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Biosynthesis of Novel Aromatic Copolyesters from Insoluble 11-Phenoxyundecanoic Acid by *Pseudomonas putida* BM01

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Two types of novel aromatic copolyesters were synthesized from 11-phenoxyundecanoic acid (11-POU) as the sole carbon source and the cosubstrates 11-POU and octanoate, respectively, by isolated *Pseudomonas putida* BM01 that is known to accumulate high concentrations of medium-chain-length polyesters. Insoluble 11-POU was recrystallized in situ in buffer by alkaline treatment and pH adjustment, followed by autoclaving. The resulting microcrystals, whose structure was different from that of the commercially available crystalline powder, suspended in media were rapidly consumed by the bacterium. Synthesized polymers were characterized by gas chromatography, nuclear magnetic resonance spectroscopy, and differential scanning calorimetry. The aromatic copolyesters synthesized from 11-POU were composed of two monomer units consisting of 3-hydroxy-5-phenoxyvalerate (5POHV) as the major component (72 to 85 mol%) and 3-hydroxy-7-phenoxyheptanoate (7POHH) as the minor component (15 to 28 mol%). The aromatic copolyesters showed a crystalline melting transition at 70°C. When the bacterium was grown on the cosubstrates 11-POU and octanoate, the bacterium synthesized the copolyesters composed of aromatic and aliphatic monomers poly(5POHV-co-7POHH-co-3-hydroxy-9-phenoxynonanoate-co-3-hydroxyalkanoates). The addition of octanoate in the feed shifted the major monomer unit in the polymer from 5POHV to 7POHH. A further-fragmented metabolite, 3-phenoxypropionate, whose concentration reached a steady state at the time of greatest polyester accumulation, was detected in the medium. The metabolic pathway of 11-POU is suggested.

Pseudomonas species such as *Pseudomonas putida* (9, 16, 17, 19), *Pseudomonas oleovorans* (3–6, 8, 10–15), *Pseudomonas citronellolis* (1), and other fluorescent *Pseudomonas* strains generally biosynthesize various types of copolyesters, principally composed of medium-chain-length (MCL) monomer units, from a wide range of carbon sources. Functional groups such as halogens (3, 10, 13), olefins (1, 6, 9, 12, 14, 17), branched alkyls (1, 5, 8), cycloalkyls (13), phenyls (4, 11, 19), and esters (15) can be introduced at or near their terminals of the long chain of pendant groups in MCL polyhydroxyalkanoates (polyesters). The introduction of the functional groups on the side chains of MCL polyesters by bacteria may allow future functional biomaterials to be produced (15), resulting from the secondary modification of the reactive groups such as olefins and other potentially interesting groups by covalently attaching active moieties with specific functions (e.g., drugs for medical treatment and chromophoric molecules for colored liquid crystals). In these respects, it may be necessary to find new bacterial polyesters with alterations in their side chains leading to different physicochemical properties useful in their applications.

First of all, it is essential to have bacterial strains accumulating high concentrations of MCL polyesters to increase the probability of introduction of unnatural functional groups. *P. putida* BM01(16), isolated in our laboratory, is thought to be a suitable strain for the study of the synthesis of functional polyesters because it accumulates MCL polyesters, including poly(3-hydroxy-5-phenylvalerate) (19). The introduction of planar benzene rings into the side chain R group may result in polyesters with unusual properties because of the probable strong interactions among aromatic rings. The bacterial aromatic polyester first reported was found in *P. oleovorans* cells grown on the cosubstrates of 5-phenylvalerate and nonanoic acid (11). It was reported that the isolated sample was a mixture of poly(3-hydroxy-5-phenylvalerate) homopolymer and poly(3-hydroxyalkanoate).

We report here the synthesis of new aromatic copolyesters composed of two structurally related monomer units with a crystalline melting transition at 70°C and a glass transition (from glassy polymer to rubbery polymer) at 14°C. Another interesting type of copolyesters, composed of both aromatic and aliphatic monomer units, was also synthesized in this study.

MATERIALS AND METHODS

Bacterial strain and cell growth in the solid-liquid heterogeneous media. *P. putida* BM01 was isolated and characterized by a procedure from *Bergey's Manual* described previously (16). 11-Phenoxyundecanoic acid (11-POU; 97% purity) and most other chemicals were purchased from Aldrich Chemical Co. (Milwaukee, Wis.) and Sigma Chemical Co. (St. Louis, Mo.). 11-POU was dissolved in alkaline 30 mM phosphate buffer (pH 11, adjusted with 10 N NaOH), and after adjustment of the pH to 7.4, the solution turned milky. The white color was developed by precipitation of 11-POU in the form of 0.1- to 0.3-μm square flakes. Autoclaving and cooling of the neutralized solution gave similar results (see Results). Cells were grown for 40 h in M1 medium (16) suspended with microcrystalline 11-POU prepared by the treatment procedure described above. Cell growth was monitored by measuring the biomass increase every 4 h. 11-POU is soluble in methanol. Therefore, insoluble 11-POU was separated from cells by washing the centrifugate (15,000 × *g*, 10 min; Supra 22K, Hanil Science Industrial Co., Seoul, Korea) with methanol, and the cells were isolated by centrifuging (15,000 × *g*, 10 min) the cell suspension. The remaining 11-POU was measured by gas chromatographic analysis of the methyl ester. The methyl ester exhibited a retention time of 31.10 min under the following run conditions with a Hewlett-Packard HP5890A gas chromatograph equipped with an OV25 column: initial temperature, 80°C, for 4 min; heating rate, 10°C/min; final temperature, 230°C, for 15 min. The composition of aromatic monomers in polyesters was also determined by gas chromatographic analysis of the sulfuric acid-methanol-treated products under the gas chromatographic run conditions described above. The remaining $(NH_4)_2SO_4$ (initial concentration, 8 mM) was measured with Nessler's reagent (1). The other characterization methods, including determina-

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tion of the remaining octanoate, polyester isolation, and determinations of biomass, polyester content in cells, and monomer composition in the isolated polymer, have been reported elsewhere (1, 16).

Electron microscopy. Cells and substrate particles for electron microscopy were negatively stained with 2% phosphotungstic acid solution (pH 7.2) (16). Electron micrographs were taken with a Hitachi (Tokyo, Japan) H-600 electron microscope under an acceleration voltage of 75 kV.

DSC analysis of polymers. Melting transitions of the 11-POU powder and the treated 11-POU particulate suspension in buffer were measured under a dry nitrogen purge with a differential scanning calorimeter (micro-DSC; Setaram Co., Caluire, France) equipped with a data station. A slurry-suspension liquid containing 2.2 mg of 11-POU per 800 μl of phosphate buffer was placed into the sample vessel. The corresponding phosphate buffer solution (800 μl) was placed into the reference vessel. Heating was done at a rate of 1°C/min. In the DSC study of vacuum-dried polymers, the sample size was approximately 10 mg. Polymer specimens were subjected to annealing at room temperature for 8 weeks before measurement to obtain steady-state structures (1). Heating was done at a rate of 20°C/min. The scanning range was between −100 and 200°C. A Du Pont 2000 thermal analysis system (DSC V4.0B; TA Instruments Inc., New Castle, Del.) was used for several polymer samples.

Spectroscopic characterization of polymers. The ^{1}H-nuclear magnetic resonance (^{1}H-NMR) and ^{1}H-noise-decoupled ^{13}C-NMR analyses of the polyester samples were carried out on a Varian (Palo Alto, Calif.) UNITY-300 spectrometer in the pulse-Fourier transform mode. Two-dimensional heteronuclear (^{1}H-^{13}C) and homonuclear (^{1}H-^{1}H) correlation spectroscopy (COSY) spectra were recorded at 20°C in a Varian UNITY-300 spectrometer.

RESULTS

Cultivation of the bacterium in solid-liquid slurry medium. The 11-POU is a flaky, crystalline powder. As expected from the chain length of the substrate molecule, it is insoluble in water, and almost no cell growth was observed for the suspension of the untreated substrate in phosphate buffer. Increasing the pH to ~11 made it soluble in a phosphate buffer solution. After the pH was adjusted to 7.4, the solution turned opaque, and on long standing, the suspended particles precipitated. After the slurry suspension was autoclaved and then cooled, the milky phase appeared again. Thus, the cultivation of the bacterium was performed in a solid-liquid two-phase slurry medium. The electron microscopic photograph of the substrate particles showed that they were square, thin flakes with sizes ranging from 0.1 to 0.3 μm. The suspended particulate solids were crystalline, which was confirmed by DSC analysis showing a melting endotherm around 60°C for the particulate suspension (Fig. 1, thermograms B and C). The unautoclaved sample revealed a rather broadened endotherm which probably resulted from the metastable structure of the precipitated particles (Fig. 1, thermogram B). Its melting temperature is significantly lower than that of the solid sample from the manufacturer, which has a melting transition at 81°C (Fig. 1, thermogram A). Such a large difference between melting temperatures suggests that the crystalline structures of the samples are totally different in nature. According to additional electron microscopic data, bacterial cells grew surrounded by 11-POU substrate particles adhering to cell walls (data not shown). Thus, the insoluble 11-POU chains in contact with cell walls may be assimilated by bacteria.

Characterization of novel aromatic copolyester from the cells grown on 11-POU. When *P. putida* BM01 was grown on 11-POU as the only carbon source, the bacterium produced a copolyester consisting of two monomer units which were identified by gas chromatography. The retention times of the two methyl esters obtained by methanolizing the purified polyester were 20.5 and 24.9 min, respectively. The ^{1}H-NMR spectrum for the sample is shown in Fig. 2a. The strong proton signals around 7 ppm may be associated with phenoxy groups. The two well-resolved absorptions with different intensities at 5.17 and 5.36 ppm associated with backbone methine protons may suggest the existence of two monomer units in the polymer chain. The ^{13}C-NMR spectrum is shown in Fig. 2b. The four absorption peaks between 110 and 160 ppm clearly showed the presence of the phenoxy pendant group. Each of the four peaks was further separated into two peaks, indicating the presence of two types of phenoxy groups. The number of peaks in the up-field region (low chemical shifted) also implied that the polyester contained at least two monomer units. A detailed assignment of the chemical shifts pertaining to each carbon is given in Table 1. Further exact assignments were made through an analysis of the 300-MHz ^{1}H-^{1}H homonuclear COSY spectrum (Fig. 3). The two sequential orders 2-3-4-5-6 and b-c-d-e-f-g in the corresponding monomer unit were confirmed in the ^{1}H-^{1}H homonuclear COSY spectrum. The assignment of carbon signals in Fig. 2b agreed well with the

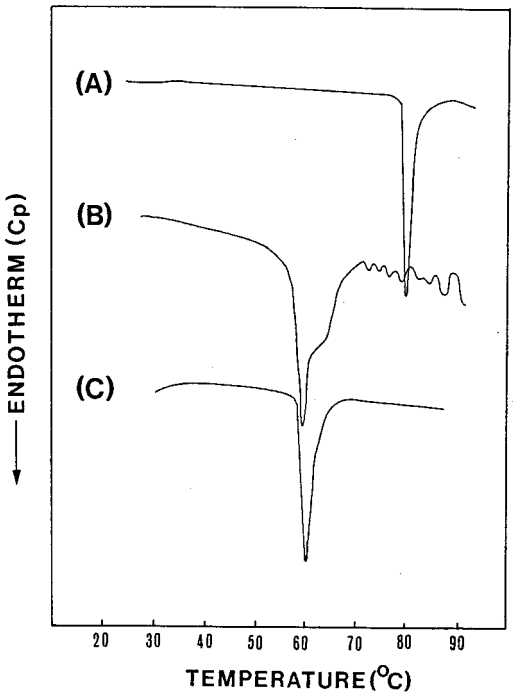

FIG. 1. DSC thermograms of nontreated 11-POU crystals (A) and 11-POU microcrystals in M1 medium after alkaline treatment and neutralization (B) and after autoclaving the sample described for panel B and cooling (C).

TABLE 1. Assignment of chemical shifts of carbon atoms in the 75-MHz ^{13}C-NMR spectrum of the polyester synthesized by *P. putida* BM01 grown on 11-POU as the sole carbon source

Carbon atom[a]	Absorption (ppm) of carbon in polyester	
	5POHV	7POHH
1 (a)	169.18	169.15
2 (b)	39.04	39.04
3 (c)	68.56	70.65
4 (d)	33.20	33.50
5 (e)	63.71	21.71
(f)		28.94
(g)		67.32
6-i (h-i)	158.51	158.95
6-o (h-o)	114.50	114.44
6-m (h-m)	129.46	129.42
6-p (h-p)	120.87	120.55

[a] Numerals are for repeating unit 5POHV, and letters are for repeating unit 7POHH.

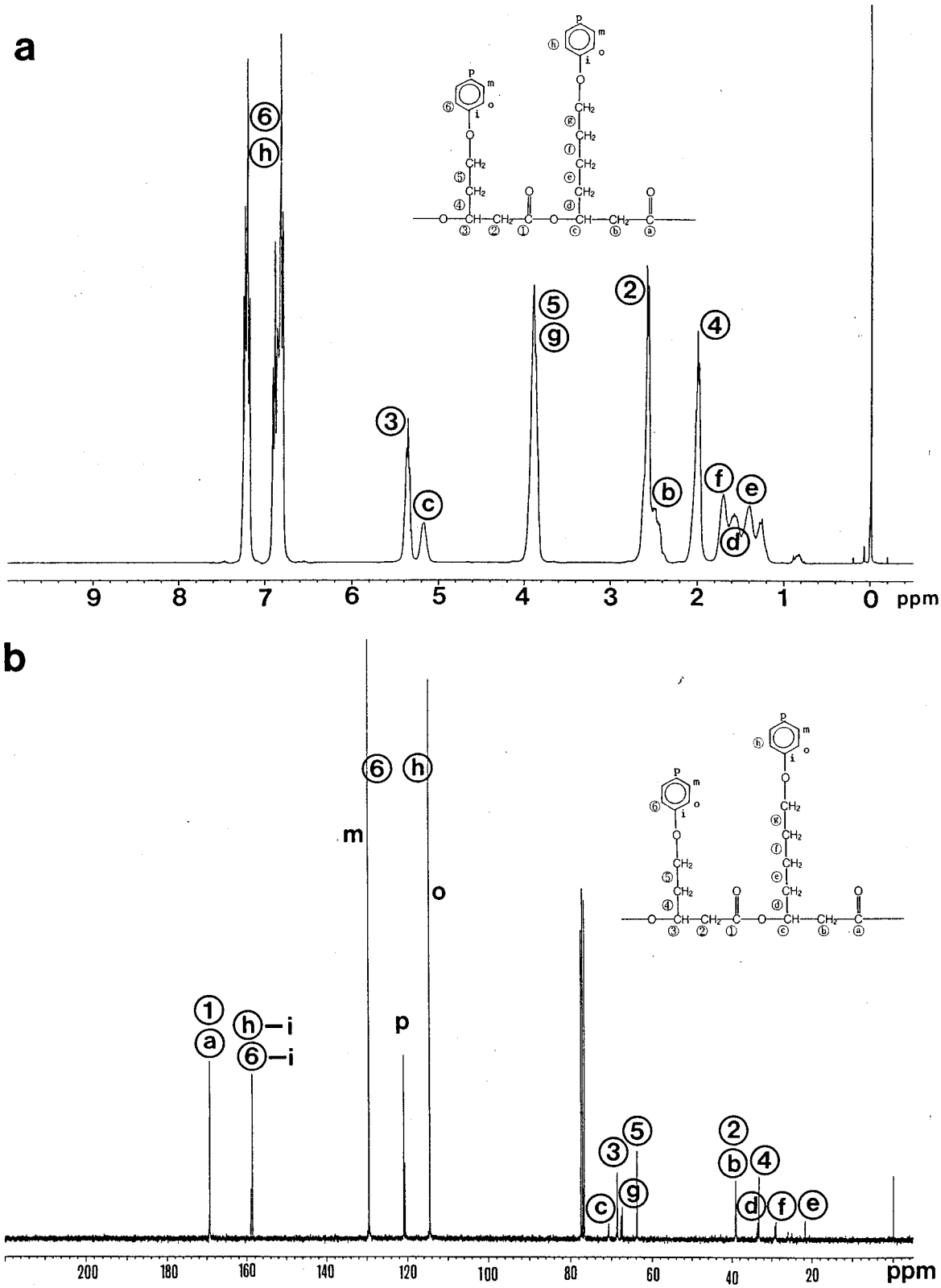

FIG. 2. (a) ^{1}H-NMR spectrum (300 MHz) of the aromatic copolyester synthesized by *P. putida* BM01 grown on 15 mM 11-POU. (b) ^{13}C-NMR spectrum (75 MHz) of the aromatic copolyester synthesized by *P. putida* BM01 grown on 15 mM 11-POU.

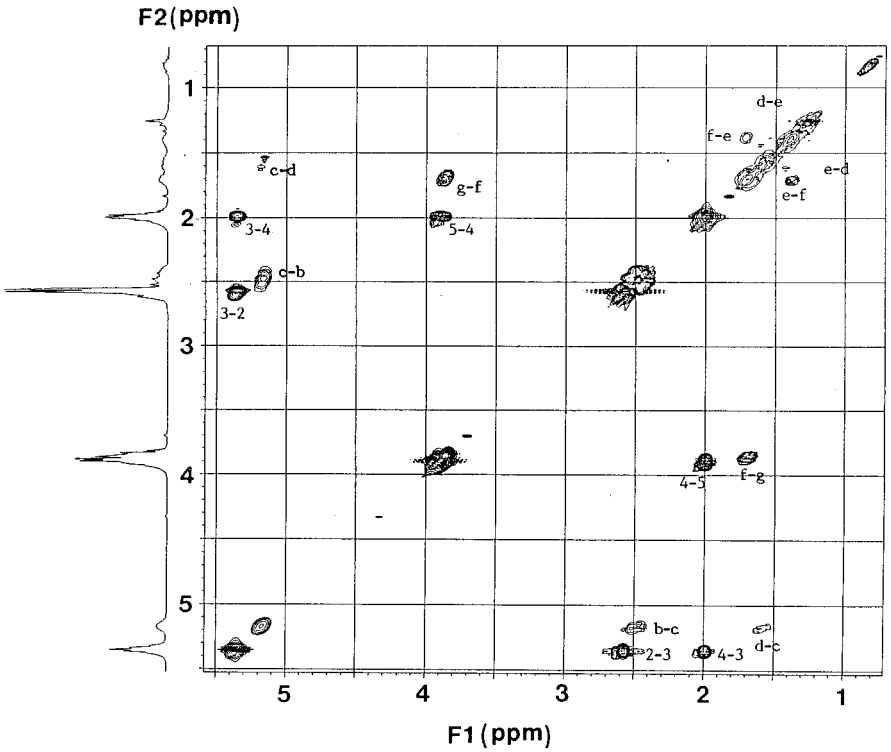

FIG. 3. ^{1}H-^{1}H homonuclear COSY spectrum (500 MHz) of the aromatic copolyester synthesized by *P. putida* BM01 grown on 15 mM 11-POU.

two-dimensional ^{1}H-^{13}C COSY spectrum (data not shown). The integrated area ratios of proton resonances in the ^{1}H-NMR spectrum were internally consistent with one another and in complete agreement with the proposed structure of the aromatic copolyester. Thus, the copolyester may be named poly(3-hydroxy-5-phenoxyvalerate-co-3-hydroxy-7-phenoxyheptanoate) [P(5POHV-co-7POHH)].

Biosynthesis of aromatic copolyesters at various feed levels of 11-POU. 11-POU is insoluble in water. However, the feeding level of particulate 11-POU suspension in the medium could be increased by a procedure similar to that described in the first paragraph of Results. Despite the heterogeneity of substrate in the medium, the polyester accumulation exhibited a substrate concentration dependence (Table 2). Cells were grown for 40 h during which 11-POU was almost completely depleted. The initial NH_4^+ concentration was 1.2 g/liter for all experiments described in Table 2. Both dry cell weight and polyester content in cells increased as the content of 11-POU in the medium was increased. An increase of 11-POU in the medium resulted in a decrease of the molar ratio [5POHV]/

TABLE 2. Polyester production of *P. putida* BM01 grown on various concentrations of 11-POU and octanoate

Concn (mM) of carbon source		Dry cell wt (g/liter)	Polyester content in dry cells (% [wt/wt])	Polyester composition (mol%)[a]				Rate of conversion[b]	Molar ratio ([5POHV]/[7POHH]) in polyesters
OA[c]	11-POU			3HAs	5POHV	7POHH	9POHN		
0	5.0	1.16	13.1	0.0	84.7	15.3	0.0	0.15	5.5
0	10.0	1.42	16.4	0.0	78.5	21.5	0.0	0.12	3.7
0	15.0	1.68	20.4	0.0	72.8	27.2	0.0	0.12	2.7
0	20.0	2.00	22.9	0.0	71.6	28.4	0.0	0.12	2.5
20	2.5	2.46	22.7	88.0	7.9	4.1	0.0	0.18	1.9
20	5.0	2.92	42.1	74.0	15.1	8.9	2.0	0.36	1.7
20	7.5	3.05	36.0	65.8	14.6	14.9	4.7	0.28	0.96
20	10.0	3.82	42.5	59.3	15.3	17.4	7.3	0.35	0.95
5	10.0	2.03	27.9	22.1	41.4	34.5	2.0	0.20	1.2
15	10.0	3.02	35.9	51.4	20.8	22.6	5.2	0.28	0.92
30	10.0	3.96	46.8	79.1	10.3	8.7	1.9	0.12	1.2
40[d]	10.0	2.25	31.5	0.0	41.9	46.7	11.4	0.35	0.90

[a] Calculated from gas chromatographic analysis. 3HAs include all aliphatic monomer units in polyesters (e.g., 3HC, 3HO, and 3HD).
[b] The rate of conversion of 11-POU to the monomer units in polyesters is defined by moles of aromatic monomer units converted divided by moles of 11-POU fed.
[c] OA, octanoate.
[d] Butyric acid, instead of octanoic acid, was added.

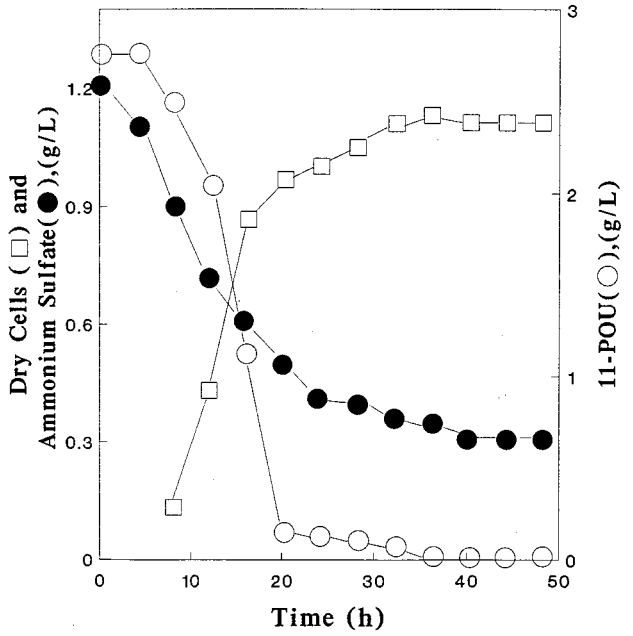

FIG. 4. Consumption of 11-POU and ammonium and accumulation of biomass when *P. putida* BM01 was grown on 10 mM 11-POU.

[7POHH] in polyester chains. However, it is interesting to note that the rate of conversion of 11-POU to the aromatic comonomer units is constant regardless of the concentration of 11-POU.

Figure 4 shows the growth kinetics of the bacterium grown on 10 mM 11-POU. Almost complete depletion of 11-POU occurred after 20 h of cultivation, while 40% of the NH_4^+ remained unconsumed. Dry biomass reached the maximal value after 30 h of cultivation. The active polyester accumulation occurred between 10 and 30 h of cultivation (data not shown), which corresponds to the exponential phase of biomass accumulation (Fig. 4). This means that cell growth and polyester accumulation occurred simultaneously. The ratio of comonomer units (5POHV and 7POHH) was relatively constant throughout the cultivation except during the early phase of growth. The increase in the concentration of 11-POU to 15 mM with the NH_4^+ concentration unchanged (1.2 g/liter) delayed cell growth by 10 h (data not shown). The carbon and nitrogen were completely consumed within 30 to 35 h of cultivation. The growth kinetics were similar to those in the case of 10 mM 11-POU-grown cells except for the delay of growth.

The thermal transition behavior of P(5POHV [73 mol%]-co-7POHH [27 mol%]) obtained from 15 mM 11-POU-grown cells is shown in Fig. 5. Despite the presence of two comonomer units with different lengths of side chain spacers, the copolymer exhibited a melting transition at 70°C although the melting enthalpy was rather small (0.7 cal/g [ca. 2.9 J/g]). The copolymer had a sharp glass transition between 9.4 and 18.8°C (midpoint, 14.1°C). It was stiff and tough below the glass transition temperature. However, it was rather sticky and soft above that temperature.

Biosynthesis of copolyesters from the cosubstrates of 11-POU and octanoate. If two substrates or more can be utilized for cell growth and polyester accumulation, the utilization of one substrate may be affected by the other substrate. Thus, the relative utilization of 11-POU and octanoate depended on the relative amount of the aromatic cosubstrate in the mixture (Table 2). Twenty millimolar octanoate was added to one liter of the medium containing various concentrations of 11-POU. An increase in 11-POU concentration in the medium resulted in increases in both dry biomass and intracellular polyester content. The molar ratio of aromatic monomers (5POHV, 7POHH, and 3-hydroxy-9-phenoxynonanoate [9POHN]) to aliphatic monomers in polyesters is linearly proportional to the molar ratio of 11-POU to octanoate in the medium. The methyl ester of 9POHN had a gas-chromatographic retention time of 32.2 min under the run conditions described in Materials and Methods. Data for the molar conversion of 11-POU to the aromatic monomers clearly demonstrate that cofeeding of octanoate with the aromatic substrate expedites the incorporation of unnatural aromatic monomer units into polyester chains. An increase in 11-POU concentration also changed the ratio between aromatic monomers in polymers, especially by not only shifting the major monomer unit to 7POHH but also by introducing the longer unit 9POHN into the polymer chain.

A similar polyester synthesis trend was observed when various amounts of octanoate were combined with 10 mM 11-POU (Table 2). An increase in octanoate content in the medium resulted in an increase in dry biomass, intracellular polyester content, and moles percent aliphatic monomer units in polyesters. The ratio of octanoate to 11-POU was relatively well conserved in the molar ratios of the corresponding aliphatic monomers to aromatic monomers. The ratios of the two aromatic monomers 5POHV and 7POHH were relatively constant with an increase in octanoate content in the feed. The ratios of three aliphatic monomers, 3-hydroxycaproate (3HC), 3-hydroxyoctanoate (3HO), and 3-hydroxydecanoate (3HD), were also relatively invariable with the change in the level of octanoate in the medium (data not shown). The average molar composition was 13.1 mol% 3HC, 83.1 mol% 3HO, and 3.7 mol% 3HD. When butyric acid, instead of octanoate, was added to the medium containing 10 mM 11-POU, *P. putida* BM01 produced a copolyester composed of only aromatic monomer units with a significant increase in 7POHH content in comparison with that in cells grown on only 11-POU.

Time courses for the consumption of carbon and nitrogen and biomass increases when cells were grown on the mixed substrates of 15 mM octanoate and 10 mM 11-POU are shown

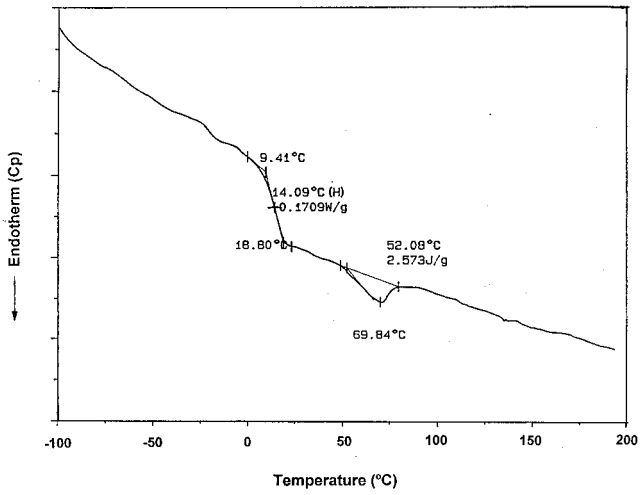

FIG. 5. DSC thermogram of the polyester synthesized by *P. putida* BM01 grown on 15 mM 11-POU.

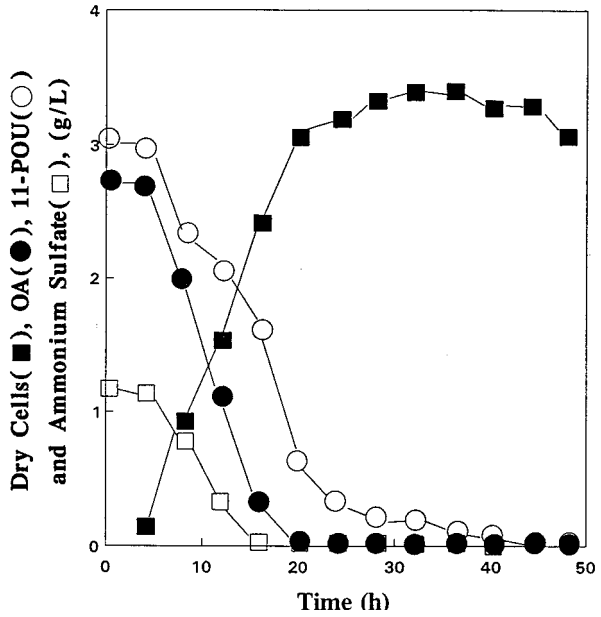

FIG. 6. Consumption of octanoate (OA), 11-POU, and ammonium and accumulation of biomass when *P. putida* BM01 was grown on a mixture of 10 mM 11-POU and 15 mM octanoate.

in Fig. 6. At these similar levels of the two carbon sources, 11-POU was slightly less preferentially utilized than octanoate. Dry biomass reached a maximum after 20 h of cultivation. The maximal weight percent of intracellular polyester occurred after 17 h of cultivation (data not shown). The specific rate of consumption of octanoate was 0.14 g liter^{-1} h^{-1} for the initial 20 h of cultivation, while the rate for 11-POU consumption was 0.1 g liter^{-1} h^{-1}, slightly lower than 0.12 g liter^{-1} h^{-1}, the value when grown on 10 mM 11-POU as a single source.

A twofold increase in octanoate concentration appreciably delayed the utilization rate of 11-POU. The twofold increase in octanoate concentration also induced the depletion of the NH_4^+ in the medium later than when grown on 15 mM octanoate and 10 mM 11-POU. The final increase of biomass during 20 to 30 h of cultivation correlates well with the rapid consumption of 11-POU in the later growth period (data not shown).

Although the specific rates of consumption of the two carbon sources were similar at the ratio of 10 mM 11-POU/15 mM octanoate, the rates of incorporation of the aliphatic and aromatic monomers into polymer chains showed a time lag between them (Fig. 7). In the early growth phase, the aliphatic-monomer-rich polymers were produced, while the aromatic monomers, whose incorporation reached the maximum at 30 h of cultivation, were incorporated rapidly in the later growth phase. The incorporation rate of 5POHV and 7POHH changed with cultivation time in a different way. 5POHV was incorporated faster than 7POHH in the early phase. The situation was reversed in the later growth phase. The content of 9POHN in polyesters also showed a similar sudden increase during the exponential period of polyester accumulation, and the increase was as much as approximately 10 mol% of the total content of aromatic monomers (data not shown). A further increase in octanoate concentration in the medium (30 mM octanoate and 10 mM 11-POU) resulted in increased heterogeneity in the monomer distribution, and the initial portion of polymers contained few aromatic monomers. The introduction of 9POHN as well as 7POHH was enhanced when NH_4^+ was depleted.

Fractionation of polyesters synthesized from the mixed substrates of 11-POU and octanoate. The polyesters synthesized by *P. putida* BM01 grown on octanoate and 11-POU consisted of several monomers such as 3HC, 3HO, 3HD, 5POHV, 7POHH, and 9POHN. Fractionation (11) of the polyester samples in mixed solvents (methanol and chloroform) was carried out to investigate whether they were copolymers or blends (i.e., mixtures of two or more separate polymers [e.g., aromatic and aliphatic copolyesters]) of polymers. A polymer blend with overall monomer composition similar to that of the separate batch sample to be tested was prepared by mixing two polymers, poly(3HC [18 mol%)-co-3HO [82 mol%]) (PHAs) and poly(5POHV [72 mol%]-co-7POHH [28 mol%]) (PPOHAs), in an appropriate ratio. When the blend was reprecipitated in the mixed solvent (methanol-chloroform, 2:1 [vol/vol]), the precipitated fraction contained the same molar amount of aromatic monomer as that in PPOHAs before blending, and no aliphatic monomers were detected in the precipitated fraction, which was identified by gas chromatography (Table 3). In the soluble fraction of the blend, few aromatic monomers were found. This means that in the mixed solvent of methanol-chloroform (2:1 [vol/vol]), the blend of the two copolyesters PPOHAs and PHAs was completely separated into component polymers without intermixing. The polyester sample with an overall monomer composition similar to that of the blend was then reprecipitated in the mixed solvent of methanol-chloroform (2.5:1 [vol/vol]). The sample was also separated into two fractions (soluble and precipitated) (Table 3). The behavior of compositional distribution was different from that observed in the blend system. Each of the two fractions contained an appreciable amount of both aliphatic and aromatic monomer units. The aliphatic-monomer-rich components remained in the soluble fraction. The aromatic-monomer-rich chains were concentrated into the precipitated fraction. Thus, the fraction-

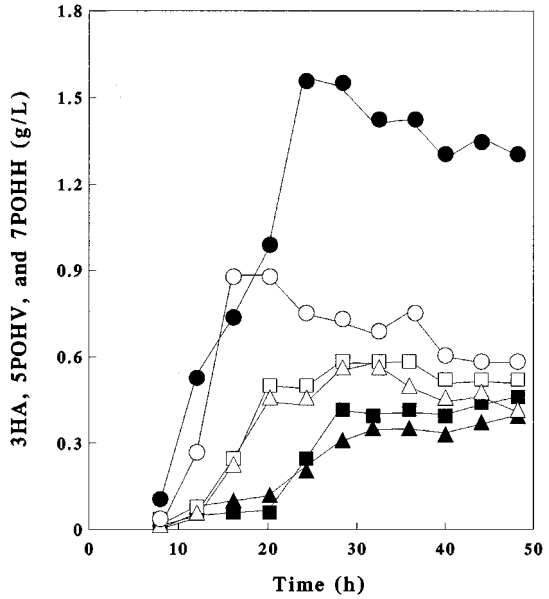

FIG. 7. Time courses of accumulation of 3-hydroxyalkanoate (3HA) (○, ●), 5POHV (△, ▲), and 7POHH (□, ■) when *P. putida* BM01 was grown on a mixture of 10 mM 11-POU and 15 mM octanoate (open symbols) or on a mixture of 10 mM 11-POU and 30 mM octanoate (filled symbols). 3-Hydroxyalkanoate includes all aliphatic monomer units such as 3HC, 3HO, and 3HD.

TABLE 3. Fractionation of the blend consisting of the aliphatic and aromatic polyesters and the copolyester with a similar overall monomer composition in the mixed solvent (chloroform and methanol)

Sample type	Amt of sample (mg)	Overall monomer composition before fractionation (mol%)[a]			Mixing ratio of methanol/chloroform (vol/vol)	Phase	Amt of recovered polyester (mg)	Monomer composition in each phase after fractionation (mol%)		
		3HAs	5POHV	7POHH				3HAs	5POHV	7POHH
Copolyester	234	69	15	16	2.5	Solution	203	72.5 ± 1.5	12.5 ± 0.5	15.0 ± 1.0
						Precipitate	24	30.5 ± 1.5	41.0 ± 1.0	28.5 ± 0.5
Blend										
PHAs[c]	68	100	0	0	2.0	Solution	66	96	1	3
PPOHAs[d]	49	0	72	28		Precipitate	44	0	75	25

[a] Calculated from gas chromatographic data. 3HAs include 3-hydroxyhexanoate, 3HO, and 3HD.
[c] PHAs, an aliphatic copolyester containing no aromatic monomer units in which 3HO was the major monomer.
[d] PPOHAs, an aromatic copolyester containing no aliphatic monomer units.

ation experiments proved that *P. putida* BM01 copolymerizes 3HC, 3HO, 3HD, 5POHV, 7POHH, and 9POHN when grown on octanoate and 11-POU.

DISCUSSION

Characteristics of solid-liquid two-phase cultivation. The successful cultivation of cells in the medium containing the insoluble substrate 11-POU was accomplished through the preparation of readily utilizable microcrystalline substrate particles whose structure was expected to be quite different from that of 11-POU in the dry crystalline state as evidenced by their different melting points. The in situ recrystallization of 11-POU in aqueous solution may lead to the formation of a loosely organized microcrystalline suspension which demonstrates a molecular structure suitable for the bacterium to easily use as a carbon source. A similar pretreatment procedure may be applied to other insoluble, crystallizable organic acids. The use of this type of insoluble substrate may be advantageous over the use of other well-dispersed oily liquid substrates in the cultivation of cells because a limited number of the dispersed substrate molecules in the medium (specifically, the solubility of 11-POU in a phosphate buffer; 3.9 mg/100 ml [0.13 mM]) may have less-toxic effects on cell growth than the detergent-like action of the soluble or oily carbons with long chains.

Despite the significantly reduced concentration of solubilized substrates in the medium available for growth, the profiles for the consumption of carbon source and polyester accumulation showed patterns similar to those usually observed in homogeneous cultivation (Fig. 4, 6, and 7). This suggests that the heterogeneous substrate particles were so well dispersed without further aggregation during cultivation that the bacterial cells could easily incorporate the substrate molecules in contact with the cell wall. The kinetics of polyester synthesis depended on the concentration of 11-POU. This may be ascribable to the increased number of substrate particles unchanged in size when the level of 11-POU as the sole carbon source was increased.

Factors that determine the conversion yield of 11-POU and the ratio of aromatic comonomers. The final molar conversion yield of the aromatic substrate to the corresponding monomers was relatively constant, with the average value of 0.12 mol/mol of the conversion yield ($Y_{p/s}$) regardless of the substrate concentration when 11-POU was used as the sole carbon source. The addition of 20 mM octanoate to the medium containing 11-POU increased the yield up to fourfold (Table 2). The increased conversion yield implies both the reduced utilization of 11-POU for cell growth and the enhanced utilization of the substrate for polyester accumulation.

5POHV-coenzyme A (5POHV-CoA) must be derived from 7POHH-CoA by the loss of 1 ethylene unit in the form of acetyl-CoA via the β-oxidation pathway to produce 5-POHV-CoA (9, 17). Similarly, 7POHH-CoA is derived from 3-hydroxy-9-phenoxynonanoyl-CoA. Thus, the addition of a more favorably utilizable substrate for cell growth than 11-POU increases the content of monomer units with longer side chains. The shift of the major monomer unit to 7POHH is also considered due to the easier utilization of octanoate in cell growth and, subsequently, the lower requirement for degrading 7POHH via the β-oxidation pathway.

The short-chain carboxylic acids (e.g., propionate, butyrate, and valerate) support only the growth of *P. putida* BM01, not polyester accumulation (16). Growth on the mixed substrates of 11-POU and the acids leads to the production of copolyesters composed of only aromatic monomers with the major

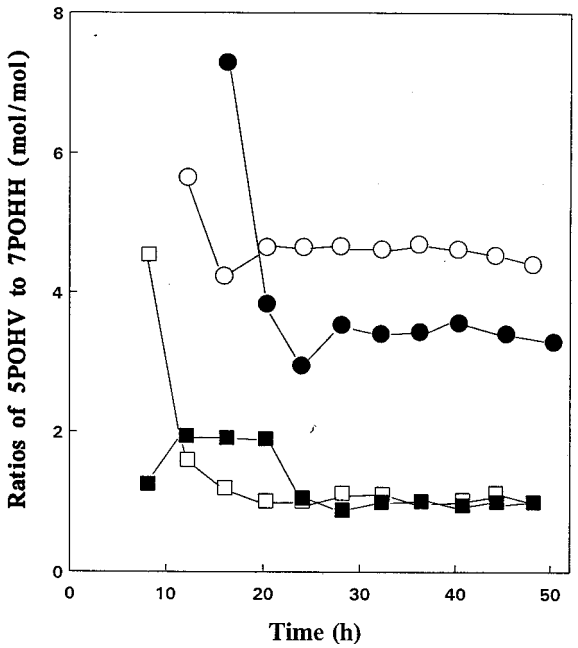

FIG. 8. Time courses of aromatic monomer ratio [5POHV]/[7POHH] in polyester when *P. putida* BM01 was grown on 10 mM 11-POU (○), 15 mM 11-POU (●), 10 mM 11-POU–15 mM octanoate (□), and 10 mM 11-POU–30 mM octanoate (■).

TABLE 4. Distribution of aromatic species during cultivation of *P. putida* BM01 grown on 10 mM 11-POU in the presence of additional substrates

Additional substrate	Culture time (h)	Concn of aromatic species in medium (mM)					Total concn of aromatic species (mM)
		11-POU	3-POP[a]	5POHV	7POHH	9POHN	
None	12	8.6	2.6	0.40[b]	0.09[b]	0.00[b]	11.7
	36	0.1	6.3	1.12	0.41	0.00	7.9
	12	8.6	1.2	0.04	0.00	0.00	9.8
	48	0.0	6.2	1.17	0.40	0.00	7.8
10 mM octanoate	12	7.7	1.4	0.50	0.39	0.09	10.1
	48	0.0	4.3	1.73	1.57	0.44	8.0
15 mM octanoate	12	7.0	0.7	0.36	0.18	ND[c]	8.2
	48	0.0	4.3	1.76	1.82	ND	7.9
20 mM octanoate	12	8.9	1.1	0.10	0.00	0.00	10.1
	48	0.0	3.4	2.22	2.54	0.71	8.9
40 mM butyrate	12	6.4	1.2	0.10	0.06	0.00	7.8
	48	0.0	3.5	2.32	1.80	0.39	8.0

[a] 3-POP, 3-phenoxypropionate.
[b] The concentration is based on the sampled culture volume.
[c] ND, not determined.

monomer unit shifted to the longer chain. The monomer composition of purely aromatic copolyester could be changed accordingly by varying the substrate ratio.

It is interesting to note an increase in the molar ratio [5POHV]/[7POHH] in the polymer as the concentration of 11-POU decreased when 11-POU was used as the sole carbon source (Table 2 and Fig. 8). The highest molar ratio was observed in the early growth phase (Fig. 8). The copolymerization rate of 3-hydroxyl CoA monomer precursors depends on their enzymatic specificities to the polyester synthase and available concentrations in the cytosol (2). However, the present synthesis data indicate that the supply rate of CoA-type monomer precursors may be a more important factor in the copolymerization in light of similar homologous structures of the aromatic monomers.

Aliphatic and aromatic monomer units are chemically linked. The melting point depression can be used as an additional criterion to determine whether two or more monomer units are copolymerized (2). The melting point for the isolated and purified polyesters decreases from 54 to 45°C as the aromatic monomer content increases from 0 to 45 mol%. A similar decrease in the enthalpy value from 4.8 ± 0.7 cal [ca. 20 ± 2.9 J]/g of polyester for PHAs to 0.3 cal [ca. 1 J]/g of polyester for the copolymer composed of 55 mol% 3-hydroxyalkanoates (e.g., 3HC and 3HO, etc.) and 45 mol% aromatic monomers was observed. A further increase in the content of the aromatic monomer units in the polymers resulted in failure to exhibit an endotherm. This indicates that the polyester synthesized by the bacterium from the cosubstrates of octanoate and 11-POU is a random copolyester and not a blend of aliphatic and aromatic polyesters such as was found previously (11) for *P. oleovorans* grown on 5-phenylvalerate and nonanoate. This also suggests that the random introduction of bulky aromatic side chains into the polymer effectively prevents crystallization. The data from the fractionating experiment previously described and the thermal analysis described above show that the aliphatic and aromatic monomers are polymerized together by the synthase in *P. putida* BM01.

Metabolic pathway of 11-POU for cell growth and polyester synthesis. In addition to the aromatic monomer components in the cells, a partially degraded form of the aromatic species was found in the medium and identified as 3-phenoxypropionate by gas chromatography (retention time of the methyl ester, 15.80 min) and NMR spectroscopy. However, this metabolite was not found in the cells. The concentration of 3-phenoxypropionate in the medium increased with cultivation time and reached a steady-state value in 24 to 28 h. The increasing profile showed an exponential pattern similar to that of the polyester accumulation (data not shown). These results indicate that 5POHV-CoA is metabolized in two ways, by incorporation into the polymer chain and by further degradation to 3-phenoxypropionate via the β-oxidation pathway to synthesize one molecule of acetyl-CoA for use in the tricarboxylic acid cycle. The total amount of aromatic species, residual 11-POU, 3-phenoxypropionate, 5POHV, 7POHH, and 9POHN did not balance the initial concentration of 11-POU throughout the cultivation except during the initial 10 h (Table 4). 3-Phenoxypropionic acid is a crystalline solid that melts at 99°C and boils at 240°C. Therefore, its loss as a result of vaporization during cultivation may be highly improbable. The deficit occurred principally in the early exponential growth phase (data not shown). This may be related to the highest molar ratio value for [5POHV]/[7POHH] occurring in the early growth phase (Fig. 8). We suggest that the deficit may have been metabolized by the breakdown of the phenoxy group via a pathway which remains to be identified. This interpretation is, of course, based on the absence of additional aromatic intermediates or products other than the four species. However, when 3-phenoxypropionate was provided as the only carbon source, no cell growth was observed.

5-Phenylvalerate can be utilized as an aromatic monomer precursor by the bacterium to synthesize only poly(3-hydroxy-5-phenylvalerate) when cofed with other substrates supporting only cell growth. The aromatic species found during cultivation were 3-hydroxy-5-phenylvalerate in cells and 3-phenylpropenic acid (*trans*-cinnamic acid) in the medium. In contrast to the case of 11-POU, the total amount of the aromatic components derived from 5-phenylvalerate exactly balanced the amount of the initial substrate. Thus, the probable breakdown of the aromatic ring on 11-POU might be due to the presence of the oxidized carbon on its aromatic ring because the breakdown of an aromatic ring by *Pseudomonas* species requires the oxidation of the aromatic carbon first (7, 18).

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