environmental microbiology

Environmental Microbiology (2015) **17**(9), 3182–3194 doi:10.1111/1462-2920.12753

The loss of function of PhaC1 is a survival mechanism that counteracts the stress caused by the overproduction of poly-3-hydroxyalkanoates in *Pseudomonas putidaΔfadBA*

José I. Obeso,[1] Beatriz Maestro,[2] Jesús M. Sanz,[2] Elías R. Olivera[1] and José M. Luengo[1]*

[1]*Departamento de Biología Molecular, Facultad de Veterinaria, Universidad de León, León 24071, Spain.*

[2]*Instituto de Biología Molecular y Celular, Universidad Miguel Hernández, Elche, Alicante 03202, Spain.*

Summary

The poly-3-hydroxylkanoate (PHA)-overproducing mutant *Pseudomonas putida* U Δ*fadBA* (*Pp*Δ*fadBA*) lacks the genes encoding the main β-oxidation pathway (FadBA). This strain accumulates enormous amounts of bioplastics when cultured in chemically defined media containing PHA precursors (different n-alkanoic or n-aryl-alkanoic acids) and an additional carbon source. In medium containing glucose or 4-hydroxy-phenylacetate, the mutant does not accumulate PHAs and grows just as the wild type (*P. putida* U). However, when the carbon source is octanoate, growth is severely impaired, suggesting that in *Pp*Δ*fadBA*, the metabolic imbalance resulting from a lower rate of β-oxidation, together with the accumulation of bioplastics, causes severe physiological stress. Here, we show that *Pp*Δ*fadBA* efficiently counteracts this latter effect via a survival mechanism involving the introduction of spontaneous mutations that block PHA accumulation. Surprisingly, genetic analyses of the whole *pha* cluster revealed that these mutations occurred only in the gene encoding one of the polymerases (*phaC1*) and that the loss of PhaC1 function was enough to prevent PHA synthesis. The influence of these mutations on the structure of PhaC1 and the existence of a protein–protein (PhaC1–PhaC2) interaction that explains the functionality of the polymerization system are discussed herein.

Received 19 September, 2014; accepted 12 December, 2014. *For correspondence. E-mail jmluer@unileon.es; Tel. (+34) 987 291228; Fax (+34) 987 291226.

Introduction

Poly-3-hydroxyalkanoates (PHAs) constitute a family of optically active, naturally occurring polyoxoesters synthesized by many species of microbes (some archaea and certain Gram-positive and Gram-negative bacteria) and accumulated as intracellular storage deposits (Huisman *et al.*, 1992; Steinbüchel and Valentin, 1995; García *et al.*, 1999; Witholt and Kessler, 1999; Luengo *et al.*, 2003; Stubbe *et al.*, 2005; Kim *et al.*, 2007; Olivera *et al.*, 2010). Although these polymers mainly serve as an energy source, PHA accumulation seems to be involved in other functions, such as survival under inorganic nutrient limitation, stress tolerance, biofilm formation and the maintenance of the redox state (Madison and Huisman, 1999; Alsor and Keasling, 2003; Pham *et al.*, 2004; Ayub *et al.*, 2009).

Most of the PHAs characterized to date contain monomers, with different chemical structures [3-, 4-, 5- or 6-hydroxy-n-(aryl)alkanoic acids], that are condensed through polymerization reactions (Lageveen *et al.*, 1988; Lenz *et al.*, 1992; Rehm *et al.*, 1998; García *et al.*, 1999; Rehm, 2003; Lütke-Eversloh and Steinbüchel, 2004; Olivera *et al.*, 2010; Escapa *et al.*, 2011; Dinjaski *et al.*, 2014). In the particular case of PHAs made up of monomers with carbon lengths between C6 and C12 (mcl-PHAs), their accumulation requires the participation of two polymerases (PhaC1 and PhaC2) (Rehm, 2003; Sandoval *et al.*, 2007; Arias *et al.*, 2008; 2013), two phasins (PhaF and PhaI) involved in granule formation (Prieto *et al.*, 1999; Sandoval *et al.*, 2007; Galán *et al.*, 2011; Maestro *et al.*, 2013) and a gene regulator (PhaD) (Klinke *et al.*, 2000; de Eugenio *et al.*, 2010). Once synthesized, PHAs are mobilized by a depolymerase (PhaZ) (de Eugenio *et al.*, 2007). All these enzymes are encoded by genes clustered within the *pha* locus (*phaC1ZC2DFI*) (Fig. 1), whereas other less specific proteins (i.e. those involved in precursors transport and synthesis are encoded by genes located outside the *pha* cluster (García *et al.*, 1999; Sandoval *et al.*, 2007).

Taking into account the physicochemical properties of PHAs (Anderson and Dawes, 1990; Peters and Rehm,

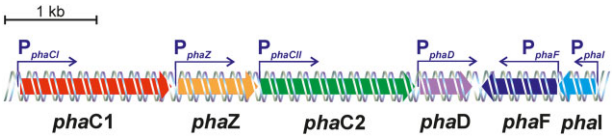


Fig. 1. Schematic organization of the *pha* cluster (genes *phaC1ZC2DFI*) in *P. putida* U. The genes *phaC1*, *phaZ*, *phaC2*, *phaD*, *phaF* and *phaI* encode polymerase 1, depolymerase, polymerase 2, a regulatory protein (PhaD) and two phasins (PhaF and PhaI) respectively. The six putative promoters are also indicated.

2005) and their broad range of biotechnological applications (Steinbüchel, 2001; van der Walle *et al*., 2001; Sevastianov *et al*., 2003; Misra *et al*., 2006; Chen, 2010; Koller *et al*., 2010), significant effort has been devoted to isolating mutants (or recombinant strains) that overproduce these polymers (Lee *et al*., 1994; García *et al*., 1999). Among them, the mutant *Pseudomonas putida* U Δ*fadBA*, a mcl-PHA-overproducer in which the genes encoding the β-oxidation pathway (*fadBA*) have been deleted (Olivera *et al*., 2001a,b), was of particular interest and was the focus of this study. Specifically, we used this mutant to examine the influence of PHA overproduction on the growth of bacteria cultured in chemically defined media containing different carbon sources. We also analysed the mechanism employed by *P. putida* U Δ*fadBA* to counteract the physiological stress caused by PHA biosynthesis and accumulation. We conclude our report with a discussion of the essential role played by PhaC1 in the biosynthesis of PHA *in vivo*.

Results

Growth of P. putida *U and* P. putida Δ fadBA *in a chemically defined medium containing different carbon sources*

Genetic engineering studies revealed that deletion of the genes encoding the β-oxidation pathway (*fadBA*) in wild-type *P. putida* U (*Pp*U) yielded a PHA-overproducing mutant, *P. putida* Δ*fadBA* (*Pp*Δ*fadBA*), which accumulated large amounts of bioplastics (Olivera *et al*., 2001a). The very large quantity of PHAs synthesized by strain *Pp*Δ*fadBA* was evident by the waxy aspect of the cultures grown on solid medium (Fig. 2B). This mutant had an unusual morphology, as it was both longer and larger than the wild type (Fig. 2).

Additional differences between *Pp*U and *Pp*Δ*fadBA* became apparent when the two strains were cultured in a chemically defined medium, containing as carbon sources glucose or 4-hydroxyphenylacetate (4-OH-PhAc), which are not PHA precursors, or octanoate, which is required for the synthesis of poly-3-hydroxyoctanoate, one of the better-known mcl-PHAs (Lageveen *et al*., 1988; Huisman *et al*., 1992). Figure 3 shows that whereas bacterial growth was similar when *Pp*U and *Pp*Δ*fadBA* were cultured in chemically defined medium (MM) containing either 4-OH-PhAc or glucose (Fig. 3A and B), there was a large difference in the growth of these strains when cultured in MM containing octanoate (OMM) (Fig. 3C).

The poor growth of *Pp*Δ*fadBA* when cultured in OMM could have been caused by the generation of a metabolic imbalance resulting from the induction of a less efficient β-oxidative pathway (β-II, which replaced FadBA; Olivera *et al*., 2001b). However, we could not rule out that the ultrastructural changes caused by abnormal PHA accumulation (Olivera *et al*., 2001a) also had an effect on cellular division and, therefore, on bacterial growth. A

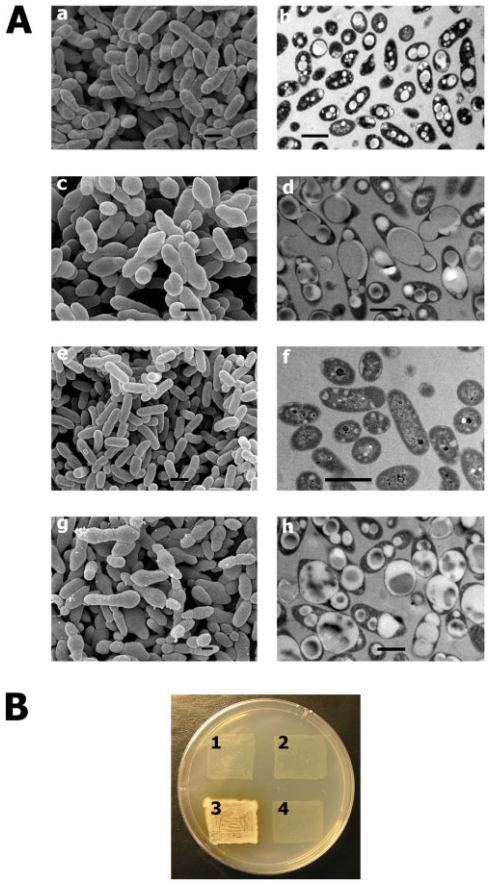


Fig. 2. (A) Scanning (a, c, e, g) and transmission (b, d, f, h) electron micrographs of *P. putida* U (a, b), *P. putida* Δ*fadBA* (c, d), *P. putida* Δ*fadBA*PHA$^-$ (e, f), and *P. putida* Δ*fadBA*PHA$^-$pMC*phaC1* (g, h) when cultured in a chemically defined medium (MM) containing 4-hydroxy-phenylacetate (4-OH-PhAc, 10 mM) and octanoate (10 mM). Bar represents 1 μm. (B) Morphological aspect of *P. putida* Δ*fadBA* PHA$^-$pMC (i); *P. putida* Δ*fadBA*PHA$^-$ (ii); *P. putida* Δ*fadBA*PHA$^-$pMC*phaC1* (iii), and *P. putida* Δ*fadBA* PHA$^-$pMC*phaC1** (4) when cultured on solid MM containing 4-hydroxy phenylacetic acid (4-OH-PhAc, 10 mM) and octanoate (10 mM). When *P. putida* Δ*fadBA* was cultured in the same medium and under the same conditions a similar waxy aspect (see Fig. 2B) was observed.

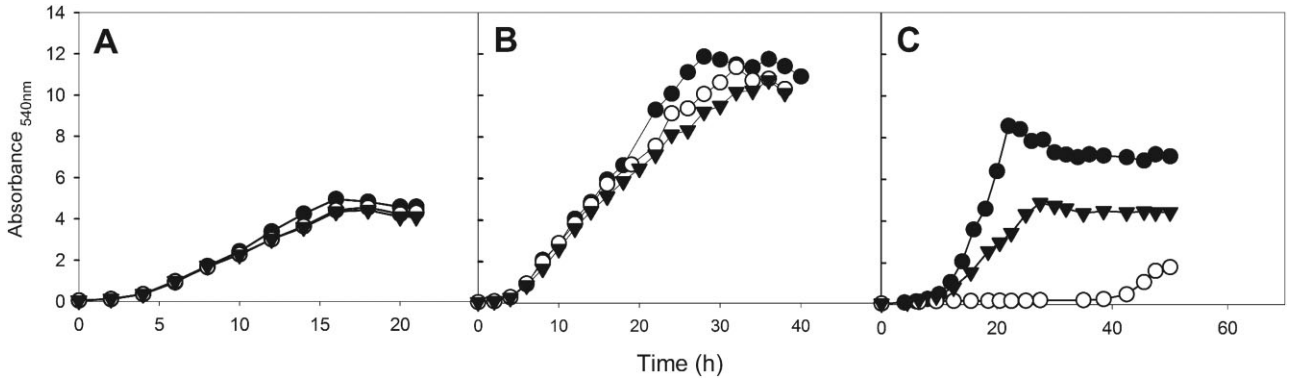


Fig. 3. Bacterial growth (A_{540nm}) during the culture of *P. putida* U (●), *P. putida* Δ*fadBA* (○) and *P. putida* Δ*fadBA*PHA⁻ (▼) in a chemically defined medium (MM) containing 20 mM 4-OH-PhAc (A), 40 mM glucose (B), or 20 mM octanoate (C) as carbon source. When *P. putida* Δ*fadBA*PHA⁻pMC*phaC1* was cultured in the same medium the growth was similar to that of *P. putida* Δ*fadBA*. All experiments were performed in triplicate and the data reported are the means of the values obtained. The differences in the absorbance values corresponding to similar determinations in repeated experiments were never higher than 13%.

modification of bacterial morphology and its influence on bacterial growth in PHA producers were previously described. Thus, Lee and colleagues (1994) showed that considerable filamentation occurred in different *Escherichia coli* recombinant strains expressing poly-3-hydroxybutyrate genes (*phb*).

Therefore, although the overproduction of PHAs in *Pp*Δ*fadBA* most likely affected basic metabolic processes that made growth in OMM difficult, the involvement of other basic mechanisms, such as those controlling cell size, cell division and, therefore, bacterial growth, was also possible. Thus, to better understand the poor growth of the mutant in OMM (Fig. 3), additional experiments were performed, as discussed below.

Isolation and identification of mutants unable to synthesize PHAs from the overproducing strain PpΔfadBA

When *Pp*Δ*fadBA* was repeatedly cultured in OMM for 24 h at 30°C, its growth increased in parallel with its loss of the ability to synthesize PHAs. To analyse this effect, *Pp*Δ*fadBA* isolated colonies were re-seeded periodically (after 24 h) on solid OMM, with the process repeated 10 times. Daily microscopic observations revealed a direct relationship between passage number and the loss of the ability to accumulate PHAs (Fig. S1). Transmission electron microscopy showed that after four passages, more than 80% of the bacteria had lost their ability to synthesize PHAs, whereas after seven passages none had accumulated detectable amounts of PHAs. The absence of PHAs in these mutants was confirmed by direct microscopic observation (see Fig. 2 and Fig. S1) and by extracting dried bacteria with chloroform and then quantifying the methanol-precipitated residues (Lageveen *et al*., 1988; Sandoval *et al*., 2007). The procedure used to isolate non-PHA-producing *P. putida* Δ*fadBA* (*Pp*Δ*fadBA*PHA⁻, briefly PHA⁻ mutants) is summarized in Fig. S1.

Furthermore, when the PHA⁻ spontaneous mutants were cultured on other media (containing or not PHA precursors), they failed to regain their ability to synthesize PHAs, suggesting the introduction of genetic changes (mutations) in *Pp*Δ*fadBA* that hindered PHA synthesis. By contrast, when similar experiments were performed with the wild-type *Pp*U, it retained its capability to synthesize PHAs, even after hundreds of passages on OMM.

Conversely, when the strain *Pp*Δ*fadBA* was repeatedly cultured on solid medium containing 10 mM octanoate and 10 mM 4-OH-phenylacetate, it never lost its ability to synthesize and overproduce PHAs. These data indicated that in the presence of an additional carbon source that restores the energy balance, PhaC1 mutations were not evident. Although these results reinforce the hypothesis that the low growth of *Pp*Δ*fadBA* in OMM is due to a metabolic dysfunction, the possibility of a combined role for both metabolic imbalance and morphological changes could not be excluded. In an attempt to distinguish between the two mechanisms, *Pp*U, *Pp*Δ*fadBA* and *Pp*Δ*fadBA*PHA⁻ were cultured in MM containing 20 mM butyrate (BMM) as the sole carbon source (Fig. 4). The three strains were expected to catabolyse butyrate via β-oxidation with *Pp*U using FadBA, and *Pp*Δ*fadBA* and *Pp*Δ*fadBA*PHA⁻ both using the β-II pathway. However, unlike octanoate, butyrate could not be used as a source of PHA precursors (3-OH-butyryl-CoA) because none of these strains had the PHB-biosynthetic pathway (García *et al*., 1999).

It is interesting to note that when cultured in OMM, *Pp*Δ*fadBA*PHA⁻ reached absorbance values that were higher than those of *Pp*Δ*fadBA* (see Fig. 3C). However, this difference was not observed when these strains were cultured in BMM (Fig. 4), suggesting that β-II-oxidation,

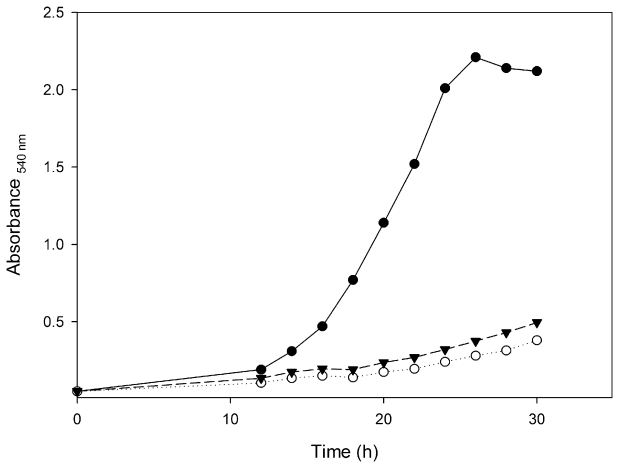


Fig. 4. Bacterial growth (A_{540nm}) during the culture of *P. putida* U (●), *P. putida* Δ*fadBA* (○) and *P. putida* Δ*fadBAPHA*$^-$ (▼) in a chemically defined medium (MM) containing 20 mM butyrate.

which replaced FadBA in *Pp*Δ*fadBA* and in *Pp*Δ*fadBA*PHA$^-$, is less efficient, especially in the catabolism of short-chain-length fatty acids. The slow degradation of the acyl-CoAs compounds generated through β-oxidation when they reached a critical size (having an acyl moiety ranging between C4 and C5) would explain why *Pp*Δ*fadBA* overproduces mcl-PHAs (Olivera *et al.*, 2001b). In summary, the poor growth of *Pp*Δ*fadBA* on OMM was probably not due to ultra-structural changes that hindered cell division, but rather to the metabolic effects imposed by the mutation.

In additional experiments in which *Pp*Δ*fadBA* was re-seeded on BMM, the mutant did not lose its ability to synthesize mcl-PHAs (data not shown). Because butyrate, like octanoate, is a ketogenic compound, these results suggest that the expression of mutations affecting PHA synthesis, as observed when *Pp*Δ*fadBA* was cultured in OMM, requires: (i) a less efficient β-oxidation, (ii) the presence in the medium of a ketogenic molecule as the sole carbon source, and (iii), concomitantly, the synthesis and accumulation of PHAs.

Identification of the sequences mutated in the pha cluster of the PHA$^-$ spontaneous mutants

To determine the nature of the mutations produced in the PHA$^-$ spontaneous mutants and to identify the mutated genes, 22 colonies of *Pp*Δ*fadBA* unable to synthesize PHAs (isolated from cultures re-seeded over a period of 10 days) were selected and the whole *pha* cluster was sequenced at least four times. When a mutation was found, the DNA fragment containing the altered sequence was polymerase chain reaction (PCR)-amplified and re-sequenced. To assure the accuracy of the information, 10 different PCRs were performed.

Although the appearance of mutations in different *pha* genes was expected, all the mutations were instead located within *phaC1*, encoding the polymerase PhaC1 (see Table 1). Surprisingly, in all of the examined mutants, none of the other *pha* genes belonging to the cluster (*phaZC2DFI*) were affected. Closer evaluation of the different *phaC1* mutations revealed that: (i) in 11 colonies, 2 nucleotide had been replaced by another (transition or transversion), yielding an amino acid change in PhaC1; (ii) in 7 cases, a stop codon had been generated; (iii) in 3 colonies, deletions of 15, 90 and 252 nucleotides, respectively, had been produced; and (iv) in 1 strain, a large duplication of 114 nucleotides (equivalent to 38 amino acids) had occurred (see Table 1). It is worth noting that different colonies coming from the same patch (Fig. S1) differed in their single mutations in *phaC1* (see colonies 3, 10 and 21 in Table 1). Thus, although many random mutations could have occurred during the same adaptive process, repeated passage resulted in the selection of only those bacteria with mutations that resulted in the loss of PHA synthesis.

Additional studies performed in OMM demonstrated the better and much faster growth of the PHA$^-$ mutants than the parental strain *Pp*Δ*fadBA* (Fig. 3C). This difference (measured as absorbance at 540 nm) was even higher than that shown in Fig. 3 because *Pp*Δ*fadBA* cells were much larger than PHA$^-$ cells (Fig. 2). Furthermore, growth of the PHA$^-$ mutants was the same as that

Table 1. Mutations found in the *phaC1* gene belonging to different spontaneous mutants unable to synthesize PHAs (*P. putida* Δ*fadBA*PHA$^-$).

Mutant	Aa change	Stop codon	Deletion	Duplication
1	I225T			
2	D232N (2)			
3	S254N (5)			
4	S254I			
5	T343P (2)			
6	M369V			
7	W376R (3)			
8	N419H			
9	N419T			
10	T432P (2)			
11	P552L			
12		E8STOP		
13		L20STOP		
14		Q116STOP (2)		
15		Y270STOP		
16		W519STOP		
17		W525STOP		
18		L60STOP		
19			84Aa 288–372	
20			30Aa 328–358	
21			84Aa 288–372	
22				38Aa 410–448

Colonies 3,10 and 21 were isolated from the same PHA$^-$ patch. The number of mutants found with the same mutation are in brackets. Aa, amino acid.

Table 2. Strains and plasmids used in this work.

Strain or plasmid	Phenotype/relevant characteristics	Reference
Pseudomonas putida		
P. putida U	Wild type.	Martínez-Blanco *et al*., 1990
P. putida Δ*fadBA*	Δ*fadBA* PHA overproducer mutant.	Olivera *et al*., 2001a
P. putida PHA$^-$ (mutants 1-22)	*P. putida* Δ*fadBA* mutants which have lost the ability to synthesize PHA.	This study
PHA$^-$ pMC	*P. putida* PHA$^-$ carrying the plasmid pBBR1MCS-3.	This study
PHA$^-$ pMC*phaC1*	*P. putida* PHA$^-$ recombinant containing in the plasmid pBBR1MCS-3 a copy of the gene *phaC1*.	This study
PHA$^-$ pMC*phaC*1*	*P. putida* PHA$^-$ recombinant containing in the plasmid pBBR1MCS-3 a mutated copy of the gene *phaC1*.	This study
PHA$^-$ pMC*phaC2*	*P. putida* PHA$^-$ recombinant containing in the plasmid pBBR1MCS-3 a copy of the gene *phaC2*.	This study
P. putida U Δ*pha*	*P. putida* mutant in which the whole *pha* cluster has been deleted.	Sandoval *et al*., 2007
Δ*pha* pMC*phaC1*	*P. putida* Δ*pha* transformed with the plasmid pBBR1MCS-3 carrying a copy of the gene *phaC1*.	Sandoval *et al*., 2007
Δ*pha* pMC*phaC1**	*P. putida* Δ*pha* transformed with the plasmid pBBR1MCS-3 carrying a mutated copy of the gene *phaC1*.	This study
Δ*pha* pMC*phaC2*	*P. putida* Δ*pha* transformed with the plasmid pBBR1MCS-3 carrying a copy of the gene *phaC2*.	Arias *et al*., 2008
Δ*pha* pMC*phaC1* phaC2*	*P. putida* Δ*pha* transformed with the plasmid pBBR1MCS-3 carrying a mutated copy of the gene *phaC1* and a copy of the gene *phaC2*.	This study
Escherichia coli		
DH10B	F–, *mcr*A, Δ*mrr-hsd*RMS-*mcr*BC), Ø80d*lac*Z1M15, Δ*lac*X74, *deo*R, *rec*A1, *end*A1, *ara*D139, Δ(*ara*, *leu*)7697, *gal*U *gal*K, λ–, *rps*L, *nup*G.	García *et al*., 1999
HB101pRK600	Cm^R, *ori*ColE1*oriV*, Mob$^+$, helper plasmid in triparental matings.	Herrero *et al*., 1990
Plasmids		
pBBR1MCS-3 (pMC)	Tc^R,*ori*BBR1, Mob$^+$,*lac* promoter *lacZ*α$^+$, broad-host-range cloning and expression vector.	Kovach *et al*., 1995
pGEM-T Easy	Ap^R, *ori*ColE1, *lacZ*α$^+$, SP6 T7, *lac* promoter, direct cloning of PCR products.	Promega (USA)
pJQ200SK	Gtm^R, *ori*p15A, Mob$^+$, *lacZ*α$^+$, *sac*B.	Quandt and Hynes, 1993
pK18*mob*	Km^R, *ori*T*oriV*, Mob$^+$, *lacZ*α$^+$, *sac*B.	Schäfer *et al*.,1994
pTZ57R/T	Ap^R,*ori*ColE1,*lacZ*α$^+$, T7 promoter, direct cloning of PCR products.	Thermo Scientific (USA)
pRK600	Cm^R,*ori*ColE1, *oriV*, Mob$^+$, auxiliar plasmid used to conjugation experiments.	Herrero *et al*.,1990

of the wild type (*Pp*U) in all media tested, with the exception of OMM. In this case, the absorbance of the *Pp*U cultures was higher (see Fig. 3C) because the wild type, unlike the PHA$^-$ mutants, accumulated PHAs intracellularly.

In summary, the higher absorbance values of the PHA$^-$ mutants (Fig. 3C) evidenced the introduction of mutations preventing PHA synthesis, which in turn provided important metabolic advantages to these bacteria.

Functional analyses of the mutated protein: restoration of PHA biosynthetic capability

To study the functionality of PhaC1, each of the different PHA$^-$ mutants (see Table 1) was transformed with a genetic construct in which the PCR-amplified *phaC1* gene from *Pp*U (pMC*phaC1*) was ligated into the plasmid pBBR1MCS-3 (pMC) (see Table 2). All recombinant strains (*P. putida* Δ*fadBA*PHA$^-$pMC*phaC1*, referred to in the following as *Pp*Δ*fadBA*PHA$^-$pMC*phaC1*) recovered their ability to synthesize bioplastics, suggesting that a correct copy of the polymerase PhaC1 was sufficient to assure PHA synthesis (see Fig. 2). However, when the corresponding mutants were transformed with constructs containing copies of the mutated *phaC1* (*phaC1**), none of them accumulated PHAs.

In earlier studies aimed at clarifying the function of mcl-PHA proteins in *Pp*U, we showed that: (i) elimination of the whole pha cluster (*P. putida* U Δ*pha*, *Pp*Δ*pha*) completely prevented PHA formation; (ii) transformation of this mutant with a plasmid harbouring the gene encoding the polymerase PhaC1 (PpUΔ*pha*pMC*phaC1*) fully restored PHA synthesis; and that (iii) PhaC1 and PhaC2 differ in their substrate specificities (Sandoval *et al*., 2007; Arias *et al*., 2008).

Thus, when the mutant *Pp*Δ*pha* was transformed with plasmids containing copies of the mutated *phaC1* (*phaC1**), the recombinants similarly failed to accumulate PHAs (Fig. 5B and D). The only recombinant able to synthesize PHAs was the one transformed with a construct containing *phaC1* obtained from the wild type (*Pp*U) (Fig. 5A).

Moreover, when *phaC2* was used to transform the PHA$^-$ mutants, none of the recombinants

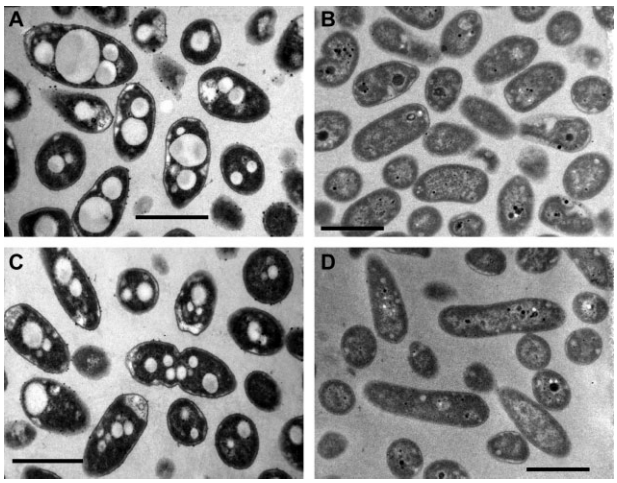


Fig. 5. Electron micrographs of (A) *P. putida* Δ*pha*pMC*phaC1*; (B) *P. putida* Δ*pha*pMC*phaC1**; (C) *P. putida* Δ*pha*pMC*phaC2* and (D) *P. putida* Δ*pha*pMC*phaC1*phaC2*. *phaC1** corresponds to a mutated form of the gene *phaC1* (PCR-amplified from the mutant *P. putida* Δ*fadBA*PHA$^-$ number 1, see Tables 1 and 2). Bars represent 1 μm.

(*Pp*Δ*fadBA*PHA$^-$pMC*phaC2*) recovered the ability to produce PHAs (data not shown). However, PHA synthesis was restored in strain *Pp*Δ*pha* transformed with the same genetic construct (pMC*phaC2*) (Fig. 5C). These data indicate that: (i) the effect of the mutation of *phaC1* could not be reversed by the expression in trans of polymerase PhaC2 (Fig. 5D) and (ii) a functional PhaC1 is required to recover the ability to synthesize PHAs. These results agree with the observation that none of the PHA$^-$ mutants was able to synthesize PHAs, despite the fact that all of them carried a functional PhaC2. Furthermore, when strain *Pp*Δ*pha* was transformed with a genetic construct containing a mutated copy of *phaC1* (mutant 1, see Table 1) together with a copy of *phaC2* (from the wild type), none of the recombinants accumulated PHAs, even though all of them had a functional copy of the polymerase PhaC2 (Fig. 5D). Because expression of the genes encoding PhaC1 and PhaC2 was driven from the plasmid promoter, the existence of protein–protein interactions between the two polymerases can be assumed (see discussion).

In silico structural analysis of PhaC1 mutations and a potential protein-protein interaction between PhaC1 and PhaC2

In a previous study, we used the PHYRE automatic fold recognition server to model part of the three-dimensional structure of PhaC1 and PhaC2 on the basis of their sequence homologies with several hydrolases (Arias *et al*., 2008). In this work, we repeated the procedure using the improved PHYRE2 homology modelling server (http://www.sbg.bio.ic.ac.uk/~phyre2/) (Kelley and Sternberg, 2009). PhaC1 and PhaC2 showed the highest similarity with human gastric lipase (Protein Data Bank code: 1hlg) and a putative esterase from *Sulfolobus solfataricus* (PDB code: 2rau) respectively. Residues 201–530 of both synthases could be modelled with > 90% accuracy. According to the prediction procedure, the modelled structures of the two polymerases acquire an α/β hydrolase fold covered by a 'lid' (residues 330–425) that buries the predicted catalytic triad (Cys296–Asp451 – His479 for PhaC1, Cys296–Asp452-His480 for PhaC2). The lid resembles the one found in several lipases and thought to be involved in interfacial activation. The lack of suitable homologues in the database prevented the modelling of residues 1–200 and 530–558.

As depicted in Fig. 6, most single-residue PhaC1-inactivating mutations mapped within or in the proximity of the predicted lid, suggesting that they interfere with its necessary opening that makes the catalytic site accessible to the substrate.

Because our results indicated that PhaC1 needs to be fully functional for PhaC2 to be active and able to synthesize PHAs, we speculated that the two proteins form a complex. To investigate this possibility, we examined the docking of PhaC1 and PhaC2 model structures using the ZDOCK protein-docking algorithm. Figure 7 shows the superimposition of the best five heterodimer complexes predicted by ZDOCK, using PhaC2 as the reference. There was a reasonable convergence of the structures towards a complex involving the solvent-exposed regions of the lids of the two polymerases, as most van der Waals contacts made use of atoms belonging to these moieties. Finally, given the similarity of the PhaC1 and PhaC2 sequences, we explored the probability of PhaC1–PhaC1 and PhaC2–PhaC2 homodimer formation and found that both complexes were likely, again through lid–lid interactions (Fig. 8).

Discussion

Pseudomonas putida U accumulates PHAs when cultured in MM containing n-alkanoic, n-phenylalkanoic acids or mixtures thereof as a carbon source (García *et al*., 1999; Olivera *et al*., 2001a). However, the relative amount of industrially important aromatic polyesters produced by the bacterium is lower than that of aliphatic polyesters (García *et al*., 1999). Thus, to improve production yields, we constructed a mutant (*Pp*Δ*fadBA*) lacking the two genes responsible for the classical β-oxidation pathway (*fadBA*).

Whereas the growth of *Pp*Δ*fadBA* and *Pp*U (wild type) was similar when the two strains were cultured in MM containing either glucose or 4-OH-PhAc, it greatly differed when the medium was OMM (Fig. 3). The poor growth of

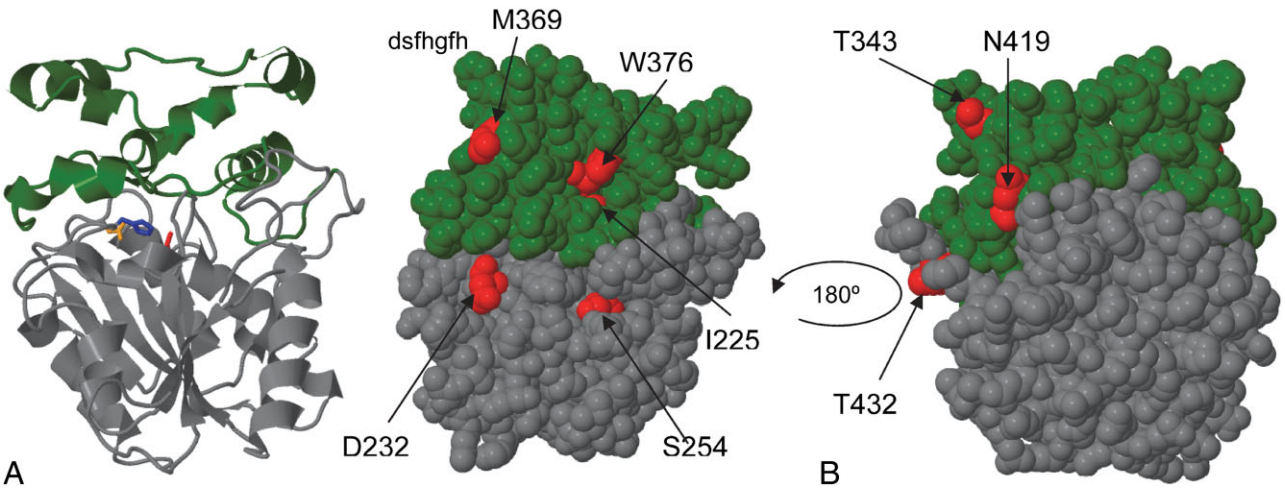


Fig. 6. Mapping of single-residue mutations in the PhaC1 model using human gastric lipase (1 hlg) as template. (A) Overall view of PhaC1 in cartoon representation. The predicted lid (residues 330–425) is depicted in green. Catalytic residues are shown in wireframe format: Cys296 (red), Asp451 (orange), and His479 (blue). (B) Two views of the van der Waals representation, with the mutated positions shown in red. The figures were rendered with Jmol 14.2 (http://www.jmol.org).

*Pp*Δ*fadBA* in OMM (Fig. 3C) can be explained by assuming that once *FadBA* is blocked, a less efficient β-oxidation pathway (replacing FadBA) is induced. Under these new metabolic conditions, the genesis of reducing power and acetyl-CoA decrease such that general metabolism will be slower. The involvement of PHA metabolism in modulating the availability of reducing equivalents has been also reported in other *Pseudomonas* strains (Ayub *et al*., 2009). In addition our hypothesis is supported by the results shown in Figs 3C and 4, which suggest that a slow assimilation of octanoate (and/or of its shorter acyl-CoA derivatives) accounts for the differences in growth of *Pp*U and *Pp*Δ*fadBA* when the strains cultured are cultured in OMM or BMM (Figs 3C and 4).

Interestingly, when *Pp*U and *Pp*Δ*fadBA* were periodically re-seeded on OMM (Fig. S1), we observed that although the wild type retained its ability to synthesize PHAs even after hundreds of re-seedings, this capacity was lost in the PHA-overproducing mutant after only a few passages (Fig. 2 and Fig. S1). Moreover, when the adapted PHA$^-$ strains were cultured in medium not conducive to PHA synthesis (MM + glucose or

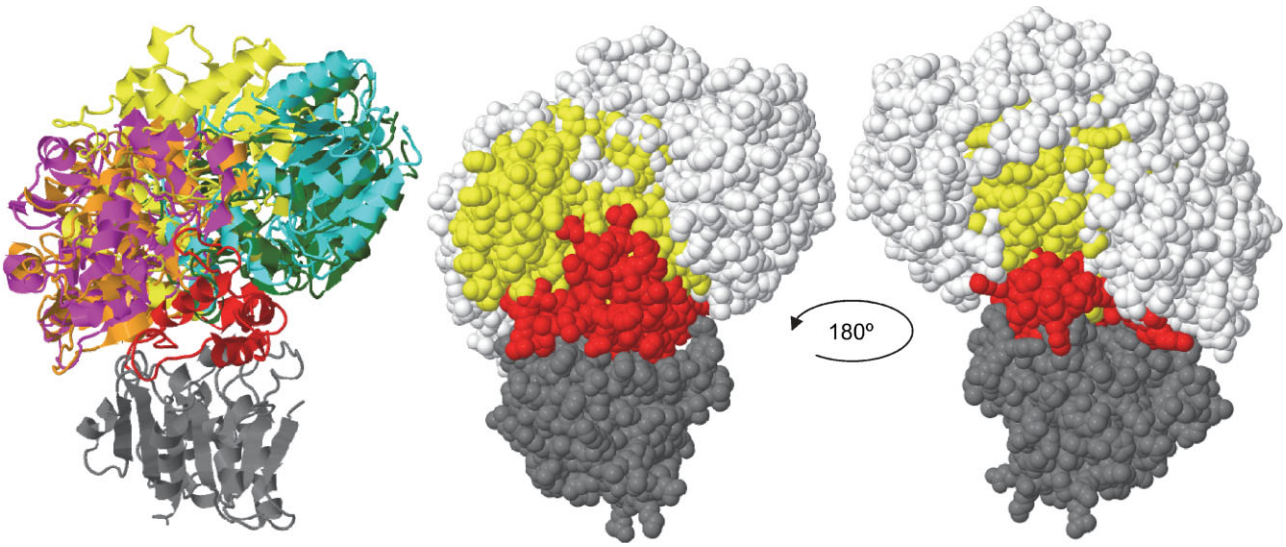


Fig. 7. Left: cartoon representation of the best five PhaC1–PhaC2 superimposed complexes predicted by ZDOCK, based on PhaC2 as the receptor. PhaC1 molecules are depicted in cyan, yellow, green, orange and magenta; the α/β hydrolase core of PhaC2 is shown in gray and its lid in yellow. Right, two views of the van der Waals representations of the superimposed complexes. Hydrolase cores are shown in white (PhaC1) and gray (PhaC2), and the 'lids' in yellow (PhaC1) and red (PhaC2).

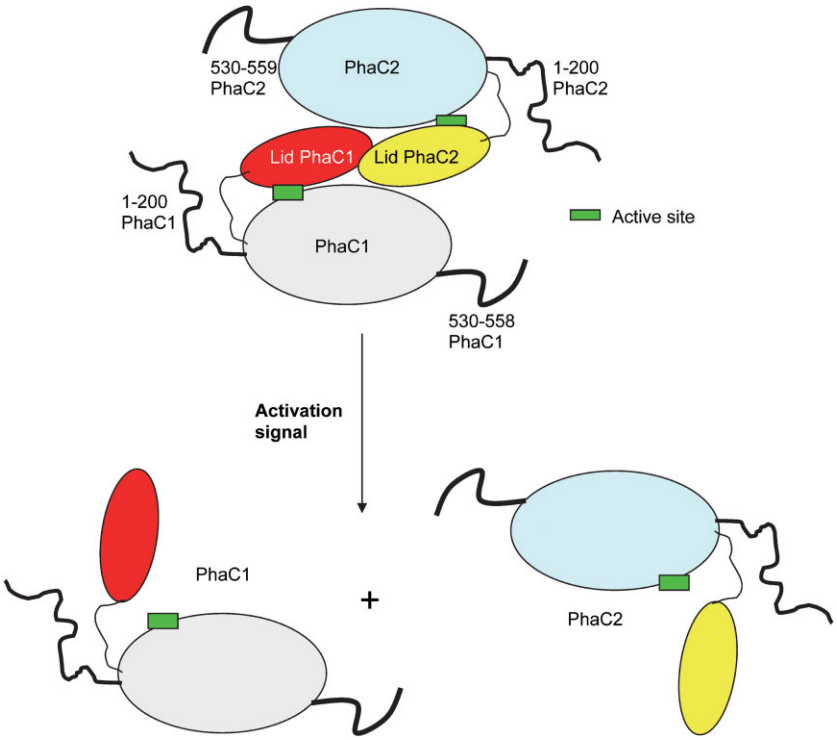


Fig. 8. Scheme proposed to explain the regulation of PhaC1/PhaC2.

MM + 4-OH-PhAc), then re-seeded for more than 100 daily passes in the same medium, and later re-seeded on OMM, all of them were still unable to synthesize PHAs, suggesting that genetic changes (mutations) hampered the production of poly-3-hydroxyoctanoate. Thus, the culture of *Pp*Δ*fadBA* in OMM seems to be trigger a survival mechanism involving the introduction of mutations in the genes making up the *pha* cluster (Fig. 1 and Table 1).

Conversely, *Pp*Δ*fadBA* did not lose the ability to synthesize PHA when subcultured in OMM supplemented with 10 mM of 4-OH-phenylacetic acid (data not shown). Furthermore, when *Pp*Δ*fadBA* was repeatedly seeded on BMM, and then inoculated on OMM, it was still able to synthesize PHAs, indicating that although the metabolic imbalance indicated above could have been caused by less efficient β-oxidation, the introduction of mutations in the *pha* genes required: (i) the utilization of a ketogenic molecule (i.e. octanoate, butyrate) as the sole carbon source and (ii) PHA overproduction. When these two conditions are met, octanoate (or the corresponding PHA precursor) flows towards a PHA pathway that effectively polymerizes the carbon arising from a short circuit of β-oxidation (β-II pathway, Olivera *et al.*, 2001b). Taking into account that fatty acid β-oxidation is a generator of reducing equivalents an acetyl-CoA, blockage of this route would deprive the cell of the ability to grow. Thus, when PHA no longer accumulates (as occurred in the PHA$^-$ mutants), the concentrations of reducing equivalents (NADH) or acetyl-CoA are restored and, therefore, growth is re-stablished (see Fig. 3C). Accordingly, PHA$^-$ mutants, due to their faster rate of growth (Fig. 3C), would displace these PHA producers (*Pp*Δ*fadBA*) from the cultures, such that the latter population is diluted with an increasing number of passages in culture. This would account for the complete absence of strains carrying mutations that did not affect PHA synthesis (or that do so only partially).

In studying the mutations in the 22 PHA$^-$ mutants, we found that all them were located in the gene *phaC1* (see Table 1). There were no mutations in the other genes belonging to the cluster (*phaZC2DFI*). The implied specific occurrence of phaC1 mutations is in stark contrast to the widely accepted mechanism underlying adaptive mutations: that they are caused by a random mutagenic mechanism. Thus, presumably, although many other mutations were no doubt introduced into the pha genes of the entire cluster, only those handicapping PHA synthesis were selected. In other words, according to our data, a lack of only PhaC1 is sufficient to completely prevent PHA synthesis.

The essential role played by PhaC1 in PHA synthesis agrees with the data reported by Arias and colleagues (2013) showing that maximal PHA accumulation correlates with a rapid and significant increase of *phaC1* transcription. Sandoval and colleagues (2007) reported that the expression of *phaC1* in a mutant carrying a deletion of the whole cluster (*Pp*UΔ*pha*) was sufficient to restore

PHA synthesis. These data would also explain our unsuccessful attempts at selecting mutants with functional alterations in the proteins PhaZ, PhaC2, PhaD, PhaF or PhaI because the presence of a functional PhaC1 would have been enough to assure normal synthesis of these biopolymers.

Nevertheless, the finding that a single mutation in PhaC1 completely blocked PHA synthesis was certainly unexpected because all of the PHA$^-$ mutants had a functional polymerase (PhaC2). Figure 5C shows that the transformation of *Pp*UΔ*pha* with a genetic construct containing the gene *phaC2* restored PHA synthesis although, as we have showed earlier, lower amounts of polymer accumulated in the recombinant *Pp*UΔ*pha phaC2* than in the recombinant PpUΔpha phaC1 (Sandoval *et al*., 2007; Arias *et al*., 2008). These results allowed us to conclude that when expressed independently, PhaC1 and PhaC2 are able to polymerize 3-OH-alkanoates. However, when PhaC1 and PhaC2 are expressed together, a functionally active PhaC1 is an absolute requirement for a functionally active PhaC2 (Fig. 5D), which in turn implies the interaction of these proteins.

Although the three-dimensional structures of the PhaC polymerases are unknown, reasonable structural models can be derived from their strong similarity with several lipases and esterases. Such models predict an α/β hydrolase core containing the active site and covered by a lid region. The formation of PhaC1–PhaC2 heterodimeric and the corresponding homodimeric complexes via extensive use of the external part of the lid moieties is suggested by *in silico* docking procedures (Fig. 7). While these models are limited to residues 201–530, the non-modelled residue stretches are likely to play a role in enzyme activation and/or regulation as well. Thus, the available data and models suggest that the importance of the lid domain stems from its involvement in interfacial-like activation and protein–protein interactions. Very similar lid–lid dimeric interactions were proposed based on the crystal structure of other α/β hydrolases such as the *Aspergillus niger* epoxide hydrolase (Zou *et al*., 2000), the macrocycle-forming thioesterase domain of *Saccharopolyspora erythraea* erythromycin polyketide synthase (Tsai *et al*., 2001), the *Streptomyces venezuelae* pikromycin thioesterase (Giraldes *et al*., 2006) and the tautomycetin thioesterase from *Streptomyces* sp. CK4412 (Scaglione *et al*., 2010). Although no biochemical data on the role of dimerization in the biological activity of these proteins are available, this dimerization scheme, through the use of the lid domains, suggest a common regulatory mechanism that deserves a future study.

Here, we identified several mutations that exclusively affect PhaC1 and that lead to the complete prevention of PHA accumulation. Because in the complete absence of PhaC1 (*P. putida* U Δ*pha*pMC*phaC2*), the PhaC2 polymerase is able to synthesize PHA (Fig. 5), then the mutations described in this work likely render mutant structures of PhaC1 that form non-productive complexes with (and thus inhibit) PhaC2. This hypothesis is depicted in Fig. 8. Accordingly, PhaC1 and PhaC2 spontaneously form heterodimers mostly through lid–lid interactions, thereby burying their active sites behind their lids and inactivating the proteins. Once an activation signal (i.e. a 3-OH-acyl-CoA derivative) reaches the complex (perhaps through the non-modelled 1–200 and 530–558 regions), the monomers separate and the lids are able to expose the active sites by a lipase-like mechanism.

The effect of PhaC1 mutations can be explained by presuming that they affect either dissociation of the inactive complex or subsequent interfacial activation steps. Thus, truncated proteins 12–18 (see Table 3) would lack the N-terminal and C-terminal parts involved in receiving activation signals, whereas in the remaining mutants (either single-residue or deletion variants) either the lid or adjacent regions would be affected, possibly hampering transmission of the activation signal that allows the dissociation-activation step to proceed.

Because the possibility of homodimers is also predicted, a similar regulation scheme may apply to those bacterial strains containing only PhaC1 or PhaC2. However, our hypothesis would argue that heterodimers prevail over homodimers; otherwise, non-mutant PhaC2 molecules would be able to replace PhaC1 in forming productive complexes. Whether the heterodimeric complexes are stronger than homodimeric ones cannot be predicted by our model, given its limitations. The existence of heteromeric PHA polymerases, integrated by different paralogues, was suggested for *Rhodospirillum*

Table 3. Amino acid sequences and length of the different PhaC1 found in PHA$^-$ mutants when stop codons are generated.

PhaC1 protein or variant	Number of amino acids	Sequence	Mw (kDa)
PhaC1(Native)	559	MSN. . ...HER	62,31
PhaC1mut.12	432 (128–559)	MTE. . ...HER	47,75
PhaC1mut.13	432 (128–559)	MTE. . ...HER	47,75
PhaC1mut.14	432 (128–559)	MTE. . ...HER	47,75
PhaC1mut.15	269 (1–269)	MSN. . ...LST	30,36
	267 (292–559)	MLG. . ...HER	29,67
PhaC1mut.16	518 (1–518)	MSN. . ...DSW	57,67
PhaC1mut.17	524 (1–524)	MSN. . ...WQS	58,50
PhaC1mut.18	432 (128–559)	MTE. . ...HER	47,75

The initial and the final amino acid for each truncated protein are in brackets. The numbers correspond to the residues of the PhaC1 (in the native protein).
Stop codons (TGAs) are generated by insertion of an A in position 9 (mut.12); by a deletion of a G at position 57 (mut. 13); by the changes C346T (mut. 14), C324A (mut. 15), G1557A (mut16), C1575A (mut17) and by the deletion of a C at position 163 (mut. 18).

rubrum, although in this case, the complexes seem to be required to achieve a more efficient catalysis (Jin and Nikolau, 2012).

The results of our study provide evidence of metabolic stress caused by PHA accumulation and describe a bacterial mechanism to counteract this effect. They also point to the pivotal role of the polymerase PhaC1 and the highly likely existence of PhaC1–PhaC2 interactions. This information expands our knowledge of mcl-PHA biosynthesis and open new approaches to the identification of heteromeric PHA polymerases. A better understanding of the structures of these complexes will provide clues to the molecular mechanism involved in PHA polymerization.

Experimental procedures

Materials

PHA precursors (n-alkanoic and n-phenylalkanoic acids) and the reagents used in this study were supplied by Sigma (USA), Merck (Germany), Alfa Aesar (USA), Pharmacia Biotech (Sweden) or other commercial firms. Molecular biology products were supplied by Invitrogen (USA), Agilent Technologies (USA) and Promega (USA). Other compounds were of analytical quality or high-performance liquid chromatography grade. Commercial DNA vectors used in the genetic work were from Thermo Scientific (USA), Stratagene (USA) and Promega (USA).

Microbial strains, plasmids and vectors

Pseudomas putida U (Colección Española de Cultivos Tipo, CECT4848) (Martínez-Blanco *et al*., 1990) accumulates aliphatic and aromatic PHAs when cultured in the presence of the appropriate precursors (García *et al*., 1999). *Pseudomas putida* U Δ*fadBA* (*Pp*Δ*fadBA*) is a mutant in which the *fadBA* genes, encoding the main β-oxidation pathway (FadBA), have been deleted. This bacterium accumulates enormous amounts of bioplastics when cultured in chemically defined medium containing different alkanoic or phenylalkanoic acids (> 90% of the cytoplasm is occupied by PHAs) (Olivera *et al*., 2001a). *Pseudomas putida* U Δ*pha* (*Pp*Δ*pha*) is a mutant in which the whole *pha* cluster has been eliminated such that it is completely unable to synthesize PHAs (Sandoval *et al*., 2007). All of these bacterial strains are from our collection.

Escherichia coli DH10B was used for plasmid propagation (García *et al*., 1999). *Escherichia coli* HB101pRK600 was the helper strain used in triparental filter mating (Herrero *et al*., 1990).

The plasmids pGEM-T Easy (Promega, USA), pTZ57R/T (Thermo Scientific, USA), pK18*mob* (Schäfer *et al*., 1994) and pJQ200KS (Quandt and Hynes, 1993) were used for subcloning genomic fragments. pBBR1MCS-3 (Tc^r) (Kovach *et al*., 1995), abbreviated as pMC, is a cloning vector that replicates autonomously in *P. putida* U; it was used to analyse the expression of different genes in this strain. DNA manipulations and sequence analyses were performed as reported (Sanger *et al*., 1977; Sambrook and Russell, 2001).

All strains and plasmids used in this work are listed in Table 2.

Culture media and growth conditions

Pseudomonas putida U and its different mutants or recombinants were maintained on a chemically defined medium (MM) (Martínez-Blanco *et al*., 1990) containing 4-hydroxyphenylacetic acid (10 mM) as carbon source and rifampicin as the selection marker. Growth slants (24 h at 30°C) were used to inoculate liquid medium (500 ml Erlenmeyer flasks containing 100 ml of MM). Each flask was seeded with 2 ml of a bacterial suspension (A_{540} = 0.5). The flasks were incubated on a rotary shaker (250 rpm) at 30°C for the time required. The carbon sources used in the cultures were octanoate, glucose or 4-hydroxy phenylacetic acid.

Escherichia coli was maintained on Luria–Bertani agar plates and cultured (37°C) overnight or for the time indicated in each experiment. When solid medium was employed, 25 g of Difco agar (USA) l^{-1} was added.

When required, the following antibiotics were added to the media: rifampicin, 25 μg ml^{-1}; ampicillin, 100 μg ml^{-1}; kanamycin, 25 μg ml^{-1}; chloramphenicol, 30 μg ml^{-1}; and/or tetracycline, 35 μg ml^{-1}.

Genetic manipulations

To analyse the function of a particular protein or a set of proteins, the respective genes were amplified (independently or in tandem), cloned into the appropriate vectors (pGEM-T Easy, pMC) and expressed in *P. putida* U or in the mutants. In some experiments a mutated copy of *phaC1* (*phaC1**) and the gene *phaC2* were cloned together into pMC. The expression of both genes (*phaC1–phaC2* or *phaC1*–phaC2*) was driven by the plasmid promoter.

PCR conditions

All the PHA$^-$ mutants isolated were analysed by PCR either to identify the mutation or to confirm the position and extent of the deletion.

The PCRs were performed in a Perkin-Elmer DNA thermal cycler 2400. Each reaction (50 ml) contained 75 mM HCl-Tris buffer, pH 9; 50 mM KCl; 20 mM $(NH_4)_2SO_4$; 100 ng of genomic DNA; 0.4 mM of each independent primer; 2 mM $MgCl_2$; 0.4 mM dNTPs; and a mixture of the thermostable DNA polymerase (two units) from *Thermus* sp. (Biotools, Spain) and Pfu DNA polymerase (one unit) from *Pyrococcus furiosus* (Promega, USA) (Miñambres *et al*., 2000). The annealing temperature was 60°C and an extension time of 2 min was used.

Polymer characterization

PHA accumulation by either *P. putida* U or by its mutants was confirmed by direct microscopic observation, as reported elsewhere (García *et al*., 1999). The synthesized polymers were extracted and quantified as indicated previously (Lageveen *et al*., 1988). Their chemical structures were

established by nuclear magnetic resonance and gas chromatography (Fritzsche *et al*., 1990; Abraham *et al*., 2001).

Electron microscopy

Bacteria were harvested from the culture medium (usually at the late-exponential phase of growth), centrifuged (7000*g*, 7 min), and then fixed with a solution of glutaraldehyde (2% by volume) in PBS (0.1 M phosphate buffer containing 0.138 mM NaCl and 2.7 Mm KCl) for 30 min at 4°C. The samples were processed as previously described (Sandoval *et al*., 2007). Ultrastructural studies were performed with a JEOL1010 transmission electron microscope.

When scanning micrographs were required, the bacteria were harvested, fixed and washed as described in Sandoval and colleagues (2007). Samples were analysed in a SEM JEOL JSM-6100 electron microscope.

Prediction of PhaC1–PhaC2 complexes

To determine the possibility that PhaC1 and PhaC2 formed a protein–protein complex, the docking of PDB files with the three-dimensional coordinates of structural models of both proteins (Arias *et al*., 2008) was analysed using ZDOCK 3.0.2 (http://www.zdock.umassmed.edu/; Pierce *et al*., 2014). The five best complexes were selected.

Acknowledgements

We gratefully acknowledge the support of the Ministerio de Economía y Competitividad (Madrid, Spain, grants No. BIO20092012-39695-C02-02 and BIO2013-47684-R) and the Consejería de Educación (Junta de Castilla y León, Valladolid, España, grant No. LE114U13).

References

Abraham, G., Gallardo, A., San, A., Román, J., Olivera, E.R., Jodrá, R., *et al*. (2001) Microbial synthesis of poly(β-hydroxyalkanoates) bearing phenyl groups from *Pseudomonas putida* U: chemical structure and characterization. *Biomacromolecules* **2:** 562–567.

Alsor, I.S., and Keasling, J.D. (2003) Process design for microbial plastic factories: metabolic engineering of polyhydroxyalkanoates. *Curr Opin Biotechnol* **14:** 475–483.

Anderson, A.J., and Dawes, E.A. (1990) Occurrence, metabolisms, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. *Microbiol Rev* **54:** 450–472.

Arias, S., Sandoval, A., Arcos, M., Cañedo, L.M., Maestro, B., Sanz, J.M., *et al*. (2008) Poly-3-hydroxyalkanoate synthases from *Pseudomonas putida* U: substrate specificity and ultrastructural studies. *Microb Biotechnol* **1:** 170–176.

Arias, S., Bassas-Galia, M., Molinari, G., and Timmis, K.N. (2013) Tight coupling of polymerization and depolymerization of polyhydroxyalkanoates ensures efficient management of carbon resources in *Pseudomonas putida*. *Microb Biotechnol* **6:** 551–563.

Ayub, N.D., Tribelli, P.M., and López, N.I. (2009) Polyhydroxyalkanoates are essential for maintenance of redox state in the Antarctic bacterium *Pseudomonas* sp. 14-3 during low temperature adaptation. *Extremophiles* **13:** 59–66.

Chen, G.-Q. (ed.) (2010) *Plastic from Bacteria: Natural Functions and Applications*, Vol. 14, *Microbiol Monographs*. New York, NY, USA: Springer.

Dinjaski, N., Fernández-Gutiérrez, M., Selvam, S., Parra-Ruiz, F.J., Lehman, S.M., García, E., *et al*. (2014) PHACOS, a functionalized bacterial polyester with bacterial activity against methicillin-resistant *Staphylococcus aureus*. *Biomaterials* **35:** 14–24.

Escapa, I.F., Morales, V., Martino, V.P., Pollet, E., Avérous, L., García, J.L., and Prieto, M.A. (2011) Disruption of β-oxidation pathway in *Pseudomonas putida* KT2440 to produce new functionalized PHAs with thioester groups. *Appl Microbiol Biotechnol* **89:** 1583–1598.

de Eugenio, L.I., García, P., Luengo, J.M., Sanz, J., San Román, 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.

de Eugenio, L.I., Galán, B., Escapa, I.F., Maestro, B., Sanz, J.M., García, J.L., and Prieto, M.A. (2010) The PhaD regulator controls the simultaneous expression of the pha genes involved in polyhydroxyalkanoate metabolism and turnover in *Pseudomonas putida* KT2440. *Environ Microbiol* **12:** 1591–1603.

Fritzsche, K., Lenz, R.W., and Fuller, R.C. (1990) An unusual bacterial polyester with a phenyl pendant group. *Makromol Chem* **191:** 1957–1965.

Galán, B., Dinjaski, N., Maestro, B., de Eugenio, L.I., Escapa, I.F., Sanz, J.M., *et al*. (2011) Nucleoid-associated PhaF phasin drives intracellular location and segregation of polyxydroxyalkanoate granules in *Pseudomonas putida* KT2440. *Mol Microbiol* **79:** 402–418.

García, B., Olivera, E.R., Minambres, 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.

Giraldes, J.W., Akey, D.L., Kittendorf, J.D., Sherman, D.H., Smith, J.L., and Fecik, R.A. (2006) Structural and mechanistic insights into polyketide macrolactonization from polyketide-based affinity labels. *Nat Chem Biol* **2:** 531–536.

Herrero, M., de Lorenzo, V., and Timmis, K.N. (1990) Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in Gram-negative bacteria. *J Bacteriol* **172:** 6557–6567.

Huisman, G.W., Wonink, E., de Koning, G., Preusting, H., and Witholt, B. (1992) Synthesis of poly(3-hydroxyalkanoates) by mutant and recombinant *Pseudomonas* strains. *Appl Microbiol Biotechnol* **38:** 1–5.

Jin, H., and Nikolau, B. (2012) Role of genetic redundancy in polyhydroxyalkanoate (PHA) polymerases in PHA biosynthesis in *Rhodospirillum rubrum*. *J Bacteriol* **194:** 5522–5529.

Kelley, L.A., and Sternberg, M.J.E. (2009) Protein structure prediction on the web: a case study using the Phyre server. *Nat Protoc* **4:** 363–371.

Kim, D.Y., Kim, H.W., Chung, M.G., and Rhee, Y.H. (2007) Biosynthesis, modification, and biodegradation of bacterial medium-chain-length polyhydroxyalkanoates. *J Microbiol* **45:** 87–97.

Klinke, S., de Roo, G., Witholt, B., and Kessler, B. (2000) Role of phaD in accumulation of medium-chain-length poly(3-hydroxyalkanoates) in *Pseudomonas oleovorans*. *Appl Environ Microbiol* **66:** 3705–3710.

Koller, M., Salerno, A., Dias, M., Reiterer, A., and Braunegg, G. (2010) Modern biotechnology polymer synthesis: a review. *Food Technol Biotechnol* **48:** 255–269.

Kovach, M.E., Elzer, P.H., Hill, D.S., Robertson, G.T., Farris, M.H., Roop, M.R., and Peterson, K.M. (1995) Four derivatives of the broad-host range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166:** 175–176.

Lageveen, R.G., Huisman, G.W., Preusting, 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-hydroxyalkenoates. *Appl Environ Microbiol* **54:** 2924–2932.

Lee, S.Y., Lee, K.M., Chan, H.N., and Steinbüchel, A. (1994) Comparison of recombinant *Escherichia coli* strains for synthesis and accumulation of poly-(3-hydroxybutyric acid) and morphological changes. *Biotechnol Bioeng* **44:** 1337–1347.

Lenz, R.W., Kim, Y.B., and Fuller, R.C. (1992) Production of unusual bacterial polyesters by *Pseudomonas oleovorans* through cometabolism. *FEMS Microbiol Lett* **103:** 207–214.

Luengo, J.M., García, B., Sandoval, A., Naharro, G., and Olivera, E.R. (2003) Bioplastics from microorganisms. *Curr Opin Biotechnol* **6:** 251–260.

Lütke-Eversloh, T., and Steinbüchel, A. (2004) Microbial polythioesters. *Macromol Biosci* **4:** 165–174.

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.

Maestro, B., Galán, B., Alfonso, C., Rivas, G., Prieto, M.A., and Sanz, J.M. (2013) A new family of intrincally disordered proteins: structural characterization of the major phasin PhaF from *Pseudomonas putida* KT2440. *PLoS ONE* **8:** e56904.

Martínez-Blanco, H., Reglero, A., Rodríguez-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 acidic sequence to the complete gene. *Biochem Biophys Res Commun* **272:** 477–479.

Misra, S.K., Valappil, S.P., Rou, I., and Boccaccini, A.R. (2006) Polyhydroxyalkanoate (PHA)/inorganic phase composites for tissue engineering applications. *Biomacromolecules* **7:** 2249–2258.

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

Olivera, E.R., Carnicero, D., García, B., Minambres, B., Moreno, M.A., Cañedo, L., *et al*. (2001b) Two different pathways are involved in theβ-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., Arcos, M., Naharro, G., and Luengo, J.M. (2010) Unusual PHA biosynthesis. In *Plastic from Bacteria: Natural Functions and Applications*, Vol. 14, *Microbiol Monographs*. Chen, G.-Q. (ed.). New York, NY, USA: Springer, pp. 133–186.

Peters, V., and Rehm, B.H.A. (2005) In vivo monitoring of PHA granule formation using GFP-labeled PHA synthesis. *FEMS Microbiol Lett* **248:** 93–100.

Pham, T.H., Webb, J.S., and Rehm, B.H. (2004) The role of polyhydroxyalkanoate biosynthesis by *Pseudomonas aeruginosa* in rhamnolipid and alginate production as well as stress tolerance and biofilm formation. *Microbiology* **150:** 3406–3413.

Pierce, B.G., Wiehe, K., Hwang, H., Kim, B.H., Vreven, T., and Weng, Z. (2014) ZDOCK server: interactive docking prediction of protein–protein complexes and symmetric multimers. *Bioinformatics* **30:** 1771–1773.

Prieto, M.A., Bühler, B., Jung, K., Witholt, B., and Kessler, B. (1999) PhaF, a polyhydroxyalkanoate-granule-associated protein of *Pseudomonas oleovorans* GPo1 involved in the regulatory expression system for pha genes. *J Bacteriol* **181:** 858–868.

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.

Rehm, B.H.A., Krüger, N., and Steinbüchel, A. (1998) A new metabolic link between fatty acid de novo synthesis and polyhydroxyalkanoic acid synthesis. *J Biol Chem* **273:** 24044–24051.

Sambrook, J., and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual*. New York, NY, USA: Cold Spring Harbor Laboratory Press.

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

Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74:** 5463–5467.

Scaglione, J.B., Akey, D.L., Sullivan, R., Kittendorf, J.D., Rath, C.M., Kim, E.S., *et al*. (2010) Biochemical and structural characterization of the tautomycetin thioesterase: analysis of a stereoselective polyketide hydrolase. *Angew Chem Int Ed Engl* **49:** 5726–5730.

Schäfer, A., Tauch, A., Jäger, W., Kalinowski, J., Thierbach, G., and Pühler, A. (1994) Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids

pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* **145:** 69–73.

Sevastianov, V.I., Perova, N.V., Shishatskaya, E.I., Kalacheva, G.S., and Velova, T.G. (2003) Production of purified polyhydroxyalkanoates (PHAs) for applications in contact with blood. *J Biomater Sci Polym Ed* **14:** 1029–1042.

Steinbüchel, A. (2001) Perspectives for biotechnological production and utilisation of biopolymers: metabolic engineering of poly-hydroxyalkanoate biosynthesis pathways as a successful example. *Macromol Biosci* **1:** 1–24.

Steinbüchel, A., and Valentin, M.E. (1995) Diversity of bacterial polyhydroxyalkanoic acids. *FEMS Microbiol Lett* **128:** 219–228.

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

Tsai, S.C., Miercke, L.J., Krucinski, J., Gokhale, R., Chen, J.C., Foster, P.G., *et al*. (2001) Crystal structure of the macrocycle-forming thioesterase domain of the erythromycin polyketide synthase: versatility from a unique substrate channel. *Proc Natl Acad Sci USA* **98:** 14808–14813.

van der Walle, G.A., de Koning, G.J.M., Weusthuis, R.A., and Eggink, G. (2001) Properties, modifications and applications of biopolyesters. *Adv Biochem Eng Biotechnol* **71:** 263–291.

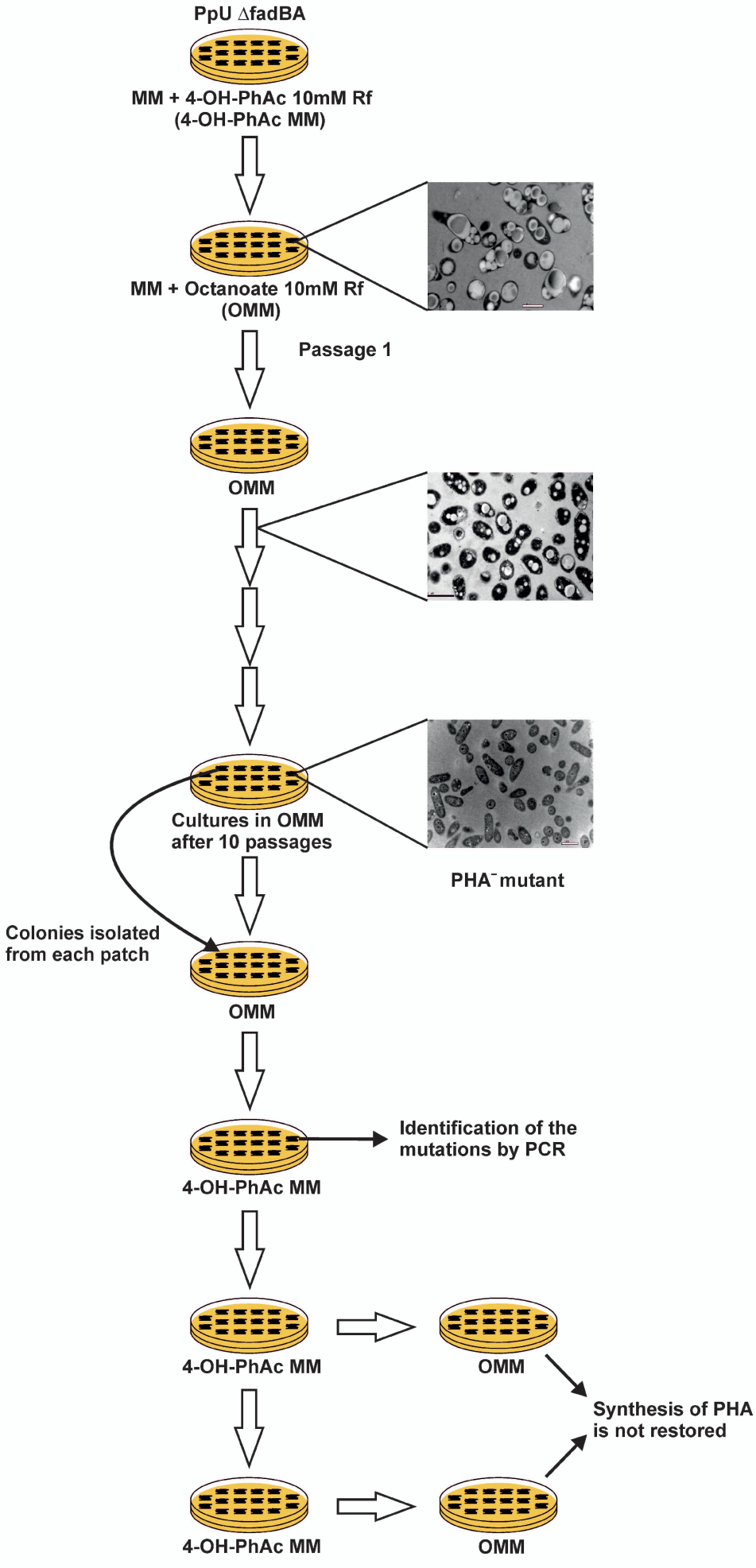
Witholt, B., and Kessler, B. (1999) Perspectives of medium chain length poly-(hydroxyalkanoates), a versatile set of bacterial bioplastics. *Curr Opin Biotechnol* **10:** 279–285.

Zou, J., Hallberg, B.M., Bergfors, T., Oesch, F., Arand, M., Mowbray, S.L., and Jones, T.A. (2000) Structure of *Aspergillus niger* epoxide hydrolase at 1.8 Å resolution: implications for the structure and function of the mammalian microsomal class of epoxide hydrolases. *Structure* **8:** 111–122.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Schematic representation of the procedure used to isolate the spontaneous mutants of *P. putida* Δ*fadBA* (*P. putida* Δ*fadBA*PHA$^-$) that lost the capability to synthesize PHAs after successive passages in solid OMM (MM containing 10 mM octanoate as the sole carbon source). The variation in the bacterial PHA content during the different stages of selection is also indicated. When similar experiments were performed with *P. putida* U, the synthesis of PHA was never lost.


PpU ΔfadBA
MM + 4-OH-PhAc 10mM Rf
(4-OH-PhAc MM)
MM + Octanoate 10mM Rf
(OMM)
Passage 1
OMM
Cultures in OMM
after 10 passages
PHA⁻ mutant
Colonies isolated
from each patch
OMM
4-OH-PhAc MM
Identification of the
mutations by PCR
4-OH-PhAc MM
OMM
Synthesis of PHA
is not restored
4-OH-PhAc MM
OMM