Poly-3-hydroxyalkanoate synthases from Pseudomonas putida U: substrate specificity and ultrastructural studies

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Summary

The substrate specificity of the two polymerases (PhaC1 and PhaC2) involved in the biosynthesis of medium-chain-length poly-hydroxyalkanoates (mcl PHAs) in Pseudomonas putida U has been studied in vivo. For these kind of experiments, two recombinant strains derived from a genetically engineered mutant in which the whole pha locus had been deleted (P. putida U Δpha) were employed. These bacteria, which expresses only phaC1 (P. putida U Δpha pMC-phaC1) or only phaC2 (P. putida U Δpha pMC-phaC2), accumulated different PHAs in function of the precursor supplemented to the culture broth. Thus, the P. putida U Δpha pMC-phaC1 strain was able to synthesize several aliphatic and aromatic PHAs when hexanoic, heptanoic, octanoic decanoic, 5-phenylvaleric, 6-phenylhexanoic, 7-phenylheptanoic, 8-phenyloctanoic or 9-phenylnonanoic acid were used as precursors; the highest accumulation of polymers was observed when the precursor used were decanoic acid (aliphatic PHAs) or 6-phenylhexanoic acid (aromatic PHAs). However, although it synthesizes similar aliphatic PHAs (the highest accumulation was observed when hexanoic acid was the precursor) the other recombinant strain (P. putida U Δpha pMC-phaC2) only accumulated aromatic PHAs when the monomer to be polymerized was 3-hydroxy-5-phenylvaleryl-CoA. The possible influence of the putative three-dimensional structures on the different catalytic behaviour of PhaC1 and PhaC2 is discussed.

Introduction

The biosynthesis of medium-chain-length poly-3-hydroxyalkanoates (mcl PHAs) in Pseudomonas putida U involves the participation of: (i) two synthases (PhaC1 and PhaC2), which polymerize different monomers with aliphatic (3-OH-n-alkanoyl-CoA) or aromatic (3-OH-n-phenylalkanoyl-CoA) structures (García et al., 1999), (ii) two additional proteins (PhaF), so-called phasins, which participate in granule formation, and (iii) a regulatory protein (PhaD) which seems to be involved in PhaF and Phal synthesis (Sandoval et al., 2007). Finally, an intracellular depolymerase (PhaZ) recently characterized (de Eugenio et al., 2007) is required for the mobilization and further catabolism of these polyesters. As occurs in other microbes (Anderson and Dawes, 1990; Lageveen et al., 1988; Steinbüchel and Füchtenbusch, 1998; Madison and Huisman, 1999; Witholt and Kessler, 1999; Hang et al., 2002; Jendrossek and Handrick, 2002; Taguchi and Doi, 2004; Stubbe et al., 2005; Ward and O’Connor, 2005), in P. putida U the genes encoding all these enzymes have been located in a DNA fragment called the pha locus (García et al., 1999; Sandoval et al., 2007). Although the role of these enzymes, their genetic organization and the nature of the polymers accumulated have been widely described (Anderson and Dawes, 1990; Lageveen et al., 1988; Steinbüchel and Füchtenbusch, 1998; García et al., 1999; Madison and Huisman, 1999; Witholt and Kessler, 1999; Hang et al., 2002; Jendrossek and Handrick, 2002; Taguchi and Doi, 2004; Stubbe et al., 2005; Ouyang et al., 2007; Sandoval et al., 2007), certain points, mainly those related to the biosynthetic enzymes, remain obscure. Thus, although some in vitro assays have been reported (Qi et al., 2000; de Roo et al., 2000), study of the substrate specificity of PHA polymerases is difficult because it would require the chemical synthesis of many different substrates (3-OH-acyl-CoA derivatives). For this reason, such studies have been approached in vivo. In this sense, most PHA polymerases studied to date have been

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PCR-amplified, cloned in several vectors, and expressed in *Escherichia coli* or in other bacteria unable to produce polyhydroxyalkanoates (Qi *et al.*, 1997; Hang *et al.*, 2002; Rehm, 2003; Ren *et al.*, 2005). Nevertheless, in all cases the results obtained should be interpreted with great caution as the bacteria used as hosts (i) are very often unable to transport some PHA precursors efficiently, (ii) lack certain enzymes that, although not belonging to the pha cluster, are required for the synthesis of PHA precursors, and (iii) lack some proteins needed for the correct intracellular location and accumulation of these polymers in the bacteria. In order to avoid these limitations as well as the lack of substrates required for the *in vitro* assays, we approached the study of the substrate specificity of the two PHA polymerases from *P. putida* U using two recombinant strains derived from a genetically engineered mutant of this bacterium (see below). Similar genetic approaches have been performed in other strains of *Pseudomonas* (Kim *et al.*, 2006; Ouyang *et al.*, 2007).

### Results and discussion

**Study of the PHAs accumulated by the recombinant strains** *P. putida* U *pha* pBBR1MCS-3-phaC1 and *P. putida* U *pha* pBBR1MCS-3-phaC2

As reserve materials, *P. putida* U (RF, Colección Española de Cultivos Tipo 4848) intracellularly accumulates different PHAs constituting by aliphatic (3-hydroxy-n-alkanoates), aromatic (3-hydroxy-n-arylalkanoates) or mixtures of aliphatic and aromatic monomers when it is cultured in a chemically defined media (Martínez-Blanco *et al.*, 1990; García *et al.*, 1999) supplemented with the PHA precursors (García *et al.*, 1999; Olivera *et al.*, 2001a,b). Using different genetic strategies, we have previously obtained different mutants (or recombinant strains) that overproduce PHAs (Olivera *et al.*, 2001a; Sandoval *et al.*, 2007) as well as other that have completely lost their ability to synthesize these polyesters (Sandoval *et al.*, 2007).

Analysis of the substrate specificity of *pha*C1 and *pha*C2 from *P. putida* U were performed in a mutant in which the whole *pha* cluster had been deleted (*P. putida* U *pha*C1ZC2DFI, henceforth abbreviated as *Pp*pha). The *pha* locus was deleted by using the methodology described by other authors, which involves a double-recombination event and selection of the required mutant by expression of the lethal *sacB* gene (Donnenberg and Kaper, 1991; Quandt and Hynes, 1993; Sandoval *et al.*, 2007). To confirm the position and extent of the deletion, the mutants were analysed by PCR using a Perkin Elmer DNA Thermal Cycler 2400 and the conditions described elsewhere (Miñambres *et al.*, 2000). When required, degenerated oligonucleotides were designed in order to introduce restriction sites (usually *Bam*HI and *Xba*I) that would facilitate the cloning of the PCR-amplified fragment into pBBR1MCS-3 (see below).

To address the study of the polymerases, the *phaC1* or *phaC2* genes were PCR-amplified, cloned into the plasmid pBBR1MCS-3 (Tc), a broad-host-range cloning and expression vector (Kovach *et al.*, 1995; Sandoval *et al.*, 2007), and used to analyse the expression of these genes in the deletion mutant Pp*pha* (see above). Henceforth, these two recombinant strains (*P. putida* U *pha* pBBR1MCS-3-phaC1 and *P. putida* U *pha* pBBR1MCS-3-phaC2) will be referred to as Pp*pha* pMC-phaC1 and Pp*pha* pMC-phaC2 respectively.

*Pseudomonas putida* U Pp*pha*, and the recombinant strains Pp*pha* pMC-phaC1 and Pp*pha* pMC-phaC2 were maintained on Trypticase Soy Agar (Difco) and growth slants (12 h at 30°C) were used to inoculate liquid media. Erlenmeyer flasks (500 ml) containing 100 ml of a chemically defined medium (MM, Martínez-Blanco *et al.*, 1990) were inoculated with 2 ml each of a bacterial suspension (A540 0.5). Incubations were carried out in a rotary shaker (250 r.p.m.) at 30°C for the time required in each set of experiments. The carbon source used to culture all these strains was 4-hydroxy-phenylacetic acid (4-OH-PhAc) (10 mM), a compound that is efficiently assimilated by *P. putida* U but that cannot be used as PHA precursor (García *et al.*, 1999). For study of the synthesis of plastic polymers, bacteria were cultured in MM + 4-OH-PhAc (10 mM) + different n-alkanoic (As) or n-phenylalkanoic acids (PhAs) (10 mM). In cases in which two PHA precursors were added to the same culture, the concentration of each was 5 mM. When required different antibiotics (RF, Tc, or both) were supplied to the cultures. In all experiments in which solid media were employed, 25 g l−1 Difco agar was added. The nature of the PHA accumulated by the different strains was established as reported elsewhere (Lageveen *et al.*, 1988; García *et al.*, 1999). The contents and composition of the different PHA accumulated were determined by gas chromatography and 13C nuclear magnetic resonance as previously reported (García *et al.*, 1999; Olivera *et al.*, 2001b).

To analyse the PHAs accumulated by Pp*pha*, Pp*pha* pMC-phaC1 and Pp*pha* pMC-phaC2, in an initial approach these strains were cultured on plates of solid MM containing 4-OH-PhAc and different aliphatic (acetic, propionic, butyric, valeric, hexanoic, heptanoic, octanoic, nonanoic and decanoic acids) or aromatic (phenylacetac, phenylpropionic, phenylbutyric, phenylvaleric, phenylhexanoic, phenylnonanoic or phenylecanoic acids) PHA precursors as carbon sources (García *et al.*, 1999). We observed that whereas Pp*pha* was unable to accumulate PHA in any media tested, Pp*pha* pMC-phaC1 and Pp*pha* pMC-phaC2 synthesize them in some media. We also found that the accumulation of PHAs in these two strains differs as a function of the precursor supplied to the bacteria.
the media. Thus, when cultured in liquid MM supplemented with alkanoic acids, \(p\Delta pha\) pMC-phaC1 accumulated PHAs in all the media that contained precursors with a carbon chains longer than five carbon atoms (from hexanoic to decanoic being acid) and the highest quantity of polymer accumulated when decanoic acid was added. In this case, the PHA content (evaluated as percentage of bacterial dry weight, w/w) was 30% (w/w), and the polymer synthesized contains a 65% of 3-OH-decanoic acid and 35% of 3-OH-octanoic acid. When nonanoic, octanoic, heptanoic or hexanoic acids were used as PHA precursors, the amount of PHA accumulated (w/w) decreased as the carbon length of the alkanoic acids (As) decreased (23%, 20%, 15% and 9% respectively), their relative composition being 60% of 3-OH-nonanoic acid and 40% of 3-OH-heptanoic acid; 97% of 3-OH-octanoic acid and 3% of 3-OH hexanoic acid; 100% of heptanoic acid and 100% of hexanoic acid respectively. These results suggest that PhaC1 does not polymerize 3-HA-CoA (3-OH-acyl-CoA) derivatives whose acyl chain contains fewer than six carbon atoms and that this enzyme recognizes 3-OH-n-acyl-CoA derivatives (3-OH-n-alkanoyl-CoA) containing eight or more carbon atoms in the acyl chain faster.

When the same experiments were performed with \(p\Delta pha\) pMC-phaC2, we observed that unlike what was found for PhaC1, the quantity of PHA synthesized (w/w) decreased (19%, 17%, 11%, 8% and 6%) when the carbon length of the As used as precursor increased (hexanoic, heptanoic, octanoic, nonanoic and decanoic acid), being maximal with hexanoic acid and heptanoic acid (19% and 17% respectively). The relative composition of the polymers accumulated were as follows: with hexanoic acid (100% of 3-OH-hexanoic acid); with heptanoic acid (100% of heptanoic acid); with octanoic acid (75% of 3-OH-hexanoic acid and 25% of 3-OH-octanoic acid); with nonanoic acid (80% of 3-OH-heptanoic acid and 20% of 3-OH-nonanoic acid); and with decanoic acid (54% of 3-OH-hexanoic acid, 37% of 3-OH-octanoic acid and 9% of 3-OH-decanoic acid).

These data revealed that although both enzymes are able to polymerize monomers with an acyl carbon length of more than five carbon atoms, PhaC2 preferentially uses 3-OH-hexanoyl-CoA, whereas the best substrate of PhaC1 is 3-OH-decanoyl-CoA.

When aromatic precursors were used, the differences in substrate specificity between PhaC1 and PhaC2 became much more evident. Thus, when \(p\Delta pha\) pMC-phaC1 and \(p\Delta pha\) pMC-phaC2 were cultured in MM + 4-OH-PhAc + different PhAs (from phenylacetic to 10-phenyldecanoic acids) it was observed that both strains failed to accumulate PHA unless the carbon length of the supplemented PhA was higher than that of 4-phenylbutyric acid (see Fig. 1).

Moreover, \(p\Delta pha\) pMC-phaC1 accumulates different PHA when cultured in media containing 5-phenylvaleric acid (PhV) (100% of 3-0H-phenylvaleric acid); 6-phenylhexanoic acid (PhH) (100% of 3-OH-phenylhexanoic acid); 7-phenyloctanoic acid (PhO) (65% of 3-OH-phenylheptanoic acid and 35% of 3-OH-phenylhexanoic acid); and 9-phenylnonanoic acid (PhN) (60% of 3-OH-phenylheptanoic acid, 35% of 3-OH-phenylheptanoic acid and 5% of 3-OH-phenylnonanoic acid); and 10-phenyldodecanoic acid (PhD) (50% of 3-OH-phenylhexanoic acid, 33% of 3-OH-phenyloctanoic acid and 17% of 3-OH-phenyldecanoic acid).

Conversely, \(p\Delta pha\) pMC-phaC2 only accumulated PHAs when cultured in media supplemented with PhAs containing an odd number of carbon atoms (5-phenylvaleric acid, 7-phenyloctanoic acid and 9-phenylnonanoic acid), and was unable to synthesize PHAs when cultured in media supplemented with 6-phenylhexanoic, 8-phenyloctanoic acid or 10-phenyldecanoic acids (see Figs 1 and 2). Furthermore, analysis of the PHA accumulated revealed that in all three cases the only polymer accumulated was poly-3-OH-phenylvalerate, suggesting that 3-OH-phenylvaleryl-CoA is the only monomer that can be polymerized by PhaC2.

Although PhaC2 does not polymerize 3-OH-phenylalkanoyl-CoAs other than 3-OH-5-phenylvaleryl-CoA (3-OH-PhV-CoA), it could be argued that in the presence of a mixture of PhV and other PhA, and once the polymerization process has started, this enzyme might be able to incorporate monomers other than 3-OH-PhV to the nascent polymer chains, thus synthesizing copolymers. To test this hypothesis \(p\Delta pha\) pMC-phaC2 was cultured in MM supplemented with 4-OH-PhAc (10 mM), PhV (5 mM) and 6-phenylhexanoic acid (PhH, 5 mM). Analysis of the polymer accumulated revealed that it was a pure homopolymer of poly-3-OH-phenylvalerate, indicating that PhaC2 is unable to polymerize 3-OH-phenylalkanoates other than 3-OH-PhV-CoA. Notwithstanding, when \(p\Delta pha\) pMC-phaC1 was cultured under the same conditions the PHA accumulated was a copolymer comprising 65% 3-OH-PhV and 35% 3-OH-PhH.

**Ultrastructural studies: structure predictions for phaC1 and phaC2**

Secondary structure predictions for PhaC1 and PhaC2 were carried out using the PROFsec program on the PredictProtein server (http://www.predictprotein.org) (Rost et al., 2004). Structure assignments were performed on those residues with a predicted accuracy higher than 82% (see Fig. S1).
Tertiary structure prediction was accomplished by homology modeling through the PHYRE server (http://www.sbg.bio.ic.ac.uk/~phyre/), selecting those models with an estimated precision higher than 95%. Figures were rendered using Jmol (http://jmol.sourceforge.net/).

PhaC1 and PhaC2 do not display a strong sequence similarity with any of the proteins of known structure contained in the PDB database (http://www.rcsb.org). However, the PHYRE server is able to find remote homologues with a similar predicted global fold. Using these utilities, we found that the residues 190–480 of both polymerases share fold similarity with several hydrolases, such as human and dog gastric lipases and the carboxylesterase from Bacillus stearothermophilus (SCOP encodes d1k8qa, d1hlga and d1tqha) (Murzin et al., 1995). That part of the protein might be modelled within a core domain of the α/β-hydrolase type covered by a ‘lid’ (residues c. 330–425) (Fig. 3A), which suggests an interfacial activation mechanism that has already been described for several lipases (Desnuelle et al., 1960). However, although the presence of the ‘lid’ was predicted in all cases, its detailed structure was found to be strongly dependent on the template used. Accordingly, we focused our analysis only on the α/β-hydrolase core.

The most revealing feature in this model is the presence in both polymerases of a putative catalytic triad at the active site (Fig. 3B and C). At one corner of the α/β-hydrolase core, facing the ‘lid’ and close to the hinge between both domains, amino acids Cys296-His479-Asp451 (PhaC1) or Cys296-His480-Asp452 (PhaC2) are in close proximity and are suggestive of the arrangement reported for many hydrolases (see Holmquist, 2000, for a review). Thus, Cys296 could act as a nucleophile, attacking the activated carboxyl group of the 3-OH-acyl-CoA, thereby displacing the coenzyme A and creating an intermediate acyl-enzyme that would subsequently react with the free hydroxyl group of another 3-hydroxyalkanoyl-CoA molecule. As shown in Fig. 3D, Cys296 lies at the bottom of an elongated crevice that could accommodate the growing polymer.

α/β-Hydrolases usually contain a serine residue as the nucleophile of the catalytic triad (Holmquist, 2000). Additionally, Cys296, Ser325 (PhaC1) and Ser326 (PhaC2) are located close to the catalytic histidine-479 residue (Fig. 3B). However, the PSI-BLAST alignment used by the PHYRE server to create the models reveal that while Cys296 is absolutely conserved in all PHA synthetases (supporting its importance), the 325/326 serine is frequently substituted by non-reactive amino acids such as alanine. Further site-directed mutagenesis experiments will be crucial to assess the exact role of these residues in the catalytic mechanism of these polymerases.
Despite exhaustive analysis, we did not find significant differences between the models for the PhaC1 and PhaC2 polymerases (at least in the active site or in the rest of $\alpha/\beta$-hydrolase core) able to account for their differences in substrate specificity. Non-conservative sequence changes were distributed more or less evenly throughout the whole structure (data not shown). Perhaps the accumulation of subtle, sparse conservative changes might be of importance for 'lid' movement or substrate accommodation. However, it should be noted that our models only spanned residues 190–480, while the remaining 260 additional residues must certainly have some important, as yet unknown, function. In order to check the whole protein sequence, in a secondary structure prediction for both proteins (see Fig. S1) we observed some differences around amino acids 115–123 (coil prediction for PhaC1, helix prediction for PhaC2) and 316–324 (beta prediction for PhaC1, coil prediction for PhaC2), the latter residues located near the ‘lid’.

In sum, the above data allow us to conclude that the PHA polymerases (PhaC1 and PhaC2) from *P. putida* show quite different substrate specificity. Thus, whereas PhaC1 is able to polymerize several aliphatic and aromatic monomers (the best substrate being 3-OH-decanoyl-CoA), PhaC2 only polymerizes an aromatic compound (3-OH-5-phenylvaleryl-CoA) and certain aliphatic ones, the best substrate being 3-OH-hexanoyl-CoA. These differences may contribute to expanding the

**Fig. 2.** Electron micrographs of *Pseudomonas putida* U Δpha pMC-phaC1 (a–d) and *P. putida* U Δpha pMC-phaC2 (e–h) when cultured in MM + 4-hydroxy-phenylacetic acid (4-OH-PhAc, 10 mM) supplemented with 10 mM 5-phenylvaleric acid (PhV) (A and E); 6-phenylhexanoic acid (PhH) (B and F); 7-phenylheptanoic acid (Phh) (C and G) or 8-phenyloctanoic acid (PhO) (D and H). Bars correspond to 1 µm.
number and characteristic of the PHAs accumulated by genetically manipulated strains (i.e. PpΔfadBA – a β-oxidation mutant – or PpΔpha pMCphaF) when transformed with plasmids containing the genes encoding PhaC1 or PhaC2.

Our ultrastructural studies revealed that although there were not significant differences between PhaC1 and PhaC2 polymerases, some changes were distributed throughout the whole structure. Thus, it could be speculated that the variations found in amino acids located near the ‘lid’ (see above) might be responsible of their different substrate specificity. However, more experimental work would be required to confirm whether such changes are indeed true determinants of specificity.

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All experiments reported in this paper comply with current Spanish legislation.

References
Anderson, A.J., and Dawes, E.A. (1990) Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol Rev 54: 450–472.
Desnuelle, P., Sarda, L., and Ailhaud, G. (1960) Inhibition of pancreatic lipase by diethyl-p-nitrophenyl phosphate in emulsion. Biochim Biophys Acta 37: 570–571.
Donnenberg, M.S., and Kaper, J.B. (1991) Construction of an eae deletion mutant of enteropathogenic Escherichia coli by using a positive-selection suicide vector. Infect Immun 59: 4310–4317.
de Eugenio, L.I., García, P., Luengo, J.M., Sanz, J., San Roman, J., García, J.L., and Prieto, M.A. (2007) Biochemical evidence that phaZ gene encodes a specific intracellular medium chain length polyhydroxyalkanoate depolymerase in Pseudomonas putida KT2442. Characterization of a paradigmatic enzyme. J Biol Chem 282: 4951–4962.
García, B., Olivera, E.R., Miñambres, B., Fernández-Valverde, M., Cañedo, L.M., Prieto, M.A., et al. (1999) Novel biodegradable aromatic plastics from a bacterial source. Genetic and biochemical studies on a route of the phenylacetyl-CoA catabolon. J Biol Chem 274: 29228–29241.
Hang, X., Zhang, G., Wang, G., Zhao, X., and Chen, G.-Q. (2002) PCR cloning of polyhydroxyalkanoate biosynthesis genes from Burkholderia caryophylli and their functional expression in recombinant Escherichia coli. FEMS Microbiol Lett 210: 49–54.
Holmquist, M. (2000) Alpha/beta-hydrolase fold enzymes: structures, functions and mechanisms. *Curr Protein Pept Sci* 1: 209–235.

Jendrossek, D., and Handrick, R. (2002) Microbial degradation of polyhydroxyalkanoates. Microbial degradation of polyhydroxyalkanoates. *Annu Rev Microbiol* 56: 403–432.

Kim, T.K., Jung, Y.M., Vo, M.T., Shioya, S., and Lee, Y.H. (2006) Metabolic engineering and characterization ofphaC1 and phaC2 genes from *Pseudomonas putida* KT2442 for the production of medium-chain-length polyhydroxyalkanoate. *Biotechnol Prog* 22: 1541–1546.

Kovach, M.E., Elzer, P.H., Hill, D.S., Robertson, G.T., Farris, M.H., Roop, R.M., and Peterson, K.M. (1995) Four new derivatives of the broad-host-range cloning vector pBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* 166: 175–176.

Lageveen, R.G., Huisman, G.W., Preusteing, H., Ketelaar, P., Eggink, G., and Witholt, B. (1988) Formation of polyesters by *Pseudomonas oleovorans*: effect of substrates on formation and composition of poly-(R)-3-hydroxyalkanoates and poly-(R)-3-hydroxyalkanoates. *Appl Environ Microbiol* 54: 2929–2932.

Madison, L.L., and Huisman, G.W. (1999) Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic. *Microbiol Mol Biol Rev* 63: 21–53.

Martinez-Blanco, H., Reglero, A., Rodriguez-Aparicio, L.B., and Luengo, J.M. (1990) Purification and biochemical characterization of phenylacetyl-CoA ligase from *Pseudomonas putida*. A specific enzyme for the catabolism of phenylacetic acid. *J Biol Chem* 265: 7084–7090.

Miñambres, B., Olivera, E.R., García, B., Naharro, G., and Luengo, J.M. (2000) From a short amino acid sequence to the complete gene. *Biochem Biophys Res Commun* 272: 477–479.

Murzin, A.G., Brenner, S.E., Hubbard, T., and Chothia, C. (1995) SCOP: a structural classification of proteins database for the investigation of sequences and structures. *J Mol Biol* 247: 536–540.

Olivera, E.R., Carnicero, D., García, B., Miñambres, B., Moreno, M.A., Cañedo, L., *et al.* (2001a) Two different pathways are involved in the beta-oxidation of n-alkanoic and n-phenylalkanoic acids in *Pseudomonas putida* U: genetic studies and biotechnological applications. *Mol Microbiol* 39: 863–874.

Olivera, E.R., Carnicero, D., Jodra, R., Miñambres, B., García, B., Abraham, G.A., *et al.* (2001b) Genetically engineered *Pseudomonas*: a factory of new bioplastics with broad applications. *Environ Microbiol* 3: 612–618.

Ouyang, S.P., Liu, Q., Fang, L., and Chen, G.Q. (2007) Construction of pha-oporon-defined knock out mutants of *Pseudomonas putida* KT2442 and their applications in poly(hydroxyalkanoate) production. *Macromol Biosci* 7: 227–233.

Qi, Q., Rehm, B.H.A., and Steinbüchel, A. (1997) Synthesis of poly(3-hydroxyalkanoates) in *Escherichia coli* expressing the PHA synthase gene phaC2 from *Pseudomonas aeruginosa*: comparison of PhaC1 and PhaC2. *FEBS Microbiol Lett* 157: 155–162.

Qi, Q., Steinbüchel, A., and Rehm, B.H.A. (2000) *In vitro* synthesis of poly(3-hydroxydecanoate): purification and enzymatic characterization of type II polyhydroxyalkanoate syntheses PhaC1 and PhaC2 from *Pseudomonas aeruginosa*. *Appl Microbiol Biotechnol* 54: 37–43.

Quandt, J., and Hynes, M.F. (1993) Versatile suicide vectors which allow direct selection for gene replacement in gram-negative bacteria. *Gene* 127: 15–21.

Rehm, B.H.A. (2003) Polyester synthases: natural catalysts for plastics. *Biochem J* 376: 15–33.

Ren, Q., van Beilen, J.B., Siervo, N., Zinn, M., Kessler, B., and Witholt, B. (2005) Expression of PHA polymerases genes of *Pseudomonas putida* in *Escherichia coli* and its effect on PHA formation. *Antonie Van Leeuwenhoek* 87: 91–100.

de Roo, G., Ren, Q., Witholt, B., and Kessler, B. (2000) Development of an improved *in vitro* activity assay for medium chain length PHA polymerase based on Coenzyme A release measurements. *J Microbiol Meth* 41: 1–8.

Rost, B., Yachdav, G., and Liu, J. (2004) The Predict Protein Server. *Nucleic Acids Res* 32 (Web Server issue): W321–W326.

Sandoval, A., Arias-Barrau, E., Arcos, M., Naharro, G., Olivera, E.R., and Luengo, J.M. (2007) Genetic and structural analysis of different mutants of *Pseudomonas putida* U affected in the poly-3-hydroxy-n-alkanoate gene cluster. *Environ Microbiol* 9: 737–751.

Steinbüchel, A., and Füchtenbusch, B. (1998) Bacterial and other biological systems for polyester production. *Trends Biotechnol* 16: 419–427.

Stubbe, A., Tian, J., He, A., Sinks, A.J., Lawrence, A.G., and Liu, P. (2005) Non-template-dependent polymerization processes: polyhydroxyalkanoate synthases as a paradigm. *Annu Rev Biochem* 74: 433–480.

Taguchi, S., and Doi, Y. (2004) Evolution of polyhydroxyalkanoate (PHA) production system by ‘enzyme evolution’: successful case studies of directed evolution. *Macromol Biosci* 4: 145–156.

Ward, P.G., and O’Connor, K.E. (2005) Bacterial synthesis of polyhydroxyalkanoates containing aromatic and aliphatic monomers by *Pseudomonas putida* CA-3. *Int J Biol Macromol* 35: 127–133.

Witholt, B., and Kessler, B. (1999) Perspectives of medium chain length poly(hydroxyalkanoates), a versatile set of bacterial bioplastics. *Curr Opin Biotechnol* 10: 279–285.

Supplementary material

The following supplementary material is available for this article online:

**Fig. S1.** Secondary structure prediction for PhaC1 and PhaC2. PhaC1 and PhaC2 sequences are shown in red and black, whereas their corresponding secondary structure predictions are shown in pink and blue respectively. Numbering is according to the PhaC1 sequence. Regions with significant discrepancies on prediction are highlighted within a green box. The residues belonging to the putative catalytic triad are shadowed in yellow. H, α-helix prediction; E, β-strand (extended conformation) prediction; L, loop prediction; dots represent other non-repetitive structures.

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