4 Molecular Basis of Medium-Chain Length-PHA Metabolism of *Pseudomonas putida*

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4.1 PSEUDOMONAS PUTIDA, A MODEL BACTERIUM FOR THE PRODUCTION OF MEDIUM-CHAIN-LENGTH PHA

Pseudomonas spp. is a continuously growing genus (approximately 300 species) of Gram-negative, aerobic, bacillus, belonging to the γ -proteobacteria class. The first description of the genus came from Professor Migula of the Karlsruhe Institute in Germany 1894–1900 [1]. The etymology of the term “pseudomonas” is derived from the Greek terminology “pseudes” (ψευδής), which means false, and “monas” (μονάς), which means one single unit. They are rod-shaped, non-sporulating, and motile with polar flagella [1–3].

One of the early discoveries of the genus is its remarkable metabolic versatility and adaptability to endogenous and exogenous stresses. In 1926, L.E. Den Dooren

first observed the ability of *Pseudomonas* to degrade a great range of substrates such as carbohydrates, organic acids, aromatics, amino acids, alcohols, and nitrogen compounds [2,4]. Furthermore, *Pseudomonas* spp. could tolerate harmful agents present in soils, such as disinfectants, detergents, organic solvents, and heavy metals [5,6], but not extreme changes in temperature and hydrogen ion concentration resulting in the absence of thermophilic and acidophilic strains in this genus [1]. Finally, some human and plant pathogens were distinguished between the varieties of *Pseudomonas* species due to the production of virulent factors such as *P. aeruginosa* and *P. syringae*, respectively [7,8].

P. putida is a “paradigm of a class of cosmopolitan bacteria” isolated from a variety of environments such as soils, plant rhizospheres, and water [6,9]. *P. putida* KT2440 (KT stands for Kenneth Timmis) strain [10–12] derives from a toluene-degrading organism initially isolated in Japan and designated as *P. arvilla* mt-2 (mt-2 stands for “meta-toluate degrader, isolate 2”) and later renamed *P. putida* mt-2 [13]. This strain harbors the TOL plasmid pWW0, which encodes a pathway for toluene and *m/p*-xylene biodegradation [14]. Recently, genomic, phylogenetic, and catabolic re-assessment of the *P. putida* clade supports the delineation of four new species, *P. allopuntida* sp. nov., *P. inefficax* sp. nov., *P. persica* sp. nov., and *P. shirazica* sp. nov. [15].

P. putida KT2440 is the best-characterized strain of its species. It was one of the first Gram-negative bacteria to be classified by the Food and Drug Regulation Administration as an HV1 (host-vector system safety level 1) certified strain, indicating that it is safe to be used in a P1 environment, but not as a food additive [16].

Since the publication and recent revision of the *P. putida* KT2440 genome [9,17], much effort has been invested in exploiting its capacity in biotechnological applications, such as the production of medium-chain-length (*mcl*) polyhydroxyalkanoates (PHA) (see Figure 4.1). Generally speaking, PHA can be classified according to the total number of carbon atoms within a PHA monomer (see Figure 4.2). For instance, short-chain-length (*scl*-PHA) such as poly(3-hydroxybutyrate) [PHB a.k.a. P(3HB)] contains 3–5 carbon atoms per monomer, while *mcl*-PHA monomers have 6–14 carbon atoms. The *mcl*-PHA are mainly produced and thoroughly studied by *Pseudomonas* spp. [18–20]. Microorganisms able to produce PHA with more than 14 carbon atoms are rarely found, such as that produced by a local isolate of *P. aeruginosa* MTCC [21]. Several *Pseudomonas* species can produce both *scl*-PHA and *mcl*-PHA [22,23]. Depending on the monomer composition and the length of the side chain, the mechanical and physicochemical properties of the resulting polymer, such as stiffness, brittleness, melting point, glass transition temperature, degree of crystallinity, and resistance to organic solvents, may vary. *Scl*-PHA tend to be stiff and brittle with a high degree of crystallinity (60%–80%), while *mcl*-PHA are elastic with low crystallinity (25%), low tensile strength, high elongation to break, low melting point, and glass transition temperature below room temperature [24,25]. The thermal and mechanical properties of two prototype PHA polymers are listed in Table 4.1.

P. putida is widely considered as a chassis for synthetic biology. There are tools available to solve synthetic biology challenges, such as the removal of non-desirable traits, enhance advantageous features, and the insertion of new activities, and understand the functionality of complex genetic and metabolic circuits [3,26].

FIGURE 4.1 PHA production and granule schematic structure. The presence of granules is observed by transmission electron microscopy (A) and phase contrast microscopy (C) pictures of *P. putida* KT2440 growing under PHA production conditions. B. Schematic representation of a PHA granule from *P. putida* KT2440. The hydrophobic polymer core (PHA) is surrounded by a layer of GAPs. The presence of phospholipids on the surface of the granule is under discussion.

FIGURE 4.2 PHA monomer chemical structure. Classification is based on the length of the aliphatic side chain X and has been divided into short-chain-length PHA, medium-chain-length PHA, and long-chain-length PHA depending on the number of carbons. All monomeric units have an asymmetric carbon center (*); *R*: enantiomer configuration.

TABLE 4.1
Thermal and Mechanical Properties of Some Representative Polymers from the PHA Family

PHA	Monomeric content	σ_t (MPa)	ϵ_b (%)	T_m (°C)	T_g (°C)	Ref.
P(3HB)	(100%)	40	3–8	173–180	5–9	[24]
P(3HHx-co-3HO)	(12%–88%)	9	380	61	–31	[25]

T_m , melting temperature; T_g , glass transition temperature; E, Young's modulus; σ_t , tensile strength; ϵ_b , elongation at break; 3HB: 3-hydroxy-butyrate; 3HHx: 3-hydroxy-hexanoate; 3HO: 3-hydroxy-octanoate.

Moreover, there are different customized plasmids for *P. putida*, like those included in the Standard European Vector Architecture database (SEVA-DB, <http://seva.cnb.csic.es>), and genome integration tools, such as Tn5 and Tn7 transposons, are also available [27–29]. In order to optimize PHA production, several gene expression and genome editing approaches have been used to reroute the carbon flux towards PHA production [30]. *P. putida*'s metabolism has been extensively characterized and seven genome-scale models (GEMs) are available including *iJN746* [31], *iJP850* [32], *PpuMBEL1071* [33], *iJP962* [34], *iEB1050* [17], *PpuQY1140* [35], and *iJN1462* [36]. These GEMs have been widely used for studying key metabolic features of *P. putida*, such as aromatic and PHA metabolism, suggesting *in silico* strategies for optimizing PHA production [31,36].

In this chapter, we focus our attention on the machinery involved in *mcl*-PHA metabolism in *P. putida*, addressing the fundamental knowledge near the pathways and enzymes involved in this metabolism as a key issue for designing sustainable and efficient production systems.

4.2 THE PHA CYCLE AND ITS KEY PROTEINS

The PHA metabolism in *P. putida* KT2440 is mediated through two PHA synthases, PhaC1 (PP_5003) and PhaC2 (PP_5005), and the PHA depolymerase, PhaZ (PP_5004), that synthesizes and degrades the PHA, respectively (see Figure 4.3). These enzymes are encoded on the *phaC1*, *phaC2*, and *phaZ* genes as part of the *pha* cluster, which is organized into two operons, *phaCIZC2D* and *phaFI* (see Figure 4.3). *PhaD* (PP_5006) encodes for a transcriptional regulator and *phaFI* (PP_5007–PP_5008) for the phasins (see later in this section). Synthases, depolymerase, acyl-CoA synthetase, and phasins are associated with the PHA granule and considered to be “granule-associated proteins” (GAPs) (see Figure 4.1).

More than 60 genes codifying for PHA synthases in 45 bacterial species have been cloned and characterized [37]. These enzymes can be divided into four classes according to the substrate specificity, amino acid primary sequence, and subunit composition [38]. In general, the synthases of class I (e.g., *Cupriavidus necator*; formally known as *Ralstonia eutropha*), class III (e.g., *Allochromatium vinosum*),

FIGURE 4.3 Regulation of *pha* gene expression in pseudomonads. Diagram of the *pha* gene cluster in *P. putida* KT2440 and its regulatory components. Both operons, *phaC1ZC2D* and *phaIF*, are under the control of the regulator, PhaD (effector-specific regulator). Despite increasing knowledge of PHA regulation, the specific roles of the global transcriptional regulators' (Crc, RpoS, and GacS/GacA) system on the synthesis and degradation of the polymer are still to be elucidated.

and class IV (e.g., *Bacillus megaterium*) preferentially use (*R*)-3-hydroxyacyl-CoA thioesters [(*R*)-HA-CoA] comprising 3–5 carbon atoms. The class II PHA synthases (PhaC1 and PhaC2) are mainly observed in pseudomonads and preferentially use CoA thioesters of various (*R*)-3-hydroxy acids comprising 6–14 carbon atoms. In *P. putida*, PHA synthases have been shown to differ in their substrate specificities [39].

A partial tertiary structure prediction of PhaC1 and PhaC2 synthases has been accomplished based on their sequence homology with several α/β hydrolases [39,40]. Residues 201–530 of both synthases were modeled with >90% accuracy as an α/β hydrolase fold core domain covered by a "lid" region (residues 330–425) that buries the predicted catalytic triad (Cys296, Asp451, and His479 for PhaC1, Cys296, Asp452, and His480 for PhaC2) (Figure 4.4A). This suggests an interfacial activation mechanism already described for several lipases, according to which the lid would open in the presence of the substrate, making the active site accessible for binding and catalytic processing. Whether lid opening is accompanied by the full dissociation of the dimer or, as suggested, for PhaC_S-CAT from *Chromobacterium* sp. USM2, the dimeric form is retained but in a more open conformation and remains to be investigated [41]. The Cys296 residue would act as a nucleophile, attacking the activated carboxyl group of the 3-OH-acyl-CoA, thereby displacing the CoA moiety and creating an intermediate acyl-enzyme that would subsequently react with the free hydroxyl group of another 3-OH-acyl-CoA molecule. Besides, Cys296 lies at the bottom of an elongated crevice that would accommodate the growing polymer. However, secondary structure predictions show some differences between the two

FIGURE 4.4 Three-dimensional structural features of synthases and depolymerases involved in PHA metabolism. A. Homology model of the PhaC1 synthase monomeric form from *P. putida* KT2440 [39]. Predicted catalytic residues are shown in van der Waals representation, and the arrow indicates the lid domain. B. Structure of dimeric PhaC from *C. necator* (PDB code 5HZ2) [42]; same indications as in A. C. Structure of dimeric PhaC_{cs}-CAT from *Chromobacterium* sp. USM2 (PDB code 5XAV) [41]. Same indications as in A. D. Homology model of the PhaZ depolymerase from *P. putida* KT2440 [48]. Predicted catalytic residues are shown in van der Waals representation. The arrow indicates the lid domain, and a dimer of the substrate (3-hydroxyoctanoate) is depicted in stick representation.

synthases around residues 115–123, 316–324, the latter stretch located near the lid region [39]. These or other sparse changes might be of importance for lid movements and/or substrate accommodation, accounting for their differences in substrate specificity.

Numerous spontaneous mutants that block PHA accumulation have been isolated, all of them accounting for changes solely affecting the *phaC1* gene within the *pha* cluster [40]. Most single-residue PhaC1-inactivating mutations were mapped within or in the proximity of the predicted lid region, similar to those isolated in *C. necator*, reinforcing the importance of this region in catalytic activation [42]. Protein-protein docking procedures *in silico* predict both homodimeric and heterodimeric complexes interacting through an extensive surface involving the solvent-exposed regions of the

lids of the two synthases. In this context, the inactivating mutations in *phaC1* near the lid might either affect the formation of a productive heterodimeric complex or subsequent interfacial activation steps. Besides, the existence of PhaC1-PhaC1 and PhaC2-PhaC2 homodimers is also possible through similar lid-lid interactions [40].

The three-dimensional structures of the catalytic domain of two highly similar class I PHA synthases, namely PhaC from *C. necator* [42,43] (PhaC_{Re} hereafter), and from *Chromobacterium* sp. USM2 [41] (PhaC_{Cs}-CAT hereafter), have been recently solved (see Figure 4.4B, C), providing valuable information that supports the predicted *P. putida* PhaC models. Remarkably, in both cases, the protein experienced proteolysis cleavage during crystallization, and only the C-terminal moiety could be studied, comprising the catalytic domain, thus accounting for the *P. putida* PhaC1 and PhaC2 regions that were modeled (Figure 4.4A). The overall structure in the two cases shares the same α/β hydrolase fold predicted for the *P. putida* synthases, with a central core displaying a left-handed super-helical twist flanked by α -helices on both sides, and also covered by a lid domain. Furthermore, the major deviations with the *P. putida* PhaC models are found in the lid region.

Both PhaC_{Re} and PhaC_{Cs}-CAT exist in solution in a monomer/dimer equilibrium, the dimer being the full catalytic active form [41–43]. The dimer interface is favored by lid-lid molecular interactions, similar to those predicted for the *P. putida* proteins, so that conformational changes involving rearrangement of the monomers would facilitate substrate entry and product formation and leaving. The catalytic triad comprises Cys291, Asp447, and His477 for *Chromobacterium* sp. and Cys319, His508, and Asp480 for *C. necator* and are located at the bottom of a crevice. The relative position of the three catalytic residues corresponds well with that of lipases, suggesting a similar mechanism by which these three residues catalyze the esterification reaction. The catalytic Cys would carry out a nucleophilic attack on the thioester of acyl-CoA, generating the acyl-Cys intermediate, whereas His would accelerate the reaction by deprotonating the thiol group of Cys, assisted by Asp. In any case, the catalytic model involves an open-closed equilibrium and comprises the use of a single active site.

Two types of enzymes responsible for the PHA depolymerization have been identified: the extracellular depolymerases and the intracellular ones. Several non-PHA producing microorganisms secrete extracellular depolymerase that allows them to obtain carbon and energy sources from the degradation of the exogenous PHA. The intracellular degradation of the *scl*-PHA was studied extensively in *C. necator* H16 and *Rhodospirillum rubrum*. Functional genomic analyses confirm the complexity of the PHB hydrolysis process [44,45].

The first intracellular *mcl*-PHA depolymerase was described in *P. putida* GPo1, and its enzymatic activity was confirmed *in vivo* on the defective *phaZ* mutant (GPo500), where PHA accumulated, and the mutant was not able to degrade the polymer in the stationary phase [46]. Similar experiments and conclusions were obtained from *P. putida* U and *P. putida* KT2442 [47–50]. The first biochemical characterization of an intracellular *mcl*-PHA depolymerase was realized for the PhaZ from *P. putida* KT2442 [48]. The PhaZ preferentially hydrolyzes *mcl*-PHA containing aliphatic and aromatic monomers. The enzyme behaves as a serine hydrolase that is inhibited by phenylmethylsulfonyl fluoride. The three-dimensional structure of PhaZ complex with a 3-hydroxyoctanoate dimer has been modeled. The enzyme presents an α/β hydrolase-type fold, constituting

a central core of an eight-stranded β -sheet and α -helices packed on both sides and capped with a lid structure (residues 133–192) built up from five α -helices connected by loops and covering a possible catalytic triad (Ser102, Asp221, and His248) near the “hinge” of the lid [48] (see Figure 4.4D). The binding surface of the core domain is highly hydrophobic, and the only polar residues surrounding the substrate are the catalytic triad and Asn35, which may take part in substrate recognition as described for the analogous Ser34 in the CumD hydrolase template [51]. Finally, the presence of a shallow surface across the core domain, followed by an elongated deep cavity facilitates the accommodation of *mcl*-hydrocarbon chains of the substrate [48].

The third type of key proteins in *mcl*-PHA machinery are the phasins; in *mcl*-PHA granules of pseudomonads, two phasins, PhaF and PhaI, have been identified as their major GAPs [52]. PhaF plays a crucial role in the spreading of PHA granules among daughter cells upon cell division by linking this event with chromosomal distribution [53]. A PhaF-deficient *P. putida* KT2440 strain showed the defective segregation of PHA granules and reduced PHA accumulation compared with the wild-type strain (33% vs. 63% PHA in cell dry mass). The organization of the granules into a needle array along the long axis of the cell, as observed in the wild-type strain, is also lost in the mutant [53]. Moreover, PhaF was demonstrated to function as a biosurfactant by separating PHA into granules in hydrophilic environments as it occurs in the cytoplasm of bacterial cells [54]. In this sense, the interfacial activity of PhaF and its PHA-binding domain confirmed the potential of these proteins to interact and stabilize the surface of very different hydrophobic materials [55]. For more detailed information about these proteins, see Chapter 2 of this volume, written by Mezzina et al.

PhaF is the major phasin in *Pseudomonas* species, and it is structurally organized in two domains. The N-terminal domain (termed as BioF in the case of the closely related *P. putida* GPol strain) shares sequence similarity with PhaI, and it is involved in PHA recognition and binding; the C-terminal moiety binds DNA in a non-specific fashion [52,53]. A structural model of PhaF has been elaborated based on sequence homology and *de novo* estimations, with the support of biophysical studies [56] (see Figure 4.5A). The model predicts an elongated shape of the phasin, with the N-terminal and PHA-binding domain acquiring a long amphipathic α -helix that interacts with the granule surface by hydrophobic interactions. However, the C-terminal domain, which contains eight AAKP-like tandem repeats, displays the characteristics of a highly positively charged, histone-like polypeptide. Both domains are linked by a leucine zipper sequence involved in protein tetramerization [56,57] (see Figure 4.5A and Figure 4.6), and are partly intrinsically disordered in the absence of their cognate ligands (PHA and DNA). Both coiled-coil-mediated oligomerization and intrinsic disorder may be common traits shared by a significant number of other phasins [56].

4.3 METABOLIC PATHWAYS INVOLVED IN *MCL*-PHA PRODUCTION IN *P. PUTIDA*

4.3.1 *MCL*-PHA SYNTHESIS FROM STRUCTURALLY RELATED SUBSTRATES

In pseudomonads, structurally, PHA-related substrates like fatty acids are catabolized through the β -oxidation pathway. The fatty acids can be incorporated into PHA

FIGURE 4.5 Three-dimensional structural features of the phasin PhaF and the transcriptional regulator PhaD. A. A theoretical model of the tetrameric form of the PhaF phasin from *P. putida* KT2440 bound to DNA [56]. B. Homology model of the PhaD regulator from *P. putida* KT2440 showing two docked molecules of 3-hydroxyoctanoyl-CoA in van der Waals representation.

FIGURE 4.6 Phasins from *P. putida* KT2440. The scheme of the PhaI and PhaF phasins structure showing three separate domains. The N-terminal PHA binding domain acquires an α -helix conformation (gray color). The leucine zipper motif of PhaI (ZIPI) and PhaF (ZIPF) consists of three heptad repeats (squared) with leucines occupying most of the *d* positions (black color) [57]. The structurally disordered C-terminal domain (DNA binding domain) comprises eight AAKP-like tandem repeats (shaded gray boxes) similar to those found in the DNA binding nucleoid-associated proteins of histone family H1. The predicted structure of the PhaF monomer is shown (bottom) [56].

directly as β -oxidation intermediates [(*R*)-HA-CoA], avoiding their complete oxidation to acetyl-CoA, yielding monomer chains of equal or shorter length than those of the carbon source applied [58]. The fatty acid catabolism and the involved enzymes in this route (Fad) are conserved in distinct bacterial species and have been widely studied in the bacterial model *E. coli*.

A detailed β -oxidation pathway in *P. putida* and its connection with PHA metabolism is shown (see Figure 4.7 and Table 4.2). In short, the fatty acids are activated into acyl-CoA through the acyl-CoA synthetase (FadD). Subsequently, an acyl-CoA dehydrogenase (FadE) catalyzes the formation of a double bond yielding an enoyl-CoA. In the next step, a tetrameric complex formed by FadBA proteins carries out the hydration, oxidation, and thiolysis processes. This complex comprises five enzymatic reactions (enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, *cis*- Δ^3 -*trans*- Δ^2 -enoyl-CoA isomerase, 3-hydroxyacyl-CoA epimerase, and 3-ketoacyl-CoA thiolase) and it is responsible for the removal of two carbon units from the acyl-chain [59]. The intermediates of the β -oxidation enoyl-CoA, (*S*)-3-hydroxyacyl-CoA, and 3-ketoacyl-CoA can be converted into (*R*)-3-HA-CoA, which are the PHA synthase substrates through a stereospecific *trans*-enoyl-CoA hydratase (PhaJ), an epimerase (FadB), or a specific ketoacyl-CoA reductase (FadG), respectively [20] (see Figure 4.7 and Table 4.2).

In *P. putida* KT2440, two fatty acid transporter genes, a long-chain fatty acid transporter (*fadL*; PP_1689) and a short-chain fatty acid transporter (PP_3124) that initialize the pathway have been identified [60]. FadD (EC 6.2.1.3) is an ATP (adenosine triphosphate), CoA, and Mg^{2+} dependent enzyme widely distributed among the organisms and exhibits a broad substrate specificity. This is a cytoplasmic membrane-associated protein that activates exogenous fatty acids into metabolically active CoA thioesters when they are transported across the cytoplasmic membrane. Two key studies have been performed on *Pseudomonas* species reporting two *fadD* gene homologs in *P. putida* U (named FadD1 and FadD2) [61] and *P. putida* GPo1 (named Acs1 and Acs2) [62]. FadD1 was proposed to be an enzyme involved in physiological fatty acid degradation, while FadD2 was a cryptic gene induced when the FadD1 was inactivated [61]. Furthermore, the Acs1 and Acs2 share 94% and 92% identity with FadD1 and FadD2 of *P. putida* U, respectively. In fluorescent microscopy experiments, the Acs1 was mainly associated with the PHA granules activating the (*R*)-HAs released by the PHA depolymerase during the PHA degradation process, whereas the Acs2 was located in the cellular membrane [62]. Finally, in *P. putida* KT2440, three genes encoding putative FadD enzymes (FadD-I: PP_4549, 94% identity with FadD1 of *P. putida* U. FadD-II: PP_4550, 91% identity with FadD2 from *P. putida* U. FadDx: PP_2213) were identified, where the FadD-I was proposed to be the principal enzyme [60]. Finally, homologous genes have also been characterized in the *P. putida* CA-3 strain, where FadD is capable of activating long-chain phenylalkanoic and alkanolic acids [63].

The second step of fatty acid degradation in *P. putida* KT2440 is realized by acyl-CoA dehydrogenases, FadE. They belong to a large family of flavoproteins and show activity towards a broad range of substrates. Depending on the substrate specificity of FadE, the enzyme can be classified into short, medium, long, or very long-chain acyl-CoA dehydrogenases. However, the boundary of classification is not tight as substrate

FIGURE 4.7 Fatty acid β -oxidation pathway and its connection with PHA metabolism. Diagram with the main metabolic steps involved in the β -oxidation pathway (black lines), the proposed connection with PHA metabolism, the responsible genes involved in these metabolic pathways (bold gray letters), and the reaction exchanges (gray color) are shown. Figure abbreviations: (S)-HA-CoA: (S)-3-hydroxyacyl-CoA, (R)-HA-CoA: (R)-3-hydroxyacyl-CoA. specificities of particular enzymes may overlap [64]. The reaction of the dehydrogenation presents the rate-limiting step in the β -oxidation since FadE has the lowest activity among all β -oxidation enzymes in *E. coli* [65]. Initial work on *P. putida* KT2440 identified PP_2216 as a specific acyl-CoA dehydrogenase for short-chain aliphatic fatty acyl-CoAs [66] and the PP_0368 as an inducible phenylacyl-CoA dehydrogenase [67].

TABLE 4.2
Gene Names, Enzymatic Activities, and Gene ID of *P. putida* KT2440
Involved in the β -Oxidation Pathway

Gene	Enzymatic activity	Annotation
<i>fadL</i>	Long-chain fatty acid transporter (LCFAs)	PP_1689
<i>fadD</i>	Acyl-CoA synthetase	PP_2213/PP_4549/PP_4550
<i>fadE</i>	Acyl-CoA dehydrogenase	PP_0368/PP_1893/PP_2039/PP_2048/PP_2216/PP_2437
<i>fadB</i>	Multienzymatic activities of the FadBA complex: enoyl-CoA hydratase 3-hydroxyacyl-CoA dehydrogenase Cis- Δ^3 -trans- Δ^2 -enoyl-CoA isomerase 3-hydroxyacyl-CoA epimerase	PP_2047/PP_2136/PP_2214
<i>fadA</i>	3-ketoacyl-CoA thiolase	PP_2137/PP_2215/PP_4636
<i>phaJ</i>	Enoyl-CoA hydratase, <i>R</i> stereoselectivity	PP_0580/PP_4552/PP_4817
<i>fabG</i>	3-ketoacyl-CoA reductase	PP_1914

Recently, *in silico* analysis of its genome sequence revealed 21 putative acyl-CoA dehydrogenases (ACADs), four (PP_1893, PP_2039, PP_2048, and PP_2437) of which were functionally characterized by mutagenesis studies. The PP_1893 (FadE) and PP_2437 (FadE2) were proposed to directly participate in fatty acid degradation, while the 19 remaining putative ACADs have a redundant role or overlap in terms of function when *P. putida* KT2440 is grown on aliphatic fatty acids [68].

The FadBA complex is encoded by two sets of genes located in two different operons in *P. putida* KT2440, *fadB* and *fadA* (PP_2136 and PP_2137) and *fadBx* and *fadAx* (PP_2214 and PP_2215). The first set seems to play a more important role since their absence causes a defective β -oxidation pathway yielding polymers with longer chain monomers [69]. Various isoenzymes have been proposed to carry out some of the steps performed by the FadBA complex [70].

As far as it concerns fatty acid catabolism regulation, in *Pseudomonas* spp., the PsrA regulator was reported to transcriptionally control the *fad* genes [71,72]. For instance, in *P. putida* KT2440, the transcriptional repressor of the β -oxidation route, PsrA, negatively regulates the expression of genes related to fatty acid metabolism. It has been suggested that PsrA binds to the promoter region of the *fadBA* operon inhibiting its expression in *P. putida*. This explains why a *psrA* mutant strain showed increased β -oxidation and a reduction in its PHA content when using fatty acids as a growth and polymer precursor [72].

4.3.2 THE CONNECTION OF THE β -OXIDATION PATHWAY TO THE PHA CYCLE

In the β -oxidation pathway, acyl-CoA is sequentially oxidized into enoyl-CoA, (*S*)-3-hydroxyacyl-CoA, and 3-ketoacyl-CoA. All of these intermediates are proposed

to be converted into a PhaC1 substrate, (*R*)-HA-CoA by a stereospecific trans-enoyl-CoA hydratase (PhaJ), an epimerase (FadB), and/or a specific ketoacyl-CoA reductase (FabG) (see Figure 4.7) [20,73].

PhaJ is one of the proposed enzymes linking β -oxidation and PHA synthesis and catalyzes a stereospecific hydration reaction from trans-2-enoyl-CoA to (*R*)-3-HA-CoA. Recent studies in pseudomonads have suggested that the PhaJ enzyme can contribute to the monomer supply for PHA biosynthesis from fatty acid precursors [74]. Four *phaJ* genes were identified in *P. aeruginosa*, where only PhaJ1 was found to be specific for *scl*-enoyl-CoAs (C4–C6) and the other three PhaJs were specific for *mcl*-enoyl-CoAs (C6–C12) [75]. From a database search, three homologs of *phaJ1*_{pa}, *phaJ3*_{pa}, and *phaJ4*_{pa} were found in *P. putida* and named *phaJ1* (PP_4552), *phaJ3* (PP_0580), and *phaJ4* (PP_4817), respectively. Only *phaJ4* showed higher expression levels after growth on fatty acids, and it was shown to produce more PHA. Additionally, the PhaJ4 enzyme substrate specificity was shown to be *mcl*-enoyl-CoAs like PhaJ4 from *P. aeruginosa* [60,74–77].

Another link between β -oxidation and PHA synthesis was previously proposed as an NADPH-dependent 3-ketoacyl-ACP reductase, FabG. This is a reversible enzyme known to catalyze the conversion of 3-ketoacyl-CoA into (*R*)-HA-CoA and vice versa [78]. Initial studies on *E. coli* have shown that FabG_{Ec} and FabG_{pa} (from *P. aeruginosa*) were capable of generating (*R*)-HA-CoA precursors from 3-ketoacyl-CoA in the presence of a PHA synthase [79,80]. In pseudomonads, its contribution to PHA biosynthesis via the β -oxidation pathway is yet unclear. In fact, overexpression of *fabG* and *phaJ* in the *P. putida* strain resulted in controversial results, with negative and positive effects on PHA biosynthesis, respectively. The *fabG* negative effect could be due to the reversible conversion of (*R*)-HA-CoA into 3-ketoacyl-CoA [74].

Finally, FadB, a putative epimerase, was proposed to connect β -oxidation and PHA synthesis by converting (*S*)-HA-CoA to (*R*)-HA-CoA. Despite the assigned epimerase activity in *E. coli* [59], its role is not physiologically relevant in pseudomonads. Thus, FadB does not seem to possess the putative epimerase function to provide the (*R*)-enantiomer of HA-CoA efficiently, and other linking enzymes are required to efficiently channel intermediates of β -oxidation towards *mcl*-PHA biosynthesis [76]. More studies need to be done to verify this.

4.3.3 DE NOVO FATTY ACID SYNTHESIS

Non-PHA related substrates, such as carbohydrates, can act as indirect precursors of PHA through their complete oxidation to acetyl-CoA and can be channeled towards the *de novo* synthesis pathway (see Figure 4.8 and Table 4.3). The genetic and biochemical characterization of the *de novo* fatty acid biosynthesis in pseudomonads has been principally analyzed in *P. aeruginosa*.

The *de novo* fatty acid intermediates are activated by the acyl carrier protein (ACP). The fatty acid synthesis starts with the acetyl-CoA carboxylation into malonyl-CoA by the acetyl-CoA carboxylase, the AccABCD complex [81]. Then, the malonyl-CoA is transesterified into malonyl-ACP by the malonyl-CoA: ACP transacylase, FabD [82]. The generated malonyl-ACP is further condensed into 3-ketoacyl-ACP by different 3-ketoacyl-ACP synthases. First, it is condensed by an acetyl-CoA molecule

FIGURE 4.8 *De novo* fatty acid biosynthesis and its connection with PHA metabolism. Diagram with the main metabolic steps involved in *de novo* fatty acid biosynthesis (black lines), the proposed connection with the PHA metabolism, the responsible genes involved in these metabolic pathways (bold gray letters), and the reaction exchanges (gray color) are shown. Figure abbreviations: (R)-HA-ACP: (R)-3-hydroxyacyl-ACP, (R)-HA-CoA: (R)-3-hydroxyacyl-CoA.

TABLE 4.3
Genes Names, Enzymatic Activities and Gene ID of *P. putida* KT2440
Involved in the *de novo* Fatty Acid Synthesis

Gene	Enzymatic activity	Annotation
<i>accABCD</i>	<i>accABCD</i> complex: Acetyl-CoA carboxylase	PP_1607/PP_0559/PP_0558/ PP_1996
<i>fabD</i>	Malonyl-CoA transacylase	PP_1913
<i>fabB</i>	3-oxoacyl-ACP-synthase	PP_4175
<i>fabF</i>	beta-ketoacyl-ACP-synthase II	PP_1916/ PP_3303
<i>fabH</i>	3-ketoacyl-ACP-synthase III	PP_4379/PP_4545
<i>fabG</i>	3-oxoacyl-ACP-reductase subunit	PP_0581/PP_1914/PP_2540/ PP_2783
<i>fabA</i>	3R-3-hydroxydecanoyl-ACP dehydratase	PP_4174
<i>fabZ</i>	3-hydroxyacyl-ACP dehydratase	PP_1602
?	Enoyl-ACP reductase	PP_1852
<i>phaG</i>	3-hydroxyacyl-CoA-ACP transferase	PP_1408
?	Medium-chain-fatty acid 3-hydroxyacyl-CoA ligase	PP_0763

by FabH, then, in successive rounds of elongation, a new molecule of malonyl-ACP is condensed with the 3-acyl-ACP formed by FabB or FabF [83,84]. FabB has also been proposed to catalyze the decarboxylation of malonyl-ACP into acetyl-ACP [85]. The following step includes the reduction of 3-ketoacyl-ACP into (*R*)-3-hydroxyacyl-ACP by a 3-ketoacyl-ACP reductase, FabG [86], and the formation of a double bond into enoyl-ACP by 3-hydroxyacyl-ACP dehydratase, FabA, or FabZ [87]. Finally, one enoyl-ACP reductase, FabI, FabK, or FabL transforms the enoyl-ACP into 3-acyl-ACP [88]. The gene that codifies for enoyl-ACP reductase activity has not been identified in *P. putida* KT2440 [84].

4.3.4 DE NOVO FATTY ACID SYNTHESIS CONNECTION TO THE PHA CYCLE

Hydroxyacyl-ACP intermediates can be transformed into (*R*)-HA-CoAs by the specific transacylase PhaG, present in most pseudomonads [89]. PhaG is reported to be a 3-hydroxyacyl-ACP-CoA transferase able to transfer the 3-hydroxyacyl group from ACP to CoA moiety in *P. putida* KT2440 and *P. aeruginosa* [90–94]. However, PhaG is proposed to function as a 3-HA-ACP thioesterase. The existence of at least one 3-hydroxyacyl-CoA ligase is needed to convert 3-hydroxyalkanoate substrates into 3-HA-CoA thioester PHA precursors [95]. Based on homology studies, the PP_0763 gene was predicted to encode a medium-chain fatty acid CoA ligase; its transcription levels were highly upregulated when cells were grown under PHA-producing conditions [95]. In order to verify this, *phaG* (PP_1408) and PP_0763 genes from *P. putida* were cloned and co-expressed with the engineered *phaC1* (STQK) synthase from *Pseudomonas* sp. 61-3 in recombinant *E. coli*. The latter strain resulted in about four-fold higher PHA production from non-fatty acid carbon sources than that in *P. putida* KT2440 [95].

4.4 PHA METABOLISM REGULATION

The *pha* gene cluster is well conserved among the *mcl*-PHA producer strains [49] and contains two operons, *phaC1ZC2D* and *phaFI*, that are transcribed in opposite directions (see Figure 4.3). However, the intergenic regions vary depending on the species (as observed in *P. corrugata*, *P. mediterranea*, and *P. aeruginosa*), suggesting that the transcription control differs between the strains [49].

PHA are widely investigated concerning their structure, versatility, physico-chemical properties, biodegradability, and sustainable and optimizable production possibilities [96–99]. However, the molecular mechanisms behind the PHA metabolism regulation are not yet clear due to the complexity of this regulation system at different levels (genetic, enzymatic, and global regulators). Furthermore, PHA regulation could depend on the carbon source(s), the specificity of the PHA synthase, and the metabolic pathways involved. The PhaC synthase substrates derive from the β -oxidation and *de novo* fatty acid synthesis. Thus, we can assume that regulating factors affecting the enzymes involved in these pathways could be involved in the PHA synthesis regulation too.

The co-existence of polycistronic transcription units was proposed by the identification of five functional promoters upstream of the *phaC1*, *phaZ*, *phaC2*, *phaF*, and *phaI* genes by using *lacZ* translational fusions. However, in RT-qPCR experiments, the P_{C1} and P_I were demonstrated to be the most active promoters [100].

PhaD was proposed to transcriptionally activate the *pha* genes by binding to 25 and 29 bp target regions of the P_{C1} and P_I promoters, respectively [100]. Both sequences contain a single binding site formed by inverted half-sites of 6 bp separated by 8 bp. Unlike most characterized members of this family, PhaD functions as an activator and not as a repressor of transcription (see Figure 4.3). A 3D model of the PhaD structure predicts a dimeric structure with each monomer conformed by two domains built up from nine α -helices [100] (see Figure 4.5B). In turn, the N-terminal domain is composed of an HTH motif comprised of $\alpha 2$ and $\alpha 3$ helices, and in the C-terminal, the $\alpha 8$ and $\alpha 9$ helices are involved in the dimerization of the protein through a four-helix bundle arrangement. Overall, a long, deep crevice enclosed by helices $\alpha 5$ – $\alpha 8$ in the C-terminal domain could be accommodated inside an elongated intermediate molecule of the fatty acid β -oxidation, hydroxyoctanoyl-CoA, that would act as the true inducer [100]. This model proposes the effector of PhaD to be a CoA intermediate of fatty acid β -oxidation or TCA (tricarboxylic acid cycle) metabolite. However, the true effector remains uncertain [100]. In different *Pseudomonas* strains, variable transcription levels of *phaF* and *phaFI* were observed related to the PhaD role [101]. A clear carbon source dependence was also demonstrated [50].

PhaF has been shown to regulate the expression of the *pha* genes, and to some extent, the entire transcriptomic profile. This effect is also believed to be derived from its DNA binding abilities and putative histone-like functionality [52,53]. The disruption of the *phaF* gene in *P. putida* KT2442 has been shown to affect PHA production (1.5-fold reduction) and the transcription of the *phaC1* and *phaI* genes. Experiments have shown that the transcription level of *phaC1* decreases approximately 3.5-fold with respect to the wild-type and that of *phaI* to reach its highest level much later in

the stationary phase [53]. Even though there is no evidence for the specific binding of PhaF to the promoter regions of *pha* genes, preliminary transcriptomic analyses of a *phaF* mutant strain has suggested an indirect regulatory effect on the expression of these genes with the mild downregulation of the *pha* cluster. A wider effect on the *P. putida phaF* mutant transcriptome has also been observed, including upregulation of acetyl-CoA acetyltransferase (Q88E32), a key enzyme in fatty acid degradation, and other proteins involved in metabolic and regulatory processes [53].

Recent evidence suggests that the expression of the genes in the *pha* cluster is also subject to the influence of global regulatory systems, although most mechanisms are not fully understood (see Figure 4.3). De la Rosa et al. [102] reported PHA synthesis to be controlled by a catabolite repression control (Crc) protein, a global regulator that optimizes carbon metabolism by inhibiting the expression of genes involved in the use of non-preferred carbon sources. In balanced C/N conditions (which are not optimal for PHA accumulation), Crc was shown to inhibit the translation of *phaCI* mRNA via its interaction with a specific sequence, *AnAAnAA*, located immediately downstream of the AUG start codon (where *n* represents any nucleotide). This results in a reduction in the PHA content of the cell [102]. The RpoS global regulator has also been suggested to be involved in the regulation of the P_{CI} promoter. Experiments on *P. putida*, using the $P_{CI}::lacZ$ translational fusion, showed that *rpoS* mutation increased the expression driven by the P_{CI} promoter. PHA synthesis is also affected by the two-component GacS/GacA global regulatory system (global antibiotic and cyanide control), as described in *P. putida* CA-3. GacS is a sensor kinase and GacA, its cognate regulator that activates the expression of small RNAs (sRNAs). These sRNAs and the sRNA-binding (RsmA) protein cooperatively regulate protein synthesis and together constitute the Gac/Rsm system. A *gacS* mutation in *P. putida* CA-3 prevents the translation of *phaCI* mRNA by a non-conventional mechanism that is yet to be fully understood [103]. As previously mentioned, a link between PHA accumulation and the transcriptional regulators of the central metabolic routes that provide substrates for PHA synthesis via the β -oxidation pathway has also been reported [72,104]. In *P. putida* KT2440, the transcriptional repressor of the β -oxidation route, PsrA, negatively regulates the expression of genes related to fatty acid metabolism by binding to the promoter region of the *fadBA* operon inhibiting its expression in *P. putida*. All these regulatory systems drive the PHA metabolism as a key cycle for maintaining the carbon and energy control in the cells. Very recently, the impact of this cycle on the robustness of *P. putida* has been highlighted.

4.4.1 THE PHA CYCLE AS A ROBUSTNESS CYCLE

Cells facing carbon excess and nutrient limitation direct the PHA metabolism to store the extra carbon in the form of polyesters by PHA synthases. Under a balanced C/N ratio, PHA metabolism is directed to the hydrolysis pathway releasing of (*R*)-HAs monomers to the extracellular medium by the action of the PhaZ depolymerase. The released monomers are then activated to (*R*)-HA-CoAs by a granule-associated acyl-CoA synthetase (FadD) via an ATP-dependent reaction (see Figure 4.9). This metabolite is a substrate for PhaC as well as the β -oxidation cycle. Depending on the metabolic state of the cell, this (*R*)-HA-CoA is either

FIGURE 4.9 The PHA cycle. The key players are the PHA synthases (PhaC1, PhaC2), PHA depolymerase (PhaZ), and Acyl-CoA synthetase (FadD). Depending on the metabolic state of the cell, the (R)-HA-CoA can either be incorporated into a nascent PHA granule or be used for fatty acid metabolism. Figure abbreviations: (R)-HA-CoA: (R)-3-hydroxyacyl-CoA, (R)-HA: (R)-3-hydroxyacyl-carboxylic acid (free monomers).

incorporated again into nascent PHA polymer chains or used by the fatty acid metabolism to make the stored carbon available to the central metabolism. Thus, PHA metabolism is far from being a unidirectional metabolic process, where the PHA are either polymerized or depolymerized, but an “ongoing” cycle, where there is simultaneous polymerization and degradation of PHA polymer to facilitate the fast turnover of the polymer [50,105]. In fact, PhaZ is active even under conditions where it would appear not to be required [105].

Due to this cyclic operational mode, the PHA cycle has been traditionally considered as a futile cycle [50,105]. Futile cycles occur in microorganisms inducing a considerable energy burden for the cell, promoting the dissipation of energy. Such cycles fall into two main categories: those involving simultaneous phosphorylation and dephosphorylation and those involving energy-driven transport reactions in the opposite direction. However, based on this canonical definition of futile cycles, PHA cannot be considered as such. It was not clear what was the metabolic advantage of this cyclic operational mode of PHA metabolism, as a great amount of energy was wasted [50,105,106]. Interestingly, microbial systems operate far from optimally in terms of efficient use of ATP. Instead, they optimize multiple objectives in which growth, but also extra production of ATP, is prioritized [107]. It has been suggested that these extra ATP levels are required for fueling proper biological responses under (un)expected environmental perturbations, thus providing metabolic robustness. The inherent property ubiquitously observed in nature, allowing systems to

maintain their functions despite external and internal perturbations, is understood as robustness [108,109]. Recent work from our group has identified a series of metabolic cycles providing robustness in *P. putida* KT2440 using the *in silico* genome-scale metabolic model *iJN1411* [110]. Such cycles are more than conventional futile cycles as in addition to acting as dissipating energy, they likely act as metabolic capacitors connecting catabolism and anabolism with central metabolism. These metabolic capacitors provide a pre-processed source of energy and anabolic building blocks while balancing and optimizing the redox state. Thus, such buffering cycles provide metabolic robustness and fitness under changing environmental conditions. The PHA cycle was identified among these metabolic cycles, thus confirming the role of cyclic PHA metabolism as a mechanism providing robustness to *P. putida*. In addition, genes encoding PHA metabolism were found to be highly expressed in an exponential growth phase irrespective of the nutritional scenario [110]. This means that during unexpected and sudden environmental perturbations, such as carbon source depletion, the cells could adapt rapidly to the new situation providing a new source of carbon and energy such as (*R*)-HA-CoA without the need to induce complex cascades of gene expression [105]. Additionally, the PHA cycle was proposed to regulate fatty acid flux towards the fatty acid oxidation pathway [105]. This scenario effectively increases *P. putida* fitness under changing environmental conditions. Supporting this novel function of PHA metabolism to a greater extent, it has been demonstrated that an active PHA cycle has a great impact on a variety of physiological processes such as cell size and number of individuals as a function of carbon/nitrogen availability and biomass formation [50,106,111]. Therefore, PHA metabolism, far from being a single carbon source storage mechanism, has an essential role in maintaining optimal growth performance and homeostasis in *P. putida*.

4.5 CONCLUSIONS AND OUTLOOK

Fueled by recent studies, PHA metabolism is no longer considered as a residual and secondary metabolism; it is starting to be considered as a central metabolic and physiological hub in *P. putida*. The more is known about the complexity of PHA metabolism, the greater the need is to go further in its study by using cutting edge integrative and systems analyses. The complete understanding of the key involved proteins, regulatory systems, and the PHA metabolism as a metabolic robustness cycle in *P. putida*, driving its known stress endurance and robustness, is of crucial importance to increase the biotechnological applications of this unique bacterial workhorse. The possibility to construct optimized strains with higher metabolic robustness based on PHA cycle engineering will position *P. putida* as an ideal biocatalyst towards the revalorization of recalcitrant residues by significantly contributing to the largely expected circular economy.

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