

## 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 biodegradable 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.

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### 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) or 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<sub>3</sub> to C<sub>14</sub> 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 at 2×10<sup>5</sup> to 3×10<sup>6</sup> 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<sub>3</sub>-C<sub>5</sub> atoms, and medium chain length polyhydroxyalkanoates (MCL-PHAs) consisting of C<sub>6</sub>-C<sub>14</sub> 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 Fuchtenbusch, 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 C<sub>3</sub> to C<sub>5</sub> monomer units, while the other group, including *P. oleovorans* and most *Pseudomonads* belonging to the rRNA homology group I can accumulate MCL-PHAs with C<sub>6</sub> to C<sub>14</sub> monomer units using 3-hydroxyacyl-CoA intermediates of  $\beta$ -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), *Rhodocyclus gelatinosus* (Liebergesell et al., 1991), and *Rhodococcus* sp. (Haywood, et al., 1991) produced terpolyesters consisting of 3HA units of C<sub>4</sub>, C<sub>5</sub>, and C<sub>6</sub> 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 polyesters were stored as separated granules within the cells (Preusting 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 C<sub>4</sub> to C<sub>12</sub> 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-hydroxyfatty acids (Rehm, 2006). The biosynthesis of PHB requires the condensation of two acetyl-CoA molecules catalyzed by the  $\beta$ -ketothiolase (*PhaA*) leading to the formation of acetoacetyl-CoA which is reduced to (R)-3-hydroxybutyryl-CoA by the (R)-specific acetoacetyl-CoA reductase (*PhaB*) (Ojumu et al., 2004). (R)-3-hydroxybutyryl-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  $\beta$ -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  $\beta$ -oxidation pathway, then the (R)-specific enoyl-CoA hydratase (*PhaJ*) 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 from 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

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.

Gene	Product	Function	Reference
<i>phaA</i>	$\beta$ -ketothiolase	precursor synthesis	Peoples and Sinskey, (1989a & b); John <i>et al.</i> , (1995)
<i>phaB</i>	acetoacetyl-CoA reductase	precursor synthesis	Peoples and Sinskey, (1989a & b); John <i>et al.</i> , (1995)
<i>phaC</i>	PHA polymerase (PHA polymerase)	polymerization	Peoples and Sinskey, 1989b; John <i>et al.</i> , (1995)
<i>phaC1</i>	PHA polymerase	polymerization	Langenbach <i>et al.</i> , (1997); Maria <i>et al.</i> , (1999)
<i>phaZ</i>	PHA depolymerase	PHA degradation	Rehm and Steinbüchel, (1999)
<i>phaC2</i>	PHA polymerase	polymerization	Qi <i>et al.</i> , (1997); Maria <i>et al.</i> , (1999)
<i>phaD</i>	GAP	not clarified yet	Rehm (2006)
<i>phaE</i>	second sub-unit of type III PHA synthase	expression of PHA synthase	Choi <i>et al.</i> , (1998); Hai <i>et al.</i> , (2004)
<i>phaF</i>	histone H1-like protein	repressor of <i>phaC1</i> , <i>phaG</i> and <i>phaI</i> gene	Hoffmann and Rehm, (2005); Pötter <i>et al.</i> , (2005)
<i>phaI</i>	GAP	not clarified yet	Rehm (2006)
<i>phaG</i>	transacylase	precursor synthesis	Hoffmann <i>et al.</i> , (2002 & 2004)
<i>phaJ</i>	(R)-specific enoyl-CoA hydratase	precursor synthesis	Fukui <i>et al.</i> , (1998)
<i>phaP</i>	phasin <i>PhaP</i>	(Promote the transcription	Pötter <i>et al.</i> , (2005)
<i>(phaP1)</i>	phasin <i>PhaP1</i>	of PHA synthetic genes	Zeller <i>et al.</i> , (2005)
<i>phaP2</i>	phasin <i>PhaP2</i>	and formation of	Pötter <i>et al.</i> , (2002 & 2004)
<i>phaP3</i>	phasin <i>PhaP3</i>	PHA granules)	Pötter <i>et al.</i> , (2002 & 2004)
<i>phaP4)</i>	phasin <i>PhaP4</i>		Pötter <i>et al.</i> , (2002 & 2004)
<i>PhaQ</i>	transcriptional regulator	repressor of <i>phaP</i>	(Tian-Renet <i>et al.</i> , 2004)
<i>phaR</i>	PHA-specific regulator protein	repressor of phasin genes	Maehara <i>et al.</i> , (2002); Pötter <i>et al.</i> , (2005)
<i>phaS</i>	GAP	not clarified yet	Rehm (2006)

### ***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 at 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 (Saegusa *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  $\sigma^{54}$  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. eutrophus* H16 are organized in an operon consisting of three genes, *phaC*, *phaA*, and *phaB*, which encode PHA synthase,  $\beta$ -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  $\sigma^1$  nuclease mapping. The promoter is similar to the *E. coli* promoter recognized by  $\sigma^{70}$  (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*), ketothiolase (*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 ketothiolase 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  $\sigma^{70}$ - and  $\sigma^{54}$ -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 *phaI* 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) (Hoffmann 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*)  $\beta$ -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*, contained 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 (Hoffmann 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  $\beta$ -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*, that 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; Hoffmann 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

- Anderson, A.J and E.A. Dawes (1990). Occurrence, metabolism, metabolic role and industrial uses of bacterial polyhydroxyalkanoates. *Microbiol. Rev.*, 54: 450-472.

- Brandl, H., E.J. Knee, R.C. Fuller, R.A. Gross and R.W. Lenz (1989). Ability of the phototrophic bacterium *Rhodospirillum rubrum* to produce various poly( $\beta$ -hydroxyalkanoates): potential sources for biodegradable polyesters. *Int. J. Biol. Macromol.*, 11: 49-55.
- Byrom, D. (1994). Polyhydroxyalkanoates. In: Plastic from microbes: microbial synthesis of polymers and polymer precursors. (D.P. Mobley eds.). Hanser Munich, pp. 5-33.
- Cevallos, M. A., S. Encarnacion, A. Leija, Y. Mora, and J. Mora (1996). Genetic and physiological characterization of a *Rhizobium etli* mutant strain unable to synthesize poly-beta-hydroxybutyrate. *J. Bacteriol.*, 178: 1646-1654.
- Choi, J., S.Y. Lee and K. Han (1998). Cloning of the *Alcaligenes latus* polyhydroxyalkanoate biosynthesis genes and use of these genes for enhanced production of poly(3-hydroxybutyrate) in *E. coli*, *Appl. Environ. Microbiol.*, 64: 4897-4903.
- Dawes, E.A and P.J. Senior (1973). The role and regulation of energy reserve polymers in micro-organisms. *Adv. Microb. Physiol.*, 10: 135-266.
- Dennis, D., C. Liebig, T. Holley, K.S. Thomas, A. Khosla, D. Wilson and B. Augustine (2003). Preliminary analysis of polyhydroxyalkanoate inclusions using atomic force microscopy. *FEMS Microbiol. Lett.*, 226: 113-119.
- DeSmet, M.J., G. Eggink, B. Witholt, J. Kingma and H. Wynberg (1983). Characterization of intracellular inclusions formed by *Pseudomonas oleovorans* during growth on octane. *J. Bacteriol.*, 154: 870-878.
- Doi, Y., Y. Kawaguchi, N. Koyama, S. Nakamura, M. Hiramitsu, Y. Yoshida and U. Kimura (1992). Synthesis and degradation of polyhydroxyalkanoates in *Alcaligenes eutrophus*. *FEMS Microbiol. Rev.*, 103: 103-108.
- Doi, Y., S. Kitamura and H. Abe (1995). Microbial synthesis and characterization of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate). *Macromol.*, 28:4822-4828.
- Du, G and J. Yu (2002). Green technology for conversion of food scraps to biodegradable thermoplastic polyhydroxyalkanoates. *Environ. Sci. Technol.*, 36: 5511-5516.
- Dunlop, W.F and A.W. Robards (1973). Ultrastructural study of poly-hydroxybutyrate granules from *Bacillus cereus*. *J. Bacteriol.*, 114: 1271-1280.
- Friedrich, B., C. Hogrefe and H.G. Schlegel (1981). Naturally occurring genetic transfer of hydrogen-oxidizing ability between strains of *Alcaligenes eutrophus*. *J. Bacteriol.*, 147:198-205.
- Fritzsche, K., R.W. Lenz and R.C. Fuller (1990). Production of unsaturated polyesters by *P. oleovorans*. *Int. J. Biol. Macromol.*, 12: 85-91.
- Fukui, T and Y. Doi (1997). Cloning and analysis of the poly(3-hydroxy-co-3-hydroxyhexanoate) biosynthesis genes of *A. caviae*. *J. Bacteriol.*, 179: 4821-4830.
- Fukui, T., N. Shiomi and Y. Doi (1998). Expression and characterization of (R)-specific enoyl coenzyme A hydratase involved in polyhydroxyalkanoate biosynthesis by *A. caviae*. *J. Bacteriol.*, 180: 667-673.
- Gabriel, J.M and C.C. Maura (1999). Polyhydroxyalkanoate inclusion body-associated proteins and coding region in *Bacillus magisterium*. *J. Bacteriol.*, 181(2): 585-592.
- Gerngross, T.U., P. Reilly, J. Stubbe, A.J. Sinskey and O.P. Peoples (1993). Immunocytochemical analysis of poly-beta-hydroxybutyrate (PHB) synthase in *Alcaligenes eutrophus* H16: localization of the synthase enzyme at the surface of PHB granules. *J. Bacteriol.*, 175: 5289-5293.
- Gerngross, T.U and D.P. Martin (1995). Enzyme-catalyzed synthesis of poly[(R)-(-)-3-hydroxybutyrate]: formation of macroscopic granules in vitro. *Proc. Natl. Acad. Sci. USA.*, 92: 6279-6283.
- Griebel, R., Z. Smith and J.M. Merrick (1968). Metabolism of polybeta-hydroxybutyrate. I. Purification, composition, and properties of native poly-beta-hydroxybutyrate granules from *Bacillus megaterium*. *Biochem.*, 7: 3676-3681.
- Hai, T., D. Lange, R. Rabus and A. Steinbüchel (2004). Polyhydroxyalkanoate (PHA) accumulation in sulfate-reducing bacteria and identification of a class-III PHA synthase (*PhaEC*) in *Desulfococcus multivorans*. *Appl. Environ. Microbiol.*, 70: 4440-4448.
- Handrick, R., S. Reinhardt and D. Jendrossek (2000). Mobilization of poly(3-hydroxybutyrate) in *Ralstonia eutropha*. *J. Bacteriol.*, 182: 5916-5918.
- Hanley, S.Z., D.J. Pappin, D. Rahman, A.J. White, K.M. Elborough and A.R. Slabas (1999). Re-evaluation of the primary structure of *Ralstonia eutropha* phasin and implications for polyhydroxyalkanoic acid granule binding. *FEBS Lett.*, 447: 99-105.
- Haywood, G. W., A.J. Anderson, G.A. Williams, E.A. Dawes and D.F. Ewing (1991). Accumulation of poly(hydroxyalkanoate) copolymer containing primarily 3-hydroxyvalerate from simple carbohydrate substrates by *Rhodococcus* sp. NICMB 40126. *Int. J. Biol. Macromol.*, 13: 83-87.
- Hocking, P.J and R.H. Marchessault (1994). *Chemistry and technology of biodegradable polymers*. (G.J.L. Griffin eds.). Blackie Academic. New York, pp. 48-96.

- Hoffmann, N., A.A. Amara, B.B. Beermann, Q. Qi, H.J. Hinz and B.H.A. Rehm (2002). Biochemical characterization of the *Pseudomonas putida* 3-hydroxyacyl ACP: CoA transacylase, which diverts intermediates of fatty acid de novo biosynthesis. *J. Biol. Chem.*, 277: 42926-42936.
- Hoffmann, N and B.H.A Rehm (2004). Regulation of polyhydroxyalkanoate biosynthesis in *Pseudomonas putida* and *Pseudomonas aeruginosa*. *FEMS Microbiol. Lett.*, 237: 1-7.
- Hoffmann, N and B.H.A. Rehm (2005). Nitrogen-dependent regulation of medium-chain length polyhydroxyalkanoate biosynthesis genes in *Pseudomonads*. *Biotechnol. Lett.*, 27: 279-282.
- Huisman, G.W., E. Wonink, R. Meima, B. Kazemier, P. Terpstra and B. Witholt (1991). Metabolism of poly(3-hydroxyalkanoates) (PHAs) by *Pseudomonas oleovorans*. *J. Biol. Chem.*, 266: 2191-2198.
- Jendrossek, D., A. Schirmer and H.G. Schlegel (1996). Biodegradation of polyhydroxyalkanoic acids. *Appl Microbiol Biotechnol.*, 46: 451-463.
- John, K., H.E. Valentin and D. Dennis (1995). Regulated expression of the *Alcaligenes eutrophus pha* biosynthesis Genes in *Escherichia coli*. *App. Environ, Microbiol.*, 61(4): 1391-1398.
- Jossek, R., R. Reichelt and A. Steinbüchel (1998). In vitro biosynthesis of poly(3-hydroxybutyric acid) by using purified poly(hydroxyalkanoic acid) synthase of *Chromatium vinosum*. *Appl. Microbiol. Biotechnol.*, 49: 258-266.
- Jossek, R and A. Steinbüchel. (1998). In vitro synthesis of poly(3-hydroxybutyric acid) by using an enzymatic coenzyme A recycling system. *FEMS Microbiol. Lett.*, 168: 319-324.
- Jurasek, L and R.H. Marchessault (2004). Polyhydroxyalkanoate (PHA) granule formation in *Ralstonia eutropha* cells: a computer simulation. *Appl. Microbiol. Biotechnol.*, 64: 611-617.
- Kim, B.S., S.Y. Lee and H.N. Chang (1992). Production of poly- $\beta$ -hydroxybutyrate by fed-batch culture of recombinant *Escherichia coli*. *Biotechnol. Lett.*, 14: 811-816.
- Klinke, S., G. de Roo, B. Witholt and B. Kessler (2000). Role of *phaD* in accumulation of medium-chain-length poly(3-hydroxyalkanoates) in *Pseudomonas oleovorans*. *Appl. Environ. Microbiol.*, 66: 3705-3710.
- Langenbach, S., B.H.A. Rehm and A. Steinbüchel (1997). Functional expression of the PHA synthase gene *phaC1* from *Pseudomonas aeruginosa* in *Escherichia coli* results in poly(3-hydroxyalkanoate) synthesis. *FEMS Microbiol. Lett.*, 150:303-309.
- Lee, E.Y., D. Jendrossek, A. Schirmer, C.Y. Choi and A. Steinbüchel (1995). Biosynthesis of copolyesters consisting of 3-hydroxybutyric acid and medium-chain-length 3-hydroxyalkanoic acids from 1, 3-butanediol or from 3-hydroxybutyrate by *Pseudomonas* sp. A33. *Appl. Microbiol. Biotechnol.*, 42: 901-909.
- Lenz, R.W., Y.B. Kim and R.C. Fuller (1992). Production of unusual bacterial polyesters by *P. oleovorans* through cometabolism. *FEMS Microbiol. Rev.*, 103: 207-214.
- Liebergesell, M., E. Hustede, A. Timm, C.Y. Choi, A. Steinbüchel, R.C. Fuller, R.W. Lenz and H.G. Schlegel (1991). Formation of poly(3-hydroxyalkanoates) by phototrophic and chemolithotrophic bacteria. *Arch. Microbiol.*, 155: 415-421.
- Maehara, A., S. Ueda, H. Nakano and T. Yamane (1999). Analyses of a polyhydroxyalkanoic acid granule-associated 16-kilodalton protein and its putative regulator in the *pha* locus of *Paracoccus denitrificans*. *J. Bacteriol.*, 181: 2914-2921.
- Maehara, A., S. Taguchi, T. Nishiyama, T. Yamane and Y. Doi (2002). A repressor protein, *PhaR*, regulates polyhydroxyalkanoate (PHA) synthesis via its direct interaction with PHA. *J. Bacteriol.*, 184: 3992-4002.
- Maria, A., M.A. Prieto, B. Bruno., K. Jung, B. Witholt and B. Kessler (1999). *PhaF*, a polyhydroxyalkanoate-granule-associated protein of *Pseudomonas oleovorans* GPo1 involved in the regulatory expression system for *pha* genes. *J. Bacteriol.*, 181(3): 858-868.
- Mayer, F., M.H. Madkour, U. Pieper-Fürst, R. Wiczorek, M.L. Liebergesell and A. Steinbüchel (1996). Electron microscopic observations on the macromolecular organization of the boundary layer of bacterial PHA inclusion bodies. *J. Gen. Appl. Microbiol.*, 42: 445-455.
- Müller, H.M and D. Seebach (1993). Poly(hydroxyalkanoates: a fifth class of physiologically important organic biopolymers? *Angew. Chem. Int. Ed. Engl.* 32:477-502.
- Ojumu, T.V., J. Yu and B.O. Solomon (2004). Production of polyhydroxyalkanoates, a bacterial biodegradable polymer. *Afri. J. Biotechnol.*, 3 (1): 18-24.
- Peoples, O.P and A.J. Sinskey (1989a). Poly-b-hydroxybutyrate biosynthesis in *Alcaligenes eutrophus* H16. Characterization of the genes encoding  $\beta$ -ketothiolase and acetoacetyl-CoA reductase. *J. Biol. Chem.*, 264:15293-15297.
- Peoples, O.P and A.J. Sinskey (1989b). Poly-b-hydroxybutyrate (PHB) biosynthesis in *Alcaligenes eutrophus* H16. Identification and characterization of the PHB polymerase gene (*phbC*). *J. Biol. Chem.*, 264:15298-15303.

- Peters, V and B.H.A. Rehm (2005). In vivo monitoring of PHA granule formation using GFP-labeled PHA synthases. *FEMS Microbiol. Lett.*, 248: 93-100.
- Pettinari, M.J., G.J. Vazquez., D. Silberschmidt, B.H.A. Rehm, A. Steinbüchel and B.S. Mendez (2001). Poly(3-hydroxybutyrate) synthesis genes in *Azotobacter chroococcum* strain FA8. *Appl. Environ. Microbiol.*, 67(11): 5331-5334.
- Pieper-Fürst, U., M.H. Madkour, F. Mayer and A. Steinbüchel (1995). Identification of the region of a 14-kilodalton protein of *Rhodococcus ruber* that is responsible for the binding of this phasin to polyhydroxyalkanoic acid granules. *J. Bacteriol.*, 177: 2513-2523.
- Pötter, M., M. Madkour, F. Mayer and A. Steinbüchel (2002). Regulation of phasin expression and polyhydroxyalkanoate (PHA) granule formation in *Ralstonia eutropha* H16. *Microbiol*, 148: 2413-26.
- Pötter, M., H. Müller, F. Reinecke, R. Wiczorek, F. Fricke, B. Bowien, B. Friedrich and A. Steinbüchel (2004). The complex structure of polyhydroxybutyrate (PHB) granules: four orthologous and paralogous phasins occur in *Ralstonia eutropha*. *Microbiol*, 150:2301-2311.
- Pötter, M., H. Müller and A. Steinbüchel (2005). Influence of homologous phasins (*PhaP*) on PHA accumulation and regulation of their expression by the transcription repressor *PhaR* in *Ralstonia eutropha* H16. *Microbiol*, 151: 825-833.
- Preusting, H., J. Kingma, G. Huisman, A. Steinbüchel and B. Witholt (1993). Formation of polyester blends by a recombinant strain of *Pseudomonas oleovorans*: different poly(3-hydroxyalkanoates) are stored in separate granules. *J. Environ. Polym. Degrad.*, 1: 11-21.
- Prieto, M.A., B. Buhler, K. Jung, B. Witholt and B. Kessler (1999). *PhaF*, a polyhydroxyalkanoate granule-associated-protein of *Pseudomonas oleovorans* GPo1 involved in the regulatory expression system for *pha* genes. *J. Bacteriol.*, 181: 858-68.
- Qi, Q., B.H.A. Rehm and A. Steinbüchel (1997). Synthesis of poly(3-hydroxyalkanoates) in *Escherichia coli* expressing the PHA synthase gene *phaC2* from *Pseudomonas aeruginosa*: comparison of *PhaC1* and *PhaC2*. *FEMS Microbiol. Lett.*, 157: 155-162.
- Qi, Q., A. Steinbüchel and B.H.A. Rehm (2000). In vitro synthesis of poly(3-hydroxydecanoate): purification and enzymatic characterization of type II polyhydroxyalkanoate synthases *PhaC1* and *PhaC2* from *Pseudomonas aeruginosa*. *Appl. Microbiol. Biotechnol.*, 54: 37-43.
- Rehm, B.H.A., N. Krüger and A. Steinbüchel (1998). A new metabolic link between fatty acid de novo synthesis and polyhydroxyalkanoic acid synthesis. The *phaG* gene from *Pseudomonas putida* KT2440 encodes a 3-hydroxyacyl-acyl carrier protein-coenzyme a transferase. *J. Biol. Chem.*, 273: 24044-24051.
- Rehm, B.H.A and A. Steinbüchel (1999). Biochemical and genetic analysis of PHA synthases and other proteins required for PHA synthesis. *Int. J. Biol. Macromol.*, 25:3-19.
- Rehm, B.H.A., Q. Qi, B.B. Beermann, H.J. Hinz and A. Steinbüchel (2001). Matrix-assisted in vitro refolding of *Pseudomonas aeruginosa* class II polyhydroxyalkanoate synthase from inclusion bodies produced in recombinant *Escherichia coli*. *Biochem. J.*, 358: 263-268.
- Rehm, B.H.A. (2003). Polyester synthases: natural catalysts for plastics. *Biochem. J.*, 376: 15-33.
- Rehm, B.H.A. (2006). Genetics and biochemistry of polyhydroxyalkanoate granule self-assembly: The key role of polyester synthases. *Biotechnol. Lett.*, 28: 207-213.
- Rodrigues, F., H.E. Valentin, P.A. Berger, M. Tran, J.M Asrar, K. Gruys and A. Steinbüchel (2000). Polyhydroxyalkanoate accumulation in *Burkholderia* sp.: a molecular approach to elucidate the genes involved in formation of two distinct short-chain homopolymers. *Appl. Microbiol. Biotechnol.*, 53: 453-460.
- Saegusa, H., M. Shiraki, C. Kanai and T. Saito (2001). Saito Cloning of an intracellular poly[D(-)-3-hydroxybutyrate] depolymerase gene from *Ralstonia eutropha* H16 and characterization of the gene product. *J. Bacteriol.*, 183: 94-100.
- Schubert, P., A. Steinbüchel and H.G. Schlegel (1988). Cloning of the *Alcaligenes eutrophus* genes for synthesis of poly-β-hydroxybutyric acid (PHB) and synthesis of PHB in *Escherichia coli*. *J. Bacteriol.*, 170: 5837-5847.
- Schubert, P., N. Krüger and A. Steinbüchel (1991). Molecular analysis of the *Alcaligenes eutrophus* poly(3-hydroxybutyrate) biosynthetic operon: identification of the N-terminus of poly(3-hydroxybutyrate) synthase and identification of the promoter. *J. Bacteriol.*, 173: 168-175.
- Shimamura, E., K. Kasuya, G. Kobayashi, T. Shiotani, Y. Shima and Y. Doi (1994). Physical properties and biodegradability of microbial poly(3-hydroxybutyrate-co-3-hydroxyhexanoate). *Macromol.*, 27: 878-880.
- Slater, S.C., W.H. Voige and D.E. Dennis (1988). Cloning and expression in *Escherichia coli* of the *Alcaligenes eutrophus* H16 poly-b-hydroxybutyrate biosynthetic pathway. *J. Bacteriol.*, 170: 4431-4436.
- Steinbüchel, A. (1991). Recent advances in the knowledge of bacterial poly(hydroxy-alkanoic acid) metabolism and potential impacts on the production of biodegradable thermoplastics. *Acta. Biotechnologica.*, 11:419-427.



- Steinbüchel, A and H.G. Schlegel (1991). Physiology and molecular genetics of poly( $\beta$ -hydroxyalkanoic acid) synthesis in *Alcaligenes eutrophus*. *Mol. Microbiol.*, 5: 535-542
- Steinbüchel, A and S. Wiese (1992). A *Pseudomonas* strain accumulating polyesters of 3-hydroxybutyric acid and medium-chain-length 3-hydroxyalkanoic acids. *Appl. Microbiol. Biotechnol.*, 37: 691-697.
- Steinbüchel, A. (1996). PHB and other polyhydroxyalkanoic acids. In: Biotechnology. (H.J. Rehm. and G. Reed eds.). VCH Publishers, Weinheim, Germany.
- Steinbüchel, A and B. Fächtenbusch (1998). Bacterial and other biological systems for polyester production. *Trend. Biotechnol.*, 16: 419-427.
- Stuart, E.S., A. Tehrani, H.E. Valentin, D. Dennis, R.W. Lenz and R.C. Fuller (1998). Protein organization on the PHA inclusion cytoplasmic boundary. *J. Biotechnol.*, 64: 137-144
- Sudesh, K., T. Fukui, and Y. Doi (1998). Genetic analysis of *Comamonas acidovorans* polyhydroxyalkanoate synthase and factors affecting the incorporation of 4-hydroxybutyrate monomer. *Appl. Environ. Microbiol.*, 64: 3437-3443.
- Tian, J., A.J. Sinskey and J. Stubbe (2005). Kinetic studies of polyhydroxybutyrate granule formation in *Wautersia eutropha* H16 by transmission electron microscopy. *J. Bacteriol.*, 187: 3814-3824.
- Tian-Ren, L., L. Jer-Sheng, S. Wang and G. Shaw (2004). *PhaQ*, a New Class of Poly- $\beta$ -Hydroxybutyrate (PHB)-Responsive Repressor, Regulates *phaQ* and *phaP* (Phasin) Expression in *Bacillus megaterium* through Interaction with PHB. *J. Bacteriol.*, 186(10): 3015-3021.
- Timm, A., D. Byrom and A. Steinbüchel (1990). Formation of blends of various poly(3-hydroxyalkanoic acids) by a recombinant strain of *Pseudomonas oleovorans*. *Appl. Microbiol. Biotechnol.*, 33: 296-301.
- Tombolini, R., S. Povolo, A. Buson, A. Squartini and M.P. Nuti (1995). Poly- $\beta$ -hydroxybutyrate (PHB) biosynthetic genes in *Rhizobium meliloti* 41. *Microbiol.*, 141: 2553-2559.
- Valentin, H and A. Steinbüchel (1993). Cloning and characterization of the *Methylobacterium extorquens* polyhydroxyalkanoic acid synthase structural gene. *Appl. Microbiol. Biotechnol.*, 39: 309-317.
- van der Leij, F.R and B. Witholt (1995). Strategies for the sustainable production of new biodegradable polyesters in plants: a review. *Can. J. Microbiol.*, 41: 222-238.
- Wong, A.L., H. Chua and P.H.F. Yu (2000). Microbial production of polyhydroxyalkanoates by bacteria isolated from oil wastes. *Appl. Biochem. Biotechnol.*, 84-86: 843-857.
- York, G.M, J., Stubbe and A.J. Sinskey (2002). The *Ralstonia eutropha* *PhaR* protein couples synthesis of the *PhaP* phasin to the presence of polyhydroxybutyrate in cells and promotes polyhydroxybutyrate production. *J. Bacteriol.*, 184: 59-66.
- Yu, J. (2001). Production of PHA from starch wastewater via organic acids. *J. Biotechnol.*, 86: 105-112.
- Zeller, M., M. Pötter, F. Reinecke, A. Steinbüchel, W.I. Burkitt, P.J. Derrick and S. König (2005). Mass spectrometry identifies homologues of phasin *PhaP1* protein of *Ralstonia eutropha* on polyhydroxybutyrate granules. *J. Biomacromol. Mass Spectrom.*, 1: 75-84.

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