Storage Polysaccharides in Prokaryotes: Glycogen, Granulose, and Starch-Like Granules

Matthieu Colpaert, Malika Chabi, Ugo Cenci, and Christophe Colleoni

Contents

Abstract Homopolymers of D-glucose represent the most successful and abundant polysaccharides found in nature. In this chapter, we will focus on α-glucan polysaccharides in particular glycogen and its derivatives (i.e., granulose, starch) that define probably one of the oldest forms of carbon storage among prokaryotes. They are made of linear chains of glucosyl units joined by α-1,4 glycosidic bonds and

M. Colpaert · M. Chabi · U. Cenci · C. Colleoni (✉)
Univ. Lille, CNRS, UMR8576-UGSF-Unité de Glycobiologie Structurale et Fonctionnelle, Lille, France
e-mail: Christophe.colleoni@univ-lille1.fr

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hooked to each other by α-1,6 glycosidic bonds, referred to as branching points. The glucan chains are organized in such manner that α-glucan polysaccharides appear mostly in the form of tiny hydrosoluble or insoluble water particles in the cytosol of bacteria. Because "Nothing in biology makes sense except in the light of evolution" to quote Dobzhansky, this chapter aims to emphasize the importance of structure-function relationship that determines the physicochemical and biochemical properties of storage polysaccharides in microorganisms.

1 Introduction

Energy homeostasis is a critical issue for any living organism. Prior to the emergence of energy-carbon-based storage compounds, several reports speculate that polyphosphate granules were probably the first form of energy storage compound that evolved in the prebiotic history of life (Achbergerová and Nahálka 2011; Albi and Serrano 2016; Piast and Wieczorek 2017).

In the prokaryotic world, energy-carbon or energy storage compounds have evolved in various forms such as polysaccharides, lipids, poly-β-hydroxybutyrate (PHB)/polyhydroxyalkanoate (PHA) and polyphosphate. Despite their distinct physicochemical properties, energy-carbon compounds share specific features such as a high molecular weight in order to reduce internal osmotic pressure within cells, and they are produced when there is an excess of energy or are degraded for the maintenance of the cells in times of starvation. For this reason, trehalose and sucrose might not be considered sensu stricto as energy-carbon storage compounds in prokaryotes but more likely as transitory carbohydrate compounds produced in response to environmental stresses (e.g., cold, salt).

In the present book chapter, we will confine our attention to glycogen, granulose, and starch-like granules, polysaccharides composed of chains of α-1,4 linked D-glucosyl units that are interconnected through α-1,6 linkages or branching points. We will exclude consideration of other polysaccharides such as dextrans, fructans, PHB/PHA, and polyphosphate. Nevertheless, energy storage functions of PHB/PHA and polyphosphate are detailed in recent reviews (Jendrossek and Pfeiffer 2014; Albi and Serrano 2016). Glycogen particles are ubiquitous in the tree life that encompasses the *Archaea*, *Bacteria*, and *Eukaryota* domains. We have to keep in mind that most of our major understanding of glycogen metabolism pathways essentially relies on the studies in *Escherichia coli* and yeast *Saccharomyces cerevisiae*, which are the usual representatives of prokaryotes and heterotroph eukaryotes, respectively (for review see Wilson et al. 2010). Nevertheless early electron microscopy observations point out that some prokaryotes substitute hydrosoluble glycogen particles by abnormal glycogen particles such as starch-like granules described in a small group of cyanobacteria or granuloses synthesized in *Clostridium* species. Fortunately, the improvement of high-throughput genome sequencing brings some insight

in storage polysaccharide metabolism network. Thus, the plethora of bacterial genomes outlines the diversity of carbon storage pathways as well as the fact that some bacterial species have lost the ability to synthesize carbohydrate storage polysaccharides. The aim of this chapter is to give an overview of basic and alternative glycogen metabolism pathways in prokaryotes and how storage polysaccharide impacts cell physiology.

2 Glycogen Particles, Starch-Like Granules, and Granulose in Prokaryotes

Although glycogen, starch, starch-like granules, and granulose are composed of glucan chains made of glucose residues linked in α-1,4 position and hooked together by α-1,6 linkages, the organization of glucan chains inside polysaccharides gives rise to distinct properties as resumed in Table 1. The glycogen synthesis occurs in the cytosol of prokaryotes and eukaryotic organisms as tiny hydrosoluble particles of 40–60 nm. Detailed structural analyses confirm that glycogen particles from eukaryotes and prokaryotes are very similar in terms of glucan chain content (chain length distribution is monomodal with a major frequency of glucan chain of 6–7 units glucose) as well as high percentage of branching points (9–12% that correspond to an average of two α-1,6-linkages per glucan chains as determined in Melendez-Hevia et al. 1993). Interestingly, taking into account these parameters, mathematical modeling predicts a size of 42 nm in granulose granule diameter and explains this self-limitation due to homogenous distribution of branching points. This particular organization leads quickly to an exponential increase in the number of non-reducing ends at the surface of polysaccharide (Fig. 1a) and then will limit the access to the catalytic sites of biosynthetic enzymes and per se the size of glycogen particles (Meléndez-Hevia et al., 1993; Meléndez et al. 1999). Interestingly, further mathematical modeling infers that glycogen particles can be considered as fractal objects, made by iteration of a single motif: one glucan chain supporting two branches. Thanks to this particular glucan chain organization, a single catabolic enzyme can release 19,000 glucose molecules in 20 s (Meléndez et al. 1997). Hence, glycogen particles are dynamic polysaccharides, which can meet the immediate need of eukaryotic and prokaryotic cells and can be used as an intermediate buffer or osmotically inert sink for carbon (Table 1).

On the contrary, starch-like granules and granulose are not soluble in water, and granule sizes are above 42 nm. We know from the structural characterization of starch-like granules produced by two unicellular diazotrophic cyanobacteria *Cyanobacterium* sp. CLg1 and *Cyanothece* ATCC51142 that those granules are similar to starch granules found in plants. Those semi-crystalline nonaqueous polysaccharides are composed of two polysaccharides: amylopectin and amylose. The former, the major fraction, is a branched polysaccharide containing 5% of branching points, while amylose, a dispensable fraction of starch, is mostly composed of slightly

Table 1 Properties of storage polysaccharides

Organisms	Polysaccharides	Size	Solubility in water	Semi-crystallinity	Polysaccharide fraction	% α-1,6	DP	MW ($\times 10^6$)	References
Bacteria	Glycogen	30–60 nm	Yes	No	100%	9–12%	6–7	2–13	Park et al. (2011)
Archaea									Meléndez et al. (1997)
Eukaryota									
Archaeplastida	Starch/starch-like	1–100μm	No	Yes	≈75% amylopectin	5–6%	11–12	10–100	Vamadevan and Bertoft (2014)
Cyanobacteria					≈25% amylose	<1%	900–3300		Nakamura et al. (2005)
Clostridium sp.	Granulose	210–270 nm	No	N.D.	N.D.	N.D	N.D	N.D	Tracy et al. (2008)
Methylacidiphilum fumariolicum	Abnormal glycogen	100–200 nm							Khadem et al. (2010)

Glycogen, starch/starch-like, granulose, and abnormal glycogen are composed of α-1,4 linked glucose residues that are interconnected through α-1,6 linkages. Glycogen is the main storage form of carbon storage in living organisms. Starch and starch-like granules have been reported in Archaeplastida lineages (plants/algae, red algae, and glaucophytes) and in a small group of unicellular nitrogen-fixing cyanobacteria. To date, granulose and abnormal glycogen have been observed in *Clostridium* sp. and *Candidatus Methylacidiphilum fumariolicum*. Despite sharing the primary structure, those homopolymers of glucose harbor various physicochemical properties in terms of size, solubility in water, and crystallinity. Starch and starch-like granules contain two polysaccharides: amylopectin fraction and branched polysaccharide that represents up to 75% (w/w) of starch granules and amylose fraction, mostly composed of long linear glucan chains. The organization of glucan chains inside polysaccharides and the frequency of branching point (α-1,6), the maximum abundance of glucan chain (degree of polymerization (DP) represents the number of glucose residues that composes glucan chain), explain the physicochemical properties of polysaccharides

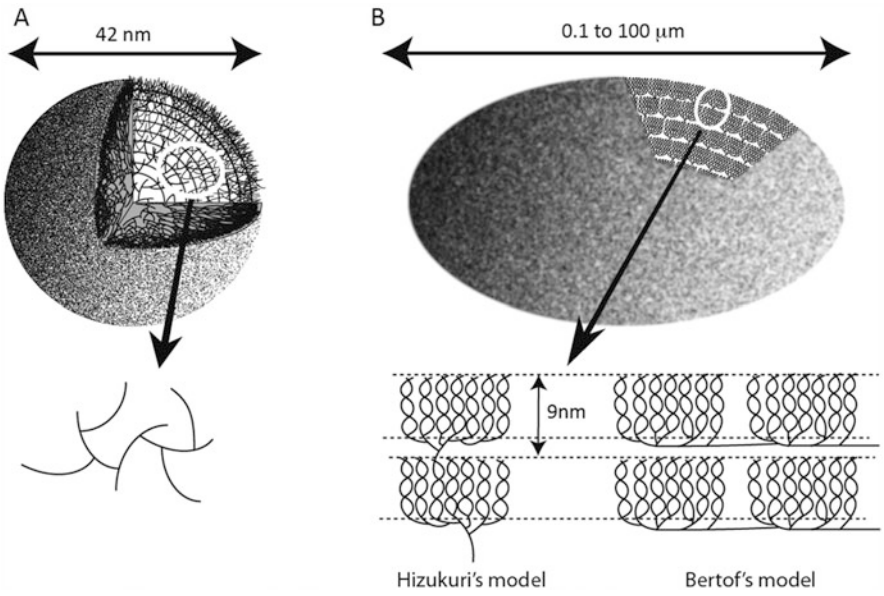
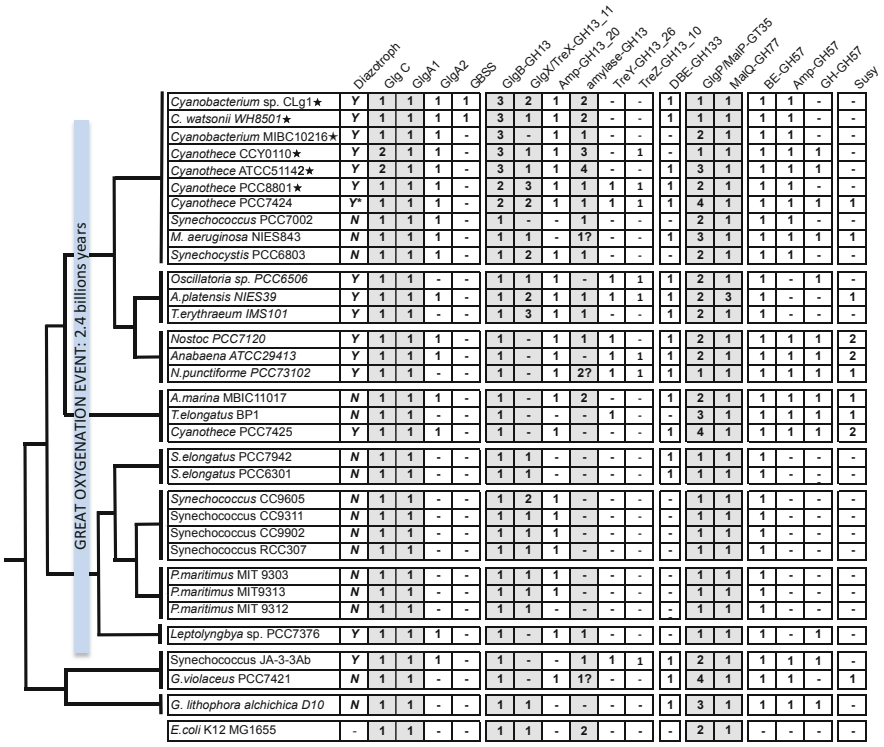


Fig. 1 Structural comparison between glycogen and amylopectin. Both polysaccharides are composed of glucan chains made of α-1,4 glucose residues and branched in α-1,6 position. (**a**) The uniform distribution of branches leads to an exponential increase that self-limits glycogen particle size to 42 nm. Mathematical modeling suggests that glycogen particles are fractal objects made of repeating patterns of glucan chains (black lines) harboring two glucan chains (intersection lines). (**b**) The size of semi-crystalline starch granule is variable depending on the source. Clusters of amylopectin are generated through the asymmetric distribution of branches, localized in the amorphous lamellae, while the intertwined glucan chains define crystalline lamellae. The sum of one amorphous and crystalline lamella is constant to 9 nm independently of amylopectin clusters examined. In the model proposed by Hizukuri (1986), a long glucan chain interconnects two clusters, whereas in the alternate model of Bertoft (2004), the clusters are anchored to a backbone consisting of a long glucan chain

branched (<1%) linear glucan chains. First proposed by Hizukuri (1986) and then modified by Bertoft (2004), these models propose a specific localization of branching points allowing the formation of double helices of glucan (Fig. 1b). Thus, this particular "cluster" organization allows more efficient storage of glucose residues without the size limitations imposed on glycogen particles. However, the downside is that synthesis and degradation of starch are more complex and less dynamic. To our knowledge, structural characterization of granulose has been limited to the measurement of absorbency of iodine-granulose complex, which appears similar to iodine-amylopectin complex (see below). Data of wide-angle X-ray diffraction analysis should discriminate between the semi-crystallinity and amorphous nature of granulose.

Table 2 Distribution and number of isoforms involved in the storage polysaccharide pathway in cyanobacteria



	Diazotroph	Glg C	GlgA1	GlgA2	GBSS	GlgB-GH13	GlgX/TreX-GH13_11	Amp-GH13_20	amylase-GH13	TreY-GH13_26	TreZ-GH13_10	DBE-GH133	GlgP/MalP-GT35	MalQ-GH77	BE-GH57	Amp-GH57	GH-GH57	Susy
Cyanobacterium sp. CLg1★	Y	1	1	1	1	3	2	1	2	-	-	1	1	1	1	1	-	-
C. watsonii WH8501★	Y	1	1	1	1	3	1	1	2	-	-	1	1	1	1	1	-	-
Cyanobacterium MIBC10216★	Y	1	1	1	-	3	-	1	1	-	-	-	2	1	1	1	-	-
Cyanothece CCY0110★	Y	2	1	1	-	3	1	1	3	-	1	-	1	1	1	1	1	-
Cyanothece ATCC51142★	Y	2	1	1	-	3	1	1	4	-	-	1	3	1	1	1	1	-
Cyanothece PCC8801★	Y	1	1	1	-	2	3	1	1	1	1	1	2	1	1	1	-	-
Cyanothece PCC7424	Y*	1	1	1	-	2	2	1	1	1	1	1	4	1	1	1	1	1
Synechococcus PCC7002	N	1	1	1	-	1	-	-	1	-	-	-	2	1	1	1	-	-
M. aeruginosa NIES843	N	1	1	1	-	1	1	-	1?	-	-	1	3	1	1	1	1	1
Synechocystis PCC6803	N	1	1	1	-	1	2	1	1	-	-	-	2	1	1	1	-	-
Oscillatoria sp. PCC6506	Y	1	1	-	-	1	1	1	-	1	1	1	2	1	1	-	1	-
A.platensis NIES39	Y	1	1	1	-	1	2	1	1	1	1	1	2	3	1	-	-	1
T.erythraeum IMS101	Y	1	1	-	-	1	3	1	1	-	-	1	2	1	1	-	-	-
Nostoc PCC7120	Y	1	1	1	-	1	-	1	1	1	-	1	2	1	1	1	1	2
Anabaena ATCC29413	Y	1	1	1	-	1	-	1	-	1	1	1	2	1	1	1	1	2
N.punctiforme PCC73102	Y	1	1	-	-	1	-	1	2?	1	1	1	1	1	1	1	1	1
A.marina MBIC11017	N	1	1	1	-	1	-	1	2	-	-	1	2	1	1	1	1	1
T.elongatus BP1	N	1	1	-	-	1	-	-	-	1	-	-	3	1	1	1	1	1
Cyanothece PCC7425	Y	1	1	1	-	1	-	1	-	-	-	1	4	1	1	1	1	2
S.elongatus PCC7942	N	1	1	-	-	1	1	-	-	-	-	1	1	1	1	-	-	-
S.elongatus PCC6301	N	1	1	-	-	1	1	-	-	-	-	1	1	1	1	-	-	-
Synechococcus CC9605	N	1	1	-	-	1	2	1	-	-	-	-	1	1	1	-	-	-
Synechococcus CC9311	N	1	1	-	-	1	1	1	-	-	-	-	1	1	1	-	-	-
Synechococcus CC9902	N	1	1	-	-	1	1	1	-	-	-	-	1	1	1	-	-	-
Synechococcus RCC307	N	1	1	-	-	1	1	1	-	-	-	-	1	1	1	-	-	-
P.maritimus MIT 9303	N	1	1	-	-	1	1	1	-	-	-	-	1	1	1	-	-	-
P.maritimus MIT9313	N	1	1	-	-	1	1	1	-	-	-	-	1	1	1	-	-	-
P.maritimus MIT 9312	N	1	1	-	-	1	1	-	-	-	-	-	1	1	1	-	-	-
Leptolyngbya sp. PCC7376	Y	1	1	1	-	1	-	1	1	-	-	-	1	1	1	-	1	-
Synechococcus JA-3-3Ab	Y	1	1	1	-	1	-	-	1	1	1	1	2	1	1	1	1	-
G.violaceus PCC7421	N	1	1	-	-	1	-	1	1?	-	-	1	4	1	1	1	-	1
G. lithophora alchichica D10	N	1	1	-	-	1	1	-	-	-	-	1	3	1	1	1	1	-
E.coli K12 MG1655	-	1	1	-	-	1	1	-	2	-	-	-	2	1	-	-	-	-

Black lines depict the evolutionary relationship between cyanobacterial species in respect to the Great Oxygenation Event (GOE). Representatives of each cyanobacteria clade were selected based on the availability of genome sequences. The number of enzyme isoforms was determined using Blast searches on NCBI, CyanoBase, and CAZy. Cyanobacteria strains labeled with a star synthesize starch-like granules. The ability of nitrogen fixation in aerobic conditions is indicated by Y (Yes) or N (No) and by Y* (Yes) in anoxic conditions. Key: ADP-glucose pyrophosphorylase (GlgC); glycogen synthase isoforms (GlgA1, GlgA2); granule-bound starch synthase (GBSS); branching enzyme GH13 family (GlgB-GH13); debranching enzyme GH13 subfamily 11 (GlgX/TreX-GH13_11); amylopullulanase-GH13 subfamily 20 (Amp-GH13_20); amylase-GH13; maltooligosyltrehalose synthase GH13 subfamily 26 (TreY-GH13_26); maltooligosyltrehalose hydrolase GH13 subfamily 10 (TreZ-GH13_10); debranching enzyme or amylo-1,6 glucosidase (DBE-GH133); glycogen phosphorylase/maltodextrin phosphorylase (GlgP/MalP-GT35); α-1,4 glucanotransferase (MalQ-GH77); putative branching enzyme GH57 family (BE-GH57); putative amylopullulanase GH57 family (Amp-GH57); putative glycosyl hydrolase family GH57 (GH-GH57). *Escherichia coli* is used as reference for the type of isoforms found in cyanobacteria (gray columns)

3 Three Biosynthesis Glycogen Pathways in Prokaryotes

Over the past decades, our understanding of glycogen biosynthetic pathway in prokaryotes relies mostly on functional approaches using *Escherichia coli*, as a model. At the present, most of the activities involving carbohydrate metabolism pathways and their regulation are well understood (for reviews see Wilson et al. 2010; Preiss 2014). In prokaryotes, the most predominant glycogen biosynthetic pathway (GlgC) is based on the use of nucleotide-sugars as activated substrates for the polymerization. Nevertheless, both high-throughput genome sequencing and comparative genome analyses have evidenced two alternative glycogen biosynthetic pathways in bacteria known as the GlgE pathway and the MalQ pathway, respectively (Fig. 2).

3.1 Nucleotide-Sugar-Based Glycogen Biosynthetic Pathway in Bacteria: GlgC Pathway

In the GlgC path, the biosynthesis of glycogen involves a suite of three enzymatic reactions for catalyzing: (1) the nucleotide-sugar synthesis mediated by ADP-glucose pyrophosphorylase (GlgC), (2) the formation of α-1,4 linkage catalyzed by glycogen synthase (GlgA), and (3) the formation of α-1,6 linkages mediated by glycogen branching enzyme (GlgB). Glycogen metabolizing genes are commonly organized in operon such as the *glgBXCAP* well conserved in Enterobacteriales and the sister lineage Pasteurales (Almagro et al. 2015). Both *glgX* and *glgP* genes encode for glycogen catabolic enzymes (see Glycogen Catabolic Paths in Prokaryotes).

Nucleotide-Sugar Synthesis: UDP-/ADP-Glucose Pyrophosphorylase (EC 2.7.7.27)

In 1957, Leloir and Cardini were the first to establish the importance of nucleotide-sugar in the biosynthesis of storage polysaccharides rather than glucose-1-phosphate which had been earlier thought to define the polymerization substrate (Leloir and Cardini 1957). In prokaryotes, genetic evidence of ADP-glucose pyrophosphorylase (GlgC) as a pivotal enzyme for glycogen biosynthesis in *E. coli* was published in 1968 (Damotte et al. 1968). Following this study, correlation between GlgC activity and glycogen biosynthesis was confirmed in many other prokaryotes as well as in cyanobacteria (Iglesias et al. 1991). In regard to its role in the glycogen pathway, ADP-glucose pyrophosphorylase is an allosteric enzyme negatively or positively regulated by fructose-1,6-bisphosphate or AMP, respectively. In cyanobacteria, the ADP-glucose pyrophosphorylase is subjected to redox regulation as well as to the ratio of 3-phosphoglycerate/orthophosphate that reflects the activity of

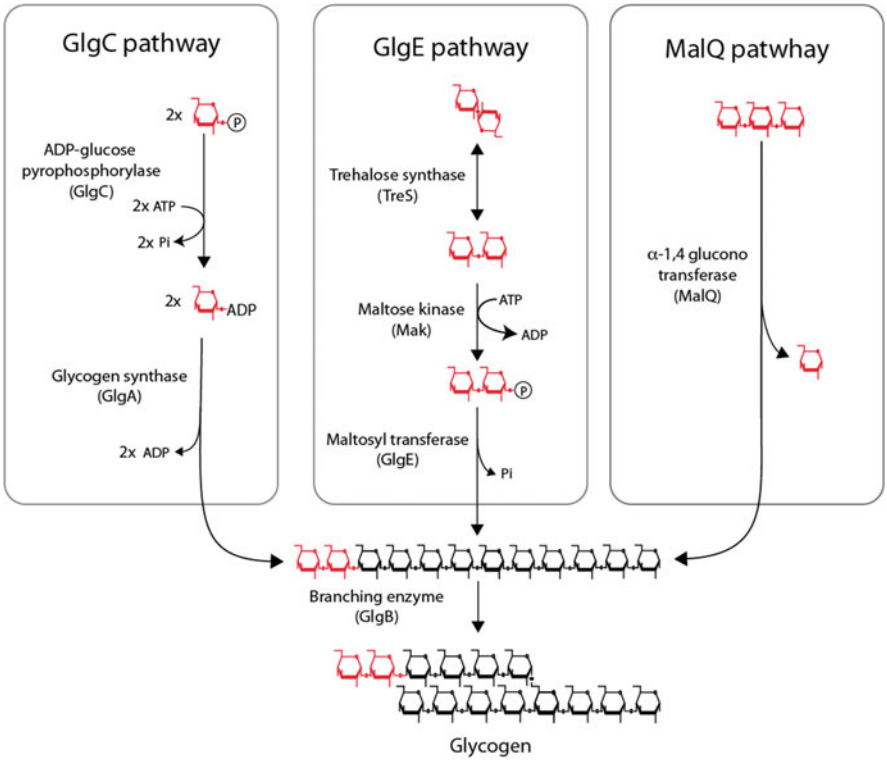


Fig. 2 Glycogen anabolism pathways in prokaryotes. The most predominant glycogen biosynthesis pathway, GlgC pathway, involves an ADP-glucose pyrophosphorylase (GlgC) and glycogen synthase (GlgA) for synthesizing the glucan chains. The former synthesizes the nucleotide-sugar, ADP-glucose, from ATP and glucose-1-phosphate (G1P). Glycogen synthase then transfers the glucose moiety of ADP-glucose onto the non-reducing end of growing glucan chain. In the GlgE pathway, non-reducing disaccharide, trehalose, is converted reversibly into maltose by trehalose synthase (TreS) followed by a phosphorylation onto the reducing end of maltose. The latter reaction processed by maltose kinase (Mak) found in some bacterial species fused with TreS activity. Maltose-1-phosphate (M1P) is incorporated onto the non-reducing end of glucan chain thanks to maltosyl transferase (GlgE). MalQ pathway, described in few species, relies onto pre-existing short glucan chains. α-1,4 Glucanotransferase (MalQ) activity disproportionates short glucan chains. Then, in both GlgC-GlgE pathways and malQ pathway, an a-1,6 linkage is produced when the neo-glucan chain fits the catalytic site of branching enzyme (GlgB). The incorporation of two glucose moieties (in red) requires 2, 1, and 0 ATP for GlgC, GlgE, and MalQ pathways, respectively

Calvin-Benson cycle (Ballicora et al. 2003; Díaz-Troya et al. 2014). It should be stressed out that some bacteria species like *Prevotella bryantii* B14, a common bacterium found in the rumen, rely apparently on UDP-glucose pyrophosphorylase activity for glycogen synthesis instead of ADP-glucose pyrophosphorylase. Accordingly, glycogen anabolism in this species depends on UDP-glucose-dependent glycogen synthase (Lou et al. 1997). Besides NDP-glucose pyrophosphorylase

activity, another possible but yet to be confirmed source of nucleotide-sugar was revealed by the study of sucrose metabolism in the filamentous nitrogen-fixing cyanobacterium *Anabaena* ATCC29213. To recall, sucrose synthesis is induced in response to osmotic stress in cyanobacteria and then catabolized either by invertase or by sucrose synthase (SuSy) activities. In plants, several studies suggested that SuSy activity was involved in the cell wall synthesis by catalyzing the release of UDP-glucose and fructose from UDP and sucrose. Unexpectedly, mutants of SuSy of *Anabaena* displayed a more complex phenotype. Indeed, SuSy null mutants harbored a decreased glycogen content, despite twofold increases of ADP-glucose pyrophosphorylase activity and are impaired in the nitrogen fixation process (Porchia et al. 1999). Further biochemical characterizations of SuSy in both *Anabaena* ATCC29413 and *Thermosynechococcus elongatus* have revealed that SuSy catalyzed preferentially the synthesis of ADP-glucose and fructose by cleaving sucrose in the presence of ADP (Porchia et al. 1999; Figueroa et al. 2013). These astonishing results contrast to the dedicated role of SuSy of plants, which is to supply in UDP-glucose for cell wall synthesis. Subsequent studies based on metabolic network modeling in *Anabaena* sp. PCC7120 may suggest that ADP-glucose pyrophosphorylase activity alone is insufficient to fulfill the need in ADP-glucose for glycogen synthesis (Cumino et al. 2007). Unexpectedly, these results suggest that sucrose in *Anabaena* sp. PCC7120 possesses an additional function besides being an osmotic protectant. It was proposed that sucrose among other metabolites might be a carrier metabolite linking the vegetative cells and the heterocyst cells. This would be similar to some plants where sucrose is a carrier metabolite that links source organs (leaves) and sink organs (tubers). Indeed, in the filamentous cyanobacterium *Anabaena*, energy costly nitrogen fixation pathway is catalyzed by an oxygen-sensitive nitrogenase activity localized in a specialized cell termed the heterocyst. In turn, to maintain an anoxic environment and high ATP level, both reductive pentose phosphate pathway and photosynthesis II activity (oxygen evolution) are shut down in this dedicated cell. In this context, the heterocyst cell may be considered as a heterotrophic cell, comparable to sink tissue cells in plants that heavily rely on neighboring photosynthetic cells for ATP and sucrose intakes.

Formation of α-1,4 Linkages: Glycogen/Starch Synthase

Glycogen/starch synthase activities transfer the glucose moiety of nucleotide-sugar onto the non-reducing end of α-glucan chains. Structural analysis of the enzymes suggests that two families exist named according to Carbohydrate Active EnZyme classification (CAZy, www.cazy.org): glycosyl transferase 3 (GT3) and glycosyl transferase 5 (GT5) (Coutinho et al. 2003). The former is exclusively found in glycogen metabolism of fungi and animal cells, while the GT5 family is widespread among Archaeplastida (plants/green algae, red alga, and glaucophytes) and prokaryotes. With the exception of *Dictyostelium discoideum*, social amoeba possesses an eukaryotic (GT3) as well as a bacterial (GT5) glycogen synthase (Ball et al. 2015). Because of the general absence of ADP-glucose in glycogen-storing

eukaryotes, most of glycogen synthases GT3 characterized in eukaryotes are UDP-glucose and oligosaccharide primer dependent (Ball et al. 2011). In prokaryotes, the specificity of glycogen synthase GT5 is not so stringent. For instance, the characterization of glycogen synthase of *Prevotella bryantii* shows clearly an elongation activity using UDP-glucose (Lou et al. 1997). More recently, Gehre and collaborators have unraveled a surprising adaptation of GlgA activity of *Chlamydia trachomatis* (Gehre et al. 2016). In etiologic agents of human and animal diseases, *Chlamydiales* have a biphasic development that requires a eukaryotic host to complete their life cycle. Inside the eukaryotic cell, they are located inside a specific inclusion vesicle, which is associated with a large accumulation of glycogen in the lumen of vesicle inclusion. Through genetic and biochemical approaches, Gehre and collaborators have evidenced that luminal glycogen pool is mostly de novo synthesized due to an evolution of GlgA activity to polymerize UDP-glucose as well as the recruitment of host UDP-glucose transporter on the inclusion membrane (Gehre et al. 2016).

While most prokaryotes possess a *glgA* gene, cyanobacteria genomes encode two isoforms named *glgA1* and *glgA2* (Ball et al. 2015). Surprisingly, both *Cyanobacterium* sp. CLg1 and *Crocosphaera watsonii* encode a third *glgA* gene, which is phylogenetically related to granule-bound starch synthase (GBSS) of plants. In plants or green algae, GBSS activities have been shown to be responsible for synthesizing the amylose fraction in starch (Delrue et al. 1992). Mass spectrometry analysis onto protein attached to starch granules of *Cyanobacterium* sp. CLg1 and incubation experiments of purified starch granules with ^{14}C-ADP-glucose have shown that GBSS-like cyanobacteria has maintained similar enzymatic properties of plants, i.e., the synthesis of long linear glucan chains giving rise to the amylose fraction of starch-like granules (Deschamps et al. 2008). The functions of GlgA1 and GlgA2 activities in glycogen biosynthesis were investigated mainly in *Synechocystis* PCC6803 (Gründel et al. 2012; Yoo et al. 2014). Single knockout mutants were unaffected in growth and glycogen biosynthesis suggesting an overlap in function of these two enzymes. However, a detailed biochemical analysis of glycogen synthases suggested that GlgA2 is responsible for long glucan chain synthesis, while the distributive activity of GlgA1 is responsible for short glucan chain (Yoo et al. 2014). More recently, the characterization of a mutant of the starch-accumulating *Cyanobacterium* sp. CLg1 strain impaired in GlgA2 activity had comforted the idea of the processive nature of GlgA2 (Kadouche et al. 2016). Indeed, the absence of GlgA2 activity resulted specifically in the disappearance of starch granules without affecting glycogen biosynthesis (Kadouche et al. 2016). The simplest explanation for this phenotype is to propose that GlgA2 activity is mandatory for the synthesis of long glucan chains indispensable for the establishment of the cluster organization of amylopectin. In comparison to the widespread distribution of the GlgA1 isoform among prokaryotes, GlgA2 is restricted to a small number of bacterial species including *Chlamydiales*. More surprisingly, GlgA2 is very similar to the genes of soluble starch synthases III and IV (SSIII and SSIV) found in Archaeplastida (Kadouche et al. 2016). This suggests that the SSIII/SSIV genes of plants have

been inherited from a prokaryote and were possibly maintained in the Archaeplastida because of its remarkable ability to synthesize long glucan chains.

Formation of α-1,6 Linkages: Glycogen/Starch Branching Enzyme

Branching enzyme (GlgB)—α-1,4-glucan: α-1,4-D-glucan 6-α glucosyl transferase—catalyzes the transglucosylation reaction by cleaving α-1,4 linkages and by transferring the generated reducing ends to C6 hydroxyls. Branching enzyme plays a critical role in determining the branching pattern of both glycogen and starch (Sawada et al. 2014). However, no clear correlation has been found between amino acid sequence of GlgB and the molecular properties of the branched glucan produced. In prokaryotes, branching enzyme activities are distributed in two CAZy families: glycosyl hydrolase 13 (GH13) and 57 (GH57) families (Suzuki and Suzuki 2016). Family GH13, also known as the α-amylase family, is the largest sequence-based family of glycoside hydrolases characterized with $(\alpha/\beta)_8$ barrel domain. Based on phylogenetic tree of family GH13, 35 subfamilies emerge containing various enzyme activities and substrate specificities acting on α-glycosidic bonds (Stam et al. 2006). GH13 branching enzymes of eukaryotic and prokaryotic cells are distributed into two subfamilies: GH13_8 and GH13_9 (Stam et al. 2006). In 2000, Binderup and his collaborators have shown that the N-terminal end of the *E. coli* branching enzyme influences the chain transfer pattern (Binderup et al. 2000). The deletion of 112 amino acids at the N-terminus led to a truncated form of *E. coli* branching enzymes, which preferentially transfers longer chains having a degree of polymerization (DP) of more than 20 residues of glucose than the unmodified branching enzyme (DP$<$14). Subsequent studies confirmed the role of the N-terminus of glycogen branching enzymes in *E. coli* as well as for other GlgB proteins of other species (Devillers et al. 2003; Palomo et al. 2009; Jo et al. 2015; Wang et al. 2015). Branching enzymes belonging to the GH57 family, first described in thermophilic bacteria *Dictyoglomus thermophilum* and *Pyrococcus furiosus*, are now described in cyanobacteria and other prokaryote species (Fukusumi et al. 1988; Laderman et al. 1993; Suzuki and Suzuki 2016). Like GH13 family, the GH57 family contains several carbohydrate-active enzyme activities: amylopullulanase, α-galactosidase, α-1,4 glucanotransferase, α-amylase, branching enzyme, and uncharacterized activities sharing a catalytic $(\beta/\alpha)_7$ barrel fold and five conserved domains (Zona et al. 2004; Murakami et al. 2006; Santos et al. 2011). In cyanobacteria, the distribution of GH57 family enzymes among species is variable, and their exact catalytic functions have yet to be defined (Colleoni and Suzuki 2012). Interestingly, putative GH57 branching enzyme sequences are well conserved among cyanobacteria including the reduced genomes of the *Synechococcus*/*Prochlorococcus* genus as well as in the early diverging cyanobacteria species such as *Gloeobacter violaceus*, *Synechococcus* JA-3-3Ab, and *Gloeomargarita lithophora.* So far the role of this GH57 family in the physiology of cyanobacteria has not yet been explored. Because the GH57 family is mainly composed of thermostable enzymes, it is tempting to suggest this family of carbohydrate-active enzymes plays an important role in

thermophile strains (e.g., *Thermosynechococcus elongatus* BP1 and *Synechococcus* JA3-3Ab) or under thermal stress conditions that require a set of stable enzymes.

The fact that most of glycogen-accumulating prokaryotes possess at least one GH13 branching enzyme activity or one GH57 branching enzyme (Suzuki and Suzuki 2016) emphasizes the mandatory role of branching enzymes in storage polysaccharide biosynthesis. Cyanobacteria species possess both GH13 and GH57 families, and for some cyanobacterial species, additional GH13 branching enzyme isoforms are observed. It is worth noting that starch-accumulating cyanobacteria harbor in total 3–4 candidate branching enzyme activities. Structural and biochemical characterization of GH13 branching enzymes of *Cyanobacterium* sp. NBRC 102756 and *Cyanothece* ATCC51142 has revealed different branching patterns that might be correlated with the ability of those strains to synthesize starch-like granules (Hayashi et al. 2015, 2017; Suzuki et al. 2015).

De Novo Biosynthesis of Glucan Chains

Following the discovery of glycogen synthase by Leloir and Cardini in 1957, the main question during many years concerned about the ability of priming glucan synthesis from nucleotide-sugar. Apparently, glycogen synthase GT3 and GT5 can be distinguished by their ability to prime de novo synthesis of a glucan. In eukaryotic cells, the initiation of glycogen synthesis appears to require two activities: (1) an UDP-glucose-specific glycosyl transferase of CAZy family GT8 (CAZy classification), which displays self-glycosylation properties (i.e., glycogenin) and is able to synthesize a short glucan made of 8 up to 36 glucose residues using UDP-glucose as nucleotide-sugar (Albrecht et al. 2004), and (2) a glycogen synthase GT3 which then elongates the glycogenin-dependent primer in order to further elongate a glucan which thereby becomes accessible for branching enzyme. The study of yeast null mutants affected in both glycogenin isoforms, Glg1p and Glg2p, supported the mandatory nature of glycogenin-controlled glucan priming for synthesis of glycogen in fungi (Torija et al. 2005). However, it should be stressed that glycogen synthesis occurs spontaneously in 2–3% of glycogenin-null mutant of yeast depending on growth conditions (i.e., nitrogen limitation) and it might reach up to 98% if the amount of UDP-glucose is upraised in the cytosol (Torija et al. 2005). More recently, the ability of GT3-glycogen synthase to initiate glycogen synthesis in the absence of glycogenin has been experimented in animals (Testoni et al. 2017). Against all odds, not only glycogenin-deficient mice (Gyg knockout) accumulate glycogen, but also an excess of large glycogen particles is observed in both homozygous and heterozygous strains. Interestingly, these results enlighten, first, that the lack of glycogenin proteins does not prevent glycogen synthesis and, second, that glycogenin might possess an unexpected function in the glycogen biosynthesis pathway such as a regulator of glycogen synthase activity through protein-protein interactions (Testoni et al. 2017).

In prokaryotes, preliminary studies reported by Krisman and his colleagues suggested that the initiation of glycogen synthesis in *E. coli* depends as in eukaryotic

cells on glucoprotein formation by a glycogenin-like protein and, therefore, is similar to the eukaryotic path (Barengo et al. 1975; Barengo and Krisman 1978). However, detailed enzymatic analyses suggested that both functions, primer initiation and elongation activity, might be probably under sole control of the *E. coli* glycogen synthase (Kawaguchi et al. 1978; Holmes and Preiss 1979). The discovery of a 7.9 kDa protein (60 amino acids) named GlgS (S stands for Stimulate) in 1997 questioned the existence of a glycogenin-like function in *E. coli* (Beglova et al. 1997). Historically, the GlgS mutant of *E. coli* was first identified as glycogen-less phenotype through random transposon insertion mutagenesis and iodine screening of cell patches (Hengge-Aronis and Fischer 1992). *glgS* gene does not belong to the *glgBXCAP* operon; its overexpression and its upregulation by sigmaS during entry into stationary phase suggested a function in the glycogen metabolism pathway. Therefore, the authors proposed that GlgS might be dedicated to glycogen initiation. However, in 2003, the expression of recombinant glycogen synthase of *Agrobacterium tumefaciens* in glycogen-less background of *E. coli* confirmed the ability of prokaryote glycogen synthase to de novo synthesize a glucan (Ugalde et al. 2003). This result was further confirmed by the restoration of starch synthesis in the double mutant SSIII/SSIV of *A. thaliana* by the transgenic expression of the *A. tumefaciens* glycogen synthase (Crumpton-Taylor et al. 2013).

Then, both transcriptomic and molecular analyses have definitely invalidated a direct function of GlgS in glycogen metabolism of *E. coli* (Rahimpour et al. 2013). It has been shown that GlgS renamed ScoR (Surface composition Regulator) controls the cell wall composition on the surface (flagella, adhesins, exopolysaccharides). Thus, in absence of ScoR or GlgS, mutant strains displayed a higher number of flagella, and synthesis of exopolysaccharides was increased at the extent of glycogen synthesis. This pleiotropic effect disappeared when GlgS is overexpressed. The authors proposed to explain glycogen-less phenotype of ScorR/GlgS mutants that both abnormal cell mobility and exopolysaccharides synthesis drained all carbon sources normally stored at the onset of stationary phase in the form of glycogen.

Although the ability of prokaryote glycogen synthase to synthesize de novo a glucan is well established, it appears that the recombinant GlgA2 isoform of *Cyanobacterium* sp. CLg1 produced in a glycogen-less background of *E. coli* is not capable of initiating a glucan synthesis by itself. The expression of GlgA2 or GlgA1 in the single *glgA* mutant background restores glycogen synthesis in *E. coli* only when cells are grown in the presence of maltose as carbon source and not in the presence of glycerol or mannitol. In addition, electrophoretic separation of enzyme followed by in situ activity assays (i.e., zymograms) indicated that the priming reaction inside a native polyacrylamide gel depends on the presence of a glucosylated protein, named X-factor, that can be supplied by *Cyanobacterium* sp. CLg1 crude extracts (Kadouche et al. 2016). Further investigation will be necessary to evidence the exact nature of this X-factor.

3.2 Alternative Glycogen Biosynthesis Pathways in Prokaryotes: The GlgE Pathway

In contrast to the nucleotide-sugar-based glycogen synthesis pathway, this alternative glycogen pathway, named the GlgE pathway, relies on a maltosyl transferase activity (GH13 subfamily 3 according to CAZy classification) that transfers maltosyl groups from maltose-1-phosphate onto the non-reducing end of glucan chains. The evidence supporting the physiological significance of this alternative pathway in prokaryotes arose from the study of *Mycobacterium tuberculosis*. Mycobacteria are pathogenic bacteria surrounded by a capsule, composed of 80% of glucan made of glucose residue linked in α-1,4 and α-1,6 positions (Lemassu and Daffé 1994). To determine whether the mycobacterial capsule contributes significantly in the virulence and depends on the putative orthologous *glgC*, *glgA*, and *glgB* genes that have been identified in the genome of *M. tuberculosis*, a series of knockout mutants were generated either in ADP-glucose pyrophosphorylase activity (Δ*glgC*) or glycogen synthase (Δ*glgA*) (Sambou et al. 2008). Surprisingly, both deletions of GlgC and GlgA genes led only to a reduction but not to the expected wipeout of both glycogen and extracellular polysaccharide formation, thus emphasizing the existence of an alternative route for glycogen synthesis. In 2016, a better understanding and clarification of glycogen metabolism in *M. tuberculosis* was achieved when Koliwer-Brandl and collaborators included null *glgE* mutants in their studies in various genetic backgrounds (Koliwer-Brandl et al. 2016). Historically, this GlgE activity was first thought to consist of a glucanase activity releasing maltose-1-phosphate (M1P) from glycogen (Belanger and Hatfull 1999). Unexpectedly, the inactivation of GlgE activity led to both an accumulation of M1P and a decrease of glycogen content suggesting a function of GlgE in the biosynthesis rather than degradation. In order to determine the source of M1P, further investigations established that GlgA is surprisingly not involved in the elongation process of glucan chain like normal glycogen synthase. On the contrary, GlgA catalyzes preferentially the formation of M1P from ADP-glucose and glucose-1-phosphate. Finally, combination of mutations points out two independent routes, GlgA-GlgC and TreS-Mak (trehalose synthase and maltose kinase) activities, that converge on the synthesis of M1P. Despite the name, trehalose synthase activity converts reversibly trehalose (alpha-D-glucose-1,1-alpha-D-glucose) into maltose. The non-reducing disaccharide trehalose functions as an important intracellular osmoprotectant in a wide range of bacterial species, fungi, and amoeba. In the GlgE pathway, maltose produced from trehalose by the TreS activity is then phosphorylated into M1P by maltose kinase activity (Mak or Pep2). Interestingly, M1P synthesis is favored by the formation of an heterocomplex between TreS and Mak (Roy et al. 2013) or by the appearance of TreS-Mak fused protein in some bacterial species (Fraga et al. 2015). M1P is then incorporated onto the non-reducing end of glucan chains via GlgE (Kalscheuer et al. 2010). The pivotal role of GlgE in the glycogen synthesis pathway of mycobacteria and the high cell toxicity of M1P explain the growing interest in the understanding of its regulation (Leiba et al. 2013) and its 3D structure (Syson et al. 2014) as a potential

target for new drugs (Leiba et al. 2013). At the present, comparative genomic analysis suggests that the GlgE pathway is not restricted to mycobacteria species but is found in 14 % of sequenced genomes from diverse bacteria such as in *Pseudomonas*, *Xanthomonas*, and *Burkholderia* species (Chandra et al. 2011). In addition, both GlgE pathway and GlgC pathway can coexist in the same bacterium cell such as the Gram-positive *Corynebacterium glutamicum*. We have little information about the coordination of these pathways in the glycogen synthesis of such bacteria. Recently, Clermont and his collaborators have noticed that glycogen synthesis in the double knockout *glgC* and *glgA* of *C. glutamicum* depended on carbon source supplied in the medium culture. Thus, no glycogen synthesis has been assayed in the presence of glucose, while normal amounts of glycogen in comparison to wild-type strain were inferred in the presence of maltose (Clermont et al. 2015). At first glance, it is tempting to suggest that *C. glutamicum* synthesizes glycogen via either GlgE pathway or the GlgC pathway depending on the carbon source available. However, since maltose is used as a precursor to the GlgE pathway as well as in the MalQ pathway (see below), further investigations are required to evaluate the contribution of these pathways in the glycogen biosynthesis of *C. glutamicum*.

3.3 Other Glycogen Biosynthesis Pathways in Prokaryotes: The MalQ Path

As third glycogen biosynthetic path in prokaryotes, the MalQ pathway relies on the ability of MalQ—an α-1,4 glucanotransferase (GH77) or amylomaltase—to disproportionate short glucans into longer glucans (Monod and Torriani 1950). When long enough to fit the catalytic site of branching enzyme, intramolecular or intermolecular transfer generates branched glucan. In the *E. coli* model, the uptake of extracellular maltose induces the transcription of several genes of the maltose metabolism. Among them both maltodextrin glucanotransferase (MalQ) and maltodextrin phosphorylase (MalP) contribute to the catabolism of maltodextrin. In response to maltose or malto-oligosaccharides (MOS: glucan chains made of 3–12 glucosyl residues), the *malPQ* operon and other genes (such as *malZ* and *malS* encoding α-glucosidase and periplasmic α-amylase, respectively) are transcriptionally activated through the regulator MalT. In contrast to glycogen phosphorylase (GlgP), MalP activity is poorly active on branched polysaccharide while it releases glucose-1-phosphate from the non-reducing end of MOS with a minimum of four residues of glucose. In the absence of MalP, *E. coli* accumulates long glucan chains responsible for the dark-blue iodine staining of cell patches (Schwartz 1967). Interestingly, the lack of MalP activity leads to abnormal cell morphology ("snake" morphology) in an *E. coli* mutant (Schwartz 1967) as well as in a mutant of *Corynebacterium glutamicum* (Seibold et al. 2009).

MalQ catalyzes the transfer of a maltosyl group from maltotriose onto the non-reducing end of maltose or onto short glucan chains made of 3–7 glucosyl units (i.e., malto-oligosaccharide (MOS)). Therefore, MalQ generates larger malto-oligosaccharides (DP>4) suitable for MalP activity. MalQ mutants of *E. coli* do not grow in the presence of maltose but can grow in the presence of MOS with a higher DP. This growth appears dependent on the MalP activity since double mutants *ΔmalPQ* are capable of growing in maltose or MOS as carbon source. By a combination of mutation involving maltose catabolizing enzymes and glycogen synthase activity, Park and his collaborators have shown that normal glycogen synthesis occurs in a *glgA* mutant background (glycogen-less strain) when MalP activity is missing (Park et al. 2011). Interestingly recent survey of glycogen gene content in sequenced genomes reveals that the artificial situation described by Park and his collaborators might arise in some bacterial species belonging to β-proteobacteria. Thus, glycogen synthesis in both *Ralstonia eutropha* H16 and *Bordetella parapertussis* 1282 relies on the *glgBXmalQ* operon, while in *Burkholderia* sp. 383 the *treZ* gene is inserted between *glgX* and *malQ* genes (Almagro et al. 2015). Besides the lack of GlgC and GlgA activities, open questions remain concerning the nature of glycogen metabolism regulation in those species.

4 Glycogen Catabolic Paths in Prokaryotes

In enterobacteria, the glycogen catabolism pathway is pretty well understood. Glycogen phosphorylase (GlgP) and glycogen debranching enzyme (direct debranching; GlgX) work in synergy with α-1,4 glucanotransferase (MalQ) and maltodextrin phosphorylase (MalP) to essentially convert glycogen to glucose-1-phosphate. Glycogen phosphorylase catabolizes the first catabolic step by releasing G-1-P from the non-reducing ends of glucan chains. This reaction stops around four residues of glucose before a branch point. Then short-branched glucans are specifically trimmed by the GlgX activity (Dauvillée et al. 2005; Suzuki et al. 2007). Short glucans released in the cytosol are further metabolized by MalP and MalQ activities. The latter disproportionates short glucans (DP<4) in longer glucans accessible to the catalytic site of MalP (Fig. 3).

4.1 Glycogen Phosphorylase

Thus, in the model organism *E. coli*, two phosphorylase activities are involved in the glycogen catabolism pathway; GlgP and MalP show clear substrate preference for glycogen and for malto-oligosaccharides, respectively. Interestingly, both GlgP and GlgX enzymes are produced during glycogen biosynthesis. In order to avoid a futile cycle, ADP-glucose acts as a competitive inhibitor with respect to G-1-P for the GlgP activity in most prokaryotes during the biosynthesis of glycogen (Chen and

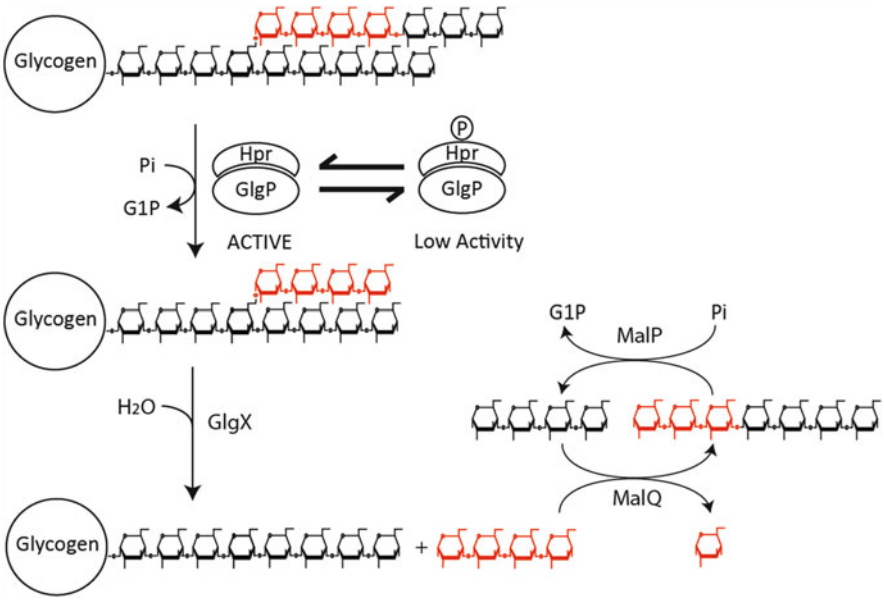


Fig. 3 Glycogen catabolism pathway. Glycogen phosphorylase (GlgP) and histidine carrier protein (Hpr) form a stable complex. Hpr proteins favor the formation of dimer, tetramer, and octamer of GlgP (not shown here for the sake of simplicity). Both phosphorylated Hpr proteins and competitive inhibitor, i.e., ADP-glucose, reduce drastically the GlgP activity during glycogen biosynthesis. The degradation of glycogen is initiated when Hpr proteins are dephosphorylated. In the presence of phosphate inorganic (Pi), GlgP activities release glucose-1-phosphate (G1P) from the non-reducing of glucan chains of glycogen. The enzymatic reactions stop four units of glucose residues before reaching an α-1,6 linkage or branching point. Debranching enzyme activities (GlgX) recognize and trim specifically short-branched maltotetraosyl residues. The new linear glucan chains produced can be then further digested by GlgP activity. Maltotetraose molecules are metabolized in glucose and G1P via the synergic action of α-1,4 glucanotransferase (MalQ) and maltodextrin phosphorylase (MalP)

Segel 1968; Takata et al. 1998). Subsequent studies on GlgP have revealed that the latter forms a tight interaction with the histidine phosphocarrier protein (Hpr) (Seok et al. 1997). The latter is a component of the phosphoenolpyruvate:sugar phosphotransferase system (PTS). This PTS system is involved in the transport and the phosphorylation of many sugars (Deutscher et al. 2006). According to the phosphorylation state of Hpr, the affinity of Hpr will change for GlgP. Thus, the phosphorylated form of Hpr (P-Hpr) displays a fourfold higher affinity for GlgP than the unphosphorylated form. However, only the Hpr form allosterically activates GlgP by 2.5-fold. Interestingly, because the concentration of Hpr is much higher than that of GlgP, this indicates that GlgP is always complexed with either P-Hpr or Hpr (Mattoo and Waygood 1983). The phosphorylation state of Hpr varies with the physiological state of the cell. Thus, at the onset of stationary phase, which coincides with glycogen operon activation and glycogen synthesis, most of Hpr is found to be phosphorylated form. Both the predominant form of P-Hpr and the increase in

ADP-glucose level will prevent the dimerization and activity of GlgP, respectively, and therefore glycogen degradation (Chen and Segel 1968; Seok et al. 2001).

In contrast to *E. coli*, the set of genes involved in the catabolic pathway varies greatly in cyanobacteria species (Colleoni and Suzuki 2012). So far, the reasons for this multiplicity of degradation pathways are unclear. The functions of phosphorylase activities were investigated in *Synechocystis* PCC8803 (Fu and Xu 2006). The characterization of knockout mutants in the two phosphorylase genes sll1367 and sll1356 revealed that GlgP (sll1367) is clearly involved in glycogen catabolism, while GlgP (sll1356) is essential for growth at high temperature. Interestingly, despite the increase of GlgP-sll1356 activity in the null GlgP-sll1367 mutant, glycogen content decreased only slightly due to mobilization at night (Fu and Xu 2006). More recently, the functions of glycogen phosphorylase isoforms were investigated in the resuscitation or awakening process, which consists in the ability of non-nitrogen-fixing cyanobacterium to exit from the dormant stage to the vegetative stage (Doello et al. 2018). In nitrogen limitation condition, light-harvesting pigments are degraded, and glycogen synthesis is induced until photosynthesis and metabolic activities are strongly reduced. Hence, this survival cell response termed "chlorosis" is maintained until the nitrogen source is supplied. The resuscitation process requires the mobilization of glycogen as energy-carbon source until the photosynthetic apparatus becomes functional. Characterization of single or double mutants of glycogen phosphorylase isoforms in *Synechocystis* PCC6803 reveals a minor role of GlgP-sll1356, while GlgP-sll1367 null mutants were impaired in the resuscitation process and revealed as a major contribution of this enzyme to glycogen catabolism during resuscitation (Doello et al. 2018). Further investigations are required to understand the function of the GlgP-sll1356 isoform. One reasonable explanation might be that sll1356 gene encodes a maltodextrin phosphorylase, which as described previously is more active on maltodextrin than glycogen. Hence, as with GlgP mutants in *E. coli* showing a defect in glycogen breakdown, the putative-MalP (sll1356) isoform cannot compensate for the loss of function of GlgP (Alonso-Casajús et al. 2006).

4.2 Glycogen Debranching Enzyme

Surprisingly, several cyanobacterial genomes, if complete, do not seem to encode any classical GlgX-GH13-type debranching enzyme (Colleoni and Suzuki 2012). However, such genomes appear to contain amylopullulanase (GH13-20 and/or GH57) or amylo-1,6 glucosidase (GH133) genes that could exert the corresponding functions. For instance, the early diverging S*ynechococcus* sp. JA-3-3Ab strain contains two candidate debranching enzymes activities: the putative amylopullulanase-GH57 and amylo-1,6-glucosidase-GH133. Little is known, however, about their roles in the glycogen catabolism pathway. Recently, an amylopullulanase type of GH13 enzyme has been characterized from the filamentous cyanobacterium *Nostoc punctiforme* (Choi et al. 2009). In contrast to the GlgX

activity, this enzyme displays hydrolysis activity toward both α-1,6 and α-1,4 linkages when incubated with soluble starch or amylopectin. Incubation experiments have shown that amylopullanase-GH13 hydrolyzes first α-1,6 linkages of long-branched glucans (4>DP>10) and chews up long glucan chains up to 8 residues of glucose from the reducing end. Because *N. punctiforme* does not contain any GlgX or TreX GH13 activity but harbors both maltooligosyltrehalose synthase (TreY) and maltooligosyltrehalose hydrolase (TreZ), it is quite possible that the amylopullulanase-GH13 substitutes for the missing TreX activity, which is required to produce the suitable malto-oligosaccharide for TreY activity. Amylo-1,6-glucosidase (GH133) shows similarity to the amino acid sequence of the C-terminal domain of indirect debranching enzymes of animals and fungi. By analogy with this eukaryotic enzyme, the function of this activity may possibly reside in debranching single α-1,6 glucose residues branched on linear glucan chains. So far, no biochemical characterization has ever been carried out on these cyanobacterial amylo-1,6 glucosidases. However, the characterization of the null GlgX mutant of *Synechococcus* sp. PCC7942 brought some insights on the putative function of the amylo-1,6 glucosidase activity (Suzuki et al. 2007).

As described above, characterizations of *glgX* mutants in both *E. coli* and *Synechococcus* PCC7942 confirm its function in the catabolism pathway. Nevertheless, characterization of *glgX*-deficient mutants of the starch-accumulating *Cyanobacterium* sp. CLg1 suggests its involvement in the crystallization process of storage polysaccharides.

5 Starch Granules in Unicellular Diazotrophic Cyanobacteria: Convergent Evolution of Glucan Trimming Mechanism in the Starch Biosynthetic Pathway of *Cyanobacterium* sp. CLg1

Until the end of the twentieth century, the mechanisms underlying the crystallization of starch were poorly understood. The characterization of mutants substituting starch by glycogen biosynthesis in green algae and different plant species surprisingly revealed a defect in the isoamylase type of debranching enzyme. In order to explain this unexpected function for hydrolytic enzymes in the amylopectin synthesis, a glucan trimming model was proposed in 1996 (Mouille et al. 1996). This model suggests that an isoamylase type debranching enzyme trims a highly branched precursor named pre-amylopectin and enables thereby the formation of closely packed double helices of glucan that precipitate out of the solution. It implies that this isoamylase preferentially trims those loosely branched glucans that prevent the clustering of double helices of glucan. In the absence of this enzyme, a highly branched hydrosoluble polysaccharide accumulates in the deficient mutants. Until 1994, starch accumulation had been described solely in lower photosynthetic eukaryotes, i.e., algae. The observation of thin sections of nitrogen-fixing

cyanobacterium *Cyanothece* ATCC51142 through day-night cycles has revealed that round-shaped materials accumulate between the cyanobacterial thylakoid membranes during the day and disappear at night (Schneegurt et al. 1994). Those large bodies were partially characterized and described as "abnormal" glycogen particles and not as starch-like material (Schneegurt et al. 1997). The authors of the study did not realize at the time that they were dealing in fact with starch-like structures. Later on, a survey of storage polysaccharides in different species of cyanobacteria reported the presence of solid granules in others cyanobacterial species (Nakamura et al. 2005). Subsequently, detailed characterization indicated that such carbohydrate granules, including those of *Cyanothece* ATCC51142, are composed of high-molecular weight polysaccharides similar to amylopectin (Suzuki et al. 2013). Around the same time, *Cyanobacterium* sp. CLg1, a new strain isolated in the tropical North Atlantic Ocean phylogenetically related to *Crocosphaera watsonii*, was shown to accumulate starch granules made of both amylopectin and amylose fractions (Falcon et al. 2002; Deschamps et al. 2008). Interestingly, as in plants, the presence of amylose was correlated with the identification of a polypeptide showing a high similarity in amino acid sequence to GBSS (granule-bound starch synthase) of plants (Deschamps et al. 2008). To the present day, less than a dozen of starch-accumulating cyanobacteria have been identified. They are all unicellular capable of nitrogen fixation and belong to the Chroococcales order (Colleoni and Suzuki 2012). Deciphering the storage polysaccharide metabolism pathways is pretty challenging since those strains are refractory to all available transformation protocols. Nevertheless in order to tackle this question, an UV mutagenesis was carried out on wild-type *Cyanobacterium* sp. CLg1. Based on the iodine staining of cell patches, more than a hundred mutants were identified and subsequently categorized according to the ratio of soluble to insoluble polysaccharide. Among them, a dozen mutants harboring an increase in the hydrosoluble glycogen-like fraction and the disappearance of starch granules were impaired in one of two of the debranching enzyme isoforms GlgX2 (Cenci et al. 2013). This striking result suggests that such as in green alga and plants, crystallization process of amylopectin relies on the presence of a debranching enzyme activity. However, detailed phylogeny analysis built with debranching enzyme sequences of Archaeplastida[1] and Bacteria indicates that the eukaryotic sequences do not have a cyanobacterial origin but are derived from obligate intracellular pathogens and symbionts of the order *Chlamydiales*. It should be stressed out that crystallization process is not clearly associated with GlgX2-like sequence among starch-accumulating cyanobacteria. For instance, the genome of starch-accumulating *Cyanothece* ATCC51142 contains a single *glgX* gene encoding a GH13-debranching enzyme (cce_3465: GH13 family according to CAZy classification) that is closely related to GlgX1 isoform and not to GlgX2 isoform of *Cyanobacterium* sp. CLg1. Interestingly the role of GlgX-GH13 of *Cyanothece*

[1]Archaeplastida phylum encompasses three photosynthetic eukaryotes lineages: plants/algae, red algae, and glaucophytes. They share a common ancestor that emerged 1.5 billion years ago when a heterotrophic eukaryote engulfs a cyanobacterium.

sp. ATCC51142 has been recently clarified in starch metabolism pathway. Indeed, knockout mutant of GlgX (cce_3465) harbors a starch granule excess phenotype similar to *E. coli* or *Synechococcus* sp. PCC7942 mutant strains impaired in the debranching enzyme activity. Hence, these data strongly suggest a function catabolic pathway rather in crystallization process (Liberton et al. 2019). If GlgX is involved in the catabolism of α-1,6 linkages, this might suggest that amylo-1,6 glucosidase (cee_3194 gene) belonging to GH133 family according to CAZy classification might have evolved to cleave off branched glucan.

5.1 Why Do Unicellular Nitrogen-Fixing Cyanobacteria Synthesize Starch-Like Granules Rather Than Glycogen?

Cyanobacteria represent one of the oldest phyla of prokaryotes on earth (Summons et al. 1999). They are unique microorganisms capable of performing oxygenic photosynthesis and for some of them to reduce molecular nitrogen through nitrogenase activity. Nitrogenase activity loses covalent bonds between two nitrogen (and two carbon or one carbon and one nitrogen) atoms in an ATP-dependent manner and requires anoxic condition. Because of the lack of substrate specificity, it is hypothesized that the function of nitrogenase activity might have evolved over time: first, by reducing the effects of toxic cyanide compounds and then by reducing atmospheric nitrogen in ammonium. Over billion years of evolution, oxygenic photosynthesis activities of cyanobacteria have changed earth's atmosphere composition, thereby generating the Great Oxygenation Event (GOE) 2.4 billion years ago (Kopp et al. 2005). The transition from a reductive to an oxidative environment was a trigger for the diversification of cyanobacteria lineages and the appearance of new traits (e.g., size and cell morphology) (Sánchez-Baracaldo et al. 2014). A remarkable adaptation was achieved in nitrogen-fixing cyanobacteria or diazotrophic cyanobacteria. Indeed, the reduction of dinitrogen to ammonium is catalyzed by a protein complex called nitrogenase that displays an extreme sensitivity to oxygen. As oxygen level rose, diazotrophic cyanobacteria developed different strategies to protect the nitrogenase activity: (1) by inhabiting anoxic biotopes, (2) by confining nitrogenase activity in specialized cells called heterocysts, and (3) by separating temporally oxygenic photosynthesis and nitrogen fixation. In diazotrophic filamentous cyanobacteria, nitrogenase activity is located in heterocysts harboring a thick cell wall and lacking a photosystem II, which is responsible for photosynthetic oxygen evolution. Neighbor cells performing normal oxygenic photosynthesis activity supply the large amount of energy required to fuel the nitrogenase complex. Hence, physical separation of two exclusive biological processes, i.e., photosynthesis and nitrogen fixation, is possible through the evolution of dedicated cell. Consistent with this view, it was believed that nitrogen fixation could not occur in unicellular cyanobacteria. The discovery of two unicellular cyanobacteria species, *Gloeothece* sp. and *Cyanothece* sp., capable of fixing dinitrogen challenged this

paradigm and, for the first time, provided evidence that in microorganisms primary metabolic processes are regulated by the circadian clock regulation (Wyatt and Silvey 1969; Singh 1973). In contrast to filamentous diazotrophic cyanobacteria that perform nitrogen fixation during the day, unicellular diazotrophic cyanobacteria carry out this activity exclusively at night. Unicellular cyanobacteria thus have developed a temporal separation of those two incompatible processes, which take place for some of them exclusively in micro-aerobic and for a small group of cyanobacteria also in aerobic conditions. In the latter case, unicellular nitrogen-fixing cyanobacteria exhibit high rates of dark respiration, which both provide the energy required for nitrogen fixation and while lowering the oxygen levels through respiratory consumption eventually lead to microaerophilic or anoxic conditions (Compaoré and Stal 2010). In order to achieve the required high rates of dark respiration, unicellular diazotrophic cyanobacteria are speculated to have evolved a more efficient storage polysaccharide allowing an increase in carbon storage in the light phase. Recently, the measurement of nitrogen fixation in six *Cyanothece* species under various growth and incubations conditions strengthened this assumption. In this report, starch-accumulating *Cyanothece* spp. exhibit a higher rate of nitrogen fixation than glycogen-accumulating *Cyanothece* species in aerobic growth conditions (Bandyopadhyay et al. 2013). This indirect proof reinforces the idea that the GOE, 2.4 billion years ago, was probably a driving force for the transition from glycogen to semi-crystalline storage polysaccharides. It should be stressed out that distribution of GlgX/isoamylase type debranching enzyme sequences does not necessarily correlate with the accumulation of starch in cyanobacteria. This suggests that the transition from glycogen to starch occurred in these cyanobacteria by either recruiting another debranching enzyme or calling for a different, yet to be described, mechanism of granule aggregation. Another striking observation from these bioinformatic genome-mining approaches consists of the absence of candidate debranching enzyme sequences of all known types in some starch-accumulating organisms. Genome analyses of starch-accumulating *Cyanobacterium* MIBC10216 and red algal-derived organisms did not reveal any gene encoding a GH13-type debranching enzyme. In such a case, the crystallization of amylopectin may indeed follow a different mechanism. A preliminary answer to this question has been addressed through the characterization of debranching enzyme and chloroplastic amylase null mutants in *Arabidopsis*. In the absence of chloroplastic amylase, DBE null mutants switch from accumulation of glycogen-like polysaccharide to synthesis of starch-like polysaccharides (Streb et al. 2008). Overall, those studies suggest that the combination of different cyanobacterial branching enzymes alone possibly offers a specific branching pattern that promotes a cluster organization of amylopectin. In turn, this could explain why usually three to four branching enzymes are usually found in these genomes.

6 Granulose: Another Example of Transition from Soluble to Insoluble Storage Polysaccharide?

Or, in other words, is there another selection pressure, which led to transition from soluble to insoluble storage polysaccharide? Few cases of abnormal glycogen/carbohydrate granules are described in the literature. It should be stressed that in the examples described below, the carbohydrate granules were not characterized structurally. Thus, this absence of information forbids us to distinguish between insoluble glycogen particles (absence of crystallinity) and true starch-like (semi-crystalline) granules. The evidence that they are actually carbohydrate granules is based on glucose assay, electronic microscope observations (size, electron density), and density (sedimentation at low speed). Therefore, further characterization of storage polysaccharides in these microorganisms should definitively improve our understanding of alternative aggregation mechanisms.

6.1 Firmicutes *Phylum:* Clostridium *sp.*

Clostridium species belong to the *Firmicutes* phylum and define Gram-positive anaerobic endospore-forming bacteria mainly used to produce acid and organic solvents (Dash et al. 2016). Back in 1950, Hopson and his collaborators reported in *C. butyricum* the accumulation of amylopectin-like polysaccharides that exert a strong interaction with iodine. The wavelength of the maximum absorbency of the iodine-polysaccharide complex (λmax) was reported to average 545 nm, which is close to the λmax value of amylopectin (550–560 nm) (Bergère et al. 1975). This material named "granulose," was found in most *Clostridium* species (Reysenbach et al. 1986). Based on electron microscopy observations, granulose sizes vary from 210 to 270 nm which is by far larger than normal glycogen particles (Tracy et al. 2008). Granulose biosynthesis occurs prior to the end of the exponential growth phase when the shift from acid to solvent production takes place while its degradation coincides with the endospore formation (Johnstone and Holland 1977). Characterization of mutants in *C. pasteurianum* and *C. acetobutylicum* ATCC824 impaired in granulose synthase harbor granulose-less phenotypes and are incapable of initiating sporulation suggesting that granuloses act as major carbon and energy source (Robson et al. 1974; Ehsaan et al. 2016). A survey of *Clostridium* genomes has evidenced the presence of one branching isoform belonging either to the GH13 family or the GH57 family (Suzuki and Suzuki 2016) and one glycogen synthase isoform named "granulose synthase." Interestingly, the latter is co-purified with native granulose (Robson et al. 1974). At the present day, the genetic determinants responsible for the synthesis of granulose in *Firmicutes* are still unknown.

6.2 Candidatus Methylacidiphilum fumariolicum

The second example concerns *Ca. M. fumariolicum*, which belongs to the *Verrucomicrobia* phylum (Pol et al. 2007). *Planctomycetes*, *Verrucomicrobia*, and *Chlamydiae* form the PVC superphylum, which to date includes two additional phyla *Lentisphaerae* and *Poribacteria*. *Ca. M. fumariolicum* is a methanotrophic bacterium that uses methane as both carbon and energy source. Interestingly, *Ca. M. fumariolicum* is able to fix nitrogen and carbon dioxide through the nitrogenase complex and the Calvin-Benson cycle, respectively (Khadem et al. 2010, 2011). In the absence of the nitrogen source (ammonium or dinitrogen gas), *Ca. M. fumariolicum* accumulates both electron-dense bodies of 100–200 nm with an elliptical or circular shape (that have been identified as abnormal glycogen particles) and soluble polysaccharide. The physiological function of the abnormal glycogen particles was further investigated by growth experiments which consisted of depletion of methane and/or nitrogen source (Khadem et al. 2012a). Overall, these experiments suggest that in absence of the major carbon dioxide and energy source (i.e., methane), the carbohydrate granules are the main source of carbon for maintaining the survival of cells. Although little is known about the fine structure of abnormal glycogen, the glycogen metabolism pathway relies on set of enzymes that is similar to that of *E. coli* (Khadem et al. 2012b).

7 Storage Polysaccharide and Fitness in Prokaryotes

A large number of studies on enterobacteria indicates an interconnection of glycogen metabolism with many cellular processes, including nitrogen, iron and magnesium metabolisms, stress responses, and RNA metabolisms. I invite readers to read the excellent review on these aspects (Wilson et al. 2010). Similar interconnections probably occur in cyanobacteria species. Furthermore, both photosynthetic activity and carbon fixation add a layer of complexity in terms of regulation and cross talks between metabolic networks. At the present, there is compelling evidence that glycogen is critical for abiotic stress responses (e.g., salinity, temperature) and for survival under day-night growth conditions (Miao et al. 2003; Suzuki et al. 2010; Gründel et al. 2012). Why do glycogen-less cyanobacteria grow normally in continuous light and not under day-night conditions? The answer starts with the characterization of *rpaA*-null mutant in *Synechococcus.* RpaA is an important transcriptional factor interconnecting circadian clock and different metabolic pathways. It activates the transcription of key genes belonging to the glycogen catabolism pathway (glycogen phosphorylase, *glgP*), glycolysis (glyceraldehyde-3-phosphate dehydrogenase, *gap*; fructose-1,6-bisphosphatase, *fbp*), and the oxidative pentose phosphate pathway (glucose-6-phosphate dehydrogenase, *zwf*; 6-phosphogluconate dehydrogenase, *gnd*). Like glycogen-less mutants, *rpaA*-null mutants grow at the same rate as the wild-type strain in continuous light, but they are

not viable under day-night cycle. Furthermore, despite normal GlgC and GlgA activities, the amount of glycogen is strongly reduced in *rpaA* mutants (Puszynska and O'Shea 2017). Two independent research groups investigated the *rpaA* mutant phenotype in *Synechococcus*. First, Diamond and colleagues posit that abnormal accumulation of reactive oxidative species (ROS) produced at night in *rpaA* mutants might explain day-night lethality. Their hypothesis is sustained by the fact that by modulating light intensity per se by modifying ROS levels or/and by reducing NADPH,H+ consuming pathways (knockout of valine, leucine, and isoleucine biosynthetic pathways), the *rpaA* mutant is enabled to grow under day-night cycle (Diamond et al. 2017). Thus, they propose that glycogen degradation and the OPP pathway fuel the cell in reducing power (NADPH,H+) at night which prevent the accumulation of ROS. Further investigations, conducted by Puszynska and O'Shea (2017), suggest that the inability of *rpaA* mutant to maintain the adenylate energetic charge at night might explain the lethality. In order to validate their hypothesis, the genes for the glucose transporter (GalP) as well as for GlgP, Gap, and Zwf activities were expressed in *rpaA* mutant. The authors correlate the phenotype rescue with a restoration of adenylate energetic charge in the *rpaA* mutant. Unfortunately, no information is available concerning the ROS levels in the rescued *rpaA* mutant. Nevertheless, both articles from Diamond et al. (2017) and Puszynska and O'Shea (2017) emphasize the critical role of carbon stores at night. Like in enterobacteria, under macro-element (i.e., N, P) limitation, non-nitrogen-fixing cyanobacteria accumulate a large amount of glycogen between thylakoid membranes. Nitrogen starvation triggers the degradation of photosynthetic pigments in many photosynthetic organisms. This mechanism named the "chlorosis response" induces dormant stage where only basal metabolic and photosynthesis activities occur as long as the nitrogen source is not available. Interestingly, only 48 h is required for a dormant cyanobacterium to be fully metabolically active and perform photosynthesis activity again when nitrogen is supplied (Klotz et al. 2016). In addition, there is now compelling evidence that both chlorosis response and survival at the dormant stage depend explicitly on glycogen metabolism and not on alternative carbon source such as poly-β-hydroxybutyrate stores (Damrow et al. 2016). More recently, Doello and his collaborators investigated the importance of glycogen pool during this resuscitation or awakening process. This study sheds light on the importance of glycogen catabolism in the few hours of awakening when the photosynthetic apparatus is not active yet to supply the cells in ATP (Doello et al. 2018). Glycogen is degraded when the glycogen phosphorylase isoform, GlgP-slr1367, is activated. The glycogen phosphorylase isoform is synthesized during the biosynthesis of glycogen. So far, the mechanisms that control glycogen phosphorylase (GlgP-slr1367) activation or inhibition during degradation and synthesis, respectively, are still unknown. In addition, this work outlines that the main glycolytic pathway routes consist of both oxidative pentose phosphate pathway and Entner-Doudoroff (ED) pathways, recently discovered in cyanobacteria, and not as expected, the Embden-Meyerhof-Parnas (EMP) pathway that yields twice more ATP than ED pathway (Chen et al. 2016). Despite the absence of alternative sources of ATP (i.e., photosynthesis

activity) during the awakening process, the thermodynamic constraints of the EMP pathway might favor the use of the ED pathway (Flamholz et al. 2013).

7.1 The Accumulation of Abnormal Glycogen Affects the Aging of Prokaryotes

Another intriguing aspect concerns the interconnection between glycogen metabolism and prokaryotic aging or senescence process. The latter is defined by an accumulation of cellular damage within the mother cell, which leads to a decrease in reproductive rates and increase of mortality with age. First described in eukaryotes including yeast, bacteria were considered immortal and free of aging because it was supposed that the cell division gives rise to two identical cells. However, early in 2000, two studies reveal that aging processes occur in bacteria as well (Ackermann et al. 2003; Stewart et al. 2005). Asymmetric partitioning of cellular components in rod-shaped bacteria such as *E. coli* and *Caulobacter crescentus* enables mother and daughter cells to be distinguished. Thus, by tracking cell divisions of rod-shaped bacteria and more precisely by following the polar cells, it appears that bacteria are affected by aging like most cells. More recently, thanks to microfluidic system devices, Boehm and his collaborators have pointed out that the replicative lifespan of *E. coli* is genetically controlled and is linked to the glycogen metabolism pathway (Boehm et al. 2016). In this study, a mutation in the carbon storage regulator gene (*csrA*) led to a drastic reduction of cell division numbers from 150 to 5 for a mother's cell. Null *csrA* mutants in *E. coli* caused an increase both in gluconeogenesis and glycogen biosynthesis and an inhibition of glycolysis as well (Sabnis et al. 1995). That this *csrA* phenotype is rescued in glycogen-less strain mutants deleted in the glycogen synthase gene (*glgA*) further emphasizes the importance of glycogen in the aging process. How can we explain that an excess of glycogen accumulation is responsible for a decrease of replicative lifespan in *E. coli*?

Based on previous reports on effect of partitioning of protein aggregates or inclusion bodies in the aging process in *E. coli* cells (Lindner et al. 2008), the authors localized glycogen particles upon cell division. Interestingly, they observed through fluorescent probe GlgA-GFP in both wild-type and *csrA* mutant that most of glycogen particles occur at the pole of the mother cell, while new released cell is free of glycogen. In the case of *csrA* mutant, the glycogen accumulation gradually increases from the pole of the mother cell more rapidly after each cell division than wild-type cell. Interestingly GglA-GFP fluorescence signal was observed across the entire cell usually after the fifth cell division; *csrA* mutant seems then deprived of the genome, which would explain why these cells then stop growing and dividing (Boehm et al. 2016).

8 Conclusion and Perspectives

The survival of free-living cells depends on their ability to maintain their energy status when faced with fluctuating environmental conditions. This critical issue requires the synthesis of energy-carbon storage compounds such as α-polysaccharides, for fueling the cell in the absence of exogenous energy. Glycogen particles represent the most primitive and abundant form of carbon storage in the living world. Described in species of all domains of life, it seems clear that glycogen particles are optimized to meet the specific needs of the cell and that evolution has spawned various biochemical pathways achieving its synthesis. Hence, mathematical modeling and biological evidence outline the tight relationship between structure and function of glycogen particles. In addition, a large body of evidence suggests that any structural alteration of glycogen leads to deleterious effects for glycogen-accumulating cells. In some cases, however, the glycogen particle was not designed to fulfill the cell requirement. Thus, transition state occurs from tiny hydrosoluble polysaccharide, glycogen to insoluble water polysaccharide starch-like. First described in Archaeplastida lineages (plants/green algae, red algae, glaucophytes), unicellular nitrogen-fixing cyanobacteria synthesize amylopectin/starch granules as well. Although there is no direct proof, it is tempting to suggest that both the ATP cost and anoxia required by nitrogen fixation pathway at night were the main driving force to substitute glycogen toward a better storage polysaccharide like starch. We can hypothesize that the same selection pressure led the nitrogen-fixing *Candidatus Methylacidiphilum fumariolicum* to synthesize carbohydrate granules rather than glycogen particles. Interestingly, the energy cost of endospore formation during solvent production in *Clostridium* might be, in that case, the driving force to substitute glycogen by granulose. Although little is known about the degradation pathway of carbohydrate granules produced in both organisms, those reflect again a compromise between storage capacity and availability of carbon where solid granules, starch and granulose, are optimized to store a maximum of glucose moieties in a limited space.

In addition, we must bear in mind that occurrence of storage polysaccharides is usually associated with the presence of other polymeric materials, such as polyphosphate granules, lipids, and poly-β-hydroxybutyrate, that can be used as energy-carbon store or as energy store exclusively (Rao et al. 2009; Achbergerová and Nahálka 2011; Achbergerová and Nahálka 2011). Thus, it is worth noting that some organisms are completely devoid of storage polysaccharide. In 2002, Henrissat and his associates pointed out the loss of carbohydrate-active enzymes concerns human or mammalian pathogenic bacteria (Henrissat et al. 2002). This observation can be extended to symbiotic bacteria either with bugs (Degnan et al. 2009; Nikoh et al. 2011) or with eukaryotes (Newton et al. 2007).

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