The Cyanophycin Granule Peptide from Cyanobacteria

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Contents

Abstract Cyanophycin (multi-L-arginyl-poly-L-aspartic acid) is a biopolymer of industrial interest, which naturally occurs in most cyanobacterial species and in a few heterotrophic bacteria. It is a non-ribosomally synthesized polyamide consisting of L-Arginine and L-Aspartate and serves as a biological nitrogen/carbon storage compound.

This chapter provides an overview about the natural occurrence of cyanophycin in cyanobacteria. Herein, we focus on the physiological function of cyanophycin in filamentous and unicellular cyanobacteria. Information about the enzymes involved

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in cyanophycin metabolism and their regulations are also provided. Due to its unique properties, cyanophycin could be used in various industrial applications. This chapter also summarizes the current state of biotechnological cyanophycin production.

1 Introduction

Cyanobacteria are Gram-negative prokaryotes that perform oxygenic photosynthesis and fix carbon dioxide through the Calvin-Benson Cycle to produce reduced carbohydrates (Rippka et al. 1979). For the synthesis of cellular building blocks, required for cell growth, Cyanobacteria assimilate various inorganic and organic nitrogen sources, such as nitrate, ammonium, urea or a few other organic nitrogen compounds (e.g., amino acids, cyanate). The preferred nitrogen source is ammonium that is converted to glutamate via the coupled reactions of glutamine synthetase and glutamine-oxoglutarate aminotransferase (GS/GOGAT pathway) (Flores and Herrero pp.; Luque and Forchhammer 2008). According to their morphology, cyanobacteria are subdivided into five divisions (Rippka et al. 1979). Unicellular forms belong to the divisions I (order *Chroococcales*) and II (order *Pleurocapsales*). Cyanobacteria of division III (order *Oscillatoriales*) and IV (order *Nostocales*) form filaments of vegetative cells, with the latter being able to differentiate specialized cells. Division V cyanobacteria (order *Stigenomaticales*) are similar to division IV, but the filaments are branched. The capability to fix dinitrogen (N_2), called diazotrophy, is found in both unicellular and filamentous forms of cyanobacteria (Rippka et al. 1979; Fredriksson and Bergman 1997; Bandyopadhyay et al. 2013). In cyanobacteria of the order *Nostocales* and *Stigenomaticales*, nitrogen fixation takes place in specialized cells termed heterocysts (Herrero et al. 2016).

Cyanobacteria are widespread and well adapted to changing environmental conditions in terms of light conditions and nutrient supply. They colonize nearly all light-exposed habitats ranging from aquartical, including fresh- and seawater to terrestrial habitats (Whitton 2012). Some species can even tolerate extreme environments like hot springs, deserts or the arctic tundra (Miller and Castenholz 2000; Whitton 2012). Cyanobacteria are very interesting targets for biotechnological research because of their ability to synthesize reduced carbon compounds and various biopolymers, only by using water, carbon dioxide and light as an energy source. This chapter will focus on the biopolymer cyanophycin, a major nitrogen storage compound produced by many cyanobacteria.

2 Cyanophycin: A Nitrogen-Rich Storage Compound

Cyanophycin is a branched non-ribosomally synthesized polypeptide first found in cyanobacteria (Borzi 1887). Due to its composition of aspartate and arginine, cyanophycin has a high nitrogen to carbon ratio of 2:1, making it an excellent nitrogen storage substance (Simon and Weathers 1976). It serves as a dynamic nitrogen reservoir in most diazotrophic and non-diazotrophic cyanobacteria as well as in some heterotrophic bacteria (Ziegler et al. 2002; Füser and Steinbüchel 2007). Cyanophycin accumulates in the form of opaque and light-scattering cytoplasmic granules and was therefore mentioned cyanophycin granule peptide (CGP). Electron microscopic studies described CGP granules as electron dense, highly structured and membrane-less inclusions (Lang et al. 1972; Allen and Weathers 1980).

The polymer is synthesized by a single enzyme termed cyanophycin synthetase (CphA) (see below for details). Genes homologous to cyanobacterial *cphA* are found in a wide range of prokaryotes (Krehenbrink et al. 2002), indicating that cyanophycin has a broader biologicals significance, which is, however, mostly unexplored to date. In general, accumulation of the polymer increased under unbalanced growth conditions, e.g., in stationary growth phase or stress condition (high light, high CO_2 or nutrient stress) but only when nitrogen is available (Allen et al. 1980; Allen and Weathers 1980; Carr 1988; Herrero and Burnat 2014; Watzer and Forchhammer 2018b). These findings stand for a putative role of the polymer as a temporary nitrogen reserve molecule (Li et al. 2001).

CGP has been noticed for potential biotechnological use. Biodegradable polyamino acids like CGP could substitute synthetical polymers with similar properties in the fields of medical, food, feed and pharmaceutical industry (Frommeyer et al. 2016). Further, CGP can serve as a natural source of amino acids such as arginine and aspartate, and the poly-aspartate backbone of this polymer could also be used as a biodegradable substitute for polyacrylates (Obst and Steinbüchel 2004; Sallam et al. 2009a, b).

3 Structure of Cyanophycin

The nitrogen-rich polymer CGP consists of two amino acids, aspartate and arginine in equimolar amounts. The aspartic acid residues were linked by peptide bounds forming a poly-L-aspartic acid backbone. Arginine is linked by iso-peptide bond to the backbone, forming the branched polymer also called [multi-(L-arginyl-poly-L-aspartic acid)] (Simon and Weathers 1976). One building unit of the polymer consists of the dipeptide β-Asp-Arg (Richter et al. 1999). Circular dichroism (CD) spectroscopy data suggest that a substantial fraction of CGP has β-pleated sheet structure (Simon et al. 1980). The individual CGP chains have no fixed length but show polydisperse molecular masses ranging from 25 to 100 kDa in cyanobacteria. This is in contrast to the native CGP producing heterotrophic bacteria

Acinetobacter sp. ADP1, which synthesizes CGP with a lower molecular weight ranging from 21 to 28 kDa (Elbahloul et al. 2005). Cyanophycins, produced by genetically modified microorganisms (GMOs) harboring cyanobacterial CGP synthesizing enzymes, have molecular masses in the range of 25–45 kDa (Frey et al. 2002; Steinle et al. 2009). Possible explanations for these differences could be that different ratios of CGP synthase to precursor molecules exist in GMOs or that CGP synthesis in a cyanobacterial background involves additional factors contributing the polymer length. These additional factors should also be absent in *Acinetobacter* sp. ADP1 (Watzer and Forchhammer 2018b). Cyanobacterial CGP is exclusively composed of aspartate and arginine. By contrast, CGP of recombinant microorganisms, further amino acids like lysin, citrulline and ornithine, have been detected (Steinle et al. 2009; Ziegler et al. 1998) (see below).

CGP granules were isolated for the first time in 1971 from *Anabaena cylindrical* by differential centrifugation (Simon 1971). Along with this study, CGP has shown its unique solubility behavior. CGP is insoluble under physiological conditions but soluble in either acidic, basic or highly ionic solutions. CGP can be solubilized by SDS but not by non-ionic detergents such as Triton X-100 (Wiefel and Steinbüchel 2014; Lang et al. 1972). Routinely, CGP is extracted by acidic treatment and subsequent precipitation at neutral pH (Frey et al. 2002).

4 Physiological Function of Cyanophycin in the Context of Nitrogen Fixation

CGP is produced by many different cyanobacteria, both diazotrophic and non-diazotrophic strains of unicellular or filamentous nature (Fig. 1). In diazotrophic strains, the formation of CGP is directly linked to the nitrogen-fixation process, whereas in non-diazotrophic strains, the biological significance remained elusive until recently (see below).

In principle, nature has evolved two solutions to the problem that oxygenic photosynthesis and nitrogen fixation are incompatible processes due to the extremely high oxygen sensitivity of the nitrogenase enzyme. Therefore, the two processes cannot take place at the same time at the same place. To solve this issue, the two processes may be separated on a temporal basis or may be confined to different compartments. Simple filamentous (Sect. 3) and unicellular nitrogen-fixing cyanobacteria temporarily separate nitrogen fixation and photosynthesis on a diurnal basis, as shown in the cyanobacterium *Cyanothece* sp. ATCC 51142; the cells perform photosynthesis during the day where they accumulate fixed carbon in the form of glycogen. This storage material is catabolized during the night to support nitrogen fixation with energy and carbon skeletons (Sherman et al. 1998). The nitrogen fixation products are immediately converted to CGP, which accumulates during the night. In the subsequent day period, when photosynthesis is active again, CGP is degraded to mobilize the fixed nitrogen for anabolic reactions to support cell
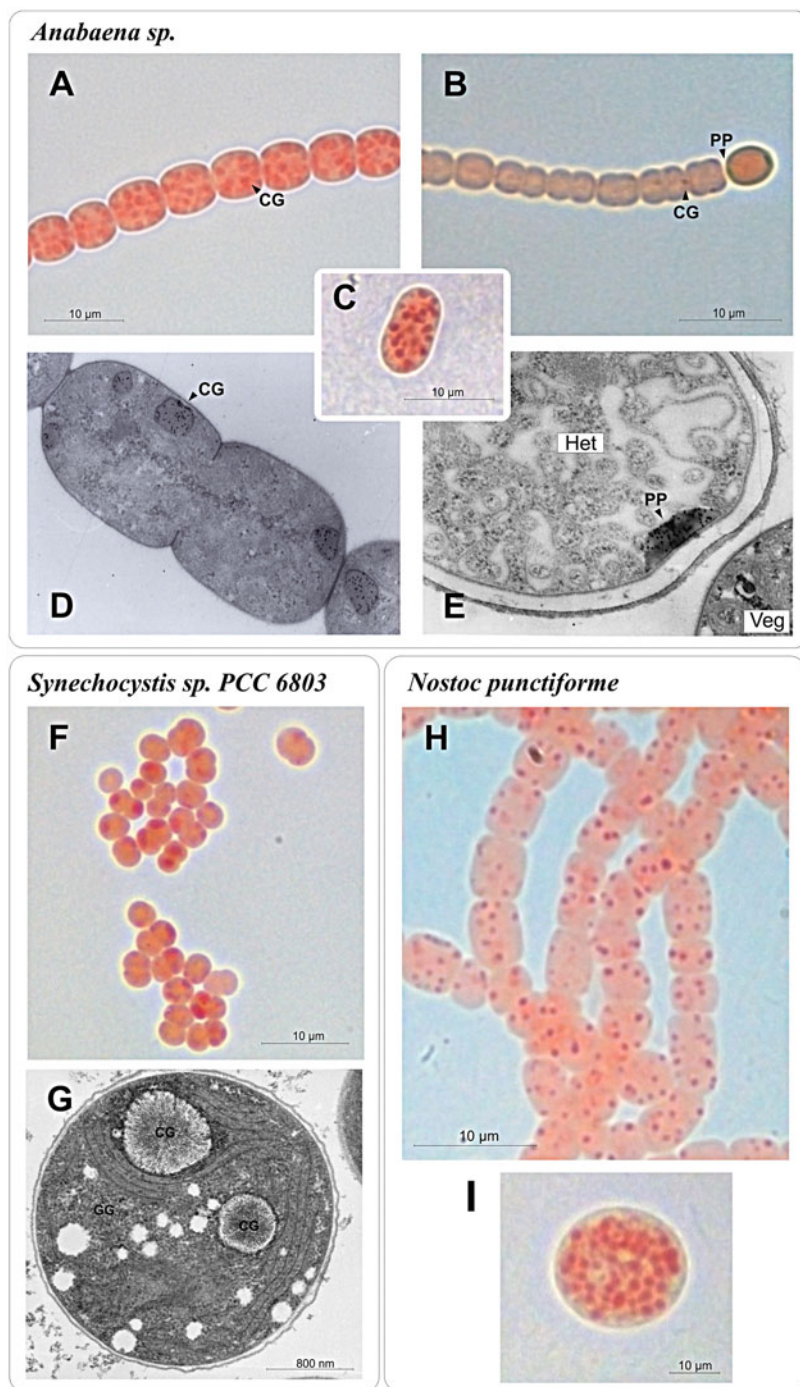
Fig. 1 Light and electron microscopic pictures of *Anabaena* sp. (**a–e**), *Synechocystis* sp. PCC 6803 (**f–g**) and *Nostoc punctiforme* ATCC 29133 (H-I) under CGP accumulating conditions. In light

growth (Sherman et al. 1998). A similar diurnal CPG accumulation during dark periods was also reported in the filamentous cyanobacterium *Trichodesmium* sp., which is the dominant diazotrophic cyanobacterium in tropical and subtropical seas and therefore of global importance in N and C cycling (Finzi-Hart et al. 2009).

Multicellular cyanobacteria of the order *Nostocales* are characterized by a spatial separation of photosynthesis and nitrogen fixation through a cell differentiation process which results in the formation of specialized cells for nitrogen fixation, the heterocysts.

4.1 Cyanophycin in Heterocyst-Forming Cyanobacteria

Heterocystous cyanobacteria comprise the orders *Nostocales* and *Stigenomaticales*. They are filamentous and true multicellular organisms in which two differently specialized cell types have a division of labor. Both processes, photosynthesis and nitrogen fixation, take place in different cells. Nitrogen fixation occurs only in the heterocysts, which are terminally differentiated cells specialized for nitrogen fixation (Kumar et al. 2010; Maldener and Muro-Pastor 2010). On the other hand, oxygenic photosynthesis takes place only in the vegetative cells. When no combined nitrogen sources are available, the multicellular organisms differentiate between 5 and 10% of cells into heterocysts.

The heterocysts provide a microoxic compartment for the activity of nitrogenase (Adams and Carr 1981). To achieve this functional state, numerous morphological and metabolic changes take place during the transition of vegetative cells into heterocysts, which are based on cell-specific gene expression occurring in the differentiated heterocysts (Wolk et al. 1994; Golden and Yoon 1998; Maldener et al. 2014; Flores et al. 2019). Concerning the photosynthetic apparatus, heterocysts show remarkably differences to the vegetative cells (Magnuson and Cardona 2016): The oxygen-producing photosystem II is absent and phycobilisomes are largely

Fig. 1 (continued) microscopic pictures, CGP was stained using the Sakaguchi reaction (Watzer et al. 2015). The intensity of the red color indicates the amount of arginine; therefore dark red dots are CGP granules. (**a**) Phosphate starved *Anabaena variabil*s ATT 29413 in the presence of nitrate as nitrogen source. (**b**) Diazothrophic growing filament of *Anabaena sp*. PCC7120 with terminal heterocyst containing polar plaques. (**c**) Mature akinete of *Anabaena variabilis* ATCC 29413. (**d–e**) Electron micrographs of *Anabaena variabilis* cells. Ultrastructural investigations were performed using electron microscopy following the immunocytochemical visualization of CGP with antisera raised against cyanophycin and a gold coupled anti-rabbit immunoglobulin G antibody (by Uwe Kahmann, Universität Bielefeld) (**d**) vegetative cells of *Anabaena variabilis,* magnification 28,500x; (**e**) heterocyst [Het] of *Anabaena variabilis,* magnification 50,000x. (**f–g**) Phosphate starved *Synechocystis* sp. PCC 6803 in light and transmission electron microscopy, respectively. (**h**) *Nostoc punctiforme* ATCC 29133 under phosphate starvation and nitrogen supplementation. (**i**) Mature akinete of *Nostoc punctiforme* ATCC 29133 (Watzer and Forchhammer 2018b). [CG] CGP granules, [PP] polar plaques, [GG] glycogen granules, [Het] heterocyst

degraded. Thylakoid membranes are re-organized and support cyclic electron transport via the photosystem I, to provide ATP (Wolk et al. 1994). In addition, respiration takes place in the thylakoid-derived honeycomb-membranes, where the molecular oxygen is reduced to water (Wolk 1996). In the microoxic environment of heterocysts, instead of CO_2 fixation by RubisCO, atmospheric N_2 is fixed by the key enzyme for nitrogen fixation, the nitrogenase. The nitrogenase encoded by *nif*HDK genes is cell specific and exclusively active in mature heterocysts (Golden et al. 1985; Fay 1992). The enzyme is very oxygen sensitive (Gallon 1981) and is irreversibly inactivated by oxygen (Hill et al. 1981; Golden et al. 1985; Fay 1992). It reduces molecular nitrogen to ammonium using reduction equivalents and ATP (Wolk et al. 1994; Kumar et al. 2010). The high energy demand for nitrogen fixation is partially provided from products of oxygenic photosynthesis performed in the neighboring vegetative cells of the filament. The division of labor between heterocysts and vegetative cells requires an efficient exchange of metabolites and signals along the filament (Wolk 1968; Wolk et al. 1974). Molecular diffusion of small molecules from cytoplasm to cytoplasm takes place through special cell-cell connections, so-called "septal junctions" (Maldener and Muro-Pastor 2010; Flores et al. 2016). The structure of the septal junctions has been resolved recently in more detail in *Anabaena sp*. PCC 7120 (Weiss et al. 2019). Each septal junction complex comprises a channel that spans the gap through the periplasmic space in the septal region and traverses the septal peptidoglycan though approx. 20 nm nanopores to connect two adjacent cells. On the cytoplasmic site of each entrance point, the channel is covered by a cap and plug structure that enables closure of the septal junctions (Weiss et al. 2019).

Mature heterocysts are characterized by a specialized cell wall composed of polysaccharide and glycolipid layers, which minimizes the diffusion of molecular oxygen into the specialized cell (Walsby 2007; Nicolaisen et al. 2009). A distinct morphological feature of heterocysts is so-called "polar plaques" or "polar nodes," which are specific structures containing CGP (Sherman et al. 2000) closely located to the neck and the adjacent vegetative cell. The polar nodes are easily recognized by light microscopy, and as such, they are characteristic morphological signatures of heterocysts (Ziegler et al. 2001) (Fig. 1). It was assumed that CGP located at these heterocystous structures is a key intermediate in the storage of fixed nitrogen products in heterocysts and acts as a dynamic nitrogen reservoir (Carr 1988; Sherman et al. 2000). CGP in the polar nodes could serve as a sink for fixed nitrogen in the heterocyst to avoid feedback inhibition of nitrogen assimilatory reactions from soluble products of nitrogen fixation (Burnat et al. 2014; Forchhammer and Watzer 2016).

Vegetative cells support the metabolism of heterocysts by supplying them with glutamate and reduced carbohydrates, e.g., alanine and the chemically inert disaccharide sucrose (Jüttner 1983; Curatti et al. 2002; Vargas et al. 2011) as a source for energy, reductant power, and the required carbon skeletons for assimilation of fixed nitrogen. Invertases degrade the disaccharides into glucose and fructose in heterocysts (Lopez-Igual et al. 2010; Vargas et al. 2011), which can be further catabolized via the oxidative pentose phosphate cycle to generate reducing power for nitrogen
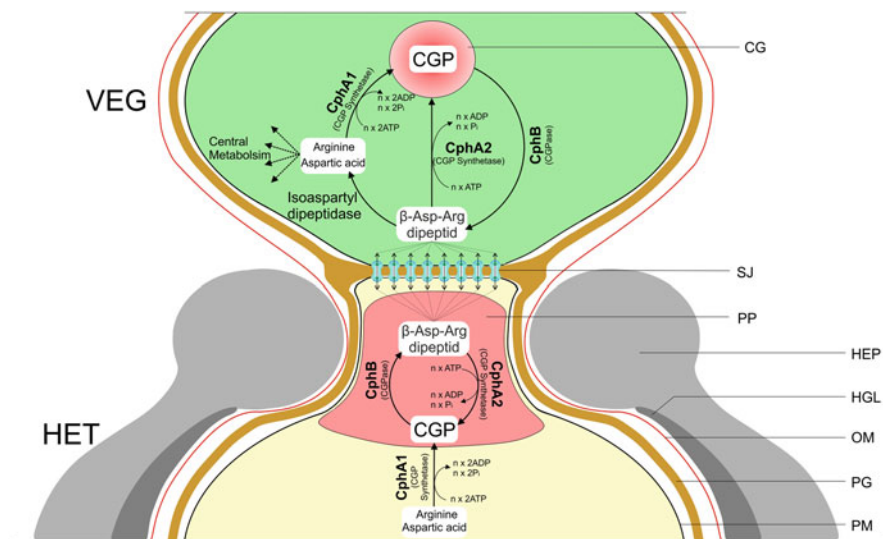
Fig. 2 Scheme of cyanophycin-related nitrogen transport between a heterocyst (HET) and a vegetative cell (VEG). Cyanophycin (CGP) is synthesized by CphA and accumulated in the polar plaques (PP). The degradation of CGP releases β-aspartyl-arginine dipeptides, which can be used to resynthesize CGP by CphA2 (see chapter "The Anammoxosome Organelle: The Power Plant of Anaerobic Ammonium-Oxidizing (Anammox) Bacteria") or transported to the adjacent vegetative cell by passing through the septal junctions (SJ). In the vegetative cells, the isoaspartyl dipeptidase releases monomeric arginine and aspartate, which flow into the central metabolism. Furthermore, monomeric amino acids or β-aspartyl-arginine dipeptides can be used by CphA1 or CphA2, respectively, to synthesize CGP, building a CGP granule (CG). *PM* Plasma membrane, *PG* Peptidoglycan, *OM* Outer membrane, *HGL* Heterocyst glycolipid layer, *HEP* Heterocyst envelope polysaccharide layer

fixation and respiration. Ammonium produced by nitrogenase activity is immediately assimilated by glutamine synthetase, whereas the GOGAT reaction takes place in adjacent vegetative cells (Martin-Figueroa et al. 2000), which requires an efficient shuffling of glutamate and glutamine between heterocysts and vegetative cells.

Vegetative cells are supplied by adjacent heterocysts with reduced nitrogen such as glutamine and the dipeptide β-aspartyl-arginine (Thomas et al. 1977; Burnat et al. 2014). Therefore, CGP in the outer region of the polar nodes is cleaved by the action of cyanophycinase into β-aspartyl-arginine dipeptides. CGP catabolic enzymes are present at significantly higher levels in vegetative cells than in heterocysts, indicating a sophisticated spatial organization of nitrogen metabolism (Burnat and Flores 2014; Burnat et al. 2014) (Fig. 2).

Surprisingly, under standard laboratory conditions, this mode of metabolic organization appears to be dispensable, since mutants of *Anabaena* sp. PCC 7120 or *Anabaena variabilis,* lacking the CGP synthetic genes, were little affected in diazotrophic growth under usual cultivation conditions (Ziegler et al. 2001). In this case, arginine and aspartate might be transferred directly to the vegetative cells,

without an intermediate storage in CGP. However, mutants lacking the CGP synthetic genes were affected in diazotrophic growth under stressful high light conditions (Ziegler et al. 2001; Klemke et al. 2016), indicating that cyanopyhcin plays a role in coping with metabolic stress.

Moreover, diazotrophic growth is significantly decreased in strains, which are unable to degrade CGP (Burnat and Flores 2014; Burnat et al. 2014). These results identified β-aspartyl-arginine dipeptides as a nitrogen vehicle in heterocyst-forming cyanobacteria, next to glutamine, arginine, or aspartate alone. A benefit of β-aspartyl-arginine dipeptides as a nitrogen transport substance is avoiding the release of free arginine and aspartate back in the heterocyst. This indicates that CGP metabolism has evolved in heterocyst-forming cyanobacteria to increase the efficiency of nitrogen fixation (Watzer and Forchhammer 2018b; Flores et al. 2019a; Burnat et al. 2014, 2019).

Several of the heterocyst-forming cyanobacteria can differentiate another type of specialized cells, the akinetes. These are resting cells able to survive long periods of unfavorable conditions. During akinete development, the cells transiently accumulate CGP in addition to other storage compounds such as glycogen or lipid droplets (Perez et al. 2016) (Fig. 1). In mature akinetes, CGP disappears gradually, and when the akinetes germinate, CGP reappears transiently (Perez et al. 2018). Apparently, synthesis of CGP seems inessential, since *Anabaena variabilis* akinetes lacking CGP granules are not impaired in germination. This behavior agrees with early observations suggesting that CGP is not the direct nitrogen source for protein biosynthesis in germinating akinetes (Sutherland et al. 1985).

4.2 Cyanophycin in Non-diazotrophic Cyanobacteria

In non-diazotrophic cyanobacteria, CGP can only be detected under unbalanced growth conditions. In exponentially growing cells, its amount is usually less than 1% of the cell dry mass. However, when growth is retarded or arrested due to unfavorable conditions under nitrogen supply, (antibiotic treatment; sulfate, phosphate or potassium starvation), the cells may start to build up CGP, which can amount up to 18% of the cell dry mass (Allen et al. 1980; Watzer et al. 2015; Simon 1973). When *Synechocystis* cells recover from prolonged nitrogen starvation, by adding nitrate to the starved cells, they transiently accumulate CGP (Klotz et al. 2016; Allen and Hutchison 1980; Watzer and Forchhammer 2018a). This raised the question regarding the biological significance of transient CGP accumulation. This issue was recently solved by Watzer and Forchhammer (Watzer and Forchhammer 2018a). The transient accumulation of CGP becomes beneficial to the cells under conditions of fluctuating or limiting nitrogen supply. Whereas wild-type *Synechocystis* cells are able to fully recover from prolonged nitrogen chlorosis when they are provided with pulses of nitrate, recovery is severely delayed in a mutant deficient in CGP synthesis (*cphA* mutant, see below). This phenotype becomes even more prominent when cells are cultivated in 12 h day/night cycles. From these findings, it can be concluded that

under natural conditions, characterized by fluctuating nutrient and light supply, cyanophycin synthesis allows intracellular sequestration of nitrogen assimilation products, so that under periods of nitrogen shortage, CGP degradation provides anabolic precursors to continue growth. Even under constant illumination and a constant but limited nitrogen supply, the wild type has a growth advantage over the *cphA* mutant. This suggests that CGP synthesis optimizes the nitrogen assimilation process.

The recent discovery of the ornithine–ammonia cycle (OAC) in cyanobacteria emphasizes the role of CGP as dynamic nitrogen storage (Zhang et al. 2018). The pathway starts with carbamoyl phosphate synthesis and ends with the conversion of arginine to ornithine, CO_2, and ammonia by a novel arginine dihydrolase (ArgZ). Disruption of the OAC-pathway by mutating *argZ* leads to impaired cell growth under fluctuating nitrogen supply. This demonstrates that the OAC-pathway allows rapid remobilization of nitrogen reserves under temporal starvation conditions. Since the *cphA*-deficient mutant showed similar growth defects under fluctuating nitrogen supply, it can be concluded that nitrogen, which is stored in CGP, can be remobilized by the OAC-pathway (Zhang et al. 2018).

5   Enzymes of Cyanophycin Metabolism

Enzymes of the CGP metabolism occur in a wide of range of phototrophic and chemolithoautotrophic bacteria (Füser and Steinbüchel 2007). In general, CGP is synthesized from aspartate and arginine by the cyanophycin synthetase (CphA1) in an ATP-depending elongation reaction. Intracellular CGP degradation is catalyzed by the cyanophycinase (CphB). The β-Asp-Arg dipeptides resulting from the cleavage of CGP are further hydrolyzed by isoaspartyl dipeptidase, releasing aspartate and arginine (Watzer and Forchhammer 2018b) (Fig. 3). The following paragraphs provide detailed information about the enzymes involved in CGP metabolism.

5.1   *Cyanophycin Synthetase (CphA)*

The non-ribosomal biosynthesis of cyanophycin takes place, as long as the cell has sufficient nitrogen and energy available by a single enzyme called cyanophycin synthetase (CphA) (Simon 1976). In the synthesis the amino acids aspartate and arginine condense in two steps with one ATP consumption per step (Simon 1976; Berg et al. 2000). Additionally, the reaction requires potassium ions, magnesium ions, cyanophycin primers (low-molecular-mass CGP), and a thiol reagent (such as β-mercaptoetanol or dithiothreitol) (Simon 1976). The requirement of low-molecular mass CGP in the reaction of CphA leads to the conclusion that CphA catalyzes the elongation of a CGP-filament but not its de novo synthesis.
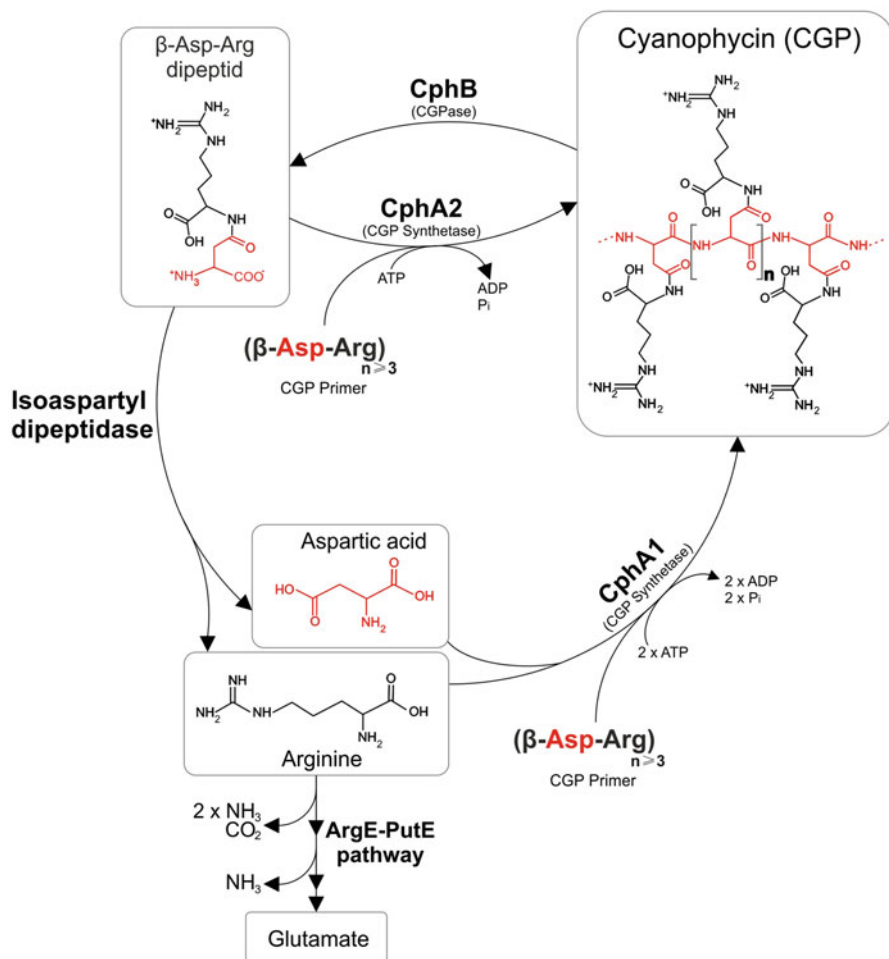
Fig. 3 Scheme of cyanophycin metabolism. Cyanophycin (CGP) is synthesized from aspartate and arginine by CGP synthetase (CphA1) in an ATP-depending elongation reaction using CGP primers, containing at least three Asp-Arg building blocks. The degradation of CGP by the cyanophycinase CphB releases β-aspartyl-arginine dipeptides, which can be further hydrolyzed by isoaspartyl dipeptidase to aspartate and arginine. In many nitrogen-fixing cyanobacteria, an additional CGP synthetase is present, termed CphA2. CphA2 can use β-aspartyl-arginine dipeptides to resynthesize CGP (Watzer and Forchhammer 2018b). The recently elucidated AgrE-PutA pathway allows rapid nitrogen mobilization by the catabolizes of arginine to glutamate, with concomitant release of ammonium (Burnat et al. 2019)

By using synthetic primers, Berg et al. could show that a single building unit (β Asp-Arg) does not serve as an efficient primer for the CphA1 elongation reaction. CphA1 needs at least an Asp-Arg trimer (β Asp-Arg)$_3$ as a primer for activity (Berg et al. 2000). Other peptides, like the peptidoglycan-pentapeptide or other cellular components, have been suggested to serve as an alternative priming substance,

Table 1 Overview of *cphA*-genes from different organisms

Gene names	Uniprot ID	Organism	References
cphA$_{6803}$; *slr2002*	P73833	*Synechocystis* sp. (strain PCC 6803/Kazusa)	Ziegler et al. (1998)
cphA$_{6308}$	P56947	*Geminocystis herdmanii* (strain PCC 6308) (*Synechocystis* sp. (strain PCC 6308))	Steinle et al. (2008)
cphA$_{MA19}$	Q8VTA5	*Synechococcus* sp. MA19	Hai et al. (1999)
cphA$_{7120}$; *all3879*	P58572	*Nostoc* sp. (strain PCC 7120/SAG 25.82/UTEX 2576)	Voss et al. (2004)
cphA$_{29413}$; *ava1814*	O86109	*Trichormus variabilis* (strain ATCC 29413/ PCC 7937) (*Anabaena variabilis*)	Ziegler et al. (1998)
cphA2$_{29413}$; *ava0335*	Q3MGC5	*Trichormus variabilis* (strain ATCC 29413/ PCC 7937) (*Anabaena variabilis*)	Klemke et al. (2016)
cphA2$_{7425}$; *cyan7425 4446*	B8HK06	*Cyanothece* sp. (strain PCC 7425/ATCC 29141)	Klemke et al. (2016)
cphA$_{NE1}$	A7YEP1	*Nostoc ellipsosporum* NE1	Hai et al. (2006)
cphA$_{TE}$; *tlr2170*	B9A0M2	*Thermosynechococcus elongatus* (strain BP-1)	Arai and Kino (2008)
cphA$_{ADP1}$; *ACIAD1279*	Q6FCQ7	*Acinetobacter baylyi* (strain ATCC 33305/ BD413/ADP1)	Krehenbrink et al. (2002)
cphA$_{DH}$; *dhaf0085*	B8FY49	*Desulfitobacterium hafniense* (strain DCB-2/ DSM 10664)	Ziegler et al. (2002)

which allows CphA1 activity in recombinant bacteria without ability to produce native CGP primers (Hai et al. 2002; Obst and Steinbüchel 2006). These alternative priming substances are also present in yeasts like *Saccharomyces cerevisiae* or *Pichia pastoris* but absent in higher plants (Steinle et al. 2008, 2010; Nausch et al. 2016). The primer-independent CphA of *Thermosynechococcus elongatus* BP-1 allows CGP production in higher plants (Arai and Kino 2008). Challenges and peculiarities of CGP production in plants have been reviewed by Nausch et al. (2016).

Up to date, several CphA1 enzymes from different bacteria, including cyanobacteria and heterotrophic bacteria, have been characterized (Table 1) (Ziegler et al. 1998; Arai and Kino 2008; Hai et al. 2002; Füser and Steinbüchel 2007; Aboulmagd et al. 2001a; Elbahloul et al. 2005; Du et al. 2019). The molecular masses of the characterized CphA1 enzymes range between 90 and 130 kDa. CphA1 from *Synechocystis* sp. PCC6803 and *Anabaena variabilis* PCC 7937 forms most likely a homodimer, while the primer-independent CphA1 from *Thermosynechoccus elongatus* forms a homotetramer (Ziegler et al. 1998; Aboulmagd et al. 2001a; Arai and Kino 2008). The primary structure of cyanobacterial CphA1 can be divided in two regions; each region contains an active site and an ATP binding site (Ziegler

et al. 1998; Berg et al. 2000). Experiments revealed that arginine is probably bound at the C-terminal and aspartate at the N-terminal active site (Berg 2003).

The putative mechanism of CphA1 has been suggested by Berg et al. by measuring the step-wise incorporation of amino acids to the C-terminus of a CGP primer. First, the carboxylic acid group at the C-terminus of the polyaspartate backbone becomes activated by phosphorylation. Subsequently, one aspartate is bound at the C-terminus by its amino group, forming a peptide bound. The intermediate (-β-Asp-Arg)$_n$—Asp is transferred to the second active site of the N-terminal region of CphA1. The β-carboxyl group of aspartate becomes phosphorylated and is then linked to the α-aminogroup of arginine, forming an isopeptide-bound (Berg et al. 2000).

For CphA1 of *Synechocystis* sp. PCC 6308, the apparent K_M value in vitro for aspartate is 450 μM, for aginine 49 μM, for ATP 200 μM and for CGP as priming substance 35 μg/ml, indicating a higher affinity toward arginine compared to aspartate. During the in vitro reaction, CphA1 converts 1.3 ± 0.1 mol ATP per mol incorporated amino acid (Aboulmagd et al. 2001a).

Previous microscopical studies revealed different localizations of CphA depending on its activity in the non-diazortphic cyanobacterium *Synechocystis* sp. PCC 6803 (see below for more details) (Watzer and Forchhammer 2018a). Under balanced growth conditions, in which no CGP synthesis occurs, CphA is equally distributed in the cytoplasm. Immediately after the induction of CGP synthesis, CphA aggregates in foci that were randomly localized in the cell. These foci increase in size due to the cyanophycin synthesizing reaction of CphA and become visible as CGP granules. Doing this, CphA is exclusively attached on the granule surface. As CGP accumulates, the number of granules per cell continuously decreases (Watzer and Forchhammer 2018a). Electron micrographs could show that large CGP granule fuse when they collide, forming larger amorphous aggregates (Watzer et al. 2015). During CGP degradation, the localization of CphA changes from the granule surface back to the cytoplasm. This suggests that as CGP is degraded, CphA dissociates from the surface and converts to the inactive form, where it remains silent until CGP accumulation is triggered again.

In addition to the main enzyme for CGP synthesis CphA1, there is a second enzyme, CphA2 (Picossi et al. 2004). The gene for the enzyme CphA2 has been identified predominantly in diazotrophic cyanobacterial species that also possess the gene for CphA1 (Klemke et al. 2016). Therefore, in heterocystous cyanobacteria of Sect. 4, 71% and 82% of Sect. 5 contain a *cphA2* gene. CphA2 is a truncated enzyme version of CphA1, showing only a high homology in amino acid sequence to the N-terminal part of CphA1, but not to the C terminal part. The function of CphA2 is the addition of β-Asp-Arg dipeptide units to the growing CGP chain using one molecule of ATP (Klemke et al. 2016). CphA2 was found directly attached to the polymer like CphA1 in vegetative cells and heterocysts. By enzymatic activities of CphA2 and the degradation enzyme cyanophycinase (see below), a continuous pool of the dipeptide β-Asp-Arg in the filament is provided. If needed, CGP can be polymerized directly from β-Asp-Arg. A *cphA2* deletion mutant displays only a minor decrease in the overall CGP content. However, such a mutant showed defects

under diazotrophic growth at high light conditions like a CphA1-deficient mutant. This observation implies that CGP hydrolysis and re-polymerization by CphA1 and CphA2 could run as a "futile cycle" in case of high photosynthetic activity (Fig. 3). Furthermore, CphA2 may be involved in regulating the cellular β-Asp-Arg pool and thereby restrict the degradation of the dipeptide by the isoaspartyl dipeptidase into the amino acids arginine and aspartate (Forchhammer and Watzer 2016).

5.2 Cyanophycinase (CphB)

CGP is resistant against hydrolytic cleavage by several arginases and proteases, probably due to its branched structure (Simon and Weathers 1976; Obst and Steinbüchel 2006). In 1999, Richter et al. reported a CGP hydrolyzing from the unicellular cyanobacterium *Synechocystis* sp. PCC 6803, called CphB (Richter et al. 1999). CphB is a 29.4 kDa, dimeric C-terminal exopeptidase, catalyzing the enzymatic cleavage of CGP to β-Asp-Arg dipeptides (Richter et al. 1999; Law et al. 2009). Based on sequence analysis, its sensitivity to serine protease inhibitors and site-directed mutagenesis, CphB can be classified as serine-type exopeptidase with the catalytic Ser at position 132 (Richter et al. 1999; Law et al. 2009). Structural modeling indicated that the substrate specificity of CphB for CGP is based on an extended conformation of the active site. The unique conformation of the active site requires β-linked aspartyl peptides for binding and catalysis, preventing CphB from non-specific cleavage of other polypeptides (Law et al. 2009; Watzer and Forchhammer 2018b).

In addition to the intracellular CphB, other versions of CGP hydrolyzing enzymes exist, catalyzing the extracellular cleavage of CGP. In 2002, Obst et al. isolated several Gram-negative, heterotrophic bacteria from different habitats, which were able to utilize CGP as a carbon, nitrogen, and energy source (Obst et al. 2002). One stain was identified as *Pseudomonas anguillisptica* strain BI. A novel CGPase was identified and called CphE in culture supernatant of this strain (Obst et al. 2002). CphE exhibits an amino acid sequence identity of 27–28% to cyanobacterial CphB. The primary degradation product of CGP digested with CphE are β-Asp-Arg dipeptides. Sensitivity to serine protease inhibitors has been reported, indicating that the catalytic mechanism of CphE is related to serein-type proteases (Obst et al. 2002). Today, extracellular CGPases have been reported in several bacteria ranging from Gram-positive to Gram-negative, from areobic to an anaerobic. This indicates that utilization of CGP as a carbon, nitrogen, and energy source seems to be a common principle in nature (Obst et al. 2002, 2004, 2005; Obst and Steinbüchel 2004; Sallam and Steinbüchel 2008; Watzer and Forchhammer 2018b).

5.3 Aspartyl-Arginine Dipeptidase

The last step of CGP catabolism is the cleavage of β-Asp-Arg dipeptides to monomeric amino acids. The ORF *sll0422* from *Synechocysis* sp. PCC 6803 and *all3922* from *Anabaena* sp. PCC 7120 are annotated on the basis of sequence similarity as "plant-type asparaginase" (PTA). In 2002, Hejazi et al. characterized several PTAs for substrate specificity, showing that *sll0422* and *all3922* are able to hydrolyze a wide range of isoaspartyl dipeptides including β-Asp-Arg.

The mature PTA of *Synechocysis* sp. PCC 6803 and *Anabaena* sp. PCC 7120 consists of two subunits, which are generated by autocleavage of the primary translation product (Hejazi et al. 2002). The native enzyme has a molecular mass of approximately 70 kDa, suggesting a subunit structure of $\alpha_2\beta_2$ (α derived from N-terminal part and β derived from the C-terminal part of the immature translation product).

A deletion mutant of gene *all3922* (coding for PTA in *Anabaena* sp. strain 7120) accumulated CGP and β-Asp-Arg and was strongly impaired in diazotrophic growth (Burnat et al. 2014). Further results showed that PTA was present in vegetative cells more than in heterocysts, although β-Asp-Arg was found in substantial amounts in heterocysts. It can be assumed that the dipeptide produced by cyanophycin degradation in heterocysts should be transferred to vegetative cells.

Furthermore, a compartmentalization was also found for arginine catabolizing enzymes. The arginine decarboxylase pathway that produces *sym*-homospermidine is also more present in vegetative cells than in nitrogen-fixing heterocysts (Burnat and Flores 2014). Distortion of the arginine decarboxylase pathway in *Anabaena* sp. PCC 7120 through the deletion of agmatinase *(speB)* led to the accumulation of high amounts of CGP and to impairment of diazotrophic growth (Burnat and Flores 2014). The arginine catabolic pathway of *Anabaena* sp. PCC 7120 has recently been elucidated by Burnat et al. 2019. This pathway consists of two genes, the arginine-guanidine removing enzyme *agrE* (*alr4995*) and a proline oxidase *putA* (*alr0540*). The AgrE-PutA pathway catabolizes arginine to produce proline and glutamate, with concomitant release of ammonium. Both genes, *agrE* and *putA*, were found to be expressed at higher levels in vegetative cells than in heterocysts, implying that arginine is catabolized mainly in the vegetative cells (Burnat et al. 2019; Flores et al. 2019a).

All these results suggest that CGP is degraded in the heterocysts to the dipeptide β-Asp-Arg and the dipeptide could serve as a mobile nitrogen reserve for the vegetative cells in addition to other molecules such as glutamine (Burnat et al. 2014, 2019; Flores et al. 2019a).

6   Genetic Organization and Expression of *cphA* and *cphB*

Genes involved in CGP metabolism are usually organized in clusters (Füser and Steinbüchel 2007). In the genome of *Synechocystis* sp. PCC 6803 *cphA* and *cphB* are localized side by side but are expressed independently (Mitschke et al. 2011). A hypothetical protein with unknown function (orf *slr2003)* is located downstream of *cphA* and both are transcribed in a polysistronic unit (Mitschke et al. 2011).

Anabaena sp. PCC 7120 contains two clusters, *c*ph1 and *cph2,* both containing cyanophycin biosynthetic genes with *cphA* and *cphB* (Picossi et al. 2004). The *cph1* cluster contains *cphB1* and *cphA1,* which are cotranscribed and form an operon. In addition, *cphA1* can be expressed from two independent promotors, of which one is constitutive and the other regulated by the global transcription factor NtcA (Picossi et al. 2004). The *chp1* operon was expressed under nitrate- and ammonia-supplemented growth, but the expression increased at diazotrophic growth conditions in heterocysts and vegetative cells. Cell-specific expression under diazotrophic conditions of the *cph1* gene cluster showed a global expression of *cphA1* and *cphB1* in vegetative cells and heterocysts, but an increased expression level was observed in heterocysts (Picossi et al. 2004). A higher enzyme activity of CphA1 and CphB1 was also observed in heterocysts than in vegetative cells (Carr 1988), which suggests a role of cyanophycin as a dynamic reservoir for fixed nitrogen (Li et al. 2001).

In the *cph2* cluster, *cphA2* and *cphB2* were found in opposite orientations and both were expressed monoistronically. Both genes were expressed under ammonia-, nitrate-, and N_2-supplemented growth, but the expression was higher in the absence of ammonia. Generally, the expression of *cph1* was higher compared to *cph2* (Picossi et al. 2004).

In addition to *cph1* and *cph2* clusters, a third cluster containing putative *cphA* and *cphB* genes was found in *Nostoc punctiforme* PCC 73102 and *Anabaena variabilis ATCC 29413* (Füser and Steinbüchel 2007).

7   Biotechnological Production of Cyanophycin

The natural compound cyanophycin has up to now no industrial application whereas chemical derivatives of CGP are of potential biotechnological interest. CGP can be converted via hydrolytic β-cleavage to poly(α-L-aspartic acid) (PAA) and free arginine. Arginine is a valuable product and PAA is biodegradable and has a high number of negatively charged carboxylic groups, making PAA a possible substituent for polyacrylates (Obst and Steinbüchel 2004; Sallam et al. 2009a, b; Khlystov et al. 2017). PAA can be employed as an anti-sealant or dispersing ingredient in many fields of applications, including washing detergents or suntan lotions. Furthermore, PAA has potential application areas as an additive in paper, paint, building, or oil industry (Sallam et al. 2009a, b; Obst and Steinbüchel 2004; Watzer and Forchhammer 2018b).

In addition to its use as polymer, CGP can also serve as a source for constituent dipeptides and amino acids in food, feed, and pharmaceutical industry. The amino acids arginine (semi-essential), aspartate (non-essential) and lysine (essential) derived from CGP have a broad spectrum of nutritional or therapeutic applications. Large-scale production of these amino acids, as mixtures or dipeptides, is established in the industry, with various commercial products already available on the market (Sallam and Steinbüchel 2010; Watzer and Forchhammer 2018b).

7.1 Cyanophycin Production Using Recombinant Production Hosts

Previous approaches to produce CGP mainly focused on heterotrophic bacteria, yeasts, or plants as production hosts. These recombinant production hosts were generated by transforming CGP synthetase genes, mostly from cyanobacterial origin. Therefore, biotechnologically established production strains like *E. coli*, *Cupriavidus necator* (formally known as *Ralstonia eutropha*), *Pseudomonas putida*, *Corynebacterium glutamicum,* or *Saccharomyces cerevisiae* were used for heterologous production of CGP (Aboulmagd et al. 2001b; Steinle et al. 2008; Frey et al. 2002; Watzer and Forchhammer 2018b; Du et al. 2019). In addition to production in microorganisms, production of CGP has also been attempted in several crop plants (reviewed by (Nausch et al. 2016).

In 1998, Ziegler et al. produced the first recombinant CGP by heterologous expression of *cphA* from *Synechocystis* sp. PCC 6803 in *E. coli*. CGP isolated from this recombinant producer strain showed some differences to those from cyanobacteria. In particular, a reduced polydispersity ranging from 20 to 40 kDa and an altered amino acid composition, containing low amounts of lysin next to arginine and aspartate, haven been reported (Ziegler et al. 1998). Based on this finding, several CGP producer strains have been generated in the following years to establish CGP for biotechnological industry.

E.coli DH1 harboring *cphA* from *Synechocystis* sp. PCC 6803 was used in the first large-scale CPG production study in a culture volume up to 500 liter, allowing CGP quantities in kilogram scale. During process optimization, the highest observed CGP amount was 24% (w/w) per cell dry mass (CDM). However, this optimal production rate required Terrific-Broth-Medium. In mineral salts medium, CGP production only occurred in the presence of additional casamino acids (Frey et al. 2002). An engineered CphA from *Nostoc ellipsosprum* could further increase the maximal CGP production up to 34.5% (w/w) of CDM, but also required complex growth media to yield such high amounts (Hai et al. 2006).

Cupriavidus necator and *Pseudomonas putida* are industry-established producer stains for several products and have therefore been considered as candidates for biotechnological CGP production (Aboulmagd et al. 2001b; Diniz et al. 2006). CPG synthase genes from *Synechocystis* sp. PCC 6308 or *Anabaena* sp. PCC 7120 have

been used to enable CGP production in these species (Voss et al. 2004; Aboulmagd et al. 2001b). Metabolic engineering and process optimization studies showed that accumulation of CGP mainly depends on the origin of *cphA* gene, the accumulation of other storage compounds like polyhydroxyalkanoates (PHA), the availability of intermediates derived from the tricarboxylic acid cycle (TCA-cycle), and the addition of CGP precursor components like arginine to the medium (Voss et al. 2004; Diniz et al. 2006). Using this knowledge, it was possible to obtain CGP amounts up to 17.9% (w/w) per CDM at 30 liter scale using mineral salt medium (Diniz et al. 2006). CGP accumulation of *Cupriavidus necator* is also strongly affected by the *cphA* expression system. A stabilized multi-copy *cphA* expression system, using the 2-keto-3-deoxy-6-phospho-gluconate (KDPG) aldolase gene-dependent addiction system, allows cultivation without antibiotic selection. The multi-copy expression of *cphA* results in an increased CGP production between 26.9% and 40% (w/w) per CDM. The high amount of 40% (w/w) of CDM could be also observed in a 30- and 500-liter scale. Without the addition of amino acids to the medium, the amount of CGP was still between 26.9 and 27.7% (w/w) of CDM (Voss and Steinbüchel 2006).

The industry-established host *Saccharomyces cerevisiae* has also been considered for biotechnological CGP production (Steinle et al. 2008, 2009). By expressing *cphA* from *Synechocystis* sp. PCC 6803, it was possible to obtain CGP in amounts up to 6.9% (w/w) of CDM. In this case, two species of CGP could be observed: water-soluble and the typical water-insoluble CGP. Depending on the cultivation conditions, the ratio between water-soluble and insoluble CGP-species could be varied. Furthermore, the isolated polymer contained 2 mol% lysine, which can be increased up to 10 mol% by adding lysin to the medium (Steinle et al. 2008). During metabolic engineering studies, several arginine biosynthesis mutants of *Saccharomyces cerevisiae* have been characterized concerning their abilities to accumulate CGP. Next to arginine, aspartate, and lysine, further non-proteinogenic amino acids like citrulline and ornithine could be detected in isolated CGP derivates (Steinle et al. 2009). Depending on these results, customized CGP derivates with altered amino acid composition could also be produced using *Pseudomonas putida* ant the yeast *Pichia pastoris* as a production host (Wiefel et al. 2011; Frommeyer et al. 2016; Steinle et al. 2010). Customized CGP derivates are important sources for β-dipeptides, which can be used in several applications. The extracellular CGPase, CphE from *Pseudomonas alcaligens*, enables large-scale production of these customized β-dipeptides from CGP derivates (Sallam et al. 2009a, b; Sallam and Steinbüchel 2010).

The biotechnologically well-established bacterium *Corynebacterium glutamicum* has so far not been considered for CGP production due to low CGP quantities and difficulties in polymer extraction. Very recently, Wiefel et al. re-evaluated the capability of *Corynebacterium glutamicum* as a CGP producer strain, by using different expression vectors, *cphA*-genes, and cultivation conditions (Wiefel et al. 2019). It was possible to create strains which produce CGP up to 14% of the CDM, including water-soluble CGP containing more than 40 mol % of lysin as a side-chain. Additionally, a strain was generated which incorporated up to 6 mol % glutamic acid into the backbone as a substitution of aspartate. Interestingly, a

previous study already reported a CGP-species from nitrogen-limited *Synechocystis* sp. PCC6308 which contained high amounts of glutamic acid (Merritt et al. 1994); however, the position in the polymer could not be resolved. The re-discovery of glutamic acid in the backbone of CGP in recombinant *Corynebacterium glutamicum* raises again the question of the biological significance of this peculiar CGP species in Cyanobacteria (Merritt et al. 1994; Wiefel et al. 2019).

7.2 Dependence of CGP Production from Arginine Biosynthesis

Generally, CGP accumulation is triggered by unfavorable conditions, such as entry into stationary phase of growth, limitation of macronutrients (with the exception of nitrogen starvation) or inhibition of translation by adding antibiotics like chloramphenicol (Allen et al. 1980; Simon 1973; Watzer and Forchhammer 2018b). All these conditions result in a reduced or arrested growth. In the exponential growth phase, amino acids like arginine and aspartate are mostly used for de novo protein biosynthesis. Under growth-limiting conditions, the protein biosynthesis is slowed down, resulting in an excess of free amino acids. This enrichment of monomeric amino acids allows CGP accumulation.

Acinetobacter calcoaceticus ADP1 accumulates only 3.5% (w/w) CGP of the cellular dry matter (CDM) when grown with ammonia as a nitrogen source and under phosphate starvation (Elbahloul et al. 2005). During process optimization it was shown that the CGP production of *A. calcoaceticus* ADP1 during growth with arginine as a sole carbon and nitrogen source increased drastically to 41.4% (w/w) of CDM. Surprisingly, a combined supply of arginine and aspartate had a much lower stimulating effect as arginine alone (Elbahloul et al. 2005). In the following metabolic engineering studies of *A. calcoaceticus* ADP1, several genes related to the arginine biosynthesis were modified to yield higher amounts of arginine. In consequence, significant higher amounts of CGP could be detected (Elbahloul and Steinbüchel 2006).

Another link between the arginine biosynthesis and CGP metabolism was observed during transposon mutagenesis studies in the filamentous cyanobacterium *Nostoc ellipsosorum*. Here, an arginine biosynthesis gene, *argL*, was interrupted by a transposon, leading to a partially impaired arginine biosynthesis. Without arginine supplementation, heterocyst failed to fix nitrogen, akinetes were unable to germinate and CGP granules did not appear. However, when arginine is provided, mutant could form CGP granules and was able to differentiate functional akinetes (Leganes et al. 1998).

These results point towards arginine as main bottleneck of CGP biosynthesis, while aspartate plays a minor role. High concentrations of arginine surpass the *Km* value of CphA, triggering CGP production. In agreement, the *Km* value for arginine is much lower compared to aspartate, indicating a higher sensitivity of CphA

towards arginine in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (Aboulmagd et al. 2001a). Whether *CphA* is subjected to additional activity control remains to be elucidated (Watzer and Forchhammer 2018a, b).

7.3 Overproduction of Cyanophycin by Genetic Engineering the P_{II} Signaling System

Since arginine synthesis is a limiting factor for CGP accumulation, overproduction of CGP requires enhanced arginine synthesis. Arginine synthesis is strictly controlled by the nutritional status of the cyanobacteria, mainly through tight regulation of the committed step of ornithine synthesis, the N-acetylglutamate kinase (NAGK) reaction. The NAGK enzyme is feedback controlled by arginine levels, and in addition, NAGK activity and arginine-feedback inhibition are modulated by the P_{II} signal transduction protein. The P_{II} signaling protein responds to the effector molecules 2-oxoglutarate (which reports the C/N metabolic balance) and to the adenylnucleotides ATP or ADP, which compete for the same binding site. Thereby, the P_{II} signaling protein integrates the energy status of the cells (ATP/ADP ratio) with the C/N metabolic balance, where high 2-OG levels indicates carbon-sufficient but nitrogen-limiting conditions (for review see (Forchhammer and Lüddecke 2016; Forcada-Nadal et al. 2018). P_{II} in the ATP-complexed state binds to NAGK, thereby enhancing its catalytic activity and tuning down arginine-feedback inhibition. This activation by P_{II} results in an increased metabolic flux into the arginine pathway (Maheswaran et al. 2004; Maheswaran et al. 2006; Watzer et al. 2015). A single amino acid replacement in the cyanobacterial P_{II} signaling protein (Ile86 to Asp86) generated a P_{II} variant (P_{II}-I86N) that constitutively binds to NAGK in vitro and enhances its activity (Fokina et al. 2010). Replacing the wild-type allele for P_{II} in *Synechocystis* by this variant resulted in a strain, which has constitutively high NAGK activity and ten-fold increased levels of arginine (BW86) (Watzer et al. 2015). Already under balanced growth conditions with nitrate as a nitrogen source, strain BW86 accumulated up to $15.6 \pm 5.4\%$ CGP relative to the cell dry mass (CDM), while CGP in the wild type was barely detectable. Phosphate or potassium starvation further increased the CGP content of strain BW86 up to $47.4 \pm 2.3\%$ or $57.3 \pm 11.1\%$ per CDM, respectively (Watzer et al. 2015). The cyanophycin, which is produced by strain BW86, showed a high polydispersity ranging from 25 to 100 kDa, similar to the polydispersity of wild-type cyanobacterial CGP (Simon 1971). This contrasted CGP from recombinant producer strains using heterologous expression systems such as heterotrophic bacteria, yeasts, or plants, whose recombinantly produced CGP shows polydispersity ranging from 25 to only 45 kDa (Frey et al. 2002; Steinle et al. 2008). The novel cyanobacterial producer stain for cyanophycin may provide a valuable resource for the synthesis of large quantities of natural long-chain cyanophycin with so-far unexplored properties.

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