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Pseudomonas oleovorans as a Source of Poly(β-Hydroxyalkanoates) for Potential Applications as Biodegradable Polyesters

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Pseudomonas oleovorans **was grown in homogeneous media containing *n*-alkanoic acids, from formate to decanoate, as the sole carbon sources. Formation of intracellular poly(β-hydroxyalkanoates) was observed only for hexanoate and the higher *n*-alkanoic acids. The maximum isolated polymer yields were approximately 30% of the cellular dry weight with growth on either octanoate or nonanoate. In most cases, the major repeating unit in the polymer had the same chain length as the *n*-alkanoic acid used for growth, but units with two carbon atoms less or more than the acid used as a carbon source were also generally present in the polyesters formed. Indeed, copolymers containing as many as six different types of β-hydroxyalkanoate units were formed. The weight average molecular weights of the poly(β-hydroxyalkanoate) copolymers produced by *P. oleovorans* ranged from 90,000 to 370,000. In spite of the higher cell yields obtained with octanoate and nonanoate, the use of hexanoate and heptanoate yielded higher-molecular-weight polymers. These copolyesters represent an entirely new class of biodegradable thermoplastics.**

The ability of bacteria to form intracellular storage granules composed of poly(β-hydroxyalkanoates) (PHA) in general, and poly(β-hydroxybutyrate) (PHB) in particular, has been used as a parameter for the taxonomic classification of the genus *Pseudomonas* (26, 29). Many different types of pseudomonads are able to produce this storage polyester, which is usually formed under nutrient-limiting conditions (8, 27). Recently, it became of industrial interest to evaluate these polyesters as biodegradable thermoplastics for a wide range of possible applications, such as surgical sutures, long-term carriers for drugs, or molded plastics and films (16–18). PHB homopolymer and copolymers of β-hydroxybutyrate and β-hydroxyvalerate are currently being industrially produced by ICI Ltd. for this purpose by the use of *Alcaligenes eutrophus*. These polymers are commercially available under the trade name Biopol (P. A. Holmes, European patent application 0 052 459, Oct. 1981). In addition to *A. eutrophus*, some cyanobacteria (e.g., *Aphanothece* sp.) are also known to form poly(β-hydroxybutyrate-co-β-hydroxyvalerate), but their PHA content is usually very low (5). Physical and mechanical properties of these copolymers, such as stiffness, melting point, extension to break, and resistance to organic solvents, can change considerably as a function of the monomer composition (2, 14, 31).

PHA extracted from sewage sludge and marine sediments showed the presence of β-hydroxyalkanoate units other than β-hydroxybutyrate and β-hydroxyvalerate (13, 25, 31), which suggests the presence of microbial populations in these environments that are capable of producing other types of PHA. However, only *Bacillus megaterium* (13) and *Pseudomonas oleovorans* (9; R. Lageveen, Ph.D. dissertation, University of Groningen, The Netherlands, 1986) are known to incorporate repeating units longer than four or five carbon atoms into their storage polyester.

In the present study, we investigated the ability of *P. oleovorans* to produce various types of PHA homopolymers or copolymers and to incorporate different monomers into its storage polymers. The objectives of this study were: (i) to optimize the growth and culture conditions for PHA production by *P. oleovorans*, including an evaluation of NH_4^+ and a variety of organic substrates for further control of polymer production; (ii) to isolate the various polyesters produced and determine their monomer composition and molecular weights; and (iii) to gain information about the metabolic pathways of the various carbon sources which were used in this study for both growth and PHA production.

MATERIALS AND METHODS

Stock cultures of *P. oleovorans* (ATCC 29347) were maintained on a slightly modified E* medium described previously (Lageveen, dissertation). The medium contained the following (per liter): $(NH_4)_2HPO_4$, 1.1 g; K_2HPO_4, 5.8 g; KH_2PO_4, 3.7 g. Ten milliliters of a 100 mM $MgSO_4$ solution and 1 ml of a microelement solution were added. The microelement solution contained the following (per liter of 1 N HCl): $FeSO_4 \cdot 7H_2O$, 2.78 g; $MnCl \cdot 4H_2O$, 1.98 g; $CoSO_4 \cdot 7H_2O$, 2.81 g; $CaCl_2 \cdot 2H_2O$, 1.67 g; $CuCl_2 \cdot 2H_2O$, 0.17 g; $ZnSO_4 \cdot 7H_2O$, 0.29 g. The carbon source was added to give a final concentration of 10 to 20 mM. The pH was adjusted to 7.0, and the medium was autoclaved. For the stock cultures, 10 mM sodium octanoate was used as the sole carbon source. Solid medium was prepared by addition of 1.5% agar. Cells were grown aerobically in liquid culture for 24 h. A sample was transferred to agar plates and grown for another 24 h. The plates were then stored at 4°C for up to 3 months. Cells from these plates were transferred to liquid medium which was used as an inoculum for all further growth experiments.

Generally, growth experiments were performed under aerobic conditions in 200-ml or 1-liter cultures in a temperature-controlled shaker (New Brunswick Scientific Co., Inc.; 31°C; 150 rpm). After the medium was autoclaved,

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non-water-soluble growth substrates such as octane were added from a nonsterile stock, giving a concentration of 10% (vol/vol). Different water-soluble *n*-alkanoic acids from formate to decanoate were used as carbon sources. One percent (vol/vol) of a culture pregrown on octanoate was added as an inoculum. After a growth experiment, the cells were harvested by centrifugation (Sorvall RC2-B; 4°C; 16,300 × *g*). The pellet was resuspended and washed once with 10 mM Tris-hydrochloride buffer (pH 7). The harvested cells were lyophilized and transferred to screw-cap scintillation vials which were stored in a freezer at −18°C until further processing.

During a growth experiment, samples were taken from the culture medium for determination of metabolic intermediates. One milliliter of the culture was pipetted into an Eppendorf tube and centrifuged for 3 min. The supernatant was filtered through a 0.2-μm-pore-size filter (Gelman Sciences, Inc.; ACRO LC13). Ten microliters was analyzed at room temperature by high-pressure liquid chromatography (Varian 5000) on an HPX-87H fast-acid column (Bio-Rad Laboratories; 100 by 7.8 mm) with 10 mN H_2SO_4 as the eluent (1.2 ml/min). Organic acids were detected at 210 nm and identified by comparison of their retention times with those of commercially available *n*-alkanoic acids. Under these conditions, the retention times of the different standards were as follows (min): acetate, 2.25; propionate, 3.05; butyrate, 3.83; valerate, 5.67; hexanoate, 8.98; heptanoate, 15.62; octanoate, 28.92; nonanoate, 44.3.

To determine the polymer content of lyophilized whole cells, approximately 4 mg of these cells was reacted in a small screw-cap test tube with a solution containing 1 ml of chloroform, 0.85 ml of methanol, and 0.15 ml of sulfuric acid for 140 min at 100°C in a thermostat-equipped oil bath (3; Lageveen, dissertation). This method degrades the intracellular PHA by methanolysis to its constituent β-hydroxycarboxylic acid methyl esters. After the reaction, 0.5 ml of distilled water was added and the tube was shaken vigorously for 1 min. After phase separation, the organic phase (bottom layer) was removed and transferred to a small screw-cap glass vial. Samples were stored in the freezer at −70°C until further analysis. The methyl esters were assayed by gas chromatography (GC) with a Perkin-Elmer 8500 gas chromatograph equipped with a Durabond-Carbowax-M15 megabore capillary column (15 m by 0.54 mm; J & W Scientific) and a flame ionization detector. A 2-μl portion of the organic phase was analyzed after splitless injection. Helium (17 ml/min) was used as the carrier gas. The temperatures of the injector and detector were 230 and 275°C, respectively. A temperature program was used which efficiently separated the different β-hydroxyalkanoic acid methyl esters (80°C for 4 min; temperature ramp of 8°C per min; 160°C for 6 min). Under these conditions, the retention times of the different β-hydroxyalkanoic acid methyl ester standards were as follows (min): C-4, 4.22; C-5, 5.82; C-6, 7.40; C-7, 9.19; C-8, 10.71; C-10, 13.46; C-11, 14.81; C-12, 16.61 (C-x represents the β-hydroxyalkanoic acid methyl ester with a chain length of x carbon atoms).

Since all of the β-hydroxyalkanoic acid methyl esters other than methyl β-hydroxybutyric acid were not commercially available, a series of racemic β-hydroxyalkanoic acids was synthesized by reaction of the dilithio salt of acetic acid with various-chain-length *n*-alkyl aldehydes (1, 22, 23). The resulting β-hydroxyalkanoic acids were then reacted with diazomethane to form the corresponding methyl esters. The methyl β-hydroxyalkanoic acids were characterized by ^1H and ^{13}C nuclear magnetic resonance, infrared spectroscopy, and elemental analysis, all of which were consistent with the expected structures. GC analyses showed that the purity of all compounds synthesized was >98%. Infrared spectra were recorded on a Perkin-Elmer model 283 spectrometer. ^1H and ^{13}C nuclear magnetic resonance spectra were obtained in deuterated chloroform with tetramethylsilane as an internal standard with Varian XL-200 (200 MHz) and XL-300 (75.4 MHz) spectrometers, respectively. Elemental analyses were performed by the Microanalysis Laboratory at the University of Massachusetts, Amherst.

Standard curves were constructed for the synthesized methyl esters with chain lengths from 5 to 12 carbon atoms. From measurements made on whole cells, the total PHA per cell dry weight was determined by summing the absolute amount of all of the β-hydroxyalkanoate monomer units detected.

PHA was extracted from lyophilized cells into chloroform by using a Soxhlet extractor. After a reflux period of 6 h, the chloroform solution was concentrated with a rotary evaporator. The solution was then passed through a hot glass funnel containing a cotton plug into rapidly stirred methanol. The ratio of methanol to chloroform was 10:1. The precipitated polymer was separated by centrifugation (Sorvall RC2-B; 4°C; 10,000 × *g*), washed twice with methanol, and dried in vacuo (1 mm Hg [133.3 Pa]) for 16 h at room temperature. The resultant material was then dissolved in acetone, filtered again, and subsequently added dropwise into rapidly stirred methanol. The polymer was again separated by centrifugation and finally dried as described above. The total amount of PHA was determined gravimetrically and calculated as the percentage of cellular dry weight.

All molecular weight data on the PHA isolated were obtained by gel permeation chromatography. The instrument used included a Waters model 6000A delivery system and a model 401 refractive index detector with 10^5-, 10^4-, 10^3-, 10^2-, and 50-nm ultrastyragel columns in series. Tetrahydrofuran was used as the eluent at a flow rate of 1.0 ml/min. Sample concentrations of 0.1 to 0.2% (wt/vol) and an injection volume of 300 μl were used. Polystyrene standards with low polydispersity were purchased from Polysciences, Inc., and used to generate a calibration curve.

RESULTS

Initially, *P. oleovorans* was grown on various insoluble carbon sources (Table 1), forming two-phase media as previously reported (9, 28). Intracellular PHA was observed when cells were grown on octane or octene. PHA produced (9) when cells were grown on octane contained primarily β-hydroxyoctanoate units instead of β-hydroxybutyrate units, which are normally believed to be present in PHA formed by many other microorganisms (8). Other eight-carbon substrates were also evaluated for cell growth and PHA production (Table 1). The water-insoluble substrates (octyne, 1-bromooctane, octylamine, and octanol) were used to incorporate functional groups into the polymer, but there was either no or only slight cell growth with these compounds. No granular polymer was seen to be formed from observations by light microscope.

In an attempt to overcome the difficulties of using a biphasic alkane-water medium, a homogeneous aqueous system was prepared by using sodium octanoate as the carbon source. The optimal concentration for cell growth on octanoate was determined to be between 10 and 20 mM, which gave cell yields of up to 1.5 g/liter within a growth

TABLE 1. Growth of *P. oleovorans* on eight-carbon substrates[a]

Substrate (concn)	Growth[b]	Cell yield (g/liter)	PHA formation[c]
Water insoluble			
Octane (10%)	+++	0.89	+
Octene (10%)	+++	0.68	+
Octyne (10%)	−	ND[d]	ND
1- Bromooctane (10%)	+	ND	ND
Octylamine (10%)	−	ND	ND
Octanol (10%)	+	0.02	−
Water soluble			
Sodium octanoate (10 mM)	+++	1.16	+
1,2,7,8-Octanetetrol (10 mM)	−	ND	ND
Sodium suberate (5 mM)	−	ND	ND

[a] After incubation for 45.5 h.
[b] +++, Good growth; +, poor growth; −, no growth.
[c] Observed as visible granules under a light microscope. +, PHA granules observed; −, no granules observed.
[d] ND, Not determined.

period of 20 h. No growth was observed within 7 days on concentrations higher than 50 mM. Also, when *P. oleovorans* was grown on the water-soluble substrates 1,2,7,8-octanetetrol and sodium suberate (sodium octanedioate) no growth was detected (Table 1).

The cell morphology of *P. oleovorans* during growth on 10 mM octanoate was observed by light microscopy and was studied as a function of the cellular PHA content. Immediately after inoculation, the culture contained only short, fat rods. During log-phase growth, the cells elongated 5 to 10 times as they accumulated PHA. This observation was also described previously when the organism was grown on octane or octene (10). As the culture grown on octanoate reached the stationary phase, PHA apparently was consumed intracellularly and was no longer visible in the microscope. The cells then reestablished their original shape.

Normally, PHA is accumulated when a nutrient or growth factor becomes limiting (4, 21), so PHA formation was studied under nitrogen-limiting conditions with 10 mM octanoate as a carbon source (Table 2). The PHA composition of cells grown on NH_4^+ concentrations between 0 and 50 mM did not vary significantly. It contained 9 to 11% β-hydroxyhexanoate, 85 to 89% β-hydroxyoctanoate, and 2 to 4% β-hydroxydecanoate.

To determine the ability of *P. oleovorans* to use a variety of substrates and to incorporate repeating units other than β-hydroxyoctanoate into its storage polyester, cells were grown on 20 mM concentrations of other *n*-alkanoic acids, including all acids from formate to decanoate. Growth was supported by all of these carbon sources, with the exception of formate (Fig. 1). The highest cell yields (up to 1.2 g [dry weight]/liter) were obtained after incubation for 44 h on octanoate and nonanoate. Generally, the cell yields increased with increasing carbon chain length of the growth substrate.

TABLE 2. Effect of ammonium concentration on growth and PHA production of *P. oleovorans* with 10 mM octanoate as a carbon source after growth for 20 h

NH_4^+ concn (mM)	Cell yield (mg [dry wt]/liter)	PHA content (% dry wt)	PHA yield (mg/liter)
0	47	37.2	18
5	680	35.6	240
10	800	30.8	265
25	865	30.1	260
50	795	24.2	190

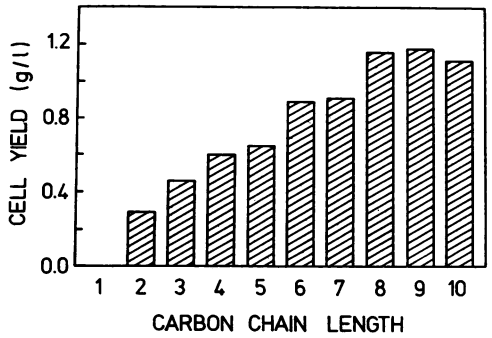

FIG. 1. Growth of *P. oleovorans* on a 20 mM concentration of different *n*-alkanoic acids, from formate to decanoate, measured as the yield of dry cells after a growth period of 44 h.

When *P. oleovorans* was grown on *n*-alkanoic acids containing four carbon atoms or more, formation of acetate (in concentrations of up to 15 mM), apparently as a metabolic intermediate, was observed in the medium. The acetate so formed was utilized later when the cultures reached the stationary phase. Small amounts of other intermediates, which were two or four carbon atoms shorter than the acid used for growth, were also found in the medium. However, other than acetate, the intermediates detected contained only an odd number of carbon atoms when the growth substrate was an odd-numbered chain length acid and an even number of carbon atoms when the carbon source was an even-numbered chain length acid. These results suggest that, during cell growth, two-carbon fragments are cleaved from the initial carbon substrate by β-oxidation, which would provide energy for bacterial cells via the tricarboxylic acid cycle (15, 24). All of the intermediates detected in the medium during growth were utilized by the organism in the early stationary phase.

When *P. oleovorans* was grown on various *n*-alkanoic acids, intracellular PHA production was detected only on acids containing six or more carbon atoms. Lyophilized cells of *P. oleovorans* grown on the acids hexanoate to decanoate were extracted with chloroform to isolate the polymers produced, which are present in amounts as high as 28% of the cellular dry weight (Fig. 2). The maximum polymer content was obtained when cells were grown on either octanoate or nonanoate. The polymer yield, as determined by solvent extraction, was almost identical to that calculated on the basis of the β-hydroxyalkanoic acid content estimated by GC of whole cells. These results showed that the recovery of PHA by solvent extraction was quantitative. From these results and from the observation that optimal cell growth was obtained with the eight- and nine-carbon *n*-alkanoic acid substrates shown in Fig. 1, it can be concluded that the highest PHA yields were obtained with these two carbon sources. Under these conditions, *P. oleovorans* produced approximately 330 mg of PHA per liter.

The polymer compositions obtained when *P. oleovorans* was grown on carbon substrates ranging from hexanoate to decanoate were determined (Table 3). All analyses were performed by GC on solvent-extracted PHA. In all cases, with the exception of decanoic acid as the carbon source, the major monomer unit found in the polymer had the same

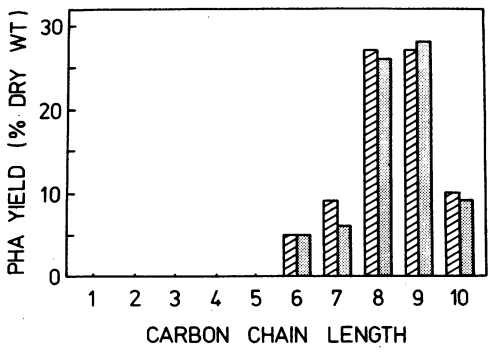

FIG. 2. PHA content of *P. oleovorans* grown on different *n*-alkanoic acids (formate to decanoate; 20 mM each). Symbols: ▨, PHA content estimated by GC of whole cells; □, PHA content estimated by solvent extraction and subsequent gravimetric measurement.

number of carbon atoms as the *n*-alkanoic acid used for growth. However, when the cells were grown on octanoate, nonanoate, and decanoate, a large fraction of the repeating units in the polymer contained two carbons less than the *n*-alkanoic acid used for growth. These results would be expected from the intermediates which were observed in the medium as discussed earlier. That is, the intermediates formed during growth were also incorporated into the polymers.

When hexanoate was used as the carbon source, the polymer contained 18% of the β-hydroxyoctanoate monomer along with 82% of the β-hydroxyhexanoate monomer. When heptanoate and octanoate were used as carbon sources, again the polymer contained significant quantities of the monomer units two carbon atoms longer than the growth substrate (Table 3). It appears, therefore, that *P. oleovorans* is capable of producing acetate from the *n*-alkanoic acid carbon source by β-oxidation, which is then used to build longer-chain-length compounds by addition to the original growth substrate or to other intermediates. These monomers are then also incorporated into the PHA.

When an odd-chain-length acid was used as the sole carbon source, small amounts of even-chain-length repeating units were found in the storage polymer. When an even-chain-length acid was used as the growth substrate, small quantities of odd-chain-length monomer units were also detected in the storage polymer. This result suggests the presence of a decarboxylation mechanism by which CO_2 is produced from the growth substrate to form intermediates which are one carbon atom shorter than the original substrate. If so, these intermediates are also incorporated into the polymers formed. However, while such monomers were detected in the polymers produced, the corresponding *n*-alkanoic acids were never found in the growth medium. The same phenomenon was recently observed by Doi and coworkers (11). When *A. eutrophus* was grown on propionate as the sole carbon source, four- and five-carbon chain length monomer units were found in the polymer, suggesting that partial decarboxylation of propionate and subsequent incorporation of acetate into the storage polymer occurred.

TABLE 3. Composition of PHA produced by *P. oleovorans* grown on different *n*-alkanoic acids[a]

Substrate	Amt of monomer found in polymer (mol%)[b]					
	C-6	C-7	C-8	C-9	C-10	C-11
Hexanoate	81.5	0.7	17.5	—	0.3	—
Heptanoate	0.8	93.5	1.2	3.6	0.2	0.7
Octanoate	9.6	—	86.1	—	4.3	—
Nonanoate	1.1	31.2	6.1	58.4	2.8	0.4
Decanoate	19.6	—	62.9	1.4	11.1	3.0

[a] Analyses were performed on solvent-extracted samples by GC. —, Monomer not detected.

[b] C-x, β-Hydroxyalkanoate monomer unit with a chain length of x carbon atoms.

TABLE 4. Molecular weight measurements of PHA produced by *P. oleovorans* grown on *n*-alkanoic acids[a]

Substrate	M_w	M_n	M_w/M_n
Hexanoate	330,000	150,000	2.2
Heptanoate	370,000	150,000	2.5
Octanoate	210,000	94,000	2.2
Nonanoate	190,000	85,000	2.2
Decanoate	92,000	46,000	2.0

[a] Analyses were performed on solvent-extracted samples by gel permeation chromatography.

The molecular weights of the polymers isolated from *P. oleovorans* grown on acids ranging from hexanoate to decanoate were measured by gel permeation chromatography. Molecular weights by weight average (M_w) and number average (M_n) were calculated. All of the PHA samples had high molecular weights, with values of M_w from 90,000 to 370,000 and polydispersities (M_w/M_n) ranging from 2.0 to 2.5 (Table 4). The higher molecular weights of PHA samples from *P. oleovorans* grown on hexanoate and heptanoate than those measured when cells were grown on octanoate or nonanoate were unexpected. Whereas the mechanism of the enzymatic polymerization of these long-chain *n*-alkanoic acids is unknown, it is possible that the higher yields of PHA which were obtained with octanoate and nonanoate as carbon substrates resulted from higher constants of binding of the corresponding monomer units to the polymerase enzyme systems, and this effect, in turn, could lead to higher rates of propagation relative to the rates of chain termination and chain transfer in the polymerization reaction to give polymers with higher molecular weights. These results suggest that this is not the case. The less efficient PHA production of *P. oleovorans* when grown on either hexanoate or heptanoate favored the formation of PHA with relatively higher molecular weights.

DISCUSSION

It is known from previous studies that *P. oleovorans*, which was originally isolated from non-water-soluble cooling fluids in metal manufacturing industries (19), produces poly-β-hydroxyoctanoate when grown on octane in a two-phase medium (9). When *n*-alkanes with 6 to 12 carbon atoms were used as substrates, it was found that *P. oleovorans* accumulated various copolymers (Lageveen, dissertation). However, because the biphasic nature of the medium used for polymer production has certain disadvantages, such as possible inhibition of microbial metabolism by the organic solvent or inadequate rates of mass transfer between the liquid phases, it has proved desirable to use a homogeneous water-based medium. Our results demonstrate the production of various types of PHA by *P. oleovorans* in homogeneous media. Furthermore, the use of homogeneous aqueous media would also facilitate physiological studies of

polymer production in a chemostat, and once the optimal conditions are established in such a study, continuous controlled synthesis of specifically designed PHA may be accomplished.

The results of the present study clearly demonstrate that *P. oleovorans* is able to produce a variety of different PHA, with units ranging from 6 to 11 carbon atoms, depending on the substrate used for growth. All of the polymers obtained were copolyesters, and none of the isolated PHA samples from this bacterium contained either a four-carbon unit or a five-carbon unit. This new class of polymers obtained from *P. oleovorans* needs to be further characterized, and detailed analysis must be performed on their physical and chemical properties. The present results expand the field of microbially produced PHA and offer the challenge of finding industrial applications for these polyesters as a possible alternative to traditional, petrochemically based plastics.

So far, in all of the growth experiments performed, the cultures were harvested when the cells reached the early stationary phase and the polymer composition was determined. Nothing is known about the time-dependent change of monomer composition of the PHA during cell growth. Since the substrate composition of the medium changes during bacterial growth because of buildup and subsequent utilization of metabolic intermediates, one would expect a change in the monomer composition of the PHA as a function of the age of the culture. The growth time, therefore, could be a controlling factor in the production of biodegradable polymers with specifically defined properties.

Several microorganisms, such as *A. faecalis* or *P. lemoignei* (20, 30), are known to degrade PHB by secretion of an esterase and to use the polymer as a carbon source. PHB was also degraded by mixed bacterial cultures which were enriched from soil (6). So far, there are no data available as to the biodegradability of the novel PHA isolated from *P. oleovorans*. In contrast to PHB, PHA with longer-chain-length pendant groups might be subjected to slower degradation rates in environments such as sewage sludge or sediment. Generally, low-molecular-weight carbon compounds are mineralized more rapidly, whereas stereochemically more complex structures have much lower rates of mineralization (7, 12). Therefore, it might be possible that PHA with long pendant groups are degraded more slowly than PHB. The lifetime of plastic articles made of PHA derived from *P. oleovorans* might thus be prolonged. By specific control of the incorporation of different-chain-length monomer units, the degradation rates of the polymers may also be controlled.

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