The protective role of PHB and its degradation products against stressful conditions in bacteria

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Running Head: PHB cycle as a stress reliever in bacteria

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Abstract

Many bacteria produce storage biopolymers that are mobilized under conditions of metabolic adaptation, for example, low nutrient availability and cellular stress. Polyhydroxyalkanoates (PHA) are often found as carbon storage in *Bacteria* or *Archaea*, and polyhydroxybutyrate (PHB) is the more frequent. Bacteria usually produce PHB upon availability of a carbon source and limitation of another essential nutrient. Therefore, it is widely believed that the function of PHB is to serve as a mobilizable carbon repository when bacteria face carbon limitation, supporting their survival. However, recent findings indicate that bacteria switch from PHB synthesis to mobilization under stress conditions such as thermal and oxidative shock. The mobilization products, 3-hydroxybutyrate and its oligomers, show a protective effect against protein aggregation and cellular damage caused by reactive oxygen species and heat shock. Thus, bacteria should have an environmental monitoring mechanism directly connected to the regulation of the PHB metabolism. Here, we review the current knowledge on PHB physiology together with a summary of recent findings on novel functions of PHB in stress resistance. Potential applications of these new functions are also presented.

Keywords

Polyhydroxybutyrate, PhaC, PhaZ, PHB cycle, 3-hydroxybutyrate oligomers, stringent response, oxidative-stress resistance, plant-bacteria interaction.

INTRODUCTION

Poly-3-hydroxybutyrate (PHB) is an aliphatic polyester member of the polyhydroxyalkanoates (PHA) family, synthesized by many prokaryotes as a carbon and reducing equivalents storage (Anderson and Dawes 1990; Steinbüchel and Valentin 1995; Madison and Huisman 1999). Usually, bacteria produce PHB upon carbon excess and low levels of nitrogen, phosphate or oxygen (Hervas *et al.* 2009). In 1988, Edwin Alfred Dawes, who dedicated his scientific career to studying microbial biochemistry, published the review *Polyhydroxybutyrate: An intriguing biopolymer* (Dawes 1988). Now, thirty-two years later, the physiological role of polyhydroxybutyrate (PHB) in bacteria is still intriguing, due to recent advances and discoveries made in the field. Beyond the eminent biotechnological potential as bioplastics with similar physicochemical properties to petrochemical materials, while highly degradable in the environment, PHB has been demonstrated to be a critical biopolymer for microbial physiology (Kim *et al.* 2013; Alves *et al.* 2016; Koskimäki *et al.* 2016; Nowroth *et al.* 2016; Batista *et al.* 2018). Several studies have associated PHB synthesis and degradation to positive fitness in bacteria and protection against abiotic and biotic stressors (Ayub *et al.* 2009; Nowroth *et al.* 2016; Madueño *et al.* 2018; Obruca *et al.* 2018; Alves *et al.* 2020; Tribelli *et al.* 2020). Herein, we present an overview of the biogenesis of PHB granules and their protective role against stressors, the stress-relieving mechanisms, and recent data on how bacteria control the cycle of PHB synthesis and degradation, opening an important window of opportunity to engineer unique microbes for bioprocesses, bioremediation of pollutants, and biofertilization of plants. However, the physiological roles of low molecular weight PHB and complexed PHB are not subject of this overview (for a detailed review on this topic please refer to (Reusch 2012)).

PHB SYNTHESIS AND SUPRAMOLECULAR STRUCTURE OF GRANULES

To understand the complexity of cellular mechanisms involved in PHA metabolism, we will first review the composition of PHA and PHB granules. PHA are polymers synthesized by esterification of coenzyme-A activated hydroxyalkanoate monomers (Anderson and Dawes 1990). Occurrence of PHA in bacteria was reported for the first time by Maurice Lemoigne in 1926 in *Bacillus megaterium* as a polymer generated by dehydration and polymerization of 3-hydroxybutyrate (Lemoigne 1926). The polymer was named poly-3-hydroxybutyrate (PHB), which is produced by bacteria as an intracellular carbon reserve (Bourque, Pomerleau and Groleau 1995; Jendrossek 2009; Ochsner *et al.* 2015). PHB is classified as scl-PHA (short chain length PHA, 3 to 5 carbon atoms in the monomers). Some bacteria, such as *Pseudomonas* species, synthesize mcl-PHA (medium chain length PHA), esterifying hydroxyalkanoate monomers of 6 to 14 carbon atoms (for a detailed review, please refer to (Anderson and Dawes 1990) and (Madison and Huisman 1999)).

The PHA polymers are generally stored intracellularly in the form of water-insoluble inclusions, often designated as PHA granules. Due to the complexity of proteins on the PHA granule surface, and their importance for bacterial physiology, the granules were also named carbonosomes (for a review, please refer to Jendrossek 2009). We will first review the most recent findings on PHA granules biogenesis achieved by studying *Ralstonia eutropha* H16 (also designated as *Hydrogenomonas eutropha* H16, *Alcaligenes eutrophus* H16, *Wautersia eutropha* H16, and *Cupriavidus necator* H16). Most statements apply to PHB granules of other species and to PHA granules consisting of mcl-PHA (for earlier overviews on PHB/PHA granules, see the following references (Pötter and Steinbüchel 2006; Kuchta *et al.* 2007; Jendrossek and Pfeiffer 2014; Prieto *et al.* 2016; Tarazona *et al.* 2020).

PHB granules *in vivo* do not have phospholipids

PHB granules of all prokaryotic species studied consist of a polymer core and a surface layer of a species-dependent number of proteins. The exact composition of this surface layer has been discussed controversially. Previously, it was assumed that there is a phospholipid monolayer around the PHB granules. However, the assumed phospholipid layer was shown to be an in vitro artefact that had occurred during PHB granule preparation (for detailed background see Bresan *et al.*, 2016, 2017). The current model of a PHB granule is shown in Fig. 1.

PHB granule-associated proteins

PHB granules *in vivo* are covered by a surface layer comprising of four classes of proteins. These so-called PHB granule-associated proteins (PGAPs) can be categorized into different functional groups: (i) the key enzyme of PHB biosynthesis, the PHA synthase (PhaC1) is covalently linked to the growing PHB molecules via a cysteine residue in the active site (Cys319 in case of PhaC1 of *R. eutropha*) (Stubbe *et al.* 2005; Wittenborn *et al.* 2016; Kim *et al.* 2017), therefore being the most important PGAP. Recent findings have shown that PhaC can detach from PHB granules at later stages of granule growth (Bresan and Jendrossek 2017). The detachment is thought to represent an aging phenomenon, preventing disruption of the cell by indefinitely growing granules under permanent PHB-permissive culture conditions.

(ii) The intracellular PHA depolymerases (PhaZs) represent the second group of catalytically active PGAPs. PhaZs are responsible for hydrolysis (cleavage with water) and thiolysis (cleavage of PHB with coenzyme A to 3HB-coenzyme A) of PHB under carbon starvation (Handrick *et al.* 2000; Uchino *et al.* 2007; Uchino *et al.* 2008; Eggers and Steinbuchel 2013; Adaya *et al.* 2018). *R. eutropha* can express seven PHB

depolymerase isoenzymes, most of which are bound to PHB granules *in vivo* (Kobayashi and Saito 2003; York *et al.* 2003; Uchino *et al.* 2008; Sznajder and Jendrossek 2014).

(iii) The third group of PGAPs constitutes the phasins (PhaPs) (Wieczorek *et al.* 1995; Pötter *et al.* 2004; Pfeiffer *et al.* 2011; Pfeiffer and Jendrossek 2012; Mezzina and Pettinari 2016). Phasins are amphiphilic polypeptides of low molecular mass, but their function is still unknown. Recently, the tridimensional structure of PhaP from *Aeromonas hydrophila* was solved, revealing that each subunit has two opposite hydrophobic and hydrophilic surfaces (Zhao *et al.* 2016). The phasin PhaP1 is the major PGAP in *R. eutropha* and determines the surface to volume ratio of formed PHB granules. It is assumed that phasins mediate between the hydrophobic core of the PHB granules and the hydrophilic cytoplasm, thus preventing other proteins with hydrophobic surfaces from binding to PHB. Up to seven phasins have been identified in *R. eutropha* (PhaP1 – PhaP7) (Pfeiffer and Jendrossek 2012; Sznajder *et al.* 2015). Comparative proteome analysis has revealed that additional proteins of so far unknown function are present on PHB granules *in vivo* in *R. eutropha* (Sznajder *et al.* 2015). One of them (H16_A0225) has a patatin-like phospholipase motif, but the function of this protein is unknown. A patatin-like enzyme has also been identified as a PGAP in the archaeon *Haloferax mediterranei* (Liu *et al.* 2015).

The last group of PGAPs refers to proteins that have two physiologically relevant locations, they can bind to DNA in addition to binding to the PHB granule surface. In the case of *R. eutropha*, these are PhaR and PhaM. PhaR is a transcriptional repressor of phasin gene *phaP1* (Pötter *et al.* 2002; York *et al.* 2002). PhaM is a multifunctional protein that binds not only to the PHB granule (via binding to PhaC) but also to the nucleoid, thus linking both (Pfeiffer *et al.* 2011; Pfeiffer and Jendrossek 2014; Bresan and Jendrossek 2017). Consequently, PHB granules are attached to the nucleoid and

distributed to daughter cells during cell division (Wahl *et al.* 2012). These 16 PGAPs constitute the PHB proteome in *R. eutropha*, representing highly organized multifunctional units (carbonosomes) (Jendrossek 2009; Jendrossek and Pfeiffer 2014) (Figure 1).

Identification of proteins attached to PHB granules of other species of bacteria suggest that even more PGAPs might be associated with PHB granules (Tirapelle *et al.* 2013; Narancic *et al.* 2018; Moreno *et al.* 2019). The PHA granules of mcl-PHA accumulating species have a similar structure consisting of a polymer core and several polymer surface-attached proteins. Most studies on the structure and composition of mcl-PHA granules have been made on *Pseudomonas putida* (Prieto et al. 2016; Tarazona *et al.*, 2020).

THE PROTECTIVE ROLE OF PHB AGAINST ABIOTIC AND BIOTIC STRESSORS

PHB has been considered a carbon storage for bacteria since its discovery (Lemoigne 1926), but several studies have correlated the synthesis and degradation of the polymer with stress resistance. For instance, the importance of PHB for bacterial colonization in carbon-limited environments has been well documented (Matin *et al.* 1979; James *et al.* 1999; Handrick *et al*. 2000; Jendrossek and Handrick 2002; Lopez *et al.* 2015). The ability to produce and store PHB is generally linked with an improved survival under stressful conditions, or competition (Kadouri *et al.* 2003; Zhao *et al.* 2007; Ratcliff *et al.* 2008; Aurass *et al.* 2009; Tribelli *et al.* 2012), and the capacity for PHB biosynthesis is a common trait for bacteria adapted to abiotic (Kadouri *et al.* 2003; Zhao *et al.* 2007; Tribelli *et al.* 2012; Nowroth *et al*. 2016) or biotic stresses (Aneja *et al.* 2005; Aurass *et al.* 2009; Kim *et al.* 2013;

Balsanelli *et al.* 2016; Quelas *et al.* 2016). The association of PHB with bacterial stress-resistance in harsh environments, and during plant colonization, has been reported in several studies (Tal and Okon 1985; Kadouri, Jurkevitch and Okon 2003; Aneja, Zachertowska and Charles 2005; Calderon-Flores *et al.* 2005; Ratcliff *et al.* 2008; Ayub *et al.* 2009; Juengert *et al.* 2017; Obruca *et al.* 2020).

The synthesis of PHB is increased in bacterial cultures exposed to moderately elevated levels of oxidative stress ($\leq$ 10 mM H_2O_2) (Obruca *et al.* 2010b, 2010a), and heavy-metal stress (Cu^{2+}) (Kamnev *et al.* 2012). However, after a certain threshold, a decrease of PHB content is typically observed in response to a greater severity of the stress (Obruca *et al.* 2010a). The *ntrC* mutant of *Herbaspirillum seropedicae* defective in the master transcriptional activator of nitrogen regulation (Ntr) stress response of the NtrBC two-component system, accumulates more PHB and survives better than the wild type when challenged with H_2O_2 (Sacomboio *et al.* 2017). Furthermore, mobilization of PHB in *H. seropedicae* SmR1 was activated by a heat shock at 45°C, and mutants that lacked the capacity to synthesize or mobilize PHB were more susceptible to heat shock (Alves *et al.* 2020). Recent results showed that *Pseudomonas extremaustralis*, a highly stress-resistant strain, exhibits a high UV radiation resistance in conditions favoring PHB accumulation. However, the PHB-deficient mutant, and a mutant incapable of producing mcl-PHAs, were sensitive towards UVA exposure (Tribelli *et al.* 2020).

When *Escherichia coli*, which is unable to synthesize PHB, was bioengineered for PHB biosynthesis and degradation, the strain showed an improved tolerance against various stresses (Wang *et al.* 2009). The authors suggested two possible factors to justify the better stress-tolerance phenotype in *E. coli* synthesizing and degrading PHB: (i) PHB (or their degradation products) could increase synthesis of

193 the alarmone ppGpp (guanosine tetraphosphate) and mRNA translation of stationary
194 phase sigma factor *rpoS* (Brown *et al.* 2002) and (ii) the PHB accumulation could
195 activate some endogenous chaperone mechanism. During stressful conditions, *E. coli*
196 and other bacteria increase the cellular concentration of RpoS. RpoS up-regulates
197 expression of genes at high concentration, leading to general stress resistance in
198 bacteria (Battesti, Majdalani and Gottesman 2011). Supporting this, mcl-PHA
199 degradation was positively correlated with ppGpp levels in *Pseudomonas oleovorans*
200 under carbon starvation (Ruiz *et al.* 2001).
201
202 **Involvement of ppGpp in PHB synthesis and degradation**
203 Deletion of the genes *spoT1* and *spoT2* encoding enzymes with ppGpp
204 synthetase activity in *R. eutropha* H16 resulted in inability to synthesize detectable
205 levels of ppGpp and accumulation of minor amounts of PHB (Juengert *et al.* 2017).
206 The effect of ppGpp on PHB accumulation in *R. eutropha* was dependent on PhaZa1
207 depolymerase, whereas in the Δ*spoT1*+Δ*spoT2* mutant, deletion of *phaZa1* restored
208 the PHB levels. In contrast, the Δ*spoT1*+Δ*spoT2* mutant of *R. eutropha*
209 overexpressing *spoT2* (encoding a ppGpp synthase without ppGpp hydrolase
210 activity) had high ppGpp levels and accumulated extremely high amounts of PHB
211 (Juengert *et al.* 2017). Involvement of ppGpp in PHA degradation has been reported
212 in *P. oleovorans* (Ruiz *et al.* 2001), suggesting that ppGpp has various species-
213 specific effects on PHB metabolism.
214
215 **Phasins acting as chaperones in *E. coli***
216 Accumulation of PHB in *E. coli* is stressful, revealed by high expression of
217 several heat shock proteins (HSP) and binding on the surface of the PHB granules

(Han *et al.* 2001; Han *et al.* 2006; Tessmer *et al.* 2007). The hydrophobic surface of the granules potentially interacts with cytoplasmic proteins of *E. coli*, resulting in protein denaturation. Association of HSPs with PHB granules reduces protein aggregation and denaturation, and high HSP expression could therefore help *E. coli* to resist the heat shock stress (Han *et al.* 2006). *E. coli* cells expressing the granule-associated phasin gene *phaP* from *Azotobacter* sp. FA8 grows better and produces more PHB using glycerol as a carbon source (de Almeida *et al.* 2007). Furthermore, expression of *phaP* from *Azotobacter* sp. FA8 in a non-PHB accumulating *E. coli* strain showed that PhaP protects the cells against heat shock and oxidative stress caused by paraquat (N,N′-dimethyl-4,4′-bipyridinium dichloride), a redox-active heterocycle compound widely applied as herbicide (de Almeida *et al.* 2011). The expression of *phaP* also reduced RpoH levels during heat shock, indicating reduction in the levels of misfolded proteins (de Almeida *et al.* 2011).

When *phaP* from *Azotobacter* sp. FA8 was co-expressed with GFP (*phaP*$_{Az}$-*gfp*) in *E. coli* expressing the PD domain, an insoluble domain of TolR from *Azoarcus* sp. CIB, the PhaP$_{Az}$-GFP colocalized with inclusion bodies of PD. The expression of *phaP*$_{Az}$ significantly reduced the content of PD inclusion bodies and increased the solubility of thermal aggregates of citrate synthase *in vitro* (Mezzina *et al.* 2015). These findings suggest that PhaP can protect bacteria against heat shock by chaperone activity. Apart from PHB granules, the protective effect of PhaP has not yet been addressed in naturally PHB-producing bacteria, because of the challenge of avoiding the association with PHB granules (York *et al.* 2001, 2002; Pötter *et al.* 2002; Neumann *et al.* 2008). This could be accomplished by using mutants defective in synthesis of PHB and synthetic promoters controlling the expression of *phaP*.

PHB METABOLISM IN METHYLOTROPHIC BACTERIA AND ITS ANTIOXIDANT ROLE

Methylotrophs represent an important bacterial group typical for having the capacity for PHB biosynthesis. Methylo- and methanotrophic bacteria are globally important for their ability to fix the greenhouse gas, methane, in addition to metabolizing methanol and other plant volatiles as carbon sources (Fall 1996; Freyermuth *et al.* 1996; Kip *et al.* 2010). For example, bacteria of the genus *Methylobacterium* naturally synthesize PHB from methanol, presenting a growing interest to couple sustainable production of chemicals with methane emission mitigation. In optimized nitrogen-limited fed-batch cultures, PHB production by *Methylobacterium* strains can reach up to 149 grams per liter (64% of dry cell weight) (Suzuki *et al.* 1986; Bourque, Pomerleau and Groleau 1995; Ochsner *et al.* 2015). The key enzyme enabling methylotrophic growth is methanol dehydrogenase (MDH), which is responsible for the oxidation of methanol to form formaldehyde in the bacterial periplasmic space (Goodwin and Anthony 1998). Formaldehyde is then either oxidized to CO_2, or assimilated through the serine cycle, or channeled into polyhydroxybutyrate (Chistoserdova *et al.* 2003). There are 15 genes involved in the oxidation of methanol in *M. extorquens* strains AM1 and PA1 (Chistoserdova *et al.* 2003; Nayak and Marx 2014). Upstream of the *mxaF* gene, there is a methanol-inducible promoter controlling transcription of the whole 14-gene cluster as a single operon (Zhang and Lidstrom 2003). The operon consists of genes for the large and small subunits (MxaFI) of MDH, and for proteins involved in transport, assembly, and electron transfer (Chistoserdova *et al.* 2003; Zhang and Lidstrom 2003). Previous studies on methylotrophic bacteria have revealed that the *mxaF* promoter is highly active in plant epiphytic or nodule-bound lifestyles (Jourand *et al.* 2005; Sy *et al.* 2005). The methylotrophic bacteria can store the methanol-fixed carbon in high quantities as

endogenous PHB granules by PhaC-catalyzed polymerization of the PHB precursor 3-hydroxybutyryl coenzyme (Valentin and Steinbüchel 1993; Bourque, Pomerleau and Groleau 1995).

PHB and ME-3HB oligomers as antioxidant defense

PHB can work as a sink of reducing equivalents and maintain redox homeostasis by controlling cellular NAD(P)H/NAD(P)$^+$ ratios (Senior *et al.* 1972; Senior and Dawes 1973). Haces *et al.* (2008) showed that the monomer of PHB, 3-hydroxybutyrate (3HB), possesses antioxidant activity. Recently, a new trait for products derived from degradation of PHB was discovered. The degradation products, methyl-esterified 3-hydroxybutyrate (ME-3HB) oligomers, were found with antioxidative activity against the hydroxyl radical (Koskimäki *et al.* 2016). The hydroxyl radical is the most cytotoxic among ROS species, and there is no known enzymatic system for its detoxification. In general, bacterial enzymatic ROS defenses, such as catalases, peroxidases, and SODs, together with metal chelating or transporting systems, are responsible for preventing the formation of hydroxyl radical in living cells. However, in stressful conditions, these cellular ROS defenses can be overwhelmed, resulting in oxidative damage (Imlay 2013).

The ME-3HB di- and trimers, isolated from *Methylobacterium extorquens* DSM13060, showed 3- and 2.8-times higher HO· scavenging activity than glutathione (GSH) in a fluorometric hydroxyl-radical scavenging capacity (HOSC) assay. In this assay, the antioxidative activity of ascorbic acid (AA) and 3-hydroxybutyrate (3HB) was more than 10-times weaker than that of ME-3HB oligomers (Koskimäki *et al.* 2016). When the cytoprotective potential of ME-3HB oligomers was tested using hydroxyl radical growth-arrest bioassays on yeast cells,

293 the ME-3HB oligomers, at concentrations of 50-200 µM, protected cells from
294 HO· stress. Yeast mutants deficient in GSH synthesis (*gsh1Δ* and *gsh2Δ*), displayed
295 approximately a ten-fold higher hypersensitivity to hydroxyl radical stress when
296 cultured in a medium not supplemented with these antioxidants (Koskimäki *et al.*
297 2016).
298
299 **ME-3HB oligomers are products of PHB degradation**
300 The ME-3HB oligomers are most likely produced in *M. extorquens* mainly by
301 degradation of the PHB polymer, due to a significant increase in expression of the
302 bacterial depolymerases *phaZ1* and *phaZ2* and degradation of PHB granules in
303 bacterial cells upon hydroxyl radical stress application. However, a concurrent
304 biosynthesis of PHB due to elevated activity of the PHB synthase gene *phaC* was
305 observed in the bacteria (Koskimäki *et al.* 2016). Earlier, a parallel degradation and
306 biosynthesis of PHA have been reported for *Pseudomonas putida* and *R. eutropha*
307 (Doi *et al.* 1990; Taidi *et al.* 1995; Ren *et al.* 2009).
308 The biosynthesis of PHB involves several enzymes and a carbon source,
309 whereas mobilization of the pre-synthesized PHB requires only one depolymerizing
310 enzyme (Fig. 2). The structural characteristics that govern the specificity of PHA
311 depolymerases are not yet fully understood (Jendrossek *et al.* 2013). Bacterial PHA
312 depolymerases produce various hydrolysis products that can be easily separated and
313 detected after mild derivatization with bromo-phenacyl bromide (Gebauer and
314 Jendrossek 2006). Depending on species, the products are typically monomers only,
315 or monomers and dimers, or a mixture of oligomers (Lee *et al.* 1999; Jendrossek and
316 Handrick 2002). In addition, some bacterial strains can express hydrolases that cleave
317 the 3HB-oligomers and 3HB-dimers to the monomeric end-product (Sugiyama *et al.*

2004). The rapid degradation of the substantial amounts of cellular PHB, to produce significant antioxidative power, can provide a fast adaptation to stressful conditions for bacteria. The ME-3HB oligomers are produced in the cells of *M. extorquens* DSM13060 at high concentrations of 200-500 μM, even without optimization of culture conditions for carbon accumulation (Koskimäki *et al.* 2016). Considering the reports of depolymerized PHB content being able to reach 146 mM of released 3HB in optimized cultures (Kawata *et al.* 2012), the functional PHB cycle can likely maintain an endogenous 3HB and oligomer pool higher than 100 mM (Obruca *et al.* 2016), providing a robust protective buffer against stress.

The 3HB belongs to the group of ketone bodies in mammalian cells, being produced as an energy source in the liver during starvation (Klocker *et al.* 2013). Besides involvement in protection from oxidative stress (Haces *et al.* 2008), 3HB has been linked with other cellular mechanisms, such as epigenetic regulation (Shimazu *et al.* 2013), anti-inflammatory signaling (Youm *et al.* 2015), mitochondrial protection (Maalouf *et al.* 2007), and prevention of apoptosis in mammals (Cheng *et al.* 2013). In addition to the various cytoprotective capacities found for 3HB, its methylated form (ME-3HB, 3-hydroxybutyrate methyl ester) shares similar capacities (Zhang *et al.* 2013). Methyl-esterification can prevent re-polymerization of 3HB and its oligomers and increase the stability of these molecules (Park *et al.* 2004). The hydroxyl group of the 3HB molecule was earlier suggested to be responsible for antioxidant capacity (Haces *et al.* 2008). However, like 3HB, there is only one hydroxyl group present in the ME-3HB dimer and trimer molecules yet having a significantly higher hydroxyl-radical scavenging capacity than 3HB. Therefore, a different explanation must exist for the higher activity of the oligomeric forms.

HOW DO BACTERIA SENSE OXIDATIVE STRESS AND MOBILIZE PHB AS A RESPONSE?

Due to the important role as an energy reservoir, mobilization of PHB is likely initiated in parallel with other bacterial adaptations, such as the stringent response. In *R. eutropha*, ppGpp-mediated stringent response leads to PHB production or mobilization very rapidly, depending on ppGpp levels (Juengert *et al.* 2017). The non-phosphorylated form of regulatory protein EIIANtr of the phosphotransferase system (PTS) interacts with the bifunctional ppGpp synthase/hydrolase SpoT1 (Karstens *et al.* 2014). Since the EIIANtr phosphorylated to non-phosphorylated ratio is influenced by the level of the phosphoryl donor phosphoenolpyruvate (PEP), it is likely that the level of PHB synthesis and degradation is controlled by the cellular energy status and the metabolic rate, as these processes directly interfere with carbon and nitrogen availability, as well as ppGpp synthesis and degradation (Ronneau *et al.* 2016) (Fig. 3).

In *R. eutropha* H16, the Δ*ptsI* and Δ*ptsH* mutants accumulated lower amounts of PHB when cultivated with gluconate as carbon source. The genes *ptsI* and *ptsH* encode EI and HPr components of the PTS system, respectively (for a detailed review on the PTS system, please refer to Deutscher et al. (2014). The Δ*ptsI* mutant accumulated 1.5-fold less PHB at the early stationary phase and degraded PHB faster in the stationary phase than the wild type, and the Δ*ptsH* mutant presented a similar pattern when cultivated with gluconate (Kaddor and Steinbüchel 2011). However, when *H16_A0384*, a homologue of *ptsN* encoding the EIIANtr protein of PTS was mutated, the mutant accumulated 10% more PHB than wild type at the early stationary phase and degraded PHB slower than the Δ*ptsI* and Δ*ptsH* mutants (Kaddor and Steinbüchel 2011). These results suggest that unphosphorylated EIIANtr favors PHB degradation, while its phosphorylated form supports PHB synthesis. The increase of PHB has also been determined for *ptsN* mutants

in *Azotobacter vinelandii* (Noguez *et al.* 2008) and *Pseudomonas putida* (Velázquez *et al.* 2007). In *A. vinelandii*, RpoS is necessary for expression of PHB synthesis genes (Hernandez-Eligio *et al.* 2011), and the RpoS levels decrease in a Δ*ptsP* mutant due to degradation by ClpAP protease (Muriel-Millan *et al.* 2017). Deletion of *ptsN* restores the levels of RpoS and PHB accumulation, indicating that unphosphorylated EIIANtr induces ClpAP and proteolysis of RpoS (Muriel-Millan *et al.* 2017).

Since PHB synthase and PHB depolymerase are constitutively expressed, allosteric regulation and posttranslational modification of PhaC and PhaZa1 are regulatory mechanisms to control PHB synthesis and mobilization (Juengert *et al.* 2018). Recently, Juengert and co-workers demonstrated that the Thr373 of PhaC1 from *R. eutropha* is phosphorylated in the stationary growth phase but unmodified in the exponential and PHB accumulation phases. Activity of a phosphomimetic Thr373Asp PhaC1 variant was significantly lower than that of wild type, showing the phosphorylation is relevant for PHB synthesis. The Ser35 of *R. eutropha* H16 PhaZa1 was phosphorylated during the exponential and stationary growth phases. Strains carrying the PhaZa1 Thr26Asp and Ser35Asp phosphomimetic variants exhibited reduced PHB mobilization in the stationary growth phase (Juengert *et al.* 2018). Post-translational control of PhaC and PhaZ activities would be an appropriate switch for rapid modulation of the PHB metabolism in bacteria. Identification of the phosphorylase and phosphatase acting in the PhaC and PhaZ modifications will allow a deeper understanding of the PHB metabolism control at the molecular level.

PHB accumulation and mobilization is important for bacteria colonizing specific environments

PHB accumulation has been reported as a common trait among bacteria from soil and aquatic environments (Balsanelli *et al.* 2016; Props *et al.* 2019). Also, *Archaea* adapted to colonize extreme environments often accumulate PHB (Liu *et al.* 2013). Koskimäki et al. (2016) found that *phaC* and *phaZ* genes are typically present in bacteria of the plant-associated families *Rhizobiaceae, Bradyrhizobiaceae, Phyllobacteriaceae, Xanthobacteraceae, Rhodospirillaceae, Burkholderiaceae,* and *Pseudomonadaceae* (Koskimäki *et al.* 2016). In addition, *phaC* and *phaZ* genes were identified in genomes of bacteria adapted to extreme environments, such as high salinity, high metal concentrations, low pH, high or low temperatures. These bacteria belonged to genera *Acidiphilium, Cupriavidus, Glaciecola, Halomonas, Janthinobacterium, Magnetospirillum, Marinobacter, Oceanicola, Polaromonas, Sulfitobacter,* and *Thiothrix*. The *phaC* and *phaZ* genes were also typical for human intracellular pathogens of the genera *Bordetella, Burkholderia, Legionella, Mycobacterium, Rickettsia,* and *Vibrio* (Koskimäki *et al.* 2016).

Transcription of *phaC* and *phaZ* genes has been found under stress in *Aromatoleum aromaticum, Dinoroseobacter shibae, Ensifer meliloti* and *Pseudomonas oleovorans* (Ruiz *et al.* 2001; Krol and Becker 2004; Trautwein *et al.* 2008; Wang *et al.* 2014), suggesting that the protective mechanisms based on PHB are widespread. Specifically, in cold environments, the increased solubility of oxygen and the stability of oxygen radicals force bacteria to adapt and survive high levels of oxidative stress (Medigue *et al.* 2005; D'Amico *et al.* 2006; Ayub *et al.* 2009). Ayub *et al.* (2009) showed that the Δ*phaC* mutant of the cold-resistant *P. extremaustralis* cannot grow in temperatures below 10°C. The cold-shock increased lipid peroxidation by 25-fold due to oxidative stress in the mutant compared to wild type bacteria. When antioxidants, such as glutathione, were added to the cultures, the cold sensitivity could be reversed. There was also a rapid mobilization

of intracellular PHB reserves in the wild type during cold shock (Ayub *et al.* 2009). Similarly, the Δ*phaC* mutant of *R. eutropha* is reported to be more sensitive to low temperature than the wild type (Nowroth *et al.* 2016).

Alves et al. (2020) demonstrated that PHB mobilization is the key to survival of *H. seropedicae* under heat shock, as addition of excess 3HB to Δ*phaZ1.2* culture rescued the mutant. Heat sensitivity was also observed in Δ*phaC1*, indicating that the complete PHB cycle is needed for protection of *H. seropedicae* against heat stress. The heritability of PHB granules is also affected by stress. When cells of field-isolated *Bradyrhizobium* were starved, PHB reserves were asymmetrically shared between replicating cells (Muller and Denison, 2018). Similar bet-hedging has previously been shown in *Sinorhizobium meliloti* (Ratcliff and Denison, 2010). Although the isolates with higher PHB content depolymerized more PHB within the first month, they maintained a PHB reserve during dormancy, which suggests that PHB supports both short and long-term adaptation (Muller and Denison, 2018).

Recent works have addressed the importance of PHB for bacterial colonization of plants. Attached bacteria of *H. seropedicae* SmR1, colonizing wheat roots, expressed the *phaC1* 1.5-fold higher than the planktonic bacteria in hydroponic medium (Pankievicz *et al.* 2016). However, the attached bacteria expressed *phaZ1* 2-fold higher than planktonic ones for three days after inoculation, indicating a fast mobilization of the stored PHB. Likely, the quantity of stored and mobilized PHB by plant-attached bacteria or bacteria colonizing internal tissues is dependent on carbon sources exudated by the host and the extent of host defenses faced by bacteria in the early stages of infection. For example, during the early stages of pine host colonization, hydroxyl radicals generated by Fenton reaction accumulated in the host tissue, and concurrent activation of the promoters of *phaC* and *phaZ1* and degradation of PHB granules was observed in *M. extorquens*

DSM13060 (Koskimäki *et al.* 2016) (Fig. 4). Similarly, several species of rhizobia deplete stored PHB during plant colonization (Charles *et al.* 1997), and PHB degradation is important to sustain bacterial infection and cell proliferation before forming the symbiosome between legumes and rhizobia (Trainer and Charles 2006; Wang *et al.* 2007). *H. seropedicae* colonizing the surface of the C4 model grass *Setaria viridis* roots expressed *phaC1::gfp* four days after inoculation, while the *phaZ1::gfp* and *phaZ2::gfp* were expressed only ten days after inoculation. In this case, PHB synthesis was active at the initial steps of colonization, and PHB mobilization was activated later to support bacterial survival within the plant (Alves *et al.* 2019).

Due to the importance of PHB in plant colonization, bacteria with high quantities of PHB should perform better at colonizing host plant. The *S. meliloti phaZ* deletion mutant was equally competent to wild type in colonizing alfalfa (*Medicago sativa*) roots from rhizosphere (Trainer *et al.* 2010), but *S. meliloti* strains typically have ≥ 2 copies of *phaZ* gene, and likely another isoform was able to compensate for the deleted one. In *Sinorhizobium fredii* NGR234 the double mutant lacking PHB synthases genes *phbC1* and *phbC2* showed reduced shoot dry weight of inoculated *Vigna unguiculata* plants, whilst strains with single gene deletions of *phbC1*, *phbC2* or *phaZ* (encoding a PHA depolymerase) had no effect (Sun *et al.* 2019).

Transcriptome sequencing of the grass-endophyte *H. seropedicae* SmR1 showed 12 - 16-fold induction of *phaZ* gene expression at the early stages of host infection. The mutants unable to synthesize PHB demonstrated 32-fold and 18-fold lower capacity for epiphytic and endophytic colonization, respectively (Balsanelli *et al.* 2016).

Availability of PHB reserves upon plant colonization not only affects bacterial but also plant fitness. Alves et al. (2019) inoculated several *H. seropedicae* mutants with various levels of accumulated PHB into *S. viridis*. The strains producing high quantities

of PHB significantly increased root area and the number of lateral roots of the host compared to the PHB-negative mutants. Interestingly, the double mutant Δ*phaZ1*+Δ*phaZ2* colonized *S. viridis* plants, but with a significant reduction of root area and number of lateral roots in the host compared to the parental strain. Field experiments of grasses inoculated with *Azospirillum brasilense* have also shown that high PHB-accumulating strains promote plant growth on wheat, maize, rice, sorghum, and barley (Fibach-Paldi *et al.* 2012; Oliveira *et al.* 2017), and the shelf life of the inoculant is longer (Kadouri, Jurkevitch and Okon 2003).

MEDICAL APPLICATIONS OF 3HB OLIGOMERS AS ANTIOXIDANT COMPOUNDS

The finding of ME-3HB oligomers with antioxidant capacity has drastically changed our understanding of bacterial physiology regarding PHB, potentially explaining how PHB-producing bacteria can retain their cellular homeostasis in extreme conditions and during infection of host cells (Koskimäki *et al.* 2016; Batista *et al.* 2018). Similarities between plant and animal early defense responses to microbial infection can permit extending the knowledge on ME-3HB oligomers and their antioxidant activity to the PHB-producing bacteria involved in human diseases. Many human pathogens belonging to the genera *Bordetella*, *Burkholderia*, *Legionella*, *Mycobacterium*, *Rickettsia*, and *Vibrio* with capacity for PHB synthesis can cause persistent intracellular infections that are difficult to treat (James *et al.* 1999; Sikora *et al.* 2009; Grant *et al.* 2012). Therefore, the knowledge on the importance of PHB for intracellular infection can provide new targets for antibacterial therapies. From another point of view, a recent study identified antimicrobial activity against non-PHB producer strains by hydroxybutyrate oligomers

(Ma *et al.* 2019), which suggests that these compounds will have numerous applications in medicine.

In mammalian *in vitro* and *in vivo* models, the therapeutic effect of 3HB is associated with free radical scavenging and improvement of mitochondrial respiration (Kashiwaya *et al.* 2000; Maalouf *et al.* 2007; Haces *et al.* 2008; Shimazu *et al.* 2013). Specifically, 3HB has been shown to inhibit histone deacetylases in human HEK293 cells and mouse models (Shimazu *et al.* 2013), and to protect rat mesencephalic and hippocampal cells from oxidative stress (Kashiwaya *et al.* 2000). Moreover, 3HB inhibits cell apoptosis under glucose deprivation and rescues activities of mitochondrial respiratory chain complexes in mouse Parkinson's disease model (Tieu *et al.* 2003), in rat PC-12 cells, and mouse Alzheimer's disease model (Zhang *et al.* 2013). The 3HB is patented and under development as treatment for Parkinson's and Alzheimer's diseases (Clarke and Veech; Henderson; Veech).

Like Alzheimer's and Parkinson's diseases, oxidative cellular damage is obvious in many ophthalmic disorders. The complex visual signal transduction in the retina creates a need for high energy, which makes the eye specifically vulnerable to oxidative stress. For example, age-related oxidative stress ultimately results in pathologies such as glaucoma or age-related macular degeneration (AMD) (Payne *et al.* 2014). Another ophthalmic disorder involving oxidative stress at the ocular surface is dry-eye-disease (DED). DED progress leads to visual disturbance and considerably lowers the quality of life. Some therapies currently exist to improve DED symptoms, mainly based on topical cyclosporine treatment with significant side effects (Yu *et al.* 2011). Due to strong antioxidative effects against the hydroxyl radical, the ME-3HB oligomers are being developed for the prevention of ophthalmic disorders such as macular degeneration and DED. Our unpublished data show that the human retinal cells are protected by ME-3HB

oligomers against oxidative stress and suggest that the observed cellular protection is induced through several innate cellular mechanisms (Koskimäki et al., unpublished).

CONCLUSIONS AND PERSPECTIVES

Herein, we demonstrate that PHB is important not only as a carbon stock but in supporting bacterial survival under adverse conditions. Thermal and oxidative shock protection emerge as important secondary roles for the PHB metabolism. The 3HB produced by degradation of PHB increases solubility of proteins susceptible to aggregation during thermal shock, whereas ME-3HB oligomers have high antioxidant activity. We also presented insights into PHB metabolism regulation and how the nucleotide ppGpp may be involved in the control of PHB synthesis and degradation. A complete picture of the regulatory mechanisms acting in the PHB cycle will be the key to engineer bacteria surviving better in hostile environments. Several PHB accumulation mutants exhibit lowered capacity of plant colonization, and the metabolism of PHB could be engineered towards more efficient bacterial symbionts interacting with plants. On the other hand, PHB metabolism provides new co-targets for severe (or persistent) bacterial infections. Bacteria of the genus *Aeromonas*, which infect and colonize the human intestine, also synthesize and store PHB. However, currently there is no knowledge on the role of PHB metabolism in the intestinal environment. Thus, the recent data reviewed here could be extrapolated to various models and applications both in human and plant health.

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