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Genomics of cyanobacteria: New insights and lessons for shaping our future

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Abstract

Cyanobacteria, the only known photosynthetic prokaryotes, are ancient organisms regarded as the producers of the Earth's oxygenic atmosphere and the ancestors of plant chloroplasts. Contemporary cyanobacteria have evolved as widely-diverse organisms in colonizing most aquatic and soil biotopes, where they face various environmental challenges and competition or symbiosis with other organisms. Cyanobacteria exhibit a wide morphological diversity (unicellular/multicellular, cylindrical/spherical shapes) and many species differentiate specialized cells for growth and survival under adverse conditions. They convert captured solar energy at high efficiencies, to fix an enormous amount of carbon from CO₂ into a huge biomass that sustains a large part of the food chain, and they tolerate high CO₂ content in gas streams. They also synthesize a vast array of biologically active metabolites with great interests for human health and industries. Hence, they are viewed as promising "low-cost" cell factories for the carbon-neutral production of chemicals, thanks to their simple nutritional requirements, their metabolic robustness and plasticity, and the powerful genetics of some model strains.

In 2013 when ABR vol 65 entitled "Genomics of Cyanobacteria" was published, approximately 70 cyanobacteria had their genomes fully or partially sequenced. Since then, the number has increased up to about 2000 (<https://genome.jgi.doe.gov/portal/>), genome editing studies using CRISPR/Cas have been reported in several model cyanobacteria (Gale et al., 2019;

☆Footnote: Follow-up of Volume 65: Genomics of Cyanobacteria.

Pattharaprachayakul, Lee, Incharoensakdi, & Woo, 2020), and the knowledge that was presented in each of the eight chapters of the vol 65 book has greatly improved, as described below.

In the first chapter entitled “Structural, Mechanistic and Genomic Insights into OCP-Mediated Photoprotection,” Cheryl Kerfeld and Diana Kirilovsky reported on the mechanisms used by cyanobacteria to protect themselves against high light fluence (Kerfeld & Kirilovsky, 2013). When the amount of light energy exceeds what is needed for assimilation of inorganic-nutrients (CO_2 , NO_3 , PO_4 , SO_4 , *etc.*), the photosynthetic electron transport chain becomes stalled in a reduced state, leading to the formation of reactive oxygen species (ROS) that generate severe cell damages. Cyanobacteria have evolved a rapid photoprotective mechanisms to cope with abrupt changes in the quality and intensity of light. Light activates a soluble orange carotenoid protein (OCP), which interacts with the light-harvesting antenna (phycobilisomes), to increase thermal dissipation of absorbed energy, thereby decreasing energy arriving at the reaction centers. The increase of thermal dissipation also causes a decrease of phycobilisome fluorescence creating a non-photochemical fluorescence quenching (Kerfeld & Kirilovsky, 2013).

Our knowledge of OCP-dependent process has greatly progressed as summarized in the recent reviews by Cheryl Kerfeld’ and Diana Kirilovsky’ laboratories (Dominguez-Martin & Kerfeld, 2019; Muzzopappa & Kirilovsky, 2020). OCP binds one ketocarotenoid, which spans both N- and C-terminal OCP domains (NTD and CTD). Upon photoactivation, the carotenoid is translocated to the NTD and the color of the protein changes to red. Then, the domains move away from one another and OCP becomes active. The red OCP binds to the phycobilisome, inducing thermal dissipation of excess energy.

OCP is composed of two globular domains, the α -helical N terminal domain (NTD; residues 18–165) and the α -helix/ β -sheet C-terminal domain (CTD; residues 190–317), of the latter presenting homologies to the nuclear transport factor 2 (NTF2) superfamily, which are connected by a long, flexible (largely unstructured) loop linker (approximately 25 residues). In the dark, OCP is orange and has a compact (closed) globular conformation. This conformation is stabilized by strong inter-domain interactions in the middle of the protein, and a ketocarotenoid, 3'-hydroxyechinenone (hECN), which spans both domains. The carotenoid is a sensor of light intensity. A strong blue-green light, absorbed by the carotenoid, induces conformational changes in the carotenoid (it moves by 12 Å)

and in the two protein domains and the orange inactive form (OCP^O) is converted into a metastable red active form (OCP^R). The OCP^R state is characterized by large-scale structural changes that involve dissociation and separation of C-terminal and N-terminal domains accompanied by carotenoid translocation into the N-terminal domain (Konold et al., 2019). Only OCP^R can bind to PBS, while OCP^O is unable to bind.

When low-light conditions return, the interaction of OCP with the fluorescence recovery protein (FRP), a non-chromophore protein, is needed to block photoprotection under low light and increase the effective size of the antenna in cells containing OCP1 (Gupta et al., 2019). In the absence of FRP, the cells remain in a quenched state, even in the dark. FRP is an α -helix elongated protein that is stable as a dimer in solution. FRP binds to CTD of OCP^R as a dimer but then monomerizes. It has been proposed that FRP binding to OCP weakens the interaction between the monomers, which can lead to spontaneous monomerization of FRP; alternatively, the binding of a second OCP to the FRP dimer may provoke a clash between the two OCP molecules, causing the splitting of the 2OCP:2FRP complex into two OCP:FRP complexes. The monomerization is not essential for FRP activity but can improve its efficiency, especially at elevated concentrations of OCP^R under high light intensities (Muzzopappa & Kirilovsky, 2020). FRP has two different activities, being essential for the OCP^R-to-OCP^O conversion reaction and for detaching OCP^R from PBS. In cells, the concentration of FRP is much lower than that of OCP, which is important since high FRP concentrations completely inhibit the PBS fluorescence quenching. Thus, the concentration of FRP not only controls the rate of PBS fluorescence recovery but also the amplitude of PBS fluorescence quenching (Muzzopappa & Kirilovsky, 2020).

Genomic studies identified three paralog families of OCP, namely, OCP1, OCP2 and OCPX. Most OCPs (approximately 65% of available sequences) belong to the OCP1 family, to which the well-characterized *Synechocystis* PCC 6803, *Arthrospira maxima*, and *Anabaena* PCC 7120 OCPs belong. Only one OCP2 (*Tolypothrix* PCC 7601) and one OCPX (*Scytonema hofmanni* PCC 7110) have been characterized (Muzzopappa & Kirilovsky, 2020). The *frp* gene is absent in strains containing only OCP2 or OCPX. In these strains, OCPX or OCP2 is able to detach from PBS and allows fluorescence recovery in the absence of FRP. Moreover, the rate of OCP^R-to-OCP^O conversion of these OCPs is fast and does not need to be accelerated by FRP. The FRP dimer interacts with high affinity with CTD of OCP1^R (Muzzopappa & Kirilovsky, 2020).

In addition to OCPs, many cyanobacterial strains contain homologs of the NTD and CTD domains of OCP. The family of NTD-like proteins, named helical carotenoid proteins (HCPs), contains at least nine different subclades (HCP1 to HCP9), while the family of CTD-like proteins (CTDHs) contains only two subclades (CTDH1 and CTDH2). Both HCPs and CTDHs are carotenoid proteins that preferentially bind CAN. While several HCPs from different subclades can coexist in cells of some strains, only one CTDH is present. While HCPs are monomers, CTDHs are dimers, sharing one canthaxanthin (CAN) molecule. HCPs from different subclades have different activities, at least in vitro. HCP2 proteins from *Anabaena* and *Tolypothrix* and HCP3 from *Anabaena* are good singlet oxygen quenchers, and HCP1 can be a carotenoid carrier. CTDHs are transient carotenoid proteins, which are able to transfer the carotenoid from the membrane to HCPs and OCP (Slonimskiy et al., 2019).

OCP and the paralogs of its domains, HCPs and CTDHs, are also excellent quenchers of singlet oxygen, decreasing the risks of oxidative damage. HCPs seem to help decrease the oxidative stress under specific stress conditions, such as desiccation. The principal role of CTDH is to transport the carotenoid from the membrane to HCP and, perhaps, to OCP. The study of these proteins allowed the discovery of a unique protein-to-protein carotenoid transfer mechanism, which could potentially be applicable to other processes involving water-soluble carotenoid proteins. These findings may also inspire the construction of stable carotenoid nanocarriers capable of targeted delivery of the antioxidant power to specific tissues, such as the macula lutea, in which the lutein-carrying NTF2-like StAR-related lipid transfer domain containing 3 (STARD3) protein plays a role in photoprotection (Dominguez-Martin & Kerfeld, 2019; Muzzopappa & Kirilovsky, 2020).

Although OCP is important for cyanobacterial photoprotection other processes exist since about one-third of cyanobacteria have no *ocp* genes, such as the PBS-lacking cyanobacteria (including *Prochlorococcus marinus* strains) and the light-tolerant model species *Synechococcus* PCC 7942 and *Thermosynechococcus elongatus* BP1 (Muzzopappa & Kirilovsky, 2020).

In the second chapter entitled “Genomics of Salt Acclimation: Synthesis of Compatible Solutes among Cyanobacteria” Martin Hagemann reviewed the existing mechanistic and genomic knowledges about cyanobacterial salt acclimation, an important process for future biotechnological applications, some of which could benefit from being performed in abundant marine waters (Cui, Sun, Chen, & Zhang, 2020; Hagemann, 2013).

Besides light, the availability of water and the amounts of dissolved ions (total salinity) are important environmental factors determining the occurrence of cyanobacteria in specific water and terrestrial environments. Because total salinity and water amount are closely linked, e.g. during desiccation, the amount of water is decreasing in parallel with the increase in total salt concentration, it is not surprising that salt and desiccation have common detrimental effects such as inhibition of photosynthesis (Yang, Wang, Liu, & Sui, 2020). In both cases, the maintenance of water and turgor pressure inside the cell is one of the central issues during the acclimation. Because water uptake is a passive process following the water potential gradient, growing cells need to establish a low water potential inside the cell relative to the surrounding medium. This is achieved by regulating the cellular osmotic potential via varying amounts of low molecular compounds. The main difference between pure water, or osmotic stress and salt stress is the additional direct ion effects on metabolic activities in the latter case. In nature, the large variations in the amount and composition of inorganic salts clearly affect the distribution of cyanobacteria. In addition to the problem that high total ion contents generally make it difficult to maintain water and turgor inside the cell, many ions are toxic for living cells. This direct toxicity is not only true for heavy metals, but for any ion at non-physiological high-cellular concentrations. Among cyanobacterial strains, three main salt tolerance groups can be distinguished: low and moderate halotolerant cyanobacteria as well as hypersaline strains. Regardless of the final salt tolerance, all cyanobacteria apply two basic strategies for a successful acclimation to high salt concentrations: accumulation of compatible solutes combined with active export of toxic ions, particularly Na^+ and Cl^- (Hagemann, 2013). These processes have been well-studied in the model cyanobacteria (Cui et al., 2020; Kirsch, Klähn, & Hagemann, 2019).

Maintaining the intracellular ion balance by expressing related-transporters is one important resistance strategy against salt stress. A series of salt-tolerance relevant transporters have been identified and characterized in cyanobacteria, such as different Na^+/H^+ antiporters, Mrp-system, and Na^+ -pumping ATPase (Cui et al., 2020). It responds to increasing salinities mainly by accumulating glucosylglycerol (GG) and sucrose, whereas the (freshwater) model cyanobacterium *Synechococcus* PCC 7942 accumulates only sucrose (Liang et al., 2020).

Salt tolerance is also closely related to the primary compatible solutes in cyanobacteria. Early studies found that cyanobacterial strains with poor salt tolerance usually accumulate sucrose or trehalose, while strains with

moderate salt tolerance accumulate glucosylglycerol (GG), and highly halotolerant strains synthesize the glycine betaine (GB), and glutamate betaine, respectively (Kirsch et al., 2019; Liang et al., 2020). High-salt induced accumulation of the main compatible solute GG is achieved by de novo synthesis. The key target of regulation is the enzyme GG-phosphate synthase (GgpS) and involves transcriptional, posttranscriptional, and biochemical mechanisms. Recently, the GG-degrading enzyme GG hydrolase A (GghA) was identified, which is particularly important for GG degradation during exposure to decreasing salinities. The inversely ion-regulated activities of GgpS and GghA could represent a main model for effectively tuning GG steady state levels according to external salinities. Similar to GG, the intracellular amount of sucrose is also salt-regulated and seems to be determined by the balance of sucrose synthesis via sucrose-phosphate synthase (Sps) and its degradation via invertase (Inv). In addition to their role as stress protectants, both compatible solutes also represent promising targets for biotechnology. Hence, the increasing knowledge on the regulation of compatible solute accumulation not only improves our understanding of the stress physiology of cyanobacteria but will also support their future biotechnological applications (Kirsch et al., 2019). Interestingly, a series of cyanobacteria are able to accumulate lipids and alkanes under salt stress. For example, lipid contents were enhanced in *Synechococcus* PCC 7942 under salt stress (Cui et al., 2020).

Another aspect of salt stress is that it increases the intracellular content of reactive oxygen species (ROS), which can damage the photosynthetic apparatus, inhibit the synthesis of photosystem-related proteins and destroy the thylakoid membrane structure, leading to inhibited photosynthesis (Yang et al., 2020). Consequently part of the cell responses to salt stress consist in an increase in the production of the superoxide dismutase (SOD) enzyme and the above-mentioned OCP (orange carotenoid) photoprotection protein (Yang et al., 2020). Furthermore, a series of secondary messengers have been recently identified, such as the cyclic-di-AMP and cyclic di-GMP, which play essential roles in cellular responses to environmental stresses (Cui et al., 2020).

In the third chapter entitled “Iron in Cyanobacteria” Nir Keren and Enrico Schleiff described the processes used by cyanobacteria to provide large amounts of the essential micronutrient iron (Fe) to their Fe-rich photosynthetic apparatus, and a wealth of other Fe-requiring enzymes crucial to cell metabolism (Kranzler, Rudolf, Keren, & Schleiff, 2013). Although Fe is the fourth most plentiful element in the Earth’s crust, it is frequently a

growth-limiting nutrient in large regions of the ocean and in many freshwater environments. This is due to both Fe concentration and chemistry. In aqueous solutions, iron has two environmentally-relevant oxidation states: Fe(II) and Fe(III). Prior to the evolution of oxygenic photosynthesis, reducing environmental conditions resulted in iron existing primarily in its reduced form, ferrous ions Fe(II). Fe(II) are relatively soluble and thereby readily bioavailable at the circumneutral pH range. Upon evolution of oxygenic photosynthesis, molecular oxygen accumulation in the atmosphere led to oxygenation of aquatic environments where Fe(II) species rapidly oxidized to ferric ions Fe(III). Fe(III) are poorly soluble at the circumneutral pH range and precipitate out of solution as ferric oxyhydroxides which are poorly bioavailable.

Fe, alone or complexed with sulfur under the form of iron–sulfur (Fe–S) clusters, serves as the cofactor of many (electron transfer) proteins involved in important physiological processes, including respiration, photosynthesis, nitrogen fixation, amino acid and purine metabolism, RNA modification and DNA replication, as well as repair and regulation of gene expression (Cassier-Chauvat & Chauvat, 2014; Gao, 2020). Hence, cyanobacteria in relying on photosynthesis to power up their whole metabolism have much larger iron requirements than non-photosynthetic organisms (Kranzler et al., 2013).

Cyanobacteria utilize multiple strategies to maintain Fe levels within a desired range. Depending on the chemistry and environmental bioavailability of Fe species, these processes include (i) synthesis, export and re-import of powerful ferric ion chelators called siderophores (Årstøl & Hohmann-Marriott, 2019); (ii) dedicated energy-consuming uptake systems; (iii) sequestration of temporary extra Fe atoms in storage proteins (ferritins) (Moparthi et al., 2016); (iii) replacing iron-containing enzymes with iron-free analogs; and (iv) degradation of abundant Fe-rich non-essential proteins in response to Fe starvation to release Fe atoms for subsequent incorporation into crucial Fe-requiring metabolic enzymes (Gao, 2020; Houot et al., 2007). These processes require a tight coordination with the homeostasis of all metal ions (Mg, Mn, Zn, Cu, etc.) that serve as cofactors for a wealth of metalloenzymes operating in cell metabolism, as occurs in most biological organisms (Foster, Osman, & Robinson, 2014; Salomon & Keren, 2015).

Siderophores are biologically produced low-molecular-weight (400–1000 kDa) compounds with an extremely high affinity for iron. Increased iron bioavailability can also be achieved by the complex

formation of Fe(III) with common metabolites including citric acid and humic acids. Typically, siderophores form a strong hexadentate octahedral complex with ferric iron (Fe³⁺). This complex can then be transported inside the cell. Once inside the cell, iron has to be removed from siderophore. This can happen either through the reduction of iron from Fe(III) to Fe(II) by ferric siderophore reductases or by ferric siderophore hydrolases or by the photolysis of iron-bound siderophores, as described for synechobactin. Based on the primary oxygen-donating ligands that bind the iron, siderophores are divided into four different types. These are the hydroxamates, catecholates, and carboxylates, as well as siderophores with mixed types of these functional groups. All cyanobacterial siderophores that have been identified so far are either hydroxamates or catecholates, which have been named: synechobactin, schizokinen and anachelin (Årstøl & Hohmann-Marriott, 2019).

The only characterized siderophore export system in cyanobacteria is that of *Anabaena* PCC 7120. In this organism, the inner membrane MFS protein SchE (all4025) is required for the secretion of schizokinen, along with the TolC-like outer membrane protein hgdD (alr2887), with the two proteins likely working in tandem. The import of siderophore-iron complexes into the periplasmic space is enabled by receptor proteins, TonB-dependent transporters (TBDT) located in the outer membrane. In the periplasm, Fe-schizokinen is recognized and transported to the cytoplasm via the hydroxamate system FhuBCD (all0387, all0388, and all0389, respectively). TBDTs are unevenly distributed in cyanobacteria. In general, TBDTs seem to outnumber the amount of TonB-proteins, indicating some functional flexibility in TonB-complexes. The genome of *Anabaena* PCC 7120 contains 22 TBDTs, while no candidate genes were identified in the marine *Prochlorococcus*. *Synechococcus* PCC 7002, which synthesizes synechobactin, contains 6 TBDTs, with two exhibiting sequence similarity to resemble known schizokinen transporters, and two found more broadly homologous to hydroxamate transporters. The large number of TBDTs in some organisms is partially explained by the possibility of uptake of siderophores which are not produced by the organism (xenosiderophores), known as siderophore piracy. For example, the non-siderophore-producing *Synechocystis* PCC 6803 is capable of utilizing both schizokinen and the unnamed siderophore of *Anabaena variabilis* ATCC 29413 and can grow with iron-siderophore complexes as their only source of iron. (Årstøl & Hohmann-Marriott, 2019).

In the fourth chapter entitled “Functional Genomics of Metalloregulators in Cyanobacteria” Maria Fillat reviewed the major families of metalloregulators in cyanobacteria (Botello-Morte, González, Bes, Peleato, & Fillat, 2013). The major metal regulator system is composed of Fur-type regulators that comprise three paralogs named FurA (Fur, iron homeostasis), FurB (Zur, Zinc homeostasis) and FurC (PerR, responses to oxidative stress), which control the expression of a wealth of genes involved in exopolysaccharide biosynthesis, phycobilisome degradation, chlorophyll catabolism and nitrogen fixation (Cassier-Chauvat & Chauvat, 2016; González, Sevilla, Bes, Peleato, & Fillat, 2016; Rachedi, Foglino, & Latifi, 2020). These metal-sensing proteins, which harbor metal-binding and DNA-binding domains, are usually allosteric proteins. Their reversible interaction with the regulatory metal drives conformational changes that affect DNA binding and the subsequent regulation of various genes involved in various processes. These findings revealed interesting connections between metabolic networks and interactivity between major regulons. They provide a better understanding of cyanobacterial physiology including maintenance of metal homeostasis in a highly variable environment, and of the strategies to deal with different stress-generated cell damages, as follows. Shortly after the publication of vol 65, the group of Dr. Fillat has studied the molecular mechanism by which FurA from the cyanobacterium *Anabaena* PCC 7120 specifically binds to “iron boxes” in P_{FurA}, FurA binds P_{FurA} using a sequential mechanism: (i) a monomer specifically binds to an “iron box” and bends P_{FurA}; (ii) two situations may occur, i.e. a second FurA monomer covers the free “iron box” or that joins to the previously used forming a dimer which would maintain the DNA kinked; (iii) trimerization in which the DNA is unbent; and (iv) finally undergoes a tetramerization; the next coming molecules covering the DNA strands un-specifically. In summary, the bending appears when an “iron box” is bound to one or two molecules and decreases when both “iron boxes” are covered. These results suggest that DNA bending contributes to the first steps of FurA repression promoting the recruitment of new molecules resulting in a fine regulation in the Fur-dependent cluster associated genes (Pallarés et al., 2014).

Besides the FUR regulators cyanobacteria have other metal regulator operating in the homeostasis of manganese (Eisenhut, 2020), nickel (Foster et al., 2017) or zinc (Pérez et al., 2017), or the tolerance to cadmium (Sun et al., 2017), mercury (Singh, Lingaswamy, Koduru, Nagu, & Jogadhenu, 2019), or the repair of Fe-S cluster (Vuorijoki, Tiwari, Kallio, & Aro, 2017).

In addition to transcription factors, many non-coding RNAs have been identified that play crucial roles in the acclimation responses of cyanobacteria to changes in the environment. Some of these regulatory RNAs are conserved throughout the cyanobacterial phylum, while others exist only in a few taxa (Muro-Pastor & Hess, 2020). A recent study on *Synechocystis* PCC 6803 showed that the gene expressing the small regulatory RNA Isar1 (Iron Stress-Activated RNA 1) is repressed by Fur. Isar1 accumulates in response to iron starvation and controls at the posttranscriptional level the expression of several genes involved in central cellular processes (photosynthesis, (iron-sulfur) cluster biogenesis, citrate cycle and tetrapyrrole biogenesis). Homologs of IsaR1 are largely conserved in the genomes of cyanobacteria, suggesting that the involvement of this riboregulator in the iron-dependent control of photosynthesis might be conserved in the cyanobacterial phylum (Rachedi et al., 2020).

In the fifth chapter entitled “Genomics of the Pleiotropic Glutathione System in Cyanobacteria” (Narainsamy, Marteyn, Sakr, Cassier-Chauvat, & Chauvat, 2013) we reported the variety of the processes used by cyanobacteria to protect themselves against oxidative stress, emphasizing on glutathione (the gamma-glutamyl-cysteinyl-glycine tripeptide, GSH) and glutathione-dependent enzymes, because they are well conserved during evolution (Cassier-Chauvat & Chauvat, 2016; Fahey, 2013). Because of their photoautotrophic lifestyle, cyanobacteria are continuously challenged with toxic reactive oxygen species (ROS) present in our oxygenic atmosphere (ozone, O₃), or generated by the metal-dependent cellular processes photosynthesis, respiration and cell metabolism. These oxidative agents are namely: singlet oxygen (¹O₂), the superoxide anion (O₂^{•-}), hydrogen peroxide (H₂O₂), and the hydroxyl radical (OH[•]). The ROS oxidants can be detoxified by various metabolites (ascorbate, carotenoids, glutathione, vitamins, etc.) and several enzymes, such as the superoxide dismutase (SOD), catalase and peroxidase, which sequentially convert the superoxide anion to hydrogen peroxide (SOD) and then to water (catalase and peroxidase). Glutathione occurs under two forms: the reduced (GSH) and the oxidized, dimeric (GSSG) forms. GSH, the major form, maintains the intracellular compartment in a reduced state. GSH can form a Fe(II)GSH complex, which likely supplies Fe for the synthesis of Fe cofactor or (Fe-S) clusters of metalloenzymes involved in photosynthesis, respiration or other cellular functions (Berndt & Lillig, 2017). GSSG, the oxidized form of glutathione generated upon cell detoxication by GSH, can be reduced back to GSH by various factors, such as the NADPH-using enzyme glutathione reductase

(GR) that occurs in many, but not all organisms (for example some cyanobacteria have no GR) (Narainsamy et al., 2013). In addition, GSH serves in the biosynthesis of ergothioneine (EGT; 2-mercaptohistidine trimethylbetaine), which has been proposed to function as a stable antioxidant and cytoprotectant (Narainsamy et al., 2016).

Among ROS-generated damages, cysteines can be oxidized to form sulfenic acid (-SOH) and disulfide (-S-S-) by a two-electrons transition; sulfinic acid (-SO₂H) by a four-electrons transition; and eventually sulfonic acid (-SO₃H) by a six-electrons transition. In *Synechocystis* PCC 6803, the redox state of ~2100 Cys residues from 1060 proteins were found to be affected by light/dark cycles (Guo et al., 2014). Two types of disulfides can be distinguished considering whether they link two cysteinyl residues of, the same or different, proteins (disulfide bridges); or from a protein and a molecule of GSH (glutathione-protein mix disulfide, also termed glutathionylation). The glutathionylation process is regarded as a transient protection of critical cysteines against irreversible oxidation (sulfinic and sulfonic acids) during oxidative stress, and as a post-translational regulatory modification. The reverse reaction, deglutathionylation is catalyzed by the NAD(P)H-dependent oxidoreductase thioredoxins (Trx), or the GSH-dependent enzymes GST or glutaredoxins (Grx). Using the well-studied unicellular strain *Synechocystis* PCC 6803 as a model, we recently identified 383 proteins that can be glutathionylated in vitro (Chardonnet et al., 2015). These glutathionylable proteins participate in a wide range of cellular processes and metabolic pathways, such as carbon and nitrogen metabolisms, cell division, stress responses and hydrogen production. The glutathionylation of several proteins, namely, the antioxidant peroxiredoxin II, the mercuric reductase, the AbrB2 transcription factor, and the 3-phosphoglycerate dehydrogenase, was confirmed by biochemical studies (Chardonnet et al., 2015).

Very little was known about GSTs in cyanobacteria, even though these prokaryotes are regarded as the inventor of the oxygen-evolving photosynthesis, as well as the GSH and the GSH-dependent enzymes, such as GST, to protect themselves against the ROS that they massively produce by their active photosynthesis (Fahey, 2013; Hamilton, Bryant, & Macalady, 2016; Narainsamy et al., 2016; Schopf, 2011). Attesting to the importance of GST in cyanobacteria, the well-studied model *Synechocystis* PCC 6803 possesses six GSTs: Sll0067, Sll1147, Sll1545, Sll1902, Slr0236, and Slr0605 (Kammerscheit, Chauvat, & Cassier-Chauvat, 2019a; Pandey, Chhetri, et al., 2015; Pandey et al., 2017; Pandey, Singh, Chhetri, Tripathi, &

Singh, 2015). We showed that both Sll1545 and Slr0236 operate in the protection against photo-oxidative stress triggered by high light or H₂O₂ (Kammerscheit et al., 2019a) and that Sll1147 and its human orthologs play a prominent role in the tolerance to membrane stresses triggered by heat, cold or lipid peroxidation (Kammerscheit, Chauvat, & Cassier-Chauvat, 2019b). We also reported that Sll0067 catalyzes the conjugation of GSH with MG, the first step in the detoxification of methylglyoxal (MG), a toxic metabolite by-product that causes diabetes in humans, by the Glx enzymes. These findings are important because the conjugation of GSH with MG is always described as spontaneous (non-enzymatic) in all organisms (Kammerscheit, Hecker, Rouhier, Chauvat, & Cassier-Chauvat, 2020).

In addition, ROS species, more particularly H₂O₂, can also operate in signaling, which is an important physiological process. Indeed, H₂O₂ possesses the required properties to be a secondary messenger in being enzymatically produced and degraded by the SOD and catalase enzymes, respectively. Furthermore, H₂O₂ oxidizes protein thiols in disulfides, which can be reduced back to thiols are thereby relevant as thiol redox switches for signaling (Mironov, Sinetova, Shumskaya, & Los, 2019).

Concerning the Grx enzymes, the analysis of 503 amino acid sequences from 167 cyanobacterial species led to the identification of four Grx classes (Mondal, Kumar, & Singh, 2020). Class I and II Grxs are single module oxidoreductase while class III and IV Grxs are absent in cyanobacteria. Class V and VI Grxs are multimodular proteins having additional modules at their C-terminal and N-terminal end, respectively. Furthermore, class VI Grxs are exclusively present in marine cyanobacteria. All four classes of cyanobacterial Grxs comprise several subgroups having variable dithiol (CxxC) and/or monothiol (Cxxx) catalytic active site motives (Mondal, Kumar, & Singh, 2020). However, class II Grxs possess the CGFS-type highly conserved monothiol catalytic active site motif (Daniel, Faruq, Laura Magdalena, Manuela, & Christopher Horst, 2020; Picciocchi, Saguez, Boussac, Cassier-Chauvat, & Chauvat, 2007).

The sixth chapter entitled “A Genomic View of Secondary Metabolite Production in Cyanobacteria” written by Annick Mejean and Olivier Ploux reviewed the connections between cyanobacterial secondary metabolites and their biosynthetic genes, with emphasis on the most significant cases like cyanotoxins, sunscreens and alkanes (Méjean & Ploux, 2013).

An important aspect of cyanobacteria is that they produce a wide range of secondary metabolites with diverse chemical structures and biological

activities (vitamins, sunscreens, antibiotics, anti-cancer, toxins, *etc.*) (Cassier-Chauvat, Dive, & Chauvat, 2017; Demay, Bernard, Reinhardt, & Marie, 2019; Jeong et al., 2020; Mondal et al., 2020). For instance, at least 800 different secondary metabolites have been identified in marine cyanobacteria so far, a number likely representing a small fraction of the natural product repertoire. The functions of most of these secondary metabolites are usually unknown, but it is assumed that their production gives some advantage to the producers in complex ecosystems. It has also been proposed that these molecules might be communication molecules although there are no firm experimental data on this issue. Some of the cyanobacterial secondary metabolites appeared to be cytotoxic, neurotoxic or dermatotoxic to animals and/or humans. In the past 10 years, many biosynthesis of cyanobacterial secondary metabolites have been deciphered, at the genetic and biochemical level. Thanks to the advent of genomic data on cyanobacterial genomes and to new powerful bioinformatic tools, about 30 clusters of genes responsible for the production of cyanobacterial secondary metabolites have been identified, including the cyanotoxins: microcystin, cylindrospermopsin, saxitoxin and anatoxin-a. Almost all cyanobacterial secondary metabolites are the products of polyketide synthases, non-ribosomal peptide synthases or hybrid thereof. However, ribosomal peptides are also produced by cyanobacteria, like the cyanobactins and recent genome mining data suggest that these metabolites are more represented than initially thought in cyanobacteria.

Recently, genome sequencing and mining has revealed that natural cyanobacteria have the capacity to produce many more secondary metabolites than have been characterized (Cassier-Chauvat et al., 2017; Pearson et al., 2016). Most cyanobacterial natural products are non-ribosomal peptides (NRPS, such as lyngbyatoxin), polyketides (PKS, such as anatoxin-A), or hybrid peptide-polyketide compounds (for examples apratoxin, curacin A, microcystin), which are sequentially synthesized by NRPS and/or PKS enzymes. For example, anatoxin-A, the potent cyanobacterial neurotoxins are biosynthesized in cyanobacteria from proline and acetate by a pathway involving three polyketide synthases (Kust, Méjean, & Ploux, 2020). The corresponding assembly lines comprise several modules, each carrying out the addition of a single substrate to the lengthening molecule. Other cyanobacterial secondary metabolites are peptides synthesized by ribosomes and post-translationally modified, such as the *N*-acetylated tri- and tetradecapeptides microviridins (serine protease inhibitors) produced by *Anabaena*, *Microcystis*, *Nodularia*, *Nostoc*, and *Planktothrix* (Dittmann, Gugger, Sivonen, & Fewer, 2015; Pearson et al., 2016). These cascades of modular synthesis,

assembly and modification activities produce a large array of secondary metabolites, some of which occurring in multiple variants (100 variants in the case of microcystin). Cyanobacteria use about 5–6% of their genome length to direct the production of secondary metabolites. For example, it is predicted that the genome of *Moorea producens* PAL-8-15-08-1 carries 18 NRPS Biosynthetic Gene Clusters (Jeong et al., 2020). These gene clusters are often large (Cassier-Chauvat et al., 2017; Dittmann et al., 2015; Pearson et al., 2016). For example, the size of the *Moorea producens* 3L (formerly *Lyngbya majuscula* 3L) gene clusters encoding curacin A (an anti-cancer inhibiting tubulin polymerization) and barbamide (a molluscicidal metabolite) are 64 and a 26 kb longs, respectively. The synthesis of aeruginosin (a protease inhibitor) is encoded by a 34-kb gene cluster in *Planktothrix* and *Microcystis*, while the synthesis of saxitoxin (a neurotoxic alkaloid, which blocks the sodium (Na⁺) channels in shellfish) is directed by a 25.7 kb gene cluster in *Raphidiopsis brookii* D9. The corresponding panoply of enzymes (polyketide synthases and non-ribosomal peptide synthases) of interest for synthetic biology can still be increased through gene manipulations with the tools available for the few genetically manipulable strains (Cassier-Chauvat et al., 2017; Jeong et al., 2020). Thus, cyanobacteria, which combine the ability to synthesize a vast array of bioactive molecules with powerful photosynthesis, radiation resistance and effective synthetic biology tools for several model strains, are well suited for future cheap production of a vast array of uniformly ¹⁴C-labeled bioactive compounds, in order to screen for new drugs and analyze their absorption, distribution, metabolism, and excretion properties (Cassier-Chauvat et al., 2017).

In the seventh chapter entitled “Assembly and Export of Extracellular Polymeric Substances (EPS) in Cyanobacteria: A Phylogenomic Approach” the groups of Roberto De Philippis and Paula Tamagnini provided a first insight on the phylogenetic history of the EPS-related genes, and constitute a robust basis for subsequent studies aiming at optimizing EPS production in cyanobacteria (Pereira, Mota, Santos, De Philippis, & Tamagnini, 2013). In addition to secondary metabolites, many cyanobacteria produce extracellular polymeric substances (EPSs), mainly composed of polysaccharides, which can remain associated to the cells (capsular EPS) or be released into the surrounding environment (released EPS).

As the primary colonizers of constrained environments, such as desert soils and lithic and exposed substrates, cyanobacteria are the first contributors to the synthesis of the EPSs constituting the extracellular polymeric matrix, frequently associated with peptides, DNA and fatty acids, which favors the formation of microbial associations with varying levels of complexity called

biofilms (Rossi & De Philippis, 2015). Thus, cyanobacteria play key roles in soil properties and functions. Cyanobacterial biofilms bind soil aggregates and create a stable surface layer that facilitates the path for colonization by other biocrust organisms such as lichens and mosses (Chamizo, Adessi, Torzillo, & De Philippis, 2020).

Thanks to their negatively-charged surface, cyanobacterial EPSs usually show a high affinity for metal cations and other positively-charged or polar molecules (Bhunia et al., 2018; Jittawuttipoka et al., 2013; Planchon et al., 2013b). On the other hand, the presence of deoxy-sugars (rhamnose and fucose), peptides and ester-linked acetyl groups can also confer a contemporary hydrophobic character, significantly affecting their rheological and emulsifying properties, as well as the capability to adhere to solid surfaces (Planchon et al., 2013a; Rossi & De Philippis, 2015; Vourc'H et al., 2018). Sugars commonly found in cyanobacterial EPS include the hexoses glucose, galactose, mannose, and fructose; the pentoses ribose, xylose, and arabinose; the deoxyhexoses fucose and rhamnose; the acidic hexoses glucuronic and galacturonic acids, the amino sugars glucosamine, galactosamine, *N*-acetyl galactosamine, and *N*-acetyl glucosamine (Bhunia et al., 2018). Many cyanobacterial EPS also possess two different uronic acids (from 2% up to 80% of the total EPS dry weight, commonly between 15% and 30%), which is a rare feature in microbial EPS. In addition, they usually contain sulfate groups, which are usually present in the EPS produced by archaea and eukaryotic EPS but absent in those produced by bacteria. These last two features, and the presence of numerous different monosaccharides (up to 13) contributing to the overall anionic charge of the cyanobacterial polymers, making them suitable for a vast array of biotechnological applications, such as metal chelation, flocculating, emulsifying or rheology agents with valuable biological activities (e.g., antiviral, antimicrobial, anticoagulant, antitumor) (Pereira et al., 2019; Rossi & De Philippis, 2015).

The mechanisms of EPS assembly and export are relatively conserved, and require the involvement of polysaccharide copolymerase and outer membrane polysaccharide export proteins. In cyanobacteria, the genes encoding these proteins occur in multiple copies, scattered throughout the genome, either isolated or in small clusters. It is also necessary to identify other EPS production genes, and understand their genomic distribution and regulation of expression (Allen, Rittmann, & Curtiss, 2019; Cui et al., 2018; Jittawuttipoka et al., 2013; Ohki et al., 2019). To better exploit the biotechnological potential of cyanobacterial EPS, there are still challenges to

address, including (i) a better understanding of the relationship between central metabolism and the synthesis of sugar nucleotides, (ii) the identification and characterization of other key components of the EPS production machinery, and (iii) elucidation of the regulatory networks of the EPS production process (Bhunia et al., 2018; Pereira et al., 2019).

Last, but not least, many cyanobacteria display multicellularity and cell differentiation. In the eighth chapter entitled “Gene Expression during Heterocyst Differentiation,” Antonia Herrero and Enrique Flores summarized the biochemical and morphological properties of the heterocysts and the program of gene expression that supports the process of differentiation and its regulation (Herrero, Picossi, & Flores, 2013). Many cyanobacteria can also fix atmospheric dinitrogen (N_2). Because the N_2 -fixing enzyme nitrogenase is irreversibly inactivated by O_2 , the N_2 -fixing (diazotrophic) cyanobacteria developed several strategies to protect their nitrogenase from O_2 . Unicellular cyanobacteria (*Chroocosphaera*, *Gloeotheca* and *Cyanotheca*) temporally separate photosynthesis (production of O_2) during the day from N_2 fixation during the night (when O_2 is no longer produced by photosynthesis and is consumed by respiration). Many diazotrophic pluricellular (filamentous) cyanobacteria, such as *Nostoc* (*Anabaena*) sp., respond to nitrogen depletion by differentiating (irreversibly) some of their vegetative cells into heterocysts, the non-diving, photosynthetically inactive, cells that fix atmospheric (inorganic) nitrogen (N_2) (Harish & Kunal, 2020; Herrero et al., 2013). This differentiation process results in a heterocyst pattern consisting of two heterocysts separated by about 10 to 15 vegetative cells (Herrero, Stavans, & Flores, 2016). The thick-walled heterocyst cells provide a micro-oxic environment for the oxygen-sensitive nitrogenase enzyme to perform nitrogen fixation. Vegetative cells and heterocysts exchange metabolites, with heterocysts providing vegetative cells with fixed nitrogen, while vegetative cells provide heterocysts with photosynthates.

Recently, intercellular molecular exchange was found to occur by simple diffusion through proteinaceous structures, known as septal junctions, which connect the adjacent cells in the filament and traverse the septal peptidoglycan through perforations known as nanopores. Proteins that are necessary to produce, and that may be components of, the septal junctions—SepJ, FraC and FraD—have been identified in the heterocyst-forming cyanobacterium *Anabaena* PCC 7120 model. Additionally, several proteins that are necessary to produce a normal number of nanopores and functional septal junctions have been identified, including AmiC-type amidases, peptidoglycan-binding proteins and some membrane transporters.

Recently, two novel ABC transporter components involved in the formation of the glycolipid layer of the heterocyst envelope have been identified (Herrero & Flores, 2019). Available reports and re-evaluation of inter-cellular molecular transfer data for some mutants of *Anabaena* suggest that the septal junctions can be regulated, likely by a mechanism of gating (Flores, Nieves-Mori3n, & Mullineaux, 2019). The cyanobacterial septal junctions resemble the metazoan gap junctions, and more precisely their constituent connexons, which are protein complexes that allow the diffusion of small compounds and ions between cells and can be regulated by gating. Septal and gap junctions represent therefore a wonderful example of convergent evolution, in which different proteins and structures perform the same function (cell–cell communication) in extremely distant organisms (Flores, Nieves-Mori3n, & Mullineaux, 2019).

At the metabolite level, heterocyst differentiation starts as a response to a persistent high-cellular carbon-to-nitrogen balance signaled by the level of the 2-oxoglutarate. In turn, this results in activation of the global transcriptional CRP-family regulator NtcA followed by increased expression, mainly localized to differentiating cells, of the genes *ntcA* and *hetR*, which encodes HetR, the differentiation-specific transcription factor. The expression of genes encoding proteins that transform the vegetative cell into a heterocyst is then activated with a spatiotemporal specificity to produce a mature functional heterocyst. A few other regulators of gene expression participate at specific differentiation steps, and a specific transcription factor, CnfR, activates *nif* genes expression under the micro-oxic conditions of the heterocyst (Flores, Picossi, Valladares, & Herrero, 2019). Recently, the role of small RNA and interruption DNA elements that influence the heterocyst formation and function has also been identified (Harish & Kunal, 2020).

Heterocysts depend on a wide variety of metals to maintain their specific functions and cellular metabolism. N₂ fixation requires a significant number of metalloproteins of the photosynthetic and respiratory metabolism, which have high Fe and Cu requirements, to provide energy and electrons to this essential biological process for the nitrogen cycle. Nitrogenases contain 38 atoms of Fe and either two Molybdenum (Mo) or two vanadium (V) atoms. Other metalloenzymes are crucial to manage efficiently reactive oxygen species generated during the metabolism of heterocysts, while some metalloproteins are especially important in Fe storage, which is the most required transition metal in heterocysts. These sets of metalloproteins are aimed at creating the optimal working conditions for the central metabolic enzyme of heterocysts, the nitrogenase complex, but also associated

hydrogenases required to metabolize efficiently the H₂ byproduct generated during the N₂ reduction reaction to recover reducing equivalents that, otherwise, would be wasted. Moreover, the uptake hydrogenase accounts for 12 atoms of Fe and one Ni atom, while the bidirectional hydrogenase requires 27 atoms of Fe and one Ni atom. The biological relevance of the metals Fe, Cu, Mo, Mn, Ni, V and Zn metals in heterocysts and their use by metalloproteins has been recently discussed (Pernil & Schleiff, 2019).

Finally, the oxygen-free environment offered by the heterocysts, in combination with an increasing arsenal of molecular biology tools for heterocyst-specific expression, has led some authors to propose that heterocysts could be exploited as cell factories for the production of hydrogen and/or chemicals (Avilan et al., 2018; Liu, Ni, Xu, & Tao, 2018; Magnuson, 2019).



1. Concluding remarks

Once again, we express our gratitude to all contributing authors of the previous vol 65 book for their dedicated efforts to clearly expose their fields of expertise to researchers and students. We noticed with great pleasure that these authors have made great contributions to the important advancement of the knowledge on cyanobacteria presented here. We also encourage cyanobacterial researcher to study new cyanobacteria endowed with specific morphological and/or physiological interests in comparison to model cyanobacteria, in order to better analyze the large biodiversity of cyanobacteria, and deploy them as industrial cell factories for the production of a wide range of chemicals in local industrial sites (Chenebault et al., 2020; Mukherjee, Madhu, & Wangikar, 2020). In this prospect it will be important to determine if, and when, the required genetic toolbox developed for the few well-studied cyanobacterial models can be adapted to the “new” cyanobacteria emerging from such investigations (Behler, Vijay, Hess, & Akhtar, 2018; Gale et al., 2019; Yao et al., 2020).

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