APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1988, p. 2924–2932
0099-2240/88/122924-09$02.00/0

Vol. 54, No. 12

Formation of Polyesters by *Pseudomonas oleovorans*: Effect of Substrates on Formation and Composition of Poly-(*R*)-3-Hydroxyalkanoates and Poly-(*R*)-3-Hydroxyalkenoates

ROLAND G. LAGEVEEN,* GJALT W. HUISMAN, HANS PREUSTING, PETER KETELAAR, GERRIT EGGINK, AND BERNARD WITHOLT

Department of Biochemistry, Groningen Biotechnology Center, University of Groningen, Nijenborgh 16, 9747 AG Groningen, The Netherlands

Received 25 April 1988/Accepted 6 September 1988

Pseudomonas oleovorans **grows on C_6 to C_{12} *n*-alkanes and 1-alkenes. These substrates are oxidized to the corresponding fatty acids, which are oxidized further via the β-oxidation pathway, yielding shorter fatty acids which have lost one or more C_2 units. *P. oleovorans* normally utilizes β-oxidation pathway intermediates for growth, but in this paper we show that the intermediate 3-hydroxy fatty acids can also be polymerized to intracellular poly-(*R*)-3-hydroxyalkanoates (PHAs) when the medium contains limiting amounts of essential elements, such as nitrogen. The monomer composition of these polyesters is a reflection of the substrates used for growth of *P. oleovorans*. The largest monomer found in PHAs always contained as many C atoms as did the *n*-alkane used as a substrate. Monomers which were shorter by one or more C_2 units were also observed. Thus, for C-even substrates, only C-even monomers were found, the smallest being (*R*)-3-hydroxyhexanoate. For C-odd substrates, only C-odd monomers were found, with (*R*)-3-hydroxyheptanoate as the smallest monomer. 1-Alkenes were also incorporated into PHAs, albeit less efficiently and with lower yields than *n*-alkanes. These PHAs contained both saturated and unsaturated monomers, apparently because the 1-alkene substrates could be oxidized to carboxylic acids at either the saturated or the unsaturated ends. Up to 55% of the PHA monomers contained terminal double bonds when *P. oleovorans* was grown on 1-alkenes. The degree of unsaturation of PHAs could be modulated by varying the ratio of alkenes to alkanes in the growth medium. Since 1-alkenes were also shortened before being polymerized, as was the case for *n*-alkanes, copolymers which varied with respect to both monomer chain length and the percentage of terminal double bonds were formed during nitrogen-limited growth of *P. oleovorans* on 1-alkenes. Such polymers are expected to be useful for future chemical modifications.**

Many bacteria are able to accumulate intracellular reserve materials, varying from internal *n*-alkane pools to polyphosphate (20, 22). Most common is poly-3-hydroxybutyric acid (PHB), originally discovered by Lemoigne (13). PHB is accumulated by a large number of bacteria, generally under conditions of a nutrient limitation in the presence of excess carbon source, and may account for up to 80% of the bacterial cell dry weight. PHB is now available on the market as a biodegradable plastic under the trade name Biopol, produced by Imperial Chemical Industries with *Alcaligenes* species (11; K. A. Powell, B. A. Collinson, and K. R. Richardson, Imperial Chemical Industries Ltd. European Patent Application no. 80300432.4, 1980). The polymer PHB has a number of interesting characteristics and can be used in various ways similar to many conventional synthetic plastics now in use: it can be molded, reinforced with inorganic fillers, spun into a fiber, or formed into a film with excellent gas barrier properties (12). With *Pseudomonas* strains, this polyester could even be produced on the inexpensive carbon source methanol (25–27).

We have recently reported that *Pseudomonas oleovorans* also produces an intracellular polyester when grown on *n*-octane (3). *P. oleovorans* is able to grow on medium-chain-length *n*-alkanes because it contains the catabolic OCT plasmid, which encodes an alkane hydroxylase complex. This enzyme complex enables *P. oleovorans* to oxidize *n*-alkanes to terminal alkanols (8). In our search for conditions which would permit reasonably high production levels of these oxidized alkanes, we found that cells grown on *n*-octane produce an intracellular polymer which we provisionally identified as poly-3-hydroxyoctanoate (3). To determine unequivocally the chemical structure of this material, Ketelaar et al. (10) synthesized optically pure (*S*)-methyl-3-hydroxyalkanoates as reference compounds. In this paper, we use these standards and show that the polymer accumulated by *P. oleovorans* during growth on *n*-octane contains (*R*)-3-hydroxyoctanoate and (*R*)-3-hydroxyhexanoate monomers, indicating a strong relationship between polymer monomers and the alkane substrate. Since *n*-alkanes are oxidized to the corresponding fatty acids by *P. oleovorans*, and these are subsequently catabolized via the β-oxidation pathway, this result suggested that excess 3-hydroxy fatty acids are diverted to polymer synthesis. If so, it might be expected that poly-3-hydroxyalkanoates (PHAs) of varying composition can be produced by *P. oleovorans* during growth on different hydrocarbons.

In this paper, we show that PHAs are mainly accumulated under nutrient-limiting conditions and that the monomer composition of these polyesters is a reflection of the substrate used (patent applied for in several countries).

MATERIALS AND METHODS

Bacterial strain, media, and growth conditions. *P. oleovorans* ATCC 29347 was used throughout the experiments. Cells were precultured overnight at 30°C in 250-ml Erlenmeyer flasks containing 50 ml of E medium (29) supple-

* Corresponding author.

mented with 0.1% (vol/vol) MT microelements and 2% (vol/vol) *n*-octane (1 liter of MT stock contains 2.78 g of $FeSO_4 \cdot 7H_2O$, 1.98 g of $MnCl_2 \cdot 4H_2O$, 2.81 g of $CoSO_4 \cdot 7H_2O$, 1.47 g of $CaCl_2 \cdot 2H_2O$, 0.17 g of $CuCl_2 \cdot 2H_2O$, and 0.29 g of $ZnSO_4 \cdot 7H_2O$ in 1 N HCl). The resulting cultures, induced for alkane oxidation (8), were used to start growth and production experiments, which were performed in small, stirred tank reactors with a working volume of 500 to 1,000 ml. The medium in these reactors consisted of two phases: an aqueous phase of E2 medium and an apolar hydrocarbon phase (20%, vol/vol). E2 medium consists of 3.5 g of $NaNH_4HPO_4 \cdot 4H_2O$, 7.5 g of $K_2HPO_4 \cdot 3H_2O$, and 3.7 g of KH_2PO_4 per liter supplemented with 10 ml of 100 mM $MgSO_4 \cdot 7H_2O$ and 1 ml of MT microelements stock solution. E2 medium is a modified E medium which does not contain citrate so that alkanes and 1-alkanes can be used as sole carbon source. Hydrocarbons were added directly to the culture medium without further sterilization.

Stirrer speeds were kept constant at 600 rpm in all experiments. The pH of the culture was maintained at 7.0 by computer-controlled addition of base (5% NH_4OH or 2 N KOH) and acid (2 N H_2SO_4). The dissolved oxygen tension was measured with a galvanic electrode (Pb/Ag) and maintained above 50% air saturation by a microcomputer via a valve in the air inlet (average gas flow rate, 120 ml/min).

In all cases, the total reactor volume was 700 ml at the start of an experiment. The medium was inoculated with an overnight alkane-induced culture to give a starting density of about 0.1 mg (dry weight)/ml.

Growth was followed by measuring the optical density of the water phase at 450 nm in a Zeiss spectrophotometer. To that end, samples of 1 to 5 ml were withdrawn from the culture, and after phase separation by standing for 5 min at room temperature in test tubes, the organic phase was removed and the optical density of the water phase was measured in a 10- to 100-fold dilution with demineralized water. Cell densities, expressed as milligrams of cell dry weight per milliliter of water phase, were calculated as described previously (31). For nitrogen-limiting conditions, the pH was regulated by addition of 2 N KOH instead of NH_4OH. The amount of nitrogen in the medium allows growth to a cell density of about 2 mg/ml.

Characterization of PHAs. To confirm our preliminary characterization of the *P. oleovorans* polyester, PHA was isolated from cells containing about 20% (wt/wt) polymer by either the method yielding PHA granules (3) or solvent extraction. Cells (25 g, dry mass) were lyophilized and suspended in 500 ml of chloroform (Jankee & Kunkel Ultra-turrax). The suspension was refluxed for 4 h, after which it was cooled down to room temperature and filtered. After evaporation to 100 ml, the filtrate was filtered again. This solution was slowly added to 1,000 ml of 96% ethanol with stirring. After 1 h without stirring, the ethanol-chloroform mixture was decanted and the gel-like precipitate was dissolved in 10 to 20 ml of chloroform. The polymer solution was filtered and precipitated again in 200 ml of 96% ethanol. After 1 h, the precipitate was dissolved in 20 ml of chloroform and the volume was reduced to 5 to 10 ml by evaporation. After 1 to 2 days, a film of polymer was obtained. The purified polymer was treated with methanol as described below. The resulting methylesters were compared with the chiral standards described by Ketelaar et al. (10), using gas-liquid chromatography (GLC), GLC-mass spectrometry (MS), and optical rotation measurements.

Assay for PHAs in cell cultures. The assay for PHAs in culture samples of *P. oleovorans* was based on a variation of the gas chromatographic assay for PHB in microbial cell mass as developed by Braunegg et al. (2).

Small culture samples (0.1 to 4 ml) with known cell dry mass were centrifuged in an Eppendorf centrifuge (5 min, 9,800 × *g*, 4°C). After the liquid phase was decanted, pellets were freeze-dried and kept at −20°C until further use.

The amount of PHA was determined by methanolysis of the monomers followed by GLC analysis. To this end, the lyophilized pellets were suspended in 2 ml of methanol containing 15% (vol/vol) concentrated H_2SO_4 and put in a screw-cap test tube with a magnetic stirrer bar. After addition of 2 ml of chloroform, the tubes were closed tightly and incubated at 100°C in an oil bath under magnetic stirring for 140 min, followed by rapid cooling on ice. After the addition of 1 ml of demineralized water and extensive vortexing of the mixture, phases were separated by centrifugation (5 min, 4,000 × *g*). The organic phase was dried over sodium sulfate and analyzed by GLC.

The linearity of this PHA assay was tested by using increasing amounts of cell mass. The assay is linear up to about 15 mg of polymer dry mass. The minimum amount of polymer which could reliably be determined was about 0.15 mg of PHA.

Analytical techniques. For analysis of the organic phases, a capillary gas chromatograph (Packard Instrument Co., Inc.) equipped with an autosampler and an integrator (Shimadzu) was used. A 25-m CP-Sil 5 CB capillary column (Chrompack) was used. Samples were injected by splitless injection. The column temperature program started at 80°C for 2 min and increased at a rate of 5°C/min during 8 min, after which it was raised to 300°C for 20 min to remove all nonvolatile components.

Samples were prepared by mixing 400 μl of the organic phase from the assay mixture with 1 ml of chloroform containing 0.5 mg of benzoic acid methylester per ml as an internal standard.

For peak analysis, capillary GLC-MS was performed on a Finnigan 3300 GC-MS-G110/MS/data system combination (Finnigan). Samples were ionized by either electron impact (70 eV) or chemical ionization with NH_3 as the reactant gas. Column and temperature programs were identical to those used for standard GLC analysis.

Optical rotations were determined with a Perkin-Elmer 241 polarimeter.

Infrared spectra were recorded on an SP3-200 infrared spectrophotometer (Pye Unicam). Samples were prepared by dissolving the lyophilized polymer in boiling chloroform, which was layered on a sodium chloride plate. After the chloroform was evaporated, the polymer film was subjected to infrared analysis.

Electron and light microscopy. For thin-section electron microscopy, cells were harvested by centrifugation, washed with phosphate-buffered saline, and suspended in 0.1% phosphate buffer (pH 7.2) containing 6% glutaraldehyde (25%, EM grade; Polysciences). After fixation for 1 h at room temperature, the cells were rinsed in phosphate buffer and postfixed with 1% OsO_4 in 0.1% phosphate buffer for 90 min. The cells were then washed in distilled water and stained with 1% uranyl acetate in distilled water for 1 h. The specimens were dehydrated through ethanol and embedded in Spurr resin (24).

Thin sections were obtained by cutting with a diamond knife on a microtome (LKB) and collected on a copper grid (300 mesh) coated with a Formvar-carbon film. The sections were contrasted with Reynolds lead citrate (18) followed by

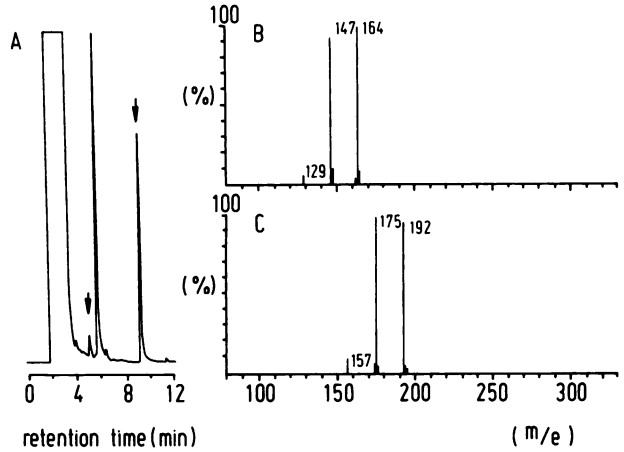

FIG. 1. GLC and GLC-MS analysis of the methanol-treated monomers of PHA produced by *P. oleovorans* on *n*-octane. *P. oleovorans* was grown on *n*-octane. After a 15-h stationary phase due to nitrogen limitation, the cells were harvested and the accumulated PHA was isolated and analyzed as described in Materials and Methods. (A) GLC pattern of isolated and methanol-treated PHA from octane-grown cells. Peaks 1 and 2 originate from the polymeric material. These peaks were further subjected to MS to determine the molecular weights (B and C). Peak 3 is derived from the internal standard benzoic acid methylester. (B) MS pattern of peak 1: peaks at 147 and 164 are the protonated and ammonium derivatives of 3-hydroxyhexanoate methylester, respectively. The peak at 129 indicates the dehydrated derivative of 3-hydroxyhexanoate-methylesters. (C) MS pattern of peak 2: the peak at 175 is the protonated form of 3-hydroxyoctanoate methylester; the peak at 192 indicates NH_4^+-hydroxyoctanoate methylester. The peak at 157 indicates the dehydrated derivative of 3-hydroxyoctanoate methylester.

2% uranyl acetate and observed in a Philips 210 electron microscope operating at 60 or 80 kV.

Freeze-fracture electron microscopy was carried out as described previously (3).

RESULTS

Identification of polymer produced by *P. oleovorans* on *n*-octane. We have described previously the biosynthesis of polymeric material by *P. oleovorans* and have preliminarily identified this polymer as poly-3-hydroxyoctanoate based on elemental analysis, infrared spectroscopy, and GLC analysis of esterified monomers (3). To identify definitively the esterified monomers on GLC, a series of chiral standards [(*S*)-(+)-methyl-3-hydroxyalkanoates] has been prepared (7).

A typical gas chromatogram of methanolized PHA isolated from *n*-octane-grown *P. oleovorans* is shown in Fig. 1A. Two different peaks were found with retention times of 4.6 and 8.9 min, which correspond to those found for the chemically synthesized (*S*)-3-hydroxyhexanoate methylester and (*S*)-3-hydroxyoctanoate methylester, respectively. The molecular weights (Fig. 1B and C) of the two compounds were confirmed by GLC-MS analysis. The major peak in panels B (*m/e* = 147) and C (*m/e* = 175) are due to protonated forms of the 3-hydroxyalkanoic acid methylesters, while the heavier peaks are due to the corresponding ammonium derivates. The peaks at *m/e* 129 in panel B and *m/e* 157 in panel C are consistent with the dehydrated form of 3-hydroxyhexanoic acid methylester and 3-hydroxyoctanoic acid methylester, respectively. The structure of the monomers was further established by electron impact MS.

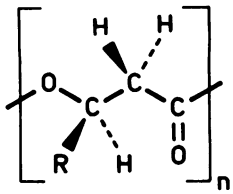

FIG. 2. Structural formula of PHA produced by *P. oleovorans* on octane. PHA formed on *n*-octane is a copolymer of two monomers, (*R*)-3-hydroxyoctanoate and (*R*)-3-hydroxyhexanoate, in a 9:1 ratio. *R* is C_5H_{11} and C_3H_7, respectively.

The major ions for the 3-hydroxyoctanoate methylester were found at *m/e*'s of 156, 100, and 74. The peak at 156 was due to the dehydrated form of the monomer (M-18), while those at 100 and 74 were due to a McLafferty rearrangement of the fatty acid methylester. A similar pattern was found for the 3-hydroxyhexanoate methylester. The ratio of the monomers 3-hydroxyhexanoate and 3-hydroxyoctanoate in PHA was found to be 1:9. The optical rotation of the methanol-treated monomers of PHA was negative (-21.9 $[\alpha]_{578}^{20}$) and opposite to those found for the chemically synthesized (*S*)-(+)-methyl-3-hydroxyhexanoate ($+18.9$ $[\alpha]_{578}^{20}$) and (*S*)-(+)-methyl-3-hydroxyoctanoate ($+23.6$ $[\alpha]_{578}^{20}$) (10).

Thus, *P. oleovorans* produces a copolymer of 3-(*R*)-hydroxyhexanoate and 3-(*R*)-hydroxyoctanoate in a ratio of 1:9 when grown on *n*-octane. The structural formula of this polymer is given in Fig. 2. Based on the optical rotation data given above and assuming that (*R*)-methyl-3-hydroxyoctanoate and (*R*)-methyl-3-hydroxyhexanoate are present in approximately the same enantiomeric excess, we conclude that the enantiomeric excess of the R form is at least 90%.

Formation of PHA in growing and starved *P. oleovorans* cells. Figure 3 shows typical growth curves of *P. oleovorans* on E2 medium with 20% (vol/vol) *n*-octane, using ammonia or KOH for pH adjustment. Only a small amount of polymer (about 5 to 6% of the cell dry weight) was formed during exponential growth. When the pH was regulated with ammonia, growth continued after the cessation of exponential growth, but the polymer content did not increase during this time.

When KOH was used to regulate the pH, growth stopped at a cell density of about 2 mg/ml and the amount of PHA increased to 25% of the cell dry mass. As the incubation continued, the amount of intracellular PHA decreased. When nitrogen was added to such cultures, growth resumed, indicating that the cells stopped growing due to nitrogen limitation when KOH was used to regulate the pH. When cells were limited for other essential elements (phosphorus, sulfur, or magnesium), growth curves and accumulation of PHA similar to those shown in Fig. 3 were observed (results not shown). Throughout these experiments, the monomer composition of the polyester remained constant, with a 3-hydroxyhexanoate/3-hydroxyoctanoate ratio of 1:9.

At higher levels of intracellular PHA, polymer granules could easily be visualized by electron and light microscopy. The polymer is accumulated intracellularly in vesicles or as granules. When cells are disrupted, such polymer granules can be collected and purified by density gradient centrifugation (3). Freeze-fracture electron microscopy (Fig. 4A) shows that purified granules consist of a plastic material, which can be stretched (and sometimes torn) during the fracturing process at -100°C, and have the same morphology seen for granules found in freeze-fractured whole cells

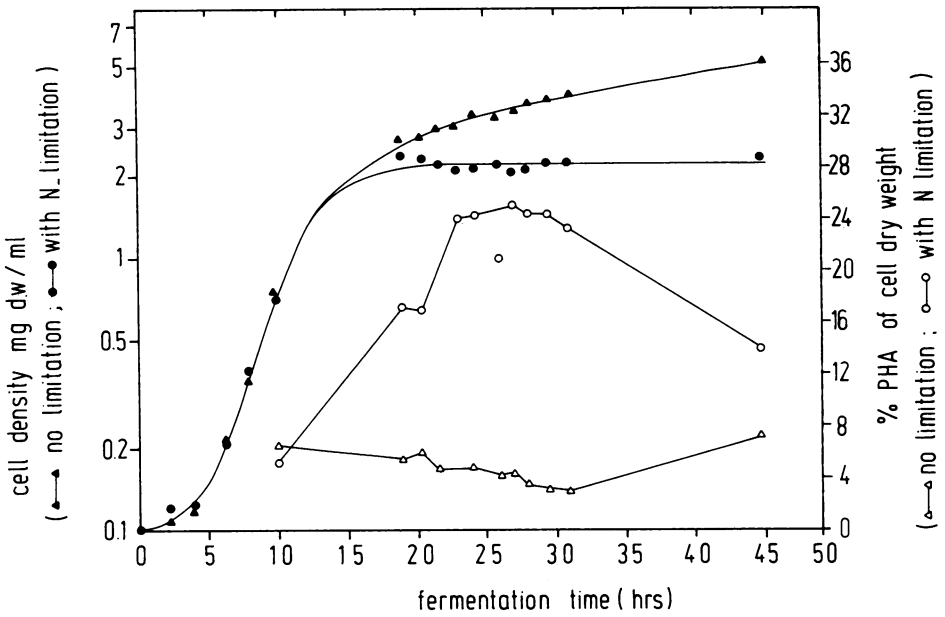

FIG. 3. PHA production by *P. oleovorans* on 20% (vol/vol) *n*-octane during growth in excess and limiting nitrogen. *P. oleovorans* was cultivated on E2 medium with 20% (vol/vol) octane. The pH was regulated with either ammonia (nitrogen excess) or KOH (nitrogen limitation). During growth and in the stationary phase, samples of the cultures were analyzed for cell dry weight (dw) and PHA content.

(3). Analysis of isolated granules showed them to consist of PHA (3), thus strongly suggesting that the intracellular granules observed by electron microscopy also consist of PHA.

These intracellular granules appear as large electron-transparent structures in *P. oleovorans* thin sections (Fig. 4B). Figure 4C shows that PHA granules can also easily be seen as white spots by phase-contrast light microscopy and that most of the cells in a population contain such spots. Figure 4D indicates that the white spots of Fig. 4C are not an optical artifact, since they are rarely seen in *P. oleovorans* cells containing little PHA.

PHA formation during growth on different *n*-alkanes. The broad substrate specificity of the alkane hydroxylase system (14, 15) enabled us to study PHA formation and composition during cultivation of *P. oleovorans* on *n*-alkanes and 1-alkenes in the range of C_6 to C_{12}.

Table 1 shows the polymer production of *P. oleovorans* when C_6 to C_{12} alkanes were used as carbon sources, while nitrogen was limiting. In all cases polymer synthesis started after exhaustion of the nitrogen source. The time required in the stationary phase for maximal polymer formation increased as higher alkanes were used as carbon sources. The total amount of polymer produced also depended on alkane chain length. Only traces of PHA were found when hexane was the substrate, whereas polymer levels of >20% (wt/wt) were found for nonane and decane, comparable to the levels observed for octane. Incubation on heptane, undecane, and dodecane resulted in intermediate levels of PHA.

The amount of polymer given in Table 1 is the sum of the different monomers found in the PHA assay. Although the total amount of polymer synthesized and the time needed to reach maximum polymer levels differed considerably for the different *n*-alkanes used, the monomeric composition of the polymers appeared to be constant for each carbon source during the entire formation process (results not shown). Interestingly, homopolymers were only formed when cells were cultured on hexane and heptane. The monomer C-chain lengths of these polymers corresponded to that of the carbon source used. In all other cases copolymers were formed (Table 1).

Thus, growth on C-even *n*-alkanes resulted in copolymers with C-even monomers which differed in C-chain length by units of C_2. The largest monomer was as long as the substrate, while the shortest monomer found was 3-hydroxyhexanoate. 3-Hydroxyoctanoate generally predominated. No C-odd monomers were found following growth on C-even carbon sources.

When C-odd *n*-alkanes were used as the carbon source, similar results were obtained, but in these cases the monomer chain lengths ranged from 3-hydroxyundecanoate to 3-hydroxyheptanoate. 3-Hydroxynonanoate predominated. With C-odd *n*-alkanes, no C-even monomers were found

PHA formation during growth on 1-alkenes. Besides saturated *n*-alkanes, unsaturated substrates such as 1-octene can be used by *P. oleovorans* as carbon and energy sources (4, 5, 19). After entering the cell, 1-octene is either converted to 1,2-epoxyoctane, which is released as such into the medium, or converted to 8-hydroxy-1-octene. Interestingly, when *P. oleovorans* was grown on 10% (vol/vol) 1-octene, it produced an intracellular polymer. Similar to the results with *n*-octane, this polymer was only synthesized in the stationary phase. GLC of the methanol-treated polymer showed two distinct groups of double peaks (Fig. 5).

GLC-MS analysis revealed that the first peak of each pair consisted of an unsaturated methylester, while the second peak consisted of the corresponding saturated methylester. Thus, the peaks with the highest retention time of each pair correspond to methyl-3-hydroxyhexanoate and methyl-3-hydroxyoctanoate, whereas the first peak of each pair corresponds with methyl-3-hydroxyhexenoate and methyl-3-hydroxyoctenoate, respectively. Based on the GLC data, it could be estimated that, during growth of *P. oleovorans* on *n*-octene, a polymer was synthesized which contained unsaturated and saturated monomers in a 54:46 molar ratio (see also Table 2).

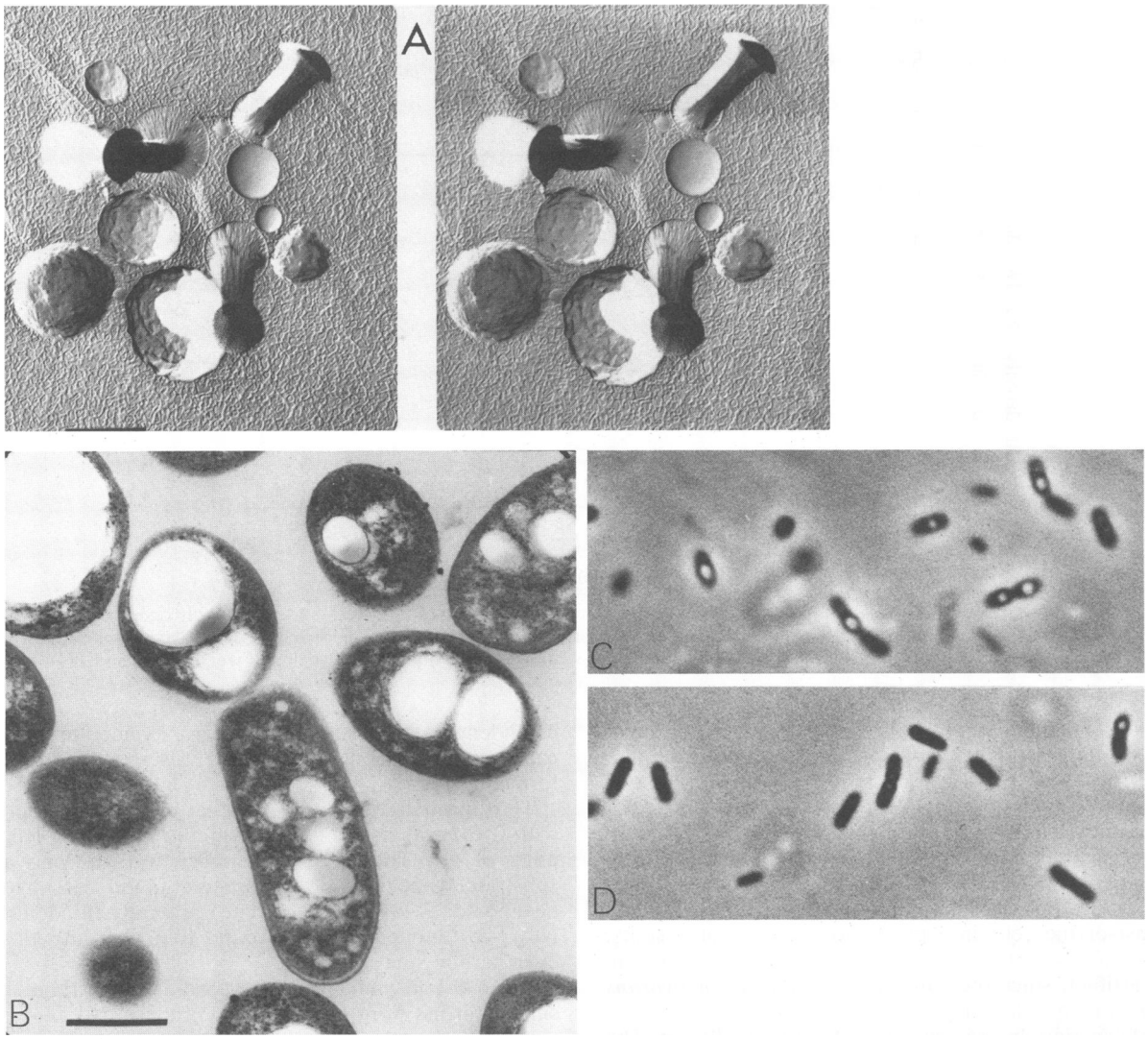

FIG. 4. Electron and phase-contrast microscopy of *P. oleovorans* containing PHA granules. (A) Stereomicrograph of freeze-fractured PHA granules isolated from *P. oleovorans*. Note that some of the granules have been stretched during the fracturing process. Bar, 0.5 μm. (B) Thin section of *P. oleovorans* grown under nitrogen-limiting conditions and containing about 20 to 25% PHA relative to cell dry mass. Bar, 0.5 μm. (C) Phase-contrast light micrograph of *P. oleovorans* containing about 25% PHA. (D) Phase-contrast light micrograph of *P. oleovorans* containing about 4% PHA.

TABLE 1. Amount and composition of PHA formed by *P. oleovorans* on *n*-alkanes

Carbon source	Amt of PHA (g/100 g of cell dry wt)	Fermentation time (h)[a]	Relative monomer composition of PHA[b]						
			$C_{6:0}$	$C_{7:0}$	$C_{8:0}$	$C_{9:0}$	$C_{10:0}$	$C_{11:0}$	$C_{12:0}$
Hexane	2.0	22	1.00						
Heptane	11.4	22		1.00					
Octane	25.3	27	0.11		0.89				
Nonane	24.3	27		0.37		0.63			
Decane	21.9	31	0.10		0.66		0.24		
Undecane	14.3	54		0.23		0.63		0.14	
Dodecane	5.8	93	0.02		0.31		0.36		0.31

[a] Time to reach maximal amount of PHA.

[b] $C_{6:0}$, 3-Hydroxyhexanoate; $C_{7:0}$, 3-hydroxyheptanoate; $C_{8:0}$, 3-hydroxyoctanoate; $C_{9:0}$, 3-hydroxynonanoate; $C_{10:0}$, 3-hydroxydecanoate; $C_{11:0}$, 3-hydroxyundecanoate; $C_{12:0}$, 3-hydroxydodecanoate.

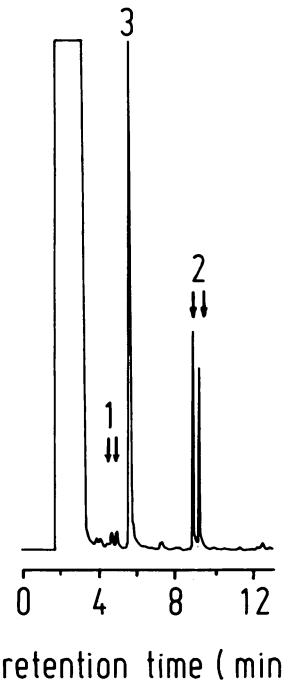

FIG. 5. GLC analysis of PHA formed by *P. oleovorans* after growth on 1-octene. The peaks indicated by double arrow 1 are due to 3-hydroxyhexenoate and 3-hydroxyhexanoate. The second pair demonstrates the presence of 3-hydroxyoctenoate and 3-hydroxyoctanoate. The unsaturated compounds have the shortest retention time in each pair as determined by GLC-MS analysis. Peak 3 at 5.6 min is derived from the internal standard benzoic acid methylester.

The presence of monomers with a double bond in the intact isolated polymer was also shown by infrared analysis. In the octene polymer spectrum (Fig. 6), three extra absorption peaks were found which were not present in the octane

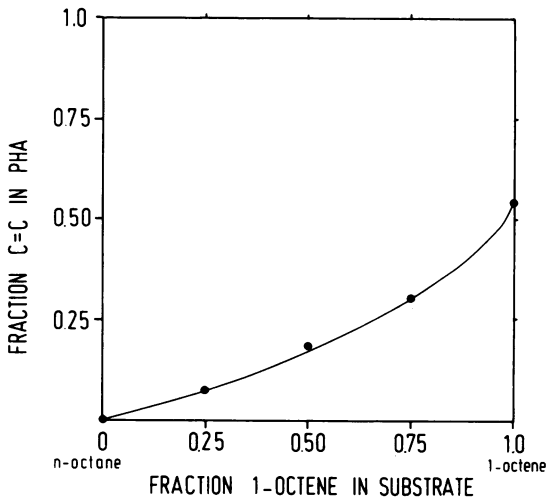

FIG. 7. Fraction of unsaturated monomers in PHA. *P. oleovorans* was grown on mixtures of *n*-octane and 1-octene and analyzed for PHA composition, as described in Materials and Methods.

polymer: at 3,080 cm^{-1} due to C=C bending, at 1,640 cm^{-1} due to C=C–H stretching vibration, and at 910 cm^{-1} resulting from H_2C=C–H bending. These peaks indicate the presence of terminal C=C bonds in the polymer.

To determine the relationship between the percentage of unsaturated monomers in PHA and the percentage of 1-octene in the growth substrate, *P. oleovorans* was grown on mixtures of *n*-octane and 1-octene. We were able to vary the percentage of unsaturated monomers in PHA between 0 and 54% with such mixtures (Fig. 7). From these experiments it can be concluded that the saturated monomers in the PHA formed by *P. oleovorans* grown on 1-octene cannot be derived from the 1.5% octane present in the 1-octene phase.

In addition to 1-octene, we also examined polymer syn-

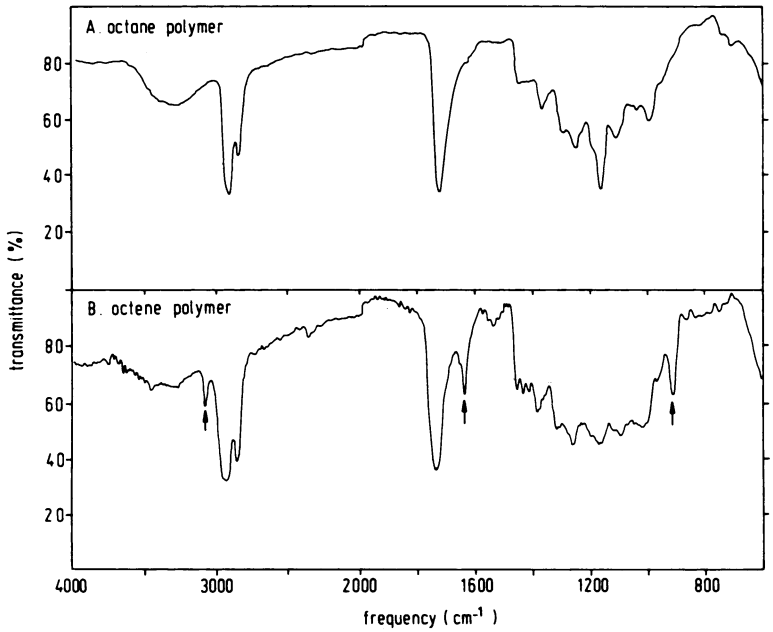

FIG. 6. Infrared spectra of PHA formed on *n*-octane and 1-octene. (A) PHA isolated from *P. oleovorans* cells grown on *n*-octane. (B) PHA isolated from *P. oleovorans* cells grown on 1-octene. Arrows indicate absorptions due to the terminal double bond in approximately 50% of the monomers.

TABLE 2. Amount and composition of PHA formed by *P. oleovorans* on 1-alkenes

Carbon source	Amt of PHA (g/100 g of cell dry wt)	Fermentation time (h)[a]	Relative monomer composition of PHA[b]							
			$C_{6:0}$	$C_{6:1}$	$C_{8:0}$	$C_{8:1}$	$C_{9:0}$	$C_{9:1}$	$C_{10:0}$	$C_{10:1}$
Hexene	ND[c]									
Octene	13.4	25	0.06	0.06	0.39	0.49				
Nonene	10.7	24					0.61	0.39		
Decene	5.1	28			0.33	0.34			0.21	0.12
Dodecene	ND									

[a] Time to reach maximal amount of PHA.
[b] $C_{6:0}$, 3-Hydroxyhexanoate; $C_{6:1}$, 3-hydroxyhexenoate; $C_{8:0}$, 3-hydroxyoctanoate; $C_{8:1}$, 3-hydroxyoctenoate; $C_{9:0}$, 3-hydroxynonanoate; $C_{9:1}$, 3-hydroxynonenoate; $C_{10:0}$, 3-hydroxydecanoate; $C_{10:1}$, 3-hydroxydecenoate.
[c] ND, Not detected.

thesis on a number of other 1-alkenes (Table 2). *P. oleovorans* formed polymers during growth on 1-nonene and 1-decene, as well as on 1-octene, but no polymers were detected during growth on 1-hexene and 1-dodecene.

The polymer compositions observed after growth on 1-nonene and 1-decene (Table 2) are a reflection of the carbon source used. The absence of the hexanoate and hexenoate monomers in the decene polymer contrasts with the presence of hexanoate monomers in the decane polymer. Similarly, no heptanoate and heptenoate monomers were detected in the polymer formed by nonene-grown cells, while the polymer produced by nonane-grown cells did contain heptanoate monomers.

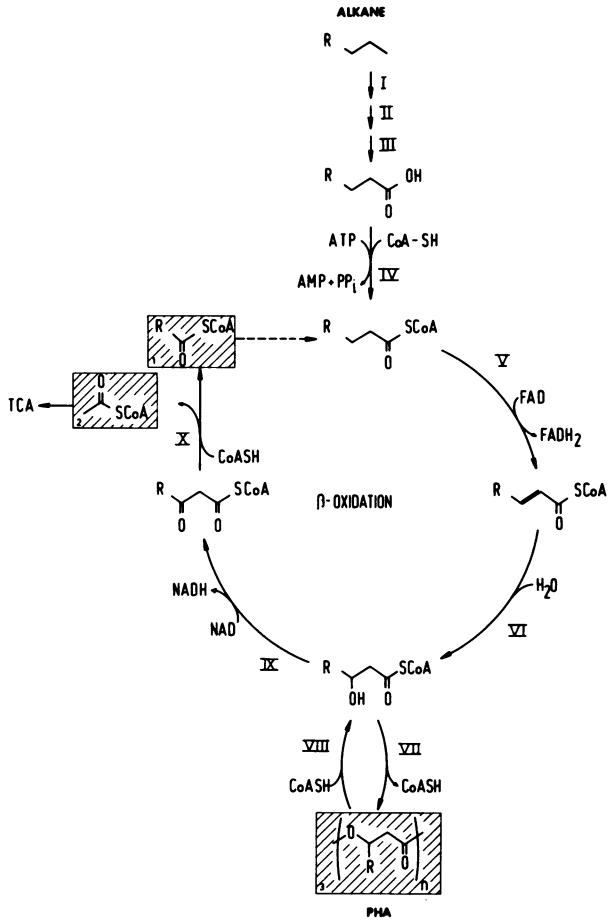

FIG. 8. Proposed synthesis route for PHAs in *P. oleovorans* after growth on *n*-alkanes and 1-alkenes. 1, Acyl-CoA; 2, acetyl-CoA; 3, PHA. I, Alkane hydroxylase; II, alkanol dehydrogenase; III, alkanal dehydrogenase; IV, acyl-CoA synthetase; V, acyl-CoA dehydrogease; VI, enoyl-CoA hydratase; VII, PHA synthetase; VIII, PHA depolymerase; IX, 3-hydroxyacyl-CoA dehydrogenase; X, 3-ketoacyl-CoA thiolase. TCA, Tricarboxylic acid cycle.

DISCUSSION

We have shown previously that *P. oleovorans* produces a bioplastic during cultivation on *n*-octane (3). In this paper, we have characterized this bioplastic and shown that it is not a homopolymer but a copolymer of (*R*)-3-hydroxyhexanoate and (*R*)-3-hydroxyoctanoate in a 1:9 ratio. Similar findings have been reported for PHB; Wallen and Rohwedder (30) found that PHB is not a homopolymer, while Findley and White (6) showed that PHB isolated from activated sludge was 95% 3-hydroxybutyric acid monomers, with 5% being a mixture of up to 11 different 3-hydroxy acids.

Formation of PHA in *P. oleovorans* was induced by nutrient limitations. Highest levels were found when nitrogen was limiting, as also seen for PHB production in *Alcaligenes* spp. by Lorenzo (H. Lorenzo, Imperial Chemical Industries Ltd. European Patent Application no. 81303373.5, 1981) and in *Pseudomonas* spp. by Suzuki et al. (25–27).

Experiments to maximize polymer production showed that the amount of intracellular bioplastic increased from 5% (wt/wt) during growth to >25% (wt/wt) of the cell dry weight during nitrogen limitation. These results are similar to those obtained for PHB formation in various microorganisms (9, 23; Lorenzo, Imperial Chemical Industries Ltd. European Patent Application no. 81303373.5, 1981).

The decrease in amount of biopolymer produced by *P. oleovorans* after the maximal amount is reached has to our knowledge never been reported for PHB. Perhaps the polyester, which is presumably a storage material, is depolymerized and degraded via fatty acid metabolism during the late stationary phase. This might also explain the limited maximum levels of polymer we observed. For increased polymer production, this breakdown process should be avoided, perhaps by elimination of a depolymerase activity.

***n*-Alkanes other than *n*-octane as a substrate.** Using different alkanes as the carbon and energy source for *P. oleovorans*, we found that a range of different polymers could be synthesized. All of the polymers produced (except those produced on hexane and heptane) are copolymers of a family of monomers presumably derived from the fatty acid degradation route. Alkanes are degraded by terminal oxidation to their corresponding fatty acids (8) and metabolized further by β-oxidation (7, 17). This results in the sequential

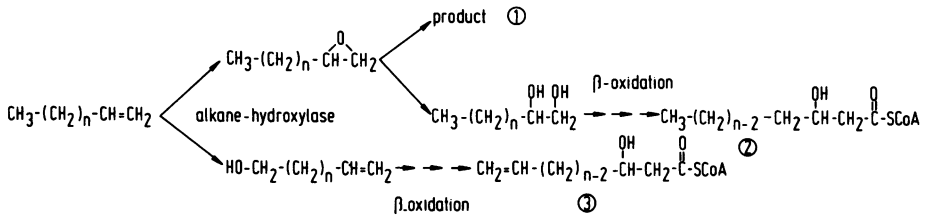

FIG. 9. Postulated degradation routes of 1-alkenes in *P. oleovorans* leading to epoxides and PHA. 1, Epoxide produced and released into the medium; 2, saturated 3-hydroxyalkanoate-CoA; 3, 3-hydroxyalkenoate-CoA. Compounds 2 and 3 can both be incorporated into PHA.

removal of C_2 units as acetyl-coenzyme A (CoA). The 3-hydroxyacyl-CoA intermediates are probably used in assembling the polymer (Fig. 8). Since the 3-hydroxyacyl-CoA units in the β-oxidation have the *S* configuration (17), while the 3-hydroxy fatty acids in PHA have been shown to have the *R* configuration, the asymmetric center of the 3-hydroxyacyl units has to be inverted. It remains to be determined whether this inversion takes place during the polymerization or an intermediate reaction occurs, catalyzed by a 3-hydroxyacyl-CoA epimerase.

This proposed biosynthetic pathway for PHAs differs substantially from the pathway for PHB synthesis. PHB is formed via condensation of two molecules of acetyl-CoA to acetoacetyl-CoA, which is then reduced to (*R*)-3-hydroxybutyryl-CoA (16). The latter compound is polymerized to PHB with retention of configuration at the asymmetric center. Since *P. oleovorans* does not form PHB on substrates such as glucose, it seems to lack the enzymes involved in formation of 3-hydroxybutyryl-CoA from acetyl-CoA. Whether the polymerase and depolymerase reactions in *P. oleovorans* and in PHB-synthesizing strains are catalyzed by identical or related enzymes remains to be determined.

The relatively high content of 3-hydroxyoctanoate and 3-hydroxynonanoate in the copolymers might be a reflection of the specificity of the PHA polymerase. Clearly the substrate specificity of the enzymes involved in the β-oxidation may also influence the monomeric composition. Units smaller than 3-hydroxyhexanoate, including 3-hydroxybutyrate, were never detected in the polymers, indicating that the smaller acyl-CoA units are used exclusively for metabolite synthesis and energy generation.

PHA formation during growth on 1-alkenes. *P. oleovorans* has long been known to grow on 1-octene with the simultaneous production of of 1,2-epoxyoctane (1, 4, 19, 28). The metabolism of 1-octene has been assumed to occur as shown in the lower pathway of Fig. 9: when the substrate is oxidized at the saturated end, the resulting 8-hydroxy-1-octene can be metabolized further to yield energy and cell mass. In contrast, when the unsaturated end is oxidized, 1,2-epoxyoctane is formed (upper pathway of Fig. 9), which has generally been thought to accumulate extracellularly (1, 4, 5, 19) or to react with cellular proteins and perhaps other susceptible compounds, causing extensive cell damage and eventually total loss of cell viability (4, 5). Thus, we had expected that, during growth on 1-octene, the resulting polymer would consist of monomers which all contain a terminal C double bond. Instead, our experiments show that, at most, 54% of the monomers is saturated. Thus, either *P. oleovorans* is able to oxidize 1,2-epoxyoctane further to 3-hydroxyalkanoate or some of the 1,2-epoxyoctane is hydrolyzed spontaneously, resulting in a metabolizable substrate for *P. oleovorans* (middle pathway of Fig. 9).

By varying the 1-octene/*n*-octane ratio, the percentage of unsaturated monomers could be changed. There is a clear relation between the mole fraction of 1-octene in the substrate mixture and the percent double bonds in the polymer (Fig. 7). Thus, we are able to produce a set of polymers with a variable amount of unsaturated monomers.

Comparing the results of polymer formation on *n*-alkanes and 1-alkenes, it is apparent that less polyester was formed when 1-alkenes were the growth substrate. In fact, when *P. oleovorans* was grown on 1-hexene or 1-dodecene, no polymer could be detected. Moreover, in contrast to the polymers formed on nonane and decane, polymers formed on 1-nonene and 1-decene contained no 3-hydroxyheptanoate or 3-hydroxyhexanoate.

Future applications. The PHA-synthesizing system of *P. oleovorans* can be used to produce a range of chiral polymers under nutrient-limiting conditions (patent applied for in several countries). This range of polyesters needs additional characterization. To this end, higher production levels will be useful, after which polymer characteristics can be compared with those of other polyesters, including PHB. Their chirality and biodegradability should make the poly-(*R*)-3-hydroxyalkanoates suited for a large number of applications (21), which can be extended even further by chemical modification of the double bond present in the polyester when unsaturated alkanes are included in the growth substrate.

ACKNOWLEDGMENTS

We thank A. P. Bruins and A. te Nijenhuis for GLC-MS analysis, M. de Hoop for electron microscopic work, N. Panman for drawing the figures, and S. M. Karelsz for typing the manuscript.

This research was supported by the Dutch Program Committee for Biotechnology.

LITERATURE CITED

1. **Abbott, B. J., and C. T. Hou.** 1973. Oxidation of 1-alkenes to 1,2-epoxyalkanes by *Pseudomonas oleovorans*. Appl. Microbiol. **26**:86–91.
2. **Braunegg, G., B. Sonnleitner, and R. M. Lafferty.** 1978. A rapid gaschromatographic method for the determination of poly-3-hydroxybutyric acid in microbial biomass. Eur. J. Microbiol. Biotechnol. **6**:29–37.
3. **de Smet, M. J., G. Eggink, B. Witholt, J. Kingma, and H. Wijnberg.** 1983. Characterization of intracellular inclusions formed by *Pseudomonas oleovorans* during growth on octane. J. Bacteriol. **154**:870–878.
4. **de Smet, M. J., J. Kingma, H. Wijnberg, and B. Witholt.** 1983. *Pseudomonas oleovorans* as a tool in bioconversions of hydrocarbons: growth, morphology and conversion characteristics in different two-phase systems. Enzyme Microb. Technol. **5**:352–360.
5. **de Smet, M. J., H. Wijnberg, and B. Witholt.** 1981. Synthesis of 1,2-epoxyoctane in two-phase system containing high concentrations of 1-octene. Appl. Environ. Microbiol. **42**:811–816.
6. **Findley, R. H., and D. C. White.** 1983. Polymeric beta-hydroxyalkanoates from environmental samples and *Bacillus megaterium*. Appl. Environ. Microbiol. **45**:71–78.
7. **Fulco, A. J.** 1983. Fatty acid metabolism in bacteria. Prog. Lipid

Res. **22**:133–160.

8. **Grund, A., J. Shapiro, M. Fennewald, P. Bacha, J. Leahy, K. Markbreiter, M. Nieder, and M. Toepfer.** 1975. Regulation of alkane oxidation in *Pseudomonas putida*. J. Bacteriol. **123**:546–556.
9. **Heinzle, E., and R. M. Lafferty.** 1980. A kinetic model for growth and synthesis of poly-3-hydroxybutyric acid (PHB) in *Alcaligenes eutrophus* H16. Eur. J. Microbiol. Biotechnol. **11**: 8–16.
10. **Ketelaar, P. E. F., E. G. J. Staring, and H. Wijnberg.** 1985. Asymmetric synthesis of (S)-(+)-1-methyl-3-hydroxyalkanoates from ketene and 2,2-dichloroaldehydes via 4-(1′,1′-dichloroalkyl)-2-oxetanones. Tetrahedron Lett. **16**:4665–4668.
11. **King, P. P.** 1982. Biotechnology. An industrial view. J. Chem. Technol. Biotechnol. **32**:2–8.
12. **Lafferty, R. M., and E. Heinzle.** 1977. Extraktion eines Thermoplasten aus Bakterien. Chem. Rundsch. **41**:14–16.
13. **Lemoigne, M.** 1926. Produit de deshydration et de polymerisation de l'acide beta-oxybutyrique. Bull. Soc. Chim. Biol. **8**:770–782.
14. **May, S. W., and B. J. Abbott.** 1973. Enzymatic epoxidation. II. Comparison between the epoxidation and hydroxylation reactions catalyzed by the ω-hydroxylation system of *Pseudomonas oleovorans*. J. Biol. Chem. **248**:1725–1730.
15. **McKenna, E. J., and M. J. Coon.** 1970. Purification and properties of the ω-hydroxylase of *Pseudomonas oleovorans*. J. Biol. Chem. **245**:3882–3889.
16. **Merrick, J. M.** 1978. Metabolism of reserve materials, p. 199–219. *In* R. K. Clayton and W. R. Sistrom (ed.), The photosynthetic bacteria. Plenum Publishing Corp., New York.
17. **Nunn, W. D.** 1986. A molecular view of fatty acid catabolism in *Escherichia coli*. Microbiol. Rev. **50**:179–192.
18. **Reynolds, E. S.** 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. **17**: 208–212.
19. **Schwartz, R. D.** 1973. Octene epoxidation by a cold-stable alkane-oxidizing isolate of *Pseudomonas oleovorans*. Appl. Microbiol. **25**:574–577.
20. **Scott, C. C. L., and W. R. Finnerty.** 1976. Characterization of intracytoplasmic hydrocarbon inclusions from the hydrocarbon-oxidizing *Acinetobacter* species HOI-N. J. Bacteriol. **127**:481–489.
21. **Sherwood, M.** 1983. Bacterial plastic comes to market. Biotechnology **1**:388–389.
22. **Shively, J. M.** 1974. Inclusion bodies of procaryotes. Annu. Rev. Microbiol. **28**:167–187.
23. **Sonnleitner, B., E. Heinzle, G. Braunegg, and R. M. Lafferty.** 1979. Formal kinetics of poly-3-hydroxy-butyric acid (PHB) production in *Alcaligenes entrophus* H16 and *Mycoplana rubra* R14 with respect to the dissolved oxygen tension in ammonium-limited batch cultures. Eur. J. Appl. Microbiol. Biotechnol. **7**:1–10.
24. **Spurr, A. R.** 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26**:31–43.
25. **Suzuki, T., T. Yamane, and S. Shimuzu.** 1986. Mass production of poly-3-hydroxybutyric acid by fully automatic fed-batch culture of methylotroph. Appl. Microbiol. Biotechnol. **23**:322–329.
26. **Suzuki, T., T. Yamane, and S. Shimuzu.** 1986. Kinetics and effect of nitrogen source feeding on production of poly-β-hydroxybutyric acid by fed-batch culture. Appl. Microbiol. Biotechnol. **24**:366–369.
27. **Suzuki, T., T. Yamane, and S. Shimizu.** 1986. Mass production of poly-β-hydroxybutyric acid by fed-batch culture with controlled carbon/nitrogen feeding. Appl. Microbiol. Biotechnol. **24**:370–374.
28. **van der Linden, A. C.** 1963. Epoxidation of α-olefins by heptane-grown *Pseudomonas* cells. Biochim. Biophys. Acta **77**:157–159.
29. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithinase of *E. coli*: partial purification and some properties. J. Biol. Chem. **218**:97–106.
30. **Wallen, L. L., and W. K. Rohwedder.** 1984. Poly-beta-hydroxyalkanoates from activated sludge. Environ. Sci. Technol. **8**:576–579.
31. **Witholt, B.** 1972. Method for isolating mutants overproducing nicotinamide adenine dinucleotide and its precursors. J. Bacteriol. **109**:350–364.