BACTERIAL BIODEGRADABLE PLASTIC: BIOSYNTHESIS, BIOCHEMISTRY AND GENETICS OF POLYHYDROXYALKANOATES

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ABSTRACT

Bacterial biodegradable plastic, polyhydroxyalkanoates (PHAs) composed of hydroxy fatty acids, are carbon storage intracellular polymers synthesized by various bacteria and archaea. These carbon storage polymers are accumulated as water insoluble cytoplasmic nano-sized inclusions in response to nutrient limitation and degraded when limiting nutrient is supplied again. The PHA particles are spherical and composed of a polyester core surrounded by phospholipids and proteins. The key enzymes of PHA biosynthesis and particle formation are the PHA synthases, which catalyze the formation of PHA. Various metabolic routes have been identified and established in bacteria to provide substrate for PHA synthases. Based on their functions, the genes which encode these PHA synthases are grouped into pha biosynthetic, regulatory, and granule associated proteins and depolymerizing genes. In most of PHA producing bacteria, pha genes especially biosynthetic genes are colocalized in operons but not under transcriptional control of a single promoter and diversity is there in this respect. Bacterial PHAs are currently considered as biocompatible and degradable biomaterials with numerous potential applications particularly in medicine, medical and packaging. Thus offering a solution for the existing ecological and environmental problems caused by conventional plastic and petroleum-based products.

Key words: PHA biosynthesis, granules formation, pha genes, biodegradable plastic, polyhydroxyalkanoates.

INTRODUCTION

The biosynthesis and accumulation of PHAs is a common phenomenon in many species of bacteria as hydrophobic inclusions of carbon and energy storage compounds in the cytoplasm (Dawes and Senior, 1973) as an electron sink mechanism for redundant reducing power under the condition of limiting nutritional elements such as N, P, S, O, or Mg in the presence of excess carbon source (Steinbüchel, 1991; Yu, 2001; Du and Yu, 2002). It was first discovered in 1926 as constituent of the bacterium Bacillus megaterium (Wong et al., 2000). The stored PHA can be degraded by intracellular depolymerases and metabolized as carbon and energy source as soon as the supply of the limiting nutrient is restored (Byrom, 1994). The majority of PHAs are composed of R(-)-3-hydroxyalkanoic acid monomers ranging from C_3 to C_14 carbon atoms with variety of saturated or unsaturated and straight or branched chain containing aliphatic or aromatic side groups (Doi et al., 1992; DeSmet et al., 1983). Approximately 150 different hydroxyalkanoic acids are now known to occur as constituents of PHAs. The molecular weight of the PHA is in the range of 2x10^6 to 3x10^8 Da, based on the type of microorganism and growth conditions. PHAs are accumulated in the cells as discrete granules, the size and number per cell varies depending on the different species (Byron, 1994). The granules appear as highly refractive inclusion under electron microscopic observation. PHAs can be divided into two broad groups based on the number of carbon atoms in the monomer units; the short chain length polyhydroxyalkanoates (SCL-PHAs), which consist of C_7-C_9 atoms, and medium chain length polyhydroxyalkanoates (MCL-PHAs) consisting of C_10-C_14 atoms. This grouping is due to the substrate specificity of the PHA synthesis that only accepts 3-hydroxyalkanoates (3HAs) of a certain range of carbon length (Anderson and Dawes, 1990). The PHA synthase of Alcaligenes eutrophus can only polymerize 3Has (SCL) while that of Pseudomonas oleovorans only polymerize 3HAs (MCL). For SCL-PHAs, the monomer units are oxidized at positions other than the third carbons while for MCL-PHAs, all the monomers units are oxidized at the third position except in few cases (Ojumu et al., 2004). A lot of PHAs (MCL) containing various functional groups such as olefins, branched alkyls, halogens, aromatic and cyano have been reported (Fritzsche et al., 1990; Kim et al., 1992; Ojumu et al., 2004). This flexibility of PHA biosynthesis makes it possible to design and produce related biopolymers having useful physical properties ranging from stiff and brittle plastic to rubbery polymers (Anderson and Dawes, 1990). PHAs have thermoplastic properties, they are biodegradable, and can be synthesized from renewable resources such as carbohydrates and lipids as the fermentation feedstocks. All these properties make this class of microbial polyester very attractive as a source of alternative materials to conventional petrochemical based plastics (Jendrossek et al., 1996). Therefore, they are considered for several applications in the packaging industry, medicine, pharmacy, agriculture and food industry, or as raw materials for the synthesis of enantiomerically pure chemicals and the production of paints (Müller and Seebach, 1993; Hocking and Marchessault, 1994; Steinbüchel,
1996; Ojumu et al., 2004). The aim of this review is to present a comparative study of PHA biosynthesis, genes organization and their functions in reported bacteria.

PHA Producing bacteria

PHAs are rather complex class of aliphatic/linear polyesters that are synthesized by most genera of bacteria and members of the family Halobacteriaceae of the Archaea (Steinbüchel and Füchtenbusch, 1998). Over 250 different bacteria, including Gram-negative and -positive species, have been reported to accumulate various PHAs (Steinbüchel, 1991; Lenz et al., 1992; Ojumu et al., 2004). The PHA-producing bacteria can be broadly divided into two groups according to the number of carbon atoms in the monomeric units of the PHAs produced (Steinbüchel, 1996). One group of bacteria, including Ralstonia eutropha (formerly A. eutrophus), produces SCL-PHAs with C3 to C5 monomer units, while the other group, including P. oleovorans and most Pseudomonads belonging to the rRNA homology group I can accumulate MCL-PHAs with C6 to C14 monomer units using 3-hydroxyacyl-CoA intermediates of β-oxidation pathway when cultured on various alkanes, alkanols, or fatty acids. (Anderson and Dawes, 1990; Steinbüchel, 1991). Although the majority of bacteria accumulate either SCL-PHAs or MCL-PHAs, several bacteria have been found to synthesize PHAs containing both short and medium chain length 3-hydroxyalkanoic acids (3HA). The bacteria Rhodospirillum rubrum (Brandl et al., 1989), Rhodococcus gelatinosus (Liebergesell et al., 1991), and Rhodococcus sp. (Haywood, et al., 1991) produced terpolymers consisting of 3HA units of C6, C5, and C6 from hexanoate. Aeromonas caviae produced a random copolymer of 3-hydroxybutyrate (3HB) and 3-hydroxyhexanoate (3HHx) (Friedrich et al., 1981; Shimamura et al., 1994; Doi et al., 1995). Pseudomonas strain GP4BH1 produced PHA containing 3HB and 3-hydroxyoctanoate (3HO) from octanoate and PHA containing 3HB, 3HO, and 3-hydroxydecanoate (3HD) from gluconate (Steinbüchel and Wiese, 1992). A recombinant strain of P. oleovorans expressing R. eutropha poly(3HB) [P(3HB)] biosynthesis genes has been shown to synthesize a blend of a P(3HB) homopolymer and a copolymer of 3HHx and 3HO units when grown on octanoate (Timm et al., 1990). Both polymers were stored as separated granules within the cells (Preusteg et al., 1993). In addition, P. fluorescens and several other Pseudomonas strains were found to produce a poly(3HB-co-3HA) [P(3HB-co-3HA)] copolymer consisting of 3HA units of C4 to C12 from 3HB and 1,3-butanediol (Lee et al., 1995). Although Thiocapsa pfennigii accumulated only a P(3HB) homopolymer from various carbon sources, a recombinant P. putida strain harboring the PHA synthesis genes of T. pfennigii produced a P(3HB-co-3HHx-co-3HO) terpolymer from octanoate (Liebergesell et al., 1993).

Biosynthesis of PHA

Bacteria synthesize PHAs by several enzymatic reactions as intracellular reservation material from acetyl-CoAs under some conditions that carbon source is present in excess but any growth factor is lack (Yu, 2001; Du and Yu, 2002). The majority of bacteria synthesize poly(3-hydroxybutyric acid). PHB and the second major class of PHA is composed of MCL-(R)-3-hydroxy fatty acids (Rehm, 2006). The biosynthesis of PHB requires the condensation of two acetyl-CoA molecules catalyzed by the β-ketothiolase (PhaA) leading to the formation of acetoacetyl-CoA which is reduced to (R)-3-hydroxybutyril-CoA by the (R)-specific acetoacetyl-CoA reductase (PhaB) (Ojumu et al., 2004). (R)-3-hydroxybutyril-CoA is substrate for the PHA synthase (PhaC) and the direct precursor of PHB biosynthesis (Rehm, 2006). In contrast, PHAs composed of medium-chain length (R)-3-hydroxyfatty acids are synthesized converting intermediates of fatty acid metabolism to (R)-3-hydroxyacyl-CoA. If the carbon source is oxidized to acetyl-CoA excluding the fatty acid β-oxidation pathway, then fatty acid de novo biosynthesis intermediates are diverted towards PHA biosynthesis catalyzed by the transacylase (PhaG). This specific transacylase catalyses the transfer of the (R)-3-hydroxyacyl moiety of the respective ACP (acyl carrier protein) thioester to CoA (Hoffmann et al., 2002; Rehm et al., 1998). If the carbon source is oxidized through the fatty acid β-oxidation pathway, then the (R)-specific enoyl-CoA hydratase (Phal) catalyses the oxidation of enoyl-CoA to (R)-3-hydroxyacyl-CoA (Fukui et al., 1998). (R)-3-hydroxyacyl-CoA is substrate for the PHA synthase (PhaC) and the direct precursor of PHA biosynthesis.

In vitro formation of PHA particles

Gerngross and Martin, (1995) were first to demonstrate in vitro synthesis of PHB and self-assembly of spherical granules by only using purified polyester synthase and substrate. This study clearly defined that the PHA synthases possessed all the features required for self-organization into spherical particles. This was further supported by establishment of in vitro PHA synthesis using purified PHA synthases form other microorganisms, such as e.g. from Cupriavidus necator, Allochromatium vinosum, P. aeruginosa (PhaC1 and PhaC2) and P. oleovorans (PhaC1) (Jossek et al., 1998; Jossek and Steinbüchel, 1998; Rehm et al., 2001). The class II polyester synthases were only
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recently purified and provision of 3-hydroxydecanoyl-CoA as substrate was sufficient for in vitro synthesis of poly(3-hydroxydecanoate) (Qi et al., 2000; Rehm et al., 2001).

Table 1. Genes involved in synthesis and regulation of PHA in bacteria.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Product</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>phaC1</td>
<td>PHA polymerase</td>
<td>polymerization</td>
<td>Langenbach et al., (1997); Maria et al., (1999)</td>
</tr>
<tr>
<td>phaZ</td>
<td>PHA depolymerase</td>
<td>PHA degradation</td>
<td>Rehm and Steinbüchel, (1999)</td>
</tr>
<tr>
<td>phaC2</td>
<td>PHA polymerase</td>
<td>polymerization</td>
<td>Qi et al., (1997); Maria et al., (1999)</td>
</tr>
<tr>
<td>phaD</td>
<td>GAP</td>
<td>not clarified yet</td>
<td>Rehm (2006)</td>
</tr>
<tr>
<td>phaF</td>
<td>histone H1-like protein</td>
<td>repressor of phaC1, phaG and phaI gene</td>
<td>Hoffmann and Rehm, (2005); Pötter et al., (2005)</td>
</tr>
<tr>
<td>phaI</td>
<td>GAP</td>
<td>not clarified yet</td>
<td>Rehm (2006)</td>
</tr>
<tr>
<td>phaJ</td>
<td>(R)-specific enoyl-CoA hydratase</td>
<td>precursor synthesis</td>
<td>Fukui et al., (1998)</td>
</tr>
<tr>
<td>phaP1</td>
<td>phasin PhaP1</td>
<td></td>
<td>Zeller et al., (2005)</td>
</tr>
<tr>
<td>phaP4</td>
<td>phasin PhaP4</td>
<td>repressor of phaP</td>
<td>(Tian-Renet al., 2004)</td>
</tr>
<tr>
<td>PhaQ</td>
<td>transcriptional regulator</td>
<td>repressor of phaP</td>
<td></td>
</tr>
<tr>
<td>phaR</td>
<td>PHA-specific regulator protein</td>
<td>repressor of phasin genes</td>
<td>Maehara et al., (2002); Pötter et al., (2005)</td>
</tr>
<tr>
<td>phaS</td>
<td>GAP</td>
<td>not clarified yet</td>
<td>Rehm (2006)</td>
</tr>
</tbody>
</table>

In vivo formation of PHA particles

Two models of PHA granule formation have been described: (i) the micelle model and (ii) the budding model (Rehm, 2006). Both models consider the defined location of the polyester synthase and to some extent the phasin protein on the surface of the granule. The micelle model is certainly supported by PHA granule formation in vitro and in the absence of membranes. However, electron microscopy studies showing membrane-like material
surrounding PHA granules in intact cells (Dunlop and Robards, 1973) or isolated granules (Mayer et al., 1996) provided evidence for the budding model. Tian et al., (2005) showed that early stage granules are not randomly distributed in the cytoplasm and close to the inner cell membrane, as would be anticipated from the two models of granule formation. They found that emerging granules arose from only the centre of the cell at unknown mediation elements suggesting a new model for PHA granule formation. Dennis et al., (2003) observed large structures (35nm) on the surface of PHB-containing granules from C. necator cells using atomic force microscopy which might function as synthesis-degradation centers. Recent fluorescence microscopic studies employing GFP-labeled PHA synthase, i.e. GFP was fused to the N-terminus of class I and class II PHA synthases, respectively, without affecting PHA particle formation, enabled in vivo monitoring of PHA granule formation as well as subcellular. In this study early stage granules were found to be localized at the cell poles suggesting that granule formation starts at the cell poles according to the budding model. Localization of granule formation was found to be dependent on nucleoid structure suggesting that nucleoid occlusion occurred. It remains unclear whether PHA chain synthesis is required for subcellular localization of granule formation. Interestingly, this study led to the observation that small emerging granules are rapidly oscillating between the cell poles. This might play a role in equal distribution of storage materials between the daughter cells (Peters and Rehm, 2005). Overall, these in vivo studies using GFP-labeled polyester synthase supported the budding model by localizing granule formation close to the cytoplasmic membrane sat the cell poles.

**Structure of PHA granules**

The PHA granules are surrounded by a phospholipids membrane (Griebel et al., 1968) with embedded or attached proteins (Stuart et al., 1998) composed of the polyester synthase (Gerngross et al., 1993), the intracellular PHA depolymerase (Handrick et al., 2000), amphipathic phasin proteins (Pieper-Fürst et al., 1995), PHA-specific regulator proteins (Maehara et al., 2002; Prieto et al., 1999; York et al., 2002) and additional proteins (Klinke et al., 2000) with unknown function. The intracellular depolymerases are required for mobilization of the reserve polyester and are attached to the granule surface (Sægusa et al., 2001). The phasin proteins play a role as structural proteins non-covalently attached to the polyester core of granules (Hanley et al., 1999). Phasins promote PHA biosynthesis and their copy number has impact on PHA granule size (Pötter et al., 2005). Simulation of the self-assembly process showed that phasins might impact on the kinetics of granule formation by reducing the lag phase (Jurasek and Marchessault, 2004). Various PHA-specific regulators such as PhbR from C. necator (Pötter et al., 2002, York et al., 2002). PhaF from Pseudomonads (Hoffmann and Rehm, 2004 & 2005) and PhaR from Paracoccus denitrificans (Maehara et al., 1999 & 2002) were found to bind non-covalently to the PHA granules. These cytosolic levels of these repressor proteins are supposed to be low, when PHA granules are formed. In PHB synthesizing bacteria, the repressor PhbR was suggested to repress transcription of genes encoding phasins, whereas in Pseudomonads the repressor PhaF seems to repress transcription of the PHA synthase gene (phaC1) and the transacylase gene (phaG) (Pötter et al., 2005). The alternative sigma factor r^4 encoded by rpoN was found to play an important role in transcriptional regulation of PHA biosynthesis genes in Pseudomonads, (Hoffmann and Rehm, 2004 & 2005). Additional proteins (PhaI, PhaD, PhaS) were found to be granule-associated and co-regulated in Pseudomonads, which function has not been clarified, yet (Hoffmann and Rehm, 2005; Klinke et al., 2000). All of these non-covalently attached proteins are not essential for PHA granule formation, but serve various biological functions such as e.g. PHA granule structure, PHA biosynthesis gene regulation and PHA mobilization (Rehm, 2006).

**Organization and function of PHA genes**

The PHA synthase genes and genes for other proteins related to the metabolism of PHA are often clustered in an operonic organization in the bacterial genomes (Rehm, 2003). But, relatively little is known about the genetic regulation of PHA biosynthesis and intracellular PHA mobilization, however, the known functions and role of identified PHA biosynthetic and regulatory genes in bacteria have been described in Table 1. The B. megaterium 11561 that produces PHA carries a 4,104-bp cluster of five pha genes, phaP, -Q, -R, -B, and -C. The phaP and -Q genes were shown to be transcribed in one orientation, each from a separate promoter, while immediately upstream, phaR, -B, and -C were divergently transcribed as a tricistronic operon (Gabriel and Maura, 1999), phaP codes for phasin, an abundant granule-associated protein, forms a boundary layer on the PHA surface to sequester hydrophobic PHA from the cytoplasm. phaQ gene, which is located upstream of the phasin-encoding phaP gene, codes for a new class of transcriptional regulator that negatively controls expression of both phaP and -Q (Table 1) (Tian-Ren et al., 2004). The PHA biosynthetic genes of A. eutroficus H16 are organized in an operon consisting of three genes, phaC, phaA, and phaB, which encode PHA synthase, β-ketothiolase, and NADPH-dependent acetoacetyl-CoA reductase, respectively (Table 1) (Peoples and Sinskey, 1989ab; Schubert et al., 1991). The
transcriptional start site was determined by S1 nuclease mapping. The promoter is similar to the E. coli promoter recognized by σ70 (John et al., 1995; Schubert et al., 1991). In R. eutropha, which has been studied in most detail with respect to PHA biosynthesis, the genes for PHA synthase (phaC), ketohiostase (phaA) and NADPH-dependent acetoacetyl-CoA reductase (phaB) also constitute the phaCAB operon (Schubert et al., 1988; Slater et al., 1988; Steinbuchel and Schlegel, 1991). Approximately 4kbp downstream of this operon, a second ketohiostase gene (bktB) was recently identified (Slater et al., 1998). BktB is in contrast to PhaA able to synthesize 3-ketovaleryl-CoA. Besides these, Burkholderia sp, Chromobacterium violaceum and Comamonas acidovorans possess a phaCAB operon (Sudesh et al., 1998), whereas in Acinetobacter sp, Pseudomonas sp and Vibrio cholerae these genes are also clustered, but phaA, phaB or other genes related to PHA metabolism are not directly linked to the phaCs in the genomes of these bacteria except A. caviae (Qi et al., 1997; Fukui et al., 1998). In this bacterium the gene encoding an enoyl-CoA hydratase is located downstream of phaC. The pha gene cluster of P. oleovorans and P. aeruginosa, which encodes the proteins involved in PHA metabolism, consists of four open reading frames phaC1ZC2D transcribed in the same direction (Maria et al., 1999). phaC1 and phaC2 genes, which encode PHA synthases (or PHA polymerases); the phaZ gene, which codes for a PHA depolymerase; and the phaD gene, which encodes a peptide of unknown function (Table 1) (Huisman et al., 1991; Maria et al., 1999). In the case of P. oleovorans, it has been reported that there are two promoters, both located upstream of the phaC1 gene, which resemble the consensus sequences for σ70- and σ54-dependent promoters. The corresponding transcriptional start sites are located respectively 198- and 112bp upstream of the ribosomal binding site (RBS) of the phaC1 gene (van der Leij and Witholt, 1995). Nevertheless, it is not known whether this promoter region drives the expression of only the phaC1 gene or of the whole pha cluster as an operon. In this sense, two putative transcription terminators have been found downstream of the phaZ and phaD genes (Huisman et al., 1991). Further, Maria et al., (1999) reported two more PHA genes phaF and phal in P. oleovorans, located downstream of the phaC1ZC2D gene cluster. phaF encodes for a regulatory protein which is associated with PHA granules and controls the expression of the phaC1, phaG and phaI gene (Table 1) (Hofmann and Rehm, 2005; Pötter et al., 2005). phaI coding for a newly identified granule-associated protein (GAP) but its function is not clarified yet (Rehm, 2006). Although the capacity of Azotobacter strains to accumulate PHAs is well-known, but to date, a cluster of three genes phbBAC responsible for the synthesis of poly(3-hydroxybutyrate) (PHB) in Azotobacter sp. FA8 was reported which have sequence similarity with genes coding for acetoacetyl-coenzyme A reductase (phbB) β-ketothiolase (phbA) and PHB polymerase gene (phbC) respectively (Pettinari et al., 2001). In all bacteria, which possess a two-component PHA synthase, phaC and phaE are directly linked in the genomes constituting most probably single operons (Hai et al., 2004). Interestingly, PHA-accumulating bacteria belonging to the Proteobacteria, such as Caulobacter crescentus, Azorhizobium caulinodans, Rhizobium etli, Sinorhizobium meliloti, P. denitrificans and Methylobacterium extorquens, contain the class I PHA synthase, genes are not colocalized with other PHA biosynthesis genes (Valentin and Steinbuchel, 1993; Tombolini et al., 1995; Cevallos et al., 1996). Only a few exceptions, such as Zoogloea ramigera, A. caviae and Nocardia corallina, not belonging to Proteobacteria have been described, which do not contain colocalized PHA biosynthesis genes (Peoples and Sinskey, 1989a; Fukui and Doi, 1997). Some species such as P. denitrificans possessed adjacent to the PHA synthase further genes like phaP (encoding phasin) and phaR (encoding regulator protein) related to PHA biosynthesis (Maehara et al. 1999 & 2002; Pötter et al., 2005). Among the Proteobacteria PHA accumulating bacteria, such as R. eutropha, Burkholderia sp, A. latus and C. acidovorans, an operonic organization of PHA biosynthesis genes, which are related to the SCL-PHA biosynthesis (class I PHA synthase gene) is found (Schubert et al., 1991; Choi et al., 1998; Rodrigues et al., 2000). Genes encoding for the metabolically linking enzymes PhaG and PhaJ are not co-localized with the PHA synthase genes, but are co-regulated (Hofmann et al., 2002 & 2004). In Pseudomonas sp and A. caviae the PHA biosynthesis (R)-specific enoyl-CoA hydratase gene (phaJ) was identified, which catalyzes conversion of the fatty acid β-oxidation intermediate (S)-3-hydroxyacyl-CoA to (R)-3-hydroxyacyl-CoA, the substrate of the PHA synthase (Fukui et al., 1998; Rehm, 2006). Moreover, phaG gene has only been found in Pseudomonads encoding the linking PHA biosynthesis enzyme, the transacylase phaG, which catalyzes the conversion of the fatty acid biosynthesis intermediate (R)-3-hydroxyacyl-ACP to (R)-3-hydroxyacyl-CoA, which serves as direct precursor of PHA biosynthesis (Table 1) (Rehm et al., 1998; Hofmann et al., 2002 & 2004). The elucidation of various genes, which are involved in PHA biosynthesis, is allowing us to develop new metabolic engineering strategies for the efficient production of PHAs.

REFERENCES


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