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Cometabolism of ω-Phenylalkanoic Acids with Butyric Acid for Efficient Production of Aromatic Polyesters in *Pseudomonas putida* BM01

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Abstract Poly(3-hydroxy-5-phenylvalerate) [P(3HPV)] was efficiently accumulated from 5-phenylvalerate (5PV) in *Pseudomonas putida* BM01 in a mineral salts medium containing butyric acid (BA) as the cosubstrate. A novel aromatic copolyester, poly(5 mol% 3-hydroxy-4-phenylbutyrate *-co-* 95 mol% 3-hydroxy-6-phenylhexanoate) [P(3HPB-*co*-3HPC)] was also synthesized from 6-phenylhexanoate (6PC) plus BA. The two aromatic polymers, P(3HPV) and P(3HPB-*co*-3HPC), were found to be amorphous and showed different glass-transition temperatures at 15°C and 10°C, respectively. When the bacterium was grown in a medium containing 20 mM 5PV as the sole carbon source for 140 h, 0.4 g/l of dry cells was obtained in a flask cultivation and 20 wt% of P(3HPV) homopolymer was accumulated in the cells. However, when it was grown with a mixture of 2 mM 5PV and 50 mM BA for 40 h, the yield of dry biomass was increased up to 2.5 g/l and the content of P(3HPV) in the dry cells was optimally 56 wt%. This efficient production of P(3HPV) homopolymer from the mixed substrate was feasible because BA only supported cell growth and did not induce any aliphatic PHA accumulation. The metabolites released into the PHA synthesis medium were analyzed using GC or GC/MS. Two β-oxidation derivatives, 3-phenylpropionic acid and *trans*-cinnamic acid, were found in the 5PV-grown cell medium and these comprised 55–88 mol% of the 5PV consumed. In the 6PC-grown medium containing BA, seven β-oxidation and related intermediates were found, which included phenylacetic acid, 4-phenylbutyric acid, *cis*-4-phenyl-2-butenoic acid, *trans*-4-phenyl-3-butenoic acid, *trans*-4-phenyl-2-butenoic acid, 3-hydroxy-4-phenylbutyric acid, and 3-hydroxy-6-phenylhexanoic acid. Accordingly, based on the metabolite analysis, PHA synthesis pathways from the two aromatic carbon sources are suggested.

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A large number of bacteria accumulate poly(3-hydroxyalkanoates) (PHAs) in cells as energy reserve materials [1, 7]. PHAs are an excellent storage material as they are highly reduced and exert a low osmotic pressure, since they are accumulated in granules. The microorganisms that produce PHAs are divided into two classes, short-chain-length (SCL) and medium-chain-length (MCL) PHA-producing organisms [5, 28]. SCL-PHAs have 1 or 2 carbons in their side chains whereas MCL-PHAs have 3 or more carbon atoms. MCL-PHAs have attracted attention because of the possible incorporation of many functional groups, such as phenyls, phenoxy groups, alkenes, etc., into their side chains [2-4, 6, 9, 11, 13-16, 23, 27], thereby resulting in the improved properties such as the increased transparency of the films cast from a phenyl-group containing PHA [Yoon and Choi, unpublished result] and the functionalization of olefinic PHA by a chemical modification of the double bond attachment of a biologically active molecule or by crosslinking between the inter-chain double bonds to enhance the material elasticity [3].

However, some precursors containing a phenyl or modified phenyl [e.g., 5-phenylvalerate (5PV), 5-(4-tolyl)-valerate, etc.] group are not readily utilized by bacteria when fed as the sole carbon source [6, 8, 13-15]. Even under optimum conditions, a cell yield of only 0.33 g/l is obtained when *P. oleovorans* is grown on 5PV as the sole carbon [8]. In order to increase the utilization capability of recalcitrant carbon compounds, the cometabolism method has been suggested [16].

Since the first paper on the synthesis of a bacterial polyester with a phenyl pendent group (specifically, poly(3-hydroxy-5-phenylvalerate) [P(3HPV)]) appeared in literature [8], several polyesters containing modified phenyl groups

have been reported [2, 6, 9, 13, 15, 23, 27]. *Pseudomonas oleovorans* and *Pseudomonas putida* are typical bacteria that can produce aromatic polyesters from phenyl-group-containing carboxylic acids. Most studies have employed the cometabolism method in which a good polymer-producing substrate (e.g., octanoic or nonanoic acid) has usually been used as the cosubstrate [2, 6, 13, 14]. Accordingly, since the resulting polymer is usually a mixture of an aromatic homopolymer, an aromatic/aliphatic copolymer, and an aliphatic polymer, the isolation and purification of pure aromatic homopolyesters from the recovered mixed polymer sample requires a tedious fractionation procedure using repetitive solution/precipitation steps [6, 14]. Therefore, it is necessary to develop a culture technique for preparing pure aromatic polyesters in large quantities. It has been found that in *P. putida* BM01, several low carboxylic acids (C_2~C_5), fructose, and glucose induce little PHA production, yet can support a large cell growth [26]. The use of these carbon sources in cometabolism with ω-phenylalkanoic acids is thus expected to result in a high production of pure aromatic polyesters free from aliphatic monomer units. The development of the high production technique of the pure aromatic polyesters is considered to be very important, because of their uses in enzymatic measurment of PHA depolymerases [12] toward the aromatic PHAs as well as other potential uses in future applications.

In this study, butyric acid (BA) was used as the cosubstrate for a high yield production of cell biomass in the cometabolic production of pure aromatic polyesters. Two phenyl substituted alkanoic acids, 5PV and 6-phenylhexanoic acid (6PC), were used as the precursors of aromatic monomers. The two phenyl-containing precursors were expected to be metabolized via different pathways, because their final β-oxidation products were structurally irrelevant. For a clearer understanding of cometabolism, the metabolic intermediates released into the media were analyzed. Several studies on molecular genetics [17, 29], substrate (presumed β-oxidation intermediates) utilization [22], and enzyme activity assays [21] have provided evidence that the intermediates of the β-oxidation of nonsubstituted alkanoates are channeled to PHA synthesis. However, no direct observation of PHA-synthesis-related β-oxidation intermediates present in a medium has been reported. Thus, on the basis of a direct observation of the related metabolites, putative PHA synthesis pathways are suggested. To the best of our knowledge, this is the first direct detection of β-oxidation and related intermediates relevant to PHA synthesis.

Materials and Methods

Microorganism and Reagents

Pseudomonas putida BM01 isolated in the authors' lab [26] was used throughout the experiments. Several carbon sources and their derivatives, including BA, 5PV, 6PC, 3-phenylpropionic acid (3PP), *trans*-cinnamic acid (CA), phenylacetic acid, 4-phenylbutyric acid, and *trans*-styrylacetic acid were obtained from Sigma Chemical Co. and used without further purification.

Quantitative Assay of Polyesters in Cells and Metabolites in the Medium

Precultures grown in a nutrient broth were grown in a 500 ml mineral salts medium [25-27] containing ω-phenylalkanoic acid or a mixture of BA, ω-phenylalkanoic acid, and 10 mM ammonium sulfate in a 2-l shake flask at 30°C under aerobic conditions. The cell growth was monitored by measuring the optical density at 660 nm. After cultivation for 30–40 h, the cells were isolated by centrifugation (7,000 rpm, 10 min) of the cell suspension, washed with methanol, and dried under a vacuum for 2 days. The supernatant was collected to analyze aromatic metabolites. An appropriate amount of magnesium sulfate was added to saturate the solution to efficiently transfer the metabolites to the organic phase. The metabolites in the solution were extracted with chloroform (extraction volume ratio=1:1), and the extracted metabolites were then esterified in a mixture of methanol and sulfuric acid [27]. The resulting methyl esters in the organic phase were analyzed using a Hewlett-Packard HP5890A gas chromatograph equipped with a HP-1 capillary column and flame ionization detector [27]. The remaining carbons in the medium were analyzed in a similar way. A typical GC running condition was as follows: initial temperature 80°C, 2 min; heating rate 10°C/min; final temperature 230°C, 2 min; carrier (He) flow rate, 3 ml/min; injector temperature, 230°C; detector temperature, 280°C. The composition of aromatic monomers in the polyesters was also determined by gas chromatographic analysis of the sulfuric acid/methanol-treated products using the above running conditions. For quantitative GC analyses, P(3HPV) was isolated, purified, and used as the standard. 5PV, 3PP, and CA were also used as standards after esterifying them under the same reaction conditions as in the treatment of metabolites. The remaining NH_4^+ was measured using Nessler's reagent [27].

Structural Characterization of Metabolites and Polymers

The structural identification of the esterified metabolites was carried out by a GC/MS (Fisons Instrument Trion1000) instrument. Helium was used as the carrier gas (2 ml/min). One-tenth microliters of the chloroform phase was analyzed after a splitless injection. The temperatures of the injector and detector were 230 and 275°C, respectively. The mass spectrometer was auto-tuned with perfluorotributylamine.

Since Fisons Instrument Data System had no mass spectral datum for *trans*-4-phenyl-3-butenoic acid methyl ester, a detailed assignment of the fragmented species of the compound was made for its exact identification (Fig. 1). The methyl esters of three structural isomers, *cis*-4-

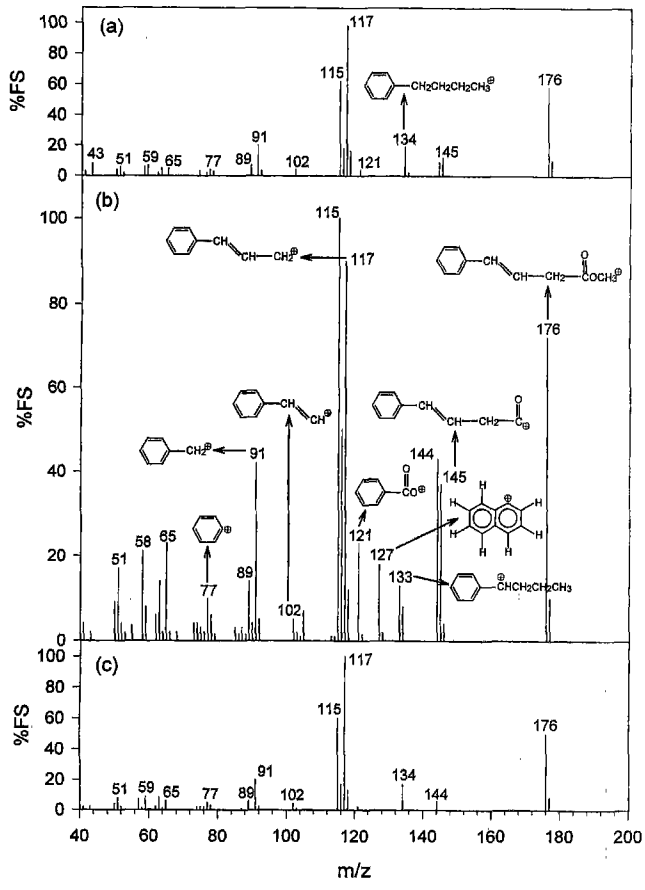

Fig. 1. Electron impact mass spectra for methyl esters of three isomeric metabolic intermediates.
(a) cis-4-phenyl-2-butenoic acid, (b) trans-4-phenyl-3-butenoic acid, and (c) trans-4-phenyl-2-butenoic acid found in the medium when *P. putida* BM01 was grown on 6PC plus BA, and chemical structural assignment for fragmented species.

phenyl-2-butenoic acid, *trans*-4-phenyl-2-butenoic acid, and *trans*-4-phenyl-3-butenoic acid, showed similar mass spectrometric fragmentation patterns (Fig. 1). The characteristic difference was a peak at m/z 127 that was seen for the methyl ester of *trans*-4-phenyl-3-butenoic acid. The molecular ion appeared at m/z 176 for all three isomers, as expected. The unexpected fragmented species at m/z 121, 127, 134, etc., seemed to result from rearrangements during flight in the mass analyzer. Specifically, the peak at m/z 127 could be ascribed to the rearranged species, naphthalene cation, which was presumably derived from the cyclization of the 1,3-butadien-4-phenyl cation. For the two other isomers, such cyclization presumably did not occur, because the double bond in the alkyl chain was separated two single bonds away from the phenyl ring (nonconjugated).

The ^{1}H and ^{13}C NMR spectra of the isolated polymers were obtained in a $CDCl_3$ solution using a Varian XL-300 NMR spectrometer. The solvent, chloroform-d, and tetramethylsilan were used as the internal chemical shift references for the ^{1}H and ^{13}C NMR spectra, respectively.

Thermal characterization of the isolated polyesters was carried out using a DuPont 2000 thermal analyzer (DSC V4.0B) equipped with a data station system. The room-temperature-annealed (for at least one month or more) PHA samples were heated at a rate of 20°C/min from −100 to 150°C. The glass transition temperatures (T_g) were reported for the midpoint of the T_g process.

RESULTS AND DISCUSSION

High Production of P(3HPV) Homopolyester from the Mixed Substrate of 5PV and BA

P. putida BM01 did not grow well on 5PV when it was fed as the only carbon source. When the bacterium was grown on 20 mM 5PV for 170 h, only 0.41 g/l of dry biomass was obtained and 18 wt% of the P(3HPV) homopolymer was accumulated in the cells (Table 1). To determine the effect of the nitrogen level on the PHA content and amount of total biomass, the cultivation was carried out at three ammonium ion concentrations, 8, 10, and 20 mM. Over the concentration range tested, no significant effect was

Table 1. Culture time dependence of metabolism of 5PV (20 mM) for cell growth and PHA production at 30°C.

Nitrogen source: $(NH_4)_2SO_4$ (mM)	Culture time (h)	Dry cell weight (g/l)	P(3HPV) (wt%)	Amount (mM) of intermediates:					O.D. at 660 nm	Remaining ammonium (mM)
				3HPV[a]	3PP[b]	CA[b]	5PV[b]	Total		
10	50[c]	–[d]	–	–	2.2	0.8	16.6	19.6	0.596	8.3
	80[c]	–	–	–	2.3	1.8	11.9	16.0	0.623	7.1
	105[c]	–	–	–	2.8	2.8	8.9	14.5	1.131	4.9
	130[c]	–	–	–	3.3	4.4	6.6	14.3	1.371	3.2
	150[c]	–	–	–	3.5	6.3	4.2	14.0	1.366	2.5
	170[c]	0.41	18	0.4	4.1	8.5	1.3	14.3	1.312	2.1
20	170	0.43	21	0.5	3.6	4.9	4.7	13.7	1.283	10.7

[a]3HPV, 3-hydroxy-5-phenylvalerate which corresponds to the monomer unit in P(3HPV). Its molar concentration was calculated assuming the culture volume as the total volume.[b]3PP, 3-phenylpropionate; CA, *trans*-cinnamic acid; 5PV, 5-phenylvalerate. The three species were detected in the cell-free supernatant. [c]All experimental values were determined at the indicated culture time for the culture grown on a mineral salts medium containing 20 mM 5PV as the sole carbon source.[d]Not determined.

Table 2. Metabolism of 5PV (20 mM) with various amounts of butyric acid for cell growth and PHA production[a].

Butyric acid (mM)	Dry cell weight (g/l)	P(3HPV) (wt%)	Amount (mM) of intermediates:					O.D. at 660 nm	Remaining ammonium (mM)
			3HPV[b]	3PP[c]	CA[c]	5PV[c]	Total		
10	0.976	36	2.0	5.0	11.0	1.5	19.5	2.91	2.3
20	1.650	28	2.6	4.9	7.5	1.7	16.7	5.22	0
30	1.795	40	4.1	4.8	7.2	1.1	17.2	7.19	0
40	2.030	56	6.5	4.3	7.5	2.0	17.3	8.23	0
50	2.487	47	6.6	4.6	4.4	4.2	19.8	9.12	0
octanoate 20[e]	2.856	(27)[f]	4.4	2.9	7.7	0.8	15.8	10.36	0

[a]Cells were grown at 30°C for 40 h. [b]3HPV, 3-hydroxy-5-phenylvalerate which corresponds to the monomer unit in P(3HPV). Its molar concentration was calculated assuming the culture volume as the total volume.[c]3PP, 3-phenylpropionate; CA, *trans*-cinnamic acid; 5PV, 5-phenylvalerate. The three species were detected in the cell-free supernatant. [d]The initial concentration was 10 mM in the form of $(NH_4)_2SO_4$ for all cultures. [e]20 mM Octanoic acid was added to the PHA synthesis medium instead of butyric acid. [f]The monomers constituting the polyester were 6.2 mol% 3-hydroxycaproate, 27.3 mol% 3-hydroxyoctanoate, 2.1 mol% 3-hydroxydecanoate, and 64.4 mol% 3HPV.

observed. In particular, even with the complete depletion of NH_4^+ at 8 mM NH_4^+, little effect was observed (data not shown). A similar pattern of PHA synthesis behavior was also reported previously in *P. oleovorans* when cultivated with only 5PV [8].

Table 2 shows an enhanced P(3HPV) homopolymer production in *P. putida* BM01 when grown with mixtures of 5PV and BA for 40 h. An increase of the BA content in the feed, in which the initial feed concentration of 5PV was 20 mM, induced an increase in the amount of total biomass up to 2.5 g/l, and the percentage of PHA with 56% of the dry cell weight in the cells peaked at 40 mM BA. With an exception of 10 mM BA, the ammonium added (10 mM) was completely depleted together with all other levels of BA content during cultivation. The isolated polymer was a P(3HPV) homopolymer free from aliphatic monomer units, as identified by NMR and GC (data not shown), thereby indicating that few aliphatic monomer units were derived from BA and incorporated into the polymer.

Synthesis of Novel Polyester from Mixed Substrate of 6PC and BA

When the bacterium was cultivated on a mixture of 30 mM BA and 10 mM 6PC in a mineral salts medium for 40 h, 1.45 g/l dry biomass was recovered and 30% of polyester in the dry cells was accumulated. The isolated and purified polymer was characterized by 75 MHz ^{13}C-NMR (Fig. 2). In addition to 10 major resonance lines associated with 10 different types of carbon atoms in the 3-hydroxy-6-phenylhexanoate-unit [(3HPC)-unit] in the polyester, several minor peaks were also observed. The absorption peaks between 120 and 145 ppm clearly showed the presence of a phenyl pendant group (C_i, δ=141.9 ppm; C_o and C_m, δ= 128.4 ppm; C_p, δ=125.9 ppm). The carbonyl resonance appeared at 169.3 ppm. The absorption at 70.5 ppm was ascribed to the backbone methine carbon (C_3). The four resonance lines appearing in the up-field region between 20 and 40 ppm were due to four types of methylene carbons (C_2, C_4, C_5, and C_6) in the 3HPC-unit. The probable minor monomer unit (less than 5 mol%) was identified as 3-hydroxy-4-phenylbutyrate (3HPB) by a GC/MS analysis (data not shown). Thus, the resulting polymer from the mixed carbon sources BA plus 6PC was P(5 mol% 3HPB -*co*- 95 mol% 3HPC). This suggests that, in the polymerization of 3-hydroxy-ω-phenylalkanoic acids, the *P. putida* BM01 synthase is able to incorporate the 3-hydroxyalkanoate with a phenyl group attached one methylene-unit carbon length away from the main backbone chain. A careful analysis of the polymer synthesized by *P. putida* BM01 from octanoic acid previously showed that the polymer contains approximately 2 mol% 3-hydroxybutyrate [26]. However, it has also been reported that *P. putida* U can synthesize a P(3HPC) homopolymer from 6PC fed as the sole carbon source [9]. The incorporation of a 3HPB-unit was also

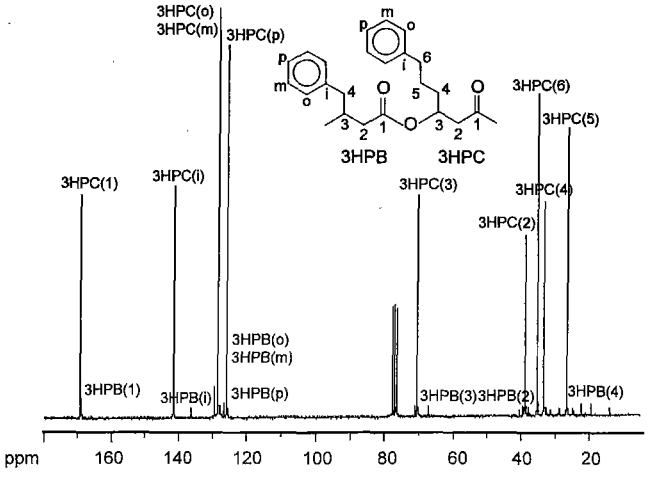

Fig. 2. 75 MHz ^{13}C-NMR spectrum of polyester synthesized by *P. putida* BM01 grown on a mixture of 30 mM BA and 10 mM 6PC.

3HPB and 3HPC stand for 3-hydroxy-4-phenylbutyrate- and 3-hydroxy-6-phenylhexanoate-units, respectively.

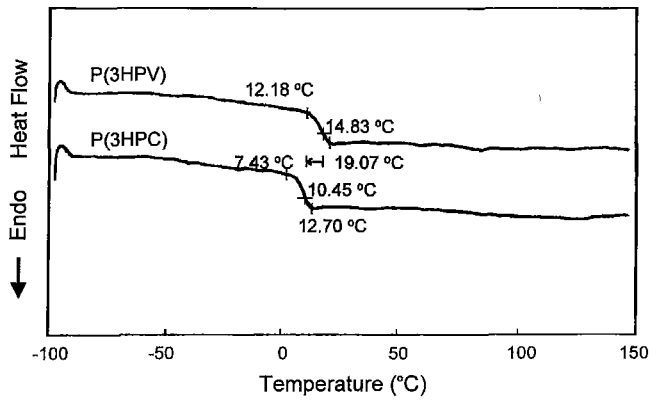

Fig. 3. Differential scanning calorimetric traces of poly(3-hydroxy-5-phenylvalerate) [P(3HPV)] homopolymer and poly(5 mol% 3-hydroxy-4-phenylbutyrate-*co*-95 mol% 3-hydroxy-6-phenylhexanoate) [P(3HPB-*co*-3HPC)] synthesized by *P. putida* BM01.

found in *Pseudomonas citronellolis* when grown in 6PC as the sole carbon source containing a mineral salts medium (unpublished result). In the latter case, an appreciable amount of aliphatic monomers (specifically, 3-hydroxyoctanoate and 3-hydroxydecanoate) was also found in the polymer (data not shown).

Glass Transition Temperatures of Isolated Polymers

The isolated P(5 mol% 3HPB -*co*- 95 mol% 3HPC) was analyzed using differential scanning calorimetry (Fig. 3). The polymer exhibited a glass transition at 10°C, that is, 5°C lower than that for P(3HPV). The glass transition at a lower temperature may reflect a decreased intermolecular interaction between the backbone chains, thereby resulting in an increased backbone chain mobility, caused by the lengthening of the side-chains by one methylene unit [10]. The two polymers P(3HPV) and P(5 mol% 3HPB -*co*- 95 mol% 3HPC) did not exhibit any melt endothermic peak. This suggests that the substitution of a phenyl group for hydrogen at the ω-position impedes the formation of a crystalline domain, probably due to a steric hindrance of the phenyl group.

Catabolism of 5PV and 6PC in PHA Synthesis

P. putida BM01 grew faster in the medium containing 6PC as the sole carbon source than on 5PV. A dry cell yield of 2.44 g/l was obtained and the PHA content was 17% of the dry biomass when the bacterium was cultured on a mineral salts medium containing 30 mM 6PC for 48 h. The GC analysis of the whole cells showed that the inclusion polymer was mainly composed of 3HPC (98 mol%). Other monomer units such as 3HPB (2 mol% or less), 3-hydroxyoctanoate (barely detectable), and 3-hydroxydecanoate (1 mol% or less) were detected as minor components. The significant difference in the assimilation rate between 5PV and 6PC suggests different metabolic routes. The metabolites released into the medium were analyzed using GC or GC/MS to investigate the metabolic pathways of 5PV and 6PC during the PHA synthesis. However, the PHA synthesis pathway inferred from the chemical analysis of the released metabolites may be acceptable only if the intracellular PHA degradation does not occur during the culture period. Otherwise, the released metabolites derived from the intracellular PHA degradation may contribute to the metabolites in the medium. However, such a probable contribution can be excluded because of no degradation of the aromatic PHA in cells during the sampling period (15 to 40 h) [Yoon *et al.*, to be submitted].

The gas chromatogram for the mixture of methyl esters obtained by esterifying the metabolic intermediates present in a culture medium containing 20 mM BA and 20 mM 5PV revealed 3 peaks at 13.4, 14.9, and 16.2 min of retention time (chromatogram not shown). The peak at 16.2 min was ascribed to the methyl ester of 5PV. The GC/MS analysis showed that the other two peaks at 13.4 and 14.9 min were identified as the methyl esters of 3PP and CA, respectively. To monitor the catabolism of 5PV, the concentrations of the metabolites were determined as a function of culture time (Fig. 4). After 40 h, the 5PV in the feed was almost depleted. Most of the 5PV consumed was converted into the phenyl-group-containing compounds such as the (R)-3-hydroxy-5-phenylvalerate unit in the polymer, and 3PP and CA in the medium (Fig. 4 and Table 2). The latter two intermediates in the medium must have been produced after one round of β-oxidation. The metabolic rate of 5PV in the media co-fed with BA was much faster than that in the 5PV-only medium. CA was the major aromatic metabolite found in most 5PV-containing media except for the medium

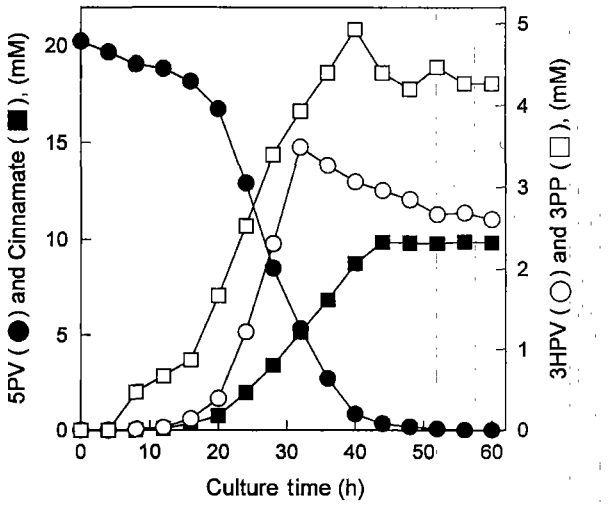

Fig. 4. Time courses for consumption of 5PV, release of 3PP and CA into medium, and accumulation of P(3HPV) in cells.
P. putida BM01 was grown in a mineral salts medium containing 20 mM BA and 20 mM 5PV. 3HPV in the figure legend indicates the monomer unit in the polymer P(3HPV).

containing 50 mM BA plus 20 mM 5PV. Although it remains to be identified, the probable low specificity of enoyl-CoA hydratase toward cinnamyl-CoA could be a factor in the slow conversion of CA, thereby causing the release of CA into the medium.

The total molar concentration for all the aromatic species detected revealed a deficit in comparison with the feed molar concentration of 5PV for both cosubstrate (BA plus 5PV) and 5PV-only feedings (Tables 1 and 2). A higher deficit occurred with the culture grown on the 5PV-only medium, indicating more breakdown of the phenyl ring than on the BA plus 5PV cosubstrate medium. Thus, the addition of BA to the medium generally released the pressure to degrade the phenyl ring moiety for cell growth. The deficit for the culture grown on the 5PV-only medium increased gradually with culture time (Table 1). All of these suggests that CA must be further degraded to remove two more carbons via the β-oxidation pathway, then the resulting benzoic acid, which is the final β-oxidation product from the ω-phenyl-substituted carboxylic acids with an odd numbered of carbon atoms, can be degraded via the benzoic acid degradation pathway [9]. This is supported by the fact that *P. putida* BM01 grew on 30 mM benzoic acid in a mineral salts medium without leading to PHA accumulation. Another strain, *P. putida* U, has also been reported to be able to catabolize benzoic acid [9]. However, *P. putida* BM01 did not grow on CA or 3PP in a mineral salts medium containing 15 mM ammonium sulfate. The inability of cell growth may be ascribed to the absence of the transport system for CA and 3PP in *P. putida* BM01, which is evident from no decrease in the level of the two metabolites in the later growth period, as shown in Fig. 4 and Table 1.

The production of a P(3HPV) homopolymer from 5PV in *P. putida* BM01 and from 7-phenylheptanoic acid or 9-phenylnonanoic acid in *P. putida* [9] suggested the inactivity of PHA synthases toward (R)-3-hydroxy-3-phenylpropionyl CoA or low epimerase activity toward (S)-3-hydroxy-3-phenylpropionyl CoA.

A gas chromatographic analysis was performed on the methyl esters obtained through the esterification reaction of the chloroform extract of the medium, in which the bacterium was grown on a mixed substrate of 20 mM 6PC and 30 mM BA for 30 h. Seven major peaks on the chromatogram (obtained on a Trion-1000 GC) appeared at 5.05, 6.72, 6.80, 7.12, 7.28, 7.65, and 9.13 min, and they were identified by mass spectrometry as the methyl esters of phenylacetic acid, 4-phenylbutyric acid, *cis*-4-phenyl-2-butenoic acid, *trans*-4-phenyl-3-butenoic acid, *trans*-4-phenyl-2-butenoic acid, 3-hydroxy-4-phenylbutyric acid, and 3-hydroxy-6-phenylhexanoic acid, respectively (Fig. 5). The intensities of phenylacetic acid and the two 3-hydroxyacids were significantly reduced during an extended cultivation of 48 h, whereas those of the other four acids remained

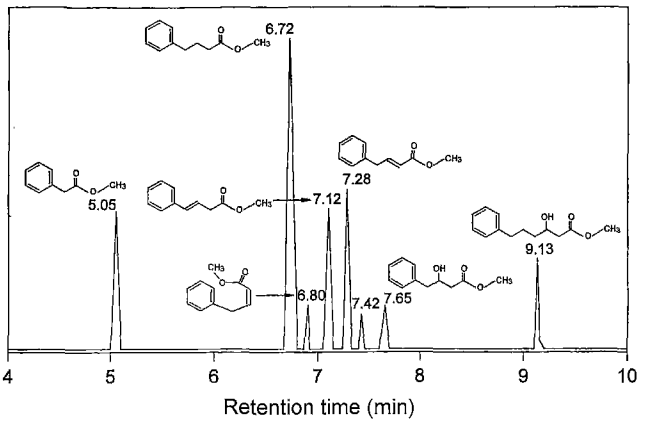

Fig. 5. GC/MS identification of methyl esters obtained after esterifying the chloroform extract of the culture medium in which *P. putida* BM01 was grown for 30 h.
The mineral salts medium contained 20 mM 6PC and 30 mM BA.

unchanged. This may indicate that the four acids, 4-phenylbutyric acid, *cis*-4-phenyl-2-butenoic acid, *trans*-4-phenyl-3-butenoic acid, and *trans*-4-phenyl-2-butenoic acid released into the medium can not be further metabolized by the bacterium. The detection of the *cis*- and *trans*-isomers of 4-phenyl-2-butenoic acid suggests the presence of a *cis-trans* isomerase for the compound. In contrast with 5PV, 3-hydroxyacids were found in the medium. Optical activities of the 3-hydroxyacids were not measured, therefore, it is not certain whether the 3-hydroxyacids were (S)-forms or (R)-forms or a mixture of the two forms.

All the acids detected, except for *cis*-4-phenyl-2-butenoic acid and *trans*-4-phenyl-3-butenoic acid, were the expected intermediates of the β-oxidation degradation of 6PC. Thus, on the basis of the metabolite analysis, a putative metabolic pathway of 6PC is suggested (Fig. 6). After being transported into the cell, 6PC is converted to 6-phenylhexanoyl-CoA by acy-CoA synthetase, and the resulting 6-phenylhexanoyl-CoA enters the β-oxidation pathway. Some of the (S)-3HPC-CoA formed is used in the polymerization after being converted to (R)-3HPC-CoA by 3-hydroxyacyl-CoA epimerase or via two more enzymatic reactions by (S)-3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA reductase, then the rest is converted to 4-phenylbutyryl-CoA and acetyl-CoA via two more enzymatic reaction steps ((S)-3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase). An additional short-cut pathway leading to PHA synthesis from the β-oxidation intermediate enoyl-CoA has also been established [18, 29]. 6-Phenylhexenoyl-CoA may be converted to (R)-3HPC-CoA via (R)-specific hydration catalyzed by (R)-specific enoyl-CoA hydratase, then, (R)-3HPC-CoA is subsequently polymerized into PHA. 4-Phenylbutyryl-CoA is further degraded to phenylacetyl-CoA and acetyl-CoA in the second round of β-oxidation. Similarly, some of the (S)-3HPB-CoA formed in the second round of β-oxidation is converted to (R)-3HPB-CoA. This

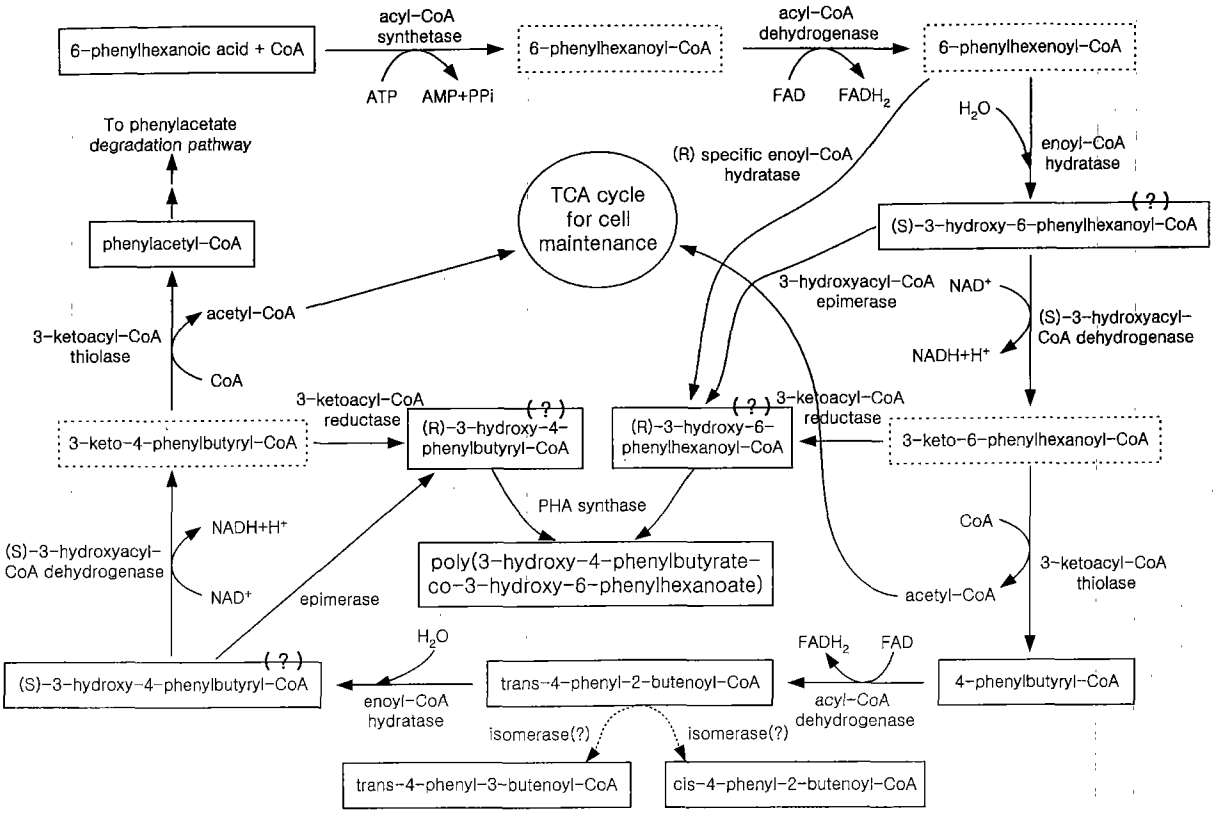

Fig. 6. Putative metabolic pathway of 6-phenylhexanoic acid for synthesis of poly(3-hydroxy-4-phenylbutyrate *-co-* 3-hydroxy-6-phenylhexanoate) in *P. putida* BM01.

The compounds in the solid-line boxes were released in the form of free acids into the culture medium and detected in the supernatant of the medium. The question marks on the solid-lines for the four 3-hydroxyacids indicate that the chirality of the GC-detected 3-hydroxyacids has not yet been determined (see text for details).

shorter (R)-form is incorporated into the polymer chain less efficiently than the longer (R)-3HPC-CoA, probably because of the high specificity of the PHA synthase. In addition, when considering the very low incorporation of (R)-3HPB-CoA, the *P. putida* PHA synthase is thought to be inactive against (even if it is available in cells) the shorter (R)-3-hydroxy-3-phenylpropionyl-CoA derived from 5PV. The concentration of the final β-oxidation product, phenylacetic acid, increased initially during cultivation, reaching a maximum at 28 h, and then decreased gradually with culture time (data not shown). Furthermore, *P. putida* BM01 grew on phenylacetic acid. These data suggest that, similar to *P. putida* U [19, 20, 24], *P. putida* BM01 also has the genes involved in the aerobic degradation of phenylacetic acid.

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