HO (▲), 3HD (◆), 3HDD (□).

cates that two components of P(3HB) and P(3HB-*co*-3HA) are simultaneously produced within cells from dodecanoic acid. The molecular weights and copolymer compositions of polyesters remained almost unchanged during the forma-

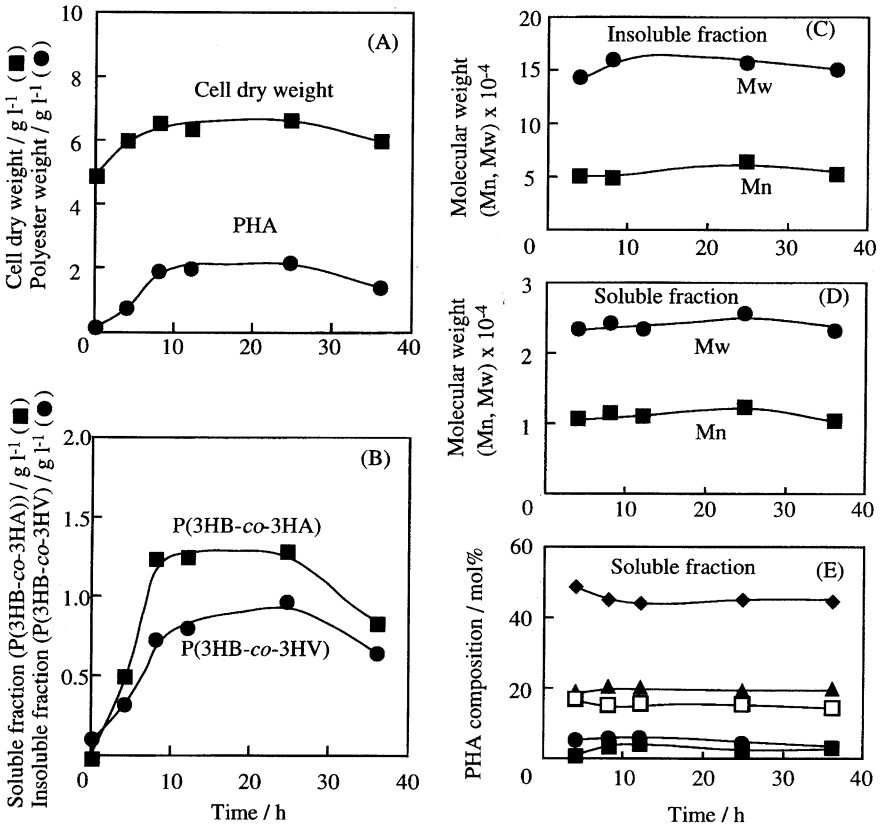

Fig. 2. Time courses of cell weight and PHA accumulation during the batch fermentation of *Pseudomonas* sp. 61-3 in a nitrogen-free medium containing tridecanoic acid (5 g dm^{-3}) at 30 °C. (E) Copolymer composition in the acetone-soluble fraction; 3HB (■), 3HV (●), 3HHp (▲), 3HN (◆), 3HUD (□).

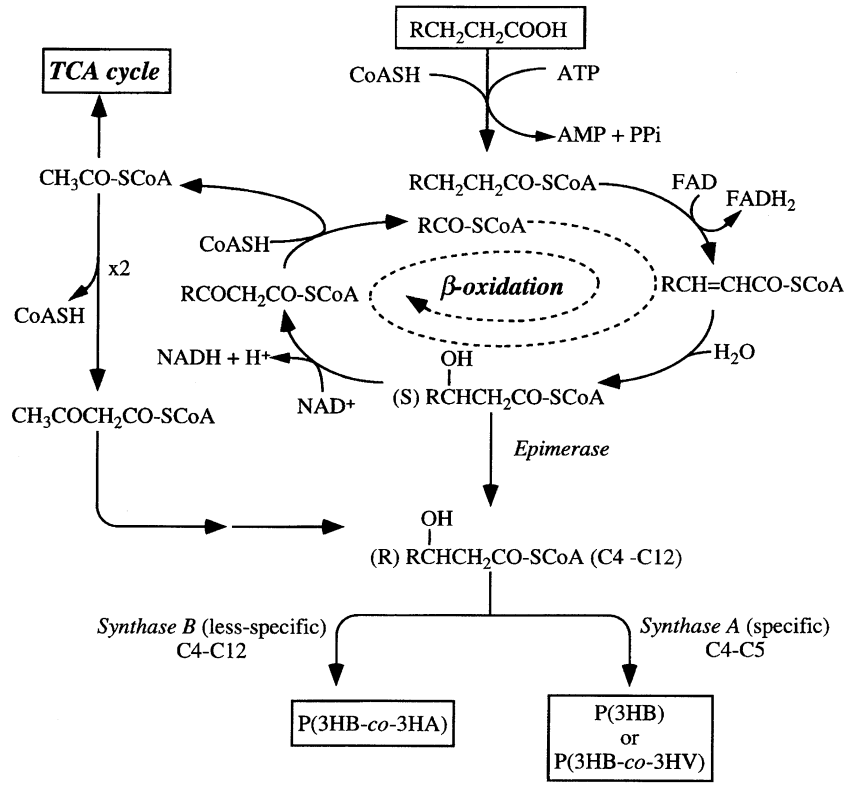

Fig. 3. Proposed biosynthetic pathways of polyester blends from a normal alkanoic acid (C6–C18) in *Pseudomonas* sp. 61-3. TCA cycle and CoASH denote the tricarboxylic acid cycle and coenzyme A, respectively.

tion of polyester blends. As Fig. 2 (B) shows, two types of copolymers of P(3HB-*co*-3HV) and P(3HB-*co*-3HA) were simultaneously formed within cells from tridecanoic acid under nitrogen-free conditions.

The formation of a polyester blend from medium- and long-chain alkanoic acids (C6—C18) suggests that *Pseudomonas* sp. 61-3 has two types of PHA synthases with different substrate specificities. As shown in Fig. 3, an acetone-insoluble fraction of P(3HB) homopolymer or P(3HB-*co*-3HV) copolymer may be formed by a PHA synthase A with a similar substrate specificity with the synthase of *A. eutrophus*, while an acetone-soluble fraction of P(3HB-*co*-3HA) copolymer may be formed by a PHA synthase B with a wide range of substrate specificity.

The pathway of polyester synthesis in this strain is linked to the cyclic β-oxidation and thiolytic cleavage of alkanoic acids. The 3-hydroxyacyl-CoA in the β-oxidation cycle has the *S* configuration, while the 3-hydroxyalkanoate units in polyester have the *R* configuration.[8] The (*R*)-3-hydroxyacyl-CoA may be formed from (*S*)-stereoisomer by the action of epimerase and polymerized into two types of polyesters in the presence of PHA synthases A and B. In all experiments, 3HA units with two fewer carbon atoms are formed because of β-oxidation and thiolytic cleavage of the alkanoic acid used as the carbon substrate. It is of interest to note that 3HB unit of C4 is incorporated into polyesters from the alkanoic acids of odd carbon numbers. The (*R*)-3-hydroxybutyryl-CoA may be formed via the condensation of acetyl-CoA. We are now attempting to isolate the PHA synthase gene(s) in *Pseudomonas* sp. 61-3.

This study was supported by the Special Coordination Research Fund of the Science and Technology Agency, Japan.

References

1) P. A. Holmes, "Developments in Crystalline Polymers-2," ed by D. C. Bassett, Elsevier, London (1988), p. 1.

2) Y. Doi, "Microbial Polyesters," VCH Publishers, New York (1990).

3) A. J. Anerson and E. A. Dawes, *Microbiol. Rev.*, **54**, 450 (1990).

4) A. Steinbüchel, "Biomaterials," ed by D. Byrom, Macmillan Publishers, Basingstoke (1991), p. 123.

5) H. M. Müller and D. Seebach, *Angew. Chem., Int. Ed. Engl.*, **32**, 477 (1993).

6) M. J. De Smet, G. Eggink, B. Witholt, J. Kingma, and H. Wynberg, *J. Bacteriol.*, **154**, 870 (1983).

7) H. Brandl, R. A. Gross, R. W. Lenz, and R. C. Fuller, *Appl. Environ. Microbiol.*, **54**, 1977 (1988).

8) R. D. Lageveen, G. W. Huisman, H. Preusting, P. Ketelaar, G. Eggink, and B. Witholt, *Appl. Environ. Microbiol.*, **54**, 2924 (1988).

9) K. Fritzsche, R. W. Lenz, and R. C. Fuller, *Int. J. Biol. Macromol.*, **12**, 85 (1990).

10) Y. Doi and C. Abe, *Macromolecules*, **23**, 3705 (1990).

11) C. Abe, Y. Taima, Y. Nakamura, and Y. Doi, *Polym. Commun.*, **31**, 404 (1990).

12) Y. B. Kim, R. W. Lenz, and R. C. Fuller, *Macromolecules*, **25**, 1852 (1992).

13) K. Fritzsche, R. W. Lenz, and R. C. Fuller, *Int. J. Biol. Macromol.*, **12**, 92 (1990).

14) Y. B. Kim, R. W. Lenz, and R. C. Fuller, *Macromolecules*, **24**, 5256 (1991).

15) E. Shimamura, M. Scandola, and Y. Doi, *Macromolecules*, **27**, 4429 (1994).

16) Y. Doi, M. Kunioka, Y. Nakamura, and K. Soga, *Macromoleculs*, **21**, 2722 (1988).

17) S. Nakamura, Y. Doi, and M. Scandola, *Macromolecules*, **25**, 4237 (1992).

18) Y. Doi, A. Tamaki, M. Kunioka, and K. Soga, *Makromol. Chem., Rapid Commun.*, **8**, 631 (1987).

19) H. Abe, Y. Doi, T. Fukushima, and H. Eya, *Int. J. Biol. Macromol.*, **16**, 115 (1994).

20) E. Y. Lee, D. Jendrossek, A. Schirmer, C. Y. Choi, and A. Steinbüchel, *Appl. Microbiol. Biotechnol.*, **42**, 901 (1995).

21) Y. Doi, A. Segawa, and M. Kunioka, *Int. J. Biol. Macromol.*, **12**, 106 (1990).

22) Y. Saito and Y. Doi, *Int. J. Biol. Macromol.*, **16**, 99 (1994).

23) H. Mitomo, N. Morishita, and Y. Doi, *Macromolecules*, **26**, 5809 (1993).

24) Y. Doi, M. Kunioka, Y. Nakamura, and K. Soga, *Macromolecules*, **19**, 2860 (1986).