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Synthesis of Poly-3-Hydroxyalkanoates Is a Common Feature of Fluorescent Pseudomonads

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The fluorescent pseudomonads are classified as a group, one characteristic of which is that they do not accumulate poly-3-hydroxybutyrate (PHB) during nutrient starvation in the presence of excess carbon source. In this paper we show that prototype strains from this subclass, such as *Pseudomonas aeruginosa*, *Pseudomonas putida*, and *Pseudomonas fluorescens*, do accumulate poly-3-hydroxyalkanoates (PHA) when grown on fatty acids. These PHAs are composed of medium-chain-length (C_6 to C_{12}) 3-hydroxy fatty acids. The ability to form these polyesters does not depend on the presence of plasmids. A specificity profile of the enzymes involved in the biosynthesis of PHA was determined by growing *Pseudomonas oleovorans* on fatty acids ranging from C_4 to C_{18}. In all cases, PHAs were formed which contained C_6 to C_{12} 3-hydroxy fatty acids, with a strong preference for 3-hydroxyoctanoate when C_{even} fatty acids were supplied and 3-hydroxynonanoate when C_{odd} fatty acids were the substrate. These results indicate that the formation of PHAs depends on a specific enzyme system which is distinct from that responsible for the synthesis of PHB. While the fluorescent pseudomonads are characterized by their inability to make PHB, they appear to share the capacity to produce PHAs. This characteristic may be helpful in classifying pseudomonads. It may also be useful in the optimization of PHA production for biopolymer applications.

Recently we reported that *Pseudomonas oleovorans* synthesizes a range of poly-3-hydroxyalkanoates (PHAs) after growth on C_6 to C_{12} linear *n*-alkanes and 1-alkenes in a controlled fermentation process (12). We found that the polymer composition reflects the growth substrate used in the fermentation. When, for instance, *n*-decane was the substrate, *P. oleovorans* formed a polymer which contained 3-hydroxydecanoate, 3-hydroxyoctanoate, and 3-hydroxyhexanoate. Since for all of the substrates tested, the monomers were directly derived from the growth substrate or had been shortened by one or more C_2 units, we concluded that poly-3-hydroxyalkanoates are polymerized from intermediates in the fatty acid oxidation pathway (12).

Only one PHA, poly-3-hydroxybutyrate (PHB), has been studied extensively since it was first observed by Lemoigne in 1926 (13). PHB is a lipid reserve material accumulated by many microorganisms under conditions in which an essential nutrient other than the carbon source is exhausted (4). Carbon sources used for PHB formation, such as glucose and methanol, are all converted to acetyl-coenzyme A (acetyl-CoA), two molecules of which are condensed to acetoacetyl-CoA by the biosynthetic thiolase. Subsequently the acetoacetyl-CoA reductase forms (*R*)-3-hydroxybutyryl-CoA, which is the substrate for the polymerization reaction (4).

Therefore, although the higher PHAs formed by *P. oleovorans* are structurally related to PHB, they appear to be synthesized by different routes. This is in accord with the observation that *P. oleovorans* does not accumulate PHB under conditions which result in optimal PHB synthesis in other microorganisms (K. A. Powell, B. A. Collinson, and K. R. Richardson, Imperial Chemical Industries Ltd. European Patent Application no. 80300432.4, 1980). In fact, one of the characteristics of the fluorescent pseudomonads, of which *P. oleovorans* is one, is their inability to accumulate PHB (7).

Given the newly established ability of *P. oleovorans* to synthesize PHA with medium-chain-length monomers rather than PHB, we felt it worthwhile to investigate whether other fluorescent pseudomonads share this characteristic.

In our previous studies we investigated the biosynthesis of PHA by *P. oleovorans* in two-liquid phase systems containing an alkane phase (12). However, most pseudomonads are unable to metabolize these hydrocarbons because they lack the OCT plasmid, which encodes the enzymes involved in the first steps of alkane oxidation (8). We have therefore analyzed the synthesis of the polyester by *P. oleovorans* during growth on related substrates, such as alkanols and fatty acids. Subsequently we analyzed PHA formation by prototype strains from the subclass of fluorescent pseudomonads. In this paper we show that all of the fluorescent pseudomonads tested accumulate PHA when grown on fatty acids. Thus, the synthesis of PHA appears to be a general characteristic of this group of pseudomonads.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains used are listed in Table 1. *Pseudomonas* strains were grown on E (20), E2 (12), or 0.5*E2 medium. The concentrations of $NaNH_4HPO_4$, K_2HPO_4, and KH_2PO_4 in the 0.5*E2 medium are half of the corresponding amounts in E2. Fatty acids (final concentrations: butyrate, valerate, hexanoate, and heptanoate, 20 mM; octanoate, nonanoate, decanoate, and undecanoate, 10 mM; dodecanoate [laureate], tridecanoate, tetradecanoate [myristate], pentadecanoate, *cis*-9-octadecenoate [oleate], *trans*-9-octadecenoate [elaidate], and *cis*-6,9,12-octadecatrienoate [γ-linolenate], 5 mM; hexadecanoate [palmitate], heptadecanoate, octadecanoate [stearate], and *cis*-13-docosenoate [erucate], 2 mM) were dissolved as 10-times-concentrated stocks in 10% Brij 58, adjusted to pH 7.0 by addition of 1 N KOH, and filter sterilized (11).

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TABLE 1. Bacterial strains used in this study

Strain	Characteristics[a]	Reference
P. oleovorans		
GPo1	OCT	18
GPo12	OCT⁻	Kok[b]
P. putida		
PpG1		9
KT2442	TOL⁻, Rif[r]	1
P. aeruginosa PAO1	Prototype	10
P. fluorescens	Prototype	19; DSM 50090[c]
P. lemonnieri		19; DSM 50415[c]
P. testosteroni	Prototype	14

[a] Abbreviations: OCT, OCT plasmid; TOL, TOL plasmid; Rif[r], rifampin resistance.
[b] M. Kok, Ph.D. thesis, University of Groningen, The Netherlands, 1988.
[c] DSM, Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH, Braunschweig, Federal Republic of Germany.

3-Hydroxybutyrate was added to 0.7%. Growth on minimal 0.5*E2 medium agar plates on 1-octanol was performed at 30°C in closed tins saturated with 1-octanol vapor. For liquid medium with 1-octanol or octanal as the carbon source, the substrate (3%, vol/vol) was added directly to the culture. To prevent cell damage by these substrates, 17% dibutylphthalate was added.

Determination of PHA. For a qualitative analysis of the presence of PHA, cells were examined by phase-contrast microscopy. For a quantitative analysis, cells were grown on 0.5*E2 agar plates or in 50-ml 0.5*E2 cultures and assayed for the presence and composition of PHA (12). Polymer composition was also determined on isolated polymers obtained from 1-liter cultures after chloroform extraction (12).

In the assay, cells are harvested, lyophilized, and subsequently treated with 15% sulfuric acid in methanol-chloroform at 100°C for 140 min to convert fatty acids to their corresponding methyl esters. The methyl esters are then analyzed by gas-liquid chromatography (GLC) (12). Adjustment for the measurement of methyl-3-hydroxybutyrate was made by starting the GLC program at 68°C. After 2 min at this temperature, the column was heated at a rate of 5°C/min for 20 min. The column temperature was then raised to 278°C at a rate of 10°C/min to remove all high-molecular-weight components. The cell density (21) and the peak area ratios of the monomers and the internal standard (methylbenzoate) allow determination of the amount of polymer accumulated by the cells and its composition.

RESULTS

Formation of PHA by *P. oleovorans* on intermediates of the alkane degradation pathway. *P. oleovorans* GPo1 was tested for synthesis of PHA in 50-ml 0.5*E2 cultures on 1-octanol, octanal, or octanoate as the substrate. *P. oleovorans* GPo1 is the prototype alkane-oxidizing strain which was used in previous studies on PHA formation (2, 5, 12). Carbon sources were added to final amounts of 3% (vol/vol), or 10 mM for octanoate. Since 1-octanol and octanal are toxic to the cells, such cultures were supplemented with dibutylphthalate (17%) to solubilize these substrates in a relatively apolar phase (9).

P. oleovorans was able to synthesize PHA when 1-octanol or octanoate was the substrate. No growth was observed on octanal. After growth on octanoate for 20 h, the amount of polymer produced was 8.7% of the cell dry weight, while growth on 1-octanol resulted in 3.1% PHA. The composition of the polymers formed on octanol and octanoate was determined. 3-Hydroxyoctanoate constituted 90% of the monomers, the remainder being 3-hydroxyhexanoate. These values are in close agreement with our previous findings for PHA formation following growth on octane (12). The oxidation state of the substrate therefore appears to have little or no effect on polymer composition.

Occurrence of PHA in *P. putida* strains. To determine whether the genetic information for PHA synthesis is located on the OCT plasmid of *P. oleovorans* GPo1 or on the chromosome, we examined three other *Pseudomonas putida* strains for PHA synthesis. *P. oleovorans* GPo12 is an OCT⁻ derivative of GPo1. PpG1 is a *P. putida* strain isolated from soil that contains no plasmid (9). *P. putida* KT2442 is a rifampin-resistant, TOL plasmid-cured derivative of *P. putida* mt-2 (1). None of these strains is able to grow on alkanes.

The different *Pseudomonas* strains were grown on minimal medium agar plates on octanol vapor and analyzed microscopically for the presence of PHA. Normally, polymer accumulates intracellularly in granules, which are seen as transparent (white) dots by phase-contrast microscopy (12). From pictures such as those shown in Fig. 1, we concluded that all of the strains examined formed a reserve material.

To determine the amount and composition of the reserve material, cells were collected from the plates and analyzed for PHA. All the strains examined except *P. putida* PpG1 synthesized PHA consisting of about 90% 3-hydroxyoctanoate and 10% 3-hydroxyhexanoate (Table 2). Interestingly, the PHA produced by *P. putida* PpG1 was nearly a homopolymer, containing about 96% C_8 and only 4% C_6 monomers. Compared with *P. oleovorans* GPo1 and GPo12, *P. putida* PpG1 and KT2442 accumulated PHA to higher intracellular levels.

Since *P. oleovorans* GPo12, the plasmid-cured derivative of *P. oleovorans* GPo1, synthesizes PHA, the genes responsible for PHA formation must be located on the chromosome. The fact that several other *P. putida* isolates which contain no plasmids also synthesize PHA suggests that PHA formation is a general characteristic of *P. putida* strains, which they may share with other pseudomonads. We therefore examined PHA synthesis in various strains related to *P. putida*.

Occurrence of PHA in fluorescent *Pseudomonas* strains. Pseudomonads have been classified according to rRNA homology (6, 17), similarity of enzymes involved in the biosynthesis of aromatic amino acids (3), and metabolic characteristics as described in *Bergey's Manual of Determinative Bacteriology* (7). The fluorescent *Pseudomonas* strains are all members of the same group within the different classification methods. A common characteristic of these strains is their inability to synthesize PHB (7).

To determine to what extent the capacity of accumulating PHA is found among fluorescent *Pseudomonas* strains, we examined several well-established prototype strains. *P. aeruginosa* PAO (10), *P. fluorescens* (19), *P. lemonnieri* (19), *P. testosteroni* (14), and the *P. putida* strains described above were grown on 10 mM octanoate or 0.7% 3-hydroxybutyrate in 50-ml 0.5*E2 cultures. The cells were harvested, and PHA content was determined for whole cells (Table 3).

The *P. putida* strains were grown in E2 medium on 30 mM octanoate or 0.7% 3-hydroxybutyrate in 1-liter fermentors, and the polyesters were isolated. The composition of the polymer was determined and shown to be identical to that obtained after whole-cell analysis. The PHAs from octanoate-grown cells were always composed of 3-hydroxy-

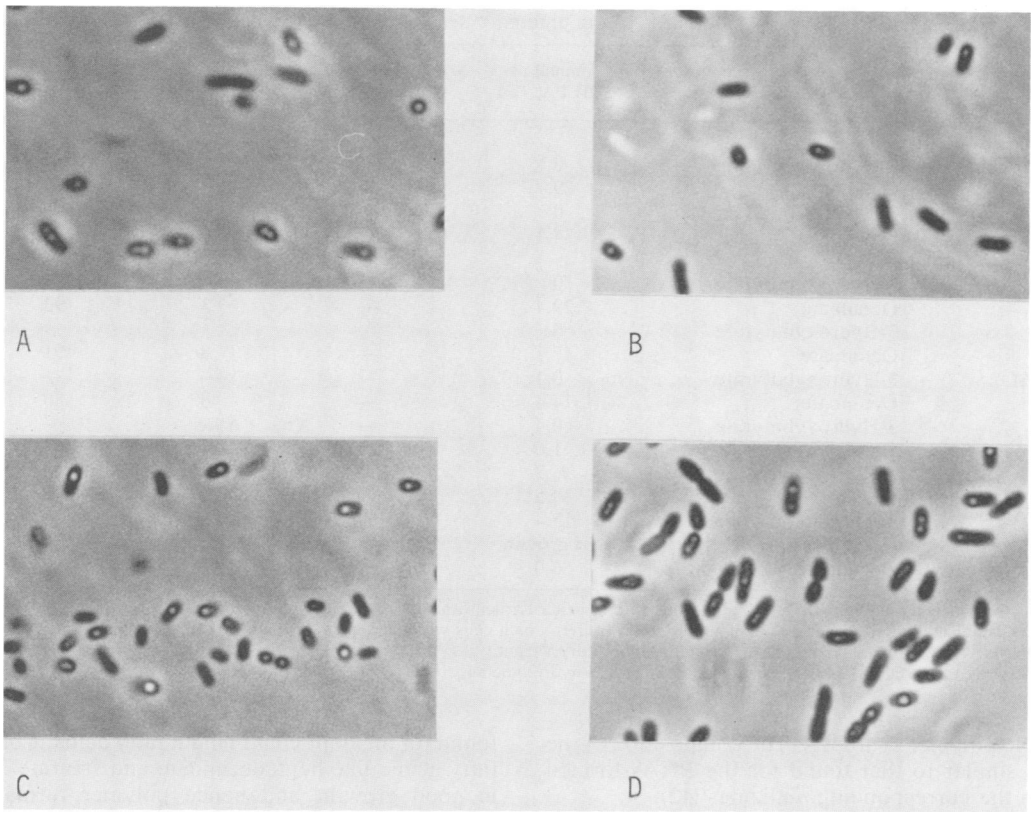

FIG. 1. Phase-contrast micrographs (×1,250) of *P. putida* strains grown on 1-octanol vapor on solid medium. (A) *P. oleovorans* GPo1; (B) *P. oleovorans* GPo12; (C) *P. putida* PpG1; (D) *P. putida* KT2442.

hexanoate, 3-hydroxyoctanoate, and small amounts of 3-hydroxydecanoate. No C_{odd} 3-hydroxy fatty acids were detected. After growth on 3-hydroxybutyrate, no 3-hydroxy fatty acids could be found either in culture samples or in the material which was obtained after precipitation of a chloroform extract of the whole cells.

P. aeruginosa, *P. fluorescens*, and *P. lemonnieri* all synthesized PHA following growth on octanoate, but no polymer was formed after growth on 3-hydroxybutyrate. In contrast, *P. testosteroni*, which was unable to grow on octanoate, produced PHB when grown on 3-hydroxybutyrate or decanoate. On the latter substrate, only 13% of the monomers were not 3-hydroxybutyrate. The amount of PHA which was formed by the different species differed considerably.

The *P. putida* strains, which include *P. oleovorans*, accumulated 30 to 50% PHA, *P. aeruginosa* accumulated about 15%, and *P. fluorescens*, the prototype of the fluorescent pseudomonads, accumulated only 1 to 2% PHA. However, *P. lemonnieri* is a *P. fluorescens* biotype IV strain (7) and accumulated PHA to almost 40% of its cell dry weight. These numbers should be regarded as only a general indication of the PHA synthesis potential of these strains, since culture conditions may affect the PHA yield (12) and we have not yet optimized PHA synthesis in all of the strains examined.

TABLE 2. PHA synthesis by *P. oleovorans* and *P. putida* strains

Strain[a]	Cellular PHA content (%)[b]	Fraction 3OH-C_6[c] (%)
P. oleovorans		
GPo1	2–5	9.5 ± 1.0
GPo12	2–5	9.4 ± 1.5
P. putida		
PpG1	10–20	4.0 ± 0.7
KT2442	10–20	9.0 ± 1.1

[a] Strains were grown on 1-octanol on 0.5*E2 plates.
[b] The amount of polymer is given as percentage of the cell dry weight.
[c] The composition is shown as the fraction of 3-hydroxyhexanoate monomers (3OH-C_6) in the polyester, the remainder being 3-hydroxyoctanoate.

Effect of growth substrate on synthesis of PHA by *P. oleovorans*. In our previous study (12), we found that the monomer composition of the polyesters was closely related to the growth substrate when *P. oleovorans* was grown on C_6 to C_{12} *n*-alkanes. Nevertheless, with substrates larger than octane or nonane, 3-hydroxyoctanoate and 3-hydroxynonanoate were always the major constituents of the copolymers. To determine whether these two components remain major polymer constituents independently of the growth substrate, we analyzed PHA formation on a number of C_4 to C_{22} fatty acids. *P. oleovorans* GPo1 was grown in 50-ml cultures containing 0.5*E2 medium supplied with the appropriate fatty acid. Cells were harvested and assayed for the presence of PHAs.

With small fatty acids such as 3-hydroxybutyrate, butyrate and valerate PHA formation was limited (Table 4). Despite the limited production (<1.5%), the PHAs formed contained C_8, C_{10}, and C_{12} monomers. No monomers smaller than C_8 were observed. Medium-chain-length carboxylic acids, such as hexanoate, heptanoate, octanoate, nonanoate, decanoate, and undecanoate, did however sup-

TABLE 3. Accumulation and composition of PHAs in different *Pseudomonas* strains grown on different carbon sources

Strain[a]	Substrate	Amount of 3OH FA[b] (%)	Composition[c] (% of total 3OH FA)			
			3OH-4	3OH-6	3OH-8	3OH-10
P. oleovorans						
GPo1	3-Hydroxybutyrate	0				
	Octanoate	29.7	—	7	90	3
GPo12	3-Hydroxybutyrate	0				
	Octanoate	34.1	—	6	91	3
P. putida						
PpG1	3-Hydroxybutyrate	0				
	Octanoate	29.4	—	3	93	4
KT2442	3-Hydroxybutyrate	0				
	Octanoate	47.1	—	8	91	1
P. aeruginosa PAO	3-Hydroxybutyrate	0.1	—	—	—	100
	Octanoate	14.4	—	8	86	7
P. fluorescens	3-Hydroxybutyrate	0.4	—	—	—	100
	Octanoate	1.6	—	14	63	22
P. lemonnieri	3-Hydroxybutyrate	0.7	—	—	14	86
	Octanoate	38.5	—	6	91	3
P. testosteroni	3-Hydroxybutyrate	15.4	99	—	—	1
	Octanoate	No growth				
	Decanoate	6.0	87	3	9	2

[a] *P. putida* strains were grown on octanoate or 3-hydroxybutyrate in 1-liter fermentors, and culture samples were analyzed for PHA. Presence of polymer in the other strains was established after growing cells in 50-ml cultures, after which cells were harvested, lyophilized, and assayed for PHA.
[b] Amount of 3-hydroxy fatty acids (3OH FA) as a percentage of cell dry weight.
[c] 3OH-4, 3-Hydroxybutyrate; 3OH-6, 3-hydroxyhexanoate; 3OH-8, 3-hydroxyoctanoate; 3OH-10, 3-hydroxydecanoate. —, <0.5%.

port considerable polymer synthesis. The composition of the polyesters was similar to that found for the PHAs formed after growth on the corresponding *n*-alkanes (12).

Growth on the longer-chain-length fatty acids (LCFAs) also resulted in PHA synthesis. With laureate, tridecanoate, myristate, pentadecanoate, and palmitate as the carbon source, polymer accumulation was in the same range as found for medium-chain-length fatty acids. Longer saturated fatty acids, like heptadecanoate and stearate, did not result in good growth, and hence polymer formation was not detected. However, the unsaturated C_{18} carboxylic acids supported both growth and PHA formation, while erucate, a C_{22} unsaturated fatty acid, did not.

Interestingly, the composition of the polyesters formed on

TABLE 4. Formation of PHAs by *P. oleovorans* on C_4 to C_{22} fatty acids

Substrate[a]	3OH FA[b] (%)	Composition[c] (% of total 3OH FA)						
		3OH-6	3OH-7	3OH-8	3OH-9	3OH-10	3OH-11	3OH-12
Saturated fatty acids								
3-Hydroxybutyrate	1.2	—	—	22	—	57	—	21
Butyrate	0.6	—	—	—	—	33	—	67
Valerate	0.7	—	—	—	—	35	—	65
Hexanoate	3.3	95	—	5	—	—	—	—
Heptanoate	2.3	—	100	—	—	—	—	—
Octanoate	8.7	8	—	91	—	1	—	—
Nonanoate	9.1	—	35	—	65	—	—	—
Decanoate	12.5	8	—	75	—	17	—	—
Undecanoate	9.8	—	28	—	59	—	13	—
Dodecanoate	6.6	6	—	57	—	32	—	5
Tridecanoate	5.4	—	32	—	48	5	14	—
Tetradecanoate	10.6	7	—	59	—	30	—	4
Pentadecanoate	5.3	—	32	—	47	8	13	—
Hexadecanoate	3.4	8	—	50	—	30	—	12
Heptadecanoate	No growth							
Octadecanoate	No growth							
Unsaturated fatty acids[d]								
Oleate	7.4	9	—	57	—	28	—	6
Elaidate	11.2	10	—	56	—	27	—	7
γ-Linolenate	5.9	10	—	57	—	30	—	4
Erucate	No growth							

[a] *P. oleovorans* was grown on different fatty acids in 50-ml 0.5*E2 cultures.
[b] The amount of 3-hydroxy fatty acids (3OH FA) is given as a percentage of the cell dry weight.
[c] 3OH-6, 3-Hydroxyhexanoate; 3OH-7, 3-hydroxyheptanoate; 3OH-8, 3-hydroxyoctanoate; 3OH-9, 3-hydroxynonanoate; 3OH-10, 3-hydroxydecanoate; 3OH-11, 3-hydroxyundecanoate; 3OH-12, 3-hydroxydodecanoate; —, <0.5%.
[d] Oleate, *cis*-9-Octadecenoate; elaidate, *trans*-9-octadecenoate; γ-linolenate, *cis*-6,9,12-octadecatrienoate; erucate, *cis*-13-docosenoate.

the LCFAs was not substrate dependent. There was substrate dependence only when the carbon backbone of the substrate was smaller than C_{12}, in which case the polymer was composed of monomers, of which the largest had the same carbon chain length as the substrate. When laureate or longer fatty acids were used, the largest monomer was either 3-hydroxyundecanoate or 3-hydroxydodecanoate, depending on the number of carbon atoms in the substrate. The composition of the polyesters formed on C_{even} fatty acids ($>C_{11}$) was approximately 1.3:8.9:4.7:1 for the C_6, C_8, C_{10}, and C_{12} monomers, respectively. After growth on C_{odd} fatty acids ($>C_{10}$), the composition was 2.3:3.4:1 for the C_7, C_9, and C_{11} monomers, respectively. Polymers formed on the larger fatty acids are thus identical to the "dodecanoate" or "tridecanoate" polymer.

DISCUSSION

We have previously described the accumulation of PHA composed of medium-chain-length 3-hydroxy fatty acids by *P. oleovorans* (5, 12). In this paper we extend these observations and show that other fluorescent pseudomonads are also capable of synthesizing PHAs under specific fermentation conditions (patent applied for).

P. oleovorans GPo1 was grown on 1-octanol and octanoate to determine the role of the alkane hydroxylase complex in the biosynthesis of PHA. These substrates supported both growth and polymer synthesis. *P. oleovorans* GPo12, which has been cured of the OCT plasmid, was also shown to produce PHA when grown on octanol and octanoate (Tables 2 and 3). The composition of the polymer resembled that found for the polymer formed by *P. oleovorans* GPo1 grown on octane (12) and on 1-octanol and octanoate. From these experiments, it has become clear that the enzymes involved in the synthesis of PHA are chromosomally encoded.

P. oleovorans is a *P. putida* strain, and the capacity of other *P. putida* strains to form PHA was investigated. When grown on octanol or octanoate, *P. putida* PpG1 and KT2442 were both able to accumulate PHA (Fig. 1). Like *P. oleovorans*, these species are both typed as *P. putida* biotype A strains (19).

In *Bergey's Manual* (7), *P. oleovorans* is included in section I of the pseudomonads, which comprises species unable to accumulate PHB. The strains of section I include the saprophytic fluorescent pseudomonads, such as *P. aeruginosa*, *P. putida*, and *P. fluorescens*. We found that *P. aeruginosa*, *P. fluorescens*, and *P. lemonnieri* (*fluorescens* biotype IV) synthesize PHA when grown on octanoate. In these polymers, 3-hydroxyoctanoate was the predominant monomer.

When *P. testosteroni*, a representative of group III of the pseudomonads (7), was grown on 3-hydroxybutyrate, PHB was formed. Interestingly, during growth on decanoate *P. testosteroni* also formed a polyester containing mainly 3-hydroxybutyrate. This indicates that the ability to synthesize polyesters with medium-chain-length monomers is not a characteristic of all pseudomonads.

The above results lead to the conclusion that PHA formation is neither a general pseudomonad characteristic nor restricted to *P. oleovorans*. Instead, PHA formation appears to be a common trait for a number of fluorescent pseudomonads. This new characteristic may be of interest for taxonomical studies and classifications.

Influence of substrate carbon chain length on the composition of the polyester. In the copolyesters formed by *P. oleovorans* on alkanes, 3-hydroxyoctanoate and 3-hydroxynonanoate are the predominant monomers (12). This preference might be a reflection of the fact that *P. oleovorans* is able to grow only on C_6 to C_{12} *n*-alkanes. Therefore, we tested whether alkanols and alkanoates are suitable substrates for PHA formation. Both alkanols and fatty acids were shown to support growth and polyester synthesis.

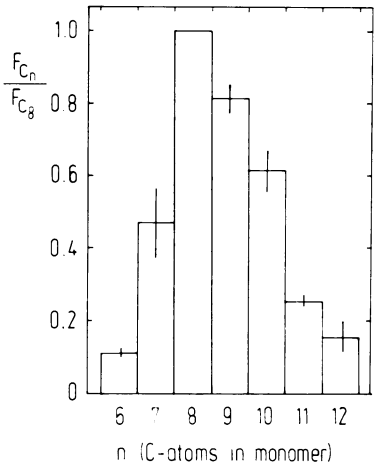

FIG. 2. Specificity of the enzyme system involved in PHA biosynthesis. *P. oleovorans* was grown on LCFAs in 50-ml 0.5*E2 cultures, and the composition of the polyesters formed was analyzed. The specificity of the system for each monomer (F_{C_n}) is shown relative to that for the 3-hydroxyoctanoate monomer (F_{C_8}). The fraction of 3-hydroxyoctanoate (F_{C_8}) in the PHAs formed on C_{even} LCFAs was taken as 1. The fraction of the other C_{even} monomers was calculated with respect to the C_8 monomer. Fractions of C_{odd} monomers were calculated likewise with respect to 3-hydroxynonanoate. The ratios between C_{even} and C_{odd} monomers were calculated from PHAs formed on equimolar mixtures of dodecanoate and tridecanoate and of tridecanoate and oleate, respectively. Values for relative specificity are means of 7 experiments. Bars indicate the range in which the values were found.

The synthesis of PHA by *P. oleovorans* with fatty acids as substrates was studied in more detail. No clear PHA was found when small-chain fatty acids ($<C_6$) were supplied. The small amount of 3-hydroxydecanoate and 3-hydroxydodecanoate found in the analysis of whole cells is partly due to fatty acids derived from lipopolysaccharides (16). As expected, we observed polymer synthesis when medium-chain fatty acids (C_6 to C_{12}) were used. The composition of the PHAs formed by *P. oleovorans* grown on these medium-chain fatty acids was substrate dependent, as in the case of alkanes (12).

Interestingly, when *P. oleovorans* was grown on C_{12} to C_{16} fatty acids, polyester was formed in considerable amounts. When C_{18} unsaturated fatty acids were added as the growth substrate, both growth and considerable PHA formation were observed. All polyesters formed after growth of *P. oleovorans* on LCFAs ($>C_{12}$) showed similar compositions. 3-Hydroxyoctanoate and 3-hydroxynonanoate were always predominant. In C_{even} LCFA-grown cells, the other PHA monomers were the C_6, C_{10}, and C_{12} 3-hydroxyalkanoates. In C_{odd} LCFA-grown cells, the other PHA monomers were 3-hydroxyheptanoate and 3-hydroxyundecanoate. Apparently the LCFAs are first degraded via β-oxidation (15) until intermediates are formed which can act as a substrate for the PHA-synthesizing system.

These findings indicate that the composition of the polyester formed by *P. oleovorans* (and probably other fluores-

cent pseudomonads) is determined both by the specificity of the enzymes in the synthesis of PHAs and by the structure of the substrate. The polymerizing enzyme system apparently shows a preference for C_8 and C_9 monomers, while longer and smaller monomers in the C_6 to C_{12} range are incorporated less efficiently (Fig. 2).

Synthesis of PHA and PHB. Although PHB and PHA are structurally related, they are not necessarily synthesized by a single bacterial strain or a common enzyme system. The specificity of the PHA-biosynthetic system may have evolved from C_4 3-hydroxy fatty acids to medium-chain-length 3-hydroxy fatty acids in strains which are able to grow on substrates with a long carbon backbone. For such strains, it may have been preferable to accumulate PHA instead of PHB, since formation of PHB requires degradation of the fatty acid to C_2 units and subsequent resynthesis of acetoacetyl-CoA, a process which results in considerable energy loss. Polymerization of longer 3-hydroxy fatty acids prevents the breakdown of energy-rich compounds during starvation and allows this energy to be used at a later stage, when growth is once again possible.

It remains to be determined how the PHA and PHB biosynthesis pathways differ and where in the PHA synthesis pathway the specificity for C_8 and C_9 3-hydroxy fatty acids is exerted. This requires a more complete understanding of the pathway and the enzymes involved in PHA formation. At present we are approaching this question by isolating mutants with altered PHA synthesis properties in order to study the genetics of PHA formation.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. **Bagdasarian, M., R. Lurz, B. Ruckert, F. C. H. Franklin, M. M. Bagdasarian, J. Frey, and K. N. Timmis.** 1981. Specific-purpose plasmid cloning vectors. II. Broad host range, high copy number, RSF1010-derived vectors for gene cloning in *Pseudomonas*. Gene **16:**237–247.
2. **Brandl, H., R. A. Gross, R. W. Lenz, and R. Clinton Fuller.** 1988. *Pseudomonas oleovorans* as a source of poly(3-hydroxyalkanoates) for potential application as biodegradable polyesters. Appl. Environ. Microbiol. **54:**1977–1982.
3. **Byng, G. S., J. L. Johnson, R. J. Whitaker, R. L. Gherna, and R. A. Jensen.** 1983. The evolutionary pattern of aromatic amino acid biosynthesis and the emerging phylogeny of pseudomonad bacteria. J. Mol. Evol. **19:**272–282.
4. **Dawes, E. A., and P. J. Senior.** 1973. Energy reserve polymers in microorganisms. Adv. Micro. Physiol. **10:**135–266.
5. **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.
6. **de Vos, P., and J. de Ley.** 1983. Intra- and intergenic similarities of *Pseudomonas* and *Xanthomonas* ribosomal ribonucleic acid cistrons. Int. J. Syst. Bacteriol. **33:**487–509.
7. **Doudoroff, M., and N. J. Palleroni.** 1974. Genus I: *Pseudomonas* migula, 1894, 237, p. 217–243. In R. E. Buchanan and N. E. Gibbons (ed.), Bergey's Manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
8. **Eggink, G., P. H. van Lelyveld, A. Arnberg, N. Arfman, C. Witteveen, and B. Witholt.** 1987. Structure of the *Pseudomonas putida alkBAC* operon. Identification of transcription and translation products. J. Biol. Chem. **262:**6400–6406.
9. **Grund, A., J. A. 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.
10. **Holloway, B. W.** 1969. Genetics of pseudomonads. Bacteriol. Rev. **33:**419–443.
11. **Jenkins, L. J., and W. D. Nunn.** 1987. Genetic and molecular characterization of the genes involved in short-chain fatty acid degradation in *Escherichia coli*: the *ato* system. J. Bacteriol. **169:**42–52.
12. **Lageveen, R. G., G. W. Huisman, H. Preusting, P. E. F. Ketelaar, G. Eggink, and B. Witholt.** 1988. Formation of polyesters by *Pseudomonas oleovorans*: the effect of substrates on the formation and composition of poly-(*R*)-3-hydroxyalkanoates and poly-(*R*)-3-hydroxyalkenoates. Appl. Environ. Microbiol. **54:**2924–2932.
13. **Lemoigne, M.** 1926. Produits de deshydration et de polymerisation de l'acide β-oxybutyrique. Bull. Soc. Chim. Biol. **8:**770–782.
14. **Marcus, P. I., and P. Talalay.** 1956. Induction and purification of α- and β-hydroxy steroid dehydrogenases. J. Biol. Chem. **218:**661–674.
15. **Nunn, W. D.** 1986. A molecular view of fatty acid catabolism in *Escherichia coli*. Microbiol. Rev. **50:**179–192.
16. **Oyaizu, H., and K. Komagata.** 1983. Grouping of *Pseudomonas* species on the basis of cellular fatty acid composition and the quinone system with special reference to the existence of 3-hydroxy fatty acids. J. Gen. Appl. Microbiol. **29:**17–40.
17. **Palleroni, N. J., R. Kunisawa, R. Contopoulou, and M. Doudoroff.** 1973. Nucleic acid homologies in the genus *Pseudomonas*. Int. J. Syst. Bacteriol. **23:**333–339.
18. **Schwartz, R. D., C. J. McCoy.** 1973. *Pseudomonas oleovorans* hydroxylation-epoxidation system: additional strain improvements. Appl. Microbiol. **26:**217–218.
19. **Stanier, R. Y., N. J. Palleroni, and M. Doudoroff.** 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. **43:**159–271.
20. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218:**97–106.
21. **Witholt, B.** 1972. Method for isolating mutants overproducing nicotinamide adenine dinucleotide and its precursors. J. Bacteriol. **109:**350–364.