Biogenesis of Medium-Chain-Length Polyhydroxyalkanoates

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Abstract

Medium-chain-length polyhydroxyalkanoates (mcl-PHA) are biotechnologically useful natural products found in many bacteria. This biopolymer functions as a carbon and energy storage reservoir in cells but has physical and mechanical properties that make it a promising bioplastic with applications ranging from adhesives to medical implants. Therefore, there is much interest in understanding the biology of mcl-PHA synthesis and metabolism. Increased knowledge of PHA biology serves as a foundation for the bioengineering of PHA and its eventual use as a biologically derived product. This chapter covers the state of knowledge on

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mcl-PHA, including its synthesis and its central role in cellular metabolism. Moreover, this chapter discusses methods for bioengineering mcl-PHA production in bacteria as well as synthetic biology methods for its study and production in the natural mcl-PHA producer, *Pseudomonas putida*.

1 Introduction

Polyhydroxyalkanoate (PHA) production is widespread in bacteria, being found in many Gram-negative and Gram-positive genera, as well as some archaea (Han et al. 2010; for a complete list of bacterial genera with references, see Lu et al. 2009). PHAs are hydrophobic polymers that accumulate in intracellular inclusions termed PHA granules or carbonosomes. The traditional view is that PHA serves an energy storage function with favorable conditions for its production commonly being a nutrient imbalance, including the availability of a suitable carbon source coupled with an imbalance in at least one other nutrient. These other lacking nutrients vary, with one commonly being nitrogen, but can include phosphate, oxygen, sulphate, or various elements (Kim and Lenz 2001). Recently, a more nuanced view presents PHA as not solely having an energy storage function but as a key component in the cycle of carbon storage and energy expenditure by the cell (Escapa et al. 2012).

While numerous organisms have been studied for their natural or recombinant production of short-chain-length PHA (scl-PHA), i.e., poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxyvalerate) (PHV), fewer organisms are natural producers of medium-chain-length PHA (mcl-PHA), with *Pseudomonas* spp. being the best studied. Mcl-PHAs have elastomeric and thermal properties that generally make them superior bioplastics for rubber and viscoelastic applications when compared to scl-PHA (Sudesh et al. 2000). The following sections will focus on mcl-PHA-producing *Pseudomonas* species with descriptions from the scl-PHA-producing *Cupriavidus necator* (formerly named *Ralstonia eutropha*) or other scl-PHA producers presented for comparison.

2 mcl-PHA Biogenesis

mcl-PHA is synthesized by the enzymatic polymerization of *(R)*-3-hydroxyacyl-CoA monomers containing between six and 14 carbons (3-hydroxyhexanoate and 3-hydroxytetradecanoate, respectively). PHA molecules are highly variable in regard to their number of monomeric repeats and the length and composition of their side chains. For example, a PHA homopolymer made up of solely 3-hydroxyhexanoate (poly(3-hydroxyhexanoate)) has a side chain of three carbon atoms, whereas a copolymer or heteropolymer consisting of 3-hydroxybutyrate and 3-hydroxyoctanoate (poly(3-hydroxybutyrate-co-3-hydroxyoctanoate)) has side chains of one carbon and five carbon atoms, respectively. Several factors determine whether a copolymer is random or contains blocks of monomers, including the metabolic state of the bacteria and the

time of exposure to various carbon sources, both of which influence what precursor substrates are available to the cell for PHA production. PHA polymer composition varies widely both among bacterial species and within individual bacteria as it depends on the carbon source(s), the metabolic pathway utilized, and the enzymes used to supply monomers. Additional variety in PHAs stems from the ability of some PHA synthases to naturally incorporate 4-, 5-, and 6-hydroxyacids or unsaturated hydroxyacids into the polymer, in addition to 3-hydroxyacids (Lageveen et al. 1988; Saito et al. 1996; Madison and Huisman 1999).

Three metabolic pathways generate precursors for PHA synthesis (Fig. 1) (reviewed in Prieto et al. 2016). Typically, scl-PHAs such as PHB are synthesized through the glycolysis of sugars to produce acetyl-CoA (Fig. 1, Pathway I). Two acetyl-CoA molecules are then combined by β-ketothiolase (PhaA) creating acetoacetyl-CoA, which is then reduced into 3-hydroxybuturyl-CoA (3HB) by acetoacetyl-CoA reductase (PhaB). The 3HB monomers are then polymerized by

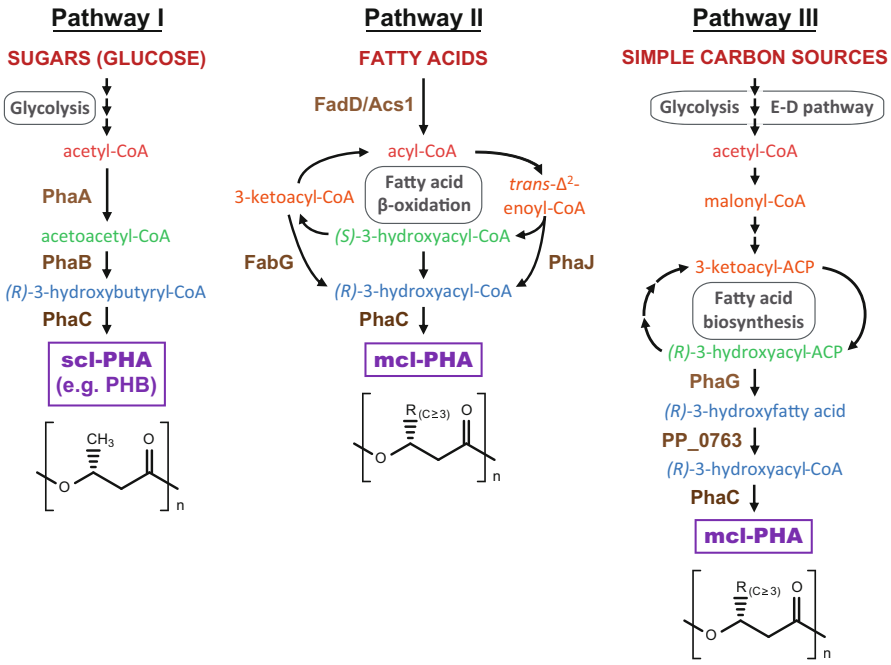

Fig. 1 Three metabolic pathways involved in the synthesis of PHA Three metabolic pathways used by bacteria to create PHA precursors. Pathway I is predominantly used in PHB-producing organisms, such as *C. necator*, while Pathways II and III are present in mcl-PHA producing pseudomonads. Enzymes are indicated in *bold* adjacent to arrows; processes involving many steps are indicated by rounded *rectangles*: glycolysis, the Entner–Doudoroff (E-D) pathway (used in pseudomonads), as well as the cycles for β-oxidation and biosynthesis of fatty acids. PHAs produced are indicated within rectangles with the chemical structure of the resulting PHA monomer given below. Note that Pathways II and III have the ability to produce mcl-PHAs from 3-hydroxyhexanoate and longer chain hydroxyacids resulting in polymers containing side chains (R-groups) with three or more carbons

PHA synthase (PhaC). This is the typical scl-PHA synthesis pathway utilized in bacteria such as *C. necator*.

In contrast, Pathways II and III are commonly used for mcl-PHA synthesis in *Pseudomonas* spp. Longer carbon chain monomers required for mcl-PHA synthesis are generated through Pathway II by the degradation of fatty acids by the β-oxidation cycle (Fig. 1, Pathway II). β-oxidation oxidizes fatty acids into *trans*-Δ^2-enoyl-CoA, *(S)*-3-hydroxyacyl-CoA, and 3-ketoacyl-CoA. These intermediates are then converted into *(R)*-3-hydroxyacyl-CoA (3HA-CoA) monomers, respectively, by a stereospecific enoyl-CoA hydratase (PhaJ), an epimerase, and a 3-ketoacyl-CoA reductase (FabG) (Tortajada et al. 2013). As with Pathway I, these mcl-3HA-CoA monomers are incorporated into PHA polymers by PhaC.

mcl-PHA monomers can also be synthesized by de novo fatty acid biosynthesis (Fig. 1, Pathway III). The de novo biosynthesis of fatty acids has special role in PHA biosynthesis as this pathway can provide various types of 3-hydroxyacyl (3HA) precursors from simple carbon sources such as glucose, fructose, gluconate, glycerol, ethanol, and acetate (Tortajada et al. 2013). These precursors are oxidized into acetyl-CoA, followed by their conversion by a series of reactions into malonyl-CoA and culminating in (*R*)-3HA-acyl carrier protein (ACP). This forms the first unit that is further elongated by successive two-carbon units. (*R*)-3HA-ACP intermediates were thought to be subsequently transformed into (*R*)-3HA-CoA thioesters by a specific CoA transferase (PhaG) found in most pseudomonads (Rehm et al. 1998). Recently, Wang et al. reported that PhaG functions as a (*R*)-3HA-ACP thioesterase that produces free (*R*)-3-hydroxyfatty acids rather than (*R*)-3HA-CoA (Wang et al. 2012). Evidence was presented showing that the PP_0763 gene product in *P. putida* functions as the medium-chain fatty acid-CoA ligase necessary to incorporate (*R*)-3-hydroxyfatty acids derived from fatty acid biosynthesis into the PHA polymer chain.

PHA production and catabolism are connected through a continuous cycle in which PHA synthesis and depolymerization are simultaneously active, resulting in constant turnover of PHA (Fig. 2a) (Ren et al. 2009a; de Eugenio et al. 2010a; Arias et al. 2013). PHA depolymerases (PhaZ) are continuously releasing 3-hydroxyfatty acids from PHA granules. These released 3-hydroxyfatty acids are then converted into 3HA-CoAs by the FadD/Acs1 acyl-CoA synthetase and can then be either catabolized via β-oxidation (Fig. 1, Pathway II) or directly reincorporated to the granule by PHA synthase.

2.1 PHA Synthases

PHA synthases are α-/β-hydrolase family enzymes that catalyze the polymerization of *(R)*-3HA-CoA thioester monomers. PHA synthases are grouped into four classes. Class I and class II synthases require a single subunit, PhaC, for activity, while classes III and IV require an additional subunit, PhaE or PhaR, respectively (Table 1). The substrate specificities vary among the classes: classes I, III, and IV synthases have high affinities for 3–5 carbon scl-3HA-CoA precursors to form scl-PHA, with class I and III synthases generally having additional low affinities

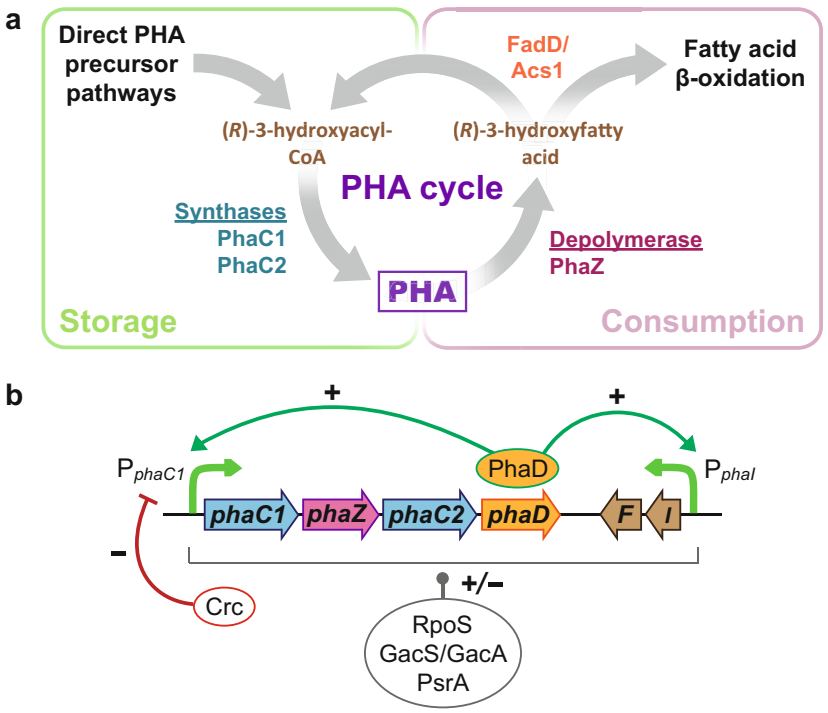

Fig. 2 The PHA cycle and regulation of *pha* gene expression in pseudomonads. (**a**) PHA production and utilization proceeds in a continuous cycle that is altered depending upon the energy and carbon needs of the cell. In response to an imbalance of specific nutrients, PHA is synthesized by the PHA synthases, PhaC1, and PhaC2 as a means to store carbon and reducing equivalents from precursors obtained from pathways II and III. Conversely, when consumed, PHA is depolymerized by the PHA depolymerase, PhaZ. The released 3-hydroxyfatty acids can then be recycled into 3-hydroxyacyl-CoAs by the acyl-CoA synthetase, FadD/Acs1, thus completing the PHA cycle, or can be used in the fatty acid β-oxidation pathway. (**b**) Schematic of the primary *pha* gene cluster in *P. putida* KT2440 and its regulatory elements. The two operons, *phaC1ZC2D* and *phaIF*, are under the partial control of the regulator, PhaD, which enhances transcription across the cluster. The global transcriptional regulator Crc has an inhibitory effect on *phaC1* transcription, and additional transcriptional regulators have been implicated to modulate *pha* gene transcription, yet their specific effects are to be determined

for ≥6 carbon mcl-3HA-CoA precursors, yet this varies by species. Notably, the class I synthases from *Aeromonas caviae* and *Rhodospirillum rubrum* or the class III synthase from *Thiocapsa pfennigii* are capable of producing mcl-PHAs containing 3-hydroxyhexanoate or 3-hydroxyoctanoate when provided with carbon sources of these lengths (Brandl et al. 1989; Fukui and Doi 1997; Liebergesell et al. 2000). However, the class II PHA synthases found in *Pseudomonas* species are clearly different than the other classes in regard to their substrate specificity as these synthases readily generate mcl-PHAs from mcl-3HA-CoA precursors (Huisman et al. 1989; Matsusaki et al. 1998).

Table 1 PHA synthase classes and representative PHA-producing species

PHA synthase class	Subunits	Representative species	Characteristics	Preferred substrate[a]	PHAs produced[b]		References[c]
					scl-PHA 3-5C	mcl-PHA ≥6C	
I	PhaC	*Cupriavidus necator*, *C. necator* H16		3HB-CoA, mcl-3HA-CoA (low affinity)	P(3HB), P(3HB-co-3HV)		(Antonio et al. 2000; Zhang et al. 2001; Reinecke and Steinbüchel 2009)
		Aeromonas caviae	Uncommon class I PHA synthase	scl- and shorter mcl-3HA-CoA	P(3HB)	P(3HB-co-3HHx) P(3HB-co-3HA)	(Doi et al. 1995; Fukui and Doi 1997)
		Rhodospirillum rubrum		scl- and shorter mcl-3HA-CoA	P(3HB), P(3HV)	P(3HHx)	(Brandl et al. 1989; Jin and Nikolau 2012)
II	PhaC	*Pseudomonas putida*, *P. aeruginosa*		mcl-3HA-CoA	P(3HB), P(3HV)	P(3HA)	(Huisman et al. 1989, 1992; Timm and Steinbüchel 1990)
		Pseudomonas sp. 61-3		3HB-CoA, mcl-3HA-CoA	P(3HB)	P(3HB-co-3HA)	(Matsusaki et al. 1998)
III	PhaC PhaE	*Allochromatium vinosum*		3HB-CoA, mcl-3-HA-CoA (low affinity)	P(3HB), P(3HB-co-3HV)		(Liebergesell and Steinbüchel 1992; Yuan et al. 2001)
		Thiocapsa pfennigii	Uncommon class III PHA synthase	scl- and shorter mcl-3HA-CoA	P(3HB), P(3HV), P(4HV)	low P(3HHx), P(3HO)	(Liebergesell et al. 2000; Gorenflo et al. 2001)
IV	PhaC PhaR	*Bacillus* spp.		3HB-CoA	P(3HB), P(3HB-co-3HV)		(McCool and Cannon 2001; Shamala et al. 2003; Singh et al. 2009)

[a]As determined by enzymatic analysis of purified PHA synthase
[b]Examples of PHAs produced, products vary depending on carbon source. P(3HB), poly(3-hydroxybutyrate); P(3HV), poly(3-hydroxyvalerate); P(4HV), poly(4-hydroxyvalerate); P(3HHx), poly(3-hydroxyhexanoate); P(3HO), poly(3-hydroxyoctanoate); P(3HA), poly(3-hydroxalkanoates) ≥ 6 carbons
[c]References are relevant examples and are not exhaustive

Pseudomonas species commonly have two genes encoding PhaC synthase, *phaC1*, and *phaC2*. When expressed separately in recombinant *E. coli*, the two enzymes had similar substrate specificities with decanoate or dodecanoate being the carbon sources that produced the highest yield of PHA when *phaC1* or p*haC2* were expressed, respectively (Langenbach et al. 1997; Qi et al. 1997). Likewise, the composition of the PHA produced by PhaC1 or PhaC2 were similar with the formation of 3-hydroxydecanoate being predominant (50–75 mol %) when cells were provided with decanoate or dodecanoate.

Much interest is centered on engineering the single subunit class I and class II PHA synthases to improve their activities, substrate specificities, product polymer characteristics, and stabilities (for review, see Nomura and Taguchi 2007). This and other bioengineering synthetic biology approaches are further discussed below in Sect. 3.

PHA synthase genes, along with the other genes involved in PHA metabolism, are often organized in operons, though their organization varies among species (reviewed in Taguchi et al. 2005). Several common patterns exist. One pattern has the *phaC* gene contained within the same locus as the *phaA* and *phaB* genes, as found in the *phaCAB* operon of *C. necator*. A second pattern is found in many pseudomonads wherein the two synthase genes, *phaC1* and *phaC2,* flank the *phaZ* depolymerase gene, forming the *phaC1ZC2* operon (Fig. 2b). Some pseudomonads, such as *Pseudomonas* sp. 61-3, contain a hybrid of these two patterns, with one locus containing the *phaC1ZC2* operon and another containing the *phaBAC* operon (Matsusaki et al. 1998).

2.2 The Carbonosome Is the Subcellular Structure for mcl-PHA Production

Bacteria producing PHA form intracellular, roughly spherical inclusions (carbonosomes) that contain densely packed hydrophobic PHA (Fig. 3) (Jendrossek and Pfeiffer 2014). The size, number, and cellular distribution of carbonosomes vary considerably among bacterial species. In pseudomonads, carbonosomes can account for nearly 90% of cell dry weight and fill the majority of the intracellular space (Jendrossek 2009; Galán et al. 2011). Carbonosomes are generally concentrated around the center of the longitudinal axis of the cell with a 500 nm median diameter and distributions ranging from one to approximately ten inclusions per cell with a mean of 5–6 (Beeby et al. 2012). Little is known regarding the precise steps leading to the development of carbonosomes, including whether fission or fusion of carbonosomes occurs. Several models have been proposed to describe the intracellular distribution of carbonosomes (Jendrossek and Pfeiffer 2014). Among these, the scaffold model is currently the best supported and posits that carbonosomes are arranged around a yet to be confirmed scaffold that determines their localization and mediates their segregation during cell division. The nucleoid is suspected to comprise this scaffold, as supported by both the observation that carbonosomes are concentrated around the cell center in most species as well as the ability of granule-associated proteins (phasins) to interact with DNA (see below) (Galán et al. 2011; Pfeiffer et al. 2011). The scaffold-mediated

arrangement of carbonosomes around the nucleoid could provide a motive underlying the observation that carbonosomes are faithfully segregated into both daughter cells during cell division, as occurs with replicated nucleoid.

2.2.1 PHA Granule Composition

The first determined chemical composition of isolated PHB carbonosomes was from *Bacillus megaterium*, which contained 97.7% PHB, 1.87% protein, and 0.46% lipids (Griebel et al. 1968). However, the exact fraction and composition of the lipid component remain elusive (Steinbuchel et al. 1995; Bresan et al. 2016). Electron and atomic force microscopy studies revealed that carbonosomes are composed of a thin, approximately 4 nm lattice-like surface layer that overlies a denser core of packed hydrophobic PHA (Fig. 3c) (Mayer and Hoppert 1997; Dennis et al. 2003, 2008). Carbonosomes appear to function to segregate nonpolar PHA molecules aggregated by the hydrophobic effect away from the aqueous environment of the cytoplasm. This oil-in-water model of carbonosomes posits that the surface coat functions as an amphipathic interface, yet its exact composition remains to be fully determined. A recent microscopic study to detect phospholipids on carbonosomes in *P. putida* and *C. necator* failed to show their presence in situ, casting doubt on whether carbonosomes contain both protein and phospholipids on their surface (Bresan et al. 2016).

Carbonosomes isolated from a variety of bacterial species were found to contain a number of surface-bound proteins when separated by electrophoresis (Fuller et al. 1992; Steinbuchel et al. 1995; Stuart et al. 1998) and/or detected by mass spectrometry (Jendrossek and Pfeiffer 2014). Further studies identified specific carbonosome

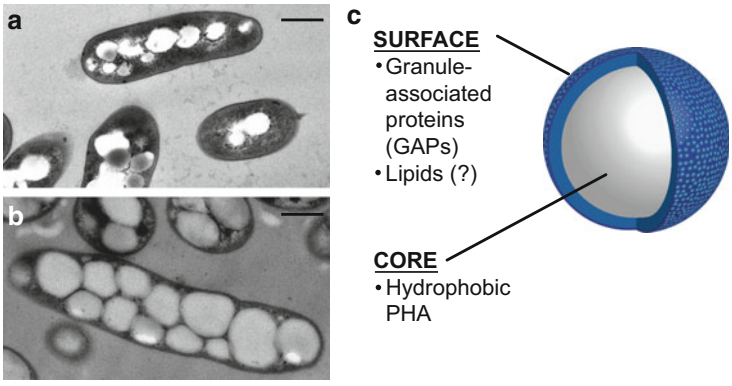

Fig. 3 Carbonosomes in bacteria producing PHA. Electron micrographs of Gram-negative bacteria producing PHA with white carbonosome spheroids visible in (**a**) *P. putida* KT2440 and (**b**) *C. necator* H16 (scale bar = 0.5 μm). (**c**) Depiction of the proposed carbonosome structure. In *Pseudomonas* spp., the granule-associated proteins (GAPs) include PHA synthase, PhaC1; PHA depolymerase, PhaZ; acyl-CoA synthetase, Acs1; and the phasins, PhaF and PhaI. The precise composition of the lipid component remains to be resolved

granule-associated proteins (GAPs). In *Pseudomonas* spp., these include PHA synthase, PhaC1; PHA depolymerase, PhaZ; acyl-CoA synthetase, Acs1; and phasins (see below) (Stuart et al. 1996; Ruth et al. 2008; Ren et al. 2009a, b). This compliment of enzymes belies the metabolic cycle of PHA synthesis for carbon and energy storage and depolymerization to release stored PHA resources. The presence of the acyl-CoA synthetase Acs1 on the surface of carbonosomes likely functions to CoA-activate 3-hydroxyfatty acids released by the action of the PHA depolymerase PhaZ thereby making them available for degradation by β-oxidation for use as the PHA-derived energy source (Fig. 2a) (Ruth et al. 2008). Additionally, a unique class of proteins, the phasins, is found on the surface of carbonosomes, which in pseudomonads are PhaF and PhaI (Prieto et al. 1999; Sandoval et al. 2007; Ren et al. 2009b). These small proteins are the predominant protein component of carbonosomes and comprise a unique class of intrinsically disordered proteins (Pötter et al. 2004; Neumann et al. 2008; Maestro et al. 2013). Phasins have been most thoroughly investigated in *P. putida*, with two identified phasins, and in *C. necator* H16, which has seven identified phasins (Kuchta et al. 2007; Pfeiffer and Jendrossek 2011, 2012).

A representative and well-studied phasin is PhaF from *P. putida*. Biophysical analyses of purified PhaF and its structural modeling revealed that it is likely a tetramer in solution with each monomer adopting a secondary structure of predominantly alpha-helices that are interspersed by regions of random coil (Maestro et al. 2013). Structural modeling predicted an extended amino-terminal alpha-helical region as well as a separate central region. The central region was predicted to contain amphipathic alpha-helices and leucine residues contained within heptad repeats with the potential to form a leucine zipper dimerization domain. The carboxy-terminal region contains AAKP repeats that are found in DNA-binding proteins, such as eukaryotic histone H1 and AlgP from *Pseudomonas aeruginosa* (Timm and Steinbüchel 1992). Further structure-function studies of PhaF revealed that the amino-terminal region forms a stable structure that is necessary for carbonosomes interaction, whereas the carboxyl-terminal region was capable of binding DNA in a sequence-independent manner (Moldes et al. 2004; Galán et al. 2011). The smaller phasin protein in *P. putida*, PhaI, is homologous to the amino-terminal region of PhaF but lacks the carboxy-terminal DNA-binding region (Prieto et al. 1999).

The function of phasins was revealed through genetic studies. The deletion of *P. putida phaF* resulted in a twofold reduction in PHA production, a decrease in the number and size of PHA granules, as well as their aberrant localization and mis-segregation during cell division (Prieto et al. 1999; Galán et al. 2011; Dinjaski and Prieto 2013). The phenotype of the Δ*phaF* mutant was mitigated to some extent by the presence of *phaI,* which likely has some redundant functions. The deletion of *phaI* alone had a similar reduction in PHA accumulation as seen with Δ*phaF*, yet the deletion of both *phaF* and *phaI* led to an eightfold reduction in PHA accumulation compared to wild type. This suggests that both phasins are required for proper PHA accumulation. These results of *P. putida* phasins are mirrored by studies of phasin-like proteins from other PHA-producing bacteria (Pfeiffer et al. 2011).

2.3 PHAs and Central Metabolism

2.3.1 Regulatory Network of the mcl-PHA Cycle

Due to the tight connection between PHA biosynthesis and cellular metabolism, its regulation is quite complex and dependent upon several factors, including the carbon source(s), the specificity of the PHA synthase, and the metabolic pathways involved. PHA accumulation is under systemic regulation that involves indirect PHA precursor routes that link the catabolism of carbon sources to PHA metabolism, fatty acid β-oxidation and de novo fatty acid synthesis, and specific PHA metabolism (the PHA cycle) (Fig. 2a). Furthermore, the regulation of PHA metabolism takes place at different levels, at the transcriptional and posttranscriptional levels by specific and global regulatory factors and through regulation of the carbon flow of different pathways that provide PHA precursors.

Transcriptional regulation is one means for regulating PHA synthesis. In *P. putida* and other pseudomonads, the *pha* cluster is organized into two main operons, *phaC1ZC2D* and *phaIF,* that are under the regulation of the TetR-like transcriptional activator, PhaD, in response to β-oxidation metabolites (Fig. 2b) (Klinke et al. 2000; de Eugenio et al. 2010b). Additionally, the global regulator Crc negatively regulates the expression of the *pha* genes under balanced C/N conditions (La Rosa et al. 2014). It was demonstrated that Crc activity is influenced not only by the carbon source but also by the C/N ratio of the culture medium. Other global transcriptional regulators, including RpoS, PsrA, and the GacS/GacA system, also affect PHA accumulation (Ruiz et al. 2004; de Eugenio et al. 2010b; Fonseca et al. 2014).

As mentioned above, active turnover in PHA accumulation and degradation is linked to central carbon metabolism. Intracellular ratios of acetyl-CoA/free CoA and $NADH/NAD^+$ orchestrate the carbon flux of 3HA-CoA with cellular demand by regulating the PHA cycle (Ruth et al. 2008; de Eugenio et al. 2010a; Zinn et al. 2011). Therefore, the control of carbon flux influences PHA metabolism by regulating the availability of PHA precursors.

2.3.2 Impact of the mcl-PHA Cycle on Central Carbon Metabolism

PHA allows cells to cope with carbon starvation by acting as a reservoir of carbon and energy. The direct link between PHA and carbon metabolism permits bacteria to use PHA as a carbon store that can be directed to central carbon metabolism under situations of carbon stress. PHA metabolism represents a bidirectional continuous process whereby PHA synthase and depolymerase are simultaneously active, ensuring PHA turnover (Fig. 2a) (de Eugenio et al. 2010a). The PHA cycle is thus linked to the metabolic network of the cell by adapting the carbon flow to cellular demand. Furthermore, PHA acts as a sink for reducing equivalents. PHA production is controlled by intracellular levels of NADH, NADPH, and acetyl-CoA and thus is under the regulation of the redox state of the cell (Schlegel and Gottschalk 1962; Schubert et al. 1988; Budde et al. 2010).

The PHA cycle has a strong impact on cell physiology as it balances global biomass (including PHA carbon/energy storage), cell division, and energy spillage (Escapa et al. 2012). It is important to stress that the PHA cycle also links carbon and

nitrogen metabolism. *P. putida* unable to accumulate PHA grown under a high C/N ratio produced the same PHA-free biomass as wild-type cells (de Eugenio et al. 2010a). However, the number of cells was tenfold more than the wild type. Thus, the number and size of cells were related to the ability to accumulate PHA. Furthermore, Martinez and coworkers showed that the cell size of a *P. putida pha* mutant was inversely related to the C/N ratio of the culture medium (Martinez et al. 2013).

Finally, in *Pseudomonas* spp. and *C. necator*, PHB has been proposed as a potential metabolic strategy to cope with cold environments, since the PHA cycle enhances the resistance of bacterial cells to low temperature and freezing conditions (Ayub et al. 2009; Obruca et al. 2016).

3 Bioengineering mcl-PHA Production

Much effort is being extended to optimize PHA production and recovery from bacteria. Many of the potential points for optimization are listed in Table 2, which focuses mainly on optimizations carried out in pseudomonads or optimizations that could be adapted from other microbiological systems for use in pseudomonads. The following subsections highlight in brief some of the strategies in use or conceived to rationally design mcl-PHA production for exploitation with an eye toward producing a defined and consistent product. The final subsection is an overview of research needs and synthetic biology tools that are available for bioengineering the mcl-PHA producer, *P. putida*.

3.1 Cultivation

Work is under way to understand the precise growth conditions needed to optimize mcl-PHA production while maximizing economy. Likely the most important consideration during cultivation is the carbon source provided. *Pseudomonas* spp. provided with solely glucose as the carbon source produce low amounts of mcl-PHAs and generally require fatty acids to generate longer-chain PHAs (Huijberts et al. 1992). Thus, various co-feeding strategies have been developed to produce mcl-PHAs of higher quality through the inclusion of two carbon sources in the media with one being a fatty acid (Sun et al. 2007; Kenny et al. 2012; Jiang et al. 2012). This allows for the employment of rational co-feeding to produce mcl-PHAs of defined composition. Moreover, there are many studies testing the feasibility of growth on a variety of inexpensive waste materials to produce mcl-PHAs. These growths generally yield varied results depending on the composition of substrates contained within the feedstock (Nikodinovic-Runic et al. 2013; Muhr et al. 2013). Furthermore, combining genetic manipulations of metabolic pathways along with cofeeding provides a promising strategy that was demonstrated in one study to increase PHA production by twofold (Escapa et al. 2013). It is likely that the future of pseudomonal mcl-PHA production will require similar genetic metabolic

Table 2 Points of technological optimization for mcl-PHA production

	Point or idea for optimization	Details	Example references
Cultivation	Media components/ carbon source	Many parameters, e.g., cofeeding strategies	(Sun et al. 2007; Kenny et al. 2012; Jiang et al. 2012)
Cellular metabolic engineering	Elimination of bystander metabolic pathways	Manipulation of a metabolic pathway	(Escapa et al. 2013)
	Carbon source utilization	Glucose as sole carbon source	(Poblete-Castro et al. 2013)
Improving PHA synthesis	PHA synthase mutagenesis	Altering substrate specificity, e.g., 2-hydroxyacids, lactyl-CoA; improving enzyme activity	(Taguchi and Doi 2004; Takase et al. 2004; Taguchi et al. 2008; Yang et al. 2010)
	Elimination of depolymerase activity	Mutations of *phaZ* depolymerase, recombinant production in non-PHA producing species	(de Eugenio et al. 2010a; Eggers and Steinbüchel 2014)
	Custom-tailored PHAs	Consistent composition, unsaturations, modifications	(Olivera et al. 2001; Liu et al. 2011; Escapa et al. 2011; Wang et al. 2011; Tortajada et al. 2013)
	Cell volume	Larger cells could accommodate more or larger carbonosomes	(Jiang et al. 2015; Kadoya et al. 2015)
	In vitro PHA synthesis	Cell-free system	(Qi et al. 2000; Tajima et al. 2016)
PHA recovery	Autolysis	Inducible or automatic expression of a lytic gene	(Hori et al. 2002; Martínez et al. 2011)
	Fragile cells	Cells engineered for easy rupture	N/A
	Lysis by predator	Use of *Bdellovibrio* sp.	(Martínez et al. 2013, 2016)

Gray shading indicates that optimization likely requires genetic engineering of the PHA-producing bacteria. *N/A* theoretical and lacks support by published results

manipulation and/or a combination of strategies to create high-quality product from inexpensive and readily available carbon sources.

3.2 Metabolic Engineering

PHA diversity – reflected in monomers, homopolymers, random copolymers, block copolymers, functional polymers, graft polymers, as well as the ranges of molecular weights and combinations of the above – is usually achieved by different bacterial

species having PHA synthases with different specificities, different substrate preferences, and possibly different metabolic pathways. It has become increasingly possible to generate PHA diversity in a single bacterial species as embodied by the PHAome concept (Chen and Hajnal 2015). For example, *P. putida* can be engineered to produce various types of PHA homopolymers, including various scl- and mcl-PHA random copolymers, mcl-PHA random copolymers containing adjustable monomer ratios, PHA block copolymers, as well as functionalized PHAs (Tortajada et al. 2013; Chen et al. 2015).

The emerging field of systems metabolic engineering combines systems biology and metabolic engineering approaches in order to identify metabolic pathway bottlenecks and provide solutions to optimize and reconstruct metabolic pathways. This requires the use of omics datasets from transcriptomic, proteomic, and metabolic studies for in silico genome-scale metabolic reconstruction. Once created in silico, the metabolic model allows one to study the cell as global network (Nogales et al. 2008; Puchałka et al. 2008; Peplinski et al. 2010). This strategy is contributing to the design of new strains and fermentation strategies aimed to increase PHA production, permitting the use of alternative carbon sources and an augmentation in PHA synthesis.

The in silico metabolic reconstruction of *P. putida* (*i*JN746) expanded the pool of potential substrates for use as carbon sources for the production of PHA precursors to include a large set of non-glycolytic substrates, such as aromatic compounds (Nogales et al. 2008). A further use of computational metabolic modeling was carried out by Poblete-Castro et al. who reengineered the metabolic network of *P. putida* toward more efficient PHA production when glucose was the carbon source (Poblete-Castro et al. 2013, 2014).

Thanks to the increasing number of omics datasets, combined with statistical tools, metabolic reconstruction is boosting our knowledge about cell metabolism and physiology. For example, Sohn and coworkers (Sohn et al. 2010) implemented in silico flux analysis in *P. putida* metabolic models (*i*JN746 and *i*JP815) and showed that both the de novo fatty acid biosynthesis and β-oxidation pathways contributed simultaneously to the synthesis of different monomers of PHA. The authors also reported that varying ratios of $NADH/NAD^+$ affected not only the accumulation of PHA but also the rate of substrate consumption. Thus, computational pathway prediction algorithms will provide a systematic framework to redesign metabolic pathways and to predict novel engineering strategies oriented to boost PHA yields. Although natural PHA producers such as *C. necator* or pseudomonads can accumulate high concentrations of PHA polymer (up to 60–90% of cell dry weight), the ability to engineer non-PHA-producing bacteria to produce PHA could be an advantage over natural producers due to their lack of PHA depolymerases and absence of tight regulation over PHA synthesis (Jambunathan and Zhang 2016). In this sense, much effort has been carried out to engineer *E. coli* to synthesize PHB by transferring the PHB biosynthetic pathway from other organisms such as *C. necator* (Slater et al. 1988). In these transgenic strains, PHB synthesis depends on the amount of free acetyl-CoA and does not require any nutrient limitations. A broad range of studies has been carried out directed toward increasing the availability of PHB

precursors such as acetyl-CoA and/or NADPH in recombinant *E. coli* (Wang et al. 2013).

Pioneering studies in strain engineering for PHA production were reports that *E. coli* strains blocked in the 3-ketoacyl thiolase (FadA) or 3-HA-CoA dehydratase (FadB) enzyme activity of the fatty acid β-oxidation pathway were able to accumulate mcl-PHA with solely the *phaC1* or *phaC2* synthase gene from *P. putida* GPo1 heterologously expressed (Klinke et al. 1999; Ren et al. 2000). Since then, the production of various copolymers of scl-PHA and mcl-PHA has been achieved in recombinant *E. coli* (Park and Lee 2004; Phithakrotchanakoon et al. 2013; Tappel et al. 2014). Additional examples include the production of poly(3HB-co-3HA) copolymers containing mcl-3HA units in *E. coli* with glucose as the carbon source (Tappel et al. 2014), or by coexpression of PhaG and the PP_0763 gene product coding for a fatty acid-CoA ligase (Wang et al. 2012), or expressing engineered PHA synthase from *Pseudomonas* sp. 61-3 (Taguchi and Doi 2004). These are examples of metabolic engineering strategies in non-PHA-producing bacteria to control PHA precursor production by redirecting central metabolism toward PHA accumulation.

3.3 Improving PHA Synthesis

An idea for improving PHA synthesis is the direct mutagenic engineering of PHA synthases. Targeted mutation of active site residues was carried out in order to alter substrate specificity sufficiently to produce new-to-nature product chemistries, an example of which is the incorporation of 2-hydroxyacids into the PHA polymer. Additionally, active site residues of a pseudomonal PhaC1 were mutated to permit the use of lactyl-CoA as a substrate to create poly(lactate-co-3HB) copolymer (Taguchi et al. 2008; Yang et al. 2010). Further mutagenic strategies of pseudomonal PhaC enzymes have resulted in increased production of PHA when compared to the wild-type enzyme, specifically as a means to produce poly(3HB-co-3HA) (Takase et al. 2004).

A further technological optimization is the development of consistent homopolymeric or functionalized PHAs containing unsaturations, modifications, and novel chemistries. Pseudomonads provided with fatty acids produce PHA precursors via the β-oxidation pathway (Fig. 1, Pathway II). Because β-oxidation shortens the fatty acid chain by two carbon atoms with each cycle, this results in PHAs containing an ensemble of precursors shorter than the provided fatty acid by multiples of two carbons. A solution to this problem was the creation of strains mutated in the β-oxidation pathway that resulted in the synthesis of PHAs with the same chain length as the provided fatty acid (Olivera et al. 2001; Liu et al. 2011; Escapa et al. 2011; Wang et al. 2011). This also allowed for the synthesis of block PHA copolymers of defined chain lengths by including specific fatty acids in the culture. An extension of this approach is the ability to make customized PHAs that contain any number of functional groups including double and triple bonds and/or phenyl, carbonyl, epoxy, etc. modifications. These specialized PHAs have unique and

novel physical properties making them tailorable to specific needs, including medical applications, packaging, and adhesives. The problem is to find a way to synthesize these functionalized PHAs in a consistent manner as current methods for providing precursors for PHA functionalization to cells result in the formation of diverse polymers with generally low yields (Tortajada et al. 2013).

Addressing the problem of augmenting PHA synthesis from another angle would be increasing the total cellular volume, thus providing increased space for PHA carbonosomes. For example, an increase in PHB content was demonstrated in recombinant *E. coli* by deleting genes involved in peptidoglycan synthesis or an actin-like protein, which resulted in larger cells (Jiang et al. 2015; Kadoya et al. 2015). Another solution would be to eliminate the need to use cells as factories through the development of an in vitro synthesis system for mcl-PHA production. Currently, the metabolic pathways required for the production of PHA precursors from carbon source substrates are far too complex to carry out extensive in vitro metabolism using existing technology, yet some progress has been made. A chemoenzymatic system using purified recombinant enzymes was shown to be capable of synthesizing PHB and poly(lactate-co-3HB) copolymer from 3-hydroxybutyrate and lactic acid substrates (Tajima et al. 2016).

3.4 PHA Recovery

An obstacle to scaling up PHA production is finding methods to simplify the recovery of PHA from cells. Current methods generally use organic solvents and/or physical disruption strategies to extract PHA. The use of solvents is especially damaging as many solvents can hydrolyze PHA, decreasing both the yield and consistency of the product. Thus streamlining PHA recovery through improved techniques is a priority. Several innovative ideas exist to extract PHA from cell factories. One strategy is cell autolysis, wherein a lytic enzyme is inducibly or automatically expressed to lyse the cells and simplify PHA harvesting (Hori et al. 2002; Martínez et al. 2011). Another lytic strategy is the use of a predatory bacterium that can be added to a cell culture and cause lysis, allowing for PHA to be more easily recovered from the media (Martínez et al. 2016). A final theoretical possibility is to produce PHA in cells modified to be fragile and rupture as the carbonosomes expand in size during PHA accumulation. Cells could be modified to have inherent structural fragility in the cell envelope through chemical exposure or mutations.

4 Research Needs and Synthetic Biology Tools

P. putida can produce a large variety of mcl-PHAs under highly controlled laboratory conditions. However, manipulating *P. putida* to create mcl-PHAs of consistently high yield and quality and to be able to harvest the product in a sustainable manner remains elusive. Maximizing cell growth as well as PHA yield in PHA-producing

media conditions requires the channeling of metabolic resources into PHA production while not sacrificing the resources required to increase biomass. Solving this puzzle remains a key obstacle for scaling up microbial PHA yields. Promise comes via the use of strain designs that optimize entire metabolic pathways that are derived from computational metabolic models based on omics data. However, constructing these strains requires the targeted editing of a number of genes to create multiple mutations. Fortunately, much effort is being directed toward developing *P. putida* into a general chassis suitable for a wide variety of synthetic biology applications due to its metabolic flexibility, tolerance to various toxins, and it being generally regarded as safe (GRAS certified) (Timmis 2002; Nikel et al. 2014). The recent development of a genome-minimalized strain of *P. putida* KT2440 is progress toward streamlining the use of this bacteria to produce PHA on a large scale (Martínez-García et al. 2014b). This strain lacks 4.3% of its genome and displays superior growth characteristics and improved expression of heterologous genes. An extension on this theme is the work currently being carried out by the EmPowerPutida international consortium of researchers toward the development of a *P. putida* chassis for production of value-added products, which could be used as a new platform for PHA production (http://www.empowerputida.eu/).

The choice of using *P. putida* as a PHA production chassis is made easier by its ability to maintain plasmids containing common broad-host-range multicopy replicons, for example, pBBR1, pRO1600/ColE1, and RK2. However, due to the high level of natural tolerance to antibiotics found in pseudomonads, care must be taken in selecting the bacterial resistance markers present on these vectors (Martínez-García and de Lorenzo 2011). Numerous chemical inducer-mediated expression systems function in *P. putida*, including those that rely upon a native activator such as P*m*/XylS induced by 3-methylbenzoate or a heterologous activator, e.g., P_{lac}/LacI with IPTG (Loeschcke and Thies 2015). More recently, a set of genome-integrated promoters was developed that allow the fine tuning of the level of constitutive expression of a gene (Zobel et al. 2015). Taken together, these tools allow for the easy manipulation of expression of genes and for rapid screening of potential genetic constructs involved in PHA production.

In order to carry out large-scale genomic engineering to optimize cellular metabolism and/or PHA synthesis, the ability to rapidly create multiple genomic changes needs to be developed in *P. putida*. This bacterium has a low rate of homologous recombination, thus the use of genome-integrating, mini-transposon-containing vectors is heavily relied upon in order to make insertions. Both Tn*5*- or Tn*10*-based random knock-in genome editing (de Lorenzo and Timmis 1994; Martínez-García et al. 2014a) as well as site-directed Tn*7*-mediated insertions are possible (Lambertsen et al. 2004; Choi and Schweizer 2006; de las Heras et al. 2008). Owing to the creativity of the scientists in this field, several other genome engineering technologies are being developed that could be scalable to carry out medium- to high-throughput editing. These include homing nuclease-based knockout technology (Martínez-García and de Lorenzo 2011), λ/Red-based deletions (Luo et al. 2016), deletions via custom mini-Tn*5* transposons combined with the Flp-*FRT* recombination system (Leprince et al. 2012), as well as significant steps toward

oligonucleotide-based recombineering (Aparicio et al. 2016). Moreover, two systems using counterselectable markers function in pseudomonads for creating markerless genome edits (Schweizer 1992; Wang et al. 2015). Finally, gene expression knockdowns through the use of CRISPRi to engineer PHA composition in recombinant *E. coli* have been described and are expected to function similarly in *P. putida*, yet the deployment of CRISPRi in *P. putida* remains to be reported (Lv et al. 2015).

Despite the numerous techniques mentioned above that have been established to carry out genome editing in *P. putida*, a pressing need remains for the development of high-throughput genome engineering strategies to carry out large-scale manipulation of metabolic pathways for tuning them toward PHA production. One possibility is the adaptation of multiplex automated genomic engineering (MAGE) technology for use in *P. putida*, which has been demonstrated in *E. coli* to simultaneously mutate dozens of loci (Carr et al. 2012). A recent development in MAGE involves the transient suppression of the highly conserved DNA mismatch repair system, which makes MAGE possible in many bacterial species (Nyerges et al. 2016). Other methods for rationally designing metabolic pathways include adaptive laboratory evolution to directionally evolve a strain toward a desired phenotype, for example, to enhance growth on a particular carbon source (Ibarra et al. 2002). Additional mutagenic approaches exist to reprogram gene expression dynamics either at the transcriptional level with global transcription machinery engineering (gTME) or through altering global translation using ribosome engineering (Hosokawa et al. 2002; Alper and Stephanopoulos 2007). These technologies could be used to implement PHA yield-biased metabolic pathway optimizations based on computational models, as well as the ability to simultaneously create a panel of orthogonal engineered strains for head-to-head comparisons of PHA production.

Related to the need to metabolically tune bacteria in order to improve mcl-PHA production is the desire to ascertain the most inexpensive and readily available carbon source that allows for the production of the desired PHA. It is a tantalizing prospect to be able to produce mcl-PHAs through bioremediation by growing on waste or hazardous materials. This objective requires metabolic engineering techniques in order to adapt bacteria to a particular waste carbon source. Interesting progress has been made toward the upcycling of petrochemical-derived plastic waste for use as a feedstock for PHA production. Examples include the conversion of pyrolyzed polystyrene into mcl-PHA by *P. putida* CA-3, the production of predominantly poly(3-hydroxydecanoate) and poly(3-hydroxydodecanoate) from several *Pseudomonas* isolates fed with terephthalic acid from waste polyethylene terephthalate (PET) plastic, and the production of mcl-PHAs from pyrolyzed polyethylene (PE) waste (Ward et al. 2006; Kenny et al. 2008; Guzik et al. 2014). However, advances in these plastic waste-utilizing *Pseudomonas* strains need to be made in order to develop strains fully capable of upcycling plastic waste – a goal currently under development by the European research consortium, P4SB: From Plastic Waste to Plastic Value Using *P. putida* Synthetic Biology (http://www.p4sb.eu/).

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