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Production of a novel copolyester of 3-hydroxybutyric acid and medium-chain-length 3-hydroxyalkanoic acids by *Pseudomonas* sp. 61-3 from sugars

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Abstract *Pseudomonas* sp. 61-3 (isolated from soil) produced a polyester consisting of 3-hydroxybutyric acid (3HB) and of medium-chain-length 3-hydroxyalkanoic acids (3HA) of C_6, C_8, C_{10} and C_{12}, when sugars of glucose, fructose and mannose were fed as the sole carbon source. The polyester produced was a blend of homopolymer and copolymer, which could be fractionated with boiling acetone. The acetone-insoluble fraction of the polyester was a homopolymer of 3-hydroxybutyrate units [poly (3HB)], while the acetone-soluble fraction was a copolymer [poly(3HB- *co*-3HA)] containing both short- and medium-chain-length 3-hydroxyalkanoate units ranging from C_4 to C_{12}:44 mol% 3-hydroxybutyrate, 5 mol% 3-hydroxyhexanoate, 21 mol% 3-hydroxyoctanoate, 25 mol% 3-hydroxydecanoate, 2 mol% 3-hydroxydodecanoate and 3 mol% 3-hydroxy-5-*cis*-dodecenoate. The copolyester was shown to be a random copolymer of 3-hydroxybutyrate and medium-chain-length 3-hydroxyalkanoate units by analysis of the ^{13}C-NMR spectrum. The poly(3HB) homopolymer and poly (3HB-*co*-3HA) copolymer were produced simultaneously within cells from glucose in the absence of any nitrogen source, which suggests that *Pseudomonas* sp. 61-3 has two types of polyhydroxyalkanoate syntheses with different substrate specificities.

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Introduction

Polyhydroxyalkanoates (PHA) are produced as a storage material by a number of bacteria under restricted growth conditions. They have been shown to occur in over 90 genera of bacteria, and 40 different constituents of PHA have been identified as various hydroxyalkanoic acids with 3–14 carbon atoms (Steinbüchel 1991). The bacteria PHA can be broadly divided in two groups. One group of bacteria including *Alcaligenes eutrophus* produces short-chain PHA with C_3-C_5 monomer units, while the other group including *Pseudomonas oleovorans* produces medium-chain PHA with C_6-C_{14} monomer units (Anderson and Dawes 1990; Steinbüchel 1991).

Although the majority of bacteria synthesize either short-chain PHA (C_3-C_5) or medium-chain PHA (C_6-C_{14}), several bacteria have been shown to accumulate polyesters containing both short- and medium-chain-length 3-hydroxyalkanoic acids (3HA). The bacteria *Rhodospirillum rubrum* (Brandl et al. 1989), *Rhodocyclus gelatinosus* (Liebergesell et al. 1991) and *Rhodococcus ruber* (Haywood et al. 1991) produced terpolymers consisting of 3HA units of C_4, C_5 and C_6 from hexanoate. *Aeromonas caviae* produced a random copolymer of 83 mol% 3-hydroxybutyrate (3HB) and 17 mol% 3-hydroxyhexanoate from olive oil (Shimamura et al. 1994). A citronellol-utilizing bacterium *Pseudomonas* sp. GP4BH1 produced a polyester containing 88 mol% 3HB and 12 mol% 3-hydroxyoctanoate when grown on octanoate, while it accumulated a polyester containing 10 mol% 3HB, 17 mol% 3-hydroxyoctanoate and 73 mol% 3-hydroxydecanoate from gluconate (Steinbüchel and Wiese 1992); it was suggested that a polymer blend rather than a copolymer is synthesized in the cell. *Pseudomonas resinovorans* also produced a polyester containing 8 mol%, 3HB 62 mol% 3-hydroxyhexanoate, 23 mol% 3-hydroxyoctanoate and 7 mol%

3-hydroxydecanoate when grown on hexanoate (Ramsay et al. 1992). Very recently, *Pseudomonas fluorescens* and other *Pseudomonas* strains were found to accumulate a copolyester of 3HB and various medium-chain-length 3HA units of C_6, C_8, C_{10} and C_{12} from 3-hydroxybutyric acid or from 1,3-butanediol (Lee et al. 1995).

A recombinant strain of *P. oleovorans* harboring the PHA synthase of *A. eutrophus* was shown to produce a blend of poly (3HB) homopolymer and a copolymer of 3-hydroxyhexanoate and 3-hydroxyoctanoate units when grown on octanoate (Timm et al. 1990). Both polyesters were deposited in separate granules within the cells (Preusting et al. 1993). Recently, a recombinant strain of PHA-negative mutant *Pseudomonas putida* harboring the PHA synthase of *Thiocapsa pfennigii* was shown to accumulate a copolyester containing 49 mol% 3HB, 47 mol% 3-hydroxyhexanoate and 4 mol% 3-hydroxyoctanoate when grown on octanoate (Liebergesell et al. 1993). In addition, the recombinant *P. putida* was shown to produce a terpolymer of 29 mol% 3HB, 40 mol% 3-hydroxyhexanoate and 31 mol% 4-hydroxyhexanoate in the presence of 4-hydroxyhexanoic acid (Valentin et al. 1994). These results were surprising since *T. pfennigii* synthesized only the poly (3HB) homopolymer from various carbon sources.

In a previous paper (Abe et al. 1994) we reported that *Pseudomonas* sp. 61-3 produced a polyester consisting of 3HA units of C_4, C_6, C_8, C_{10} and C_{12} when grown on gluconate. In this paper we report that a random copolymer of 3HB and medium-chain-length 3HA units of C_6, C_8, C_{10} and C_{12} is produced together with poly (3HB) homopolymer by *Pseudomonas* sp. 61-3 from sugars under nitrogen-free conditions.

Materials and methods

Bacterial strain and culture media

Pseudomonas sp. 61-3 (FERM P-13108) was isolated from the soil 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 microscopy clearly showed the presence of polar flagella. Although DNA·DNA hybridization of the strain 61-3 was carried out with *Pseudomonas aeruginosa* (IFO 12689), *P. fluorescens* (IFO 14160), *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 because of low homology intensities (13%–27%). The taxonomic assignation of this strain was carried out in the Japan Food Research Laboratories.

The medium used for polyester accumulation was a mineral salts medium containing a carbon source and ammonium chloride as nitrogen source. The composition of the mineral salts medium was as follows (l^{-1} distilled water) : 0.36–7.2 g NH_4Cl, 3.8 g Na_2HPO_4, 2.65 g KH_2PO_4, 0.2 g $MgSO_4$, and 1 ml microelement solution. The microelement solution contained 9.7 g $FeCl_3$, 7.8 g $CaCl_2$, 0.218 g $CoCl_2{\cdot}6H_2O$, 0.156 g $CuSO_4{\cdot}5H_2O$, 0.118 g $NiCl_3{\cdot}6H_2O$ and 0.105 g $CrCl_3{\cdot}6H_2O$ l^{-1} 0.1 M HCl. The pH of the medium was adjusted to 7.0.

Cell growth and polyester synthesis

Inocula were grown in 5-ml test-tubes containing nutrient-rich medium [1% polypeptone, 1% yeast extract, 0.5% meat extract and 0.5% $(NH_4)_2SO_4$]. 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 5 ml inoculum solution was transferred to a mineral salts (100 ml) in a 500-ml Sakaguchi flask containig 1.0–2.0 g carbon source. Cells were grown for a given time depending on the amounts of carbon and nitrogen sources. Cell growth was monitored spectrophotometrically at 600 nm. Cells were then harvested at 4 °C by centrifugation in a Hitachi Himac centrifuge (6000 rpm for 10 min), washed with distilled water, and lyophilized. The polyester was extracted from the 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 dry cells was subjected to methanolysis with a solution consisting of 1.7 ml methanol, 0.3 ml 98% sulfuric acid and 2.0 ml chloroform at 100 °C for 140 min to convert the constituents to their methyl esters (Braunegg et al. 1978). Addition of 1 ml 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 × 0.25 mm) and a flame-ionization detector.

All molecular mass data were obtained by gel-permeation chromatography at 40 °C, using a Shimadzu 6A gel permeation chromatography 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}, and sample concentrations of 1.0 mg ml^{-1} were applied. 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, 27 100-Hz spectral width, 16 000 data points and 22 000 accumulations. Tetramethylsilane (Me_4Si) was used as an internal shift standard.

Differential scanning calorimetry data of polyesters were recorded in the temperature range of −150 °C to 200 °C on a Shimadzu DSC-50 system 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 differential scanning calorimetry 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 °C to 200 °C at a heating rate of 20 °C min^{-1}. T_g was taken as the midpoint of the heat capacity change.

The concentrations of ammonium ion in culture solutions were determined by the Berthelot method. The concentrations of glucose in culture solutions were determined at 25 °C by the UV method with a biochemical analysis kit (Boehringer Mannheim GmbH Biochemica) on a Hitachi U-2000 spectrophotometer.

Table 1 Production of polyesters from various sugars by *Pseudomonas* sp. 61-3 for 48 h at 30°C. C/N is the molar ratio of carbon atoms to nitrogen atoms in the carbon and nitrogen sources. The polyester content is that of dry cells. The polyester composition was determined by GC analysis: 3*HB* 3-hydroxybutyrate, 3*HH* 3-hydroxyhexanoate, 3*HO* 3-hydroxyoctanoate, 3*HD* 3-hydroxydecanoate, 3*HDD* 3-hydroxydodecanoate

Carbon source	Concn. (g/l)	NH_4Cl (g/l)	C/N (mol/mol)	Cell dry weight (g/l)	Polyester yield (g/l)	Polyester content (% by wt.)	Polyester composition (mol%)				
							3HB	3HH	3HO	3HD	3HDD
Glucose	20	7.2	5	2.17	0.26	12	100	–	–	–	–
	20	3.6	10	2.11	0.27	13	100	–	–	–	–
	20	2.4	15	2.19	0.22	10	98	–	2	–	–
	20	1.8	20	2.37	0.28	12	96	–	2	2	–
	20	1.2	30	3.02	0.79	26	91	–	6	3	–
	20	0.9	40	3.24	0.62	19	87	–	7	6	–
	20	0.72	50	3.31	0.70	21	87	2	5	6	–
	10	0.36	50	1.38	0.23	17	80	3	10	7	–
Fructose	20	7.2	5	2.80	0.22	8	100	–	–	–	–
	20	3.6	10	3.30	0.53	16	98	–	1	1	–
	20	2.4	15	3.43	0.65	19	98	–	1	1	–
	20	1.8	20	3.60	0.72	20	96	1	1	2	–
	20	1.2	30	4.73	0.61	13	82	2	6	10	–
	20	0.9	40	4.43	0.53	12	80	2	6	12	–
	20	0.72	50	2.68	0.46	17	78	2	6	12	2
	10	0.36	50	1.37	0.23	17	87	2	5	6	–
Mannose	20	0.72	50	2.30	0.49	22	92	4	2	2	–
	10	0.36	50	2.39	0.27	11	87	8	2	3	–

Determination of enzyme activities

Approximately 0.2–1.0 g (wet weight) cells was suspended in 3 ml 50 mM TRIS/HCl buffer (pH 7.5) and disrupted by sonication for 5 min by a Branson (150 W) ultrasonic disintegrator. The cell debris and unbroken cells were removed by centrifugation for 20 min at 12 000 rpm and 4 °C to obtain the crude cell-free extract. The activities of β-ketothiolase and acetoacetyl-CoA reductase in the extract were determined spectrophotometrically according to the methods of Oeding and Schlegel (1973) and of Lynen and Wieland (1955) respectively. Protein concentration was estimated according to Bradford (1976).

Results

Production of polyesters on various sugars

Polyester synthesis was carried out by a single-stage cultivation of *Pseudomonas* sp. 61-3 on various sugars under aerobic conditions at 30 °C for 48 h. Glucose, fructose and mannose were used as the sole carbon source, and the initial molar ratio of carbon to nitrogen sources in a mineral medium was varied from 5 mol/mol to 50 mol/mol. The composition of polyester produced was determined by GC analysis. The result is listed in Table 1. At low C/N molar ratios (below 10) in culture media, *Pseudomonas* sp. 61-3 accumulated a poly(3HB) homopolymer within the cells. In contrast, the strain produced a polyester containing 3HB and medium-chain-length 3HA units of C_6, C_8, C_{10} and C_{12} at high C/N molar ratios (above 10), and the 3HB fraction in the polyester decreased with an increase in the C/N molar ratio. The polyester contents in the cells were 8–26% by weight, depending of the C/N molar ratio. Thus, *Pseudomonas* sp. 61-3 produced an interesting polyester consisting of both short- and medium-chain-length 3HA units ranging from C_4 to C_{12} under nitrogen-limited conditions.

Characterization of polyesters

The polyesters produced could have been a blend of homopolymer and copolymer, so the polyester containing short- and medium-chain-length 3HA units was fractionated for 5 h with boiling acetone. As shown in Table 2, the acetone-insoluble fraction (28% by weight) was a poly(3HB) homopolymer, while the acetone-soluble fraction (72% by weight) contained saturated and unsaturated 3HA units ranging from C_4 to C_{12}. In the acetone-soluble fraction, seven different 3HA units were identified by NMR and GC analyses as follows: 44 mol% 3HB, 5 mol% 3-hydroxyhexanoate, 21 mol% 3-hydroxyoctanoate, 25 mol% 3-hydroxydecanoate, 2 mol% 3-hydroxydodecanoate and 3 mol% 3-hydroxy-5-*cis*-dodecenoate. Table 3 shows the molecular masses and thermal properties of the two fractions of polyesters. The acetone-insoluble fraction had melting and glass-transition temperatures of 176°C and 4°C respectively, which are identical to those of the poly(3HB) homopolymer (Marchessault et al. 1981). In

contrast, the acetone-soluble fraction was an amorphous polymer, and a single glass-transition temperature was observed at $-43^{\circ}C$. Thus, the fusion of the poly(3HB) homopolymer was not detected in the differential scanning calorimetry curve of the acetone-soluble fraction, suggesting that the fraction is a random copolymer of seven monomer units of C_4 to C_{12}.

Figure 1 shows the 100-MHz ^{13}C-NMR spectrum of the acetone-soluble fraction, together with the chemical-shift assignments for each carbon resonance and an expanded spectrum of carbonyl resonances. The chemical-shift assignments of all carbon resonances are the same as those reported in previous papers (Eggink et al. 1992; Huijberts et al. 1994; Lee et al. 1995). The carbonyl carbon resonances (169.1–169.5 ppm) are clearly resolved into three groups of peaks, arising from different diad sequences of connecting 3HB and the other six 3HA units of medium-chain-length: C_6 to C_{12}. The peak at 169.10 ppm is assignable to the carbonyl resonance in the 3HB*–3HB sequence, since the chemical shift is consistent with that of the carbonyl resonance in the poly(3HB) homopolymer. The peak at 169.42 ppm is assigned to the carbonyl resonance in 3HA*–3HA sequences, since the chemical shift is almost consistent with that of the carbonyl resonance in the medium-chain poly(3HA) of C_6 to C_{12}. The peak at 169.28 ppm may be assigned to the carbonyl resonances in the 3HB*–3HA and 3HA*–3HB sequences of connecting short- and medium-chain-length units. The diad-sequence distribution of 3HB and medium-chain-length 3HA units in the acetone-soluble fraction was determined from the peak areas of carbonyl resonances. The result is given in Table 3.

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 $F_{ij} = F_i F_j$. The diad fractions calculated from the mole fractions of 3HB and 3HA units are in good agreement with the observed values (see Table 3). It has been concluded that the acetone-soluble fraction of the amorphous polymer has a statistically random distribution of 3HB and medium-chain-length 3HA units.

Table 2 Fractionation of ployester produced with boiling acetone for 5 h. Polyester was produced for 48 h at 30°C from glucose (20 g/l) in the presence of NH_4Cl (0.72 g/l). The polyester composition was determined by GC analysis: 3*H5DD* 3-hydroxy-5- *cis*-dodecenoate, 3*H7TD* 3-hydroxy-7-*cis*-tetradecenoate, *ND* not detected, *Tr* trace amounts (< 1 mol %)

Fraction	Yield (mg)	Polyester composition (mol%)						
		3HB	3HH	3HO	3HD	3HDD	3H5DD	3H7TD
Whole polymer	363	87	2	5	6	ND	ND	ND
Acetone-insoluble	101	100	—	—	—	—	—	—
Acetone-soluble	262	44	5	21	25	2	3	Tr

Accumulation of poly(3HB) and poly(3HB-co-3HA) blend

The growth characteristics of *Pseudomonas* sp. 61-3 and conditions of poly(3HB) and poly(3HB-*co*-3HA) synthesis from glucose were investigated at 30°C. Figures 2 and 3 show the time courses of cell growth and polyester accumulation during the batch cultivation of *Pseudomonas* sp. 61-3 in mineral media containing 20 g/l glucose and 0.36–0.72 g/l NH_4Cl. The initial C/N molar ratios of carbon to nitrogen sources were 50 mol/mol and 100 mol/mol, as shown in Figs. 2 and 3 respectively. The cell dry weight increased rapidly with time during the initial stage of fermentation in the presence of an excess of nitrogen source (NH_4^+), while little polyester accumulated within cells. The accumulation of poly(3HB) was initiated under nitrogen-limited conditions. After the nitrogen source in the medium had been completely exhausted, poly(3HB-*co*-3HA) copolymer was produced together with poly(3HB) homopolymer. Thus, the two components of poly(3HB) and poly(3HB-*co*-3HA) were simultaneously produced

Table 3 Properties and diad sequence distributions of fractionated polyester samples. 3*HB* 3-hydroxybutyrate (C_4), 3*HA* medium-chain-length 3-hydroxyalkanoate units (C_6–C_{12}). Observed relative intensities were determined from relative peak areas of carbonyl carbon resonances; calculated values were obtained by Bernoullian statistics. *ND* not detected

Fraction	Molecular mass		Thermal properties				Sequence		
	M_n	M_w/M_n	T_g (°C)	T_m (°C)	ΔH_m (J/g)	Relative intensities	3HB*-3HB	3HB*-3HA + 3HA*-3HB	3HA*-3HA
Acetone-insoluble	526 000	2.5	4	176	83	Observed	1.00	—	—
Acetone-soluble	40 000	4.4	−43	ND	0	Observed	0.18	0.47	0.35
						Calculated	0.19	0.49	0.32

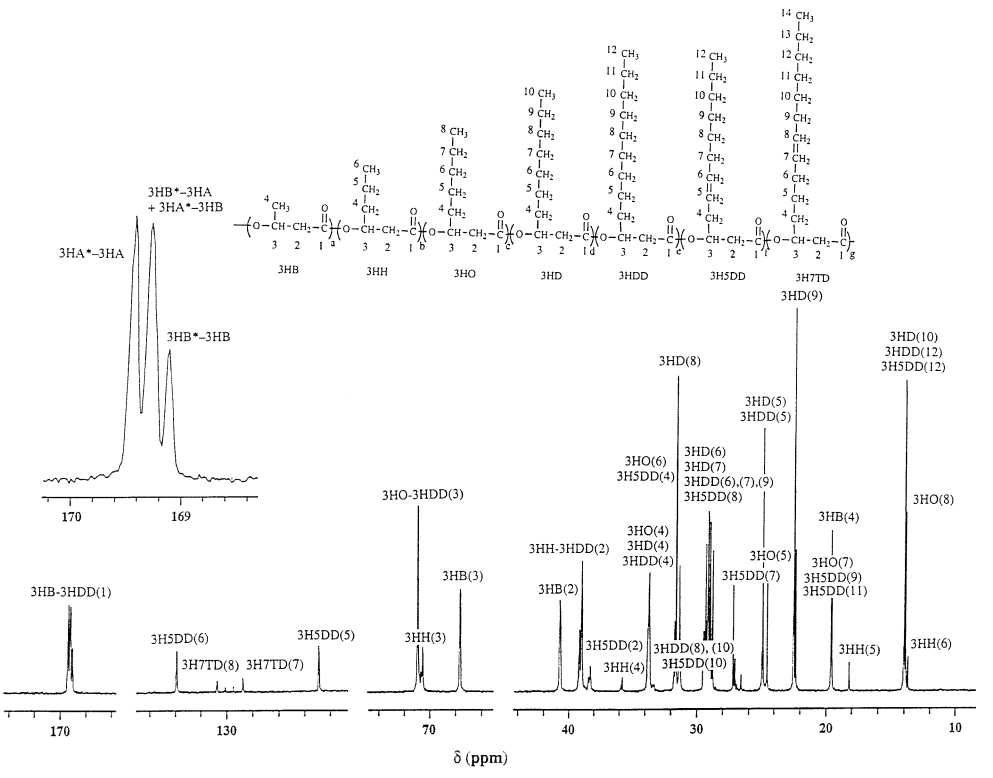

Fig. 1 100-MHz ^{13}C-NMR spectrum of the acetone-soluble fraction of polyester in chloroform. 3*HB* 3-hydroxybutyric acid, 3*HA* 3-hydroxyalkanoic acids, 3*HH* 3-hydroxyhexanoate, 3*HO* 3-hydroxyoctanoate, 3*HD* 3-hydroxydecanoate, 3*HDD* 3-hydroxydodecanoate, 3*H5DD* 3-hydroxy-*cis*-5-dodecenoate, 3*H7TD* 3-hydroxy-*cis*-7-tetradecenoate

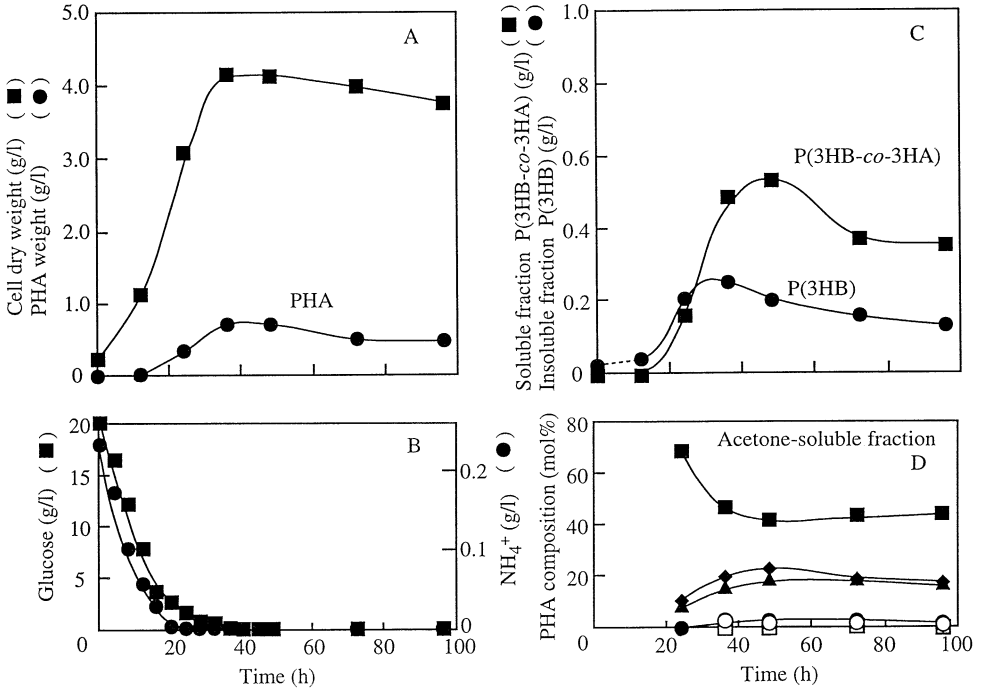

Fig. 2A–D Time courses of cell growth and polyhydroxyalkanoate (*PHA*) accumulation during the batch cultivation of *Pseudomonas* sp. 61-3 in a mineral medium containing glucose (20 g/l) and NH_4Cl (0.72 g/l) (C/N = 50) at 30°C. **D** Copolymer composition in the acetone-soluble fraction; 3HB (■), 3HH(●), 3HO (▲), 3HD (◆), 3HDD (□), 3H5DD (○) *P* poly

within cells from glucose in the absence of any nitrogen source. After glucose had been exhausted, both poly(3HB) and poly(3HB-*co*-3HA) were gradually degraded with time, suggesting that the polyesters were utilized for energy generation under conditions of carbon starvation.

The time-dependent changes in copolymer composition of the acetone-soluble fraction poly(3HB-*co*-3HA)

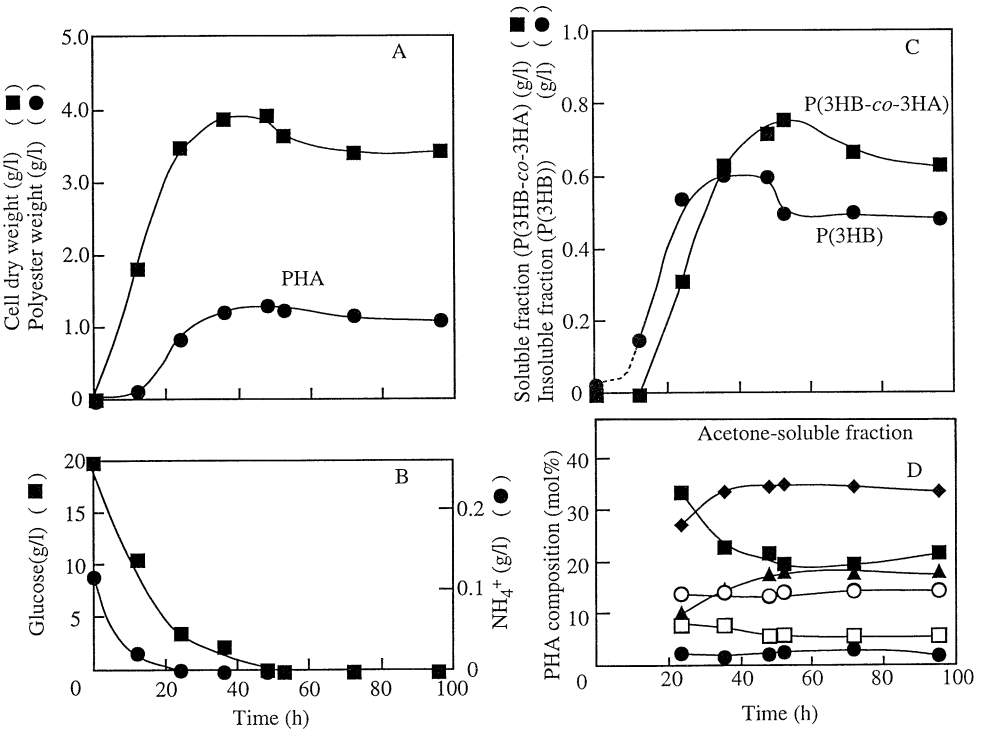

Fig. 3A–D Time courses of cell growth and PHA accumulation during the batch cultivation of *Pseudomonas* sp. 61-3 in a mineral medium containing glucose (20 g/l) and NH_4Cl (0.36 g/l) (C/N = 100) at 30°C. **D** Copolymer composition in the acetone-soluble fraction; 3HB (■), 3HH(●), 3HO (▲), 3HD (■), 3HDD (□), 3H5DD (○). *P* poly

are shown in Figs. 2D and 3D. The 3HB fraction in the copolymer decreased from 70 mol% to 44 mol% with time (Fig. 2D), while the 3-hydroxyoctanoate and 3-hydroxydecanoate fractions increased to 21 mol% and 25 mol% with time respectively. A similar tendency in the copolymer was observed (Fig. 3D), in which the 3HB fraction decreased from 34 mol% to 20 mol% with time.

Discussion

The data reported here demonstrate that *Pseudomonas* sp. 61-3 accumulates poly(3HB) homopolymer from glucose under nitrogen-limited conditions and that a random copolymer of 3HB and medium-chain-length 3HA units [poly(3HB-*co*-3HA), C_4-C_{12}] is produced in addition to poly(3HB) after cell growth has been stopped by the exhaustion of the nitrogen source. It is of interest to note that the pseudomonad strain produces both polymers poly(3HB) and poly(3HB-*co*-3HA) simultaneously under nitrogen-free conditions. The production of a polyester blend from glucose suggests that this strain has two types of PHA synthases with different substrate specificities. Poly(3HB) homopolymer may be formed by a PHA synthase A with a similar substrate specificity to that of the synthase of *A. eutrophus*, while poly(3HB-*co*-3HA) copolymer may be produced by a PHA synthase B with a wide range of substrate specificity. A recombinant strain of *P. oleovorans* harboring the genes for poly(3HB) synthesis of *A. eutrophus* was shown to produce a blend of short-chain poly(3HB) and medium-chain poly(3HH-*co*-3HO) when grown on octanoate (Timm et al. 1990), which supports the presence of two PHA synthases having quite different substrate specificities in *Pseudomonas* sp. 61-3.

Figure 4 shows the schematic pathway of poly(3HB) and poly(3HB-*co*-3HA) synthesis from glucose in *Pseudomonas* sp. 61-3. Table 4 shows the enzymatic activity of β-ketothiolase and acetoacetyl-CoA reductases in *Pseudomonas* sp. 61-3 grown on glucose. This strain showed no NADPH-dependent acetoacetyl-CoA reductase activity and very low β-ketothiolase activity, suggesting that all the (*R*)-3-hydroxyalkanoic acid monomer units are supplied via the *de novo* fatty acid biosynthesis pathway. The *de novo* fatty acid biosynthesis from acetyl-CoA results in the formation of (*R*)-3-hydroxyacyl-ACP (acyl carrier protein) with C_4, C_6, C_8, C_{10} and C_{12} substituents. The (*R*)-3-hydroxyacyl-ACP intermediates may be converted into (*R*)-3-hydroxyacyl-CoA (3HA-CoA), followed by the polymerization into poly(3HB) and poly(3HB-*co*-3HA) through synthases A and B. However, at present, it is not clear whether the acyl moieties of (*R*)-3-hydroxyacyl-ACP are directly incorporated into the polymer or whether a transfer to CoA prior to polymerization is required (Saito and Doi 1993). A lag time was observed in the accumulation phase of poly(3HB)

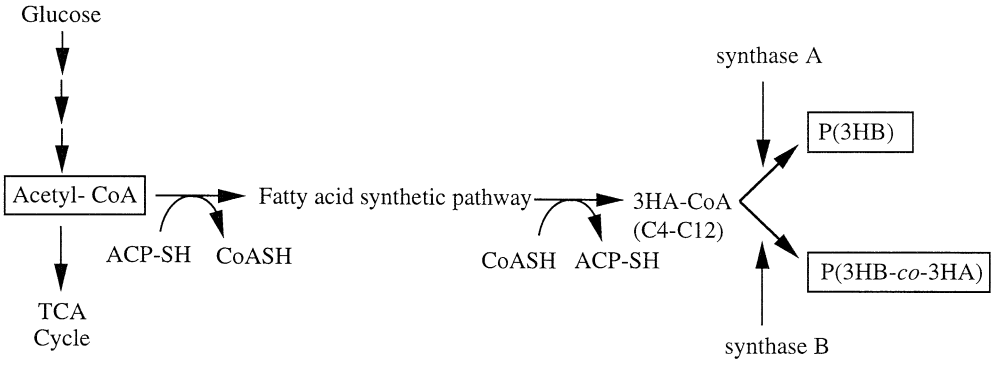

Fig. 4 Proposed pathways of poly(3HB) and poly(3HB-*co*-3HA) syntheses from glucose in *Pseudomonas* sp. 61-3. *TCA* Cycle the tricarboxylic acid cycle

Table 4 Enzymatic activities of β-ketothiolase and acetoacetyl-CoA reductases in *Pseudomonas* sp. 61-3. C/N is the molar ratio of carbon atoms to nitrogen atoms in the carbon and nitrogen sources. *ND* not detected

C/N (mol/mol)	Time (h)	Total protein (mg)	Specific activity (U/mg)		
			β-Ketothiolase	NADPH-dependent acetoacetyl-CoA reductase	NADH-dependent acetoacetyl-CoA reductase
5	12	5.910	0.0071	ND	0.28
50	12	0.835	ND	ND	3.06
50	72	0.613	1.31	ND	2.27

and poly(3HB-*co*-3HA); production of poly(3HB) was initiated at a low ammonium ion concentration, while poly(3HB-*co*-3HA) was produced after the nitrogen source had been completely exhausted. This fact suggests not only the presence of two types of PHA synthases but also of individual control systems for the activities of two PHA synthases, depending on the concentration of the nitrogen source. PHA synthase A (specific toward 3HB units) may be induced or activated below a certain concentration of nitrogen source, while PHA synthase B (widely specific toward 3HA units of C_4–C_{12}) may function under nitrogen-free conditions. It has been reported that *P. oleovorans* (Huisman et al. 1991) and *P. aeruginosa* (Timm and Steinbuchel 1992) have two PHA synthase genes in the *pha* operon, that transcription of these genes is controlled by a common promoter region, and that the substrate specificities of two synthases seem to be very similar (Huisman et al. 1992). The polyester formation of two different types of poly(3HB) and poly(3HB-*co*-3HA) in *Pseudomonas* sp. 61-3 was accounted for in terms of the presence of two different PHA synthases. Another reason for the occurrence of two different polyesters may be the provision of hydroxy fatty acid CoA thioesters as substrates for the PHA synthase, which may be quite different at different growth phases.

To our knowledge, *Pseudomonas* sp. 61-3 is the first strain identified that produces a blend of poly(3HB) and copolymers of short- and medium-chain-length 3HA units. The results reported here suggest an interesting biosynthetic pathway of polyesters with two types of PHA synthase in this strain. Furthermore, the PHA synthase in this bacterium that is capable of forming poly(3HB-*co*-3HA) seems to have the broadest substrate specificity (C_4–C_{12}) among all PHA synthases reported previously. The authors are now attempting to isolate the PHA synthase gene(s) to prove the validity of the model.

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