APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1990, p. 3354–3359
0099-2240/90/113354-06$02.00/0

Vol. 56, No. 11

Accumulation of a Polyhydroxyalkanoate Containing Primarily 3-Hydroxydecanoate from Simple Carbohydrate Substrates by *Pseudomonas* sp. Strain NCIMB 40135

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Received 2 May 1990/Accepted 15 August 1990

A number of *Pseudomonas* species have been identified which accumulate a polyhydroxyalkanoate containing mainly 3-hydroxydecanoate monomers from sodium gluconate as the sole carbon source. One of these, *Pseudomonas* sp. strain NCIMB 40135, was further investigated and shown to accumulate such a polyhydroxyalkanoate from a wide range of carbon sources (C_2 to C_6); however, when supplied with octanoic acid it produced a polyhydroxyalkanoate containing mainly 3-hydroxyoctanoate monomers. Polymer synthesis occurred in batch culture after cessation of growth due to exhaustion of nitrogen. In continuous culture under nitrogen limitation up to 16.9% (wt/wt) polyhydroxyalkanoate was synthesized from glucose as the carbon source. The monomer units are mainly of the *R*-(−) configuration. Nuclear magnetic resonance studies confirmed the composition of the polymer. Differential scanning calorimetry suggested that the solvent-extracted polymer contained a significant proportion of crystalline material. The weight-average molecular weight of the polymer from glucose-grown cells was 143,000.

Poly(3-hydroxybutyrate) (PHB) can be synthesized by many bacteria, and a number of carbon sources are known to be suitable substrates (4). *Alcaligenes eutrophus* will accumulate PHB from medium containing acetate, butyrate, or glucose (8). Polyhydroxyalkanoates (PHAs) containing 3-hydroxybutyrate (3HB) together with other hydroxyacid monomer units are produced by *A. eutrophus* only when carbon sources that are precursors of these monomers are included in the medium. Thus, PHAs containing 3-hydroxyvalerate (3HV), 4-hydroxybutyrate (4HB), or 5-hydroxyvalerate (5HV) monomers, in addition to 3HB units, accumulate when valeric acid, 4-hydroxybutyric acid, or 5-chlorovaleric acid, respectively, is provided (6–8). The photosynthetic bacterium *Rhodospirillum rubrum* accumulates PHA containing 3-hydroxyhexanoate (3HHx) monomers, in addition to 3HB and 3HV units, when hexanoic acid is the sole carbon source (2).

Pseudomonas oleovorans and a number of other *Pseudomonas* species accumulate PHAs containing C_5 to C_{12} 3-hydroxyacids from medium containing an *n*-alkane, *n*-alcohol, or *n*-alkanoic acid as the sole carbon source (1, 11, 15). These polymers invariably contain, as a major monomer unit, a 3-hydroxyacid of the same carbon chain length as the accumulation substrate. Significant amounts of other monomers possessing two fewer and two more carbon atoms than the substrate are also incorporated into the polymer. The failure of fluorescent pseudomonads to accumulate PHB, coupled with their ability to produce PHAs from *n*-alkanoic acids, may be of taxonomic value (13).

This paper describes the accumulation by *Pseudomonas* sp. strain NCIMB 40135 of a PHA containing predominantly 3-hydroxydecanoate (3HD) monomer units (together with a proportion of 3-hydroxyoctanoate [3HO] units) from a wide range of unrelated carbon sources.

Since this work was carried out, Timm and Steinbüchel have confirmed and extended our observations to show that the ability to accumulate PHA containing 3HD is shared by many strains of *P. aeruginosa* and certain other *Pseudomonas* species (20).

(Part of this work was presented at the NATO Advanced Research Workshop on New Biosynthetic Biodegradable Polymers of Industrial Interest from Microorganisms, Sitges, Spain, 26 to 31 May 1990, p. 119–129.)

MATERIALS AND METHODS

Culture conditions. PHA accumulation in shake flask batch cultures was achieved as described for *A. eutrophus* (12), except that a range of carbon sources (10 g liter^{-1}) was used for both growth and accumulation stages.

Pseudomonas sp. strain NCIMB 40135 was grown in continuous culture under conditions previously described for *A. eutrophus* (10), except that the carbon source was provided at 30 or 5 g liter^{-1} for glucose and 10 or 5 g liter^{-1} for octanoic acid to achieve nitrogen- and carbon-limiting growth conditions, respectively. In certain experiments, other parameters were adjusted as described. For growth in a pH-controlled batch fermentor, nitrogen-limiting growth medium was used with a glucose concentration of 30 g liter^{-1} and an $(NH_4)_2SO_4$ concentration of 1.11 g liter^{-1}.

Analysis and isolation of PHA. The composition of PHA in freeze-dried bacteria was determined as described previously (11). PHA was extracted from freeze-dried bacteria as described by Brandl et al. (1).

Examination of the physical properties of PHA. Polymer samples were dried in vacuo over P_2O_5 prior to analysis.

The optical rotation of the methyl 3-hydroxyacid monomers produced by methanolysis (15) of the polymer was determined at 589 nm in chloroform solution (15 g liter^{-1}).

Nuclear magnetic resonance (NMR) measurements were

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TABLE 1. Bacteria which produce PHA containing 3HD as the principal monomer unit from sodium gluconate as the sole carbon source

Strain	PHA (%, wt/wt)	Polymer composition (mol%)	
		3HO	3HD
Pseudomonas sp. strain NCIMB 40135	26	15	85
P. putida NCIMB 8865	17	20	80
P. putida NCIMB 9571	9	24	76
P. aeruginosa NCIMB 9904	2.5	0	100
P. aeruginosa NCIMB 8626	1.5	15	85
P. fluorescens NCIMB 9520	0.2	0	100

TABLE 2. Production of PHAs by *Pseudomonas* sp. strain NCIMB 40135 from various carbon sources

Carbon source	PHA (%, wt/wt)	Polymer composition (mol%)	
		3HO	3HD
Acetate	5	15	85
Glycerol	5	10	90
Lactate	9	15	85
Succinate	1.3	53	47
Glucose	8	24	76
Gluconate	17	20	76
Fructose	16	17	83
Octanoate	66	95	2

made on a JEOL GX 270 spectrometer operating at 270.17 MHz for ^1H and 67.94 MHz for ^{13}C. Spectra were recorded in $CDCl_3$ and were referenced to tetramethylsilane for ^1H work and $CDCl_3$ (77.1 ppm) for ^{13}C work.

The glass transition temperature (T_g), melting temperature (T_m), and heat of fusion (ΔH_m) of PHA were measured by differential scanning calorimetry (Perkin Elmer DSC-II). PHA samples (10 mg) were heated at a rate of 20°C min^{-1} from −60 to 200°C, quickly cooled, and then scanned a second time over the same temperature range. Data used for T_g, T_m and ΔH_m are reported for the first scan. T_g was taken as the onset temperature, and T_m was taken as the peak of the melting endotherm.

The molecular weights of PHA samples were determined by gel permeation chromatography with two PL gel columns (column backing material, 10 μm) (Polymer Laboratories Ltd.) and an infrared detector. Chloroform was used as the eluant, and calibration was effected with polystyrene standards of low polydispersity.

Analytical methods. Ammonia was measured by the indophenol method (3). Glucose was estimated by using a commercial kit (GOD-Perid; Boehringer). Gluconic acid and 2-ketogluconic acid were determined as described by DeMoss (5).

RESULTS AND DISCUSSION

Production of novel PHA. Several bacteria were found to accumulate PHA containing 3HD as the principal monomer when supplied with sodium gluconate as the sole carbon source (Table 1). Sodium gluconate was a more suitable growth substrate than glucose, as growth on glucose was accompanied by a significant decrease in pH, even in strongly buffered medium. No 3HB units were detected in any of these organisms. A number of organisms known to accumulate PHB were also examined (*A. eutrophus* NCIMB 11599, *Bacillus megaterium* NCIMB 9521, and *Bacillus subtilis* NCIMB 9590), and no 3HD-containing polymer was

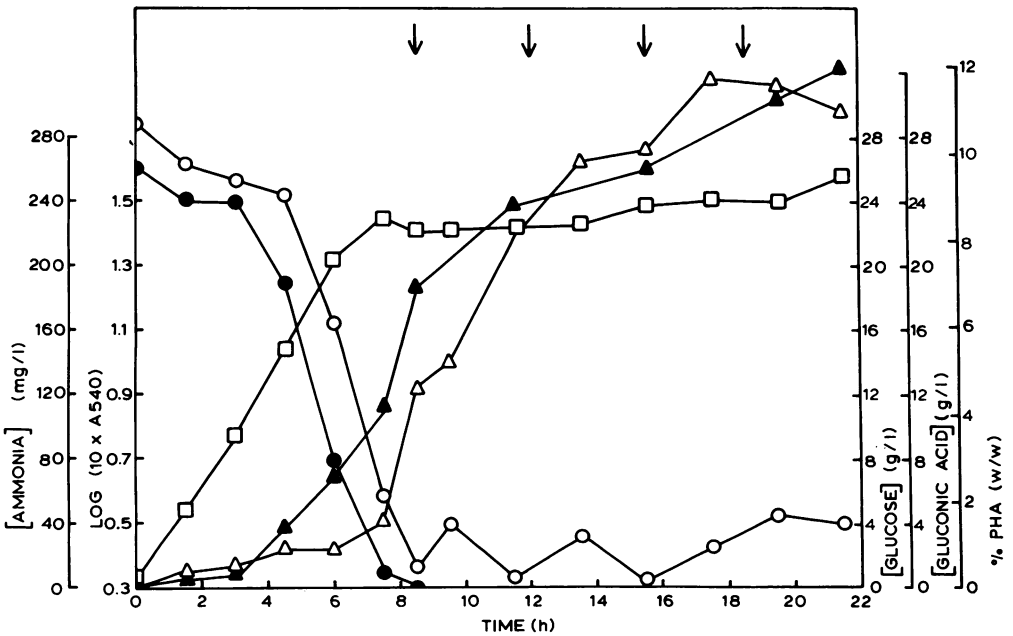

FIG. 1. Production of PHA containing primarily 3-hydroxydecanoate by *Pseudomonas* sp. strain NCIMB 40135. *Pseudomonas* sp. strain NCIMB 40135 was grown in aerobic batch culture at 30°C in defined medium with glucose as the carbon source and supplied with further glucose at intervals after nitrogen exhaustion to encourage polymer synthesis. The pH was maintained at 7.0 by addition of 2 M KOH. Symbols: □, log (10 × A_{540}); ○, glucose; ●, ammonia; △, total PHA; ▲, gluconic acid. Arrows indicate glucose (1%, wt/vol) additions. The final sample (21.5 h) contained 11% (wt/wt) PHA, consisting of the following 3-hydroxyacids (by gas-chromatographic analysis): 79 mol% HD, 20 mol% HO, and 1 mol% HHx.

TABLE 3. Accumulation of PHA by *Pseudomonas* sp. strain NCIMB 40135 in continuous culture

Carbon source	Dilution rate (h^{-1})	Dissolved oxygen[a]	Limiting substrate	PHA (%, wt/wt)	Monomer composition (mol%)	
					3HO	3HD
Glucose	0.1	70	Ammonia	4.4	20	80
Glucose	0.035	70	Ammonia	17	22	78
Glucose	0.1	1	Ammonia	7	14	86
Glucose	0.1	0	Oxygen	0		
Glucose	0.1	70	Glucose	0		
Octanoate	0.1	70	Octanoate	5.6	100	0
Octanoate	0.1	70	Ammonia	23	99	1

[a] Percent air saturation.

detected. The organisms in Table 1 accumulate PHAs from *n*-alkanoic acids and in certain cases *n*-alcohols and *n*-alkanes (11). *P. oleovorans* ATCC 29347, which is known to accumulate PHA from *n*-alkanes (15) and *n*-alkanoic acids (1), did not produce a 3HD-containing polymer from sodium gluconate under our standard conditions.

Pseudomonas sp. strain NCIMB 40135 synthesizes a PHA containing mainly 3HD monomer units from a wide range of growth and accumulation substrates, regardless of the number of carbon atoms (C_2 to C_6) in the substrate (Table 2). The proportions of the monomer units can be altered by the addition of low concentrations of *n*-alkanoic acid to the accumulation medium (e.g., hexanoic acid or octanoic acid).

Attempts to produce polymers containing 3HB, 3HV, or 4HB monomers in *Pseudomonas* sp. strain NCIMB 40135 have been unsuccessful under our conditions. From the data in Tables 1 and 2 it is clear that PHAs containing 3HD as the principal monomer can be produced by certain pseudomonads from a range of substrates that are not precursors of this monomer. The conditions for growth and polymer accumulation have not been optimized for the strains used, and further work is required to determine that best combination of substrate and organism for PHA production.

The growth of *Pseudomonas* sp. strain NCIMB 40135 with glucose as the sole carbon source in a pH-controlled batch fermentor resulted in the accumulation of a substantial concentration of gluconate (Fig. 1), and copious amounts of alkali were required to maintain a pH value of 7.0. The oxidation of glucose to gluconate and, in some organisms, 2-ketogluconate (catalyzed by glucose and gluconate dehydrogenases, respectively), occurs in *Pseudomonas* species (16). 2-Ketogluconate was present in stationary-phase cultures of *Pseudomonas* sp. strain NCIMB 40135 grown on glucose (data not shown). Accumulation of gluconate and 2-ketogluconate is responsible for the substantial decrease in pH in shake flask cultures grown with glucose as the carbon source. Significant accumulation of PHA was not observed until the nitrogen source was exhausted, as has been reported for organisms that accumulate PHB (18) and PHAs derived from hydrocarbons (15).

The accumulation of PHA by *Pseudomonas* sp. strain NCIMB 40135 in continuous culture can clearly be con-

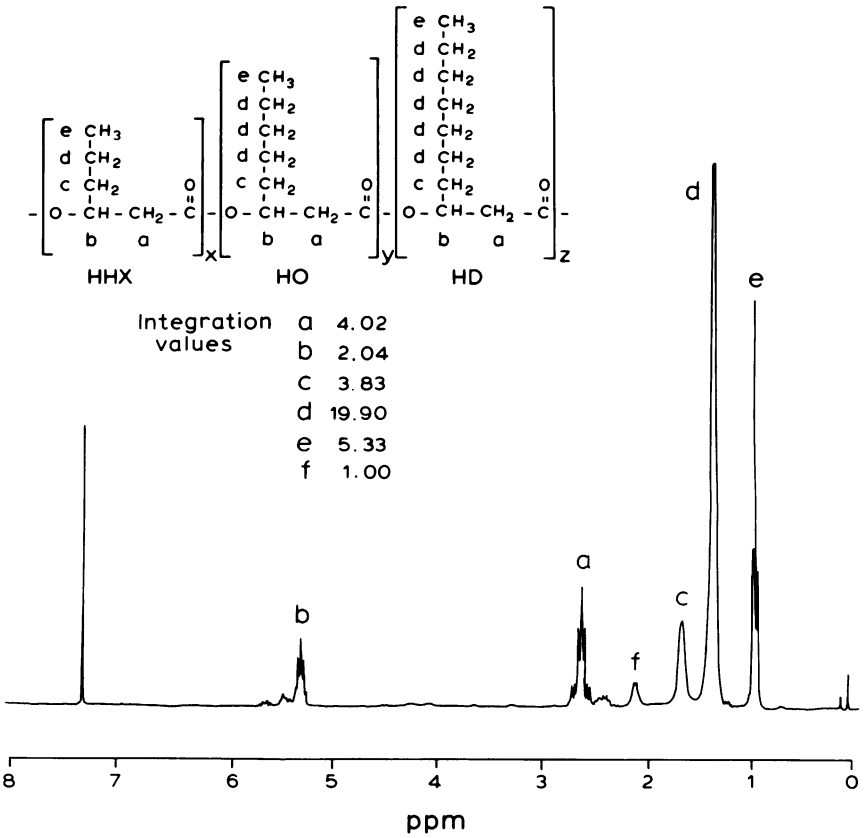

FIG. 2. 270-MHz ^1H NMR spectrum, recorded at 21°C in $CDCl_3$, of the PHA extracted from glucose-grown *Pseudomonas* sp. strain NCIMB 40135. The PHA sample contained (by gas-chromatographic analysis) 79 mol% HD, 20 mol% HO, and 1 mol% HHx.

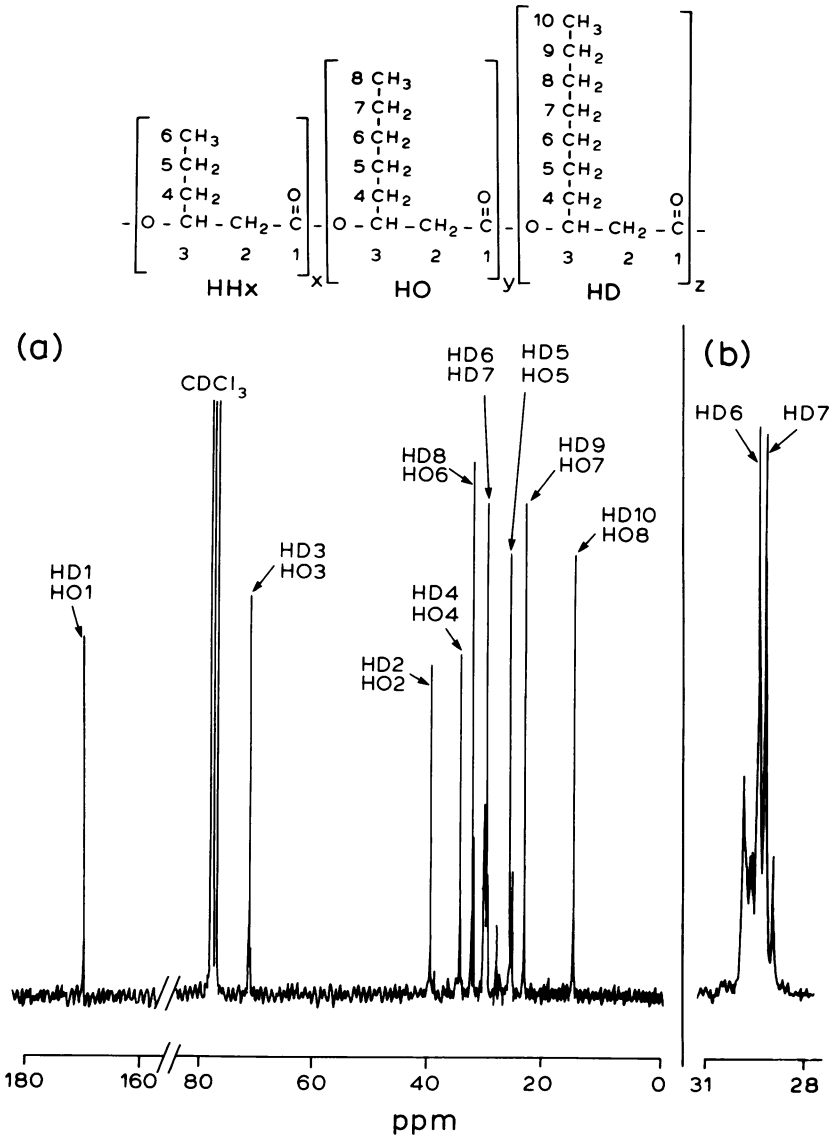

FIG. 3. (a) 67.9-MHz ^{13}C NMR spectrum, recorded at 21°C in $CDCl_3$, of the PHA extracted from glucose-grown *Pseudomonas* sp. strain NCIMB 40135. The PHA sample contained (by gas-chromatographic analysis) 79 mol% HD, 20 mol% HO, and 1 mol% HHx. (b) Expansion of part of the side-chain methylene region of spectra.

trolled by altering the growth parameters (Table 3). During the first 4 days of continuous culture (under conditions of constant temperature, pH, dilution rate [0.1 h^{-1}], medium composition, and dissolved oxygen tension), the polymer content of glucose-grown bacteria changed with time, increasing to 18% (wt/wt) before decreasing to an apparently steady-state value of 4.4% (wt/wt). This pattern was reproduced in three separate experiments, and as yet we are unable to explain the observations. After these initial fluctuations of polymer content, subsequent changes of growth parameters were followed by establishment of apparent steady state; the polymer content was then determined.

Growth on octanoic acid as the carbon source resulted in incorporation of a high concentration of PHA into bacteria under nitrogen-limiting conditions, but even under carbon limitation significant polymer synthesis occurred, and in both cases the polymer contained mainly 3HO units. When glucose was used as the carbon source, the accumulated polymer contained mainly 3HD monomer units. Under standard conditions (dilution rate, 0.1 h^{-1}; 70% air saturation) only low polymer contents were detected (4.4%, wt/wt), but these could be increased either by decreasing the oxygen tension to 1% of air saturation (yielding 7.0%, wt/wt) or by decreasing the dilution rate to 0.035 h^{-1} when 16.9% (wt/wt) was achieved. No polymer was synthesized under oxygen-limiting conditions, even though some PHB-storing organisms such as *Azotobacter beijerinckii* and *A. eutrophus* accumulate high concentrations of PHB under such conditions (17, 19).

Physical properties of the extracted polymer. The optical rotation of the methyl esters of PHA from glucose-grown cells was negative (-6.3 $[\alpha]^{21}_{589}$) compared with published values for (*S*)-(+)-methyl-3-hydroxyoctanoate (+23.6 $[\alpha]^{20}_{578}$) and methyl esters derived from poly-(*R*)-(−)-3-hydroxyalkanoate (-21.9 $[\alpha]^{20}_{578}$), indicating that the *R*-(−) form is in enantiomeric excess.

Samples of polymer extracted from glucose-grown cells

harvested in the stationary phase after 21.5 h of growth (Fig. 1) were examined by NMR. The ^1H NMR spectrum of PHA is shown in Fig. 2. The integration values shown agree reasonably well with the proposed polymer structure, in which the ratio of peak b to peak d has a value of 9.8H, compared with 9.1H calculated from the repeating-unit composition determined by gas-chromatographic analysis. Peak f cannot be accounted for by the proposed structure and may represent a minor impurity in the purified polymer. The ^1H NMR spectra of related PHA samples studied by Gross et al. (9) show peaks at almost identical chemical shifts compared with this polymer, but the integration values of peak d are lower in their polymers.

The ^{13}C NMR spectrum of PHA is shown in Fig. 3. The spectrum has 10 major peaks for the carbon atoms of the individual monomer units, although the peaks corresponding to carbon atoms 6 and 7 can be resolved only when an expansion of the side-chain methylene carbons is examined. Expansion of the carbonyl, side-chain methylene, and methyl carbon regions showed minor peaks due to 3HO monomer units (data not shown), but these peaks were poorly resolved from the major peaks in all cases. The ratio of decanoate to octanoate is 78:22 on the basis of average peak height from several carbon lines. The chemical shift data of individual monomers are consistent with those described for other PHAs (9), although the major component in this case clearly contains 10 carbon atoms. No peaks corresponding to 3HHx monomers were observed in the spectrum. All carbon lines are sharp singlets, and there is no evidence for block structure in this polymer.

Thermal analysis of polymers from *Pseudomonas* sp. strain NCIMB 40135 was carried out by differential scanning calorimetry to determine the peak melting temperature (T_m), enthalpy of fusion (ΔH_m), and glass transition temperature (T_g). Polymer extracted from bacteria provided with glucose as the sole carbon source had T_m and ΔH_m values of 54.8°C and 22.4 J g^{-1}, respectively. This material did not exhibit a T_g under the conditions used. The polymer produced by this organism from octanoic acid-containing medium was also examined, and the values obtained were T_m = 58°C, ΔH_m = 28.2 J g^{-1}, and T_g = 31°C. Both polymers displayed a significant degree of crystallinity, as shown by the large melting endotherms. Long *n*-alkyl side chains (9) and very short *n*-alkyl side chains as in PHB (P. A. Holmes, L. F. Wright, and S. H. Collins, European patent 0 052 459, December 1985) favor crystallinity, whereas intermediate examples are less crystalline (e.g., poly-3-hydroxyhexanoate [9]). However, the degree of crystallinity is also controlled by the ratio of the individual monomers in a copolymer (14). The T_g of the polymer from *Pseudomonas* sp. strain NCIMB 40135 grown on glucose is presumably below −60°C, consistent with a polymer containing a long side-chain group.

The molecular weight data (based on polystyrene standards) of the polymers from glucose-grown cells (M_w = 143,000; M_n = 61,900; M_w/M_n = 2.32) and octanoic acid-grown cells (M_w = 169,000; M_n = 91,000; M_w/M_n = 1.86) are similar and could be accounted for by slight differences in experimental conditions. These molecular weights are, however, very low when compared with a sample of PHB purified from glucose-grown *A. eutrophus* (M_w = 1,825,000; M_n = 1,210,000; M_w/M_n = 1.50) (10).

In *P. oleovorans* the incorporation of 3-hydroxyacids possessing fewer carbon atoms than the substrate may involve the removal of C_2 units by β-oxidation, as suggested by Lageveen et al. (15). Similarly, the extension of the carbon chain involves the addition of C_2 units, possibly derived by β-oxidation of the substrate (1). Generally, with longer-chain substrates (C_{10} and above) the major component of the *P. oleovorans* polymer contains two fewer carbon atoms than the substrate.

The synthesis of PHA containing 3HD as the principal monomer by *Pseudomonas* sp. NCIMB 40135, reported in this paper, is also likely to involve reactions related to those of general fatty acid metabolism, and we are currently investigating the biosynthesis of this polymer.

ACKNOWLEDGMENTS

We thank ICI plc for funding this work under their Joint Research Scheme; Andy Webb, ICI, for performing differential scanning calorimetry and gel permeation chromatography of polymer samples; and David Byrom, ICI, for his valuable suggestions. We also thank Bob Knight for performing optical rotation measurements.

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