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Pseudomonas putida KT2442 Cultivated on Glucose Accumulates Poly(3-Hydroxyalkanoates) Consisting of Saturated and Unsaturated Monomers

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The biosynthesis of poly(3-hydroxyalkanoates) (PHAs) by *Pseudomonas putida* KT2442 during growth on carbohydrates was studied. PHAs isolated from *P. putida* cultivated on glucose, fructose, and glycerol were found to have a very similar monomer composition. In addition to the major constituent 3-hydroxydecanoate, six other monomers were found to be present: 3-hydroxyhexanoate, 3-hydroxyoctanoate, 3-hydroxydodecanoate, 3-hydroxydodecenoate, 3-hydroxytetradecanoate, and 3-hydroxytetradecenoate. The identity of all seven 3-hydroxy fatty acids was established by gas chromatography-mass spectrometry, one-dimensional ^{1}H-nuclear magnetic resonance, and two-dimensional double-quantum filtered correlation spectroscopy ^{1}H-nuclear magnetic resonance. The chemical structures of the monomer units are identical to the structure of the acyl moiety of the 3-hydroxyacyl-acyl carrier protein intermediates of de novo fatty acid biosynthesis. Furthermore, the degree of unsaturation of PHA and membrane lipids is similarly influenced by shifts in the cultivation temperature. These results strongly indicate that, during growth on nonrelated substrates, PHA monomers are derived from intermediates of de novo fatty acid biosynthesis. Analysis of a *P. putida pha* mutant and complementation of this mutant with the cloned *pha* locus revealed that the PHA polymerase genes necessary for PHA synthesis from octanoate are also responsible for PHA formation from glucose.

Many bacteria are able to accumulate poly(3-hydroxyalkanoates) (PHAs) as a carbon and energy reserve. Biosynthesis of these polymers is triggered by a combination of nutrient limitation and carbon source excess. The composition of PHAs, of which poly(3-hydroxybutyrate) (PHB) is the most common, depends on the substrate used and the specificity of the PHA synthesizing system. The enzymology and genetics of PHB synthesis have been extensively studied for *Alcaligenes eutrophus*, which can accumulate considerable amounts of PHB from a number of carbon sources such as glucose, fructose, acetate, and butyrate. PHB is synthesized from acetyl-coenzyme A (CoA) by a sequence of three reactions catalyzed by 3-keto-thiolase, NADPH-dependent acetoacetyl-CoA reductase, and PHB synthase. Molecular cloning and nucleotide sequence analysis revealed that the PHB biosynthetic genes are clustered and presumably organized in one single operon (16–18, 21). The PHB polymerizing system appears to be highly specific, and, in addition to 3-hydroxybutyrate, only a limited number of other monomers, such as 3-hydroxyvalerate, 4-hydroxybutyrate (7), and 5-hydroxyvalerate (8), can be incorporated into the polymer.

Fluorescent *Pseudomonas* strains are unable to accumulate PHB, but they share the ability to produce medium-chain-length PHAs during unbalanced growth on medium- and long-chain alkanols and fatty acids (13). The PHA biosynthetic pathway is not yet fully elucidated, but it has been proposed that 3-hydroxyacyl-CoA intermediates of the β-oxidation pathway for the degradation of medium- and long-chain alkanols and fatty acids are substrates for the PHA polymerizing system (15). During growth on medium-chain-length fatty acids, *Pseudomonas putida* accumulates PHA-containing 3-hydroxy fatty acids which are, with respect to the carbon chain length, either directly derived from the substrate or shortened by one or more C_2 units.

Recently, Huisman et al. (14) described the cloning and characterization of the *P. oleovorans pha* locus, which was found to encode two PHA polymerases and, most likely, a PHA depolymerase. So far, no clear differences in function or specificity have been observed for the two PHA polymerases. Overexpression of the cloned polymerase genes in *P. putida* resulted in the formation of polymers with significantly higher amounts of monomers with the same carbon chain length as the substrate. Apparently, higher levels of PHA polymerase increase the flux of β-oxidation intermediates to PHA synthesis, thereby reducing the proportion of intermediates which are shortened by one or more C_2 units via continued β-oxidation.

It was generally assumed that PHAs could only be formed in *Pseudomonas* cells growing on medium- or long-chain fatty acids. We have found that *P. putida* also accumulates PHA during unbalanced growth on glucose and other substrates which are unrelated to alcohols or fatty acids. During the course of this study, the same phenomenon was reported by Timm and Steinbüchel (23) and by Haywood et al. (12). These authors demonstrated that several *Pseudomonas* strains, when grown on nonrelated substrates, accumulate PHAs which consist predominantly of 3-hydroxydecanoate monomer units. Minor constituents of these polymers were 3-hydroxyhexanoate, 3-hydroxyoctanoate, and 3-hydroxydodecanoate (12, 23).

Our objective is to study PHA biosynthesis in *P. putida* and its relationship with fatty acid metabolism. In this report, we focus on PHA formation from nonrelated substrates. Detailed gas chromatography-mass spectrometry (GC-MS) and two-dimensional double-quantum filtered cor-

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TABLE 1. Composition of PHA synthesized by *P. putida* KT2442 during growth on different carbon sources[a]

Substrate	% PHA[b]	Relative amt of monomers is purified PHA (%, wt/wt)[c]						
		C_6	C_8	C_{10}	$C_{12:1}$	C_{12}	$C_{14:1}$	C_{14}
Glucose	16.9	tr[d]	6.9	74.3	8.8	7.7	1.6	tr
Fructose	24.5	0.5	12.6	70.8	8.5	5.7	1.6	0.3
Glycerol	22.0	1.7	21.4	63.6	8.6	3.8	0.8	0.1
Decanoate	27.6	5.3	52.3	42.3	ND[e]	ND	ND	ND

[a] Cells were cultivated in 250 ml of medium in 1-liter Erlenmeyer flasks with 20 g of substrate per liter except in the case of glycerol, when 40 g/liter was used. Cultivation of *P. putida* on decanoate was performed in a 3-liter fermentor containing 20 mM decanoate in 1 liter of medium. Cells were harvested after the cell dry weight had reached approximately 1 g/liter.

[b] The amount of purified polymer was determined gravimetrically and calculated as the percentage of the cell dry mass. All experiments were performed in duplicate.

[c] C_6, 3-hydroxyhexanoate; C_8, 3-hydroxyoctanoate; C_{10}, 3-hydroxydecanoate; $C_{12:1}$, 3-hydroxy-5-*cis*-dodecenoate; C_{12}, 3-hydroxydodecanoate; $C_{14:1}$, 3-hydroxy-7-*cis*-tetradecenoate; C_{14}, 3-hydroxytetradecanoate.

[d] tr, trace amounts (<0.1%), wt/wt).

[e] ND, not detectable.

relation spectroscopy (2D-DQF-COSY) ^{1}H-nuclear magnetic resonance (NMR) analyses revealed the presence of both saturated and unsaturated 3-hydroxy fatty acids in these PHAs. All monomers could be identified as intermediates which occur during de novo biosynthesis of fatty acids. Similar to the fatty acid composition of membrane lipids, the ratio of saturated to unsaturated monomers in PHA is subject to thermal regulation. Furthermore, we present evidence showing that the PHA polymerases responsible for PHA synthesis from fatty acids are also involved in PHA synthesis from glucose.

MATERIALS AND METHODS

Bacterial strains and plasmids. *P. putida* KT2442 (1); *P. putida* GPp104, a PHA$^-$ mutant of *P. putida* KT2442 (14); and *P. oleovorans* GPo1 (19) were used. The following plasmids were used: pGEc401, pLAFR1 containing *phaA*, *phaB*, and *phaC*; pGEc404, pJRD215 containing *phaC*; and pGEc405, pJRD215 containing *phaA* (14). Mobilization of recombinant plasmids from *Escherichia coli* to *P. putida* GPp104 was performed by the triparental mating procedure of Friedman et al. (10). The exconjugants were selected by replica plating on a minimal medium containing octanoate and an appropriate antibiotic (tetracycline, 15 μg/ml; kanamycin, 20 μg/ml).

Media and growth conditions. *P. putida* was grown on various carbon sources in 0.5× E2 medium, which is a minimal salts medium (15). In 0.5× E2 medium, the concentrations of $NaNH_4HPO_4$, K_2HPO_4, and KH_2PO_4 are half the concentrations of these salts in E2 medium. The amount of ammonium in this medium (0.15 g/liter) is depleted at the end of the growth phase. Glucose (20 g/liter), fructose (20 g/liter), glycerol (40 g/liter), and octanoate and decanoate (20 mM each) were the carbon sources. The cultures were inoculated from exponentially growing precultures. Cells were cultivated in Erlenmeyer flasks or in a 3-liter batch fermentor with a working volume of 1.5 liters with temperature control (Applikon, Schiedam, The Netherlands). The stirrer speed in the fermentor was kept constant at 600 rpm. The fermentor cultures were aerated at a flow of 6 liters/h. Cultures in Erlenmeyer flasks were incubated in an orbital incubator at 150 rpm (Gallenkamp) at the desired temperature. Cell densities were measured spectrophotometrically at 450 nm (24). Cultures were harvested by centrifugation, washed, and lyophilized.

PHA determination. Analysis of the PHA composition was performed essentially as described by Lageveen et al. (15). Lyophilized cells (approximately 15 mg) or purified PHA (approximately 5 mg) was dissolved in 2 ml of 15% sulfuric acid in methanol. Two milliliters of chloroform containing 0.5 mg of methylbenzoate (internal standard) per ml was added, and the mixture was heated for 140 min at 100°C under stirring in a closed screw-cap tube. The sample was subsequently cooled on ice, and 1 ml of demineralized water was added. After vortexing for 1 min, phases were separated by centrifugation for 5 min at 3,500 × *g*. The organic phase was collected, dried over Na_2SO_4, and analyzed by gas-liquid chromatography. Analysis of the methyl esters was performed on a Carlo Erba GC6000 GC (Carlo Erba, Milan, Italy) equipped with a CP-Sil 5CB column (25 m by 0.32 mm; Chrompack, Middelburg, The Netherlands) operating in a split mode (split ratio, 1:40) with temperature programming. A bacterial fatty acid methyl ester standard mixture (Supelco, Inc., Bellefonte, Pa.) was used for peak identification. GC-MS spectra were obtained by using a Carlo Erba HRGC/MS GC equipped with a CP-Sil 5CB column attached by a direct interface to a Carlo Erba QMD 1000 MS. Spectra were taken as electron impact spectra (70 eV) or as chemical ionization spectra, using isobutane as the reacting agent with a scan rate of 1 s^{-1}.

Polymer isolation and lipid extraction. PHAs were extracted from lyophilized cells (approximately 2 g) with 200 ml of chloroform in a soxhlet apparatus for 3.5 h. The extract was cooled to room temperature, and chloroform was removed with a rotary film evaporator. The polymer was redissolved in 15 ml of chloroform and precipitated with 150 ml of methanol under vigorous agitation for 5 min, after which precipitation was continued for 2 h. The methanol-chloroform mixture was decanted, and the polymer was dried on air. Phospholipids and neutral lipids were extracted as described by de Smet et al. (6). The fatty acid composition of these membrane lipids was analyzed by gas-liquid chromatography by the same procedure as described for the PHA determination. A bacterial fatty acid methyl ester standard mixture (Supelco) was used for peak identification.

^{1}H-NMR analysis. Samples were dissolved in deuterated chloroform, and ^{1}H-NMR spectra were recorded on a Bruker AMX 400 wb NMR spectrometer at a probe temperature of 27°C. For the 2D-DQF-COSY (5), 512 experiments of 1,024 complex data points were recorded. The time domain data were multiplied with a phase-shifted sine-bell. Phase-sensitive Fourier transformation was performed after zero filling to a 1,024 × 512 complex data size.

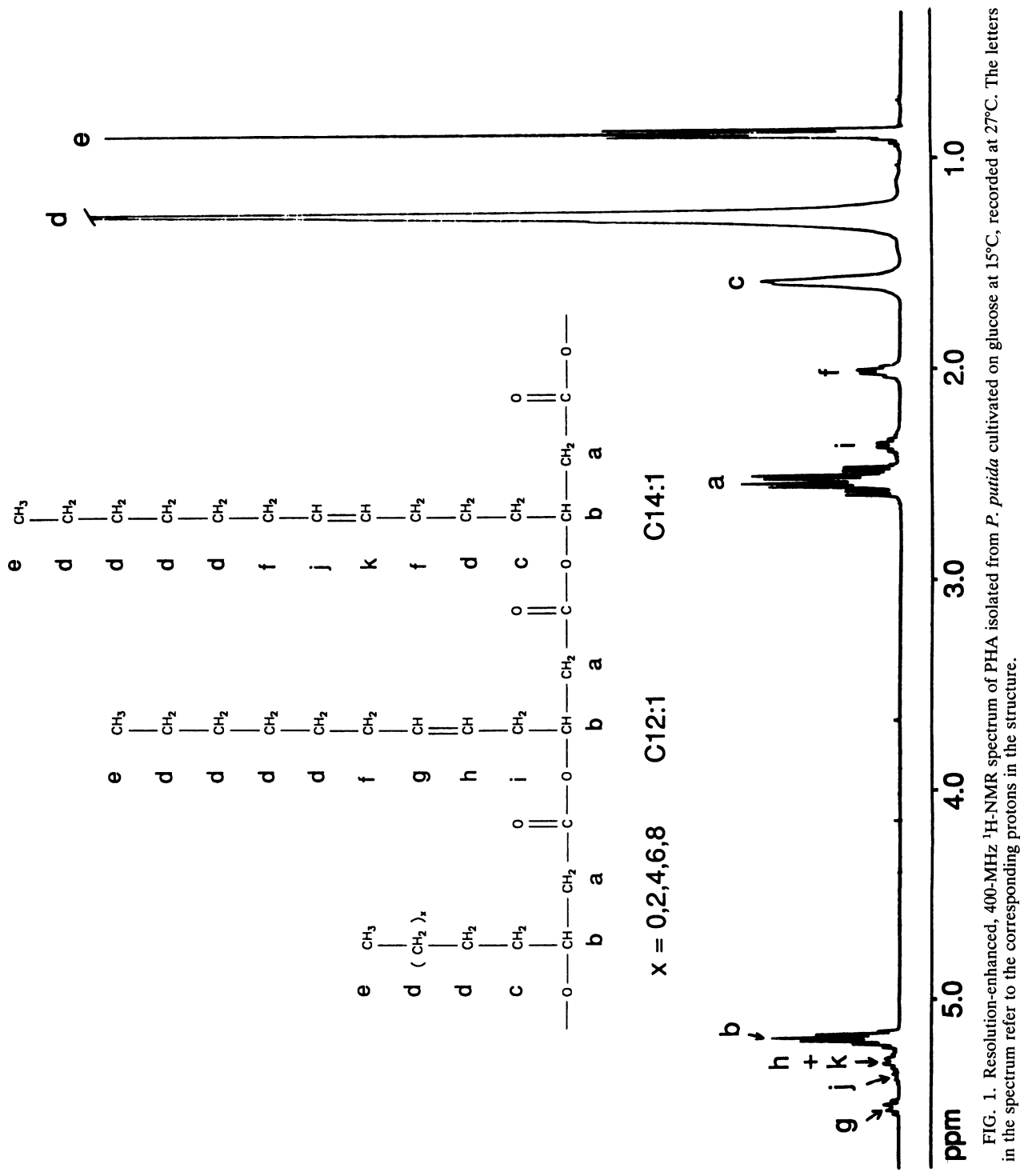

FIG. 1. Resolution-enhanced, 400-MHz ^{1}H-NMR spectrum of PHA isolated from *P. putida* cultivated on glucose at 15°C, recorded at 27°C. The letters in the spectrum refer to the corresponding protons in the structure.

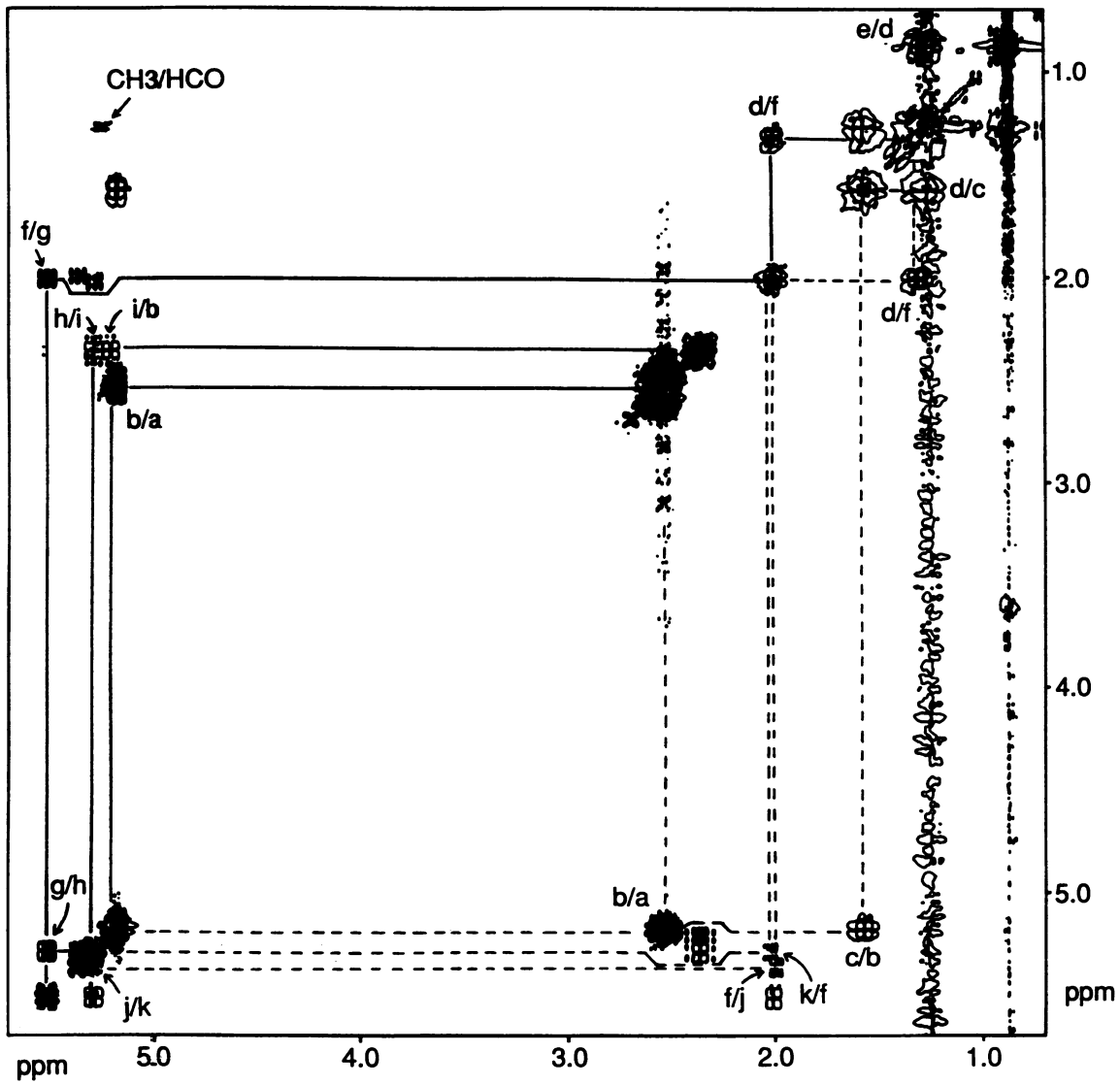

FIG. 2. 2D-DQF-COSY spectrum of PHA isolated from *P. putida* cultivated on glucose at 15°C, recorded at 27°C. The cross-peaks between protons are indicated by using the same letter code as for the one-dimensional spectrum. The assignments for $C_{12:1}$ (——) and $C_{14:1}$ (– – –) are shown.

RESULTS

Formation of PHA from nonrelated substrates. We have found that *P. putida* KT2442 is capable of producing PHAs from substrates such as glucose and glycerol. PHA accumulation is observed when the cultivation medium contains an excess of carbon source (>10 g/liter) and a limiting amount of nitrogen. To investigate PHA formation by *P. putida* on nonrelated carbon sources, we have cultivated this strain on glucose, fructose, glycerol, and decanoate. Decanoate was used as a reference substrate since PHA formation on this substrate has been well described (2, 11). Polymer accumulation in cells growing on nonrelated substrates generally occurs within 72 h. When decanoate is used as the growth substrate, PHA synthesis is seen within 48 h. The yield of PHA in *P. putida* cultivated on nonrelated substrates can amount to approximately 25% of the cell dry mass. Upon prolonged starvation, however, the polymer is metabolized again (data not shown).

To assure an accurate determination of the PHA compositions, we have purified all polymers from the *P. putida* cultures (Table 1). The methanolyzed PHAs were analyzed by GC, and the 3-hydroxy fatty acid methyl esters were identified by comparison with a standard mixture of bacterial fatty acid methyl esters. PHAs formed on glucose, fructose, and glycerol contained five saturated monomers: 3-hydroxyhexanoate, 3-hydroxyoctanoate, 3-hydroxydecanoate, 3-hydroxydodecanoate, and 3-hydroxytetradecanoate. 3-Hydroxydecanoate is the predominant compound of these polymers. Interestingly, two additional monomers were found to be present in all polymers. On the basis of gas-liquid chromatography data, we tentatively identified these compounds as 3-hydroxydodecenoate and 3-hydroxytetradecenoate. The identities of the 3-hydroxy methyl esters were further established by using electron impact and chemical ionization MS. The GC-MS spectra obtained with the standard mixture of bacterial fatty acid methyl esters were used as a reference.

TABLE 2. Influence of cultivation temperature on PHA composition in *P. putida*[a]

Substrate	Temp (°C)	% PHA[b]	Relative amt of monomers in purified PHA (%, wt/wt)[c]						
			C_6	C_8	C_{10}	$C_{12:1}$	C_{12}	$C_{14:1}$	C_{14}
Glucose, 2%	15	20.5	0.4	8.4	64.5	16.7	6.0	4.0	tr
	30	17.0	tr[d]	6.9	74.3	8.8	7.7	1.6	tr
Decanoate, 20 mM	15	48.5	5.9	50.9	43.2	ND[e]	ND	ND	ND
	30	38.1	5.3	52.3	42.4	ND	ND	ND	ND

[a] Cells were cultivated in 200 ml of medium in 500-ml Erlenmeyer flasks with 20 g of glucose per liter or 20 mM decanoate at 15°C. Cultivation of *P. putida* at 30°C on glucose and decanoate was performed 1 liter of medium in a 3-liter fermentor. Cells were harvested after the cell dry weight had reached approximately 1 g/liter.

[b] The amount of purified polymer was determined gravimetrically and calculated as the percentage of the cell dry mass. All experiments were performed in duplicate.

[c] C_6, 3-hydroxyhexanoate; C_8, 3-hydroxyoctanoate; C_{10}, 3-hydroxydecanoate; $C_{12:1}$, 3-hydroxy-5-*cis*-dodecenoate; C_{12}, 3-hydroxydodecanoate; $C_{14:1}$, 3-hydroxy-7-*cis*-tetradecenoate; C_{14}, 3-hydroxytetradecanoate.

[d] tr, trace amounts (<0.1%, wt/wt).

[e] ND, not detectable.

The molecular weights of all PHA-derived 3-hydroxyalkanoate methyl esters, including 3-hydroxydodecenoate methyl ester and 3-hydroxytetradecenoate methyl ester, were confirmed by chemical ionization GC-MS.

One-dimensional and 2D ^{1}H-NMR analysis of PHA. To establish the structure of the monomers unequivocally, PHA purified from glucose-grown *P. putida* was analyzed with ^{1}H-NMR. The ^{1}H-NMR spectrum together with the assignments of PHA are depicted in Fig. 1. Peaks a and b can be assigned to protons next to the carboxyl and hydroxy groups, respectively; peak c, to the first CH_2 of a saturated alkane side chain; and peaks d and e, to the remaining CH_2 groups and the CH_3 group, respectively. The chemical shifts of peaks g, h, j, and k indicate the presence of an unsaturated group in some of the monomers.

As a tool to assign all signals in the proton spectrum, 2D-DQF-COSY spectroscopy is a well-established technique in ^{1}H-NMR. In a 2D-DQF-COSY spectrum, cross-peaks are present between coupled proton signals belonging to neighboring protons of the structure. Going from proton to proton, it is possible to "walk" through a structure. In this way, the position of a double bond in an unsaturated alkyl chain can be established. In the 2D-DQF-COSY spectrum (Fig. 2), a cross-peak is present between peak g, which, considering its chemical shift, belongs to a C—C double bond, and peak f. For this reason, peak f is assigned to a CH_2 group next to a double bond. Compared with peak d, peak f is shifted with $\Delta\delta = 0.74$ ppm. The chemical shift of peak i, compared with that of peak d ($\Delta\delta = 1.10$ ppm), can be explained as the addition of the effects of a neighboring unsaturated group (cf. peak f) and a hydroxyl group (cf. peak c; $\Delta\delta = 0.32$ ppm). The assignment of peak i as the CH_2 group between the hydroxyl group and a double bond can be deduced from the 2D-DQF-COSY spectrum. In this way, the presence of unsaturated compounds with a double bond extending from C-5 is demonstrated. An assignment pathway for 3-hydroxydodecenoate ($C_{12:1}$) from peak d to peak a via peaks f, g, h, i, and b is indicated in the 2D-DQF-COSY spectrum. On the basis of the molecular weight as determined by GC-MS, we conclude that this compound must be 3-hydroxy-5-dodecenoate ($C_{12:1\Delta5}$). The coupling between peaks g and h of $J = 10.4$ Hz indicates a *cis* configuration of the double bond.

The integration of peak f cannot be completely ascribed to $C_{12:1}$. Instead, this signal can partly be assigned to both CH_2 groups adjacent to the double bond of 3-hydroxy-7-tetradecenoate ($C_{14:1\Delta7}$). Peak j is assigned to the double bond of $C_{14:1\Delta7}$. A cross-peak close to the diagonal in the 2D-DQF-COSY spectrum reveals the presence of a peak k coinciding with peak h. The assignment pathway for $C_{14:1}$ is also indicated in Fig. 2. Because of the different positions for peak f within $C_{14:1}$ and $C_{12:1}$, slightly different chemical shifts are found for peak f, which is evident from the 2D-DQF-COSY spectrum. From the cross-peak with peak i, it can be concluded that peak b for $C_{12:1}$ is shifted due to the vicinity of the double bond. The integration of peak j and the differences in the integrations between peak i and f and between peaks g and h indicate amounts of 4% $C_{14:1}$ and 15% $C_{12:1}$, which is in accordance with GC and GC-MS data. A cross-peak between the hydroxy region and the CH region can be ascribed to a (shifted) CH_3 attached to an HCO, indicating the presence of trace amounts of 3-hydroxybutyrate. In the one-dimensional spectrum, these signals cannot be recognized since they coincide with other stronger signals.

Influence of temperature on PHA composition. We have examined whether a change in the cultivation temperature, which controls the ratio of saturated to unsaturated fatty

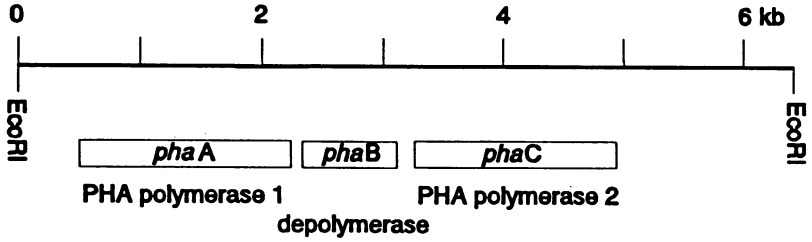

FIG. 3. PHA locus of *P. oleovorans* GPo1.

TABLE 3. Composition of PHA in *P. putida* KT2442, *P. putida* GPp104, *P. oleovorans* GPO1, and recombinant strains of *P. putida* GPp104 cultivated on glucose and octanoate[a]

Substrate	Strain	% PHA[b]	Relative amt of monomers in PHA (%, wt/wt)[c]						
			C_6	C_8	C_{10}	$C_{12:1}$	C_{12}	$C_{14:1}$	C_{14}
Glucose, 2%	KT2442	16.0	tr[d]	9.2	68.1	10.2	8.8	2.5	1.0
	GPp104	<0.1	ND[e]	ND	ND	ND	ND	ND	ND
	GPO1	<0.1	ND	ND	ND	ND	ND	ND	ND
	GPp104/*phaA*	13.5	tr	6.0	65.6	10.7	11.8	3.9	2.0
	GPp104/*phaC*	14.0	tr	9.7	66.8	11.5	12.0	tr	tr
	GPp104/*phaA*/*phaB*/*phaC*	19.7	0.5	7.8	64.7	11.8	11.0	tr	1.1
Octanoate, 20 mM	KT2442	39.0	6.3	92.0	1.7				
	GPp104	<0.1	ND	ND	ND				
	GPO1	50.5	6.0	91.7	2.3				
	GPp104/*phaA*	39.9	10.6	88.0	1.4				
	GPp104/*phaC*	19.9	2.8	94.6	2.6				
	GPp104/*phaA*/*phaB*/*phaC*	37.3	6.4	88.9	4.7				

[a] Cells were cultivated in 50 ml of medium containing either 20 mM octanoate or 20 g of glucose per liter supplemented with the appropriate antibiotic (tetracycline, 15 μg/ml; kanamycin, 20 μg/ml) in 300-ml Erlenmeyer flasks. After the cell dry weight had reached approximately 1 g/liter, cells were harvested, washed, and lyophilized.

[b] The PHA content of the cells was determined in whole cells by GC analysis and was calculated as a percentage of the cell dry weight.

[c] C_6, 3-hydroxyhexanoate; C_8, 3-hydroxyoctanoate; C_{10}, 3-hydroxydecanoate; $C_{12:1}$, 3-hydroxy-5-*cis*-dodecenoate; C_{12}, 3-hydroxydodecanoate; $C_{14:1}$, 3-hydroxy-7-*cis*-tetradecenoate; C_{14}, 3-hydroxytetradecanoate. With octanoate as the substrate, $C_{12:1}$, C_{12}, $C_{14:1}$, and C_{14} were not detectable in any of the strains tested.

[d] tr, trace amounts (<0.1%, wt/wt).

[e] ND, not detectable.

acids in membrane lipids (3), would also affect the ratio of saturated to unsaturated monomers in PHA. To this end, *P. putida* was grown on glucose and decanoate at two different temperatures (15 and 30°C) and PHA was isolated from these cells. Decanoate was used as the reference substrate because unsaturated monomers have not been found in PHA produced from this compound at 30°C.

Similar amounts of polymer were formed at both temperatures, and all previously identified monomers were found in the PHAs (Table 2). PHA isolated from cells cultivated on glucose at 15°C contained 20% unsaturated monomers, whereas PHA purified from *P. putida* grown at 30°C contained 10% unsaturated 3-hydroxy fatty acids. PHAs synthesized from decanoate contained no unsaturated monomers, and the composition of this polymer did not vary with the growth temperature.

The observed temperature effect could be the result of differences in fatty acid metabolism of *P. putida* growing on decanoate and glucose. Therefore, we have determined the ratio of saturated to unsaturated fatty acids in the membrane lipids of cells which were cultivated on these substrates. The fatty acids which we have included in our calculations are palmitic acid, palmitoleic acid, vaccenic acid, and stearic acid. We found that, in glucose-grown cells, the ratio of saturated to unsaturated fatty acids in the membranes increased from 0.5 at 15°C to 1.3 at 30°C. In cells cultivated on decanoate, this ratio increased from 0.5 at 15°C to 1.5 at 30°C, showing that the thermal regulation of membrane fatty acid composition in *P. putida* is not influenced by using glucose or decanoate as the carbon and energy source.

Genetic analysis of PHA synthesis from glucose. *P. putida* GPp104 is a mutant which was selected by Huisman et al. (14) for its inability to synthesize PHA from octanoate. The *pha* locus was cloned from *P. oleovorans* and was found to consist of two PHA polymerase genes (*phaA* and *phaC*) and a PHA depolymerase gene (*phaB*) (Fig. 3). We have examined whether the PHA polymerase genes responsible for the PHA synthesis on octanoate are also involved in the formation of PHA from glucose. First, we cultivated *P. oleovorans* GPo1, *P. putida* KT2442, and *P. putida* GPp104 on glucose and octanoate and examined the cells for polymer formation.

GPp104 was found to be unable to synthesize PHA from glucose or octanoic acid, indicating that the *pha* mutation affects the synthesis of PHA from both carbohydrates and fatty acids. Interestingly, *P. oleovorans* GPo1 also is not capable of accumulating PHA during growth on glucose. After mobilization of the *pha* genes in GPp104, we cultivated the recombinant strains on glucose and octanoate. The cells were analyzed for PHA production, and the composition of the polymer was determined (Table 3). Introduction of either *phaA* or *phaC* into GPp104 was sufficient for restoration of the PHA phenotype. The recombinant plasmids carrying the complete *pha* locus complemented the mutation as well. In the recombinant strains, the PHA yields are comparable to those in the wild-type strain. All previously identified monomers were present in the PHAs of the recombinant strains, and no major differences in composition were found. These results clearly show that the genes responsible for PHA formation on octanoate are also involved in polymer synthesis from glucose.

DISCUSSION

Formation of PHAs from nonrelated substrates. We have analyzed PHA accumulation by *P. putida* KT2442 during growth on nonrelated substrates, such as glucose, fructose, and glycerol. In addition to the predominant monomer, 3-hydroxydecanoate, six other monomers with chain lengths ranging from 6 to 14 carbon atoms were found to be present in these copolymers. Two of the monomers contain unsaturated bonds, namely, 3-hydroxy-*cis*-5-dodecenoic acid and 3-hydroxy-*cis*-7-tetradecenoic acid. All monomers were identified by combined analysis with gas-liquid chromatography, GC-MS, and NMR.

Recently, Haywood et al. (12) and Timm and Steinbüchel (23) showed that many *Pseudomonas* strains share the ability to accumulate PHA from simple carbohydrate substrates. They identified mainly 3-hydroxydecanoate and

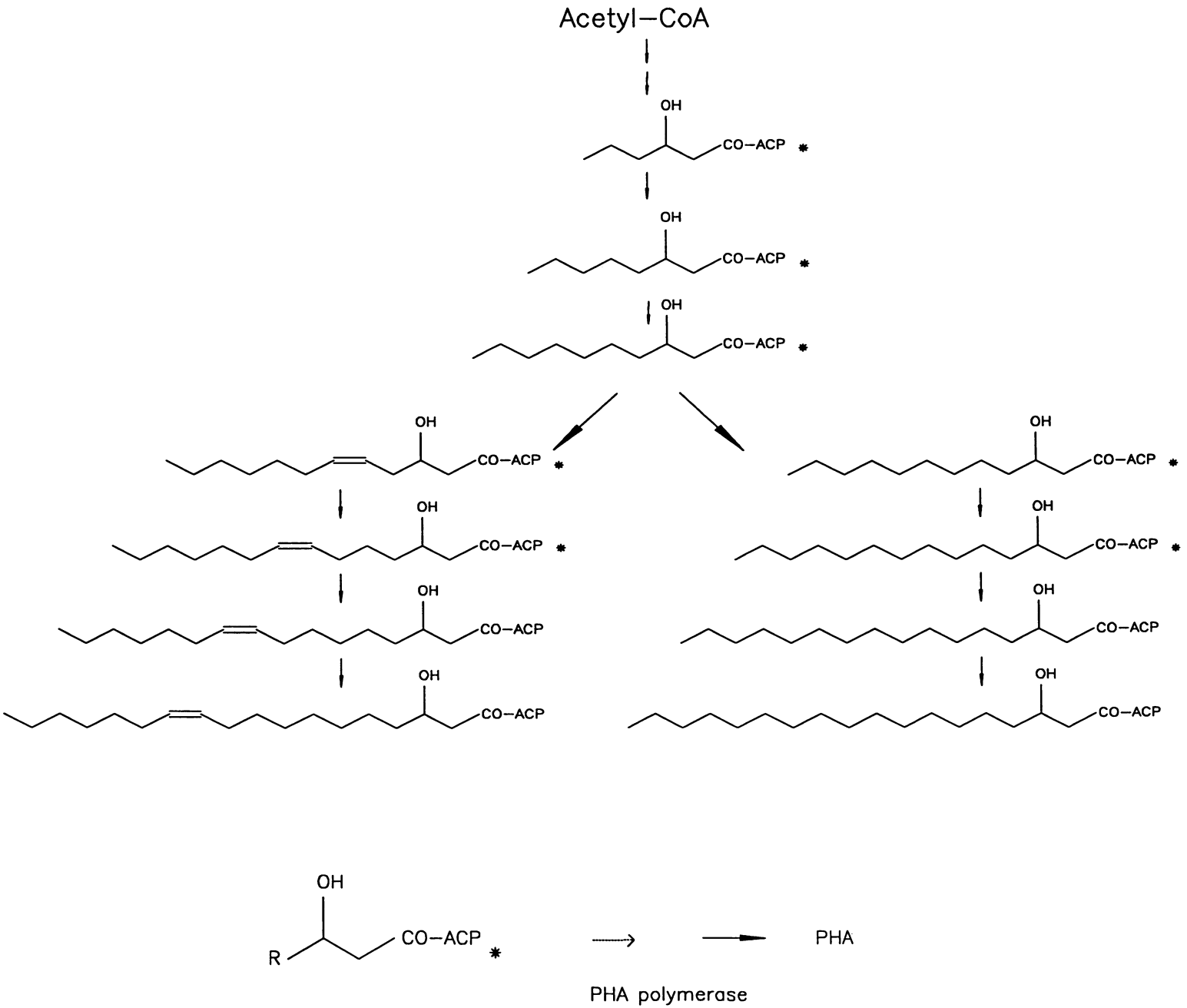

FIG. 4. Postulated relationship between fatty acid biosynthesis and PHA formation on nonrelated substrates. The upper part shows 3-hydroxyacyl intermediates which occur during elongation and diversification of the fatty acid biosynthesis. The intermediates marked with an asterisk (*) can be incorporated in PHA. At present, it is not known whether enzymes other than PHA polymerases are involved in the polymerization.

some 3-hydroxyhexanoate, 3-hydroxyoctanoate, and 3-hydroxydodecanoate in these PHAs. They did not report the presence of unsaturated monomers. Examination of their ^{1}H-NMR data for PHA isolated from strain NCIMB 40135 (12), however, reveals that the PHA formed by this strain also contains approximately 20% unsaturated monomers. We conclude, therefore, that the PHAs accumulated by *Pseudomonas* strain NCIMB 40135 and *P. putida* KT2442 during growth on carbohydrates are composed of the same saturated and unsaturated 3-hydroxy fatty acids and that the incorporation of unsaturated fatty acids is a common feature of fluorescent pseudomonads during growth on nonrelated substrates.

De novo fatty acid synthesis and PHA formation. The similarity in composition of PHA formed on different nonrelated substrates indicates that a common intermediate in the metabolism of these substrates is the precursor in the synthesis of PHA monomers. Acetyl-CoA is a likely candidate for this because of its central role in the carbohydrate metabolism of *Pseudomonas* spp. The presence of both saturated and unsaturated hydroxy fatty acids in PHA and the fact that seven PHA monomers also occur as sequential intermediates in the fatty acid biosynthetic pathway of bacteria (20) suggest a possible linkage between de novo fatty acid biosynthesis and PHA synthesis. The point at which synthesis of unsaturated fatty acids diverges from that of saturated fatty acids is clearly reflected in the monomer composition of PHA. The monomers 3-hydroxy-5-*cis*-dodecenoate and 3-hydroxy-7-*cis*-tetradecenoate exactly match the acyl moieties of 3-hydroxyacyl-acyl carrier protein intermediates of the unsaturated fatty acid biosynthetic pathway.

Thermal regulation of fatty acid and PHA syntheses. PHA synthesis on nonrelated substrates and de novo fatty acid synthesis both respond to changes in cultivation temperature. In general, microorganisms synthesize membranes with a greater proportion of unsaturated fatty acids when cultivated at low temperatures. This regulatory system is believed to control the physical state of the membrane lipids and thus to maintain optimal functioning of the membranes at various growth temperatures (3, 4). The shift towards a higher level of de novo unsaturated fatty acid biosynthesis at low temperatures changes the ratio of saturated to unsaturated fatty acids. The saturated/unsaturated fatty acid ratio in the lipids of *P. putida* decreased with decreasing growth temperature and appeared to be independent of the carbon source used (glucose or decanoate). The composition of the PHA isolated from *P. putida* grown on glucose varied in the same way with changing cultivation temperature, whereas the composition of PHA formed from decanoate remained constant. These findings reinforce the assumption that there are at least two distinct substrate supply routes for PHA synthesis in *P. putida*.

Relationship between substrate routes and PHA polymerases. The *pha* locus of *P. oleovorans* consists of two genes encoding a PHA polymerase and a third gene encoding a PHA depolymerase (Fig. 3). We have shown that each of the polymerase genes enables the synthesis of PHA in *P. putida* from octanoate as well as glucose, which implies that both genes have identical functions in the formation of PHA.

P. oleovorans GPo1 is not able to form PHA when cultivated on glucose or other nonrelated substrates. Our results indicate that this must be ascribed to differences in the physiology between *P. oleovorans* GPo1 and *P. putida* KT2442, since the *P. oleovorans pha* genes can restore PHA formation from glucose in *P. putida* GPp104. This conclusion is in agreement with the results obtained by Timm et al. (22), who demonstrated that *P. oleovorans* carrying the cloned PHB genes accumulates PHB more readily on fatty acids than on carbohydrates. Timm et al. assumed that, due to an efficient regulation of the catabolism of carbohydrates, little acetyl-CoA is accumulated in these cells.

On the basis of the results presented in this report, we propose a model for the biosynthetic route of PHA from nonrelated substrates (Fig. 4). In this model, glucose is converted to acetyl-CoA, which is directed to the de novo fatty acid biosynthesis. The intermediates in the fatty acid biosynthesis, which are (*R*)-3-hydroxyalkanoates-acyl carrier protein, can be converted to a substrate for the PHA polymerase system. At present, it is not clear whether the acyl moieties of (*R*)-3-hydroxyalkanoates-acyl carrier protein can be directly incorporated in the polymer or whether a transfer to CoA prior to polymerization is required.

During growth on fatty acids, the PHA biosynthetic route is linked to the β-oxidation cycle (11, 15). Therefore, we have to consider the possibility that PHAs can also be produced via β-oxidation of newly synthesized long-chain saturated and unsaturated fatty acids. It is, however, not very likely that during growth on carbohydrates all monomers are derived from the β-oxidation cycle, since PHAs synthesized from substrates such as oleic acid and palmitic acid consist predominantly of 3-hydroxyoctanoate and 3-hydroxydecanoate (9, 13). The principal monomer of PHAs formed on nonrelated substrates is 3-hydroxydecanoate, whereas 3-hydroxyoctanoate is only a minor constituent. Nevertheless, it remains to be determined to what extent PHA monomers are derived from intermediates of fatty acid biosynthesis and intermediates of β-oxidation. Further genetic and enzymatic studies are needed to elucidate in more detail the relationship between fatty acid metabolism and PHA biosynthesis in *P. putida*.

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