Polyhydroxybutyrate: A Useful Product of Chlorotic Cyanobacteria

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Abstract

Polyhydroxybutyrate (PHB) is a carbon polymer with diverse functions, varying greatly on the organism producing it. This microreview describes the current knowledge about PHB metabolism, structure, and different physiological roles with a special focus on cyanobacteria. Despite the physiological function of PHB in the cyanobacterial phylum still being unknown, these organisms provide the unique opportunity to directly convert atmospheric CO₂ into bioplastic using a solar-based process. Recent research on PHB metabolism in the cyanobacterial model organism *Synechocystis* revealed a sophisticated control of PHB granule formation. Novel insights about the metabolic background of PHB synthesis resulted in the engineering of the first cyanobacterial super-producer strain.

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The Characteristics and Classification of Polyhydroxyalkanoates

Polyhydroxybutyrate (PHB) was discovered in *Bacillus megaterium* in 1926 [Lemoigne, 1926]. PHB is composed of 3-hydroxybutyrate monomers. When several 3-hydroxybutyrate monomers are connected via an ester bond, they form PHB. The latter accumulates in the form of water-insoluble inclusions within the cell. Six decades after PHB was first discovered, in 1983 researchers showed that when grown on octane, *Pseudomonas oleovorans* produces poly-beta-hydroxyoctanoate granules [de Smet et al., 1983]. This was the first time that the microbial production of other polyhydroxyalkanoates (PHA) was discovered. PHAs are classified in three different groups, depending on the length of the monomer: short- (C₃–C₅), medium- (C₆–C₁₆) and long-chain-length PHA (scl, mcl, and lcl, respectively) [Reddy et al., 2003]. A selection of different PHA types is given in Figure 1.

Production of PHA is described from representatives from all three kingdoms of life: archaea, bacteria, and eukaryotes. Just recently it has been reported that also eukaryotic algae can naturally produce PHB, namely *Chlorella* [Cassuriaga et al., 2018] and *Botryococcus*

Fig. 1. General structure of PHA. Depending on the side chain R, several different types of PHA are formed. In the case of PHB (bold), the side chain corresponds to a methyl group. The monomeric units (n) of PHAs are linked via an ester bond between the hydroxy- and the carboxyl groups, forming the respective PHA.

Fig. 2. PHB synthesis pathway. Two units of acetyl-CoA are condensed to acetoacetyl-CoA via PhaA. Next, PhaB reduces it to 3-hydroxybutyryl-CoA. The latter serves as a monomer, which gets polymerized to PHB by PhaC or PhaEC, depending on the organism.

[Kavitha et al., 2016]. However, PHA production is best characterized in heterotrophic bacteria, such as *P. putida* [Timm and Steinbüchel, 1990], *Cupriavidus necator* (formerly known as *Ralstonia eutropha*) [Yabuuchi et al., 1995], *B. megaterium* [Griebel et al., 1968], *Rhodococcus ruber* [Haywood et al., 1991] or *Acinetobacter* sp. [Schembri et al., 1995]. Since PHB is by far the most widely distributed type of PHA in bacteria, this review focuses mostly on PHB. For the sake of completeness, however, some aspects related to other PHAs are also mentioned.

Due to its physical and chemical properties, PHB is often considered as a potential substitute for thermoplastic polymers, such as polypropylene. However, there are important differences compared to commonly used plas-

tics. For example, PHB exhibits low elasticity but high rigidity, which are undesirable material properties for many applications [Van der Walle et al., 2001].

PHB Metabolism

PHB synthesis starts with acetyl-CoA monomers. In the first step, the enzyme PhaA, an acetyl-CoA acetyltransferase (also termed acetoacetyl-CoA thiolase) catalyzes the condensation of two units of acetyl-CoA to acetoacetyl-CoA. In the next step, an acetoacetyl-CoA reductase (PhaB), reduces acetoacetyl-CoA to 3-hydroxyacetyl-CoA, while oxidizing one molecule of NADPH to NADP⁺. Finally, a synthase connects a 3-hydroxyacetyl-CoA to an elongated PHB poly-

Fig. 3. PHA metabolism and different metabolic pathways [Koch and Forchhammer, 2020]. Important enzymes are highlighted in red. Depending on the organism, the PHA synthase is either PhaC or PhaEC. Not shown is the PHA depolymerization, which is catalyzed by PhaZ.

mer (Fig. 2). In cyanobacteria, this synthase consists of two different dimers, namely PhaE and PhaC, making it a member of class III PHA synthases [Hein et al., 1998].

The PHB polymer can be catabolized by PHA depolymerases, termed PhaZ. This can take place either intra- or extracellularly, depending on their purpose. In *C. necator*, there are seven different depolymerases annotated, indicating the importance of a regulated mobilization of PHB [Pohlmann et al., 2006]. PhaZ degrades PHB into monomers of crotonyl-CoA [Eggers and Steinbüchel, 2013], which is then further metabolized in the β -oxidation pathway. It was furthermore shown that the phasins PhaP have a strong impact on the degradation of PHB [Eggers and Steinbüchel, 2014].

Besides the enzymes mentioned above, some species, like *P. putida*, possess further enzymes, which enable them to convert additional substrates into PHA. One enzyme is PhaJ, which converts enoyl-CoA into *R*-3-hydroxyacyl-CoA and thereby connects the fatty acid β -oxidation with the PHA synthesis [Liu et al., 2016]. PhaG, another enzyme found in *P. putida*, converts acyl-ACP to (*R*)-3-acyl-CoA and thereby links the fatty acid de novo synthesis to the PHA metabolism. The fatty acid and PHA pathways are further interconnected by enzymes, which catalyze reactions in both pathways. For ex-

ample, the enzyme FabG, which is actually part of the fatty acid biosynthesis, can also catalyze the same function as PhaB, but with lower catalytic efficiency [Zhang et al., 2017]. An overview of the most important pathways involved in the PHA metabolism is shown in Figure 3 (except the PHA depolymerization). Further details can be found in recent reviews [Zhang et al., 2020].

With all those enzymes in place, the PHA cycle is complete and acetyl-CoA can be metabolized to PHA and back again. Interestingly, some studies suggest that there could be a constant carbon flow around PHA synthesis and degradation, creating a potential futile cycle [Ren et al., 2009; de Eugenio et al., 2010]. Thereby the cells could balance metabolic intermediates and adapt the cell to changing environmental conditions. In support of this hypothesis, PHA synthase and depolymerase were shown to be simultaneously active in *P. putida* [Arias et al., 2013].

Proteins Associated with PHB Granules

Despite the apparent simplicity of PHB, the polymer is organized in highly organized structures with a spherical shape, called PHB granules. Due to this complexity, the

granules are also named carbonosomes to highlight that PHB granules are rather subcellular organelles and not just a prolonged chain of molecules [Jendrossek, 2009]. A set of different proteins, termed as granule-associated proteins (GAP), are located on the granule-surface. They serve various different purposes, such as structural, biosynthetic, catabolic, and regulatory functions.

A specific group are phasins, which are low-molecular-weight proteins attached to the surface of PHB granules [Mezzina and Pettinari, 2016]. They are very abundant and shield the hydrophobic PHB surface from the hydrophilic cytoplasm, as well as prevent the interaction with other proteins. A representative of such phasins is PhaP1 from *C. necator*, the most important phasin in this species [Pötter et al., 2004; Pfeiffer and Jendrossek, 2012]. The amount of PhaP1 is tightly regulated and corresponds with the amount of intracellular PHB [York et al., 2001]. In *P. putida*, PhaF and PhaI are the main GAP [Prieto et al., 1999].

Besides just covering the granules surface, GAP exhibit further functions. For example, PhaM in *C. necator*, which binds to PhaC as well as chromosomal DNA, ensures equal distribution among the daughter cells. Additionally, PhaM activates the PHB synthase PhaC1 and thereby directly influences the PHB metabolism [Pfeiffer and Jendrossek, 2014]. The regulator PhaR regulates *pha* genes on a transcriptional level [Pötter et al., 2002; York et al., 2002]. It was long disputed whether PHB granules are covered with a phospholipid membrane. During PHB preparations, phospholipids have often been found in vitro. However, using a phospholipid-specific fluorescent reporter, Bresan et al. [2016] could eventually demonstrate that PHB granules do not contain phospholipids on their surface in vivo. This demonstrates that phospholipids are merely contaminations of the preparation procedure.

Physiological Role of PHB in Bacteria

The ability to produce PHB is widespread in the bacterial kingdom. A phylogenetic analysis found the presence of the *phaC* gene in species from 40 different genera and within a wide range of taxonomic groups, highlighting how abundant the ability to produce PHB is [Kalia et al., 2007]. PHBs are in general considered as storage molecules of carbon and energy, which are built up in periods of carbon excess to be used in times of carbon shortage [Anderson and Dawes, 1990]. This long-time belief still holds true for many bacteria, although more and more

other physiological functions have recently been discovered. Also, for some bacteria like cyanobacteria, no physiological relevance of PHB was yet discovered. The most important roles of PHB are described below.

In many organisms, PHB accumulates under conditions of nutrient limitation or unbalanced conditions, such as nitrogen limitation [Anderson and Dawes, 1990]. However, there are also species, like *C. necator*, which do accumulate PHB even during normal growth and under balanced conditions [Jendrossek and Pfeiffer, 2014]. Nevertheless, the amount of accumulated PHB is usually higher when grown under nutrient limitation. Besides serving as a storage polymer, PHB has been suggested to increase resistance against various kinds of stresses. In general, 3-hydroxybutyrate oligomers can protect bacteria against hydroxyl-radicals [Koskimäki et al., 2016]. In *Azospirillum brasilense* for example, PHB-deficient strains are more susceptible against abiotic stresses, such as UV irradiation, heat, desiccation, osmotic pressure, and osmotic shock [Kadouri et al., 2003a]. In *Sinorhizobium* strains, an induced PHB accumulation after exposure to high salt concentrations was observed [Arora et al., 2006]. In *Aeromonas hydrophila* the production of [P(3HB-co-3HHx)] copolymers results in increased resistance against a wide variety of abiotic stresses, including UV irradiation, hydrogen-peroxide, ethanol, heat and cold treatments, and high osmotic pressure [Zhao et al., 2007]. Interestingly, a study investigating *P. oleovorans* showed that even the deletion of the PHA depolymerase PhaZ was sufficient to increase the sensitivity towards hydrogen-peroxide and heat shock [Ruiz et al., 2004]. This demonstrates that the entire PHB metabolism, including its mobilization, is important for providing stress tolerance to the PHB-producing cells, rather than the sheer presence of the polymer [Castro-Sowinski et al., 2009]. PHB was furthermore shown to increase the number of viable cells by protecting *C. necator* cells against cold stress [Nowroth et al., 2016]. Additionally, in *Herbaspirillum seropedicae*, studies demonstrated that PHB reduces intracellular redox stress, potentially by eliminating a surplus of reducing equivalents and thereby serving as an electron sink [Batista et al., 2018]. *Chromatium vinosum*, an anoxygenic phototrophic bacterium, converts glycogen to PHB under dark, anaerobic conditions. This provides the strain with the advantage over other fermenting organisms, which commonly secrete their fermentation products and thereby lose carbon [Van Gernerden, 1968]. Another role of PHB can be found in *B. cereus*: this bacterium produces most PHB just before the formation of spores and degrades it during spore germination, indicat-

ing the importance of PHB for surviving their dormant state [Valappil et al., 2007; Castro-Sowinski et al., 2009]. Several studies also found a connection between PHB production and the formation of EPS (exopolysaccharides), since impaired PHB production in some cases resulted in a higher EPS production [Wang et al., 2008], while with a lower EPS production in other strains [Kadouri et al., 2003b]. When the EPS production was higher, the authors suggested that the increased EPS production is attributable to the intracellular mobilization of carbon sources (PHB).

Interestingly, PHB producing *A. brasilense* was able to endure long periods of starvation, but it also showed certain disadvantages compared to a PHB-free mutant, such as lower motility or impaired root adhesion and EPS production [Kadouri et al., 2002]. This indicates that PHB formation is not always an advantage, but depends on the environmental conditions. Similarly, *Sinorhizobium* bacteria were shown to use a bet-hedging strategy to produce offspring with higher or lower amounts of PHB, adapted for long- or short-period starvation, respectively [Ratcliff and Denison, 2010]. Another interesting function of PHB could be the provision of nutrients to a microbial community [Prieto et al., 2016]. When a PHB producer is lysed, for example by predatory bacteria, it releases its PHB granules to the environment [Jendrossek and Handrick, 2002]. Thereby, the energy-rich carbon polymer is made available to the microbial community [Prieto et al., 2016]. In line with this, predatory *Bdellovibrio* cells have a growth advantage when preying on PHA-producing cells compared to PHB-free mutants [Martínez et al., 2013]. For more details on the physiological functions of PHB in bacteria, readers are referred to recent reviews [Castro-Sowinski et al., 2009; Müller-Santos et al., 2020; Obruca et al., 2020].

PHB in Cyanobacteria

Cyanobacteria comprise a large group of PHB-producing bacteria. They grow photoautotrophically and are the only bacteria that perform oxygenic photosynthesis. This allows cyanobacteria to occupy almost all illuminated habitats. In a recent study, 137 different cyanobacterial strains were investigated for their ability to produce PHB. Out of these, 134 were PHB producers [Kaewbai-Engam et al., 2016]. Interestingly, a phylogenetic analysis revealed that the full set of functional *phaABC* genes presumably evolved first in cyanobacteria, indicating its importance for cyanobacterial growth [Kalia et al., 2007].

Despite that, its physiological function is yet undiscovered. In recent years, this question was addressed by comparing a PHB-free *ΔphaEC* strain to a PHB-producing wild-type, but due to the absence of a clear phenotype, the function of PHB remains puzzling [Damrow et al., 2016; Koch et al., 2020a]. Cyanobacteria produce PHB mostly under unbalanced growth conditions, for example when they are grown in a medium lacking either nitrogen, phosphate, or potassium [Kaewbai-Engam et al., 2016]. Additional organic carbon sources, like acetate or fructose, can further increase the PHB production [Panda et al., 2006]. The PHB metabolism in cyanobacteria is best described during nitrogen starvation, which triggers a process called chlorosis [Klotz et al., 2016; Doello et al., 2018]. PHB slowly accumulates during the course of several weeks; depending on the cyanobacterial strain, up to 25% PHB per cell-dry-weight can be accumulated [Kaewbai-Engam et al., 2016]. PHB is commonly stored in a few granules, which are located in the middle of the cell [Hauf et al., 2015]. Compared to other PHB producers, only relatively little is known about the proteins which are involved in PHB metabolism. Although two new proteins were recently found to play a role (the phasin PhaP as well as the regulator Slr0058) [Hauf et al., 2015; Koch et al., 2020c], more proteins, such as PHB-degrading enzymes, are to be discovered. Most studies were performed in the strain *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*), which serves as a well-characterized model strain for cyanobacterial metabolism. In the following, the latest discoveries and hints towards PHB's physiological role are summarized.

The Role of PHB during Nutrient Limitation

Cyanobacteria are known for producing, in addition to PHB, a variety of different storage compounds, such as glycogen, cyanophycin, or polyphosphate. In contrast to the unknown function of PHB, the polymers glycogen and cyanophycin can clearly be linked to carbon and nitrogen storage metabolism, respectively [Doello et al., 2018; Watzel and Forchhammer, 2018]. As previous studies have shown, glycogen is the main carbon and energy storage compound under conditions of nutrient limitation [Klotz et al., 2016; Doello et al., 2018] and its biosynthetic genes were found in all cyanobacterial genomes [Beck et al., 2012]. It is remarkable that cyanobacteria produce two different carbon polymers, since most other microorganisms produce just one. This clearly indicates that the two carbon polymers have different functions. Glycogen synthesis is extremely dynamic and is immediately induced when cells experience nitrogen limitation.

Since glycogen synthesis mutants are unable to survive nitrogen chlorosis, glycogen metabolism appears to be pivotal for the acclimation to nitrogen deprivation [Klotz and Forchhammer, 2017; Doello et al., 2018]. Mutants that are deficient in the major glycogen-degrading phosphorylase GlgP2 do not accumulate PHB during chlorosis. From these analyses, it could be concluded that glycogen is slowly catabolized and converted into PHB during prolonged nitrogen starvation [Koch et al., 2019]. The cyanobacterium *Synechocystis* PCC 6803 operates at least three parallel metabolic routes to catabolize glycogen: the oxidative pentose phosphate (OPP) pathway, the Entner-Doudoroff (ED) pathway, and the Emden-Meyerhof-Parnas (EMP) pathway [Chen et al., 2016]. Analysis of mutants in which one or two of these pathways were disrupted showed that mutants in the ED and OPP pathways accumulated similar amounts of PHB as the wild-type, whereas mutants with a defect EMP pathway converted much less glycogen into PHB [Koch et al., 2019]. This effect was even more pronounced when the cells were cultivated under light/dark regime [Koch et al., 2020a]. Although *Synechocystis* normally accumulates more PHB under this condition, a mutant, which is unable to use the EMP pathway, produces almost no PHB. In contrast, the wild-type and a mutant unable to use either the ED or the OPP pathway (Δzwf), produced approximately 15% PHB per cell dry weight.

Acetyl-CoA, a key glycolytic product, serves as the precursor for the subsequent PHB synthesis, while the formation of glycogen requires phosphorylated glucose residues. This further suggests a role of glycogen as a quick-response energy and carbon storage, since glucose-1P can directly be metabolized via any of the three pathways (ED, OPP, EMP) to provide energy for the cells' anabolism. In contrast, acetyl-CoA rather serves as a building block for various anabolic reactions (e.g., in lipid metabolism) and anaplerotic reactions related to the TCA cycle. While glycogen quickly accumulates or degrades within just 2 days at the beginning or end of nitrogen chlorosis, respectively, the formation of PHB takes much longer, lasting for several weeks after the induction of nitrogen starvation [Koch et al., 2020c].

Remarkable differences between PHB and glycogen are that glycogen shows a higher solubility in water than PHB, and that PHB forms much larger granules than glycogen. It was hypothesized that the relatively small glycogen granules could serve as a quick-response carbon storage due their easy accessibility based on a larger surface-to-volume ratio, whereas the large PHB granules could instead form a long-term storage. However, under condi-

tions of nitrogen limitation, no physiological differences were ever discovered between wild-type and a PHB-free $\Delta phaEC$ mutant strain, even after prolonged starvation [Damrow et al., 2016; Klotz et al., 2016]. To test whether PHB can play a role in stress resistance, a recent study applied more than 30 different stresses to nitrogen-starved *Synechocystis* cells, including all of those stresses which are known for being relevant in other PHB-producing bacteria [Koch et al., 2020a]. However, the ability to produce PHB did not give the wild-type cells a growth advantage compared to a $\Delta phaEC$ mutant in any of the tested conditions.

To better understand the metabolic role of PHB in *Synechocystis*, a recent study attempted to identify the so far unknown PHB depolymerase [Koch et al., 2020c]. Sequence homology analysis indicated that the gene *slr0060* encodes for a putative esterase with a patatin-like domain, which is typical for some intracellular PHB depolymerases. The respective deletion mutant $\Delta slr0060$ produced slightly less PHB than a wild-type control, but showed no further distinctive phenotype. Interestingly, the same study revealed that when a source of combined nitrogen was added to chlorotic cells to induce resuscitation to vegetatively growing cells, no net degradation of PHB in the cyanobacterial culture took place: the overall amount of PHB within a culture remained constant for several days [Koch et al., 2020c]. Only through cell growth and division, the PHB level per cell decreased gradually. However, fluorescence-microscopic analysis investigating Nile red-stained PHB revealed that the granules disaggregated and were spread among dividing daughter cells. The physiological function of this behavior and why PHB does not become actively metabolized has yet to be explained. Possibly cultivation in laboratory conditions did not trigger the degradation of PHB, and the required environmental conditions which initiate PHB catabolism are still to be found. Therefore, to date it remains open whether cyanobacteria contain an intracellular PHB depolymerase as described for many other bacteria.

Potential Role of PHB in Controlling the Redox State

Besides conditions of nutrient limitation, glycogen is the essential energy and carbon storage during dark phases, where no photosynthesis can take place. Excess of carbon gets stored in the form of glycogen during the day, while during the night it releases its energy and carbon to sustain the cyanobacterial metabolism. Although no studies so far have shown a PHB accumulation during conditions of balanced growth, there are several indicators that PHB might play a role during dark phases. Tran-

scriptomic data from several studies have shown very clearly that all PHB-related genes are strongly up-regulated at the beginning of the night and down-regulated during the day [Kuchō et al., 2005; Saha et al., 2016]. It was furthermore shown that PHB-related genes are strictly controlled by the circadian clock [Köbler et al., 2018]. Interestingly, the transcripts for PHB synthesis are counter-cyclically regulated to those of the glycogen metabolism, which are up-regulated during the beginning of the day and down-regulated during the beginning of the night. This hints towards a direct connection of both polymers, as it was already shown during nitrogen starvation [Koch et al., 2019]. However, also during diurnal (day/night) cultivation, a PHB-free mutant showed no growth phenotype, while growth of glycogen-free mutants was severely impaired [Damrow et al., 2016]. Furthermore, under conditions of nitrogen limitation, diurnal cultivation resulted in higher PHB levels than continuous light cultivation [Koch et al., 2020a]. This further highlights the relevance of dark phases for the formation of PHB.

PHB was previously suggested to play a role as a potential electron sink since each PHB subunit consumes one NADPH equivalent [De Philippis et al., 1992]. This hypothesis was further corroborated by studying a *Synechocystis* mutant in the gene *slr0783* [Schlebusch and Forchhammer, 2010; Hauf et al., 2013]. The *slr0783* gene belongs to the most strongly induced genes during nitrogen starvation and is the first gene of the Nit1C operon, a highly conserved gene cluster present in cyanobacteria and many other bacterial species (including proteobacteria and actinobacteria), enabling the utilization of cyanide [Jones et al., 2018]. Many heterotrophic bacteria possessing this gene cluster are able to produce PHB and to fix nitrogen. Since the entire operon was highly up-regulated in *Synechocystis* during nitrogen starvation, a role during nitrogen-chlorosis was suspected. Further analysis revealed that the Δ *slr0783* mutant was unable to accumulate PHB due to a strongly decreased PHB synthase activity [Schlebusch and Forchhammer, 2010]. The inability of the Δ *slr0783* mutant to sustain PHB synthesis could finally be attributed to the redox state of the cells: during nitrogen starvation, the NADPH/NADP⁺ ratio steadily increased in wild-type cells, whereas in the Δ *slr0783* mutant it remained constant. Various treatments of the mutant cells that artificially increased the NADPH/NADP⁺ ratio restored PHB synthase activity and PHB synthesis [Hauf et al., 2013]. This highlights that the increased levels of NADPH during nitrogen starvation are an important trigger for PHB synthesis.

According to the abovementioned facts, the formation of PHB would be particularly beneficial whenever a surplus of NADPH cannot be metabolized, for example because respiration is not possible and anaerobic processes are required. In this scenario, the formation of a PHB precursor would serve as an intracellular electron sink with the advantage to sustain all intracellular carbon, instead of secreting it like other fermentation products, such as acetate. As mentioned above, the EMP pathway plays a crucial role for the formation of PHB. This pathway produces a lower NADPH/ATP ratio compared to the OPP or ED pathway. This is advantageous under conditions of NADPH excess, such as anaerobic growth. A previous study has shown that the EMP pathway is also the most relevant during fermentation processes in cyanobacteria [Stal and Moezelaar, 1997].

Another role of PHB could be a strategy to prevent metabolic spillover: a PHB-free *C. necator* mutant secretes pyruvate into its medium [Raberg et al., 2014]. A similar metabolic spillover was shown in *Synechocystis* mutants that are unable to synthesize glycogen and secrete pyruvate and 2-oxoglutarate under conditions of nitrogen starvation [Grundel et al., 2012]. Potentially, PHB could serve as an additional buffer for storing excess carbon, particularly because it is metabolically closer to pyruvate than glycogen. However, in contrast to the glycogen-deficient mutants, no studies have so far analyzed secreted organic acids in the supernatant of a PHB-free mutant. To further investigate this, future studies should analyze secreted metabolites under different growth conditions in a Δ *phaEC* mutant and compare it to a wild-type.

Cell Biology of PHB in Cyanobacteria

The synthesis of PHB granules is tightly controlled. Based on sequence homology to other PHB-producing bacteria, several genes were identified which encode for proteins that are putatively involved in the PHB metabolism. One of those genes is *slr0058*, which encodes for a protein that contains a domain that is similarly found in PhaF from *P. putida* [Koch et al., 2020c]. Unlike the wild-type, a Δ *slr0058* mutant produces small amounts of PHB during vegetative growth and shows delayed growth. This growth deficiency was clearly coupled to the presence of PHB, since a *phaEC* knockout in the Δ *slr0058* background recovered wild-type growth. The Slr0058 protein was furthermore shown to regulate the number of PHB granules within the cell. While a wild-type possesses around 3 granules per cell on average, the Δ *slr0058* mutant contained about twice as many, but smaller gran-

Fig. 4. Schematic overview of the current view of PHB metabolism in *Synechocystis*. After the induction of nitrogen starvation, cells fix inorganic CO₂ and store it in the form of glycogen. Throughout the course of chlorosis, glycogen is catabolized, mainly via the EMP pathway, to form acetyl-CoA, the building block of PHB. Next, PhaA and PhaB convert acetyl-CoA subunits to 3-hydroxybutyryl-CoA at the expense of one NADPH molecule, which becomes oxidized to NADP⁺. Slr0058 initiates the formation of PHB granules and detaches as the PHB granules mature. The elongation of 3-hydroxybutyryl-CoA to the polymer PHB is catalyzed by the enzyme PhaEC, while PhaP shields the surface of the PHB granule. The color change indicates the progression of chlorosis, from vegetative growth to fully chlorotic (green to yellow, respectively). The process starts with the initial formation of glycogen, which eventually results in the accumulation of PHB.

Fig. 5. Microscopic analysis of varying PHB contents in wild-type cells. **a** Fluorescence microscopy of wild-type cells after 14 days of nitrogen starvation. PHB granules are visualized by staining with Nile red. **b** TEM picture of wild-type cells after 17 days of nitrogen starvation. Representative PHB granules are indicated in two different cells by red asterisks. Figure modified from Koch et al. [2020a].

ules. Therefore, the surface area of those is higher compared to the fewer granules in the wild-type. Hence, it is likely that the $\Delta slr0058$ mutant suffers from unintended interactions between the PHB surface and the cell's interior. Protein localization using fluorescent protein-tagged Slr0058 showed that it aggregated in distinct foci during vegetative growth and these foci dispersed during the course of chlorosis. This led to the hypothesis that Slr0058 could act as an initial aggregation point for the PHB synthase PhaEC to direct the initiation of PHB synthesis and avoid uncontrolled formation of random PHB granules. Subsequently, it dissociates from maturing PHB granules.

Another protein involved in the regulation of number and size of PHB granules is PhaP (Ssl2501) [Hauf et

al., 2015]. PhaP is directly located at the surface of PHB granules and hence considered a classical phasin. A PhaP deletion mutant showed half as many PHB granules as the wild-type. At the same time, the granules in the $\Delta phaP$ strain were larger than in the wild-type. The presence of PhaP and Slr0058 shows that the formation and maintenance of PHB granules in *Synechocystis* is tightly regulated. Decreased viability, for example in a $\Delta slr0058$ mutant strain, underlines the importance of this regulation. A summary of the current understanding of the PHB metabolism in *Synechocystis* is shown in Figure 4.

Interestingly, in a nitrogen-starved *Synechocystis* population, the distribution of PHB granules among the cells is quite heterogenous (Fig. 5). Fluorescence microscopy

showed that a large number of cells contained only small-to-medium amounts of PHB, while a few cells within the same population accumulated larger quantities [Koch et al., 2020a]. This indicates that the formation of PHB could be a bet-hedging strategy of the cells to be prepared for different future conditions [Ratcliff and Denison, 2010]. This highlights that the ability to possess PHB might be relevant under only very specific scenarios. The fact that so many cyanobacterial species produce PHB and formation of the granules is controlled in such a complex manner indicates that PHB fulfils an important physiological function and provides an evolutionary advantage to cyanobacteria in natural environments. These conditions could include, for example, the complex interactions with other microbes, which are difficult to mimic under laboratory conditions.

A First Cyanobacterial Superproducer Strain

Due to its industrial relevance as a bioplastic, considerable efforts have been made in the past years to increase the intracellular PHB concentrations in cyanobacteria, mainly by using genetic engineering. Several recent reviews cover this topic [Costa et al., 2018; Markl et al., 2018]. Cyanobacteria could in principle solve several drawbacks of PHB production with heterotrophic bacteria, such as avoiding the use of agricultural feedstock that is usually used for nutrition. However, the attempts published so far showed limited success in obtaining a high yield and production rates that would allow cost-efficient PHB production in a sustainable process. Very recently, the discovery of PirC, a central carbon flow regulator that controls the flux of carbon towards PHB, now provides a new approach towards economically feasible PHB production in cyanobacteria. PirC is a small protein controlled by the PII signaling protein that exerts control over the activity of phosphoglycerate mutase [Orthwein et al., 2020]. Thereby, PirC directs the flow of carbon depending on the nitrogen availability. Mutant strains of PirC show an increased flux of newly fixed carbon towards pyruvate through lower glycolysis, thereby providing the building blocks for enhanced PHB synthesis. In combination with the overexpression of heterologous *phaAB* genes, the mutant strain PPT1 was created [Koch et al., 2020b]. Under phototrophic conditions, this strain accumulated 63% PHB per cell dry weight. Upon the addition of 10 mM acetate, PHB contents of more than 80% were reached, an amount that was so far only achieved by some heterotrophic bacteria. This demonstrates the potential of *Synechocystis* serving as a host for the sustainable production of industrial products.

Conclusion and Outlook

Despite the simple chemical structure of PHB, its granules are highly complex pseudo-organelles, with various different proteins involved in maintaining the correct function. Depending on the organism, PHB can serve numerous roles, from molecule storage to stress resistance and many more. Although the physiological function in cyanobacteria remains puzzling, the field of research is constantly expanding, thereby gaining deeper insights into its role. One of the most promising hypotheses is the role of PHB for the regulation of the intracellular redox balance. As an implication for biotechnological approaches this implies that when increased amounts of PHB are desired, cultivation conditions which favor high levels of reduction equivalents should be considered. Still, fundamental questions remain, such as a yet undiscovered PHB depolymerase. Since cyanobacteria grow photoautotrophically, they could serve as a chassis for a carbon-neutral, sustainable production of PHB. Further research in this field will hence be not only beneficial for basic research, but may also provide knowledge of industrial relevance.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Conceptualization: K.F. Writing – original draft preparation: M.K. Review and editing: M.K. and K.F.

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