Impaired polyhydroxybutyrate biosynthesis from glucose in *Pseudomonas* sp. 14-3 is due to a defective β-ketothiolase gene

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Abstract

Pseudomonas sp. 14-3 accumulates polyhydroxybutyrate (PHB) from octanoate, but not from glucose. To elucidate this unusual phenotype, genes responsible for the synthesis of PHB were cloned and analyzed. A PHB polymerase gene (*phaC*) was found downstream from genes coding for a β-ketothiolase (*phaA*), an acetoacetyl-coenzyme A reductase (*phaB*) and a putative transcriptional regulator (*phaR*). All genes were similar to *pha* genes from several related species, but differences were observed in the distal region of *phaA*. Complementation with heterologous β-ketothiolase genes from *Azotobacter* sp. FA8 or *Pseudomonas putida* GPp104 restored the capability of *Pseudomonas* sp. 14-3 to synthesize PHB from glucose, demonstrating that its β-ketothiolase was nonfunctional. Analysis of the genome sequences of other *Pseudomonas* species has revealed the existence of putative β-ketothiolase genes. The functionality of one of these thiolase genes, belonging to *P. putida* GPp104, was experimentally demonstrated. *Pseudomonas* sp. 14-3 is the first natural *phaA* mutant described, that despite this mutation accumulates high amounts of PHB when growing on fatty acids.

Introduction

Polyhydroxyalkanoates (PHAs) are carbon and energy storage compounds that are accumulated by many bacterial species under unbalanced growth conditions (Madison & Huisman, 1999). PHAs are composed of different monomeric units. According to the number of carbon atoms in the monomeric units, bacteria are said to be short (C3–C5) or medium chain length (C6–C14) PHA producers.

Synthesis and degradation of PHAs are part of a cycle in which the acyl-CoA precursors are converted by different metabolic pathways into PHAs. The first PHA studied was poly (3-hydroxybutyrate) (PHB), constituted by C4 monomer units, that is also the most widespread in nature. The PHB biosynthetic pathways and the genes coding for the corresponding enzymes have been studied in a broad range of bacteria. The most widely distributed of these pathways includes a β-ketothiolase, which condenses two molecules of acetyl-CoA to give acetoacetyl-CoA, a NADPH- or NADH-dependent reductase, that reduces this compound to give D(−)-3-hydroxybutyryl CoA, and a PHB synthase, that uses this compound as a substrate for polymerization (Steinbüchel & Hein, 2001). PHB can also be synthesized through *de novo* fatty acid biosynthesis and β-oxidation pathways from sugars and fatty acids (Aldor & Keasling, 2003).

Pseudomonas sp. 14-3, isolated from a temporary pond in Antarctica, accumulates large quantities of PHB when grown on octanoate. However, PHB production is not detected when using glucose as a carbon source (Ayub *et al.*, 2004). This species shows a high level of stress resistance when compared with other *Pseudomonas* species. In previous studies, it was demonstrated that its increased tolerance to both thermal and oxidative stress was associated with the production of PHB (Ayub *et al.*, 2004). PHB accumulation is a rare or uncommon characteristic among *Pseudomonas* species (Kessler & Palleroni, 2000) that usually accumulate PHAs composed of medium chain length monomers.

In this paper, we investigated the genes involved in PHB biosynthesis of *Pseudomonas* sp. 14-3 in order to explain the PHB-negative phenotype observed when grown in glucose. We analyzed the *pha* genes molecularly and studied their function by complementation analysis using different bacterial strains. Examination of the available genome sequences for *Pseudomonas* strains that are unable to synthesize PHB revealed the presence of putative *phaA* genes. The ability of one of these genes to participate in the synthesis of PHB was evaluated experimentally.

Materials and methods

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1.

Cloning and molecular analysis of pha genes of *Pseudomonas* sp. 14-3

Genomic DNA of *Pseudomonas* sp. 14-3 was partially digested with *XhoI* and cloned into the mobilizable cosmid pVK102 according to the method of Pettinari *et al.* (2001). The genomic library was screened for the presence of the PHB synthase gene by complementation analysis using *Cupriavidus necator* PHB$^-$4 (formerly known as *Alcaligenes eutrophus* and then as *Ralstonia eutropha* PHB$^-$4) as a host. Recombinant cosmids were purified and subjected to restriction analysis using several enzymes. DNA fragments obtained by digestion of the cosmids with *Xho*I and/or *EcoR*I were subcloned into pBBR1MCS-2, and sequenced. Additional fragments for sequencing were obtained by PCR amplification. The sequencing reactions were performed by Macrogen Inc. (Korea). Sequences were aligned, assembled and analyzed using Bioedit Sequence Alignment Editor CAP (Hall, 1999), CLUSTALX (Thompson *et al.*, 1997), BLASTP and RPS-BLAST programs (http://www.ncbi.nlm.nih.gov/BLAST/).

Plasmid construction

Plasmid pRB2 carrying the *phaB* and *phaA* genes of *Azotobacter* sp. FA8 expressed from their natural promoter (P_{Az}), was constructed by cloning an *c.* 3 kb *Sal*I fragment from a recombinant cosmid, pRAC1 (Pettinari *et al.*, 2003), carrying a genomic DNA fragment of *Azotobacter* sp. FA8 into pBluescript SK$^-$ (Stratagene, La Jolla, CA). Plasmid pCTBA2 and pCTBA carrying P_{Az} $phaBA_{Az}$ were constructed by subcloning the 3.1 kb *Sal*I and 3.1 kb *Sac*I fragments from pRB2 into pBBR1MCS-2 and pSJ33, respectively. Plasmid pCTB carrying P_{Az} $phaB_{Az}$, and the first (5′) part of *phaA*, was constructed by subcloning a 1.8 kb *Sma*I fragment from pRB_2 into pBBR1MCS-2. PCR was carried out to amplify a fragment containing the entire β-ketothiolase gene of *Pseudomonas putida* GPp104 using primers designed from *P. putida* KT 2440 (accession #: NC_002947). Primers used were as follows: paktup, 5′CGCTCGAGAGCGCCGCTTG GGGCGATTC 3′ and paktlow, 5′CGCTCGAGCGACGGC GTGCGCCCAATCA 3′ (*Xho*I restriction sites are underlined). The primers flanked a 1.5 kb fragment including the entire β-ketothiolase gene and the intergenic regions. This amplification fragment was digested with *Xho*I and cloned into pBBR1MCS-2. The identity of this fragment was verified by sequencing. The resulting plasmid, named pCTT, was introduced by transformation into competent cells of *Pseudomonas* sp. 14-3 prepared according to Lee *et al.* (2005).

Conjugations

Conjugations of *P. putida* GPp104 and *Pseudomonas* sp. 14-3 with *Escherichia coli* S17-1 harboring plasmids were performed on mineral salts medium plates (Schlegel *et al.*, 1970) supplemented with octanoate (0.25% w/v), containing tetracycline (5 μg mL^{-1}) for pVK102 and derivates, or kanamycin (10 μg mL^{-1}) for pSJ33, pBBR1MCS-2 and its derivates.

PHA content determinations

To quantitate PHA accumulation, cultures were carried out in 0.5NE2 medium (Huisman *et al.*, 1992) plus sodium octanoate (0.25% w/v), glucose (3% w/v) or sodium gluconate (3% w/v). Analysis of PHA content was performed from lyophilized cells subjected to methanolysis and hot chloroform extraction. Methyl ester derivatives were

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic	Source or reference
Pseudomonas sp. 14-3	Wild type, PHB producer	Ayub *et al.* (2004)
Pseudomonas putida GPp104	PHA-negative mutant of KT2442	Huisman *et al.* (1991)
Cupriavidus necator PHB-4	PHA-negative mutant of H16	Schlegel *et al.* (1970)
Escherichia coli S17-1	*recA*, *tra* genes	Simon *et al.* (1983)
pVK102	Tc^R cosmid	Knauf & Nester (1982)
pCT1377	pVK102 containing truncated *phaA* and *phaC* of *Pseudomonas* sp. 14-3	This study
pCT1283	pVK102 containing *phaRBAC* of *Pseudomonas* sp. 14-3	This study
pSJ33	Km^R; low-copy number, conjugative	Jaenecke & Diaz (1999)
pBBR1MCS-2	Km^R; medium-copy number, conjugative	Kovach *et al.* (1995)
pCTBA	pSJ33 containing *phaBA* of *Azotobacter* sp. FA8 under their natural promoter	This study
pCTBA2	pBBR1MCS-2 containing *phaBA* of *Azotobacter* sp. FA8 under their natural promoter	This study
pCTB	pBBR1MCS-2 containing *phaB* of *Azotobacter* sp. FA8 under their natural promoter	This study
pCTT	pBBR1MCS-2 containing a 1.5-kb fragment harboring the β-ketothiolase gene of *P. putida* GPp104	This study

analyzed by gas chromatography as described previously (Braunegg *et al*., 1978). The PHA content was expressed as a percentage of cell dry weight.

Nucleotide sequence accession number

The nucleotide sequences obtained here have been deposited in the EMBL Nucleotide Sequence Database accession #: AM262984 (corresponding to the *Pseudomonas* sp. 14-3 *phaRBAC* genes).

Results and discussion

Pseudomonas sp. 14-3 showed high PHB production (80 wt %) in overnight cultures grown in accumulation medium containing sodium octanoate as carbon source (Table 2). PHB production from octanoate has been described in a few other *Pseudomonas* species (Diard *et al*., 2002). PHB accumulation in *Pseudomonas* sp. 14-3 was also observed using longer chain fatty acids such as palmitic acid (data not shown), but, unexpectedly, PHB was not detected when using sodium gluconate or glucose (Table 2).

In order to find an explanation for this unusual phenotype, the genes responsible for the synthesis of PHB in *Pseudomonas* sp. 14-3 were studied.

A genomic library of this strain was screened for the presence of the PHB synthase gene by complementation analysis using *C. necator* $PHB^{-}4$. Two recombinant plasmids, pCT1377 and pCT1283, restored the PHB^{+} phenotype. These plasmids were purified and digested with several enzymes, giving an estimated insert size of 20 kb. The inserts in the recombinant plasmids were subcloned and sequenced as indicated in materials and methods. Sequence analysis of the *Pseudomonas* sp. 14-3 cloned region revealed a PHB polymerase gene (*phaC*) downstream from genes coding for a β-ketothiolase (*phaA*), an acetoacetyl-coenzyme A reductase (*phaB*) and a putative transcriptional regulator (*phaR*). The *phaR* gene was oriented in the direction opposite to that of the other three genes (Fig. 1). No genes related with PHB synthesis were located upstream or downstream of the *pha* gene cluster. The organization of the *pha* gene cluster in *Pseudomonas* sp. 14-3 was similar to that observed in *Azotobacter* species: *Azotobacter* sp. FA8 (Pettinari *et al*., 2001, 2003) and *Azotobacter vinelandii* (Peralta-Gil *et al*., 2002) and also in *Pseudomonas* sp. 61–3 (Matsusaki *et al*., 1998). Unlike *Pseudomonas* sp. 14-3, all these species are able to produce PHB from glucose or gluconate. Two putative PhaR-binding sites, designated R1 and R2, similar to those described for *Pseudomonas* sp. 61–3 (Matsusaki *et al*., 1998) and *A. vinelandii* (Peralta-Gil *et al*., 2002) were found associated with the intergenic *phaR–phaB* region (Fig. 1).

The deduced amino acid sequences of PhaB and PhaC of *Pseudomonas* sp. 14-3 showed a conserved size and high identity with the *pha* gene cluster products of several *Pseudomonas* and *Azotobacter* species (Table 3). The highest similarity (99% identity) was found with the recently described PhaC synthase of *Pseudomonas oleovorans* NRRL B-778 (Solaiman & Ashby, 2005). The β-ketothiolase (PhaA) was also similar to other thiolases, among them thiolases belonging to *Pseudomonas* species that do not possess a functional PHB cluster, such as *Pseudomonas fluorescens* Pf-5 and *P. putida* KT2440 (Table 3). The deduced aminoacid sequence of *phaA* was aligned with the

Table 2. Production of PHB by wild and recombinant *Pseudomonas* sp.14-3 and *Pseudomonas putida* GPp104

Strain	Introduced plasmid (relevant markers)	Carbon source	PHB content (wt %)
Pseudomonas sp.14-3	None	Octanoate	80.6 ± 9.7
		Gluconate	ND
		Glucose	ND
	pBBR1MCS-2	Glucose	ND
	pCTB (P_{Az}*phaB*$_{Az}$)	Glucose	ND
	pCTBA (P_{Az} *phaB*$_{Az}$ *phaA*$_{Az}$)	Glucose	20.2 ± 2.7
	pCTBA2 (P_{Az} *phaB*$_{Az}$ *phaA*$_{Az}$)	Glucose	27.7 ± 2.4
	pCTT (β-ketothiolase$_{Pp}$)	Glucose	38.2 ± 2.3
P. putida GPp104	None	Octanoate	1.1 ± 0.1
	pVK102	Octanoate	0.9 ± 0.0
	pCT1377 (*phaA*$_{Ps}$ *phaC*$_{Ps}$)	Octanoate	13.5 ± 1.4
		Gluconate	ND
		Glucose	ND
	pCT1377 and pCTB (*phaC*$_{Ps}$ and *phaB*$_{Az}$)	Glucose	10.9 ± 2.1
	pCT1283 (*phaR*$_{Ps}$ *phaB*$_{Ps}$ *phaA*$_{Ps}$ *phaC*$_{Ps}$)	Octanoate	36.2 ± 3.4
		Gluconate	14.4 ± 1.1
		Glucose	10.9 ± 0.7

Bacteria were grown at 30 °C for 24 h in 0.5 NE_2 medium Huisman *et al*. (1992) containing sodium octanoate (0.25% w/v), glucose (3% w/v) or sodium gluconate (3% w/v).

ND, not detected.

$_{Ps}$., *Pseudomonas* sp.14-3; $_{Az}$., *Azotobacter* sp. FA8; $_{Pp}$., *P. putida* GPp104.

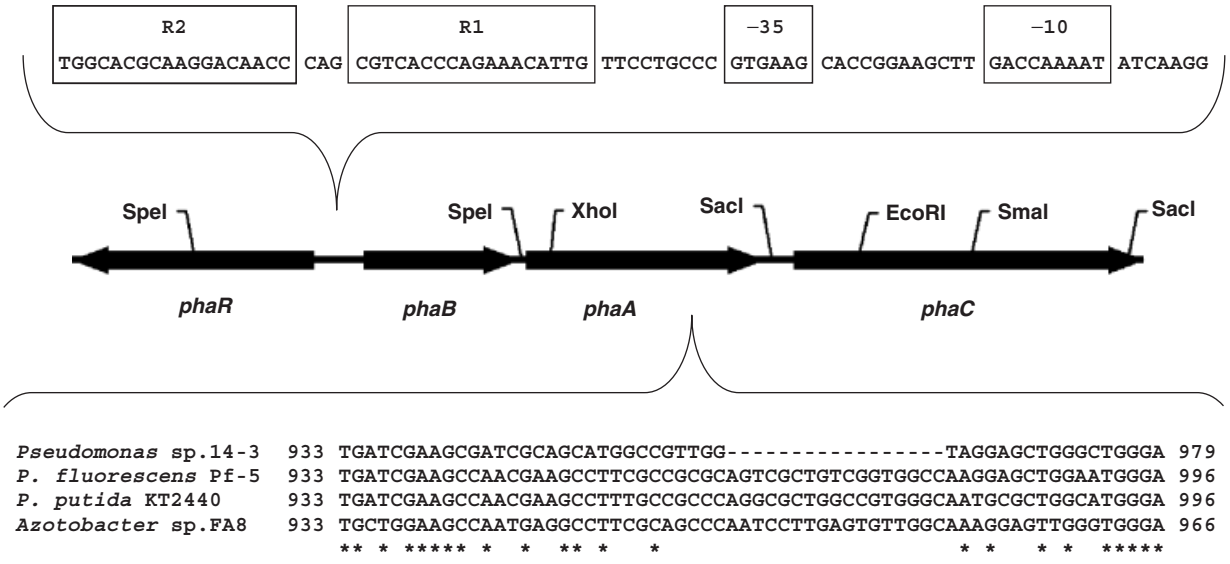

Fig. 1. Organization of the *Pseudomonas* sp.14-3 region containing *phaR*, *phaB*, *phaA* and *phaC* genes. Relevant restriction sites are indicated. The nucleotide sequence of the intergenic *phaR–phaB* region showing putative PhaR-binding sites (R2 and R1) and the sequences- −35 and −10 of a probable *phaBAC* operon σ^{70} promoter is shown at the top. Alignment of nucleotides 933–979 of *phaA* from *Pseudomonas* sp. 14-3 with the corresponding regions of *phaA* from *Azotobacter* sp. FA8, gene PFL_2321 from *Pseudomonas fluorescens* Pf-5 and gene PP_4636 from *Pseudomonas putida* KT2440, revealing the gap in the alignment is shown at the bottom.

Table 3. Similarity of the products of *pha* biosynthesis genes of *Pseudomonas* sp. 14-3 to other bacterial proteins

			Similarity to other gene products			
				Aminoacid similarity (%)		
Pseudomonas sp.14-3 gene product (aminoacid number)	Gene	Organism	No. of amino acids	Identity	Similarity	Accession #
PhaB (251)	*phbB*	*Pseudomonas* sp. HJ-2	251	90	94	AAQ72536
	phbB	*Pseudomonas* sp. 61-3	248	79	90	BAA36196
	phaB	*Azotobacter* sp. FA8	247	70	86	CAC41336
PhaA (384)	*phaA*	*Azotobacter* sp. FA8	392	71	78	CAC41637
	PFL_2321	*P. fluorescens* Pf-5	392	70	78	YP_259428
	PP_4636	*P. putida* KT2440	392	70	78	AAN70209
PhaC (566)	*phaC*	*P. oleovorans* NRRL B-778	566	99	99	AAL17612
	phaC	*Pseudomonas* sp. HJ-2	566	87	92	AAQ72537
	phbC	*Pseudomonas* sp. 61-3	567	73	84	BAA36198

thiolases of *Azotobacter* sp. FA8 (accession #: CAC41637), *P. fluorescens* Pf-5 (accession #: YP_259428), *P. putida* KT2440 (accession #: AAN70209), *Pseudomonas aeruginosa* PAO1 (accession #: AAG05389), *P. fluorescens* PfO-1 (accession #: ABA73817), *Pseudomonas syringae* pv. syringae B728a (accession #: YP_233920), and *P. syringae* pv. phaseolicola (accession #: AAZ33929) using the CLUSTALW program, showing that the first 316 amino acid residues of PhaA of *Pseudomonas* sp. 14-3 were almost identical to the other thiolases, but the rest of its aminoacid sequence showed no similarity (data not shown). Comparison of the corresponding nucleotide sequences showed a very good alignment for the first 968 nucleotides, followed by a discontinuity spanning 17 nucleotides, after which the alignment was restored. Alignment of *phaA* from *Pseudomonas* sp. 14-3 with three of the thiolase genes is shown in Fig. 1. Site mutagenesis studies performed with the β-ketothiolase of *Zooglea ramigera* have demonstrated that a cysteine residue conserved in all β-ketothiolases analyzed, Cys-378, is the active site base, and that mutations in this base result in inactive enzymes (Williams *et al.*, 1992). Sequence analysis of *phaA* from *Pseudomonas* sp. 14-3 suggested that it had suffered a deletion, giving rise to a defective thiolase. This mutant enzyme has a carboxy terminal region that is completely different from all known β-ketothiolases and does not have a Cys-378 residue, so it is unable to participate in the synthesis of the monomer necessary for PHB production from glucose.

The kind of rearrangement observed in *Pseudomonas* sp. 14-3 could be due to the activity of insertion sequences found in the vicinity of *pha* genes. The analysis of the *pha* region of *Azotobacter* sp. FA8 revealed the presence of complete and incomplete insertion sequences, that suggested a probable acquisition of *pha* genes by lateral transfer (Pettinari *et al*., 2003). Preliminary analysis of the *pha* region of *Pseudomonas* sp. 14-3 has revealed the presence of insertion sequences at both sides of the *pha* gene cluster that could be responsible for the rearrangements (data not shown).

Several complementation analysis were performed to determine if the inability to accumulate PHB from glucose was associated with a defect in the *pha* gene products of *Pseudomonas* sp. 14-3. *Azotobacter* sp. FA8 genes coding for a functional β-ketothiolase (*phaA*) and acetoacetyl-coenzymeA reductase (*phaB*) located downstream from their wild type promoter were introduced by conjugation in *Pseudomonas* sp. 14-3 using plasmids pCTBA (low-copy number) and pCTBA2 (medium-copy number) (Table 1). Both plasmids, pCTBA and pCTBA2, conferred the ability to produce PHB from glucose to the host (Table 2), reaching similar levels of PHB accumulation. This result strongly suggested that either *phaA* or *phaB*, or both, were not functionally active in *Pseudomonas* sp. 14-3. Plasmid pCTB, carrying *phaB* from *Azotobacter* sp. FA8 was introduced in *Pseudomonas* sp. 14-3, and PHB production was analyzed in the recombinants. No PHB accumulation was observed from glucose (Table 2). These results, together with sequence analysis, suggested that *Pseudomonas* sp. 14-3 has a defective *phbA* gene.

As an additional approach to analyze the functionality of the *pha* genes of *Pseudomonas* sp. 14-3, several plasmids containing different combinations of these genes were introduced into a related strain, *P. putida* GPp104, for complementation analysis. This strain is a PHA negative mutant derived from *P. putida* KT2440 (Huisman *et al*., 1991). *Pseudomonas putida* KT2440 provides medium chain length monomers through *de novo* fatty acids synthesis and β-oxidation pathways for PHA synthesis. Its PHA-negative derivative is frequently used as a host in complementation experiments involving *pha* genes (e.g. Matsusaki *et al*., 1998; Kolibachuk *et al*., 1999; Clemente *et al*., 2000; Hein *et al*., 2002).

Plasmids pCT1377, containing a truncated *phaA* and a complete *phaC*, and pCT1283, containing *phaRBAC* from *Pseudomonas* sp. 14-3, were introduced into *P. putida* GPp104, and production of PHB from different carbon sources was determined. The recombinant strains produced only PHB (Table 2) and no medium chain length monomers when using octanoate as carbon source (data not shown), like *Pseudomonas* sp. 14-3. PHB production by the transconjugants was higher when the entire *Pseudomonas* sp. 14-3 *pha* gene cluster was present, but lower than in *Pseudomonas* sp. 14-3 (Table 2). The ability of the transconjugants to accumulate PHB using the *Pseudomonas* sp. 14-3 *phaC* gene indicated that it was expressed even in the absence of the other *pha* genes. A putative sigma70 promoter was found upstream from the translational initiation site of *phaC* gene by computer analysis of the sequences (data not shown). When the complementation experiments were performed using glucose or gluconate instead of octanoate, PHB production was only restored in the transconjugants when the entire *Pseudomonas* sp. 14-3 *pha* gene cluster was present (Table 2).

Unlike the native strain, *P. putida* GPp104 harboring the *pha* genes from *Pseudomonas* sp. 14-3 was able to accumulate PHB from glucose. A possible explanation could be that *P. putida* GPp104 has a functional β-ketothiolase of its own. Support for this interpretation comes from the finding of a β-ketothiolase in the complete genome of *P. putida* KT2440 (Nelson *et al*., 2002), the parent strain of the mutant *P. putida* GPp104. In order to verify this hypothesis, we introduced plasmid pCT1377, harboring the *phaC* from *Pseudomonas* sp. 14-3, and plasmid pCTB, carrying *phaB* from *Azotobacter* FA8, in *P. putida* GPp104. PHB production from glucose was enabled in the recombinants (Table 2), demonstrating that the function of *phaA*, the only PHB biosynthetic gene that was not provided in the heterologous plasmids, was undoubtedly provided by a gene present in the host.

Mutagenesis studies have shown that only one thiolase is involved in PHB synthesis in natural PHB producing bacteria, even when multiple thiolases are present, as in *C. necator* and *A. vinelandii* (Kranz *et al*., 1997; Slater *et al*., 1998; Segura *et al*., 2000; Taroncher-Oldenburg *et al*., 2000). To investigate if the defective β-ketothiolase was solely responsible for impaired PHB production from glucose in *Pseudomonas* sp. 14-3, we introduced plasmid pCTT, carrying the β-ketothiolase of *P. putida* GPp104, into *Pseudomonas* sp.14-3, and analyzed its ability to accumulate PHB when grown in glucose. The recombinants accumulated PHB from glucose (Table 2), demonstrating that the thiolase from *P. putida* GPp104 was able to complement the defective *phaA* gene in *Pseudomonas* sp. 14-3.

Conclusions

The deduced aminoacid sequence of the β-ketothiolase (PhaA) of *Pseudomonas* sp. 14-3 was compared with sequences present in the databases, and it was found to be similar to other thiolases, among them several thiolases belonging to *Pseudomonas* species that are unable to accumulate PHB. In this study, we have experimentally demonstrated that one of these enzymes, a β-ketothiolase identified in the complete genome sequence of *P. putida* KT2440, can

participate in monomer synthesis. Its activity in the native strain was also demonstrated, as complementation experiments showed that *P. putida* GPp104, a PHA derivative of *P. putida* KT2440, accumulated PHB when a functional reductase (PhaB) and polymerase (PhaC) were supplied.

The results presented in this work indicate that *Pseudomonas* sp. 14-3 is able to synthesize PHB in spite of having a damaged *phaA* using an alternative route for PHB production that allows it to synthesize the polymer from fatty acids, but not from glucose or gluconate. To our knowledge, this is the first report of a natural *phaA* mutant.

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