Novel biodegradable bacterial plastics, made up of units of 3-hydroxy-n-phenylalkanoic acids, are accumulated intracellularly by Pseudomonas putida U due to the existence in this bacterium of (i) an acyl-CoA synthetase (encoded by the fadD gene) that activates the aryl-precursors; (ii) a β-oxidation pathway that affords 3-OH-aryl-CoAs, and (iii) a polymerization-depolymerization system (encoded in thepha locus) integrated by two polymerases (PhaC1 and PhaC2) and a depolymerase (PhaZ). The complete assimilation of these compounds requires two additional routes that specifically catabolize the phenylacetyl-CoA or the benzoyl-CoA generated from these polymerases through β-oxidation.

Genetic studies have allowed the cloning, sequencing, and disruption of the genes included in thepha locus (phaC1, phaC2, andphaZ) as well as those related to the biosynthesis of precursors (fadD) or to the catabolism of their derivatives (acuA, fadA, and paa genes). Additional experiments showed that the blockade of eitherfadD orphaC1 hindered the synthesis and accumulation of plastic polymers. Disruption ofphaC2 reduced the quantity of stored polymers by two-thirds. The blockade ofphaZ hampered the mobilization of the polymer and decreased its production. Mutations in thepaa genes, encoding the phenylacetic acid catabolic enzymes, did not affect the synthesis or catabolism of polymers containing either 3-hydroxyaliphatic acids or 3-hydroxy-n-phenylalkanoic acids with an odd number of carbon atoms as monomers, whereas the production of polymers containing units of 3-hydroxy-n-phenylalkanoic acids with an even number of carbon atoms was greatly reduced in these bacteria. Yield-improving studies revealed that mutants defective in the glyoxylid acid cycle (isocitrate lyase) or in the β-oxidation pathway (fadA), stored a higher amount of plastic polymers (1.4- and 2-fold, respectively), suggesting that genetic manipulation of these pathways could be useful for isolating overproducer strains. The analysis of the organization and function of thepha locus and its relationship with the core of the phenylacetyl-CoA catabolon is reported and discussed.

Polyhydroxyalkanoates (PHAs) are naturally occurring polymers synthesized by different bacteria when cultured under a wide variety of nutritional and environmental conditions (1–8). Some species accumulate these polymers only when an essential nutrient becomes limiting, whereas others store these compounds from the very early exponential phase of growth (9–11). All PHAs known to date are accumulated intracellularly as reserve materials or as a sink for reducing equivalents (2), and they start to be mobilized when the carbon or energy source is exhausted from the culture broth (4, 8, 12). Regarding the monomer content, the structure of PHAs is quite variable (more than 80 molecules have been reported as constituents of PHAs), and their composition depends on the bacterial strain and growth conditions (12). Although PHAs are commonly stored as heteropolymers or copolymers (1–8, 13), in some cases only homopolymers (poly-3-hydroxybutyric, poly-3-hydroxyhexanoic, and 3-hydroxyheptanoic acid) are accumulated (13–14). Generally speaking, most PHAs reported to date can be included in two different groups: those containing units of 3-hydroxybutyrate or close derivatives (short chain length hydroxyfatty acids) (called PHBs) (4, 15–18) and those consisting of medium chain length hydroxyfatty acids (C10–C12) or related compounds (2–4, 9, 19–28). The general structure of both species of polymers, is as shown in Scheme 1.

![Scheme 1](http://www.jbc.org)

Here R represents an aliphatic chain ranging between 1 and 9 carbon atoms.

In recent years, physicochemical studies on PHAs (mechan-
ical properties and general characteristics) have received considerable attention due to their biotechnological applications and industrial relevance. In this sense, these polyesters are used as biodegradable and as biocompatible thermoplastic materials (29–31). Furthermore, the interest in their occurrence in nature (1–8), their physiological role (32–33), and their molecular organization and polymer architecture (34–39) has led to the accumulation of an impressive body of knowledge about this topic (2–7). As a consequence, genetic, biochemical, and engineering approaches have focused on reconstituting in vitro the main enzymatic steps involved in PHA biosynthesis and/or degradation (14, 40–45) as well as on expressing the biosynthetic genes in different prokaryotic or eukaryotic systems (2, 7, 14, 43–48).

Although dozens of different bacterial PHAs have been reported in the last decade (1–6), to the best of our knowledge, only two species, *Pseudomonas oleovorans* (49–51) and *Pseudomonas putida* BM01 (52), synthesize plastic polyesters containing an aromatic ring in the monomer. These unusual polymers, poly-(3-hydroxyphenoxyalkanoates), were accumulated when this strain was cultured in a chemically defined medium containing ω-phenoxyalkanoates as carbon sources (50). However, when other structurally related compounds (n-phenylalkanoates, PhAs) were tested as polymer precursors, it was observed that *P. oleovorans* was able to synthesize only a homopolymer of 3-hydroxy-5-phenylpentoic acid when grown in the presence of 5-phenylpentoic acid (49–51).

The restricted capacity of plastic-producing bacteria to store polyesters bearing a phenyl group suggests that the synthesis of these unusual compounds requires (i) a specific uptake system for the transport of the aromatic precursors, (ii) a specific acyl-CoA synthetase (ACS), (iii) a specific polymerase or a mutated enzyme with broader substrate specificity (14), and/or (iv) the existence of an additional catabolic route, linked to the β-oxidation pathway, to ensure complete assimilation of the β-oxidation products (benzoyl-CoA or phenylacetyl-CoA) generated from the monomers (3-hydroxyphenoxyalkanoic-CoA derivatives) once released from the stored polymer (Fig. 1) (9). This complete assimilation could reduce the possibility that intermediate metabolites might inhibit the biosynthetic process.

In the present work, we describe the existence of novel natural aromatic plastics and analyze their structure for the first time. Genetic and biochemical studies were also performed in order to establish the sequence and characteristics of the genes and enzymes specifically involved in the synthesis and degradation of these polyesters (PHPhAs). The inclusion of this pathway in the phylacteyl-CoA catabolism (53) and its influence in the evolution of the catabolic potential of *P. putida* U are also discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—n-Phenylalkanoic acids, n-alkanoic acids, and [1-14C]Phenylacetic acid were supplied by Lancaster Synthesis or by Sigma. [1-14C]Octanoic acid was from American Radiolabeled Chemicals. All other products were of analytical quality or HPLC grade.

**Microorganisms and Culture Conditions**—The strain of *P. putida* U (Colección Española de Cultivos Tipo 4848) used in all of the experiments was from our collection. It was maintained on Trypticase Soy Agar (Difco), and growth slants (8 h at 30 °C) were used to inoculate liquid medium. Each 2000-ml Erlenmeyer flask containing 500 ml of the required medium was inoculated with 10 ml of a bacterial suspension (10^8 bacteria). Incubations were carried out in a rotary shaker (250 rpm) at 30 °C for 4 days in each set of experiments. The medium (MM) used for the growth of *P. putida* U and its mutants was a chemically defined one (54). When required, the carbon source (phenylacetic acid, PhAc) was replaced by a different one (several aromatic analogues or fatty acids). The concentration of the molecule used as carbon source was indicated in each set of experiments. The synthesis of plastic polymers was studied in bacteria grown for different times in a MM (54) in which PhAc had been replaced by several n-alkanoic (Aa) or PhAs at the required concentrations (between 5 and 15 mM). In some experiments, the carbon source was 4-hydroxyphenylacetic acid (4-HPhAc). This compound, which is efficiently assimilated by *P. putida* U, cannot be used as plastic precursor by this bacterium.

*Escherichia coli* HB101 containing the plasmid pPhA1, which includes the transposon Tn5, was kindly supplied by J. L. Ramos (Estación Experimental del Zaidán, Consejo Superior de Investigaciones Científicas, Granada, Spain). *E. coli* XL1-Blue (Stratagene) was supplied by the commercial firm, and it was used for overexpressing different proteins. *E. coli* fadR (a strain defective in the β-oxidation transcriptional repressor) and fadB (mutated in the gene encoding the enoyl-CoA hydratase of the 3-hydroxyacycyl-CoA dehydratase) mutants, which were used to study the functional expression of phaC1, phaC2, or the complete pha locus (phaC1-phaZ-phaC2) from *P. putida* U, were kindly supplied by C. C. DiRusso (Department of Biochemistry and Molecular Biology, Albany Medical College, Albany, NY). A different culture of the fadB E. coli mutant was also supplied by A. Steinbüchel and B. Rehm (Institut für Mikrobiologie, Westfälische Wilhelms-Universität Münster, Münster, Germany). When required, a double mutant E. coli JMU 193 (fadR, fadB) supplied by Q. Ren, Institut Für Biotechnologie, ETH, Zürich, Switzerland) was used. *Micrococcus luteus* (ATCC 9341) was used for the evaluation of acyl-CoA synthetase by bioassay (54).

**Characterization of PHPhAs**—PHPhAs were isolated following the procedure reported by Lageeven et al. (9). For these experiments, cells grown in a chemically defined medium (MM) (54) containing different concentrations of n-phenylalkanoic acids as the sole carbon source (54) were used. The contents and composition of PHPhAs were determined by gas chromatography as previously reported (49).

NMR spectral analyses were recorded at 18 °C on a Varian Unity 300 NMR spectrometer at 300 (1H) and 75 (13C) MHz, using tetramethylsilane as internal standard. Spectra were measured in CD2OD.

**Isolation of Mutants**—Mutants of *P. putida* unable to degrade octanoic acid or phenylacetic acid or those affected in the production of poly-(3-hydroxyphenoxyalkanoic) acids were selected by mutagenesis with the transposon Tn5 as reported (56). In some cases, mutants were obtained by disruption of the required gene (see below).

Mutants lacking a functional glyoxylic acid cycle or a β-oxidation pathway were characterized by enzyme assay (57), metabolically (studying their ability to grow in MM containing acetate or octanoate as the sole carbon source), and by location of the insertion. Mutants handicapped in the biosynthesis of plastic polymers were identified by the different contrast of the colonies (translucent) when properly cultured (8, 13).

The strains unable to assimilate phenylacetic acid (indicated as PhAc) were classified according to the intermediate accumulated in the culture broth (see below) or as a function of the presence or absence of phenylacetyl-CoA ligase activity in their cell-free extracts (58).

**HPLC Equipment and Chromatographic Procedure**—To determine the rate of utilization of the carbon sources and to identify the catabolic intermediates accumulated by certain mutants, samples of culture broth (50 ml) were taken at different times, centrifuged, and filtered through an Amicon refilter (10, 0.45 μm). Aliquots were analyzed on a HPLC apparatus (SP8800; Spectra Physics) equipped with a variable wavelength UV-visible detector (Waters 486), Millenium software (Waters 2010), and a microparticulate (particle size 10 μm; pore size 100 nm) reverse-phase column (Nucleosil C-18, 4.6 (inner diameter) by 250 mm; Phenomenex Laboratories). The mobile phase was as follows: A, 0.2 M KH2PO4 (pH 4.5); B, CH3CN in a linear gradient ranging from 95% A:5% B to 50% A:50% B over 1 h. Flow rate was 1 ml min⁻¹, and the eluate was monitored at 254 nm. Column temperature was 30 °C. Under these conditions, the retention times of 4-HPhAc, 2-hydroxyphenylacetic acid, 6-phenylhexanoic acid, and 8-phenyloctanoic acid were 19, 25, 30, 45, and 56 min, respectively.

**DNA Manipulations and Sequencing**—DNA manipulations, gel electrophoresis, DNA sequence analysis, primer synthesis, promoter analysis, and polymerase chain reaction amplification were performed as reported (53). To analyze the function of the genes encoding the polymerases and the depolymerase involved in the biosynthesis and mobilization of the plastic polymers, each particular gene was disrupted by mutagenesis with the transposon Tn5 as described above. In the case of the desired gene was established by polymerase chain reaction amplification (53). When required, overexpression of the different genes and proteins was carried out using the plasmid pQE-32 (Quagen Inc.)
Novel Bacterial Plastics

The collection of unusual bacterial plastics (those containing units of PHPhAs) was approached under the assumption that bacteria able to accumulate aliphatic plastics would also be able to synthesize aromatic polyesters whenever the substrate specificity of the polymerases is broad enough and when the effective degradation of the final \( \beta \)-oxidation product (phenylacetyl-CoA or benzoyl-CoA) is produced by a catabolic route. Accordingly, we first selected bacterial species able to (i) produce aliphatic plastics and (ii) grow in a chemically defined medium (MM) containing either benzoic acid or phenylacetic acid as the sole carbon source (54). These microbes would have (i) the biosynthetic pathway necessary for aliphatic polymer production and (ii) the catabolic enzymes required for the degradation of the final \( \beta \)-oxidation products: benzoyl-CoA (or benzoic acid) and phenylacetyl-CoA (or phenylacetic acid). This second requirement seems to be essential, since if \( n \)-phenylalkanoic acids are catabolized, as expected, through the \( \beta \)-oxidation pathway (Fig. 1) (66), the final product would be benzoyl-CoA or phenylacetyl-CoA if they have an odd or an even number of carbon atoms, respectively. Accordingly, only those microbes able to assimilate phenylacetic acid (PhAc) or benzoic acid via a CoA intermediate would be able to grow efficiently in MM containing \( n \)-phenylalkanoic acids as the sole carbon source.

Taking into account all the above considerations, we selected a bacterial strain, \( P. \ putida \) U, that meets the following metabolic conditions: (i) it is able to catabolize PhAc and benzoic acid aerobically (54–56), and (ii) it synthesizes different polyesters when cultured in MM containing \( C_6 \)–\( C_{12} \) alkanoic acids (60).

Ultrastructural studies revealed that \( P. \ putida \) U is able to synthesize biodegradable plastics when cultured in a chemically defined medium containing several PhAs whose aliphatic moiety ranges between 6 and 10 carbon atoms as carbon sources (see Table I). These compounds, which appeared as electron-transparent granules, were stored intracellularly as reserve materials (Fig. 2).

Although certain bacterial species only accumulate PHAs when an essential nutrient (e.g. nitrogen, phosphorus, sulfur, magnesium) becomes limiting (8, 10, 11), \( P. \ putida \) U synthesizes plastic polyesters from the very early logarithmic phase of growth (Fig. 2), suggesting that the regulatory mechanisms that control the biosynthesis of these polymers in both types of bacteria are quite different.

Bacterial growth and polymer contents were higher when PhAs containing acyl chains longer than six carbon atoms were used as carbon sources (Fig. 3 and Table I), the highest values being detected when 10-phenyldecanoic acid was supplied to the medium. When other PhAs (containing an acyl chain of less than six carbon atoms) were tested, no polyesters were produced, since \( P. \ putida \) cannot assimilate these compounds or does so very poorly (Fig. 3A).

**Structural Analysis of the PHPhAs**

The general structure of these novel polyesters, established by NMR studies, is shown in Fig. 4. A \(^1\)H–\(^{13}\)C correlation
The percentage of PHPhAs is indicated as percentage of bacterial dry weight. PHPhA relative composition is indicated as the molar fractions of the total of 3-hydroxyphenylalkanoates in the polymer. 3HPhV, 3-hydroxyphenylvaleric acid; 3HPhH, 3-hydroxyphenylheptanoate; 3HPhO, 3-hydroxyphenyloctanoate; 3HPhN, 3-hydroxyphenynonanoate; 3HPhD, 3-hydroxyphenyldecanoate. Similar results were obtained when the isocitrate lyase mutant was cultured in MM plus 8-phenyloctanoic acid (15 mM). ACS mutant did not accumulate plastic polymers when cultured in MM plus 8-phenyloctanoic acid (15 mM) plus 4-HPhAc (5 mM) and neither when 8-phenylhexanoic acid was replaced by octanoic acid (15 mM) (see Table 2). The results indicated in this table and in the following ones are the means of the data obtained in three different experiments (bacteria harvested from 2.5 L of the required MM).

| Carbon source       | PHPhA content | PHA relative composition |
|---------------------|---------------|--------------------------|
| Phenylacetic acid   | 0             | 3HPhV 3HPhH 3HPhN 3HPhD |
| 5-Phenylvaleric acid| 0             | 100                      |
| 6-Phenylhexanoic acid| 12           | 100                      |
| 7-Phenylheptanoic acid| 12           | 62.5                     |
| 8-Phenylheptanoic acid| 19           | 37.5                     |
| 9-Phenylnonanoic acid| 21           | 48.2                     |
| 10-Phenyldecanoic acid| 28           | 20.6                     |

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Analysis of PHPhAs Biosynthetic and Catabolic Enzymes

Although certain bacteria such as Alcaligenes eutrophus or P. oleovorans accumulated high amounts of plastic polymers containing 3-hydroxy-ω-alkanoic acids as monomers (2, 8, 50–51), to the best of our knowledge they do not produce polymers containing n-phenylalkanoic acids as monomers (2, 8, 50–51). Therefore, we have never observed monomers with a size longer than the fatty acid precursor, indicating that in P. putida U the precursors of these polymers are not obtained through fatty acid synthesis.
Transport System—When the uptake of [14C]octanoic acid was studied (60), we observed that 5-phenylpentanoic acid, 6-phenylhexanoic acid, 7-phenylheptanoic acid, and 8-phenyloctanoic acid strongly inhibited the uptake of octanoic acid (70, 85, 98, and 97%, respectively) when added at the same concentration (154 μM), whereas other related compounds with a lower molecular weight such as 3-phenylpropionic acid and 4-phenylbutyric acid did not cause any significant effect (zero inhibition and 10%, respectively). Furthermore, an efficient transport of octanoic acid (98%) was observed when P. putida U was cultured in MM containing phenyloctanoic acid. We therefore suggest that the uptake of n-phenylalkanoic and n-al-

Fig. 2. Electron micrographs. 1, P. putida U (wild type) cultured in minimal medium containing 8-phenyloctanoic acid (15 mM) as the sole carbon source. a, early exponential phase (× 10,000); b, early stationary phase (× 15,000); c, late stationary phase (× 10,000). 2, P. putida Oct1 \(^{-}\) (ACS \(^{-}\) mutant) (handicapped in the degradation of n-phenylalkanoic acids or n-alkanoic acids, containing an even or an odd number of carbon atoms) cultured until stationary phase in MM supplemented with 5 mM 4-hydroxyphenylacetic acid (which cannot serve as a plastic precursor) with 15 mM 8-phenyloctanoic acid (a) or 15 mM 7-phenylheptanoic acid (b) as carbon source (× 12,000). When this mutant was cultured in the same MM in which 8-phenyloctanoic acid had been replaced by other n-phenylalkanoic acids, by C\(_6\)–C\(_{10}\) n-alkanoic acids, by citrate, or by citrate and acetate, no synthesis or accumulation of plastic polyesters were observed. 3, P. putida PhaC1 \(^{-}\) mutant cultured until stationary phase in MM containing 15 mM 8-phenyloctanoic acid (× 20,000). Identical results were obtained when 8-phenyloctanoic acid was replaced by different n-phenyloctanoic acids or n-alkanoic acids. 4, P. putida PhaZ \(^{-}\) mutant grown under the same conditions as those described for PhaC1 \(^{-}\). a, early exponential phase of growth (× 15,000); b, early stationary phase (× 12,000); c, late stationary phase (× 15,000).
kanoic acids are at least tightly linked and very probably brought about through the same mechanism. Although a definitive conclusion could only be obtained after analyzing the uptake of radioactive n-phenylalkanoic acids, the lack of commercially available labeled compounds and the difficulty involved in synthesizing them did not allow further and more accurate analyses of this particular point. However, taking into account that a blockade in acyl-CoA synthetase (which activates medium chain-length fatty acids; see below) also hampers the cellular incorporation of octanoic acid and other polyester precursors (as shown after HPLC analysis of the culture broth), it seems reasonable to propose that the same transport system could be involved in the uptake of all of these compounds (Fig. 1). In fact, it has been suggested (68–70) that the acyl-CoA synthetase from E. coli activates fatty acids concomitant with transport, and it has been proposed that this enzyme would either be a component of the transport apparatus involved in the uptake of fatty acid or that it would interact, inside the inner membrane, with a hypothetical fatty acid cotransporter. More recently, several authors (71–75) have provided evidence indicating that the uptake of long chain fatty acid in E. coli requires the direct participation of an outer membrane protein and acyl-CoA synthetase.

**Acyl-CoA-activating Enzyme—**The analysis of the acyl-CoA-activating enzyme(s) was carried out. It could be speculated that two different enzymes (or more) would be involved in the activation of alkanic and phenylalkanoic acids and that, therefore, the absence of this enzyme that activates phenylalkanoic acids would explain why certain bacteria do not synthesize aromatic polyesters. To clarify this point, we analyzed the acyl-CoA-activating enzyme(s) involved in the uptake of fatty acid or that it would interact, inside the inner membrane, with a hypothetical fatty acid cotransporter. More recently, several authors (71–75) have provided evidence indicating that the uptake of long chain fatty acid in E. coli requires the direct participation of an outer membrane protein and acyl-CoA synthetase.

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altered the expression of a gene encoding an acetyl-CoA-activating enzyme (acetyl-CoA synthetase or acetate kinase) or some of the proteins involved in the glyoxylic acid cycle (65).

Among the strains included in the first group we identified a mutant (Oct1<sub>ACS</sub><sup>2</sup>, henceforth called ACS<sup>2</sup>) in which Tn<sub>5</sub> insertion occurred in a gene encoding a protein that shows

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**Fig. 5—continued**

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**FIG. 5.** Complete nucleotide sequence and derived amino acid sequence of the *pha* locus from *P. putida* A. DNA fragment encoding the two PHA/PHPhA polymerases (PhaC1 and PhaC2) and the PHA/PHPhA depolymerase (PhaZ). **B**, alignment of these proteins (designated as PSP) with those reported in *P. oleovorans* (PSO) and *P. aeruginosa* (PSA). Underlined sequences correspond to the fragments used for disrupting each specific gene (52). The box in the PhaZ indicates a lipase consensus sequence.
strong homology (accession number AF150669) with the long chain fatty acid ACSs from \textit{E. coli} and \textit{Pseudomonas fragi} \cite{77–79}. This gene (\textit{fadD}) was cloned, sequenced, and expressed in \textit{E. coli} DH5\textalpha.

The ACS\textsuperscript{2} mutant efficiently catabolized acetate and butyrate but was unable to assimilate \textit{n}-alkanoic or \textit{n}-phenylal- kanoic acids with an acyl moiety ranging between C\textsubscript{5} and C\textsubscript{10}, undoubtedly indicating that a single enzyme is responsible for the activation of all of these compounds and that, as expected, this reaction is not catalyzed by acetyl-CoA synthetase or by the acetate kinase (EC 6.2.1.1 and EC 2.7.2.1, respectively). Furthermore, study of the polyesters stored revealed that this mutant does not accumulate plastic polymers when cultured in the appropriate conditions (MM plus 4-HPhAc plus PhAs or As as carbon sources) (Fig. 2 and Table III). Additionally, we observed that the ACS\textsuperscript{2} mutant was unable to transport \textit{\textsuperscript{14}C\textsuperscript{}}octanoic acid (0.3\% of the final amount transported by the wild type), suggesting that, as mentioned above, the mechanism involved in the uptake of this acid and the activation to its CoA thioester comprises a single event or at least two closely related events (Fig. 1). Similar results were obtained when the transport of \textit{\textsuperscript{14}C\textsuperscript{}}phenylacetic acid was analyzed in mutants in which the gene encoding phenylacetyl-CoA ligase (EC 6.2.1.30), the first enzyme of the specific route involved in the catabolism of phenylacetic acid \cite{56}, had been disrupted. We observed that the \textit{P. putida} U mutants lacking this enzymatic activity were unable to take up phenylacetic acid from the broths.\textsuperscript{3} Thus, it can be concluded that in the absence of this single acyl-CoA synthetase activity the uptake of all of the substrates recognized by this enzyme, which are the precursors of the monomers used for the synthesis of polyesters, will be blocked, hence avoiding an unnecessary loss of energy, since once transported these compounds cannot be catabolized. As a consequence of this blockade, polyesters cannot be synthesized. In light of the above results, it seems that this single acyl-CoA synthetase is responsible for activation of alkanoates and phenylalkanoates.

On the other hand, it is interesting to draw attention to the lack of plastic storage in the ACS\textsuperscript{2} mutant when grown in MM plus acetate plus citrate (Fig. 2), since this result indicated that \textit{P. putida} U, unlike other related bacteria \cite{48}, does not synthesize polyesters (or does so very poorly) via fatty acid biosynthesis. This assumption is supported by the fact that when this mutant or the wild type was cultured in minimal media containing either glucose or gluconate as carbon source, synthesis of plastic polymer was not detected (data not shown).

**The Polymerizing-Depolymerizing Enzymatic System**—We have analyzed the sequence and organization of the genes and proteins involved in the polymerization and depolymerization of these polyesters in \textit{P. putida} U. For these experiments, mutants blocked in the genes involved in the synthesis of poly-3-hydroxyphenylalkanoates were isolated \cite{13}. Sequence analysis of the DNA fragment in which the transposon Tn\textsubscript{5} had been inserted revealed that (i) in \textit{P. putida} U, three genes (\textit{phaC1}, \textit{phaZ}, and \textit{phaC2}) included in the \textit{pha} locus; (ii) the promoters present in the \textit{pha} locus in \textit{P. putida} U, in \textit{P. oleovorans}, and in \textit{P. aeruginosa}.}

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\textsuperscript{3} B. Mi\textntambres, unpublished results.
possibility that a promoter could exist between the biosynthetic regions of PhaC1 and PhaZ (subtractive). This balance could be represented as follows: PhaC1 + PhaC2 - PhaZ = 365. When PhaC2 was mutated, the quantity of plastic synthesized (PhaC1 - PhaZ) was reduced to 115 mg/dwc; therefore, we conclude that the contribution of PhaC2 to the synthesis of plastic should be about 250 mg/dwc. However, when a PhaZ mutant was cultured in the same conditions, we obtained only 250 mg/dwc. This later quantity suggests that in this mutant only the PhaC1 is working, since otherwise total synthesis should have been greater. Although these data suggest that there is not an active promoter downstream from the phaZ gene, we cannot rule out the possibility that a promoter could exist between the phaC1 and the phaZ genes. Moreover, we observed that mutants in which the phaC1 gene was disrupted did not synthesize plastics at all (Table IV), which should not have happened if there had been a promoter downstream from the phaC1 gene. It is worth noting that although the depolymerase activity was lacking in the PhaZ mutant (and therefore mobilization of the polymer did not occur; see Table IV), the quantity of polymer accumulated (250 mg/dwc) was considerably lower than that found in the control (365 mg/dwc), suggesting that the polymerases and probably all three enzymes (PhaC1, PhaC2, and PhaZ) must be organized in a complex to function efficiently.

Genetic studies revealed that whereas the sequences of the genes included in the pha locus are well conserved, further differences were found when the promoter and the two intergenic regions reported in the pha locus of P. oleovorans, P. aeruginosa, and P. putida U were compared (Fig. 6). Thus, in P. putida U the lengths of the intergenic regions are shorter than in other species and lack the inverted repeat sequences found in P. oleovorans and in P. aeruginosa (13, 21). This genetic organization suggested that in P. putida U the three pha genes are organized in an operon and that translational coupling may exist.

On the other hand, the study of the putative promoter regions in the three bacterial species revealed that the sequence of P. aeruginosa is quite different from the other two species (Fig. 6A). This variation could account for a different regulation of the three pha loci. When genetic studies, using a plasmid specifically designed for testing promoters (53), were performed, we did not find any functional promoter sequences within the fragment of DNA containing the structural sequences (PhaC1, PhaZ, PhaC2) from the ATG of phaC1 to the ATG of phaZ (see Fig. 5). We revealed for the first time in Pseudomonads of rRNA homology group I that a blockage on the phaC1 prevented accumulation of plastic polymers, whereas a mutation in the phaC2 reduced the intracellular amount of polymers by two-thirds. Furthermore, disruption of the gene encoding PhaZ prevented the mobilization of the polymer accumulated intracellularly and decreased the total PHAs content, suggesting that PhaC2 is not synthesized, since it must be expressed from a promoter located upstream the gene encoding the depolymerase (see Table IV).

An analysis based on the amounts of polymer stored by the different mutants allowed us to obtain interesting conclusions about the genetic organization of the pha locus. Thus, when P. putida U (wild type) was cultured in MM plus octanoic acid until the stationary phase of growth, the amount of PHAs extracted was 365 mg/g of dry weight cells (dwc). This quantity suggests that in this mutant only the PhaC1 is working, since otherwise total synthesis should have been greater. Although these data suggest that there is not an active promoter downstream from the phaZ gene, we cannot rule out the possibility that a promoter could exist between the phaC1 and the phaZ genes. Moreover, we observed that mutants in which the phaC1 gene was disrupted did not synthesize plastics at all (Table IV), which should not have happened if there had been a promoter downstream from the phaC1 gene. It is worth noting that although the depolymerase activity was lacking in the PhaZ mutant (and therefore mobilization of the polymer did not occur; see Table IV), the quantity of polymer accumulated (250 mg/dwc) was considerably lower than that found in the control (365 mg/dwc), suggesting that the polymerases and probably all three enzymes (PhaC1, PhaC2, and PhaZ) must be organized in a complex to function efficiently.

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### Table IV

Accumulation of PHAs (or PHPhAs) by P. putida U (wild type) and by its mutants disrupted in the genes phaC1, phaC2 or phaZ when cultured in MM plus octanoic acid (or 8-phenyloctanoic acid, 15 mM) for different times

| Strains    | PHAs content | PHA content | PHPhAs content | PHPhA content |
|------------|--------------|-------------|---------------|---------------|
|            | Ex | St | Lst | Ex | St | Lst | Ex | St | Lst |
| P. putida U | 531 | 1314 | 30 | 28 | 34 | 0.8 | 80 | 174 | 0 |
| PhaC1−      | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| PhaZ        | 228 | 783 | 900 | 12 | 20 | 24 | 64 | 100 | 119 |
| PhaC2−      | 233 | 415 | 69 | 11 | 12 | 2  | 55 | 64  | 10 |

### Table V

Accumulation of PHAs (or PHPhAs) and monomer composition of the polymers synthesized by P. putida U and by the PhaC1−, PhaZ−, and PhaC2− mutants, cultured until the stationary phase of growth in MM plus different n-alkanoic or n-phenylalkanoic acids (15 mM)

| Strain | Carbon source | PHA or PHPhA content | PHA relative composition | PHPHA relative composition |
|--------|---------------|----------------------|--------------------------|---------------------------|
|        | %            | mol/100 mol          |                          |                           |
| P. putida U | Oct  | 34 | 2 | 98 | 3HH  | 3Hh  | 3HO  | 3HN  | 3HD  | 3HPV  | 3HPH  | 3HPH  | 3HPhO  | 3HPhN  | 3HPhD  |
|         | Non  | 16 | 35 | 65 |       |       |       |       |       | 100  | 62  | 38  |       |       |       |
| PhaC1− | Oct  | 0  | 0  | ND |       |       |       |       |       |       |     |     | 100  |       |       |       |
|         | Non  | 21 |       |     |       |       |       |       |       |       |     |     |       | 100  |       |       |
| PhaZ−  | Oct  | 20 | 3  | 97 |       |       |       |       |       |       | 100  | 61  | 39  |       |       |       |
|         | Non  | 11 | 34 | 66 |       |       |       |       |       |       |     |     | 100  |       |       |       |
| PhaC2− | Oct  | 12 | 2  | 98 |       |       |       |       |       |       |     |     | 100  |       |       |       |
|         | Non  | 7  | 33 | 67 |       |       |       |       |       |       |     |     | 100  |       |       |       |
|         | PhaO | 11 | 34 | 66 |       |       |       |       |       |       |     |     |       |       | 100  |       |       |
|         | PhN  | 7  | 33 | 67 |       |       |       |       |       |       |     |     |       |       |       | 100  |       |
The abnormal size is caused by the absence of septum required for a regular cell division.

Next, the general specificity of both polymerases was analyzed independently by studying the structure of the polymers synthesized and stored in the different mutants reported above. We observed that the polystyrenes accumulated by the wild-type strain and by the PhaZ and by the PhaC2 mutants were similar in monomer composition and in the percentage of aliphatic and aromatic found in \( P. \) putida \( U \); and (vi) the lack of depolymerase does not imply a greater accumulation of polymers but rather the impossibility of mobilizing them (see Table IV and Fig. 2).

Yield Improvement Studies

Since the overexpression of the \( \text{pha} \) locus from \( P. \) putida \( U \) in \( E. \) coli does not lead to a higher production of PHAs (or PHPhAs) in the recombinant strains, certain biochemical studies were also performed in order to improve the production of plastic polymers in \( P. \) putida \( U \). It is well established that the degradation of \( n \)-alkanoic acids and phenylalkanoic acids through \( \beta \)-oxidation generates several units of acetyl-CoA (66) that are later catabolized by the glyoxylate cycle (65). It could therefore be expected that the introduction of a mutation in

![Fig. 7. Micrographs of \( E. \) coli DH5a’ (harboring the plasmid pUC18 containing as an insert the gene that encodes PhaC1) when cultured in LB supplemented with 10 mM hexanoic acid and 0.5 mM acrylic acid. Shown are an optical photograph (24-h-old cultures) (a); a transmission micrograph (24-h-old (b) and 60-h-old (c) cultures); and a scanning micrograph (24-h-old cultures) (d). The abnormal size is caused by the absence of septum required for a regular cell division.](image)
some of the genes encoding enzymes belonging to the glyoxyllic acid cycle could slow down the β-oxidation route (the source of acetyl-CoA) and hence cause the accumulation of some catabolic intermediates (polyester precursors), thus channeling them to the polymeric system and increasing the amount of stored polymers. To check this hypothesis, we isolated a mutant in which the transposon Tns9 (76) had been inserted into a gene belonging to the glyoxyllic acid cycle (in the aceA gene encoding the isocitrate lyase gene, accession number AF150671). This mutant, which shows an Oct- Ac- phenotype, was unable to grow in MM containing either acetate or n-alkanoic acids whose carbon length was an even number of carbon atoms, whereas it grew well in MM containing either several n-alkanoic acids with an odd number of carbon atoms or n-phenylalkanoic acids with an even or odd number. These results indicate that unlike the catabolism of alkanoic acids containing an even number of carbon atoms, those other compounds do not require the glyoxyllic acid cycle to be assimilated (see below). Moreover, when this isocitrate lyase mutant was cultured in MM plus citrate plus acetate plus octanoate (or 8-phenylctanoate), a higher percentage of PHA (or PHPPhA) content was observed (see Table VI). To explain this result, we assume that although some octanoic acid is used as the carbon source and catabolized through β-oxidation, the presence of citrate and acetate slows down the metabolic flux of this catabolic pathway, thus channeling the intermediates (3-OH-acetyl-CoAs) to the synthesis of polymer.

Taking into account that a blockade in β-oxidation could lead to the accumulation of 3-OH-acetyl-CoA derivatives that could be used for the synthesis of plastic polymers, we analyzed the accumulation of PHAs and PHPPhAs when P. putida U was cultured in MM containing 15 mM octanoic acid or 8-phenylctanoic acid as the source of intermediates, 5 mM 4-OH-phenylacetic acid for supporting bacterial growth, and the ketothiolase inhibitor acetyl acid (0, 5 mM) (85, 86). We observed that, as Steinbüchel and co-workers reported in a E. coli recombinant strain (86), accumulation of polymer increased 2-fold (60 and 38% of bacterial dry weight, respectively) with respect to a control cultured in the absence of acetyl acid. When a PhaZ- mutant (handicapped in the mobilization of the plastic once synthesized, see Table IV) was cultured in MM supplemented with acrylic acid, the quantity of polymer accumulated was double that observed in the control (without acrylic acid) but lower (65%) than the amount of plastic accumulated by the wild-type P. putida U. These results again support the hypothesis that the polyhydroxyalkanoate biosynthetic enzymes work more efficiently as a complex enzymatic system.

To further analyze the overproduction of PHAs, the fadA gene encoding the 3-oxoacyl-CoA thiolase (accession number AF150672) involved in the β-oxidation pathway (see below) was disrupted by homologous recombination by using an internal fragment of the gene, obtained by polymerase chain reaction amplification using primers of the homologous gene of P. fragi (87). We observed that the quantity of PHAs accumulated in this mutant, when it was cultured in MM supplemented with 15 mM octanoic acid and 5 mM 4-HPhAc, was higher (68% dwc) than that observed in the parental strain, showing that a blockade in the β-oxidation contributes efficiently to improve the synthesis of plastic polymers.

**Table VI**

| Strains        | PHAs content | PHA content | PHPPhAs | PHPPhA content |
|---------------|--------------|-------------|---------|----------------|
| P. putida U   | 1570         | 28          | 180     | 20             |
| PhaZ-         | 1325         | 23          | 162     | 18             |
| ICL-          | 1503         | 38          | 220     | 28             |

According to the results presented above, we assumed that the biosynthesis and mobilization of PHPPhAs and PHAs in P. putida U must be carried out by the same enzymatic system and that it was similar to those reported in P. oleovorans and in P. aeruginosa (13, 21). However, the synthesis of aromatic polyesters is not a common event among the bacteria, although many species are able to accumulate high amounts of aliphatic plastics intracellularly (1). This difference cannot be explained by the requirement of a different acyl-CoA synthetase or different polymerases, since, as we have shown above, the same enzymes participate in the activation and in the polymerization of aromatic and aliphatic monomers. However, the degradation of PHAs containing an even number of carbon atoms (C<sub>n</sub>-2PhAs) by P. putida U seems to require two different pathways: the first, a β-oxidation route, that catalyzes PHAs to phenylacetyl-CoA, and a route that must transform this thioester into assimilable metabolites. Under this assumption, it could be argued that only bacteria able to metabolize PHAs would be able to synthesize aromatic plastics (PHPPhAs). In fact, the accumulation of these polymers would not represent any advantage if the microbe were unable to catabolize them. Thus, phenyl derivatives containing either an odd or an even number of carbon atoms will be catabolized through β-oxidation to benzyol-CoA (odd) or phenylacetyl-CoA (even), which could be further degraded to the intermediates of general metabolism. Bacteria lacking these additional pathways would only be able to partially catabolize these compounds (releasing some units of acetyl-CoA), and this incomplete degradation would afford scant energetic benefit for the microbe. However, it is not clear whether a single β-oxidation pathway exists in P. putida U or whether different pathways might be involved in the catabolism of PHAs and alkanoates in this bacterium. To clarify this point, the different mutants unable to catabolize octanoic acids (Oct<sup>-</sup>) were classified into three groups: (i) those lacking an specific acyl-CoA activating activity (ACS<sub>-</sub> also called Oct<sup>-</sup>; see above); (ii) those mutants lacking a functional glyoxylic acid cycle (Oct<sup>-</sup>), and (iii) a different group affected in the β-oxidation pathway (Oct<sup>-</sup>), obtained by genetic disruption of the ketothiolase gene (the fadA mutant). We observed that all these strains efficiently catabolized phenylacetic acid and benzoic acid, whereas those named Oct<sup>-</sup> and Oct<sup>-</sup> were unable to grow in MM containing either aliphatic or phenylalkanoic acids with a number of carbon atoms (even or odd) higher than 4 or did so very poorly. These data allow us to conclude that in P. putida U a single acyl-CoA synthetase and single β-oxidation pathway are involved in the catabolism of aliphatic and aroylalkanoic acids with the above indicated carbon length.

Recently, an additional pathway required for the specific catabolism of phenylacetic acid (via phenylacetyl-CoA) (56) has been discovered (53). We identified a piece of DNA of about 18 kilobase pairs containing 15 open reading frames that are required for the catabolism of PHAc and phenylacetyl-CoA in P. putida U (53). This catabolic pathway, called the phenylacetyl-CoA catabolon core, appears to be organized in three contiguous operons that contain five different functional units and several regulatory elements (Fig. 8). Using mutagenesis with
the transposon Tn5, different mutants of *P. putida* unable to grow in MM containing PhAc as the carbon source (PhAc− strains) were selected. We observed that all of the PhAc− mutants were able to catabolize n-As and n-PhAs containing an odd number of carbon atoms (C_{2n+1}-PhAs), whereas they assimilated C_{2n+2}-PhAs with an even number very poorly, since they were only able to utilize the acetyl-CoA molecules released by β-oxidation (Fig. 3C). Analysis of plastic polymer accumulation in these mutants grown in MM containing 4-hydroxyphenylacetic acid for supporting bacterial growth and 8-phenyloctanoic acid as a plastic precursor revealed that whereas mutations in all of the catabolic genes involved in the specific route of phenylacetic acid did not affect the production of aliphatic polymers (PHAs), most of them (with the exception of the mutants lacking phenylacetyl-CoA ligase, an enzyme that is not required for the catabolism of phenylalkanoic acids) (53) accumulated lower quantities of aromatic polymers (PPhAs) (Table III). These results suggest that both pathways (PPhAs biosynthetic/degradative route and PhAc catabolic pathway) are closely related, since a blockade in the second one causes the accumulation of some intermediate(s) that negatively affect(s) (probably by feedback control) the activities of the enzymes involved in the process of polymerization (Table III).

We also observed that all the PhAc− mutants were able to grow in MM containing benzoic acid as the sole carbon source (not shown), indicating that the β-oxidation final products of both types of PHAs (benzoyl-CoA/phenylpropionyl-CoA and phenylacetyl-CoA) are catabolized through different pathways. Similar data had been reported in *P. aeruginosa*. Both enzymes (PhaC1 and PhaC2) showed a similar substrate specificity and polymerizing rate, whereas the depolymerizing rate shown by PhaZ is about one half of the activity shown by each of these polymerases. Expression of these enzymes in recombinant *E. coli* fadB or fadR mutants does not permit the synthesis of polymers, whereas the synthesis and accumulation of PHAs occurred at a very low rate when a monopyrolyc construction was used. (iv) A blockade in a β-oxidation gene affecting the expression of ketothiolase (fadA mutant) greatly improves the accumulation of these polymers. Similar results were obtained when the ketothiolase was inhibited with acrylic acid as well as when the metabolic flux through the glyoxylic acid was altered (disruption on the gene encoding the isocitrate lyase). (v) Finally, at least two specific catabolic routes linked to a single β-oxidation pathway are required for the complete catabolism of the 3-hydroxyphenylalkanoates containing an even number of carbon atoms. Both are integrated in a complex catabolic unit (phenylacetyl-CoA catabolon) responsible for the degradation of several structurally related aromatic compounds (53).

Deeper knowledge of these biodegradable plastics could be important not only to establish the physicochemical properties of novel, nonpolluting compounds but also to obtain derivatives with new or broader applications. Moreover, expression of the genes encoding the plastic polymerases and depolymerase in other microbes, via genetic engineering (incorporation of genes encoding R-specific enoyl-CoA to the pha locus in order to efficiently channel β-oxidation intermediates to the polymeric system), could help to enhance both their biosynthetic and their catabolic potential as well as increase the quantity of biodegradable plastics that could be stored (45). Furthermore, a biochemical approach to the regulatory mechanisms controlling the expression of these genes and study of the proteins participating in both the synthesis and in the stabilization of such polymers could help in the design of genetically engineered microbes that could be employed to eliminate certain aromatic contaminants from the biosphere under a broad range of metabolic and environmental conditions. Thus, we have recently shown that the disruption of the *paaN* gene, encoding the repressor of the PhAc catabolic pathway (Fig. 8) (53), elicits a dual effect, i.e. the constitutive expression of the pathway and the suppression of the carbon catabolite repression of this route. This result shows that genetically engineered strains could be efficiently used to degrade PhAc and certain aromatic precursors even from complex media containing readily metabolizable carbon sources that in the wild-type strain would strongly repress the PhAc catabolic pathway.

**Conclusions**

In sum, from the present findings the following conclusions can be drawn. (i) *P. putida* U synthesizes and accumulates novel biodegradable plastic polymers containing as monomers 3-hydroxy-n-phenyl derivatives (10 ≤ n ≤ 5). (ii) A single acyl-CoA synthetase seems to be involved in the activation of phenylalkanoics and alkanoic acids to their CoA thioesters (which are the precursors of the different polymers), and a similar transport system seems to be required for the uptake of alkanoic and phenylalkanoic acids. The disruption of the *fadD* gene handicapped the uptake of precursors, their activation, and, therefore, the synthesis of plastics. (iii) Polymerization of the monomers is carried out by two polymerases (PhaC1 and PhaC2), which are also involved in the synthesis of polyhydroxalkanoates. These enzymes are encoded by two genes (*phaC1* and *phaC2*) organized in a single operon, which also includes a depolymerase gene (*phaC1*-phaZ-*phaC2*) and which is very similar to the *pha* locus reported for *P. oleovorans* and *P. aeruginosasa*. Both enzymes (PhaC1 and PhaC2) showed a

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