Carbonosomes

Dieter Jendrossek

Contents

Abstract The understanding of the physiology of poly(3-hydroxybutyric acid) (PHB) and related polyhydroxyalkanoic acids (PHAs) has made great progress since the identification and cloning of the first PHB synthase gene from *Ralstonia eutropha* H16 in three laboratories in the late 1980s. In particular, the identification of many proteins as part of the proteinaceous surface layer of PHB and PHA granules and their classification into PHA synthases, PHA depolymerases, phasins, PHA- and DNA-binding proteins and others has led to the conclusion that PHB and PHA granules are functional supramolecular complexes for with the designation as

D. Jendrossek (✉)
Institute of Microbiology, University of Stuttgart, Stuttgart, Baden-Württemberg, Germany
e-mail: dieter.jendrossek@imb.uni-stuttgart.de

D. Jendrossek (ed.), *Bacterial Organelles and Organelle-like Inclusions*,
Microbiology Monographs 34, https://doi.org/10.1007/978-3-030-60173-7_10

carbonosomes has been suggested. In this chapter, the current knowledge on the functions of PHA granule associated proteins (PGAPs) and the composition of carbonosomes will be summarized at the example of PHB granules from *R. eutropha* and of PHA granules from *Pseudomonas putida*.

1 Introduction

Many prokaryotic species are able to synthesise short-chain-length polyhydroxyalkanoates (scl-PHAs) such as poly(3-hydroxybutyrate), PHB, or related medium-chain-length polyhydroxyalkanoates (mcl-PHAs). The bacteria accumulate these storage compounds in the form of ≈200 to ≈600 nm insoluble, granule-like inclusions (PHA granules) when they are cultivated under unbalanced growth conditions, e.g. at high carbon to nitrogen source ratios. PHA granules consist of a polyester core and a surrounding surface layer of several (up to 16) proteins. These so-called PHA granule-associated proteins (PGAPs) have specific, in parts, unknown functions in the synthesis, maintenance and mobilisation (intracellular reutilisation by depolymerisation) of PHAs.

The complexity of PHA granules with many PGAPs at the PHA surface indicates that PHA granules have more functions than that of a carbon and energy reserve material only. This is in agreement with data from several laboratories suggesting that PHA granules are important for the adaptation of cells to oxidative and other stresses. To indicate that PHA granules are supramolecular complexes and have organelle-like properties, the designation "carbonosomes" had been proposed as generic term for PHA granules (Jendrossek 2009). This review will provide an overview on the composition of the carbonosome core and of the carbonosome surface structure using *Ralstonia eutropha* and *Pseudomonas putida* as a model species for scl-PHA- and mcl-PHA-accumulating species, respectively. The function(s) of different PGAPs in the physiology of the accumulating species will be described and discussed. The biochemical pathways leading to the precursors of PHA monomers in different species have been extensively explored and are summarised in several previous reviews (for examples, see Anderson and Dawes 1990; Steinbüchel et al. 1993b; Steinbüchel and Valentin 1995; Madison and Huisman 1999; Pötter and Steinbüchel 2006) and will be not part of this contribution.

2 Composition of the Carbonosome Core

The term "carbonosome" in this review is used for all kinds of PHA granules in the native (in vivo) form in prokaryotes. Carbonosomes are defined as supramolecular complexes that consist of a polymer core with a backbone of mainly carbon atoms

Fig. 1 General structure of PHAs. (**a**) Polyoxoester, (**b**) Polythioester

and are surrounded by a surface layer of at least three types of proteins: (1) a polymer synthase that catalyses the polymerisation of the polymer core from the respective monomers, (2) a polymer depolymerase that catalyses the depolymerisation of the polymer to monomers and or oligomers and (3) one or more other types of proteins that contribute to or even define the properties of the surface layer. In the case of PHB or PHA granules, the phasins (PhaPs) fulfil this function. Granulose and glycogen that are synthesised, e.g. by many cyanobacteria, have proteins of type (1) and (2), but, at present, no evidence is available whether phasin homolog-like proteins of type (3) are present in vivo in granulose/glycogen-forming species. If such compounds will be detected, granulose/glycogen would represent another subtype of carbonosomes (polysaccharide-type) in comparison to polyester type of carbonosomes. For similar reasons cyanophycin granules at present are not (yet) considered as carbonosomes.

The core of polyester carbonosomes consists of the PHA polyester with the general formula shown in Fig. 1. In addition to these polyoxoesters, polythioesters such as poly(3-mercaptopropionate) can be also synthesised by some species if the bacteria are cultivated in the presence of thiol-containing precursors (Lütke-Eversloh et al. 2001; Thakor et al. 2005). Remarkably, and in sharp contrast to all PHAs, such polythioesters seem to be not biodegradable (Elbanna et al. 2004).

The molecular masses of the formed polymers in most species are in the range of $\approx 10^5$ to $\approx 10^7$ Da (Suzuki et al. 1988; Kawaguchi and Doi 1992; Myshkina et al. 2008; Adaya et al. 2018). Depending on the genotypic and metabolic capabilities of the PHA-accumulating species, the type of its PHA synthase and the available growth substrate, the monomeric constituents of the formed PHAs can verify to a large extent. Nevertheless, the most prominent and most common PHA is the homopolyester PHB consisting of ester-linked 3-hydroxybutyrate units only. However, due to the low substrate specificity of most PHA synthases with respect to the number of carbon atoms of the fatty acid monomer moiety and the position of the hydroxy group, PHAs can consist of monomers with 3 to at least 14 carbon atoms. The side group R might contain halogen atoms, unsaturated carbon-carbon bonds or even contain aromatic groups (Abraham et al. 2001; Olivera et al. 2010). Furthermore, PHA synthases can polymerise various substrates into the same polymer molecules if the metabolism of the accumulating strain is able to synthesise different CoA-activated monomeric precursors simultaneously. As a consequence, the resulting polyesters consist of two or more different hydroxyalkanoic acids and are copolyesters. The (bio)physical properties of PHA copolymers largely depend on the monomeric composition. For overviews on the complexity of possible compositions of microbially produced PHAs, see previous reviews (Steinbüchel and Valentin 1995; Meng and Chen 2018). For some applications of PHAs, it might be desirable

to have homopolyesters. However, with the exception of PHB and polyhydroxyvalerate (PHV) (Steinbüchel et al. 1993a), most naturally produced PHAs are copolyesters. Homopolyester of PHAs can be produced by genetically modified organisms (GMOs), in which the β-oxidation pathway of fatty acids is the only pathway leading to CoA-activated monomers and the shortening of the substrate molecules by acetyl-CoA units is blocked by mutation (Liu et al. 2011). These GMOs accumulate PHA homopolyesters with a number of carbon atoms in the monomer that corresponds to the number of carbon atoms of the substrate fatty acid. For overview, see Meng and Chen (2018).

3 Protein Composition of the Carbonosome Surface Layer

PHA granules have a higher density than most other cellular components and form a distinct band after density gradient centrifugation of crude extracts obtained from PHA-containing cells with glycerol or sucrose gradients. PHA granules can be further purified by repeated centrifugation and washing steps. For details, see the pioneer works of J.W. Merrick and B. Witholt (Merrick and Doudoroff 1964; Griebel et al. 1968; de Smet et al. 1983); a methodical overview is given in Jendrossek (2007). The proteins of the isolated PHA granule fraction can be then analysed by SDS-PAGE and identified by proteome analysis. The first identification of PGAPs from purified carbonosomes was published in 1992 using *Chromatium vinosum* as a PHA-accumulating species (Liebergesell et al. 1992). Since then, numerous reports were published describing the isolation of PHA granules and identification of PGAPs from several species, e.g. *Pseudomonas oleovorans* (Fuller et al. 1992), *R. eutropha* (Wieczorek et al. 1995; Jendrossek and Pfeiffer 2014), *Herbaspirillum seropedicae* (Tirapelle et al. 2013), *Azotobacter vinelandii* (Adaya et al. 2018; Moreno et al. 2019) and many other species. In these publications, analysis of purified carbonosome fractions revealed the presence of several to many protein bands by SDS-PAGE. The number and intensities of these bands largely differed for carbonosome fractions obtained from different species and were also dependent on the staining method (Coomassie versus silver staining). In all cases it is not possible to decide whether a particular protein band of a carbonosome fraction represents a true PGAP in vivo or has become artificially bound to PHA granules during cell disruption and the carbonosome isolation process. That some proteins can artificially bind to PHB granules was already shown in the very first description of purified PHB granules from *C. vinosum* (Liebergesell et al. 1992) in which lysozyme, which had been added to the cells during isolation procedure, was tightly bound to purified PHA granules. Additional evidence for the artificial binding of proteins to carbonosomes comes from experiments, in which PHB granules were analysed that had been isolated from recombinant *E. coli* expressing the PHB biosynthetic *phaCAB* operon of *R. eutropha.* SDS-PAGE of this fraction revealed a large number of proteins that could not be removed even by repeated washing steps of the isolated PHB granules using buffers with different ionic strengths (Handrick

et al. 2004b). This points to a high affinity of some *E. coli* proteins to the PHB granule surface. The number of proteins, which were identified in isolated carbonosome preparations, dramatically increased up to several hundreds with the improvement of the sensitivity of proteome analysis (Tirapelle et al. 2013; Jendrossek and Pfeiffer 2014). In conclusion, the identification of a specific protein in a PHB granule preparation does not proof whether this protein is a true PGAP in the PHB-accumulating strain; it may represent a false-positive protein that has some affinity to the (hydrophobic) surface of PHA granules and binds to isolated PHA granules in vitro. Therefore, additional evidence is necessary for the identification of a true PGAP in PHA-accumulating species. One possibility is the identification of PGAPs in transmission electron micrographs of thin sections of PHA-accumulating cells by the immuno-gold technique with a PGAP-specific antibody and a gold-labelled secondary antibody. This technique has been used to identify the PHB synthase and/or the phasin proteins at the surface of PHA granules in several PHA-accumulating species (Gerngross et al. 1993; Liebergesell et al. 1994; Pieper-Furst et al. 1994; Wieczorek et al. 1995; Follner et al. 1997; Pötter et al. 2002). A second possibility is the construction of gene fusions coding for fusion proteins of a PGAP candidate fused to a green fluorescent protein variant followed by fluorescence microscopical localisation of the fusion protein in living cells. A colocalisation of the fusion protein with PHA granules that can be visible in light microscopy if the granules have a sufficient diameter or that have been stained with another fluorophore (e.g. Nile red) will then indicate whether or not the test protein is indeed a PGAP in vivo. This technique was first applied in 1999 for the localisation of a PHB synthase and a phasin protein of *Bacillus megaterium* by McCool and Cannon (1999) and later became the method of choice for the identification of a considerable number of PGAPs in many PHA-accumulating species such as *R. eutropha* (Handrick et al. 2004a; Uchino et al. 2007; Neumann et al. 2008), *Rhodospirillum rubrum* (Jendrossek 2005; Sznajder and Jendrossek 2011), *Magnetospirillum gryphiswaldense* (Schultheiss et al. 2005), *Pseudomonas putida* (Ruth et al. 2008) or *Synechocystis* sp. (Hauf et al. 2015).

However, both methods require either a time-consuming purification of the protein of interest and generation of specific antibodies or substantial molecular biological cloning work for each test protein. Such procedures might be practicable to perform for one or two key enzymes of PHA metabolism if other indirect evidence is already available that the protein most likely is a true PGAP. However, these approaches can't be easily performed for the high number of proteins that are nowadays identified by the sensitive proteome analysis methods. For example, the number of identified proteins in two independently isolated batches of carbonosome preparations from *R. eutropha* amounted 404 and 268 proteins (Jendrossek and Pfeiffer 2014; Sznajder et al. 2015). In this case, comparative proteome analysis can be applied to reduce the number of false-positive proteins. To this end, the proteomes of different cell fractions (e.g. soluble fraction, membrane fraction and PHB granule fraction) can be determined, and those proteins that are specifically present in the PHB granule fraction only are likely candidates for in vivo PGAPs. Using comparative proteome analysis with *R. eutropha*, 14 proteins were identified

to be specifically present in the PHB granule fraction (Sznajder et al. 2015). However, the two most abundant proteins of PHA granules (phasin PhaP1 and PHB synthase PhaC1) were also present in traces in the soluble fraction. The in vivo colocalisation of these 14 proteins with PHA granules was verified by fusion analysis of GFP variants. This approach enabled the identification of several new PGAPs (Sznajder et al. 2015).

4 The Carbonosome of *Ralstonia eutropha*

R. eutropha H16 was isolated in Hans G. Schlegel's lab in 1962 from the Weende spring in Göttingen, Germany, as a chemolithoautotrophic bacterium (Wilde 1962). It is also able to grow heterotrophically on some sugars, a large variety of fatty acids and many other carbon sources. It was first considered as a suitable species to produce single-cell protein from carbon dioxide and hydrogen in the 70th of the last century. However, the high content of (ribo)nucleic acids in prokaryotes can cause gout in mammals, and this prevented the use of *R. eutropha* as alternative food source. In the 1980s, the ability of *R. eutropha* to accumulate large amounts of PHB up to approximately 90% of its cellular dry weight from renewable resources came into focus (Schubert et al. 1988; Peoples and Sinskey 1989). PHB was considered as a promising biodegradable alternative to chemosynthetically produced polymers, and this was the starting point of worldwide increasing research activities on the molecular biological background of PHB biosynthesis. Although a variety of Gram-negative, Gram-positive and even some *Archaea* species were chosen to investigate the biosynthesis, maintenance and mobilisation of PHA, it was *R. eutropha* that became the model organism of PHB research, and meanwhile the PHB granules of *R. eutropha* H16 are the best characterised carbonosomes of all studied species. In the next paragraph, I will first summarise the current knowledge about the composition and properties of the PHB granules in *R. eutropha* and then contrast them with the properties of carbonosomes from mcl-PHA-accumulating species using *P. putida* as an example.

5 Proteins of the *R. eutropha* Carbonosome

Sixteen proteins have been identified as PGAPs by comparative proteome analysis of isolated PHB granules (Table 1, Fig. 2) (Sznajder et al. 2015). These are the following: the PHB synthases PhaC1 and PhaC2, the PHB depolymerase PhaZa1 and three other putative hydrolases (a putative phospholipase [H16_A0225], two putative PHB depolymerases PhaZe1 [H16_A0671] and PhaZe2 [H16_B1632]), the phasins PhaP1 to PhaP8 and PhaR and PhaM. All 16 proteins were identified in 2 separate batches of PHB granules that had been isolated from NB- or MSM-gluconate-grown cultures of *R. eutropha,* respectively. Interestingly, the abundance

Table 1 PHA depolymerases and/or PHB oligomer hydrolases

Species	Protein/ (KEGG)	Locus tag	Length (aa)	Mw [kDa]	Active site lipase box	Localisation/remark	Reference
R. eutropha H16	PhaZa1 (PhaZ1)	H16_A1150	419	47.3	$SVC_{183}QP$	nPHB	Handrick et al. (2000); Saegusa et al. (2001); Uchino et al. (2007); Eggers and Steinbüchel (2013)
R. eutropha	PhaZa2 (PhaZ2)	H16_A2862	404	44.8	$AIC_{173}QP$	Cytoplasm[b]	Leuprecht (2012)
R. eutropha	PhaZa3 (PhaZ5)	H16_B1014	407	45.2	$AVC_{175}QP$	nPHB[b]	Leuprecht (2012)
R. eutropha	PhaZa4 (PhaZ4)	H16_PHG178	245[a]	27.4[a]	$GLC_{?}EG$[a]	nPHB[b]	Leuprecht (2012)
R. eutropha	PhaZa5 (PhaZ3)	H16_B0339	412	45.2	$AIC_{182}QA$	nPHB[b]	Leuprecht (2012)
R. eutropha	PhaZb (PhaY1)	H16_A2251	718	74.3	$SVS_{320}NG$	Cytoplasm/nPHB	Kobayashi et al. (2003) Leuprecht (2012)
R. eutropha	PhaZc (PhaY2)	H16_A1335	293	31.6	$GTS_{108}MG$	Cytoplasm/nPHB	Kobayashi et al. (2005); Leuprecht (2012)
R. eutropha	PhaZd1 (PhaZ6)	H16_B2073	362	39.2	$GMS_{190}AG$	High activity, capacity to bind to nPHB[b]	Sznajder and Jendrossek (2014)
R. eutropha	PhaZd2 (PhaZ7)	H16_B2401	365	38.4	$GLS_{193}AG$	High activity, capacity to hydrolyse nPHB[b]	Abe et al. (2005); Sznajder and Jendrossek (2014)
R. eutropha	PhaZe1	H16_A0671	761	83.9	$GNC_{190}QA$	nPHB	Sznajder et al. (2015)
R. eutropha	PhaZe2	H16_B1632	773	86.4	$GNC_{212}QG$	nPHB	Sznajder et al. (2015)
A. vinelandii OP	PhaZ1	Avin_03910	914	101	$GNC_{215}QA$	nPHB	Adaya et al. (2018)

(continued)

Table 1 (continued)

Species	Protein/ (KEGG)	Locus tag	Length (aa)	Mw [kDa]	Active site lipase box	Localisation/remark	Reference
P. putida GP01	PhaZ		283	31.5	$GVS_{102}WG$	mcl-PHA	Huisman et al. (1991); de Eugenio et al. (2007)
P. putida KT2440	PhaZ	PP_5004	283	31.4	$GVS_{102}WG$	mcl-PHA	Nelson et al. (2002)

The designation of PHB depolymerase proteins in the literature and in KEGG data base and the respective locus tags and important features are given. Question marks indicate that the position of the amino acid residue is questionable due to uncertain gene start

[a]Correct gene start and gene length questionable

[b]Data obtained by artificial overexpression, protein not found in isolated nPHB granules of wild-type cells

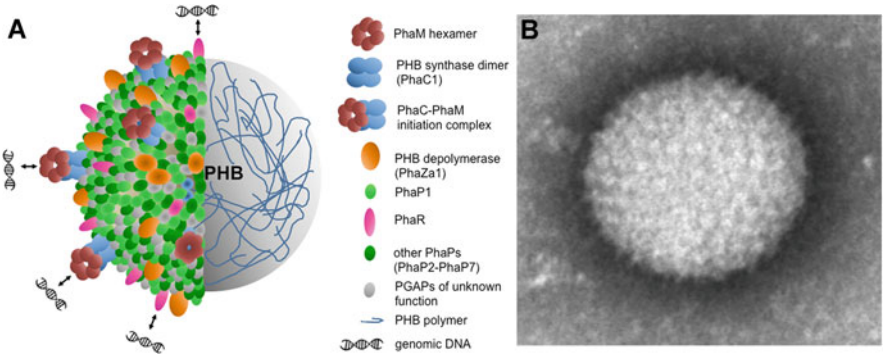

Fig. 2 PHB carbonosome. Schematic view of a PHB carbonosome in *R. eutropha* (**a**). PGAP proteins of the surface layer are symbolised in the left half of the PHB granule. Association of PhaM and of PhaR to regions on the genome is indicated by a symbol of a DNA helix. Image by F. Becker. The image in (**b**) shows a liberated PHB granule of *Caryophanon latum* after negative staining. Image modified from Jendrossek et al. (2007). The visible globular-like structures presumably represent PGAPs

of PGAPs in PHB granules of nutrient broth (NB)-grown cells was considerably higher than in PHB granules isolated from mineral salts medium (MSM)-grown cells. All identified proteins were associated with PHB granules in vivo as confirmed by the colocalisation of Nile red-stained PHB granules and fusions of these proteins with green fluorescent protein variants. The 16 identified PGAPs can be categorised into 4 subgroups: (1) PHB synthases (PhaCs), (2) phasins (PhaPs), (3) PHB depolymerases (PhaZs) and related hydrolases and (4) proteins with additional binding ability to DNA. Figure 2 shows a TEM image and a cartoon of a *R. eutropha* carbonosome.

5.1 PHB Synthases (PhaCs)

The PHA synthase is the key enzyme of PHA biosynthesis and catalyses the last step in PHA biosynthesis, i.e. the polymerisation of a hydroxyalkanoic acid from its CoA-activated monomer. In principle, the presence of a PHA synthase and a suited CoA-activated hydroxyalkanoic acid is sufficient to form a high molecular weight polymer (Gerngross and Martin 1995). In the case of *R. eutropha,* the PHB synthase is covalently linked to the growing polymer chain via the active site cysteine Cys319 (Gerngross et al. 1994; Wodzinska et al. 1996). The structure of the *R. eutropha* PHB synthase has been solved (Wittenborn et al. 2016; Kim et al. 2017a, b). The PHB-bound PHA synthase can be used as a marker protein for the identification and localisation of (native) PHA granules in vivo.

The first example of the use of fluorescent proteins for the *in vivo* co-localisation of PHA-associated proteins with PHB has been published in M. Cannon's lab

(McCool and Cannon 1999) at the example of the PHA synthase PhaC subunit and of the phasin PhaP in *Bacillus megaterium*. In this publication, fusions of PhaC and PhaP with the green fluorescent protein (GFP) were generated and formed fluorescent foci at the same position as PHB granules. Since then, several reports have been published in which PHA synthases in PHA-producing species were fused to fluorescent proteins (Jendrossek 2005; Peters and Rehm 2005; Jahns and Rehm 2009). An (almost forgotten) property of the PHB synthase PhaC1 of *R. eutropha* is the ability of the enzyme to catalyse the reverse reaction, i.e. the cleavage of PHB in the presence of free coenzyme A (CoA) to 3-hydroxybutyryl-CoA (3HB-CoA, thiolytic activity of PhaC1) (Uchino and Saito 2006). Thiolysis has been also detected for intracellular PHB depolymerase PhaZa1 (see below).

R. eutropha H16 has a second PHB synthase gene (*phaC2*). Transcription analysis and proteome determination of a PHB granule fraction and fusion analysis with eYFP confirmed that *phaC2* is expressed and that the gene product represents a true PGAP in *R. eutropha* although the degree of expression is very low (Peplinski et al. 2010; Brigham et al. 2012; Sznajder et al. 2015). PhaC2 is unable to synthesise PHB in a *ΔphaC1* background, but it can restore PHB accumulation in a *R. eutropha* background with a mutated, non-functional *phaC1* gene (strain PHB-04; for details, see Peplinski et al. (2010); Pfeiffer and Jendrossek (2012)). The function of PhaC2 in *R. eutropha*, if any, is obscure.

5.2 Phasins (PhaPs)

A common feature of carbonosomes in all investigated carbonosome-forming species is the presence of one or several so-called phasin proteins (PhaPs). Phasins are small amphiphilic proteins that cover a considerable part of the carbonosome surface and are thought to mediate between the hydrophobic polymer core and the hydrophilic cytoplasm (Steinbüchel et al. 1995). The first phasin (PhaP1) was discovered in A. Steinbüchel's laboratory by the characterisation of transposon-induced mutants that had a reduced but not completely abolished ability to accumulate PHB (so-called "PHB-leaky" phenotype) (Wieczorek et al. 1995). Phasins determine the surface-to-volume ratio of produced PHB granules: in the absence of PhaP1, *R. eutropha* synthesises less PHB compared to the wild type in the form of only one very big PHB granule, whereas an overproduction of PhaP1 leads to the formation of an increased number of unusually small PHB granules. The observation that phasins have a strong impact on the size of the produced PHB granules has been shown for many PHA-accumulating species (see Table 2 of Mezzina and Pettinari 2016) (Sun et al. 2019). Remarkably, eight phasin proteins (PhaP1–PhaP8) have been identified in PHB granules isolated from *R. eutropha* (Table 1) (Pötter et al. 2004; Kuchta et al. 2007; Pfeiffer and Jendrossek 2011; Pfeiffer and Jendrossek 2012; Sznajder et al. 2015). PhaP1 is by far the most abundant protein in PHB granule preparations, and deletion of the *phaP1* gene has a strong impact on PHB granule number and granule diameter. PhaP2, PhaP3 and PhaP4 are also present in

substantial abundances in PHB granule preparations even though in much lower amounts than PhaP1 (Table 1); however, deletion of *phaP2, phaP3* and *phaP4* (single or in combination (Kuchta et al. 2007)) or of *phaP5, phaP6, phaP7* or *phaP8* (Pfeiffer and Jendrossek 2011; Pfeiffer and Jendrossek 2012; Schulze 2018) had no detectable phenotype in granule numbers or size. The overexpression of phasin genes (*phaP5, phaP6, phaP7* or *phaP8*) fused with *eyfp* led to a more polar localisation of clusters of PHB granules (Pfeiffer and Jendrossek 2011, 2012; Sznajder et al. 2015).

5.3 PHB Depolymerases (PhaZs)

Intracellular PHB depolymerases are the key enzymes for the reutilisation (mobilisation) of the storage polymer in the PHB-accumulating strain itself and catalyse the cleavage of PHAs to monomers and/or oligomers. Intercellular PHB depolymerases share only little homology to extracellular PHB depolymerases. The latter are secreted by PHA-degrading species that are able to utilise exogenous, denatured (partially crystalline) PHB as a carbon source but must not necessarily be able to accumulate PHB.

While no extracellular PHB depolymerase activity could be detected in culture fluids of *R. eutropha* (unpublished data), this species has a high number of intracellular enzymes with PHB depolymerase activity: the intracellular PHB depolymerase PhaZa1 is the main PHB depolymerase responsible for the mobilisation of PHB in carbon-starved cells (Handrick et al. 2000; Saegusa et al. 2001; Jüngert et al. 2017). It is bound to PHB granules in vivo as shown by fusion with GFP (Uchino et al. 2007; Sznajder and Jendrossek 2011; Sznajder et al. 2015). In contrast to most extracellular PHB depolymerases that hydrolyse extracellular (partially crystalline) PHB, i.e. PHB that has been released from lysed, dying bacteria, intracellular PHB depolymerases can cleave only the so-called native PHB granules (Merrick and Doudoroff 1964) that are in the amorphous state (for overviews on PHB degradation by extracellular PHA depolymerases, see Jendrossek (2001); Jendrossek and Handrick (2002)). Extracellular PHB depolymerases have a substrate-binding domain that enables them to bind to denatured (crystalline) PHB. The binding domain of intracellular PHB depolymerases has not been defined, yet. Native PHB granules harbouring PhaZa1 hydrolyse PHB to 3HB and oligomers of 3HB at a low rate in an aqueous in vitro system (Saegusa et al. 2001). However, in the presence of added CoA, the rate of hydrolysis decreased, and 3HB-CoA was formed (Uchino et al. 2007, 2008). This indicated that PhaZa1 in vivo most likely catalyses a thiolytic cleavage of PHB, thus saving the energy of the ester bonds in PHB in the thioester bond of 3HB-CoA. The thiolytic cleavage of PHB to crotonyl-CoA (in addition to (*R*)-3HB-CoA) was shown in a PHB synthesising recombinant *E. coli* strain if *phaZa1* was co-expressed with *phaCAB* and *phaP1* (Eggers and Steinbüchel 2013). Remarkably, when these experiments were performed with native PHB granules isolated from *R. eutropha*, the amount of formed crotonyl-CoA was reduced, and (*S*)-3HB-CoA was identified as main cleavage product. It was

concluded that *R. eutropha* is able to distinguish between the biosynthesis of PHB via polymerisation of anabolically formed (*R*)-3HB-CoA to PHB and the (catabolic) mobilisation of PHB with PhaZa1 via (*S*)-3HB-CoA (Eggers and Steinbüchel 2013).

R. eutropha has four other putative PHB depolymerases (PhaZa2–PhaZa5) in addition to PhaZa1 (York et al. 2003; Pohlmann et al. 2006). All PhaZa isoenzymes share substantial amino acid similarity, and all have the same (unusual) cysteine-containing lipase box with the (putative) active site cysteine (Cys183 in PhaZa1) (Kobayashi and Saito 2003) (Table 2). The *phaZa2–phaZa5* genes are only poorly expressed under most tested culture conditions, and in agreement with transcript analysis, no evidence for the presence of detectable amounts of PhaZa2 to PhaZa5 was obtained by proteome analysis of NB and MSM cultures of *R. eutropha* (Lawrence et al. 2005; Brigham et al. 2012; Sznajder et al. 2015); the physiological functions of PhaZa2 to PhaZa5 remain unknown. Fusions of *phaZa2, phaZa3, phaZa4* and *phaZa5* with *eyfp* and overexpression in *R. eutropha* under PHB permissive conditions revealed that PhaZ3, PhaZ4 and PhaZ5 are true PGAPs in vivo (after artificial overexpression) (Leuprecht 2012). Surprisingly, PhaZa2-eYFP was detected only in the cytoplasm and did not colocalise with PHB granules.

Two 3-hydroxybutyrate oligomer hydrolases (PhaZb and PhaZc) (Kobayashi et al. 2003; Kobayashi et al. 2005) and two highly active intracellular PHB depolymerases (PhaZd1 and PhaZd2) have been additionally described for *R. eutropha* (Abe et al. 2005; Sznajder and Jendrossek 2014). However, PhaZb and PhaZc were located both in the cytosolic and PHB granule fraction in vitro (Kobayashi et al. 2003, 2005), and fusions of PhaZb or PhaZc with eYFP showed a soluble fluorescence and did not colocalise with accumulated PHB granules (Leuprecht 2012). Therefore, PhaZb and PhaZc can't be considered as PGAPs. Presumably, PhaZb and PhaZc are important for the hydrolysis of soluble 3-hydroxybutyrate dimers and/or other soluble ester compounds.

The PHB depolymerases PhaZd1 and PhaZd2—when expressed from a plasmid under control of the constitutive *phaC* promoter—hydrolysed PHB very efficiently (in vivo) so that free PHB granules were not detectable and a potential colocalisation of the PHB depolymerases with PHB granules could not been investigated. Accordingly, the purified PHB depolymerases PhaZd1 and PhaZd2 hydrolysed native PHB granules (in vitro) with extremely high specific activities (Sznajder and Jendrossek 2014). However, neither PhaZd1 nor PhaZd2 was detectably expressed in *R. eutropha* wild type, and the physiological function of these two PHB depolymerases therefore remains obscure. A colocalisation of a PhaZd1-eYFP fusion with PHB granules was observed if the catalytic site (S190) had been mutated to an alanine resulting in an inactive PhaZd1 protein. This suggested that PhaZd1 theoretically could be considered as a PGAP in *R. eutropha* if PhaZd1 would be expressed. Expression of a PhaZd2-eYFP fusion in *R. eutropha* showed that PhaZd2-eYFP did not colocalise with PHB granules and therefore a physiological function of PhaZd2 in PHB metabolism is unlikely.

Three other putative hydrolases are present in isolated PHB granules of *R. eutropha*. These are the H16_A0225, the H16_A0671 (PhaZe1) and the H16_B1632 (PhaZe2) gene products. All three proteins are putative hydrolases

Table 2 PHA granule-associated proteins (PGAPs)

Species	Protein/ (KEGG)	Locus tag	Length (aa)	Mw [kDa]	Localisation/ evidence	Reference
R. eutropha H16	PhaP1	H16_A1381	192	20	TEM-IG FM SDS-PAGE	Wieczorek et al. (1995)
R. eutropha	PhaP2	H16_PHG202	188	20	SDS-PAGE	Pötter et al. (2004)
R. eutropha	PhaP3	H16:2172	185	20	SDS-PAGE	Pötter et al. (2004)
R. eutropha	PhaP4	H16_B2021	189	20	SDS-PAGE	Pötter et al. (2004)
R. eutropha	PhaP5	H16_B1934	142	16	FM	Pfeiffer and Jendrossek (2011)
R. eutropha	PhaP6	H16_B1988	207	23	FM	Pfeiffer and Jendrossek (2012)
R. eutropha	PhaP7	H16_B2326	142	16	FM	Pfeiffer and Jendrossek (2012)
R. eutropha	PhaP8	H16_A2001	159	18	FM	Sznajder et al. (2015)
R. eutropha	PhaC1	H16_A1437	589	64	TEM-IG FM SDS-PAGE	Gerngross et al. (1993)
R. eutropha	PhaC2	H16_A2003	591	65	FM	Pfeiffer and Jendrossek (2012)
R. eutropha	PhaZa1	H16_A1150	419	47	FM	Uchino et al. (2007)
R. eutropha	PhaZe1	H16_B1632	773	86	FM	Sznajder et al. (2015)
R. eutropha	PhaZe2	H16_A0671	761	84	FM	Sznajder et al. (2015)
R. eutropha		H16_A0225	449	51	FM	Sznajder et al. (2015)
R. eutropha	PhaR	H16_A1440	183	21	SDS-PAGE[a]	Pötter et al. (2002)
R. eutropha	PhaM	H16_A0141	263	27	TH, FM	Pfeiffer et al. (2011)
A. vinelandii OP	PhaZ1	Avin_03910	914	101	SDS-PAGE	Adaya et al. (2018)
A. vinelandii UW136	OprI	Avin_23100		≈7	SDS-PAGE	Adaya et al. (2018); Moreno et al. (2019)
P. putida GP01	PhaC1		559	62	SDS-PAGE	Huisman et al. (1991); Ren et al. (2009)

(continued)

Table 2 (continued)

Species	Protein/ (KEGG)	Locus tag	Length (aa)	Mw [kDa]	Localisation/ evidence	Reference
P. putida GP01	PhaC2		560	63	SDS-PAGE	Huisman et al. (1991); Ren et al. (2009)
P. putida GPo1	PhaZ		283	31.5	SDS-PAGE	Huisman et al. (1991); Foster et al. (1996); Stuart et al. (1996); de Eugenio et al. (2007)
P. putida GPo1	PhaF		255	26	SDS-PAGE	Prieto et al. (1999)
P. putida GP01	PhaI		139	15	SDS-PAGE	Prieto et al. (1999)
P. putida GPo1	Acs1		565	62	FM	Ruth et al. (2008)
P. putida KT2440	PhaZ	PP_5004	283	31.4		Nelson et al. (2002)[b]

TH, two hybrid; FM, fluorescence microscopy; IG, immuno-gold
[a]Binding of PhaR to PHB shown in vitro using artificial PHB granules
[b]*P. putida* KT2440 and its derivative KT2442 strain have the same *pha* genes as *P. putida* GPo1

with H16_A0225 having a phospholipase signature. Fusions of each of the three genes with *eyfp* and expression in *R. eutropha* confirmed that all gene products colocalised with formed PHB granules (Sznajder et al. 2015). Purified H16_A0225 protein had no detectable PHB depolymerase activity with native PHB granules but could hydrolyse *p*-nitrophenyl-decanoic acid. The deletion of H16_A0225 had no detectable phenotype in PHB synthesis or in mobilisation of PHB (Schulze 2018). The physiological function of this PGAP remains therefore unknown. The deletion of each one or of both of the other two genes (*phaZe1* and *phaZe2*) did not substantially change the ability of the mutant to accumulate PHB. However, the two single and the double mutants without *phaZe1* and/or *phaZe2* had slightly higher PHB contents in the stationary growth phase (i.e. during PHB mobilisation), suggesting that the PhaZe1 and PhaZe2 could have PHB depolymerase activity although a biochemical proof with purified proteins is still lacking. Based on the presence of the putative cysteine containing lipase box and similarity to a thiolytic PHB depolymerase of *Azotobacter vinelandii* (Adaya et al. 2018) (Table 2), PhaZe1 and PhaZe2 are supposed to represent thiolytic PHB depolymerases (Sznajder et al. 2015).

Interestingly, H16_B1632 is the first gene of a putative four-genes operon coding for a putative phosphotransacetylase (H16_B1631, *pta1*), a putative acetate kinase (H16_B1630, *ackA*) and a putative enoyl-reductase (H16_B1629, *fabI2*). If indeed the H16_B1632 gene product encodes a thiolytic PHB depolymerase, the formed 3HB-CoA could be converted to 3-hydroxybutyryl-phosphate and subsequently to

3-hydroxybutyrate (3HB) via the action of (unspecific) Pta1 and AckA proteins. This would enable the cells to gain one ATP molecules and to save the energy of the cleaved ester bond. Fabl2 potentially could interconvert 3HB-CoA and crotonyl-CoA; evidence that this reaction is catalysed by isolated native PHB granules from *R. eutropha* has been published, previously (Eggers and Steinbüchel 2013).

5.4 Proteins Binding to PHB Granules and to DNA

Two PGAP proteins have dual subcellular locations and can bind not only to PHB granules but also to genomic DNA in *R. eutropha*. These are the regulator (repressor) of phasin synthesis PhaR and the PhaM protein. PhaR binds to the operator regions of *phaP1*, *phaP3* and *phaR* and represses their transcription (York et al. 2002; Pötter et al. 2002, 2005). However, it also binds to the surface of PHB granules, and—if doing so—it cannot repress the transcription of *phasin* genes and its own transcription. As a consequence, the repression of transcription is released, and PhaP1, PhaP3 and PhaR can be formed. Since phasin proteins have a strong binding affinity to PHB, they displace PhaR molecules from the PHB granule surface, and this in turn enhances the probability that the liberated PhaR molecules bind to the operator regions of *phaP1* and *phaP3* (and *phaR*) leading to a stop of further transcription of *phaP1*, *phaP3* and *phaR*. Thus, the dual binding ability of PhaR to PHB granules and to DNA ensures that the production of PhaP1 and PhaP3 proteins (and of PhaR) is stopped only when the surface of PHB granules is covered with phasin proteins.

The multifunctional PhaM protein does not bind directly to PHB but efficiently interacts with the PHB synthase PhaC1 (Pfeiffer et al. 2011; Ushimaru and Tsuge 2016) and can be co-purified with PhaC (Cho et al. 2012; Schulze 2018). In vitro, purified PhaC1 and purified PhaM form a high molecular weight complex presumably consisting of a PhaC1 dimer and a PhaM hexamer (unpublished data). The specific activity of the PHB synthase in the presence of PhaM is approximately threefold higher, and the lag phase of the in vitro polymerisation reaction of PhaC1 (Gerngross and Martin 1995) is abolished by added PhaM (Pfeiffer and Jendrossek 2014). PHB granule-associated PhaM interacts with and binds to the nucleoid region in vivo. Electrophoretic gel mobility shift analysis (EMSA) confirmed that purified PhaM is able to bind to DNA in vitro, however, in a sequence-unspecific manner. The C-terminal part of PhaM is responsible for this DNA-binding ability because C-terminal truncations of PhaM have lost the ability to bind to DNA. The C-terminus of PhaM is highly basic and harbours histone-like PAKKA motifs. The lysine residues of these PAKKA motifs are essential for the interaction of PhaM with DNA in vivo and in vitro as revealed by site-directed mutagenesis of these residues (Bresan and Jendrossek 2017). As a consequence of the dual binding of PhaM to PhaC1 and to DNA, PHB granules are attached to the nucleoid region. Doubling of the genomic DNA and separation of the two daughter genomes during cell division distributes the genome-attached PHB granules to both daughter cells. Accordingly,

mutants lacking *phaM* are unable to distribute formed PHB granules to both daughter cells during cell division (Pfeiffer et al. 2011; Wahl et al. 2012).

5.5 Other Proteins Bound to PHB Granules

In addition to the above-mentioned reports on true PGAPs of *R. eutropha*, several other proteins were described to be attached to PHB granules in various PHB-accumulating species (Tirapelle et al. 2013; Jendrossek and Pfeiffer 2014; Adaya et al. 2018; Moreno et al. 2019). Depending on the sensitivity of the staining procedure in SDS-PAGE of isolated PHB granule fractions (Coomassie brilliant blue or silver staining) or of the proteome analysis facility, several dozens to a few hundreds of proteins have been detected in purified PHB granule fractions. The annotated functions of these proteins (in *R. eutropha*) are diverse and include proteins involved in RNA and protein biosynthesis (RNA polymerase and ribosome components), energy metabolism (ATP synthase components), transport, C-metabolism (tri-carbonic acid cycle proteins) and others (Jendrossek and Pfeiffer 2014). It is unlikely that so many different proteins are truly bound to carbonosomes in vivo. More likely, the majority of these proteins represent false-positive PGAPs. However, it is difficult to exclude for sure that one or few of these in vitro carbonosome-attached proteins are true PGAPs in vivo. For example, in many reports on proteome analysis of isolated PHA granules, one or several proteins were identified that are annotated as outer membrane proteins. In case of *R. eutropha*, six Omp proteins were identified in the PHB granule fraction (Jendrossek and Pfeiffer 2014). Could it be that some of these Omp proteins are mis-annotated and in fact represent not yet identified PGAPs? A recent study, for example, showed that the outer membrane protein OprI of *Azotobacter vinelandii* is part of the PHB granule proteome and deletion of the o*prI* gene reduced the PHB content of cells grown on solid media (Adaya et al. 2018; Moreno et al. 2019). However, a direct in vivo proof of the presence of OprI in PHB granules, e.g. by fusion analysis with a fluorescent protein, is still missing. In conclusion, a function of Omp proteins in PHA metabolism is possible, but additional in vivo evidence is necessary to confirm this hypothesis.

In PHB-accumulating *Archaea*, two other proteins have been identified as PGAPs: a protein with a patatin-like phospholipase motif was identified in a PHB granule fraction of PHB mobilising *Haloferax mediterranei* cells (Liu et al. 2015). The purified enzyme was able to hydrolyse native PHB granules and thus represented a functional PHB depolymerase. Interestingly, the PHB-associated A0225 protein of *R. eutropha* (see above) also harbours a patatin-like phospholipase motif; however, a PHB depolymerase activity could not be shown for the purified protein (Schulze 2018). Another PHB-associated protein in *H. mediterranei* is the enoyl-CoA hydratase PhaJ1 (Liu et al. 2016).

6 The Carbonosome of *Pseudomonas putida*

The discovery that some bacteria can synthesise and accumulate polyesters consisting of mcl-hydroxyalkanoic acids (mcl-PHAs) goes back to 1983 when B. Witholt's lab published the formation of poly(3-hydroxyoctanoic acid) (PHO) by *P. putida* GPo1 (previously *Pseudomonas oleovorans* GPo1) (de Smet et al. 1983; Lageveen et al. 1988). Since then, numerous *Pseudomonas* species have been identified that are able to synthesise mcl-PHAs (for a selection of the first of those studies, see Huisman et al. (1989); Timm and Steinbüchel (1990); Huijberts et al. (1992)). Depending on the absence or presence of a 3-hydroxyacyl-CoA-acyl carrier protein transferase (*phaG* gene product that links β-oxidation with fatty acid biosynthesis (Rehm et al. 1998)), these species can synthesise mcl-PHAs only from related substrates, i.e. from medium- or long-chain fatty acids, or also from unrelated substrates such as glucose. Key enzyme for the synthesis of mcl-PHAs is the type II PHA synthase (PhaC) that differs from type I PHA synthases of PHB-accumulating bacteria (Rehm 2003; Rehm 2006; Mezzolla et al. 2018; Chek et al. 2019). A common feature of *P. putida* GP01 and other mcl-PHA-producing pseudomonads is the presence of two (related) *phaC* genes (*phaC1* and *phaC2*) on the chromosome that form a gene cluster together with a PHA depolymerase (*phaZ*) gene between *phaC1* and *phaC2* (Huisman et al. 1991; Kim et al. 2006). Meanwhile, two other related strains of *P. putida* (strain KT2440 and its derivative KT2442) have become the model species of mcl-PHA research, and most research activities on the identification of PGAPs in mcl-PHA-accumulating bacteria were performed with *P. putida* KT2440/KT2442. Below, a summary on the present knowledge on PGAPs of PHA granules in *Pseudomonas* species is provided (see also Fig. 3). It should be noted that the described results were obtained with either *P. putida* GPo1 or with *P. putida* KT2440/KT2442.

6.1 mcl-PHA Synthases (mcl-PhaCs)

All studied mcl-PHA-producing pseudomonads have two similar type II PHA synthase genes (*phaC1* and *phaC2*, separated by a PHA depolymerase *phaZ* gene) in the *pha* locus of their respective genomes. Both PHA synthases appear to be generally attached to mcl-PHA granules as revealed from proteome studies of isolated carbonosome preparations of several *Pseudomonas* species (Fuller et al. 1992; Stuart et al. 1996) although in vivo evidence by TEM-immuno-studies or by fusion with fluorescent proteins are rare (Peters and Rehm 2005). Purified PhaC1 and PhaC2 (either on isolated native PHA granules or isolated as hexa-histidine-tagged fusions) are able to catalyse the polymerisation of medium-chain-length hydroxyalkanoic acids (Qi et al. 2000); they differ in their K_m and V_{max} values (125 vs. 37 μM and 41 vs. 2.7 U/mg, respectively) with (*R*)-3-hydroxyoctanoyl-CoA as a substrate (Ren et al. 2009) as well as in their substrate specificities for mainly

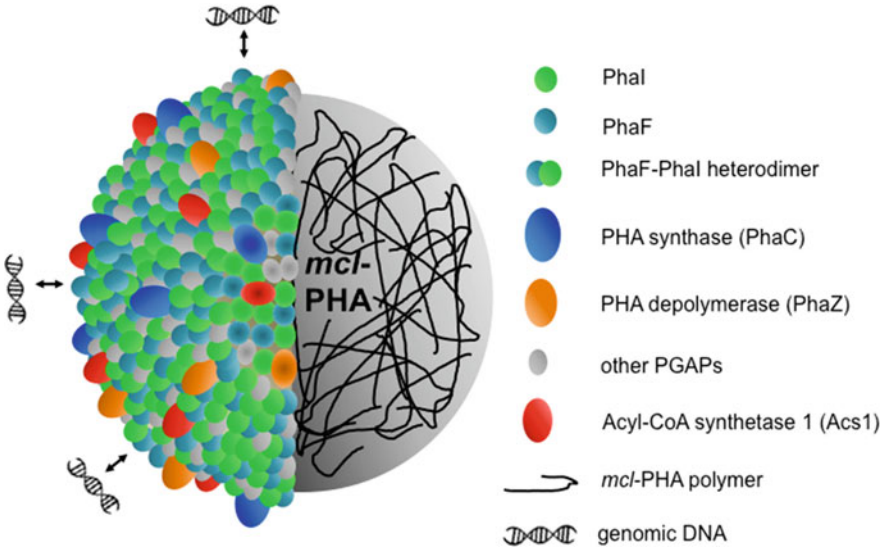

Fig. 3 mcl-PHA carbonosome. Schematic view of a PHA carbonosome in *P. putida*. PGAP proteins of the surface layer are symbolised in the left half of the PHA granule. Associations of PhaF and/or PhaF-PhaI hetero-dimers to the bacterial nucleoid are indicated. Image by F. Becker

mcl-3HA-monomers (PhaC1) or revealing a broad substrate specificity, respectively (short- to long-chain 3HA-monomers) (Chen et al. 2006).

6.2 mcl-PHA Depolymerase (mcl-PhaZs)

mcl-PHA-accumulating *Pseudomonas* species generally have a mcl-PHA depolymerase gene (mcl-*phaZ*) located between the *phaC1* and the *phaC1* genes of the respective *pha* locus. The conclusion that these mcl-PHA depolymerase proteins have indeed mcl-PHA depolymerase activity comes from *phaZ*-mutant analysis (Huisman et al. 1991; Solaiman et al. 2003; Cai et al. 2009), *phaZ* knock-out and *phaZ* overexpression analysis and by showing depolymerase activity of purified proteins with artificially prepared mcl-PHA granules (Foster et al. 1994, 1996; Jiang et al. 2004; de Eugenio et al. 2007, 2008; Isabel de Eugenio et al. 2010a; Arias et al. 2013). Proteome analysis of isolated mcl-PHA granule fractions from several *Pseudomonas* species had identified the mcl-PHA depolymerase protein, suggesting that mcl-PHA depolymerases are PGAPs in these species (Fuller et al. 1992; Stuart et al. 1996). However, in vivo studies that could unequivocally verify the subcellular location of the mcl-PHA depolymerase in vivo are missing. Intracellular mcl-PHA depolymerases of *Pseudomonas* species most likely are serine hydrolases and have a lipase box in the active centre (GxSxG in case of *P. putida* GPo1 (Huisman et al. 1991)) (Table 2). Therefore, mcl-PHA depolymerases are true

hydrolases and are unlikely to have thiolytic activity as PhaZa1 and related isoenzymes of *R. eutropha* with a cysteine as the active site (see above).

6.3 Phasins

mcl-PHA-accumulating *Pseudomonas* species have two types of phasins, namely, PhaI and PhaF. While PhaI and the N-terminal part of PhaF share substantial amino acid similarity and have both coiled coil domains, neither PhaI nor PhaF have a pronounced amino acid similarity to phasins of PHB-accumulating species (Prieto et al. 1999; Maestro et al. 2013). PhaI and PhaF in vivo interact with each other and probably form hetero-oligomers (Tarazona et al. 2019). The N-terminal domain of PhaF is responsible for the attachment to mcl-PHA carbonosomes (Moldes et al. 2004). The function of the C-terminal domain of PhaF will be discussed below.

6.4 Proteins Binding to mcl-PHA Granules and to DNA

A homolog of the PhaR transcriptional repressor protein of *R. eutropha* that would be able to bind to mcl-PHA carbonosomes and to the operator region of phasin genes is not present in *P. putida* and has not been identified in any other mcl-PHA-accumulating species. Instead, a PhaD protein is present in *P. putida* that activates the transcription of PHA biosynthetic genes under appropriate conditions (Isabel de Eugenio et al. 2010b). A binding of PhaD to mcl-PHA carbonosomes has not been described for any mcl-PHA-accumulating species. However, the PhaF phasin protein has PAKKA-like motifs in the C-terminal domain of the protein (Prieto et al. 1999), and binding of the C-terminal domain to the nucleoid of *P. putida* has been demonstrated by fusion analysis with GFP (Galan et al. 2011). The properties of PhaF in *P. putida* and of PhaM in *R. eutropha* to bind to formed carbonosomes and to the nucleoid suggest that both proteins have similar functions and enable both species to distribute the storage granules more or less equally to both daughter cells during cell division (for more details, see below). Despite this similar function, PhaF and PhaM do not share significant amino acid homology to each other (except for the PAKKA motifs).

6.5 Other mcl-PHA-Bound Proteins

One of the two acyl-CoA synthetases (Acs1) of *P. putida* is in vivo attached to PHA granules as revealed by fusion analysis with GFP (Ruth et al. 2008) (Fig. 3). This finding suggests that monomeric hydroxyalkanoic acids can be converted to

substrates of the PHA synthase in direct vicinity of PHA granule-bound PHA synthases supporting the view that PHA granules are functional units (carbonosomes).

7 Are Carbonosomes Covered by a Phospholipid Monolayer?

The presence of a phospholipid monolayer at the surface of carbonosomes has been assumed for more than 50 years: Merrick and co-workers published a work in 1967 in which they determined the constituents of a PHB granule fraction that had been purified from *Bacillus megaterium* (Griebel et al. 1968). PHB (97.7%) and protein (1.87%) were the two main components, but 0.46% lipids (including phosphatidic acid) were additionally present. Later, Horowitz and Sanders detected several phospholipids such as phosphatidyl-ethanolamine, phosphatidyl-glycerol, diphosphatidyl-glycerol and a fourth not identified compound (possibly phosphatidyl-serine) in PHB granules isolated from *R. eutropha* (Horowitz and Sanders 1994). These findings together with the determination of the thicknesses of the *R. eutropha* PHB granule boundary layer and of the *R. eutropha* cytoplasm membrane of 2.9–3.8 nm and 6.7–8.1 nm, respectively (Mayer and Hoppert 1997), were the background for the postulation of a phospholipid monolayer surrounding PHB granules. Based on these findings, most reviews on PHA metabolism and on PHA granule structure include the presence of a phospholipid monolayer in cartoons of PHB or PHA granules (Steinbüchel et al. 1995; Pötter and Steinbüchel 2006; Ruth et al. 2008; Grage et al. 2009; Rehm 2010; Dinjaski and Prieto 2013; Prieto et al. 2016). However, the assumed phospholipid layer of carbonosomes has never been shown in whole or living cells. Even in cryo-EM images of *R. eutropha* cells, no continuous surface layer could be detected at the surface of PHB granules (Beeby et al. 2012). Another remarkable point is that despite more than three decades of intensive research on PHB metabolism in *R. eutropha* and other PHB-accumulating species, no publication appeared in which a mutant in phospholipid metabolism was identified that resulted in a phenotype with altered PHB accumulation. The phasin protein PhaP1 in *R. eutropha* was discovered by the PHB-leaky phenotype in a screening approach in which strains with altered ability to accumulate PHB were screened in a transposon mutant library (Wieczorek et al. 1995). However, mutants with insertions in genes related to phospholipid biosynthesis were not detected in these studies. In 2008, the use of the phospholipid-binding C2 domain of bovine lactadherin fused to a fluorescent protein was described for its use to detect phosphatidyl-serine in eukaryotic cells (Yeung et al. 2008). This prompted Bresan et al. to use the same system in bacteria (Bresan et al. 2016). However, all attempts to find evidence for the presence of phospholipids at the surface of scl-PHA or mcl-PHA granules in different species failed, while the phospholipids of the cytoplasm membrane and the phospholipid bilayer membrane of magnetosomes in

Magnetospirillum gryphiswaldense were successfully detected by the reporter system (Dsred2EC-LactC2) (Bresan et al. 2016). Furthermore, the authors of that study could find evidence for the (artificial) in vitro binding ability of phospholipids to PHB granules in a crude cell extract of PHB-accumulating *R. eutropha* cells providing an explaining for the in vitro detection of phospholipids in earlier studies. The above-mentioned findings do not exclude—although not being likely—that single phospholipid molecules or phospholipid-related molecules differing chemically from conventional phospholipids and thus cannot be detected by phospholipid-binding proteins such as C2 domain or segment A of RNAse E (Schulze 2018) could be present in carbonosomes in vivo. In principle, phasin proteins can stably interact with phospholipid layers (Mato et al. 2019). Taken together, there is no in vivo evidence for the presence of a phospholipid (mono)-layer around PHB or PHA granules. Carbonosomes are covered by a surface layer consisting of only proteins, and the presence of a continuous monolayer of conventional phospholipids around carbonosomes is unlikely.

8 Biogenesis of Carbonosomes

The in vivo biogenesis of carbonosomes has been studied in *R. eutropha*. This species rapidly mobilises previously accumulated PHB in the stationary growth phase of cultures with nutrient broth (NB) as a carbon source. When such PHB-free cells are transferred to fresh medium and grown under PHB-permissive conditions (e.g. on NB medium with 0.2% gluconate to increase the C-to-N ratio), the cells start to synthesise one or two new PHB granules in the first 20 min after transfer. Remarkably, one of the newly formed PHB granules is always located close to one of the cell poles, while the second PHB granule originates near the other cell pole. A formation of two PHB granules in the same half of the rod-shaped cells has not been detected (as long as not more than two PHB granules have been formed). This indicates that the spatial formation of PHB granules is controlled in growing *R. eutropha* cells. The PhaM protein determines the subcellular localisation of newly formed PHB granules. PhaM forms oligomers (presumably hexamers) and is bound to the nucleoid of the cells in the absence of PHB (Pfeiffer et al. 2011). The lysine residues of the PAKKA motifs in the C-terminus of PhaM are responsible for the binding of PhaM to DNA in vitro and in vivo (Bresan and Jendrossek 2017). It is not known whether PhaM binds to a specific position on the nucleoid. DNA chip experiments revealed no binding preference of PhaM (unpublished data). Gel mobility shift experiments with purified PhaM showed that PhaM has no DNA sequence specificity in vitro. The N-terminus of PhaM interacts with the PHB synthase PhaC (Pfeiffer et al. 2011). These findings suggest that under PHB-permissive conditions (e.g. in NB-gluconate medium) nucleoid-bound PhaM oligomers recruit PHB synthase dimers and form a PHB granule initiation complex. This initiation complex is formed in each cell half near the cell pole. At the same time, the nucleoid has started to replicate and to separate. If one assumes that the genome has only one or

few (not yet identified) binding sites for the attachment of the PhaM-PhaC initiation complex, one might speculate that genome replication and segregation of the daughter chromosomes during genome replication are the explanation why always one PHB granule in each cell half is formed at the beginning of PHB granule biosynthesis. As soon as the initiation complex is formed, PhaM-attached PHB synthase dimers start to synthesise PHB (Pfeiffer and Jendrossek 2014). The hydrophobic PHB chain aggregates and grows to a microscopically detectable granule. During this process, phasins and other PGAPs bind to the growing surface of the arising PHB granule. The PHB granules can be liberated from the covalently bound PhaM-PhaC complex at later stages of PHB accumulation. Evidence for this "aging phenomenon of carbonosomes" is the identification of non-fluorescent PHB granules at later stages of PHB accumulation in strains expressing *eyfp-phaC1* or *eyfp-phaM* (Bresan and Jendrossek 2017). If the cells with liberated (PhaM-PhaC1-free) PHB granules are further kept under PHB-permissive conditions, then free PhaC1-PhaM complexes can initiate the formation of additional, new PHB granules. The release of PHB granules from the PhaC1-PhaM complex ensures that PHB granules do not grow ad infinitum if the cells are permanently kept under PHB-permissive conditions.

The PhaM protein is not essential for PHB biosynthesis. Mutants with a deletion of the *phaM* gene accumulate slightly reduced amounts of PHB compared to the wild type, and the number of granules is only 0, 1 or 2 in most cells. However the diameters of the carbonosomes are substantially enlarged similar as in *ΔphaP1* mutants (Wieczorek et al. 1995). The distribution of the PHB granules during cell division in *ΔphaM* cells is disordered. Directly after cell division, one daughter cell harbours all (one or two) PHB granules, while the other daughter cell has no PHB granule. Only under ongoing PHB-permissive conditions, the PHB-free daughter cell can synthesise an own (new) PHB granule at later stages of growth. These data suggest that a major physiological function of PhaM is to ensure an equal distribution of the accumulated carbonosomes during cell division to both daughter cells. In this respect the function of PhaM seems to be similar to PhaF in *P. putida* (Galan et al. 2009, 2011; Prieto et al. 2016). PhaF has also C-terminal located PAKKA motifs as PhaM and enables PhaF to bind to DNA. However, the N-terminal domain of PhaF does not interact with the PHA synthase but has a phasin motif and interacts with phasin PhaI (Prieto et al. 1999). Therefore, PhaF is unlikely to be part of a PHA granule initiation complex in mcl-PHA-accumulating species. Nevertheless, *P. putida* strains, in which the *phaF* gene has been deleted, have a disordered localisation of accumulated PHA granules, and equal distribution of these granules to the daughter cells during cell division is blocked (Galan et al. 2011).

In summary, the biogenesis of carbonosomes in two model organisms, in scl-PHA-accumulating *R. eutropha* and in mcl-PHA-accumulating *P. putida*, follows via a scaffold model in which the scaffold is the bacterial nucleoid. Evidence for a scaffold model in PHB granule formation in *R. eutropha* was already provided in studies of J. Stubbe's laboratory (Tian et al. 2005a, b). In these studies, the scaffold transiently was detected as "dark-stained mediation elements" in *R. eutropha*. The data of Tian et al. are in good agreement with the assumption that the mediation

elements represent the bacterial nucleoid. Previously proposed and discussed models of carbonosome biosynthesis (i.e. the budding model and the micelle model (Ellar et al. 1968; Stubbe and Tian 2003; Stubbe et al. 2005; Tian et al. 2005b; Pötter and Steinbüchel 2006)) are not supported by in vivo evidence.

9 Novel Function of Carbonosomes

The most obvious function of PHB and related carbonosomes is that of an insoluble and therefore osmotic inert reservoir for carbon and energy and has been experimentally verified for several species (for a selection of publications, see Dawes and Senior 1973; Matin et al. 1979; James et al. 1999; Handrick et al. 2000; Ratcliff et al. 2008; Wang et al. 2009). In addition, PHAs are relatively reduced compounds and can be therefore considered as sinks for electrons (Senior and Dawes 1971). In agreement with this, oxygen limitation in many PHA-producing species increases the amount of accumulated PHAs. Degradation of PHA and oxidation of the hydrolysis products can be used to promote consumption of oxygen in nitrogen-fixing species to protect the nitrogenase from inactivation by oxic conditions (Stam et al. 1986; Mandon et al. 1998). Another function of accumulated PHAs is its protective role in coping with various stresses such as oxidative stress, high/low temperatures or osmotic stress. For examples, see Kadouri et al. (2003); Zhao et al. (2007); Ayub et al. (2009); Obruca et al. (2016); Koskimäki et al. (2016); Nowroth et al. (2016); Obruca et al. (2017); Batista et al. (2018); and Sedlacek et al. (2019). It is not clear in which cases the enhanced stress resistance of PHB-containing cells is a direct protective effect of the polymer or is only an indirect effect. The function of PHB in enhancing stress resistance is a relatively young research field, and the molecular mechanisms, by which PHB can effectuate a higher level of stress resistance, are only poorly understood. In this context, an astonishing result was the finding that PHB degradation products such as 3HB and methyl esters of 3HB have strong radical scavenging activities and help PHB-accumulating plant-symbiotic bacteria (*Methylobacterium extorquens*) to tolerate oxygen radicals produced by the plant cells during contact with incoming symbionts (Koskimäki et al. 2016). Recently, metabolism of PHB was investigated in *Synechocystis* sp. PCC 6803 (Koch et al. 2019). The authors provided evidence that PHB is involved in glycogen metabolism but presumably has no (prominent) function for survival under (nitrogen) starving conditions. In summary, it is evident that, despite several decades of intensive research on PHB metabolism, still more research is necessary to understand the molecular basis of the protective roles of PHB in prokaryotic species.

Acknowledgements I thank the Deutsche Forschungsgemeinschaft for the continuous support on my work on carbonosomes. I deeply appreciate the work of my current and previous co-workers and students in the field of PHB metabolism: Libertad Adaya, Meike Andermann, Felix Becker, Astrid Behrends, Bernd-Holger Briese, Martina Backhaus, Stephanie Bresan (Schulze), Bernd-Holger Briese, Chiara Brück, Nicole Fischer, Letizia Focarete, Anja Frank, Andrea Frisse, Birgit Gebauer,

Rene Handrick, Eva-Maria Hüper, Ingrid Knoke, Siska Hermawan, Pia Kaufmann, Philipp Kimmig, Britta Klingbeil, Lea Kneissle, Janina Jüngert (Berg), Daniela Leuprecht, Heike Lill, Claudia Matz, Beate Müller, Verena Nowroth, Cameron Patterson, Elena Pluta, Thomas Reichert, Dominik Reis, Watsana Penkrue, Daniel Pfeiffer, Ute Schöber, Nora Schuth, Simone Reinhardt, Fabian Romen, Thomas Sander, Andreas Schirmer, Eva Slaninova, Thomas Stanislawski, Anna Sznajder (Sommer), Naihmeh Taheri Talesh, Ulricke Technow, Monika Tischbirek, Kay Terpe, Christian Thiel, Gianpaolo Tomasi, Keiichi Uchino, Manuela Unsinn, Tanja Veselinovic, Andreas Wahl and Lei Wang.

References

Abe T, Kobayashi T, Saito T (2005) Properties of a novel intracellular poly(3-hydroxybutyrate) depolymerase with high specific activity (PhaZd) in *Wautersia eutropha* H16. J Bacteriol 187:6982–6990. https://doi.org/10.1128/JB.187.20.6982-6990.2005

Abraham GA, Gallardo A, San Roman J, Olivera ER, Jodra R, García B, Miñambres B, Garcia JL, Luengo JM (2001) Microbial synthesis of poly(beta-hydroxyalkanoates) bearing phenyl groups from *Pseudomonas putida*: chemical structure and characterization. Biomacromolecules 2:562–567. https://doi.org/10.1021/bm010018h

Adaya L, Millán M, Peña C, Jendrossek D, Espín G, Tinoco-Valencia R, Guzmán J, Pfeiffer D, Segura D (2018) Inactivation of an intracellular poly-3-hydroxybutyrate depolymerase of *Azotobacter vinelandii* allows to obtain a polymer of uniform high molecular mass. Appl Microbiol Biotechnol 187:6982–6915. https://doi.org/10.1007/s00253-018-8806-y

Anderson AJ, Dawes EA (1990) Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol Rev 54:450–472

Arias S, Bassas-Galia M, Molinari G, Timmis KN (2013) Tight coupling of polymerization and depolymerization of polyhydroxyalkanoates ensures efficient management of carbon resources in *Pseudomonas putida*. Microb Biotechnol 6:551–563. https://doi.org/10.1111/1751-7915.12040

Ayub ND, Tribelli PM, López NI (2009) Polyhydroxyalkanoates are essential for maintenance of redox state in the Antarctic bacterium *Pseudomonas* sp. 14-3 during low temperature adaptation. Extremophiles 13:59–66. https://doi.org/10.1007/s00792-008-0197-z

Batista MB, Teixeira CS, Sfeir MZT, Alves LPS, Valdameri G, Pedrosa F de O, Sassaki GL, Steffens MBR, de Souza EM, Dixon R, Müller-Santos M (2018) PHB biosynthesis counteracts redox stress in *Herbaspirillum seropedicae*. Front Microbiol 9:472. https://doi.org/10.3389/fmicb.2018.00472

Beeby M, Cho M, Stubbe J, Jensen GJ (2012) Growth and localization of polyhydroxybutyrate granules in *Ralstonia eutropha*. J Bacteriol 194:1092–1099. https://doi.org/10.1128/JB.06125-11

Bresan S, Jendrossek D (2017) New insights in PhaM-PhaC-mediated localization of PHB granules in *Ralstonia eutropha* H16. Appl Environ Microbiol AEM:00505–00517. https://doi.org/10.1128/AEM.00505-17

Bresan S, Sznajder A, Hauf W, Forchhammer K, Pfeiffer D, Jendrossek D (2016) Polyhydroxyalkanoate (PHA) Granules Have no Phospholipids. Sci Rep 6:26612. https://doi.org/10.1038/srep26612

Brigham CJ, Speth DR, Rha C, Sinskey AJ (2012) Whole-genome microarray and gene deletion studies reveal regulation of the polyhydroxyalkanoate production cycle by the stringent response in *Ralstonia eutropha* H16. Appl Environ Microbiol 78:8033–8044. https://doi.org/10.1128/AEM.01693-12

Cai L, Yuan M-Q, Liu F, Jian J, Chen G-Q (2009) Enhanced production of medium-chain-length polyhydroxyalkanoates (PHA) by PHA depolymerase knockout mutant of *Pseudomonas putida* KT2442. Bioresour Technol 100:2265–2270. https://doi.org/10.1016/j.biortech.2008.11.020

Chek MF, Hiroe A, Hakoshima T, Sudesh K, Taguchi S (2019) PHA synthase (PhaC): interpreting the functions of bioplastic-producing enzyme from a structural perspective. Appl Microbiol Biotechnol 103:1131–1141. https://doi.org/10.1007/s00253-018-9538-8

Chen J-Y, Song G, Chen G-Q (2006) A lower specificity PhaC2 synthase from *Pseudomonas stutzeri* catalyses the production of copolyesters consisting of short-chain-length and medium-chain-length 3-hydroxyalkanoates. Antonie Van Leeuwenhoek 89:157–167. https://doi.org/10.1007/s10482-005-9019-9

Cho M, Brigham CJ, Sinskey AJ, Stubbe J (2012) Purification of polyhydroxybutyrate synthase from its native organism, *Ralstonia eutropha*: implications for the initiation and elongation of polymer formation in vivo. Biochemistry 51:2276–2288. https://doi.org/10.1021/bi2013596

Dawes EA, Senior PJ (1973) The role and regulation of energy reserve polymers in micro-organisms. Adv Microb Physiol 10:135–266. https://doi.org/10.1016/s0065-2911(08)60088-0

de Eugenio LI, Garcia P, Luengo JM, Sanz JM, San Roman J, Garcia JL, Prieto MA (2007) Biochemical evidence that phaZ gene encodes a specific intracellular medium chain length polyhydroxyalkanoate depolymerase in *Pseudomonas putida* KT2442—Characterization of a paradigmatic enzyme. J Biol Chem 282:4951–4962. https://doi.org/10.1074/jbc.M608119200

de Eugenio LI, Garcia JL, Garcia P, Prieto MA, Sanz JM (2008) Comparative analysis of the physiological and structural properties of a medium chain length polyhydroxyalkanoate depolymerase from *Pseudomonas putida* KT2442. Eng Life Sci 8:260–267. https://doi.org/10.1002/elsc.200700057

de Smet MJ, Eggink G, Witholt B, Kingma J, Wynberg H (1983) Characterization of intracellular inclusions formed by *Pseudomonas oleovorans* during growth on octane. J Bacteriol 154:870–878

Dinjaski N, Prieto MA (2013) Swapping of phasin modules to optimize the in vivo immobilization of proteins to medium-chain-length polyhydroxyalkanoate granules in *Pseudomonas putida*. Biomacromolecules 14:3285–3293. https://doi.org/10.1021/bm4008937

Eggers J, Steinbüchel A (2013) Poly(3-Hydroxybutyrate) Degradation in *Ralstonia eutropha* H16 Is Mediated Stereoselectively to (S)-3-Hydroxybutyryl Coenzyme A (CoA) via Crotonyl-CoA. J Bacteriol 195:3213–3223. https://doi.org/10.1128/JB.00358-13

Elbanna K, Lütke-Eversloh T, Jendrossek D, Luftmann H, Steinbüchel A (2004) Studies on the biodegradability of polythioester copolymers and homopolymers by polyhydroxyalkanoate (PHA)-degrading bacteria and PHA depolymerases. Arch Microbiol 182:212–225. https://doi.org/10.1007/s00203-004-0715-z

Ellar D, Lundgren DG, Okamura K, Marchessault RH (1968) Morphology of poly-beta-hydroxybutyrate granules. J Mol Biol 35:489–502. https://doi.org/10.1016/s0022-2836(68)80009-9

Follner CG, Madkour M, Mayer F, Babel W, Steinbüchel A (1997) Analysis of the PHA granule-associated proteins GA20 and GA11 in *Methylobacterium extorquens* and *Methylobacterium rhodesianum*. J Basic Microbiol 37:11–21

Foster LJ, Lenz RW, Fuller RC (1994) Quantitative determination of intracellular depolymerase activity in *Pseudomonas oleovorans* inclusions containing poly-3-hydroxyalkanoates with long alkyl substituents. FEMS Microbiol Lett 118:279–282. https://doi.org/10.1111/j.1574-6968.1994.tb06841.x

Foster LJ, Stuart ES, Tehrani A, Lenz RW, Fuller RC (1996) Intracellular depolymerase and polyhydroxyoctanoate granule integrity in *Pseudomonas oleovorans*. Int J Biol Macromol 19:177–183

Fuller RC, O Donnel JP, Saulnier J, Redlinger TE, Foster J, Lenz RW (1992) The supramolecular architecture of the polyhydroxyalkanoate inclusions in *Pseudomonas-oleovorans*. FEMS Microbiol Lett 103:279–288. https://doi.org/10.1016/0378-1097(92)90321-E

Galan B, Maestro B, Garcia JL, Sanz JM, Prieto MA (2009) Role of the bi-modular PhaF protein as DNA binding factor in *Pseudomonas putida* KT2442. New Biotechnol 25:S62. https://doi.org/10.1016/j.nbt.2009.06.292

Galan B, Dinjaski N, Maestro B, de Eugenio LI, Escapa IF, Sanz JM, Garcia JL, Prieto MA (2011) Nucleoid-associated PhaF phasin drives intracellular location and segregation of polyhydroxyalkanoate granules in *Pseudomonas putida* KT2442. Mol Microbiol 79:402–418. https://doi.org/10.1111/j.1365-2958.2010.07450.x

Gerngross TU, Martin DP (1995) Enzyme-catalyzed synthesis of poly[(*R*)-(-)-3-hydroxybutyrate]: formation of macroscopic granules *in vitro*. Proc Natl Acad Sci USA 92:6279–6283

Gerngross TU, Reilly P, Stubbe J, Sinskey AJ, Peoples OP (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 TU, Snell KD, Peoples OP, Sinskey AJ, Csuhai E, Masamune S, Stubbe J (1994) Overexpression and purification of the soluble polyhydroxyalkanoate synthase from *Alcaligenes eutrophus*: evidence for a required posttranslational modification for catalytic activity. Biochemistry 33:9311–9320

Grage K, Jahns AC, Parlane N, Palanisamy R, Rasiah IA, Atwood JA, Rehm BHA (2009) Bacterial polyhydroxyalkanoate granules: biogenesis, structure, and potential use as nano-/micro-beads in biotechnological and biomedical applications. Biomacromolecules 10:660–669. https://doi.org/10.1021/bm801394s

Griebel R, Smith Z, Merrick JM (1968) Metabolism of poly-beta-hydroxybutyrate. I. Purification, composition, and properties of native poly-beta-hydroxybutyrate granules from *Bacillus megaterium*. Biochemistry 7:3676–3681

Handrick R, Reinhardt S, Jendrossek D (2000) Mobilization of poly(3-hydroxybutyrate) in *Ralstonia eutropha*. J Bacteriol 182:5916–5918

Handrick R, Reinhardt S, Schultheiss D, Reichart T, Schüler D, Jendrossek V, Jendrossek D (2004a) Unraveling the function of the *Rhodospirillum rubrum* activator of polyhydroxybutyrate (PHB) degradation: the activator is a PHB-granule-bound protein (phasin). J Bacteriol 186:2466–2475. https://doi.org/10.1128/JB.186.8.2466-2475.2004

Handrick R, Technow U, Reichart T, Reinhardt S, Sander T, Jendrossek D (2004b) The activator of the *Rhodospirillum rubrum* PHB depolymerase is a polypeptide that is extremely resistant to high temperature (121 degrees C) and other physical or chemical stresses. FEMS Microbiol Lett 230:265–274. https://doi.org/10.1016/S0378-1097(03)00919-4

Hauf W, Watzer B, Roos N, Klotz A, Forchhammer K (2015) Photoautotrophic polyhydroxybutyrate granule formation is regulated by cyanobacterial phasin PhaP in *Synechocystis* sp. strain PCC 6803. Appl Environ Microbiol 81:4411–4422. https://doi.org/10.1128/AEM.00604-15

Horowitz DM, Sanders JKM (1994) Amorphous, biomimetic granules of polyhydroxybutyrate: preparation, characterization, and biological implications. J Am Chem Soc 116:2695–2702. https://doi.org/10.1021/ja00086a001

Huijberts GN, Eggink G, de Waard P, Huisman GW, Witholt B (1992) *Pseudomonas putida* KT2442 cultivated on glucose accumulates poly(3-hydroxyalkanoates) consisting of saturated and unsaturated monomers. Appl Environ Microbiol 58:536–544

Huisman GW, de Leeuw O, Eggink G, Witholt B (1989) Synthesis of poly-3-hydroxyalkanoates is a common feature of fluorescent pseudomonads. Appl Environ Microbiol 55:1949–1954

Huisman GW, Wonink E, Meima R, Kazemier B, Terpstra P, Witholt B (1991) Metabolism of poly (3-hydroxyalkanoates) (PHAs) by *Pseudomonas oleovorans*. Identification and sequences of genes and function of the encoded proteins in the synthesis and degradation of PHA. J Biol Chem 266:2191–2198

Isabel de Eugenio L, Escapa IF, Morales V, Dinjaski N, Galan B, Luis Garcia J, Prieto MA (2010a) The turnover of medium-chain-length polyhydroxyalkanoates in *Pseudomonas putida* KT2442 and the fundamental role of PhaZ depolymerase for the metabolic balance. Environ Microbiol 12:207–221. https://doi.org/10.1111/j.1462-2920.2009.02061.x

Isabel de Eugenio L, Galan B, Escapa IF, Maestro B, Sanz JM, Luis Garcia J, Prieto MA (2010b) The PhaD regulator controls the simultaneous expression of the pha genes involved in polyhydroxyalkanoate metabolism and turnover in *Pseudomonas putida* KT2442. Environ Microbiol 12:1591–1603. https://doi.org/10.1111/j.1462-2920.2010.02199.x

Jahns AC, Rehm BHA (2009) Tolerance of the *Ralstonia eutropha* class I polyhydroxyalkanoate synthase for translational fusions to its C terminus reveals a new mode of functional display. Appl Environ Microbiol 75:5461–5466. https://doi.org/10.1128/AEM.01072-09

James BW, Mauchline WS, Dennis PJ, Keevil CW, Wait R (1999) Poly-3-hydroxybutyrate in *Legionella pneumophila*, an energy source for survival in low-nutrient environments. Appl Environ Microbiol 65:822–827

Jendrossek D (2001) Microbial degradation of polyesters. Adv Biochem Eng Biotechnol 71:293–325

Jendrossek D (2005) Fluorescence microscopical investigation of poly(3-hydroxybutyrate) granule formation in bacteria. Biomacromolecules 6:598–603. https://doi.org/10.1021/bm049441r

Jendrossek D (2007) Peculiarities of PHA granules preparation and PHA depolymerase activity determination. Appl Microbiol Biotechnol 74:1186–1196. https://doi.org/10.1007/s00253-007-0860-9

Jendrossek D (2009) Polyhydroxyalkanoate granules are complex subcellular organelles (carbonosomes). J Bacteriol 191:3195–3202. https://doi.org/10.1128/JB.01723-08

Jendrossek D, Handrick R (2002) Microbial degradation of polyhydroxyalkanoates. Annu Rev Microbiol 56:403–432. https://doi.org/10.1146/annurev.micro.56.012302.160838

Jendrossek D, Pfeiffer D (2014) New insights in the formation of polyhydroxyalkanoate granules (carbonosomes) and novel functions of poly(3-hydroxybutyrate). Environ Microbiol 16:2357–2373. https://doi.org/10.1111/1462-2920.12356

Jendrossek D, Selchow O, Hoppert M (2007) Poly(3-hydroxybutyrate) granules at the early stages of formation are localized close to the cytoplasmic membrane in *Caryophanon latum*. Appl Environ Microbiol 73:586–593. https://doi.org/10.1128/AEM.01839-06

Jiang Y, Ye J, Wu H, Zhang H (2004) Cloning and expression of the polyhydroxyalkanote depolymerase gene from *Pseudomonas putida*, and characterization of the gene product. Biotechnol Lett 26:1585–1588. https://doi.org/10.1023/B:BILE.0000045657.93818.18

Jüngert JR, Borisova M, Mayer C, Wolz C, Brigham CJ, Sinskey AJ, Jendrossek D (2017) Absence of ppGpp Leads to Increased Mobilization of Intermediately Accumulated Poly (3-hydroxybutyrate) (PHB) in *Ralstonia eutropha* H16. Appl Environ Microbiol AEM.:00755–00717. https://doi.org/10.1128/AEM.00755-17

Kadouri D, Jurkevitch E, Okon Y (2003) Involvement of the reserve material poly-beta-hydroxybutyrate in *Azospirillum brasilense* stress endurance and root colonization. Appl Environ Microbiol 69:3244–3250. https://doi.org/10.1128/aem.69.6.3244-3250.2003

Kawaguchi Y, Doi Y (1992) Kinetics and Mechanism of Synthesis and Degradation of Poly (3-Hydroxybutyrate) in *Alcaligenes eutrophus*. Macromolecules 25:2324–2329. https://doi.org/10.1021/ma00035a007

Kim T-K, Jung Y-M, Vo MT, Shioya S, Lee Y-H (2006) Metabolic engineering and characterization of phaC1 and phaC2 genes from *Pseudomonas putida* KCTC1639 for overproduction of medium-chain-length polyhydroxyalkanoate. Biotechnol Prog 22:1541–1546. https://doi.org/10.1021/bp0601746

Kim J, Kim Y-J, Choi SY, Lee SY, Kim K-J (2017a) Crystal structure of *Ralstonia eutropha* polyhydroxyalkanoate synthase C-terminal domain and reaction mechanisms. Biotechnol J. https://doi.org/10.1002/biot.201600648

Kim Y-J, Choi SY, Kim J, Jin KS, Lee SY, Kim K-J (2017b) Structure and function of the N-terminal domain of *Ralstonia eutropha* polyhydroxyalkanoate synthase, and the proposed structure and mechanisms of the whole enzyme. Biotechnol J. https://doi.org/10.1002/biot.201600649

Kobayashi T, Saito T (2003) Catalytic triad of intracellular poly(3-hydroxybutyrate) depolymerase (PhaZ1) in *Ralstonia eutropha* H16. J Biosci Bioeng 96:487–492. https://doi.org/10.1016/S1389-1723(03)70136-4

Kobayashi T, Shiraki M, Abe T, Sugiyama A, Saito T (2003) Purification and properties of an intracellular 3-hydroxybutyrate-oligomer hydrolase (PhaZ2) in *Ralstonia eutropha* H16 and its identification as a novel intracellular poly(3-hydroxybutyrate) depolymerase. J Bacteriol 185:3485–3490. https://doi.org/10.1128/JB.185.12.3485-3490.2003

Kobayashi T, Uchino K, Abe T, Yamazaki Y, Saito T (2005) Novel intracellular 3-hydroxybutyrate-oligomer hydrolase in *Wautersia eutropha* H16. J Bacteriol 187:5129–5135. https://doi.org/10.1128/JB.187.15.5129-5135.2005

Koch M, Doello S, Gutekunst K, Forchhammer K (2019) PHB is Produced from Glycogen Turnover during Nitrogen Starvation in *Synechocystis* sp. PCC 6803. Int J Mol Sci 20:1942. https://doi.org/10.3390/ijms20081942

Koskimäki JJ, Kajula M, Hokkanen J, Ihantola E-L, Kim JH, Hautajärvi H, Hankala E, Suokas M, Pohjanen J, Podolich O, Kozyrovska N, Turpeinen A, Pääkkönen M, Mattila S, Campbell BC, Pirttilä AM (2016) Methyl-esterified 3-hydroxybutyrate oligomers protect bacteria from hydroxyl radicals. Nat Chem Biol 12:332–338. https://doi.org/10.1038/nchembio.2043

Kuchta K, Chi L, Fuchs H, Pötter M, Steinbüchel A (2007) Studies on the influence of phasins on accumulation and degradation of PHB and nanostructure of PHB granules in *Ralstonia eutropha* H16. Biomacromolecules 8:657–662. https://doi.org/10.1021/bm060912e

Lageveen RG, Huisman GW, Preusting H, Ketelaar P, Eggink G, Witholt B (1988) Formation of polyesters by *Pseudomonas oleovorans*: effect of substrates on formation and composition of poly-(*R*)-3-hydroxyalkanoates and poly-(*R*)-3-hydroxyalkenoates. Appl Environ Microbiol 54:2924–2932

Lawrence AG, Choi J, Rha C, Stubbe J, Sinskey AJ (2005) In vitro analysis of the chain termination reaction in the synthesis of poly-(R)-beta-hydroxybutyrate by the class III synthase from *Allochromatium vinosum*. Biomacromolecules 6:2113–2119. https://doi.org/10.1021/bm0501048

Leuprecht D (2012) Untersuchung des intrazellulären PHB Abbaus von *Ralstonia eutropha* H16 durch Fluoreszenzmikroskopie und Two-Hybrid-Analyse. Diploma thesis, University Stuttgart

Liebergesell M, Schmidt B, Steinbüchel A (1992) Isolation and identification of granule-associated proteins relevant for poly(3-hydroxyalkanoic acid) biosynthesis in *Chromatium vinosum* D. FEMS Microbiol Lett 78:227–232. https://doi.org/10.1016/0378-1097(92)90031-i

Liebergesell M, Sonomoto K, Madkour M, Mayer F, Steinbüchel A (1994) Purification and characterization of the poly(hydroxyalkanoic acid) synthase from *Chromatium vinosum* and localization of the enzyme at the surface of poly(hydroxyalkanoic acid) granules. Eur J Biochem 226:71–80. https://doi.org/10.1111/j.1432-1033.1994.tb20027.x

Liu Q, Luo G, Zhou XR, Chen G-Q (2011) Biosynthesis of poly(3-hydroxydecanoate) and 3-hydroxydodecanoate dominating polyhydroxyalkanoates by β-oxidation pathway inhibited *Pseudomonas putida*. Metab Eng 13:11–17. https://doi.org/10.1016/j.ymben.2010.10.004

Liu G, Hou J, Cai S, Zhao D, Cai L, Han J, Zhou J, Xiang H (2015) A patatin-like protein associated with the polyhydroxyalkanoate (PHA) granules of *Haloferax mediterranei* acts as an efficient depolymerase in the degradation of native PHA. Appl Environ Microbiol 81:3029–3038. https://doi.org/10.1128/AEM.04269-14

Liu G, Cai S, Hou J, Zhao D, Han J, Zhou J, Xiang H (2016) Enoyl-CoA hydratase mediates polyhydroxyalkanoate mobilization in *Haloferax mediterranei*. Sci Rep. https://doi.org/10.1038/srep24015

Lütke-Eversloh T, Bergander K, Luftmann H, Steinbüchel A (2001) Identification of a new class of biopolymer: bacterial synthesis of a sulfur-containing polymer with thioester linkages. Microbiology (Reading, Engl) 147:11–19. https://doi.org/10.1099/00221287-147-1-11

Madison LL, Huisman GW (1999) Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic. Microbiol Mol Biol Rev 63:21–53

Maestro B, Galan B, Alfonso C, Rivas G, Prieto MA, Sanz JM (2013) A new family of intrinsically disordered proteins: structural characterization of the major phasin PhaF from *Pseudomonas putida* KT2440. PLoS One. https://doi.org/10.1371/journal.pone.0056904

Mandon K, Michel-Reydellet N, Encarnación S, Kaminski PA, Leija A, Cevallos MA, Elmerich C, Mora J (1998) Poly-beta-hydroxybutyrate turnover in *Azorhizobium caulinodans* is required for growth and affects *nifA* expression. J Bacteriol 180:5070–5076

Matin A, Veldhuis C, Stegeman V, Veenhuis M (1979) Selective advantage of a *Spirillum* sp. in a carbon-limited environment. Accumulation of poly-beta-hydroxybutyric acid and its role in starvation. J Gen Microbiol 112:349–355. https://doi.org/10.1099/00221287-112-2-349

Mato A, Tarazona NA, Hidalgo A, Cruz A, Jiménez M, Pérez-Gil J, Prieto MA (2019) Interfacial activity of phasin PhaF from *Pseudomonas putida* KT2440 at hydrophobic-hydrophilic biointerfaces. Langmuir 35:678–686. https://doi.org/10.1021/acs.langmuir.8b03036

Mayer F, Hoppert M (1997) Determination of the thickness of the boundary layer surrounding bacterial PHA inclusion bodies, and implications for models describing the molecular architecture of this layer. J Bas Microbiol 37:45–52

McCool GJ, Cannon MC (1999) Polyhydroxyalkanoate inclusion body-associated proteins and coding region in *Bacillus megaterium*. J Bacteriol 181:585–592

Meng D-C, Chen G-Q (2018) Synthetic Biology of Polyhydroxyalkanoates (PHA). Adv Biochem Eng Biotechnol 162:147–174. https://doi.org/10.1007/10_2017_3

Merrick JM, Doudoroff M (1964) Depolymerization of Poly-beta-hydroxybutyrate by intracellular enzyme system. J Bacteriol 88:60–71

Mezzina MP, Pettinari MJ (2016) Phasins, multifaceted polyhydroxyalkanoate granule-associated proteins. Appl Environ Microbiol 82:5060–5067. https://doi.org/10.1128/AEM.01161-16

Mezzolla V, D'Urso OF, Poltronieri P (2018) Role of PhaC type I and type II enzymes during PHA biosynthesis. Polymers (Basel) 10:910. https://doi.org/10.3390/polym10080910

Moldes C, Garcia P, García JL, Prieto MA (2004) In vivo immobilization of fusion proteins on bioplastics by the novel tag BioF. Appl Environ Microbiol 70:3205–3212. https://doi.org/10.1128/AEM.70.6.3205-3212.2004

Moreno S, Castellanos M, Bedoya-Pérez LP, Canales-Herrerías P, Espín G, Muriel-Millán LF (2019) Outer membrane protein I is associated with poly-β-hydroxybutyrate granules and is necessary for optimal polymer accumulation in *Azotobacter vinelandii* on solid medium. Microbiology (Reading, Engl) 193:265. https://doi.org/10.1099/mic.0.000837

Myshkina VL, Nikolaeva DA, Makhina TK, Bonartsev AP, Bonartseva GA (2008) Effect of growth conditions on the molecular weight of poly-3-hydroxybutyrate produced by *Azotobacter chroococcum* 7B. Appl Biochem Microbiol 44:482–486. https://doi.org/10.1134/S0003683808050050

Nelson KE, Weinel C, Paulsen IT, Dodson RJ, Hilbert H, Santos dos V, Fouts DE, Gill SR, Pop M, Holmes M, Brinkac L, Beanan M, DeBoy RT, Daugherty S, Kolonay J, Madupu R, Nelson W, White O, Peterson J, Khouri H, Hance I, Lee PC, Holtzapple E, Scanlan D, Tran K, Moazzez A, Utterback T, Rizzo M, Lee K, Kosack D, Moestl D, Wedler H, Lauber J, Stjepandic D, Hoheisel J, Straetz M, Heim S, Kiewitz C, Eisen J, Timmis KN, Dusterhoft A, Tummler B, Fraser CM (2002) Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. Environ Microbiol 4:799–808

Neumann L, Spinozzi F, Sinibaldi R, Rustichelli F, Pötter M, Steinbüchel A (2008) Binding of the major phasin, PhaP1, from *Ralstonia eutropha* H16 to poly(3-hydroxybutyrate) granules. J Bacteriol 190:2911–2919. https://doi.org/10.1128/JB.01486-07

Nowroth V, Marquart L, Jendrossek D (2016) Low temperature-induced viable but not culturable state of *Ralstonia eutropha* and its relationship to accumulated polyhydroxybutyrate. FEMS Microbiol Lett 363:fnw249. https://doi.org/10.1093/femsle/fnw249

Obruca S, Sedlacek P, Mravec F, Samek O, Marova I (2016) Evaluation of 3-hydroxybutyrate as an enzyme-protective agent against heating and oxidative damage and its potential role in stress response of poly(3-hydroxybutyrate) accumulating cells. Appl Microbiol Biotechnol 100:1365–1376. https://doi.org/10.1007/s00253-015-7162-4

Obruca S, Sedlacek P, Mravec F, Krzyzanek V, Nebesarova J, Samek O, Kucera D, Benesova P, Hrubanova K, Milerova M, Marova I (2017) The presence of PHB granules in cytoplasm protects non-halophilic bacterial cells against the harmful impact of hypertonic environments. New Biotechnol 39:68–80. https://doi.org/10.1016/j.nbt.2017.07.008

Olivera ER, Arcos M, Naharro G, Luengo JM (2010) Unusual PHA biosynthesis. Microbiol Monogr 14:133–186

Peoples OP, Sinskey AJ (1989) Poly-beta-hydroxybutyrate (Phb) biosynthesis in *Alcaligenes eutrophus* H16—Identification and characterization of the PHB polymerase gene (PhbC). J Biol Chem 264:15298–15303. https://doi.org/10.1016/0014-5793%2888%2980693-8

Peplinski K, Ehrenreich A, Doering C, Boemeke M, Reinecke F, Hutmacher C, Steinbüchel A (2010) Genome-wide transcriptome analyses of the "Knallgas" bacterium *Ralstonia eutropha* H16 with regard to polyhydroxyalkanoate metabolism. Microbiology (Reading, Engl) 156:2136–2152. https://doi.org/10.1099/mic.0.038380-0

Peters V, Rehm BHA (2005) In vivo monitoring of PHA granule formation using GFP-labeled PHA synthases. FEMS Microbiol Lett 248:93–100. https://doi.org/10.1016/j.femsle.2005.05.027

Pfeiffer D, Jendrossek D (2011) Interaction between poly(3-hydroxybutyrate) granule-associated proteins as revealed by two-hybrid analysis and identification of a new phasin in *Ralstonia eutropha* H16. Microbiology (Reading, Engl) 157:2795–2807. https://doi.org/10.1099/mic.0.051508-0

Pfeiffer D, Jendrossek D (2012) Localization of poly(3-hydroxybutyrate) (PHB) granule-associated proteins during PHB granule formation and identification of two new phasins, PhaP6 and PhaP7, in *Ralstonia eutropha* H16. J Bacteriol 194:5909–5921. https://doi.org/10.1128/JB.00779-12

Pfeiffer D, Jendrossek D (2014) PhaM is the physiological activator of poly(3-hydroxybutyrate) (PHB) synthase (PhaC1) in *Ralstonia eutropha*. Appl Environ Microbiol 80:555–563. https://doi.org/10.1128/AEM.02935-13

Pfeiffer D, Wahl A, Jendrossek D (2011) Identification of a multifunctional protein, PhaM, that determines number, surface to volume ratio, subcellular localization and distribution to daughter cells of poly(3-hydroxybutyrate), PHB, granules in *Ralstonia eutropha* H16. Mol Microbiol 82:936–951. https://doi.org/10.1111/j.1365-2958.2011.07869.x

Pieper-Furst U, Madkour MH, Mayer F, Steinbüchel A (1994) Purification and characterization of a 14-kilodalton protein that is bound to the surface of polyhydroxyalkanoic acid granules in *Rhodococcus ruber*. J Bacteriol 176:4328–4337

Pohlmann A, Fricke WF, Reinecke F, Kusian B, Liesegang H, Cramm R, Eitinger T, Ewering C, Pötter M, Schwartz E, Strittmatter A, Voss I, Gottschalk G, Steinbüchel A, Friedrich B, Bowien B (2006) Genome sequence of the bioplastic-producing "Knallgas" bacterium *Ralstonia eutropha* H16. Nat Biotechnol 24:1257–1262. https://doi.org/10.1038/nbt1244

Pötter M, Steinbüchel A (2006) Biogenesis and structure of polyhydroxyalkanoate granules. Microbiol Monogr 1:1–28

Pötter M, Madkour MH, Mayer F, Steinbüchel A (2002) Regulation of phasin expression and polyhydroxyalkanoate (PHA) granule formation in *Ralstonia eutropha* H16. Microbiology (Reading, Engl) 148:2413–2426. https://doi.org/10.1099/00221287-148-8-2413

Pötter M, Müller H, Reinecke F, Wieczorek R, Fricke F, Bowien B, Friedrich B, Steinbüchel A (2004) The complex structure of polyhydroxybutyrate (PHB) granules: four orthologous and paralogous phasins occur in *Ralstonia eutropha*. Microbiology (Reading, Engl) 150:2301–2311. https://doi.org/10.1099/mic.0.26970-0

Pötter M, Müller H, Steinbüchel A (2005) Influence of homologous phasins (PhaP) on PHA accumulation and regulation of their expression by the transcriptional repressor PhaR in *Ralstonia eutropha* H16. Microbiology (Reading, Engl) 151:825–833. https://doi.org/10.1099/mic.0.27613-0

Prieto MA, Bühler B, Jung K, Witholt B, Kessler B (1999) PhaF, a polyhydroxyalkanoate-granule-associated protein of *Pseudomonas oleovorans* GPo1 involved in the regulatory expression system for pha genes. J Bacteriol 181:858–868

Prieto A, Escapa IF, Martinez V, Dinjaski N, Herencias C, la Pena de F, Tarazona N, Revelles O (2016) A holistic view of polyhydroxyalkanoate metabolism in *Pseudomonas putida*. Environ Microbiol 18:341–357. https://doi.org/10.1111/1462-2920.12760

Qi Q, Steinbüchel A, Rehm BH (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

Ratcliff WC, Kadam SV, Denison RF (2008) Poly-3-hydroxybutyrate (PHB) supports survival and reproduction in starving rhizobia. FEMS Microbiol Ecol 65:391–399. https://doi.org/10.1111/j.1574-6941.2008.00544.x

Rehm BHA (2003) Polyester synthases: natural catalysts for plastics. Biochem J 376:15–33. https://doi.org/10.1042/BJ20031254

Rehm BHA (2006) Genetics and biochemistry of polyhydroxyalkanoate granule self-assembly: the key role of polyester synthases. Biotechnol Lett 28:207–213. https://doi.org/10.1007/s10529-005-5521-4

Rehm BHA (2010) Bacterial polymers: biosynthesis, modifications and applications. Nat Rev Microbiol 8:578–592. https://doi.org/10.1038/nrmicro2354

Rehm BH, Krüger N, Steinbüchel A (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

Ren Q, de Roo G, Witholt B, Zinn M, Thöny-Meyer L (2009) Overexpression and characterization of medium-chain-length polyhydroxyalkanoate granule bound polymerases from *Pseudomonas putida* GPo1. Microb Cell Fact 8:60–69. https://doi.org/10.1186/1475-2859-8-60

Ruth K, de Roo G, Egli T, Ren Q (2008) Identification of two acyl-CoA synthetases from *Pseudomonas putida* GPo1: one is located at the surface of polyhydroxyalkanoates granules. Biomacromolecules 9:1652–1659. https://doi.org/10.1021/bm8001655

Saegusa H, Shiraki M, Kanai C, Saito T (2001) 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. https://doi.org/10.1128/JB.183.1.94-100.2001

Schubert P, Steinbüchel A, Schlegel HG (1988) Cloning of the *Alcaligenes eutrophus* genes for synthesis of poly-beta-hydroxybutyric acid (PHB) and synthesis of PHB in *Escherichia coli*. J Bacteriol 170:5837–5847

Schultheiss D, Handrick R, Jendrossek D, Hanzlik M, Schüler D (2005) The presumptive magnetosome protein Mms16 is a poly(3-hydroxybutyrate) granule-bound protein (phasin) in *Magnetospirillum gryphiswaldense*. J Bacteriol 187:2416–2425. https://doi.org/10.1128/JB.187.7.2416-2425.2005

Schulze S (2018) Neue Einlicke in die Funktionalität PHB-Granula-assoziierter Proteine in *Ralstonia eutropha* H16. PhD thesis, University Stuttgart

Sedlacek P, Slaninova E, Koller M, Nebesarova J, Marova I, Krzyzanek V, Obruca S (2019) PHA granules help bacterial cells to preserve cell integrity when exposed to sudden osmotic imbalances. New Biotechnol 49:129–136. https://doi.org/10.1016/j.nbt.2018.10.005

Senior PJ, Dawes EA (1971) Poly-β-hydroxybutyrate biosynthesis and the regulation of glucose metabolism in *Azotobacter beijerinckii*. Biochem J 125:55–66. https://doi.org/10.1042/bj1250055

Solaiman DKY, Ashby RD, Foglia TA (2003) Effect of inactivation of poly(hydroxyalkanoates) depolymerase gene on the properties of poly(hydroxyalkanoates) in *Pseudomonas resinovorans*. Appl Microbiol Biotechnol 62:536–543. https://doi.org/10.1007/s00253-003-1317-4

Stam H, van Verseveld HW, de Vries W, Stouthamer AH (1986) Utilization of poly-β-hydroxybutyrate in free-living cultures of *Rhizobium* ORS571. FEMS Microbiol Lett 35:215–220

Steinbüchel A, Valentin HE (1995) Diversity of bacterial polyhydroxyalkanoic acids. FEMS Microbiol Lett 128:219–228

Steinbüchel A, El-Mehdi D, Marchessault RH, Timm A (1993a) Synthesis and production of poly (3-hydroxyvaleric acid) homopolyester by *Chromobacterium violaceum*. Appl Biochem Biotechnol 39:443–449

Steinbüchel A et al (1993b) Molecular basis for biosynthesis and accumulation of polyhydroxyalkanoic acids in bacteria. FEMS Microbiol Lett 104:347–350

Steinbüchel A, Aerts K, Babel W, Follner C, Liebergesell M, Madkour MH, Mayer F, Pieper-Furst U, Pries A, Valentin HE (1995) Considerations on the structure and biochemistry of bacterial polyhydroxyalkanoic acid inclusions. Can J Microbiol 41(Suppl 1):94–105

Stuart ES, Foster LJ, Lenz RW, Fuller RC (1996) Intracellular depolymerase functionality and location in *Pseudomonas oleovorans* inclusions containing polyhydroxyoctanoate. Int J Biol Macromol 19:171–176

Stubbe J, Tian J (2003) Polyhydroxyalkanoate (PHA) hemeostasis: the role of PHA synthase. Nat Prod Rep 20:445–457

Stubbe J, Tian J, He A, Sinskey AJ, Lawrence AG, Liu P (2005) Nontemplate-dependent polymerization processes: polyhydroxyalkanoate synthases as a paradigm. Annu Rev Biochem 74:433–480. https://doi.org/10.1146/annurev.biochem.74.082803.133013

Sun Y-W, Li Y, Hu Y, Chen W-X, Tian C-F (2019) Coordinated regulation of size and number of PHB granules by core and accessory phasins in the facultative microsymbiont *Sinorhizobium fredii* NGR234. Appl Environ Microbiol AEM:00717–00719. https://doi.org/10.1128/AEM.00717-19

Suzuki T, Deguchi H, Yamane T, Shimizu S, Gekko K (1988) Control of molecular weight of poly-p-hydroxybutyric acid produced in fet-batch culture of *Protomonas extorquens*. Appl Microbiol Biotechnol 27:487–491

Sznajder A, Jendrossek D (2011) Biochemical characterization of a new type of intracellular PHB depolymerase from *Rhodospirillum rubrum* with high hydrolytic activity on native PHB granules. Appl Microbiol Biotechnol 89:1487–1495. https://doi.org/10.1007/s00253-011-3096-7

Sznajder A, Jendrossek D (2014) To be or not to be a poly(3-hydroxybutyrate) (PHB) depolymerase: PhaZd1 (PhaZ6) and PhaZd2 (PhaZ7) of *Ralstonia eutropha*, highly active PHB depolymerases with no detectable role in mobilization of accumulated PHB. Appl Environ Microbiol 80:4936–4946. https://doi.org/10.1128/AEM.01056-14

Sznajder A, Pfeiffer D, Jendrossek D (2015) Comparative proteome analysis reveals four novel polyhydroxybutyrate (PHB) granule-associated proteins in *Ralstonia eutropha* H16. Appl Environ Microbiol 81:1847–1858. https://doi.org/10.1128/AEM.03791-14

Tarazona NA, Maestro B, Revelles O, Sanz JM, Prieto MA (2019) Role of leucine zipper-like motifs in the oligomerization of *Pseudomonas putida* phasins. Biochimica et Biophysica Acta (BBA) - General Subjects 1863:362–370. https://doi.org/10.1016/j.bbagen.2018.11.002

Thakor N, Lütke-Eversloh T, Steinbüchel A (2005) Application of the BPEC pathway for large-scale biotechnological production of poly(3-mercaptopropionate) by recombinant *Escherichia coli*, including a novel in situ isolation method. Appl Environ Microbiol 71:835–841. https://doi.org/10.1128/AEM.71.2.835-841.2005

Tian J, He A, Lawrence AG, Liu P, Watson N, Sinskey AJ, Stubbe J (2005a) Analysis of transient polyhydroxybutyrate production in *Wautersia eutropha* H16 by quantitative Western analysis and transmission electron microscopy. J Bacteriol 187:3825–3832. https://doi.org/10.1128/JB.187.11.3825-3832.2005

Tian J, Sinskey AJ, Stubbe J (2005b) Kinetic studies of polyhydroxybutyrate granule formation in *Wautersia eutropha* H16 by transmission electron microscopy. J Bacteriol 187:3814–3824. https://doi.org/10.1128/JB.187.11.3814-3824.2005

Timm A, Steinbüchel A (1990) Formation of polyesters consisting of medium-chain-length 3-hydroxyalkanoic acids from gluconate by *Pseudomonas aeruginosa* and other fluorescent pseudomonads. Appl Environ Microbiol 56:3360–3367

Tirapelle EF, Müller-Santos M, Tadra-Sfeir MZ, Kadowaki MAS, Steffens MBR, Monteiro RA, Souza EM, Pedrosa FO, Chubatsu LS (2013) Identification of proteins associated with

polyhydroxybutyrate granules from *Herbaspirillum seropedicae* SmR1—old partners, new players. PLoS One 8:e75066. https://doi.org/10.1371/journal.pone.0075066

Uchino K, Saito T (2006) Thiolysis of poly(3-hydroxybutyrate) with polyhydroxyalkanoate synthase from *Ralstonia eutropha*. J Biochem 139:615–621. https://doi.org/10.1093/jb/mvj069

Uchino K, Saito T, Gebauer B, Jendrossek D (2007) Isolated poly(3-hydroxybutyrate) (PHB) granules are complex bacterial organelles catalyzing formation of PHB from acetyl coenzyme A (CoA) and degradation of PHB to acetyl-CoA. J Bacteriol 189:8250–8256. https://doi.org/10.1128/JB.00752-07

Uchino K, Saito T, Jendrossek D (2008) Poly(3-hydroxybutyrate) (PHB) depolymerase PhaZa1 is involved in mobilization of accumulated PHB in *Ralstonia eutropha* H16. Appl Environ Microbiol 74:1058–1063. https://doi.org/10.1128/AEM.02342-07

Ushimaru K, Tsuge T (2016) Characterization of binding preference of polyhydroxyalkanoate biosynthesis-related multifunctional protein PhaM from *Ralstonia eutropha*. Appl Microbiol Biotechnol 100:4413–4421. https://doi.org/10.1007/s00253-015-7225-6

Wahl A, Schuth N, Pfeiffer D, Nussberger S, Jendrossek D (2012) PHB granules are attached to the nucleoid via PhaM in *Ralstonia eutropha*. BMC Microbiol 12:262. https://doi.org/10.1186/1471-2180-12-262

Wang Q, Yu H, Xia Y, Kang Z, Qi Q (2009) Complete PHB mobilization in *Escherichia coli* enhances the stress tolerance: a potential biotechnological application. Microb Cell Fact 8:47–49. https://doi.org/10.1186/1475-2859-8-47

Wieczorek R, Pries A, Steinbüchel A, Mayer F (1995) Analysis of a 24-kilodalton protein associated with the polyhydroxyalkanoic acid granules in *Alcaligenes eutrophus*. J Bacteriol 177:2425–2435

Wilde E (1962) Untersuchungen über Wachstum und Speicherstoffsynthese von *Hydrogenomonas*. Arch Mikrobiol 43:109. https://doi.org/10.1007/BF00406429

Wittenborn EC, Jost M, Wei Y, Stubbe J, Drennan CL (2016) Structure of the catalytic domain of the class I polyhydroxybutyrate synthase from *Cupriavidus necator*. J Biol Chem 48:25264–25277. https://doi.org/10.1074/jbc.M116.756833

Wodzinska J, Snell KD, Rhomberg A, Sinskey AJ, Biemann K, Stubbe J (1996) Polyhydroxybutyrate synthase: evidence for covalent catalysis. J Am Chem Soc 118:6319–6320. https://doi.org/10.1021/ja961108a

Yeung T, Gilbert GE, Shi J, Silvius J, Kapus A, Grinstein S (2008) Membrane phosphatidylserine regulates surface charge and protein localization. Science 319:210–213. https://doi.org/10.1126/science.1152066

York GM, Stubbe J, Sinskey AJ (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. https://doi.org/10.1128/JB.184.1.59-66.2002

York GM, Lupberger J, Tian JM, Lawrence AG, Stubbe J, Sinskey AJ (2003) *Ralstonia eutropha* H16 encodes two and possibly three intracellular poly[D-(-)-3-hydroxybutyrate] depolymerase genes. J Bacteriol 185:3788–3794. https://doi.org/10.1128/JB.13.3788.3794.2003

Zhao YH, Li HM, Qin LF, Wang HH, Chen G-Q (2007) Disruption of the polyhydroxyalkanoate synthase gene in *Aeromonas hydrophila* reduces its survival ability under stress conditions. FEMS Microbiol Lett 276:34–41. https://doi.org/10.1111/j.1574-6968.2007.00904.x