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Genetically engineered *Pseudomonas*: a factory of new bioplastics with broad applications

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Q1 **Summary**

New bioplastics containing aromatic or mixtures of aliphatic and aromatic monomers have been obtained using genetically engineered strains of *Pseudomonas putida*. The mutation (–) or deletion (Δ) of some of the genes involved in the β-oxidation pathway (*fad*A$^-$, *fad*B$^-$ Δ*fad*A or Δ*fad*BA mutants) elicits a strong intracellular accumulation of unusual homo- or co-polymers that dramatically alter the morphology of these bacteria, as more than 90% of the cytoplasm is occupied by these macromolecules. The introduction of a blockade in the β-oxidation pathway, or in other related catabolic routes, has allowed the synthesis of polymers other than those accumulated in the wild type (with regard to both monomer size and relative percentage), the accumulation of certain intermediates that are rapidly catabolized in the wild type and the accumulation in the culture broths of end catabolites that, as in the case of phenylacetic acid, phenylbutyric acid, *trans*-cinnamic acid or their derivatives, have important medical or pharmaceutical applications (antitumoral, analgesic, radiopotentiators, chemopreventive or antihelmintic). Furthermore, using one of these polyesters (poly 3-hydroxy-6-phenylhexanoate), we obtained polymeric microspheres that could be used as drug vehicles.

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Introduction

Poly-3-OH-alkanoates, so-called PHAs, are polymers with plastic properties that are accumulated as reserve materials by different living systems (Anderson and Dawes, 1990). Although many microbes, particularly bacteria, are able to synthesize aliphatic polyesters (Witholt and Kessler, 1999; Kim *et al*., 2000; Sudesh *et al*., 2000), the synthesis of polymers containing aromatic monomers seems to be a rare event (Anderson and Dawes, 1990; Fritzsche *et al*., 1990; Steinbüchel and Valentin, 1995; García *et al*., 1999; Madison and Huisman, 1999; Witholt and Kessler, 1999). Recently, we have reported that a strain of *Pseudomonas* (*P. putida* U) accumulates and catabolizes different poly-3-OH-*n*-phenylalkanoates as a result of the existence in this bacterium of specific pathways (related to the β-oxidation route) that allow their synthesis and degradation (García *et al*., 1999). The relative amount of these aromatic polyesters in *P. putida* is lower (10–20% of the bacterial dry weight, bdw) than the quantity of the aliphatic polymers accumulated under similar culture conditions (about 38% of the bdw) and, in both cases, this accumulation is insufficient to warrant them being used for industrial purposes.

Taking into account the importance of these compounds, we have addressed different studies aimed at increasing the amount of polymer synthesized by this bacterium. Thus, in order to facilitate their accumulation, we designed a strategy based on a genetic engineering approach. Using the transposon Tn*5* as a disrupting element (Berg, 1989), different mutants affected in the β-oxidation pathway were obtained (Olivera *et al*, 2001).

Results and discussion

Different mutants of *P. putida* affected in the β-oxidation pathway were isolated by insertional mutagenesis with the transposon Tn*5*. Once characterized, we showed that all of them corresponded to two different types: (i) those in which the transposon was inserted in the *fad*D gene (GenBank AF290948), encoding the acyl-CoA synthetase involved in the activation of aliphatic and aromatic fatty acids whose acyl chain is longer than four carbon atoms (García *et al*., 1999); and (ii) those in which the transposon had disrupted the *fad*B gene (GenBank AF290949), encoding a protein with four different enzymatic activities: enoyl-CoA hydratase, 3-OH-acyl-CoA

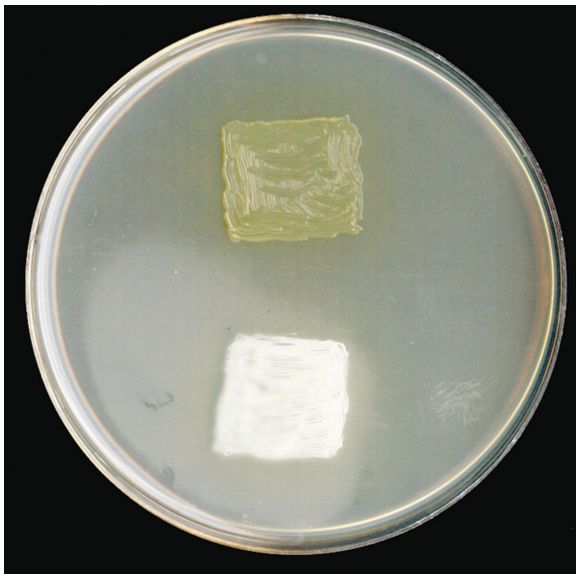

Fig. 1. Ultrastructural studies. Morphological aspect of the cultures of *P. putida* (top) and its Δ*fadBA* mutant (bioplastic overproducer strain) (bottom) grown in chemically defined solid medium containing 4-OH-phenylacetic acid (10 mM) and 7-phenylheptanoic acid (10 mM) as carbon sources.

dehydrogenase, *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase and 3-OH-acyl-CoA epimerase (Olivera *et al*., 2001). This genetic information was used specifically to disrupt (–) with the plasmid pK18::*mob* (Schäfer *et al*., 1994) or to delete (Δ) (Donnenberg and Kaper, 1991) *fadB, fadA* or both genes (*fadB*$^-$, *fadA*$^-$ Δ*fadB*, Δ*fadBA* strains) (Olivera *et al*., 2001). When *fadB*$^-$, *fadA*$^-$ Δ*fadB*, Δ*fadBA* mutants were cultured in different chemically defined media (Martínez-Blanco *et al*., 1990) containing n-alkanoates (hexanoate and/or octanoate), n-phenylalkanoates (5-phenylvalerate, 6-phenylhexanoate, 7-phenylheptanoate and/or 8-phenyloctanoate) or a combination of them as a source of monomers to build plastic polymers, and 4-OH-phenylacetic acid (a compound that cannot be polymerized) to support bacterial growth, large amounts (> 55% cell dry weight) of different polymers were synthesized. Morphological analyses revealed that these mutants accumulated such a large amount of polyesters (> 90% of the cytoplasm was occupied by the polymers) that their morphology was dramatically altered. The large amount of polymer synthesized was even observable in solid cultures of these mutants, which acquired an intense white colour. This was especially noticeable in the Δ*fad*BA mutant (see Figs 1 and 2). Taking into account that *fad*A$^-$, *fad*B$^-$ Δ*fad*A or Δ*fad*BA mutants accumulated the same polymers (with regard to both structure and degree of polymerization and amount) when they were cultured in the same medium and under identical conditions, we have selected strain Δ*fad*BA$^-$ for all the experiments reported below. Δ*fad*BA lacks both genes (*fad*A *and fad*B), and it is more stable than those mutants in which gene disruption was performed (*fad*A$^-$ or *fad*B$^-$).

When cultured under different conditions (see *Experimental procedures*), the polymers synthesized by this mutant were extracted from the bacteria (Lageveen *et al*., 1988; García *et al*., 1999) and characterized in detail (Abraham *et al*., 2001). Polymer characterization (see *Experimental procedures*) revealed that a large number of co-polymers could be synthesized, and that their monomer composition could be modified at will simply by changing the relative proportion of the fatty acids (n-alkanoic, n-phenylalkanoic or combinations thereof)

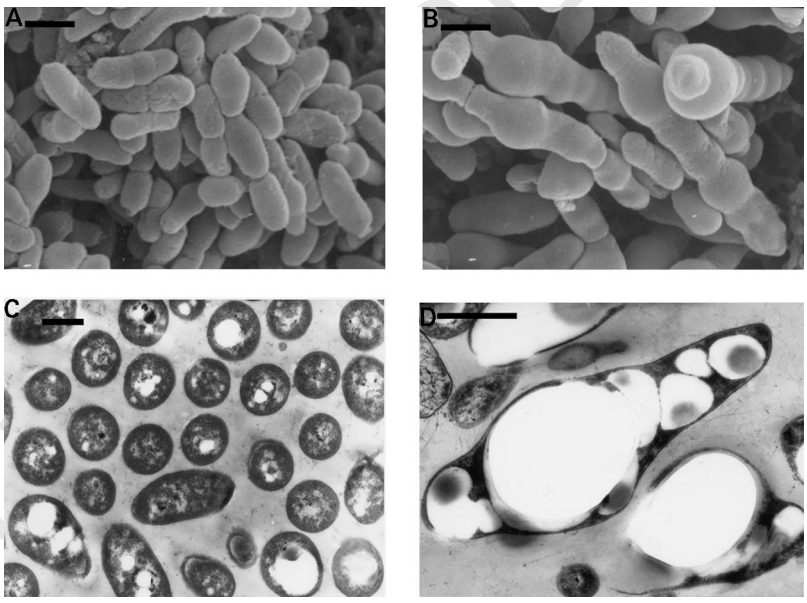

Fig. 2. Electron micrographs. Scanning (A and B) and transmission (C and D) microphotographs of *P. putida* (A and C) and Δ*fadBA* mutant (B and D) cultured in chemically defined solid medium containing 4-OH-phenylacetic (10 mM) acid and 7-phenylheptanoic acid (10 mM) as carbon sources. Bars correspond to 1 μm.

Table 1. Polymers overproduced by the Δ*fadBA* mutant of *P. putida*.

Plastic precursors	Monomer composition	Polydispersity values (Mw/Mn)	*Tg* (°C)	Structure
6-PhH	100% 3-OH-6-PhH	2.20	−1.3	Amorphous
7-PhHp	77% 3-OH-7-PhHp + 23% 3-OH-PhV	2.32	−11.2	Amorphous
6-PhH + 7-PhHp	51% 3-OH-7-PhHp + 38% 3-OH-6-PhH + 11% 3-OH-5-PhV	2.74	−8.2	Amorphous
8-PhO	73% 3-OH-8-PhO + 27% 3-OH-6-PhH	2.01	−14.8	Amorphous
8-PhO + 7-PhHp + 6-PhH + 5-PhV	24% 3-OH-8-PhO + 35% 3-OH-7-PhHp + 21% 3-OH-6PhH + 20% 3-OH-5-PhV	ND	ND	Amorphous
8-PhO + 8-O + 6-PhH + 6-H	23% 3-OH-8-PhO + 45% 3-OH-8-O + 18% 3-OH-6-PhH + 14% 3-OH-6-H	ND	ND	Amorphous

Chemical identification of the polymers and analyses of their physicochemical properties were performed as reported previously (Abraham *et al.*, 2001). ND, not determined. In all cases, the relative percentages of bioplastic as a percentage of bacterial dry weight were higher than 55%. 5-PhV, 6-PhH, 7-PhHp, 8-PhO, 6-H and 8-O correspond to 5-phenylvaleric acid, 6-phenylhexanoic acid, 7-phenylheptanoic acid, 8-phenyloctanoic acid, 6-hexanoic acid and 8-octanoic acid respectively. The molecular weight of the different polymers ranged between 156 000 and 300 000.

added as a source of monomers to the culture broth. Table 1 shows the monomer composition of some of the polyesters accumulated when different combinations of fatty acids were tested, as well as the most important characteristics of some of them (average molecular weight values, polydispersity values and thermal properties). All these polymers are amorphous (Fig. 3A and B), with glass transition temperatures ranging from −14.8°C

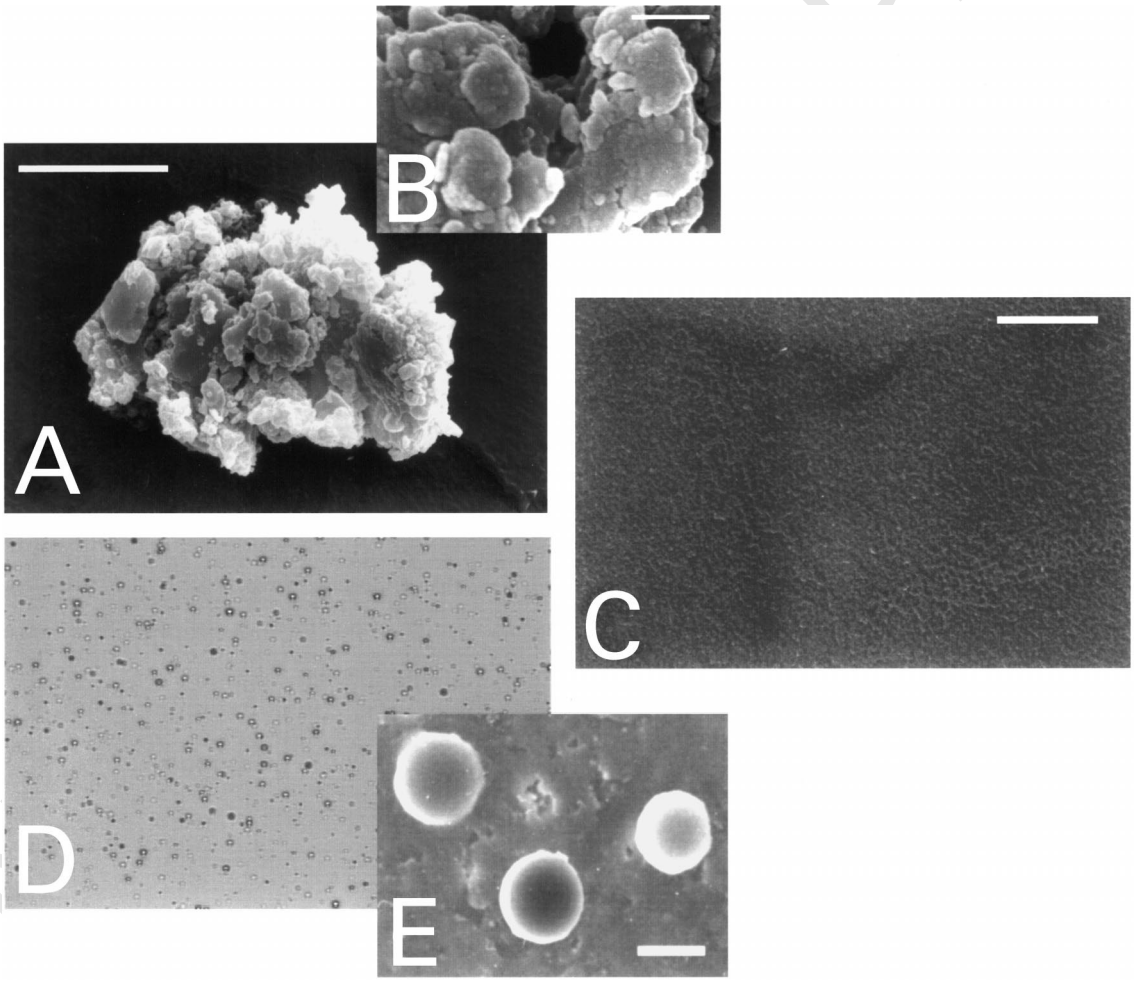

Fig. 3. Bioplastic microphotographs. Scanning microphotographs of: (A and B) a sample of the amorphous bioplastic (3-OH-6-phenylhexanoate homopolymer) and (C) aspect of a thin film of this polymer. Light (D) and scanning microphotographs (E) of the microspheres prepared with poly 3-OH-6-phenylhexanoate. Bars in (A–C) correspond to 1 μm, whereas in (E), the bar represents 10 μm.

to −1.3°C (Table 1). It should be stressed that, even though we only indicate some of them in Table 1, in fact, many more polymers were obtained, as small variations in the relative percentages of fatty acids added to the broth permitted the synthesis of polymers with different monomer compositions and probably with different physicochemical properties.

We have reported previously that, whereas the catabolism of aliphatic polyesters (PHAs) can also be carried out in these mutants by a second set of β-oxidation enzymes (β_{II}), which replace the function of the $\alpha_2\beta_2$ complex (β_I-oxidation) when a blockade in the *fad*BA of β_I exists, the complete catabolism of aromatic polyesters specifically requires the enzymes of the above indicated $\alpha_2\beta_2$ multifunctional complex, such that these mutants can only partially catabolize these polyesters to CoA thioesters containing more than five carbon atoms in their acyl moiety. These intermediates, which cannot be catabolized further, are hydrolysed to release their CoA moiety and are excreted to the broth. Thus, 4-phenyl-2-butenoic acid and *trans*-cinnamic acid, two intermediates generated through the β-oxidation of n-phenylalkanoates containing an odd or even number of carbon atoms, respectively, are released to the culture broth by these mutants (Olivera *et al*., 2001).

Furthermore, mutants disrupted in any of the genes belonging to the phenylacetic acid catabolic pathway (*paa*) (Olivera *et al*., 1998; Luengo *et al*., 2001) accumulated different intermediates as a function of the location of the mutation point. Thus, the mutants lacking any of the enzymes belonging to the second operon (ring oxidation system) accumulate phenylacetic acid (PhAc) when cultured in chemical defined medium containing 4-OH-phenylacetic acid (for supporting bacterial growth) and n-phenylalkanoates with an odd number of carbon atoms as a source of intermediates, whereas those blocked in the *paa*L (third operon, *paa*JKL) accumulate 2′-OH-phenylacetic acid. These data allowed us to conclude that *P. putida* U: (i) catabolizes the n-phenylalkanoates containing an odd number of carbon atoms to phenylacetyl-CoA and that this compound is transformed in general metabolites through a specific pathway (phenylacetyl-CoA catabolon core); (ii) degrades n-phenylalkanoates containing an even number of carbon atoms to *trans*-cinnamic acid, which cannot be catabolized further; and (iii) all these mutants could be used to obtain different catabolites of n-phenylalkanoic acids.

It has been reported that some β-oxidation catabolic derivatives of these polyesters (phenylacetic acid, phenylbutyric acid and other related compounds) are antitumoral compounds that have been shown to be very effective in the treatment of different types of cancers (colon, nasopharyngeal, myeloma, leukaemia, brain, thyroid, breast, ovarian) (Ferrandina *et al*., 1997; DiGiuseppe *et al*., 1999; Kebebew *et al*., 1999; Ozawa *et al*., 1999; Thibout *et al*., 1999; Chun *et al*., 2000; Wargovich *et al*., 2000; Witzig *et al*., 2000). However, some of these molecules, or other related compounds, cause certain toxic effects (alteration in mental status, Kussmaul respiration and metabolic acidosis with an increased anion gap) when administered intravenously to different patients (Praphanphoj *et al*., 2000). It could be expected that macromolecules that release these compounds at very low rates could be much more appropriate, as they would avoid the toxicity caused by a high concentration of the drug, at the same time retaining their pharmaceutical effect. Accordingly, studies on the chemical and biological degradation of the above-described polymers are critical to establish whether they can be used directly as implants or whether they should be used as vehicles for other pharmaceutical molecules. For this reason, we analysed the chemical degradation of one of these polymers (poly-3-hydroxy-6-phenylhexanoate homopolymer). To study the hydrolytic stability of this bioplastic, samples were maintained in buffer solutions with different pH values: 2 (HCl–glycine, 0.2 M); 7.4 (phosphate buffer, 0.04 M); and 10 (NaOH–glycine, 0.25 M) at 37°C over several periods of time (from 1 day to 2 months). We observed that hydrolysis of the homopolymer occurred at very low rates in all the buffers used, being faster at acidic pH values (pH 2.0) (see Fig. 4A). At this pH value, the polymer was chemically degraded to shorter homopolymers and, after 1 month of incubation, monomeric units (3-OH-6-phenylhexanoic acid) were released (see Fig. 4B). These findings indicate that this polymer is quite stable at a pH of around 7.0 and, therefore, it could be used as a drug vehicle (temporary implants) (Witholt and Kessler, 1999) in order to get a slow release of the active product. The fact that, at pH 2 (a pH value similar to that of the stomach), the polymer was partially hydrolysed (releasing active monomers) represents an additional therapeutic advantage, as the monomers released, which can be β-oxidized in the organism to phenylbutyric acid, phenylacetic acid or *trans*-cinnamic acid, could have important pharmaceutical effects (see below) improving or broadening the clinical effects of the drug trapped in it.

Taking into account the behaviour of poly-3-hydroxy-6-phenylhexanoate homopolymer in the above buffer solutions, we selected it as a model to obtain polymeric microspheres (Fig. 3D and E). They were obtained at atmospheric pressure according to a solvent evaporation method (see *Experimental procedures*). The easy obtaining of microspheres could facilitate their use as drug vehicles, enlarging the biotechnological applications of these polyesters. Thus, from a pharmacological point of view, these bioplastics could play a dual function: on one hand, their stability at physiological pH values could allow

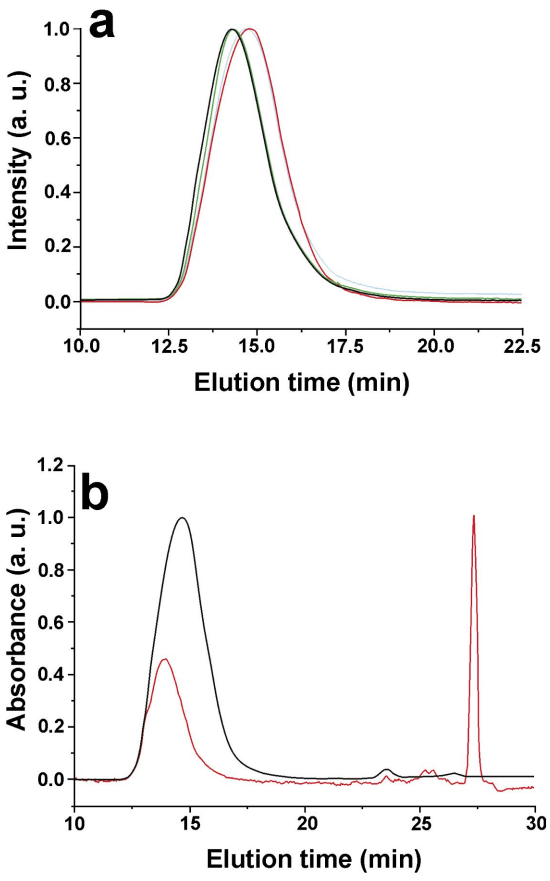

Fig. 4. Stability of poly(3-hydroxy-6-phenylhexanoate) in different buffers.A. Gel permeation profiles (using a refractive index detector) of this polymer after 1 month of incubation (37°C) in different buffers (HCl–glycine, pH 2.0: red; KH_2PO_4–Na_2HPO_4, pH 7.4: blue; and NaOH–glycine, pH 10: green). Original polymer is indicated by a black line.B. UV analysis (using a UV detector at $\lambda = 273$ nm) of the degradation product release (red line) from this polymer when incubated at pH 2 (au, arbitrary units). Peak showing an elution time of 27 min corresponds to 3-OH-6-phenylhexanoate.

their use as drug vehicles (trapping certain pharmacological substances that will be slowly released in implants) and, on the other hand, the release (as a consequence of their hydrolysis and catabolism) of phenylacetic acid, phenylbutyric acid or other β-oxidation derivatives (García *et al.*, 1999; Olivera *et al.*, 2001) that have been identified as potent antitumoral (Ferrandina *et al.*, 1997; DiGiuseppe *et al.*, 1999; Kebebew *et al.*, 1999; Ozawa *et al.*, 1999; Thibout *et al.*, 1999; Chun *et al.*, 2000; Wargovich *et al.*, 2000; Witzig *et al.*, 2000), analgesic (Martindale, 1996), radipotentiators (Miller *et al.*, 1997; Ozawa *et al.*, 1999), chemopreventive (Prasanna *et al.*, 1995; Wargovich *et al.*, 2000), antihelmintic (Martindale, 1996), nitric oxide synthase inhibitors (Pahan *et al.*, 1997), protectors against waste nitrogen excretion in patients suffering inherited urea cycle enzymes deficiencies (Simell *et al.*, 1986) or apoptosis regulators (Adam *et al.*, 1997) could increase the required pharmacological effect over months.

The easy production of different polymers by single culture variations as indicated above could allow the design of bioplastics with specific monomer composition and, hence, with the appropriate physicochemical and pharmacological properties. Further analysis of the macromolecular architecture of these new materials as well as additional studies addressing their chemical and biological degradation could be extraordinarily important for their use as drugs precursors, as drugs vehicles, as scaffolding for the regeneration of nerve axon and arteries (Witholt and Kessler, 1999) or for other therapeutic applications such as their use for supporting mammalian cell adhesion to solid supports.

In summary, the mutants reported here could be used for the industrial production of a huge number of biodegradable polymers with different characteristics and applications, opening up a plethora of potential biotechnological uses.

Experimental procedures

Production of bioplastics

Bacteria were cultured in chemically defined medium (Martínez-Blanco *et al.*, 1990) containing 4-OH-PhAc (10 mM) + n-alkanoates, n-phenylalkanoates or a combination of these as a source of monomers to build plastic polymers for different times. When only one precursor was tested, its concentration in the culture was 10 mM, whereas when more than one was supplied to this medium, the concentration of each was 5 mM. Bacterial accumulation of polyesters was followed by direct microscopic observation of the cultures.

Polymer characterization

The polymers accumulated by the different mutants were extracted from the cells as indicated elsewhere (Lageveen *et al.*, 1988; García *et al.*, 1999). Structural analyses and physicochemical properties were determined as indicated by Abraham *et al.* (2001) using a *fad*A$^-$ mutant.

^{13}C nuclear magnetic resonance (NMR) spectra were performed in a Varian XLR-500 NMR spectrometer operating at 125.70 MHz for ^{13}C measurements at room temperature. The NMR spectra were obtained from 25% (w/v) $CDCl_3$ solutions, and a delay time between pulses of 2.0 s was applied.

Average molecular weights, molecular weight distribution and polydispersity index (Mw/Mn) were determined using a Perkin-Elmer gel permeation chromatograph equipped with a refractive index detector series 200. A set of 10^4 Å, 10^3 Å and 500 Å PL-gel columns conditioned at 25°C was used to elute the samples (10 mg ml^{-1}) with high-performance liquid chromatography (HPLC)-grade chloroform (flow rate 1 ml min^{-1}). Polystyrene standards were used for calibration.

Differential scanning calorimetry (DSC) experiments were carried out in a Perkin-Elmer DSC 7. Two scans were performed using a 10°C min^{-1} heating rate and a

320°C min^{-1} cooling rate (quenching) between runs. Thermograms were obtained in the range −50°C to 75°C under nitrogen purge. The glass transition temperature (*T*g) values were taken at the onset of the transition of second scans.

Mass spectrum analyses of some β-oxidation intermediates were performed as reported previously (Olivera *et al.*, 2001).

Collection of plastic microspheres

Microspheres were obtained at atmospheric pressure according to a solvent evaporation method (Beck *et al.*, 1979; Menei *et al.*, 1994; Gallardo *et al.*, 1998). Briefly, 0.5 g of polymer was dissolved in 12 ml of dichloromethane at room temperature. The organic phase was emulsified in 100 ml of aqueous phase containing 2% (w/v) PVA under high stirring (ultra-Turrax T25 IKA at 8000 r.p.m.) for 30 min. To complete the evaporation of dichloromethane, the solution was stirred magnetically for 6 h.

Acknowledgements

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Author Queries

Q1 Author: please provide 4–6 keywords for the index.

Q2 **Abraham et al. (2001) in press**. Any more details?

Q3 **Luengo et al. (2001) in press.** Any more details?