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H. Matsusaki · H. Abe · K. Taguchi · T. Fukui · Y. Doi

Biosynthesis of poly(3-hydroxybutyrate-*co*-3-hydroxyalkanoates) by recombinant bacteria expressing the PHA synthase gene *phaC1* from *Pseudomonas* sp. 61-3

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Abstract *Pseudomonas* sp. 61-3 accumulated a blend of poly(3-hydroxybutyrate) [P(3HB)] homopolymer and a random copolymer consisting of 3-hydroxyalkanoate (3HA) units of 4–12 carbon atoms. The genes encoding β-ketothiolase ($PhbA_{Re}$) and NADPH-dependent acetoacetyl-CoA reductase ($PhbB_{Re}$) from *Ralstonia eutropha* were expressed under the control of promoters for *Pseudomonas* sp. 61-3 *pha* locus or *R. eutropha phb* operon together with $phaC1_{Ps}$ gene (PHA synthase 1 gene) from *Pseudomonas* sp. 61-3 in PHA-negative mutants *P. putida* GPp104 and *R. eutropha* $PHB^{-}4$ to produce copolyesters [P(3HB-*co*-3HA)] consisting of 3HB and medium-chain-length 3HA units of 6–12 carbon atoms. The introduction of the three genes into GPp104 strain conferred the ability to synthesize P(3HB-*co*-3HA) with relatively high 3HB compositions (up to 49 mol%) from gluconate and alkanoates, although 3HB units were not incorporated at all or at a very low fraction (3 mol%) into copolyesters by the strain carrying $phaC1_{Ps}$ gene only. In addition, recombinant strains of *R. eutropha* $PHB^{-}4$ produced P(3HB-*co*-3HA) with higher 3HB fractions from alkanoates and plant oils than those from recombinant GPp104 strains. One of the recombinant strains, *R. eutropha* $PHB^{-}4$/pJKSc46-*pha*, in which all the genes introduced were expressed under the control of the native promoter for *Pseudomonas* sp. 61-3 *pha* locus, accumulated P(3HB-*co*-3HA) copolyester with a very high 3HB fraction (85 mol%) from palm oil. The nuclear magnetic resonance analyses showed that the copolyesters obtained here were random copolymers of 3HB and 3HA units.

H. Matsusaki · H. Abe · K. Taguchi · T. Fukui · Y. Doi (✉)
Polymer Chemistry Laboratory and the RIKEN Group
of Japan Science and Technology Corporation,
RIKEN Institute, 2-1 Hirosawa,Wako-shi,
Saitama 351-0198, Japan
e-mail: ydoi@postman.riken.go.jp
Tel.: +81-48-4679402
Fax: +81-48-4674662

Introduction

Polyhydroxyalkanoates (PHAs) are accumulated in various bacteria as intracellular carbon and energy storage material during nutrient-limited conditions (Anderson and Dawes 1990; Steinbüchel 1991; Müller and Seebach 1993; Lee 1996; Madison and Huisman 1999). These bacterial PHAs are expected to become attractive alternatives for petrochemically based plastics, since they are biodegradable thermoplastics. The biosynthesis of poly[(*R*)-3-hydroxybutyrate] [P(3HB)] has been studied in detail in *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*). The biosynthesis genes of *R. eutropha* are organized in a single operon as $phbCAB_{Re}$, which are genes of polyhydroxybutyrate (PHB) synthase, β-ketothiolase, and NADPH-dependent acetoacetyl-CoA reductase, respectively (Oeding and Schlegel 1973; Haywood et al. 1988a, b). P(3HB) is a highly crystalline and stiff material, leading to brittleness and low extension to break (Holmes 1985; Doi 1995).

Pseudomonads belonging to the rRNA homology group I accumulate copolymers consisting of medium-chain-length 3HA (mcl-3HA) units ranging from 5 to 14 carbon atoms (Lageveen et al. 1988; Haywood et al. 1990; Timm and Steinbüchel 1990; Huijberts et al. 1992). However, 3HB units are incorporated not at all or at a very low fraction into PHA by these bacteria, and 3-hydroxyoctanoate (3HO) and 3-hydroxydecanoate (3HD) are major monomer units (Huisman et al. 1989; Anderson and Dawes 1990; Lee 1996; Madison and Huisman 1999). These medium-chain-length PHAs (mcl-PHAs) have a much lower level of crystallinity and are more elastic (Gross et al. 1989; Preusting et al. 1990).

Pseudomonas strain GP4BH1 and a recombinant strain of *P. oleovorans* expressing *R . eutropha* P(3HB) biosynthesis genes ($phbCAB_{Re}$) accumulated polyesters consisting of 3HB and mcl-3HA, but the PHAs produced were polymer blends of P(3HB) and mcl-PHA (Timm et al. 1990; Preusting et al. 1992; Steinbüchel and Wiese 1995), whereas *Pseudomonas* sp. A33 and

P. fluorescens were found to produce a copolymer consisting of 3HA units of 4–12 carbon atoms from 3-hydroxybutyric acid and 1,3-butanediol, in which 10–20 mol% 3HB units were incorporated (Lee et al. 1995). Although *Thiocapsa pfennigii* accumulated only a P(3HB) homopolymer, a recombinant strain of PHA-negative mutant of *P. putida* expressing the PHA biosynthesis genes of *T. pfennigii* accumulated a copolyester consisting of 3HB and 3-hydroxyhexanoate (3HHx) as main constituents and 3HO as a minor constituent from octanoate (Liebergesell et al. 1993).

We have reported that *Pseudomonas* sp. 61-3 produces a blend of P(3HB) homopolymer and a random copolymer, P(3HB-*co*-3HA), consisting of 3HA units of 4–12 carbon atoms (Abe et al. 1994; Kato et al. 1996a, b). Recently, two types of PHA biosynthesis gene loci (*phb* and *pha*) of this strain have been elucidated at the molecular level, encoding the genes of PHB synthase and PHA synthases (Matsusaki et al. 1998). Especially, heterologous expression in PHA-negative mutants of *P. putida* and *R. eutropha* has shown that PHA synthases ($PhaC1_{Ps}$ and $PhaC2_{Ps}$) of *Pseudomonas* sp. 61-3 are capable of incorporating 3HB units into a polyester chain together with mcl-3HA units, suggesting that PHA synthases from this strain are able to use (*R*)-3HB-CoA as a substrate.

In this study, we have investigated whether $PhaC1_{Ps}$ makes it possible to synthesize P(3HB-*co*-3HA) with various monomer compositions in heterologous hosts. The genes encoding β-ketothiolase and NADPH-dependent acetoacetyl-CoA reductase from *R. eutropha* were expressed to supply (*R*)-3HB-CoA for PHA synthase together with $phaC1_{Ps}$ gene from *Pseudomonas* sp. 61-3 in PHA-negative mutants of *P. putida* and *R. eutropha* to synthesize a novel P(3HB-*co*-3HA) with a high 3HB composition.

Materials and methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *P. putida* and *R. eutropha* were cultivated at 30 °C in a nutrient-rich (NR) medium (Matsusaki et al. 1998). *Escherichia coli* strains were grown at 37 °C on Luria-Bertani (LB) medium (Sambrook et al. 1989). When needed, ampicillin (50 mg/l) and kanamycin (50 mg/l) were added to the medium.

DNA manipulation and plasmid construction

Isolation of plasmids, digestion of restriction endonucleases, agarose gel electrophoresis, and transformation of *E. coli* were carried out by standard procedures (Sambrook et al. 1989) or as recommended by manufacturers. DNA restriction fragments were isolated from agarose gels by using a QIAEX II Gel Extraction Kit (QIAGEN). All other DNA-manipulating enzymes were used as recommended by the manufacturers.

The PCR product of a 5-kb *Sma*I-*Pst*I region containing $phbCAB_{Re}$ genes (Peoples and Sinskey 1989a, b) was cloned into the pGEM-T-derived vector as a *Bam*HI fragment created by PCR, after both *Nde*I and *Pst*I unique sites of the vector were eliminated by the treatment of the corresponding restriction enzymes and T4 DNA polymerase, to yield pGEM'-*phbCAB*. The 2.2-kb *Rco*RI-*Xba*I region containing $phaC1_{Ps}$ gene was introduced into pBluescript II KS^+ to give pBSEX22. From these two plasmids, three kinds of recombinant plasmids, pJBB49-*phb*, pJKSc46-*pha*, and pJKSc54-*phab*, carrying $phaC1_{Ps}$ and $phbAB_{Re}$ under the control of

Table 1 Bacterial strains and plasmids used in this study (*JCM* Japan Collection of Microorganisms, *ATCC* American Type Culture Collection, *DSM* Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH)

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
Pseudomonas sp. 61-3	Wild type	JCM 10015, Matsusaki et al. 1998
R. eutropha H16	Wild type	ATCC 17699, Schlegel et al. 1970
P. putida GPp104	PHA-negative mutant of KT2442	Huisman et al. 1991
R. eutropha PHB^-4	PHA-negative mutant of H16	DSM 541, Schlegel et al. 1970
E. coli DH5α	*deoR endaA1 gyrA96 hsdR17* ($r_K^- m_K^+$) *re1A1 supeE thi-1 Δ(lacZYA-argFV169) ϕ80ΔlacZΔM 15F-λ-*	Clontech
E. coli S17-1	*recA* and *tra* genes of plasmid RP4 integrated into the chromosome; auxotrophic for proline and thiamine	Simon et al. 1983
Plasmids		
pBluescript II KS^+	Ap^r *lacPOZ* T7 and T3 promoter	Stratagene
pGEM-T	Ap^r *lacPOZ* T7 and SP6 promoter	Promega
pBSEX22	pBluescript II KS^+ derivative; pha_{Ps} *promoter,* $phaC1_{Ps}$	This study
pGEM'-*phbCAB*	pGEM-T derivative; phb_{Re} promoter, $phbC_{Re}$, $phbA_{Re}$, $phbB_{Re}$	This study
pGEM'-P_{Re}C1AB	pGEM-T derivative; phb_{Re} promoter, $phaC1_{Ps}$, $phbA_{Re}$, $phbB_{Re}$	This study
pBS-P_{Ps}C1AB	pBluescript II KS^+ derivative; phb_{Ps} promoter, $phaC1_{Ps}$, $phbA_{Re}$, $phbB_{Re}$	This study
pBS-phbAB	pBluescript II KS^+ derivative; phb_{Re} promoter, $phbA_{Re}$, $phbB_{Re}$	This study
pBS-P_{Ps}C1-P_{Re}AB	pBluescript II KS^+ derivative; pha_{Ps} promoter, $phaC1_{Ps}$, phb_{Re} promoter, $phbA_{Re}$, $phbB_{Re}$	This study
pJRD215	Cosmid; Km^r Sm^r RSF1010 replicon Mob^+	Davison et al. 1987
pJASc22	pJRD215 derivative; $phaC1_{Ps}$	Matsusaki et al. 1998
pJBB49-*phb*	pJRD215 derivative; phb_{Re} promoter, $phaC1_{Ps}$, $phbA_{Re}$, $phbB_{Re}$	This study
pJKSc46-*pha*	pJRD215 derivative; pha_{Ps} promoter, $phaC1_{Ps}$, $phbA_{Re}$, $phbB_{Re}$	This study
pJKSc54-*phab*	pJRD215 derivative; pha_{Ps} promoter, $phaC1_{Ps}$, phb_{Re} promoter, $phbA_{Re}$, $phbB_{Re}$	This study

Pseudomonas sp. 61-3 native promoter (P_{Ps}) and/or *R. eutropha* promoter (P_{Re}) were constructed as shown in Fig. 1.

Conjugation of *P. putida* or *R. eutropha* with *E. coli* S17-1 harboring the broad-host-range pJRD215-derivative plasmids constructed as described above was performed as described by Friedrich et al. (1981).

Production of PHA

Cells grown in NR medium for 12 h were transferred to 500-ml flasks with 100 ml nitrogen-limited mineral salt (MS) medium (pH 7.0) containing 0.9 g $Na_2HPO_4 \cdot 12H_2O$, 0.15 g KH_2PO_4, 0.05 g NH_4Cl, 0.02 g $MgSO_4 \cdot 7H_2O$, and 0.1 ml trace element solution (Kato et al. 1996a) at 0.05 of initial absorbance at 600 nm and were cultivated on a reciprocal shaker (130 strokes/min) at 30 °C for 72 h. Filter-sterilized carbon sources, except plant oils, were added to the medium as indicated in the text. The grown cells were harvested, washed with methanol and/or distilled water, and lyophilized. The polyesters accumulated in the cells were extracted with chloroform for 48 h and purified by reprecipitation with methanol.

Analytical procedure

Determination of the cellular PHA content and polymer composition by gas chromatography and molecular mass measurements by gel permeation chromatography (GPC) were performed described previously (Abe et al. 1994). The 500 MHz ^{1}H- and the 125 MHz ^{13}C-nuclear magnetic resonance (NMR) spectra of $CDCl_3$ solution of copolyesters (5 mg/ml) were obtained as described previously (Abe et al. 1994).

Results

Accumulation of PHA by the recombinant strains of *P. putida* and *R. eutropha*

Tables 2 and 3 show the accumulation of PHA in transconjugants of *P. putida* GPp104 and *R. eutropha* PHB$^-$4 carrying the *phaC1*$_{Ps}$ gene from *Pseudomonas* sp. 61-3 and the *phbAB*$_{Re}$ genes from *R. eutropha*. The recombinant strains of GPp104 harboring pJBB49-*phb* and pJKSc46-*pha* produced P(3HB-*co*-3HA) with high 3HB compositions of approximately 50 mol% from gluconate. Although GPp104/pJKSc46-*pha* effectively produced copolyesters from octanoate and dodecanoate, GPp104/pJBB49-*phb* produced little PHA from fatty acids, suggesting that the expression levels of *phaC1*$_{Ps}$ and *phbAB*$_{Re}$ genes would be low under the control of the promoter (P_{Re}) for *R. eutropha phbCAB*$_{Re}$ operon in the host during cultivation with fatty acids. In comparison to the recombinant strain harboring pJBB49-*phb* or pJKSc46-*pha*, a copolyester with low 3HB composition (3 mol%) was synthesized in the gluconate-grown GPp104/pJKSc54-*phab* in which two promoters (P_{Ps} and P_{Re}) were used for the expression of *phaC1*$_{Ps}$ and *phbAB*$_{Re}$ genes, respectively. Thus, the introduction of *phbAB*$_{Re}$ gene into *P. putida* GPp104 conferred the ability to provide (*R*)-3-hydroxybutyryl-coenzyme A [(*R*)-3HB-CoA] effectively on the host, and 3HB fractions in P(3HB-*co*-3HA) copolyesters synthesized by the recombinant strains carrying *phbAB*$_{Re}$ genes were higher than that in the copolyester from GPp104/pJASc22 (Matsusaki et al. 1998). In addition, it has been verified that PhaC1$_{Ps}$ of *Pseudomonas* sp. 61-3 is capable of incorporating a wide compositional range of 3HA units of C4 to C12 into the polyester.

When gluconate was used as the sole carbon source, the PHA content (25 wt%) from GPp104/pJBB49-*phb* was higher than those of the other recombinant strains (Table 2). By contrast, GPp104 strains harboring the other plasmids (pJASc22, pJKSc46-*pha* and pJKSc54-*phab*) efficiently accumulated PHA from octanoate and dodecanoate but not from gluconate. It seems that the function of the promoters used for the expression of *phaC1* depends on carbon sources.

All recombinant strains of *R. eutropha* PHB$^-$4 produced P(3HB) homopolymer from fructose, and P(3HB-*co*-3HA) copolyesters from fatty acids (Table 3). When octanoate and tetradecanoate were used as the sole carbon source, the monomer compositions in the copolyesters were very similar among these recombinant strains and the mole fractions of 3HB unit in the copolyesters were approximately 20–35 mol% and 45–50 mol%, respectively. The P(3HB-*co*-3HA) with high 3HB compositions were obtained when the recombinant PHB$^-$4 strains were cultivated on a longer fatty acid. The amount of acetyl-CoA generated via β-oxidation from a longer fatty acid is more than that from shorter one. Hence, a large amount of (*R*)-3HB-CoA may be formed via dimerization of acetyl-CoA and reduction of acetoacetyl-CoA catalyzed by PhbA$_{Re}$ and PhbB$_{Re}$, resulting in the synthesis of copolyesters with high 3HB compositions.

The 3HB fractions of copolyesters produced by the recombinant PHB$^-$4 strains harboring pJASc22 or pJKSc54-*phab* from plant oils (olive, corn, and palm oils) were 47–56 mol%, and the additional copies of *phbAB*$_{Re}$ genes did not affect the mole fractions of 3HB unit in the copolyesters from PHB$^-$4/pJKSc54-*phab*. In contrast, 3HB compositions of more than 70 mol% were found in the P(3HB-co-3HA) copolyesters synthesized from plant oils by PHB$^-$4 strains harboring pJBB49-*phb* or pJKSc46-*pha*. It is noted that PHB$^-$4/pJKSc46-*pha* accumulated a copolyester with a high 3HB fraction (85 mol%) from palm oils. The recombinant strain of PHB$^-$4/pJBB49-*phb* efficiently accumulated P(3HB) from sugar (fructose), while it accumulated little PHA from alkanoates. A similar result was observed in GPp104/pJBB49-*phb* (Table 2). On the other hand, PHB$^-$4 strains harboring the other three plasmids, which were constructed to express *phaC1*$_{Ps}$ gene under the control of the native promoter (P_{Ps}), accumulated more PHA from alkanoates rather than from sugar. Thus, the promoters used for the expression of the genes gave a significant difference for PHA contents.

Characterization of copolyesters isolated from the recombinants of *P. putida* and *R. eutropha*

To investigate the compositions and the sequence distributions of copolyesters synthesized by recombinant

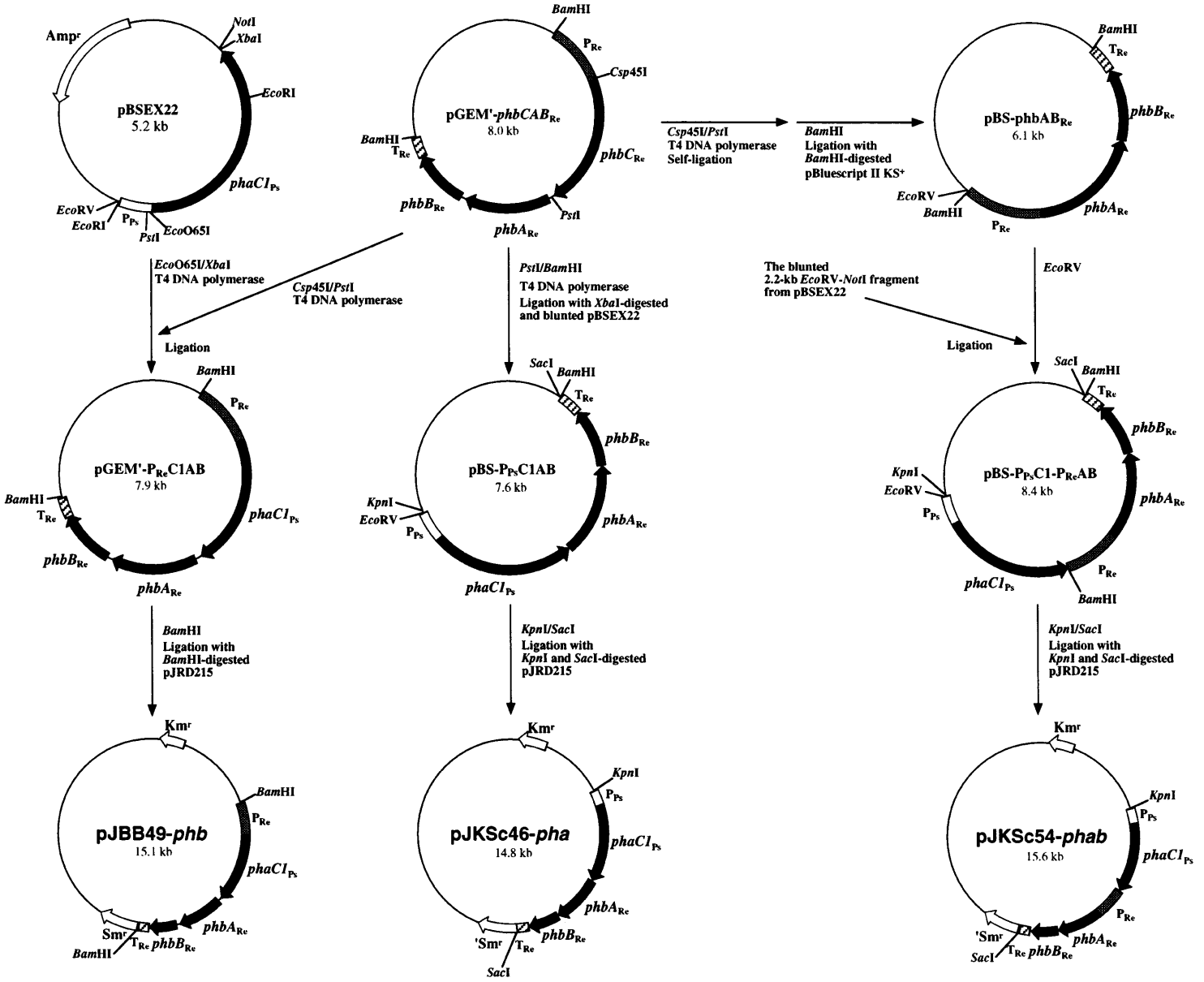

Fig. 1 Construction strategy of the plasmids used for heterologous expression of *phaC1*$_{Ps}$ and *phbAB*$_{Re}$ genes. *phaC1*$_{Ps}$, PHA synthase 1 gene from *Pseudomonas* sp. 61-3; *phbA*$_{Re}$ and *phbB*$_{Re}$, β-ketothiolase, and NADPH-dependent acetoacetyl-CoA reductase genes from *Ralstonia eutropha*, respectively; P_{Ps}, promoter region upstream of *pha* genes in *Pseudomonas* sp. 61-3; P_{Re} and T_{Re}, promoter and terminator region on *phbCAB*$_{Re}$ operon in *R. eutropha*, respectively; Km^r, kanamycin resistant gene; Sm^r, streptomycin resistant gene; 'Sm^r, 5′-truncated streptomycin resistant gene

strains of *P. putida* GPp104 and *R. eutropha* PHB^{-}4, ^{1}H- and ^{13}C-NMR analyses were performed as described previously (Abe et al. 1994; Kato et al. 1996a). The mole fractions of 3HB unit and 3HA units (C6-C12) were determined from the intensity ratio of main-chain methylene proton resonance to methyl proton resonance in the ^{1}H-NMR spectra (data not shown). The values obtained were consistent with those obtained from GC analysis of methanolyzed PHA.

Figure 2 shows the 125 MHz ^{13}C-NMR spectrum of the copolyester produced by PHB^{-}4/pJKSc46-*pha* from tetradecanoate, together with the chemical shift assignments for each carbon resonance and an expanded spectrum of carbonyl resonances. The carbonyl carbon resonances (169.1–169.5 ppm) are clearly resolved into

Table 2 Accumulation of PHA by recombinant *Pseudomonas putida* GPp104 strains harboring *phaC1*$_{Ps}$ and *phbAB*$_{Re}$ (*3HB* 3-hydroxybutyrate, *3HHx* 3-hydroxyhexanoate, *3HO* 3-hydroxyoctanoate, *3HD* 3-hydroxydecanoate, *3HDD* 3-hydroxydodecanoate, *3H5DD* 3-hydroxy-*cis*-5-dodecanoate)

Plasmid (relevant markers)	Substrate[a]	Dry cell weight (g/l)	PHA content (wt%)	PHA Composition (mol%)					
				3HB (C4)	3HHx (C6)	3HO (C8)	3HD (C10)	3HDD (C12)	3H5DD (C12′)
pJBB49-*phb* (P_{Re}, *phaC1*$_{Ps}$, *phbA*$_{Re}$, *phbB*$_{Re}$)	Gluconate	0.77	25	48	1	6	34	7	4
	Octanoate	0.48	trace						
	Dodecanoate	0.54	1	0	7	37	36	20	0
pJKSc46-*pha* (P_{Ps}, *phaC1*$_{Ps}$, *phbA*$_{Re}$, *phbB*$_{Re}$)	Gluconate	0.76	10	49	0	6	30	10	5
	Octanoate	1.03	18	9	14	75	1	1	0
	Dodecanoate	0.53	40	16	9	30	28	17	0
pJKSc54-*phab* (P_{Ps}, *phaC1*$_{Ps}$, P_{Re}, *phbA*$_{Re}$, *phbB*$_{Re}$)	Gluconate	0.84	3	3	0	22	51	15	9
	Octanoate	0.73	4	0	11	71	10	8	0
	Dodecanoate	0.53	17	9	13	35	27	17	0

[a] Cells were cultivated at 30 °C for 72 h in MS medium containing the sodium salt of gluconate (2% wt/vol), octanoate, or dodecanoate (0.5% wt/vol) as the sole carbon source

Table 3 Accumulation of PHA by recombinant *R. eutropha* PHB^{-}4 strains harboring *phaC1*$_{Ps}$ *and phbAB*$_{Re}$ (*3HB* 3-hydroxybutyrate, *3HHx* 3-hydroxyhexanoate, *3HO* 3-hydroxyoctanoate, *3HD* 3-hydroxydecanoate, *3HDD* 3-hydroxydodecanoate, *3H5DD* 3-hydroxy-*cis*-5-dodecanoate)

Plasmid (relevant markers)	Substrate[a]	Dry cell weight (g/l)	PHA content (wt%)	PHA Composition (mol%)					
				3HB (C4)	3HHx (C6)	3HO (C8)	3HD (C10)	3HDD (C12)	3H5DD (C12′)
pJASc22 (P_{Ps}, *phaCl*$_{Ps}$)	Fructose	0.82	2	100	0	0	0	0	0
	Octanoate	0.87	12	33	9	58	0	0	0
	Tetradecanoate	0.97	14	46	4	21	18	11	0
	Olive oil	0.97	21	55	5	17	15	8	trace
	Corn oil	0.85	10	55	6	19	15	4	1
	Palm oil	0.99	12	56	5	19	14	6	0
pJBB49-*phb* (P_{Re}, *phaCl*$_{Ps}$, *phA*$_{Re}$, *phB*$_{Re}$)	Fructose	1.03	32	100	0	0	0	0	0
	Octanoate	0.65	6	32	23	45	0	0	0
	Tetradecanoate	0.71	1	44	0	20	23	13	0
	Olive oil	0.52	6	76	4	8	8	4	0
	Com oil	0.61	5	78	2	7	13	0	0
	Palm oil	0.59	5	79	2	7	8	4	0
pJKSc46-*pha* (P_{Ps}, *phaCl*$_{Ps}$, *phbA*$_{Re}$, *phbB*$_{Re}$)	Fructose	0.81	4	100	0	0	0	0	0
	Octanoate	0.82	13	35	9	56	0	0	0
	Tetradecanoate	0.85	12	51	4	18	16	11	0
	Olive oil	0.65	8	74	2	12	8	4	0
	Corn oil	0.70	7	82	1	8	8	1	0
	Palm oil	1.00	6	85	0	7	6	2	0
pJKSc54-*phab* (P_{Ps}, *phaCl*$_{Ps}$, P_{Re}, *phbA*$_{Re}$, *phbB*$_{Re}$)	Fructose	0.66	5	100	0	0	0	0	0
	Octanoate	0.69	11	17	18	65	0	0	0
	Tetradecanoate	1.14	17	46	8	20	16	10	0
	Olive oil	0.87	23	50	7	18	16	8	1
	Com oil	0.87	17	47	8	22	17	4	2
	Palm oil	0.72	26	51	7	18	16	7	1

[a] Cells were cultivated at 30 °C for 72 h in MS medium containing fructose (2% wt/vol), the sodium salt of octanoate (0.1% wt/vol × 5) or tetradecanoate, olive oil, corn oil, or palm oil (0.5% wt/vol) as the sole carbon source

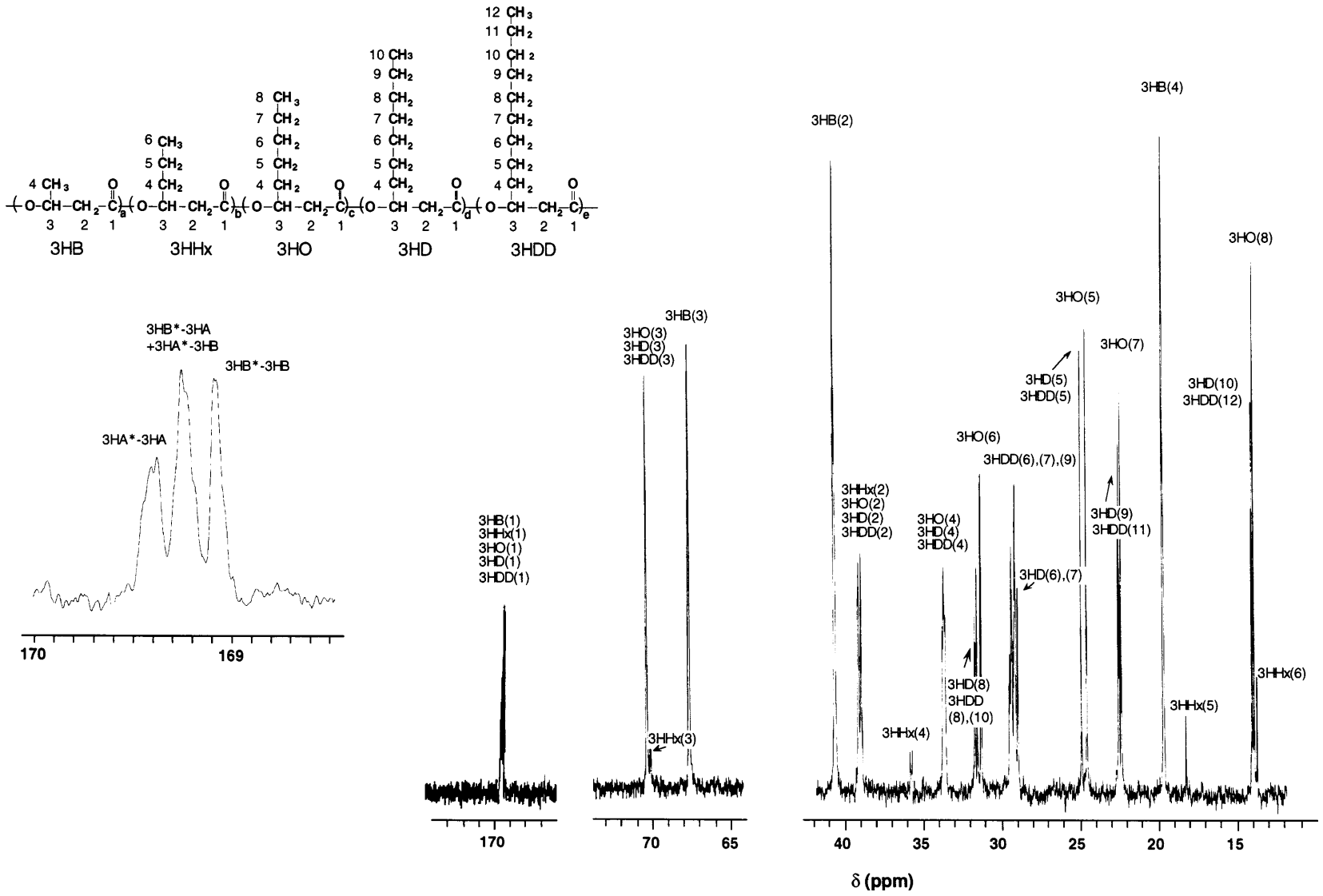

Fig. 2 125-MHz ^{13}C-NMR spectrum of P(51% 3HB-*co*-3HA) produced by recombinant *R. eutropha* PHB^{-}4 harboring pJKSc46-*pha* from tetradecanoate. 3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate; 3HD, 3-hydroxydecanoate; 3HDD, 3-hydroxydodecanoate. 3HA denotes 3-hydroxyalkanoates from C6 to C12

three groups of peaks, arising from different diad sequences of connecting 3HB and mcl-3HA units (C6–C12): 3HB*-3HB, 3HB*-3HA plus 3HA*-3HB, and 3HA*-3HA. The diad sequence distribution data of the copolyester samples with different monomer compositions obtained here were compared with Bernoullian statistics applicable to a statistically random copolymerization, suggesting that these copolyesters are mainly constituted of random copolymer (Table 4). Since the observed values were not completely consistent with the calculated ones, however, these copolyesters may contain block polymers or mixtures of 3HB- and 3HA-rich random copolymers as minor components.

Table 4 also shows the molecular weights of copolyesters synthesized by recombinant *P. putida* GPp104 and *R. eutropha* PHB^{-}4 strains. The number-average molecular weights (Mn) of copolyesters tested were in the range of 10.0×10^4 to 16.3×10^4 and the polydispersities (Mw/Mn) were 2.0–2.9.

Discussion

Recent progress in the physiology and molecular genetics of PHA synthesis has demonstrated that there are two types of polyester synthases, which are distinguished by their substrate specificities (Steinbüchel 1996; Rehm and Steinbüchel 1999), i.e., short-chain-length polyester synthase (PHB synthase) and medium-chain-length polyester synthase (PHA synthase) accepting (*R*)-3-, 4-, and 5-hydroxyalkanoyl-CoA thioesters of 3–5 carbon atoms and (*R*)-3HA-CoA thioesters of 5–14 carbon atoms as substrates, respectively. The PHA synthases of *Pseudomonas* sp. 61-3 have been proved to be capable of incorporating the 3HB unit into a polyester chain together with mcl-3HA units (Matsusaki et al. 1998). In this study, we investigated whether it was possible to synthesize P(3HB-*co*-3HA) copolyesters with higher 3HB compositions on heterologous host strains harboring $phaC1_{Ps}$ gene and $phbAB_{Re}$ genes from *Pseudomonas* sp. 61-3 and *R. eutropha*, respectively. We expect such copolyesters to be tough materials with flexibility, leading to a wide range of applications comparable to those of polyethylene and polypropylene. A recombinant *P. putida* GPp104 strain carrying $phaC1_{Ps}$ gene only accumulated mcl-PHA, and the 3HB unit is not incorporated at all or at a very low fraction (3 mol%) into PHA from gluconate or fatty acids, respectively (Matsusaki et al. 1998), suggesting that (*R*)-3HB-CoA molecules are insufficiently supplied in GPp104 strain. Therefore, introduction of $phbAB_{Re}$ genes into GPp104 strain restored the pathway for (*R*)-3HB-CoA formation. As a result, GPp104/pJBB49-*phb* or pJKSc46-*pha* accumulated P(3HB-*co*-3HA) copolyesters with about 50 mol% 3HB units from gluconate, while 3HB fractions in the copolyesters from octanoate or dodecanoate were less than 16 mol%. The reduced 3HB mole fractions may be because (*R*)-mcl-3HA-CoA molecules formed through an unknown branched pathway via β-oxidation might be efficiently supplied as substrates for PHA synthase without further removal of the C2 unit of the intermediates as acetyl-CoA in this bacterium. If this hypothetical phenomenon occurs in the cells, only a small amount of acetyl-CoA molecules could be formed, leading to fewer (*R*)-3HB-CoA molecules as a substrate for PHA synthase.

The 3HB compositions in copolyesters synthesized by GPp104/pJKSc54-*phab* were very low. The employment of two promoters (P_{Ps} and P_{Re}) for the expression of $phaC1_{Ps}$ and $phbAB_{Re}$ genes may be undesirable for the formation of (*R*)-3HB-CoA molecules.

The 3HB compositions of copolyesters produced by *R. eutropha* PHB^{-}4 harboring $phaC1_{Ps}$ gene only were much higher than those of copolyesters from *P. putida* GPp104 transconjugants, owing to the original existence of an efficient pathway providing (*R*)-3HB-CoA. Accumulation of P(3HB) homopolymer from fructose in all PHB^{-}4 recombinants is probably due to a defect in the key enzyme converting intermediates of de novo fatty acid synthesis to (*R*)-mcl-3HA-CoA as substrates for the het-

Table 4 Dyad sequence distributions and molecular weights of P(3HB-*co*-3HA) copolyester samples[a] [*3HB* 3-hydroxybutyrate, *3HA* medium-chain-length 3-hydroxyalkanoate units (C6–C12)]

PHA composition (mol%)		Relative intensities	Dyad sequence distribution			Molecular weight	
3HB(C4)	3HA (C6-C12)		3HB*-3HB	3HB*-3HA + 3HA*-3HB	3HA*-3HA	Mn ($\times 10^4$)	Mw/Mn
3	97	Observed	0	0.01	0.99	16.0	2.3
		Calculated	< 0.01	0.06	0.93		
47	53	Observed	0.26	0.38	0.36	16.3	2.0
		Calculated	0.22	0.50	0.28		
51	49	Observed	0.27	0.46	0.27	10.0	2.1
		Calculated	0.26	0.50	0.24		
65	35	Observed	0.44	0.39	0.17	12.8	2.1
		Calculated	0.42	0.46	0.12		
85	15	Observed	0.77	0.20	0.03	15.5	2.9
		Calculated	0.70	0.27	0.03		

[a] P(3HB-*co*-3HA) copolymers were obtained from recombinant *R. eutropha* PHB^{-}4 strains except P(3% 3HB-*co*-3HA) from *P. putida* GPp104 (pJASc22) grown on dodecanoate. PHA compositions were determined by ^{1}H-NMR. Observed relative intensities were determined from relative peak areas of carbonyl carbon resonances in ^{13}C-NMR spectra. Calculated values were obtained by Bernoullian statistics. Molecular weights were determined by gel permeation chromatography

erologous PHA synthase in *R. eutropha* cells. In *P. putida* KT2440, PhaG [(*R*)-3-hydroxyacyl-ACP:CoA transferase] provides (*R*)-mcl-3HA-CoA via the fatty acid biosynthesis pathway (Rehm et al. 1998). The monomer compositions in copolyesters from alkanoate-grown cells were very similar among all recombinant PHB^{-}4 strains, and the additional copies of $phbAB_{Re}$ genes did not give more 3HB units into the copolyesters. However, when plant oils were used as the sole carbon sources, copolyesters consisting of higher 3HB fractions were synthesized by PHB^{-}4 strains harboring pJBB49-*phb* or pJKSc46-*pha*. The application of inexpensive plant oils as carbon sources for PHA biosynthesis is predicted to reduce the production cost of bacterial polyesters.

Changing the promoter for the expression of $phaC1_{Ps}$ gene affected the PHA contents in both heterologous host strains, *P. putida* GPp104 and *R. eutropha* PHB^{-}4. The PHA contents in the recombinant strains expressing $phaC1_{Ps}$ gene under the control of the native promoter (P_{Ps}) were higher than those under the control of *R. eutropha* promoter (P_{Re}) when alkanoates were used as the sole carbon sources. In contrast, when sugars were used as the sole carbon sources, the PHA contents in both the host strains expressing $phaC1_{Ps}$ gene under the control of P_{Re} promoter were higher than those under the control of P_{Ps} promoter. This may be due to a difference in the expression level or period of $phaC1_{Ps}$ gene under the control of two distinct promoters which must be influenced by the carbon sources.

In conclusion, we succeeded in the biosynthesis of copolyesters consisting of 3HB and mcl-3HA units with various monomer compositions by using recombinant strains of *P. putida* GPp104 and *R. eutropha* PHB^{-}4 carrying $phaC1_{Ps}$ and $phbAB_{Re}$ genes, and achieved the synthesis of a novel P(3HB-*co*-3HA) with a higher 3HB composition (up to 85 mol%) from plant oils by recombinant *R. eutropha* PHB^{-}4 strains, indicating that $PhaC1_{Ps}$ is obviously capable of incorporating 3HB unit as well as mcl-3HA units into a polymer chain. P(3HB-*co*-3HA) copolyesters produced here were random copolymers of 3HB and 3HA units as shown by NMR analyses. The properties of such copolyesters can probably be widely varied by varying the compositions of 3HB and 3HA units. Further studies are being performed to elucidate the thermal, physical, and mechanical properties of P(3HB-*co*-3HA) copolyesters and will be reported elsewhere.

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