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# Polyhydroxyalkanoate Production Enzymes: a Survey and Biological Perspective

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*Polyhydroxyalkanoates (PHA) have attracted interest as bio-based, biodegradable biocompatible polymers. Researchers have accumulated a wide breadth of knowledge on the biosynthetic processes of these valuable polymers, with experts in the disciplines of microbiology, biochemistry, physics, medicine, and engineering contributing to the current state of knowledge of synthesis, production and application of PHA. An important perspective of PHA biosynthesis is the subset of microbial enzymes that are involved in producing this polymer and their many permutations. In this review, we survey the key enzymes involved in synthesis and homeostasis of all types of PHA. Emphasis is made on structure and function of enzymes and how enzymology has shaped knowledge of the PHA production pathway and ushered in new discoveries.*

*Keywords: bioplastics, polyhydroxyalkanoate, enzymes, granule associated proteins, polymer biosynthesis, PHA homeostasis.*

## Introduction

As natural products, polyhydroxyalkanoates (PHAs) have been intensively studied for over 50 years (Lemoigne, 1927; Merrick et al., 1965; Peoples, Sinskey, 1989a; Anderson et al., 1990; Valentin, Steinbuchel, 1994). PHAs, as bioplastics with favorable thermal and mechanical properties (Sudesh et al., 2000), have long been thought of as replacements for petroleum-based polymers. Currently, PHAs are under intense investigation

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as biocompatible and biodegradable materials for medical uses, such as resorbable sutures, tissue culture scaffolds, implants, and drug delivery devices (Shishatskaya et al., 2002; Shishatskaya, Volova, 2004; Shishatskaya et al., 2008; Shishatskaya et al., 2009). These biopolymers have also been investigated for household, agricultural, and industrial uses (Philip et al., 2007; Chen, 2009). Researching the production of these polymers, various inexpensive feedstocks, such as agricultural and food processing wastes, have been shown to be attractive as carbon sources. Also, there is a wealth of literature based on the use of gaseous feedstocks containing carbon dioxide ($CO^2$) as the main carbon source for polymer production (Volova et al., 1987; Volova et al., 1988a; Volova et al., 1988b; Ishizaki et al., 2001; Volova et al., 2001; Cramm, 2009).

PHAs are divided into three main types, based on monomer composition. Polymers containing monomers with a four- or five-carbon backbone are called short chain length PHAs (scl-PHA), and polymers with six- to fourteen-carbon backbone monomers are known as medium chain length (mcl-PHA). Polymers produced with both scl- and mcl-monomers are known as mixed chain length PHAs. Production of over 150 varieties of PHA has been demonstrated to date (Valentin, Steinbuchel, 1994), however only a select few exhibit any usefulness. The organism that has long been considered the paradigm of PHA biosynthesis, *Ralstonia eutropha*, natively produces scl-PHA. The *Pseudomonas* species, also well studied in terms of their polymer biosynthesis, typically produces mcl-PHA (Huisman et al., 1989; Timm, Steinbuchel, 1990; Huijberts et al., 1992). Some bacteria, such as *Aeromonas caviae*, produce mixed chain length PHA (Lee et al., 2000; Fukui, Doi, 1997).

Table 1 lists the main enzymes involved in scl- or mcl-PHA homeostasis. In this review, we discuss the critical enzymological discoveries that have been made over the decades involving each of these enzymes. As a result of the works discussed here, metabolic engineering and directed evolution of the PHA production pathway has become both feasible and adaptable.

Table 1. Survey of key enzymes involved in scl- and mcl-PHA homeostasis and their biochemical characterization.

| Enzyme | Known *in vitro* assay | Assay reference |
|---|---|---|
| PhaA – β-ketothiolase | Y | (Peoples, Sinskey, 1989b) |
| PhaB – Acetoacetyl-CoA reductase | Y | (Peoples, Sinskey, 1989b; Budde et al., 2010) |
| PhaJ - (*R*)-specific enoyl-CoA hydratase | Y | (Tsuge et al., 2003) |
| PhaG - (*R*)-3-hydroxydecanoyl-ACP:CoA transacylase | N | None |
| PhaC – PHA synthase | Y | (Yuan et al., 2001) |
| iPhaZ – Intracellular PHA depolymerase | Y* | (Gebauer Jendrossek, 2006; Uchino, Saito, 2006; Uchino et al., 2007) |
| ePhaZ – Extracellular PHA depolymerase | Y | (Gebauer, Jendrossek, 2006) |

*See text. Assay methods for PHB thiolysis and hydrolysis have been developed. However, a method has not yet been established for separating out individual iPhaZ enzyme activities and determining whether hydrolysis or thiolysis or both occur *in vivo*.

**Polyhydroxyalkanoate biosynthesis**

We know the value of PHA in several applications, as stated above, but why do cells natively synthesize the polymer? Typically, PHA is biosynthesized by microorganisms in response to nutrient (non-carbon) limitation. The polymer represents intracellular storage of carbon and reducing potential in many organisms (Anderson, Dawes, 1990; Brigham et al., 2011). Bacteria store PHA in intracellular inclusion bodies known as granules (Fig. 1), with the best-studied example being that of *R. eutropha*. Recently, it has been demonstrated that polyhydroxybutyrate (PHB) synthesis in *R. eutropha* is dependent on the stringent response (Brigham et al., manuscript submitted), further emphasizing the role of PHA in stress response. Also it has been demonstrated that *R. eutropha* cells containing intracellular stores of PHB are capable of withstanding other stress conditions, such as temperature and pH challenges (N. Zhila, T.G. Volova, personal communication).

It has been demonstrated that many different bacteria, as well as some archaea, can synthesize PHA. The most well-known examples are mentioned above, but many other species have been shown to synthesize intracellular polymer, while examination genome sequences of many other organisms suggests PHA biosynthesis capabilities. Some, in the case of *Legionella pneumophila,* potentially use PHB as a mechanism of persistence in nutrient-poor environments prior to infection of a host organism (Mauchline et al., 1992). This rationale could be suggested in the case of *Vibrio cholerae*,

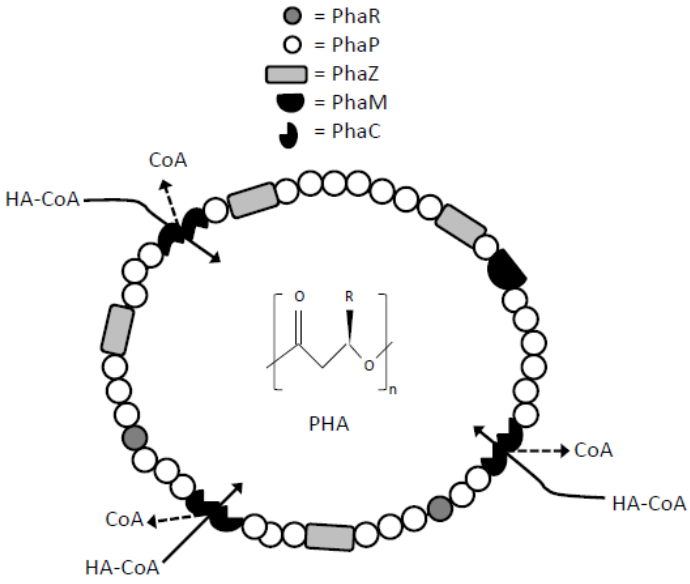


Fig. 1. Schematic drawing of an intracellular polyhydroxyalkanoate (PHA) granule. The granule, an intracellular inclusion that is sometimes considered an organelle, is surrounded by proteins and enzymes with various roles in PHA homeostasis. The phasin (PhaP) protein segregates the hydrophobic PHA from the aqueous cytoplasm (Potter et al., 2004). The regulator (PhaR) binds to PHA and/or *phaP1* promoter DNA and regulates phasin expression. The PhaM protein is thought to anchor the PHA granule non-specifically to the nucleoid (Pfeiffer et al., 2011). The depolymerases (PhaZ) mobilize PHA during nutrient excess and carbon starvation (Jendrossek, Handrick, 2002; York et al., 2003). The PHA synthases (PhaC) polymerize PHA, using hydroxyacyl-CoA (HA-CoA) molecules as monomers, resulting in the addition of an HA monomer to the nascent PHA chain and the release of a CoA moiety (Yuan et al., 2001; Cho et al., 2012)

the genome of which contains genes for PHA biosynthetic machinery. Regardless of what genus or species of microorganism synthesizes the polymer, the function of PHA in the cell is as a response to environmental stresses. This and the potential commercial value of different types of PHA make the polymer an interesting topic for study.

**Monomer supply**

Monomers for PHA biosynthesis are typically produced in one of three ways: 1) they are synthesized *de novo* from compounds like acetyl-CoA and propionyl-CoA, 2) the CoA thioesters present as intracellular intermediates of fatty acid degradation (β-oxidation) are converted to *(R)*-3-hydroxyacyl-CoA compounds for use as PHA substrates (Tsuge et al., 2000), or 3) hydroxyacyl-ACP (acyl carrier protein) conjugates are converted to *(R)*-3-hydroxyacyl-CoA molecules for use as PHA polymerization substrates (Hoffmann et al., 2000). Figure 2 shows an example PHA production pathway combining all monomer supply pathways discussed here. Method (1) above is performed by microorganisms that produce mainly scl-PHA, such as *R. eutropha*. Methods (2) and (3) are performed by organisms that produce mcl-PHA, like *Pseudomonas* species (Lageveen et al., 1988; Hoffmann et al., 2000; Chung et al., 2009).

For synthesis of short chain PHA precursors, two molecules of acetyl-CoA, or one molecule of acetyl-CoA and one molecule of propionyl-CoA, are ligated together by a β-ketothiolase enzyme

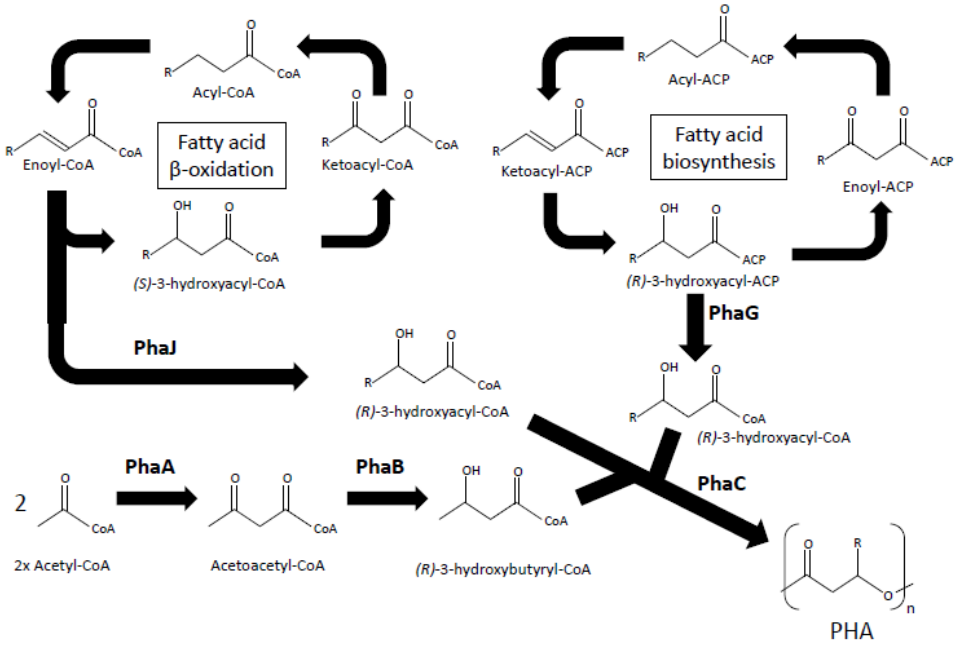


Fig. 2. Pathway diagram of PHA biosynthesis including the three routes of monomer supply discussed in this review. Medium chain length PHA (mcl-PHA) can be produced with monomers supplied from fatty acid β-oxidation (via the PhaJ enzyme (Fukui et al., 1998)) or fatty acid biosynthesis (via the PhaG enzyme (Rehm et al., 1998)). Traditional PHB production, as demonstrated in wild type *Ralstonia eutropha*, is supplied with monomers that are produced by the PhaA and PhaB enzymes, where two molecules of acetyl-CoA are ligated together (PhaA) to form acetoacetyl-CoA and then reduced (PhaB) to produce the 3-hydroxybutyryl-CoA precursor (Peoples, Sinskey, 1989b)

to produce 3-hydroxybutyryl-CoA (3HB-CoA) or 3-hydroxyvaleryl-CoA (3HV-CoA), respectively. The acetyl-CoA typically comes from carbon flow through central metabolism from the Entner-Doudoroff pathway. Propionyl-CoA results from the breakdown of odd carbon chain length fatty acids during β-oxidation; typically propionate is added to the bacterial culture to foster the production of 3-hydroxyvalerate (3HV) monomers in PHA. Other short chain length precursors of PHA include lactyl-CoA (LA-CoA) and 3-hydroxypropionyl-CoA (3HP-CoA). The LA-CoA precursors are typically produced by the ligation of a CoA moiety onto lactate from acetyl-CoA, using a CoA transferase enzyme (Matsumoto, Taguchi, 2010). Synthesis of lactate-containing polymers has not been demonstrated by wild-type organisms. 3HP-CoA can be produced in engineered organisms from glycerol (Andreessen et al., 2010; Andreessen, Steinbuchel, 2010), 3-hydroxypropionaldehyde, malonyl-CoA, or degradation intermediates of 1,7-heptanediol or 1,5-pentanediol (Andreessen et al., 2010), and from wild-type organisms by direct feeding of 3-hydroxypropionate to the growth media (Hiramatsu, Doi, 1993).

For synthesis of long chain monomers, intermediates of fatty acid degradation or fatty acid biosynthesis can be exploited. Intermediates from fatty acid β-oxidation, specifically *trans*-2-enoyl-CoA compounds, are substrates of an *(R)*-specific enoyl-CoA hydratase enzyme (PhaJ), which will convert the substrate into a PHA precursor (Tsuge et al., 2000). The exact length of the 3-hydroxyacyl-CoA (3HA-CoA) precursor produced depends on the length of the starting enoyl-CoA compound (Li et al., 2011; Wang et al., 2011). PHA precursor molecules can also be synthesized via fatty acid biosynthesis. It was demonstrated that *Pseudomonas aeruginosa* and *Pseudomonas putida* strains could produce mcl-PHA when grown on non-related carbon sources, i.e. gluconate. An *(R)*-3-hydroxydecanoyl-ACP:CoA transacylase, PhaG, was shown to convert a fatty acid biosynthesis precursor, specifically 3-hydroxyacyl-ACP, into *(R)*-3-hydroxyacyl-CoA molecules for PHA biosynthesis (Hoffmann et al., 2000).

**PhaA and BktB,**
**β-ketothiolase enzymes**

*Reaction: 2 × acetyl-CoA → acetoacetyl-CoA (PhaA and BktB), or acetyl-CoA + propionyl-CoA → β-ketovaleryl-CoA*

The β-ketothiolase enzymes produce monomers for scl-PHA. These enzymes perform "bio-Claisen" condensations on two CoA thioester molecules, thus creating a carbon-carbon bond (Masamune et al., 1989a; Masamune et al., 1989b). Masamune, Sinskey, and coworkers presented the first characterized β-ketothiolase from *Zoogloea ramigera* (Davis et al., 1987a; Davis et al., 1987b; Peoples et al., 1987). Through further examination using a point mutant β-ketothiolase enzyme, it was determined that a cysteine residue at position 89 (C89) was a critical active site nucleophile (Thompson et al., 1989). A gene encoding a similar enzyme, termed *phbA* (now known as *phaA*), was characterized in *R. eutropha*, using the *Z. ramigera* sequence as a seed. In the *R. eutropha* primary protein sequence, a highly conserved cysteine residue analogous to C89 was found, as well as a conserved cysteine residue towards the *C*-terminal end of the protein (Peoples, Sinskey, 1989b). This residue, C378, has been determined to be an active site base involved in deprotonation during the condensation reaction (Masamune et al., 1989a; Masamune et al., 1989b). The importance of C378 was further confirmed by site-directed mutagenesis in *Z. ramigera* (Palmer et al., 1991).

Slater and coworkers (1998) established the presence of multiple β-ketothiolase paralogs in *R. eutropha*. PhaA was found to catalyze only

the production of 3HB-CoA. Another enzyme, BktB, catalyzes the production of 3HB-CoA or 3HV-CoA. The examination of thiolytic cleavage activities (the reverse reaction) of PhaA and BktB show that PhaA is specific to production (or cleavage, in vitro) of C4 PHA precursors like 3HB-CoA, whereas BktB has broader specificity and can produce PHA precursors of C4-C6 chain length (Slater et al., 1998). Recently, the presence of 13 other isozymes of PhaA has been examined in *R. eutropha*.

Expression of only 5 of the 15 total isozymes was detected under PHB production conditions. Surprisingly, deletion of 8 of the 15 genes encoding PhaA isozymes (including *phaA* and *bktB*) was required to significantly decrease the intracellular PHB content in *R. eutropha*, suggesting that the analogous activities of other enzymes could at least partially substitute for the lack of PhaA activity in PHB production (Lindenkamp et al., 2010).

**PhaB, reductase enzymes**

*Reaction: acetoacetyl-CoA + NADPH → 3-hydroxybutyryl-CoA + $NADP^+$*

The enzyme acetoacetyl-CoA reductase is typically termed PhaB, in most organisms. Acetoacetyl-CoA reductase activity was measured in bacterial cell extracts as early as 1969 (Bloomfield et al., 1969). This enzyme was first purified from extracts of *Azotobacter biejerinckii*. Reductase activity of this purified enzyme preferentially used NADPH as the cofactor, and reduction proceeded optimally at a pH of ~5 (Ritchie et al., 1971). NADPH-linked acetoacetyl-CoA reductase activity was also shown in *Zoogloea ramigera* (Saito et al., 1977). The PhaB1 gene product from *R. eutropha* H16 also exhibited NADPH-dependent reductase activity (Peoples, Sinskey, 1989b). The genes *phaA* and *phaB* from the methylotrophic bacterium *Paracoccus denitrificans* were analyzed in recombinant *E. coli*. It was shown that NADH was preferred over NADPH for recombinant *P. denitrificans* PhaB activity (Yabutani et al., 1995). A PhaB homolog was characterized in the haloarchaeon *Haloarcula hispanica* (Han et al., 2009), suggesting the presence of PHA production pathways in another domain of life besides bacteria.

In *R. eutropha*, additional *phaB* genes and their respective products have been characterized. Deletion of *phaB1* (present in the *phaCAB* operon in *R. eutropha*) and *phaB3* resulted in a decreased ability to produce intracellular PHB. Deletion of a gene termed *phaB2*, however, did not result in any observable phenotype. In addition, the *phaB3* gene was found to be greatly downregulated when cells were grown on palm oil as the sole carbon source (Budde et al., 2010). This observation was confirmed in microarray studies comparing *R. eutropha* growth on fructose to growth on triacylglycerols (Brigham et al., 2010). In *R. eutropha* cells, the larger portion of acetoacetyl-CoA reductase activity is from PhaB1. Deletion of the *phaB2* gene resulted in no significant decrease in reductase activity, suggesting that PhaB2 may not be a functional acetoacetyl-CoA reductase under conditions studied. Cell extracts of all *R. eutropha* strains tested revealed that NADPH was the preferred cofactor, with no change in NADH-mediated reductase activity in any mutant strain (Budde et al., 2010).

**PhaJ, *(R)*-specific enoyl coenzyme-A hydratase**

*Reaction: Ketoacyl-CoA → **(R)**-3-hydroxyacyl-CoA*

Twenty years ago, Eggink and coauthors (1992) suggested that PHA biosynthesis in *Pseudomonas* species utilized intermediates from fatty acid biosynthesis and degradation pathways. This hypothesis was poignant because of the observation that *Pseudomonads* are able

to produce mcl-PHA regardless of carbon source used (Huisman et al., 1989; Timm, Steinbuchel, 1990; Huijberts et al., 1992). Other discoveries demonstrated that the Gram-negative soil bacterium *Aeromonas caviae* will only produce intracellular PHA when grown in the presence of triacylglycerols, fatty acids and similar compounds. *A. caviae* will not produce PHA when grown on sugars as the sole carbon source (Doi et al., 1995). The observed biosynthetic metabolism of PHA of these bacteria suggests that 3HA-CoA precursors are not constructed with acetyl-CoA as a starting material. In the case of *A. caviae*, a region in the chromosome was identified that contained all genes necessary for PHA production. Insertion of these genes in a PHB- host of *R. eutropha* conferred the ability to produce P(HB-*co*-HHx) when cells were grown on hexanoic or octanoic acids. Furthermore, it was determined that the product of one of the genes in this *A. caviae* gene cluster exhibited *(R)*-specific enoyl-CoA hydratase activity. This type of enzyme activity allows cells to utilize fatty acid β-oxidation intermediates (specifically enoyl-CoA compounds) to produce monomer precursors for PHA biosynthesis (Fukui, Doi, 1997).

Since these initial, seminal discoveries regarding the mechanism of a PHA synthetic pathway alternative to that in *R. eutropha*, genes and enzymes from *Pseudomonas* sp., *A. caviae,* and other species have been studied and utilized heterologously for their ability to incorporate mcl-3HA precursors into PHA, in order to produce polymers with interesting thermal and mechanical properties. The *phaJ* gene from *A. caviae* (*phaJAc*), encoding the *(R)*-specific enoyl-CoA hydratase was expressed in *E. coli* and characterized. Stereospecificity of the PhaJ*Ac* enzyme was demonstrated by coupled assays involving the hydration of crotonyl-CoA. In the presence of *(S)*- 3HA-CoA hydratase, minimal activity from the coupled enzyme was observed. With PhaJ activity coupled to crude extracts of *R. eutropha* in a 3HB-CoA polymerase assay, activity from the coupled enzyme system was observed, suggesting an *(R)*-stereospecificity of substrates for PhaJ (Fukui et al., 1998). In 2003, the crystal structure of PhaJ*Ac* was determined. The location of the catalytic dyad of the active site, Asp31 and His36, was determined to be deep in the substrate binding pocket. Also, residues like Leu65 and Val130 were located deep in the substrate binding pocket, suggesting preference of PhaJ for certain substrate chain lengths (Hisano et al., 2003).

Two PhaJ enzymes from *P. aeruginosa* were characterized for their substrate specificity. One, PhaJ1, showed substrate specificity for hydroxyoctanoyl-CoA, while the other, PhaJ4, demonstrated greater specificity for hydroxydecanoyl-CoA. This study also showed that both PhaJ1 and PhaJ4 from *P. aeruginosa* were active towards the production of shorter chain length 3HA-CoA molecules, like 3HB-CoA (Davis et al., 2008). Two different PhaJ enzymes have been discovered and characterized in *Pseudomonas putida*, as well. One enzyme, termed PhaJ1*Pp*, showed substrate specificity for C8 carbon substrates, while the other, PhaJ4*Pp*, demonstrated high activity for both C4 and C8 substrates. Interestingly, PhaJ1*Pp* exhibited low *(R)*-stereospecificity (Sato et al., 2011).

Both *phaJ* from *A. caviae* and *phaJ1* from *P. aeruginosa* were used to construct *R. eutropha* strains capable of producing high levels of poly(hydroxybutyrate-*co*-hydroxyhexanoate) (P(HB-*co*-HHx)) copolymer using plant oils as the sole carbon source. The P(HB-*co*-HHx) polymer produced contains high molar percentages of the C6 monomer, 3HHx (Budde et al., 2011). These results confirm that the PhaJ enzymes are capable of utilizing fatty acid β-oxidation intermediates as substrates in many different organisms and backgrounds. Recently, it was found that the

genome of *R. eutropha* strain H16 contains genes encoding *(R)*-specific enoyl-CoA hydratase. Three gene products, PhaJ4a, PhaJ4b, and PhaJ4c, so named because they displayed primary sequence similarity to PhaJ4 in *P. aeruginosa*, were shown to have *(R)*-specific enoyl-CoA hydratase activity. It was determined that PhaJ4a and PhaJ4b had 10X greater catalytic efficiency than PhaJ4c. Expression of these native *phaJ* genes, however, results in only small amounts of HHx monomer incorporated into PHA in *R. eutropha* cells (Kawashima et al., 2011).

**PhaG, a 3-hydroxyacyl-ACP:CoA transferase**

*Reaction: (R)-3-hydroxyacyl-ACP → (R)-3-hydroxyacyl-CoA*

While production of 3HA-CoA compounds from fatty acid β-oxidation is most common and most well-studied, PHA precursors can also be produced from fatty acid biosynthesis pathways (Fig. 1). The link between fatty acid biosynthesis and PHA production was first explored in detail with the characterization of the *phaG* gene from *P. putida* and its product, a 3-hydroxyacyl-acyl carrier protein (ACP):CoA transferase. Transcription of the *phaG* gene was shown to occur during PHA production using gluconate as the sole carbon source. The purified enzyme was shown to transfer an acyl moiety from 3-hydroxydecanoyl-CoA to an ACP, demonstrating its transferase activity (Rehm et al., 1998). The purified PhaG was further characterized and several conserved amino acid residues were shown to be essential for enzyme activity. Furthermore, a conserved serine (S102) and histidine (H251) were determined to be part of the catalytic triad, along with D182 (Hoffmann et al., 2002). The *phaG* gene and product have also been examined in *P. aeruginosa* (Hoffmann et al., 2000), *P. fluorescens* (Choi et al., 2009), *Pseudomonas* sp. 61-3 (Matsumoto et al., 2001), and *Pseudomonas mendocina* (Zheng et al., 2005). The *phaG* gene, along with a *Pseudomonas phaC* PHA synthase gene, have been used in studies to demonstrate *de novo* production of mcl-PHA, 3-hydroxydecanoic acid and rhamnolipids in engineered *E. coli* (Rehm et al., 2001; Zheng et al., 2004; Wang et al., 2012).

**PHA Polymerase, the Agent of PHA Biosynthesis**

*Reaction: n HA-CoA → PHA + n CoA*

The polymerization of hydroxyalkanoate monomers is catalyzed by the PHA polymerase, typically termed PhaC, PhaEC, or sometimes PhaRC. These polymerases are divided into four classes, depending on the subunit composition and the type of polymer synthesized. Class I synthases are the most well known and well studied, and include enzymes like *R. eutropha* PhaC. Class II synthases are those that polymerize mcl-PHA, and are typically expressed in *Pseudomonas* sp. Both Class I and II PHA synthases consist of one type of protein subunit, termed PhaC. Class III synthases consist of a heterodimer of the PhaC and PhaE proteins, with enzymes from *Allochromatium vinosum* and *Thiocapsa pfennigii*. The PhaC subunit is smaller in size than Class I and II PhaC proteins, with the former weighing ~40 kDa and the latter ~60-70 kDa. Class IV synthases are the least well studied of the PHA polymerase enzymes, and consist of a heterodimer of PhaC and PhaR proteins. For Class III and Class IV synthases, the PhaE and PhaR subunits, respectively, possess hydrophobic *C*-terminal faces that likely are the contact point of the synthase and the PHA granule (Rehm, 2003). The substrate specificity of the Class III and IV synthases is scl-3HA-CoA monomers. This has been shown experimentally with a recombinantly purified *A. vinosum* PhaEC, although the enzyme did exhibit low levels of activity using 4HB-CoA, 3HHx-CoA, and LA-CoA as substrates (Yuan et al., 2001).

Table 2. Comparison of consensus active site pentapeptide sequences of PHA polymerizing and depolymerizing enzymes with lipase and esterase enzymes.

| Enzyme | Active site consensus sequence | n[a] | Reference |
|---|---|---|---|
| PHA synthase | GXCXG[b] | 62 | (Rehm, 2003) |
| PHA depolymerase - intracellular | GXSXG (GVSWG) | 21 | (Jendrossek, Handrick, 2002) |
| PHA depolymerase – extracellular | GXSXG | 19 | (Jendrossek, Handrick, 2002; Shinohe et al., 1996) |
| Lipase | GXSXG | 44 | (Gupta et al., 2004; Jaeger et al., 1999) |
| Esterase | GXSXXc | 23 | (Arpigny, Jaeger, 1999) |

[a] n = number of sequences in the protein family aligned to derive consensus sequence
[b] X = any amino acid
[c] In Family IV, V, and VI esterases, the active site pentapeptide is GXSXG

Table 2 compares the active site of PHA polymerase with those of PHA depolymerases, lipase, and esterase enzymes. For the *R. eutropha* PHA synthase enzyme, immunocytochemical studies showed that PhaC was located on the surface of PHB granules (Gerngross et al., 1993). Following this, the active site of the enzyme was determined to be a cysteine moiety at position 319 (C319) (Gerngross et al., 1994). The catalytic triad of C319, D480, and H508 has been suggested for *R. eutropha* PhaC, based on site-directed mutagenesis studies. Also, these residues are conserved in all PHA synthases (Rehm et al., 2002). Furthermore, *in vitro* PHB production was demonstrated with 3HB-CoA substrate purified *R. eutropha* PhaC enzyme. The absence of any other compound in the PHB production reaction suggested that only enzyme and substrate are needed for polymer synthesis (Gerngross, Martin, 1995). Furthermore, the activity of *R. eutropha* PhaC was demonstrated to control the molecular weight and polydispersity of the resulting PHB (Sim et al., 1997). Data from this work suggests that PhaC from *R. eutropha* is not able to carry out PHB chain termination by itself (Lawrence et al., 2005). Thus, the manner in which the enzyme terminates elongation of a PHB chain still requires further study. Gerngross and Martin (1995) noted that PHB synthesis by recombinant *R. eutropha* PhaC exhibited a lag in activity that could be eliminated with sufficient amounts of a detergent, Hecameg. The lag phase was later confirmed, but it was determined that addition of Hecameg merely shortened but did not eliminate the lag in activity (Yuan et al., 2001). Studies involving acylation of *R. eutropha* PhaC using a saturated trimer (sT-CoA) analog of $[3HB]_3$-CoA demonstrated that the lag phase in activity was significantly reduced and activity was increased. It was suggested that the addition of sT-CoA to the reaction mixture facilitated dimerization when the sT moiety bound to the active site of the enzyme (Jia et al., 2001). Recently, activity of epitope-tagged PhaC, purified from the native *R. eutropha*, exhibited no lag phase. This result was due to the presence of different fractions of PHA synthase, monomer and dimer fractions that still exhibited lag in activity, and a high molecular weight (HMW) fraction that did not lag. It was shown that the HMW fraction consisted of co-purifying components like oligomeric PHB and the PhaP1 phasin protein. Thus, these "mini-granules," with PHB or oligo-3HB bound to the active site included primed, dimerized synthase enzymes, thus exhibiting no lag in enzyme activity (Cho et al., 2012). In addition,

a recent examination of a Class I PHA synthase from *Chromobacterium* sp. demonstrated an enzyme activity 5-fold greater than that of the *R. eutropha* PhaC enzyme (Bhubalan et al., 2011). This discovery, coupled with the experimentally determined broad substrate specificity of the *Chromobacterium* sp. PhaC enzyme (Bhubalan et al., 2011; Ling et al., 2011), has biotechnological implications in improvement of the PHA production process.

In their *R. eutropha* PhaC localization studies, Gerngross and coworkers (1993) proposed a model of granule biogenesis where PHA synthases containing nascent PHB chains would aggregate together at random nucleation sites to form intracellular polymer granules. This micelle model of PHB granule formation (Fig.3A) was one of three original models proposed in the literature. A scaffold model was also proposed (Fig.3C), with similarities to the micelle model, except including intracellular sites of nucleation called "mediation elements," which were dark-stained structures that appeared in electron microscopy studies of PHB granule formation in *R. eutropha* (Tian et al., 2005a; Tian et al., 2005b). The membrane budding model (Fig.3B), where hydrophobic PHA interacts with the hydrophobic lipids of the cell membrane and polymer granules nucleate at the site of the membrane, was suggested both because it is analogous to lipid body formation in eukaryotic systems (Parthibane et al., 2012; Stubbe et al., 2005) and as a result of fluorescence microscopy studies (Cho *et al*., 2012; Jendrossek, 2005; Jendrossek, 2009). The caveat of the membrane budding model is that many of the studies that led to its proposition were performed in an artificial system, using fluorescent tagged granule associated proteins for localization (Jendrossek, 2005; Jendrossek, 2009) or using *E. coli* as the host organism (Peters et al., 2007). Recently, a modified micelle model has been proposed (Fig.3D), which involves not only the PhaC enzyme in *R. eutropha*, but also the PhaP1 phasin protein. The principles of the micelle model and the modified micelle model are similar, with the exception that in the modified micelle model, nascent PHB chains are complexed with PhaP1 proteins in order to maintain solubility of the nascent granule. It has been demonstrated that another newly-discovered granule associated protein, PhaM, has both PHB binding and DNA binding capabilities (Fig.1). The PhaM protein may bind nonspecifically to the *R. eutropha* nucleoid to provide sites of PHA granule nucleation (Pfeiffer et al., 2011). It has thus been suggested that the "mediation elements" as seen in earlier electron microscopy studies are the nucleoid region of *R. eutropha*.

Class II PHA synthases, like those found in *P. putida, P. aeruginosa,* and others, preferentially utilize mcl-3HA-CoA as substrates for polymer biosynthesis. They typically consist of one type of protein subunit, PhaC, which is 60-65 kDA in size (Rehm, 2003). The region of DNA containing the PHA synthase genes from *P. aeruginosa* strain PA01 was first cloned and characterized in 1992. At that time, it was hypothesized that the cysteine moiety at position 296 (C296, analogous to C319 from *R. eutropha* PhaC) was responsible for the transesterification reaction (Timm, Steinbuchel, 1992). A catalytic triad of C296, D452, and H453, has been suggested by construction of threading models and by site-directed mutagenesis (Amara, Rehm, 2003). It was shown that expression of *P. aeruginosa phaC1*, heterologoulsly expressed in *E. coli*, conferred mcl-PHA biosynthesis capabilities. The *E. coli* strain used in this study was a *fadB* mutant strain, which resulted in the accumulation of mcl-3HA-CoA precursors for polymer synthesis (Langenbach et al., 1997). Similarly, expression of *P. aeruginosa phaC2* was shown to confer mcl-PHA synthesis capabilities in the same strain of *E. coli* (Qi et al., 1997). It has been shown in *Pseudomonas stutzieri* that PhaC1

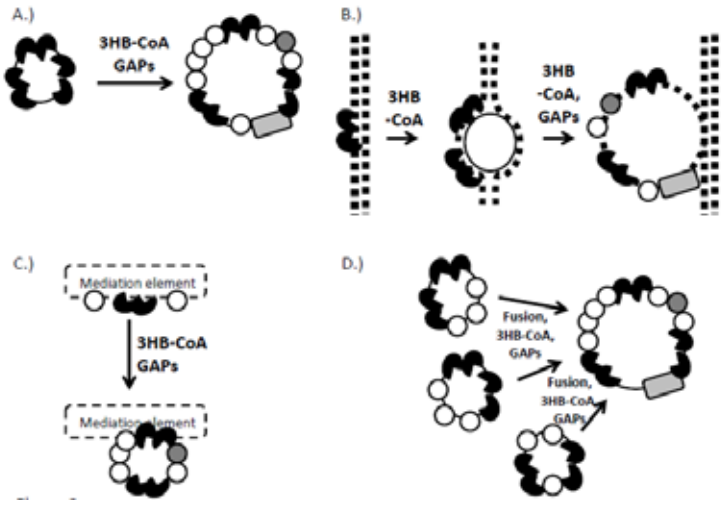


Fig. 3. *Ralstonia eutropha* granule biogenesis models. (A) The micelle model; (B) the membrane budding model; (C) the scaffold model; and (D) the modified micelle model. Symbols represent the same enzymes as in Fig. 1

and PhaC2 exhibit different substrate specificities, with PhaC1 specific to mcl-3HA-CoA substrates and PhaC2 able to utilize both scl- and mcl-3HA-CoA substrates (Chen et al., 2004; Chen et al., 2006). The enzymatic activities of recombinantly expressed and purified *P. aeruginosa* PhaC1 and PhaC2 were measured using 3-hydroxydecanoyl-CoA as the substrate. Activities of PhaC1 and PhaC2 were reported to be 0.039 activity units per mg (U/mg) and 0.035 U/mg, respectively. Free coenzyme-A was shown to inhibit the polymerization reaction with a $K_i$ of 85 μM (Qi et al., 2000). Interestingly, a PHA synthase from *Pseudomonas* sp. 61-3 has been characterized, and it has been found to exhibit broad substrate specificity (3HA monomers of 4-12 carbon atoms in length) (Matsusaki et al., 1998). Expression of *Pseudomonas* sp. 61-3 *phaC* in *R. eutropha* was shown to confer the ability to synthesize poly(hydroxybutyrate-*co*-hydroxyalkanoate) (P(HB-*co*-HA)) copolymers, where HA consisted of monomers of 6-12 carbon atoms in chain length (Matsusaki et al., 2000). Engineering of *Pseudomonas* sp. 61-3 PhaC by mutagenesis of serine residue at position 477 and a glutamine residue at position 481 resulted in increased 3HB fractions in the resulting polymer (Shozui et al., 2009), suggesting these residues play a role in substrate specificity. This synthase was also engineered to accept LA-CoA as a substrate in the seminal engineering studies of a P(LA-*co*-HB) producing bacterium (Taguchi et al., 2008).

As stated previously, *A. caviae* is able to produce P(HB-*co*-HHx) copolymer when grown on oils or fatty acids. This suggests that the PHA synthase of *A. caviae* has broad substrate specificity and can accommodate both scl- and mcl-3HA monomers. The initial isolation and characterization of the *A. caviae* PhaC showed that the active site was similar to the *R. eutropha* enzyme (Fukui, Doi, 1997). Higher enzyme activity mutants of *A. caviae phaC* have been isolated (Taguchi et al., 2001; Kichise et al., 2002). Both of these mutants displayed a concomitant increase in 3HHx monomer incorporation along with increase in activity. The mutations in the higher activity synthases were found to be in two codons that were not highly conserved among PHA synthases, N149S and D171G (Kichise et al., 2002). The genes encoding the higher activity

synthases were expressed in the *R. eutropha* mutant strain PHB-4. The PHA produced from these strains in cultures with soybean oil as the sole carbon source consisted of a maximum of 5 mol% 3HHx monomer (Tsuge et al., 2004). The two mutations, N149S and D171G, were combined on one *phaC* gene and examined for their synergistic effects. An *R. eutropha* strain expressing the double mutant synthase produced PHA with 5.2 mol% 3HHx and 18.1 mol% 3HHx when cultivated on soybean oil and octanoic acid, respectively. In both cases the double mutant exhibited an increase in 3HHx content compared to the wild type and single mutant synthases, suggesting a synergistic effect of the two mutations in terms of 3HHx incorporation into PHA (Tsuge et al., 2007). Chimeric PHA synthases were engineered using the sequences from *R. eutropha phaC* and *A. caviae phaC*. When expressed in *E. coli*, a recombinant synthase consisting of the *N*-terminal 25% (amino acids 1-154) of the *A. caviae* PhaC and the *C*-terminal 75% (amino acids 155-589) of the *R. eutropha* PhaC, the maximum amount of intracellular PHA was recovered, compared to strains expressing the other chimeric mutants and the wild type synthases. Also, 2 mol% 3HHx was incorporated into PHA, suggesting that the *N*-terminal region of *A. caviae* PhaC conferred broader substrate specificity (Matsumoto et al., 2009). This makes sense as the N149 residue, which has been shown to affect substrate specificity, is present on this *N*-terminal portion of the chimera.

**PhaZ, PHA depolymerases, and PHA Breakdown**

*Reaction: PHA + n CoA → n HA-CoA, or PHA + n H2O → HA*

Saegusa and coworkers (2001) were the first to characterize an intracellular PHA depolymerase in *R. eutropha*. Soon after this initial characterization of a depolymerase enzyme, York et al. (2003) determined that *R. eutropha* expressed multiple PHA depolymerases. Two of them, PhaZ1 and PhaZ2, played significant roles in PHB mobilization. In a recent study, intracellular PHB depolymerases were shown to have an effect on granule architecture, with PhaZ2 implicated to be involved with granule density (Brigham et al., 2012). Intracellular PHA depolymerases have been shown to be specific to native PHA (nPHA), which is found in intact intracellular granules. Typically, intracellular PHA depolymerases do not act on crystalline PHA, or polymer not present in intracellular granules (Gebauer, Jendrossek, 2006). PhaZ1 from *R. eutropha* was determined to mobilize PHB by thiolysis, using CoA as a co-substrate, resulting in the formation of 3HB-CoA from purified PHB granules. This thiolysis reaction still occurred, albeit to a lesser extent, when granules lacking PhaZ1 were used (Uchino et al., 2007). This thiolysis in the absence of PhaZ1 could indicate that other PhaZ enzymes present on the PHB granule also mobilize PHB in the presence of CoA. Furthermore, hydrolysis of PHB to 3HB has been demonstrated using *E. coli* recombinantly expressing the PHB biosynthesis operon *phaCAB* and *phaZ1* genes (Uchino et al., 2008). It is unclear at this time whether hydrolysis and thiolysis of PHB suggests conflicting evidence as to the true mechanism of PhaZ activity. It is clear that more evidence is needed, preferably resulting from standardized assays that mimic *in vivo* conditions as closely as possible, to determine the true mechanism of PhaZ-mediated intracellular PHB mobilization.

Enzymology of extracellular PHA depolymerases is more well-studied, likely because model systems have proven to be more readily characterizable. Recombinantly expressed and purified PHB depolymerase from *Alcaligenes faecalis* (now known as *Ralstonia pickettii*) was shown to cleave oligomers of 3HB

containing 3 or more subunits. It was suggested that the enzyme had 4 hydroxybutyryl binding sites, 3 of which must be occupied for cleavage to take place. Further characterization showed that two of the binding sites, flanking the site of PHB cleavage, exhibited *(R)*-specific stereoselectivity (Bachmann, Seebach, 1999). A highly active, thermotolerant depolymerase, PhaZ7, from *Paucimonas lemoignei* has been characterized biochemically, and is a serine hydrolase containing the pentapeptide motif AHSMG at the active site where the serine residue is at position 136 (S136). Alteration of this pentapeptide motif to a lipase box consensus or a PHB depolymerase box consensus had no significant effect of the activity of PhaZ7 (Braaz et al., 2003). Also, the catalytic triad of PhaZ7 has been demonstrated to be residues S136, D242, and H306 (Handrick et al., 2001). This catalytic triad suggests a mechanism of action similar to lipases and serine esterases, where S136 attacks the carbonyl carbon of a bound PHB chain (Handrick et al., 2001; Papageorgiou et al., 2008). As mentioned above, the substrate binding domain of another PHA depolymerase, $PhaZ_{RpiT1}$ from *R. pickettii* strain T1 has been described. Random mutagenesis and high throughput screening identified residues like V415, L441, and V457 that play a role in hydrophobic interactions during substrate binding (Hiraishi et al., 2006). In addition, directed mutagenesis showed that residues L441, Y443, and S445 play crucial roles in substrate binding in $PhaZ_{RpiT1}$ (Hiraishi et al., 2010). The crystal structure of *P. lemoignei* PhaZ7 has been determined to a 1.9 Å resolution. However, even with this resolution, a substrate binding pocket was not able to be identified, suggesting considerable structural rearrangement during catalysis (Papageorgiou et al., 2008). Recently, a crystal structure of PhaZ7 with a 1.2 Å resolution has been solved, revealing substrate binding information. With the updated structure, the active site is still found to be deeply buried, but conformational changes of surface loops were identified, suggesting involvement in PHA binding (Wakadkar et al., 2010).

The ability to degrade PHA in an extracellular environment is widely distributed among microorganisms. Bacteria and fungi have been characterized to degrade PHA (Jendrossek, Handrick, 2002). In an early study, lipases and PHA depolymerases from both prokaryotic and eukaryotic sources were examined for their ability to degrade PHA homopolymers. Lipases were unable to degrade PHB, but did show polymer degradation activity against polyhydroxypropionate (PHP) and poly(4-hydroxybutyrate) (P4HB). PHB depolymerases, on the other hand, were able to readily degrade PHB and demonstrated more robust activity against PHB and P4HB (Mukai et al., 1993). These results suggested that, while lipases exhibit PHA degradation capabilities, the polymers are degraded more rapidly with the aid of *de facto* PHB depolymerase enzymes. Early environmental studies of biodegradation of PHA polymers showed that PHB, and P(HB-*co*-HV) were degraded significantly in soil and aqueous environments. The rate of copolymer degradation was faster than that of PHB degradation. The PHA degrading organisms in the soil microbial communities consisted of a variety of bacteria and fungi. Common fungal genera among the fungi were *Aspergillus* and *Penicillium* (Mergaert, Swings, 1996). PHA degrading species in microbial communities have also been examined in root microbial ecosystems of coniferous and deciduous trees (Boyandin et al., 2012). While there is a wealth of ecological information in the literature regarding PHA degradation in environmental isolates and communities, very little biochemical characterization has been performed.

Table 3. Unanswered questions in the field of PHA homeostasis research.

| Enzyme activity and role in PHA homeostasis – Questions and subjects for further research |
|---|
| - Crystal structure of PHA synthase enzyme |
| - Cellular regulation of PHA production and PHA-related gene expression |
| - Characterization of PHA as a stress response molecule |
| - Correlation of PhaZ expression v. function in *R. eutropha* |
| - Defined roles of each intracellular PHB depolymerase in *R. eutropha* |
| - Characterization of intracellular PHB depolymerase reactions *in vivo* |

## Conclusions

Since the discovery and characterization of the original PHA biosynthesis pathways, many advances have been made in biochemical characterizations of the enzymes involved. There are, however, many unanswered questions in PHA pathway biochemistry, including those listed in Table 3. While crystal structures have been solved for some enzymes involved in PHA biosynthesis, the "holy grail" crystal structure of PhaC has yet to be successfully undertaken (Table 3). Also, understanding of PHA depolymerization in the intracellular and extracellular environments will shed light on PHA homeostasis on cellular and environmental levels. Further refinement of the enzymology of the PHA biosynthesis pathways can lead to production of polymers with tailor-made properties, made possible by directed evolution of enzyme active sites and substrate binding domains. These active sites and domains must be established and well understood in many of the enzymes discussed in this review before the production of tailor-made PHA becomes a reality.

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# Ферменты, участвующие в синтезе полигидроксиалканоатов: обзор и биологическая перспектива

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*Интерес к полигидроксиалканоатам (ПГА) вызван тем, что они являются биодеградируемыми биосовместимыми полимерами на биологической основе. Учёные накопили широкий спектр знаний о процессе биосинтеза этих ценных полимеров; специалисты в таких областях, как микробиология, биохимия, физика, медицина и технология, внесли свой вклад в современное состояние знаний о синтезе, получении и применении ПГА. Важное значение для перспектив биосинтеза ПГА имеет группа микробных ферментов, которые участвуют в производстве ПГА и их многочисленных разновидностей. В этом обзоре мы рассматриваем ключевые ферменты синтеза и гомеостаза всех типов ПГА. Особое внимание уделяется структуре и функционированию ферментов и тому, как энзимология сформировала знания о пути создания ПГА и известила о новых открытиях.*

*Ключевые слова: биопластики, полигидроксиалканоат, ферменты, гранулосвязанные белки, биосинтез полимера, гомеостаз ПГА.*