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Contents

Foreword: Cyanobacteria Biotechnology

Dear reader,

This book comes on the heels of a first era of development of photosynthetic cyanobacteria as microbial catalysts. We are now poised for the beginning of a second one.

Cyanobacteria have been studied for decades as model organisms for various aspects of photosynthesis, such as water oxidation, light sensing, harvesting and conversion, regulation of the Calvin–Benson–Bassham cycle, the circadian rhythm, and nutrient starvation. Although cyanobacteria have traditionally been cultivated at large scale as supplemental foodstuffs or sources of antioxidants, it was only 20 years ago that reliable genetic engineering was developed for model strains in a demonstration of ethanol biosynthesis by *Synechococcus elongatus*. It is fitting in the foreword of such a book to ask where does the field of cyanobacteria metabolic engineering stand today?

From an industrial perspective, the use of light to power CO_2 fixation and conversion is attractive, as both substrates are abundant and cheap. Furthermore, microbial cells are regenerative catalysts and already a proven and accepted technology for some products. However, microbial conversion of light energy to a desired chemical comes with, to borrow from an old proverb, "many a slip 'twixt the cup and the lip." There is an energy loss at each step of the conversion process, including light capture, electron transport, and within the stoichiometry of the native metabolic network. Introduction of additional conversion steps to a target chemical brings more inefficiency. Thus, from the same industrial perspective that prioritizes low costs, it is clear that a potential cyanobacteria process must be made to operate near the theoretical energy conversion limit, or perhaps, the conversion limit must be increased, to ensure adoption. Metabolic engineering and biotechnology aim to elucidate the mechanisms of these inefficiencies, as well as devise and test designs for mitigating them. To date, advances in these areas are not sufficient for cyanobacteria to have widespread industrial use.

However, there is hope. As this book will show, our capabilities in studying and manipulating cyanobacteria is now highly advanced and include facile gene editing, rapid design and implementation of biosynthetic pathways, and powerful techniques for mapping metabolic fluxes and photosynthetic processes. Application of these tools within basic research continuously reveals new features of cyanobacteria

metabolism, which become new targets for optimization. The discovery of novel, fast-growing strains has shown that photosynthesis *can* power CO_2 uptake and cell growth at rates significantly higher than previously thought, and many metabolic engineering strategies described in this book can now be ported to such strains. Bold, novel designs for engineering light harvesting, atmospheric nitrogen fixation, and bioreactors will also lead to improvements in productivity in the medium term. Interestingly, our efforts will likely aid by advances in crop engineering, where the chloroplast metabolism is often a target. As cyanobacteria serve as models for the chloroplast of C3 plants, plant scientists are also studying (and engineering) cyanobacteria.

This book should serve as a guide for engineering cyanobacteria and is thus intended for multiple audiences. Researchers at the beginning of their careers, such as graduate students, should find the book useful to learn what has been achieved to date, and for a given specialization, where the most lucrative research lines lie. Cyanobacteria are understood well enough to make rational modifications for purpose, but mechanisms for regulation of key pathways are not yet known. This book is also useful for non-academics, such as biotechnologists seeking to exploit photosynthesis for industrial use. There are several chapters detailing metabolism of cyanobacteria for certain chemical classes, as well as descriptions of state-of-the-art methodologies for creating new strains, analyzing them, and even scale-up.

This book is divided into three parts: Core Cyanobacteria Processes, Concepts in Metabolic Engineering, and Frontiers of Cyanobacteria Biotechnology. Each chapter is written by experts and makes extensive reference to recent literature and patent filings. The initial chapters describe key metabolic processes unique to cyanobacteria, namely, CO_2 uptake and fixation (Chapter 1 by Hagemann et al.), the photosynthetic electron transport chain (Chapter 2 by Lea-Smith and Hanke), and light harvesting (Chapter 3 by Branco dos Santos and colleagues). In keeping with the theme of this book, these chapters also describe engineering strategies to improve efficiencies of these processes.

Key concepts in metabolic engineering of cyanobacteria are introduced in the subsequent four chapters. Xiong and colleagues (Chapter 4) give an overview of how to measure and interpret metabolic fluxes, and how this technology has revealed new aspects of cyanobacteria metabolism. Hudson (Chapter 5) reviews the state of the art of synthetic biology in cyanobacteria, a rapidly growing sub-discipline, with perspective on what should be considered for an industrial process. In Chapter 6, Ducat and colleagues describe the source-sink balance in cyanobacteria and plants, namely, how do cells sense an imbalance in energy and adapt so as to dissipate it, and can this be exploited for bio-production? In Chapter 7, Ku and Lan compile examples of metabolic engineering in cyanobacteria to derive heuristics for future engineering regarding reaction driving forces, kinetics, and stability. The middle of the book explores in more detail metabolic pathways for the biosynthesis of compounds of industrial interest. The ethanol biosynthesis pathway (Chapter 8, Luan and Lu) is the most studied, has been deployed at pilot scale, and is a model for understanding limitations in large-scale production. Terpenes (Chapter 9, Rodrigues and Lindberg), storage polymers (Chapter 10, Koch and Forchhammer), and fatty acids (Chapter 11, Kawahara and Hihara) are higher value compounds that find use outside of biofuels.

In Chapter 12, Oliveira and colleagues describe our current understanding of how cyanobacteria secrete or pump compounds outside of the cell and give perspective on future engineering of product export.

The third part begins by highlighting N_2-fixing cyanobacteria (Chapter 13, Zhou and colleagues), strains that are underrepresented in the literature but have potential application for fertilizer, and production of nitrogen-rich chemicals. Chapter 14 by Wangikar and colleagues reports on recently discovered fast-growing cyanobacteria, their unique attributes in energy and carbon metabolism, and how CO_2 fixation and growth rate could be enhanced in other strains. The book is concluded by two chapters on cultivation technology. In Chapter 15, Bühler and colleagues describe cyanobacteria biofilms as a new format for high-density cultivation, but one that comes with unique challenges in terms of mass transfer to and from the biocatalyst. In Chapter 16, Touloupakis and Carlozzi summarize their work on outdoor cultivation of photosynthetic bacteria, where the day/night cycle causes periodicity in cell growth and, in this case, hydrogen production.

The book highlights several "grand challenges," in cyanobacteria biotechnology that awaits a new generation of scientists and engineers. Can we achieve a high, stable partitioning of carbon away from biomass and into a product of interest? What new metabolic pathways can mitigate the energetic costs of carbon fixation and photorespiration and can these be integrated with the native metabolism? How can we expand the PAR spectrum for cyanobacteria? How can we improve tolerance to "industrial stresses," such as high light (compounded by eventual cultivation in atmospheric CO_2), salt, and bio-contaminants? What is the optimal reactor configuration that balances cost with productivity?

One theme is that drawing parallels to heterotrophic bacteria will only take us so far in engineering cyanobacteria; these microbes have unique regulation mechanisms that we must continue to elucidate. To paraphrase a summation by Ducat in Chapter 6, cyanobacteria metabolism may be evolved and optimized to anticipate fluctuating conditions and not for continuous secretion of fixed carbon as biotechnologists would like. This book is meant to inform and inspire scientists to solve such challenges. We all are looking forward to the next era of cyanobacteria engineering.

Stockholm 2020 *Paul Hudson, Editor*

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Part I

Core Cyanobacteria Processes

1

Inorganic Carbon Assimilation in Cyanobacteria: Mechanisms, Regulation, and Engineering

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1.1 Introduction – The Need for a Carbon-Concentrating Mechanism

Cyanobacteria are oxygenic phototrophs that occur in almost all photic habitats on the Earth. During their long-lasting evolution, they adapted to a wide range of abiotic conditions. Among them, the availability of the photosynthetic substrate inorganic carbon (C_i, including CO_2 and dissolved inorganic carbon, mostly bicarbonate – HCO_3^-) can fluctuate on different timescales. It is generally accepted that oxygenic photosynthesis evolved in cyanobacteria at least 2.7 billion years ago, a time when C_i was at much higher levels in the Earth atmosphere (6000 ppm) and the oceans [1–3]. Because of the activity of photosynthetic organisms, particularly after the rise of eukaryotic algae and plants, the cyanobacterial habitats became strongly depleted of C_i, accompanied by an increase in molecular oxygen (O_2). Over the long term, cyanobacteria adapted from a C_i-rich, O_2-free atmosphere to a C_i-poor, O_2-rich atmosphere. This change had drastic consequences for C_i assimilation, in particular for ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO), the carboxylating enzyme of the Calvin–Benson–Bassham cycle (CBBC). RubisCO is not a *bona fide* carboxylase; it evolved from an enolase in the methionine salvage pathway [4]. In CBBC, RubisCO catalyzes the activation of the acceptor molecule ribulose 1,5-bisphosphate (RuBP) by changing its enol into the reactive keto conformation; this activation allows CO_2 binding to RuBP and its carboxylation. Then, the non-stable carboxylation product is hydrolytically split into two stable molecules of 3-phosphoglycerate (3PGA). Generally, RubisCO is characterized by a rather low speed and low affinity toward CO_2. Cyanobacterial RubisCO proteins, belonging to the "type 1" RubisCOs, have a particularly high K_m for CO_2 of approximately 300 μM, which is about 10-fold higher than the concentration of dissolved CO_2 in water [5]. In addition, RuBP activated by RubisCO can also react with O_2 in the oxygenase reaction, which leads to the formation of one-molecule 3PGA and one-molecule 2-phosphoglycolate (2PG). The latter product has inhibitory effects

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on the enzymes of CBBC in plants, such as triosephosphate isomerase and sedoheptulose 1,7-bisphosphate phosphatase [6, 7]. Hence, 2PG concentrations need to be kept low; this is maintained by the 2PG salvage pathway called photorespiration (see Section 1.5). In conclusion, the long-term change in the atmospheric composition introduced problems for RubisCO, which were solved by the evolution of the carbon-concentrating mechanism (CCM, see Section 1.2) in cyanobacteria, as well as in many other photosynthetic organisms [8].

C_i availability not only changes over long timescales but can also fluctuate on short timescales. The solubility of CO_2 in water is mainly influenced by pH, temperature, and total ion content. In general, high temperature decreases the solubility of gases, including CO_2. It can also be dissolved in water, leading to the formation of bicarbonate and carbonate, whereby the occurrence of the different C_i species depends on pH. For example, in the slightly alkaline water of oceans, less than 1% of C_i is in the form of CO_2 and most of the dissolved C_i is present as bicarbonate (87.5%). Carbonate dominates in alkaline lakes, while acidic waters preferentially contain only CO_2. The pH-dependent interconversion of the C_i species is rather slow and can be highly accelerated in biological systems by carbonic anhydrases (CA). Such enzymes, which belong to different enzymatic classes, are present in all cyanobacteria and many other photosynthetic organisms [9]. In addition to abiotic factors, the C_i availability is also strongly influenced by biotic factors, especially in eutrophic waters. In dense phytoplankton population, for example, in cyanobacterial surface scums that appear during the so-called bloom situations, the high photosynthetic activity will strongly deplete C_i and O_2 will accumulate to high levels [10]. Heterotrophic organisms, for example, bacteria specifically associated with cyanobacteria, may not only provide vitamins and other growth-stimulating molecules but can also locally enrich water with CO_2 released via respiration. Hence, cyanobacteria also have to acclimate to short-term variations in C_i; hence, the response to varying C_i needs to be tightly regulated at different levels.

1.2 The Carbon-Concentrating Mechanism (CCM) Among Cyanobacteria

The above-mentioned problems of RubisCO due to the long-term depletion of the atmospheric CO_2 and the enrichment of O_2 initiated diverse adaptation mechanisms. For example, RubisCO proteins of type 1 in C3 plants increased the CO_2 affinity to respond to low atmospheric CO_2 partial pressure [11]. Cyanobacteria used another strategy; they did not invest in the improvement of RubisCO but instead evolved a CCM. CCM is defined as the measures that result in higher C_i affinity of the intact photosynthetic organism/cell compared to the CO_2 affinity of its RubisCO. Hence, the CCM works to increase the CO_2 partial pressure in the vicinity of RubisCO, which allows the enzyme to efficiently perform the carboxylation reaction and at the same time suppresses the oxygenase reaction to a large extent. The cyanobacterial CCM is a so-called biophysical CCM because it mainly depends on transport/uptake of C_i by different mechanisms, leading to high intracellular

accumulation of the charged bicarbonate, which in contrast to CO_2 cannot easily diffuse from the cell through biological membranes. RubisCO is confined to the carboxysome, a bacterial micro-compartment with a protein shell containing in addition CA that efficiently converts bicarbonate into CO_2 (Figure 1.1). Hence, RubisCO operates in a highly CO_2 enriched environment inside the carboxysome, which is also believed to exclude O_2, thereby minimizing the competing oxygenase reaction. When the cyanobacterial CCM evolved among cyanobacteria is still a matter of discussion. One common assumption dates it to the low CO_2, high O_2 period about 400 million years ago [12], while much earlier dates for its appearance have also been reported [13]. Because of the notion that the CCM is functionally and structurally well conserved in the majority of cyanobacterial clades, including the basal and most distant forms, it is more parsimonious to assume that the CCM evolved much earlier than 400 million years ago in the cyanobacterial radiation [14].

1.2.1 C_i Uptake Proteins/Mechanisms

Over the past 30 years, five different C_i uptake systems have been identified in different model cyanobacteria (Figure 1.1). Their activity allows a high accumulation of bicarbonate inside the cells, which can exceed the external amount of 100–1000-fold [15].

First, the bicarbonate transporter BCT1 has been characterized [16]. BCT1 belongs to the group of primary active ATP-binding cassette (ABC) transporters, which energize the uptake of substrates via ATP hydrolysis. In cyanobacteria, BCT1 is usually encoded by the *cmp* operon, which comprises the genes for the ATP-binding subunit, the bicarbonate pore protein(s), and a periplasmic bicarbonate-binding protein that determines the high bicarbonate affinity of the system. The *cmp* operon expression is strongly stimulated by the activator protein CmpR under low C_i conditions (LC, usually ambient air), which is often encoded upstream of the structural *cmp* operon [17].

Second, the sodium-dependent bicarbonate transporter (SbtA) has been identified in *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*), which is a high-affinity bicarbonate transporter presumably executing a bicarbonate/Na^+ symport [18]. The Na^+ dependency of SbtA has been concluded from the high stimulation of the transporter by increasing Na^+ amounts and its dysfunction upon mutation of Na^+ transport protein J (NtpJ), a protein assumed to be involved in sodium export [19]. However, it turned out that NtpJ is not involved in Na^+ export but instead is part of the Ktr-type K^+ transport system [20, 21]. Hence, whether or not Na^+ and bicarbonate transport are indeed directly coupled in SbtA still needs to be verified experimentally on isolated SbtA reconstituted into liposomes. Like BCT1, *sbtA* expression is highly induced under LC conditions.

Third, BicA, another widespread bicarbonate transporter, was identified in *Synechococcus* sp. PCC 7002, which belongs to the SulP family and is also believed to couple bicarbonate uptake to Na^+ symport [22]. In contrast to BCT1 and SbtA, the BicA transporter exhibits a more constitutive, low-affinity, but highly active bicarbonate uptake.

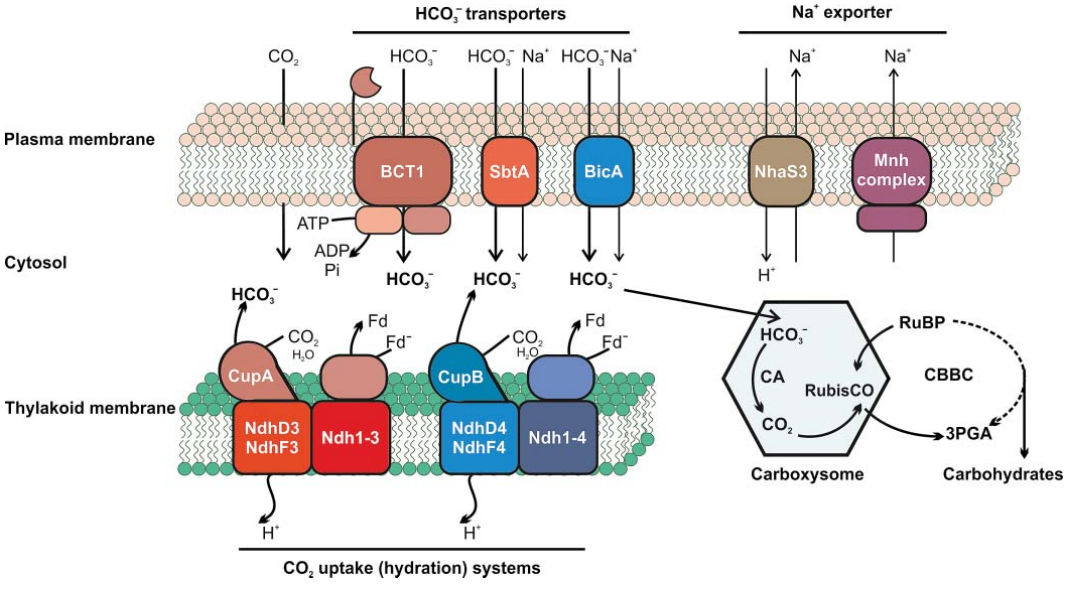

Figure 1.1 Schematic presentation of cyanobacterial carbon-concentrating mechanism (CCM). The cyanobacterial CCM utilizes three bicarbonate transporters, BCT1, SbtA, and BicA, located in the plasma membrane. The two thylakoid-embedded CO_2 uptake (hydration) systems convert cytoplasmic CO_2 to bicarbonate: Ndh1-3 and Ndh1-4 comprising the cyanobacteria-specific small subunits NdhD3/4, NdhF3/4, and CupA/B. Among them, the expression of transporters BCT1, SbtA, and Ndh1-3 complex (in warm color) are strongly induced under LC conditions, while BicA and Ndh1-4 complexes (in cold color) are constitutively expressed. The Na^+ gradients are kept by at least two Na^+ export systems: NhaS3 (brown) and Mnh complex (purple). The accumulated bicarbonate penetrates into carboxysomes, where bicarbonate is dehydrated back to CO_2 by carbonic anhydrase (CA) in proximity to RubisCO and fixed into carbohydrates through CBBC.

In addition to the three bicarbonate transporters, two CO_2 uptake (hydration) systems have been identified. CO_2 is a gas and no direct transport protein has been characterized to date. Hence, CO_2 passively diffuses inside the cell driven by its partial pressure gradient, whereby the diffusion can be accelerated by specific aquaporin-like channels [23]. Inside the cell, CO_2 is efficiently converted into bicarbonate by two specialized NDH1-like complexes located in the thylakoid membrane [19, 24–26]. The conversion of CO_2 into bicarbonate fulfills two tasks; it maintains the partial pressure gradient for CO_2, promoting inward diffusion, and reduces the diffusion of CO_2 that is not fixed by RubisCO in the carboxysome from the cell. The latter function has been demonstrated by the observation that cyanobacterial cells release CO_2 during the first minutes after illumination of darkened cells, a time span when RubisCO is still inactive because of missing activation via carbamylation, but carboxysome-located CA is already working [27].

The cyanobacterial NDH1-like complex is involved in different functions. In addition to CO_2 hydration into bicarbonate, it participates in respiration and cyclic electron transport around photosystem 1 [24, 26]. Proteomic and mutant studies revealed that these multiple tasks are fulfilled by structurally different complexes, which are associated with a different set of auxiliary subunits, leading to different complex sizes (see e.g. [25]). Among them, the two small complexes Ndh1-MS and Ndh1-MS′ are specifically involved in the CO_2 uptake [26]. In *Synechocystis*, one has been characterized as a constitutive system with low CO_2 affinity named as Ndh1-4 (NDH1-MS′), while the other represents a high-affinity system named as Ndh1-3 (NDH-MS) that is highly induced under LC conditions [19, 25]. Cyanobacteria-specific small NDH1 subunits have been identified, which also play an essential role in the CO_2 to bicarbonate conversion [26, 28, 29]. Among them, the CupA or CupB subunits, which reach into the cytoplasm on the distal membrane part of the NDH-MS complexes, are specifically involved in the CO_2 uptake [19, 30–32].

Despite the impressive progress in functional and structural studies of NDH1 complexes, some questions remained open for a long time [26]. For example, the nature of the electron donor for the cyanobacterial NDH1 has been discussed because the NAD(P)H_2-oxidizing subunits were never identified in isolated NDH1 complexes nor harbor cyanobacterial genome homologs for these proteins. Recently, the structures of the large NDH1 complex from *Thermosynechococcus elongatus* have been solved, which unequivocally showed that reduced ferredoxin donates electrons to the complex. Hence, the NDH1 complex among cyanobacteria is not a *bona fide* NAD(P)H_2 dehydrogenase complex [33]. Another open question is related to the mechanism, which permits the CO_2 conversion by the specialized NDH1-MS complexes. It is assumed that because of light-driven trans-thylakoid proton transport via the NdhD subunits [32], an alkaline region is created at the cytoplasmic site of the NDH1-MS complexes, which forms the predicted "alkaline pocket" driving the CO_2 conversion [34]. Recently, the CA EcaB was found at the thylakoid membrane, where it seems to be specifically linked to the NDH1-3 and NDH1-4 (NDH1-MS) complexes involved in CO_2 uptake in *Synechocystis* [35]. The authors concluded that EcaB might be involved in the proton extraction by the

NDH1-3 and NDH1-4 complexes to allow for continuous CO_2 to bicarbonate conversion. Moreover, the EcaB protein also seems to regulate the CO_2 uptake activity of these specialized NDH1 complexes [35]. The structure of the NDH-MS complex has recently been solved by cryo-electron microscopy, which permitted to gain a mechanistic understanding of the CO_2-hydrating reaction performed by CupA [36].

The multiple systems involved in C_i uptake among cyanobacteria seem to cooperate in the accumulation of bicarbonate inside the cell. This redundancy explains why the mutation of single bicarbonate transporters or CO_2 uptake systems causes usually no severe phenotype, i.e. these mutants can still grow at ambient air conditions (LC). Only under specific conditions do growth phenotypes of single mutants become visible, such as variation of the external pH to make CO_2 or bicarbonate the predominantly available C_i form [32]. However, the combined inactivation of several transporters, including a *Synechocystis* mutant with all five C_i uptake systems inactivated [37], resulted in the characteristic high CO_2-requiring (HCR) phenotype. The HCR phenotype of these mutants directly supports the notion that despite the existence of an intact carboxysome, the accumulation of high cellular bicarbonate concentrations is an essential part of the cyanobacterial CCM. This has also been shown in experiments expressing a highly active recombinant CA in the cytoplasm of *Synechococcus elongatus*. The activity of this human CA collapsed the internal accumulation of bicarbonate, resulting in the HCR phenotype [38].

It should be noted that not all cyanobacteria possess all the five described C_i uptake systems [39]. For example, it has been shown that *Microcystis* spp. ecotypes are characterized by variable numbers of C_i transporters, especially the SbtA protein is often absent while BicA is retained [40]. Competition experiments showed that strains having both SbtA and BicA outcompete *Microcystis* spp. with only BicA under LC conditions. Consistently, *sbtA* and *bicA* genotypes of *Microcystis* strains were found predominantly during cyanobacterial bloom development, leading to C_i-limiting conditions, while *bicA*-only genotypes were successful in seasons with enriched C_i amounts in lakes, in agreement with its low-affinity, high-flux characteristics [41]. Picoplanktonic cyanobacteria living in the open oceans are characterized by large genome reductions. Consistently, the genomes of *Prochlorococcus* spp. and marine *Synechococcus* spp. have lost several C_i uptake systems [42]. The molecular basis of C_i acquisition in these ecological important cyanobacteria, which are believed to perform approximately 20% of annual CO_2 fixation, is not well understood and needs further investigations. Bioinformatic analysis showed that their genomes harbor many yet uncharacterized proteins belonging to protein families of characterized C_i uptake systems. Hence, these proteins represent excellent candidates for the further study on new C_i uptake systems among cyanobacteria [39].

1.2.2 Carboxysome and RubisCO

The final C_i fixation by RubisCO is performed in the bacterial micro-compartments called carboxysomes (Figure 1.1). Carboxysomes are polyhedral bodies, resembling phage capsids, which became visible in all cyanobacterial cells via transmission

electron microscopy. Their specific function in CO_2 fixation was demonstrated in attempts to localize RubisCO and the site of $^{14}CO_2$-incorporation in cyanobacterial cells, both are confined to the carboxysome structures. Despite a similar function, two phylogenetically distinct carboxysomes and RubisCO types are present among cyanobacteria, distinguishing the so-called β-cyanobacteria and the so-called α-cyanobacteria [43, 44]. β-cyanobacteria, to which the majority of the cyanobacterial phylum belongs, harbor RubisCO proteins of the subclass 1B and β-carboxysomes (Ccm-type). In the model cyanobacterium *S. elongatus*, most carboxysomal proteins are encoded in one large *ccm* operon that forms a superlocus also comprising operons encoding RubisCO and some C_i uptake systems [45]. The α-cyanobacteria, which form a phylogenetic separate clade of picoplanktonic cyanobacteria dominated by oceanic *Prochlorococcus* spp. and *Synechococcus* spp. [46], contain RubisCO type 1A and α-carboxysomes (Cso-type), which were most probably acquired via horizontal gene transfer from proteobacteria [47]. Because most cyanobacteria, including all well-investigated model strains, belong to the class of β-cyanobacteria, we will here shortly describe only this type.

The structure and genesis of β-carboxysomes were intensively characterized over the past 20 years. These carboxysomes assemble from the inside out [48]. First, hexadecameric RubisCO molecules and CA assemble together with CcmM to a procarboxysome, which is subsequently surrounded by the shell proteins. In the case of *S. elongatus* and *Synechocystis*, a β-type CA named as CcaA has been identified, which is responsible for the rapid conversion of bicarbonate into CO_2 inside the carboxysomes [49, 50]. Structural analyses revealed that CcaA forms well-packed trimer-of-dimers organization. Moreover, it binds with high affinity to CcmM [51]. However, the CA-type inside carboxysomes is variable among cyanobacteria [47, 52]. CcaA homologs are only found in a rather low number of cyanobacterial genomes. This implied the question, which CA is replacing the CcaA in other cyanobacterial strains. In the filamentous strain *Nostoc* (*Anabaena*) sp. PCC 7120, one isoform of CcmM has been shown to exhibit CA activity [53]. This CA-like domain is well conserved in cyanobacterial CcmM proteins, in addition to RbcS-like domains. The structure showed that this CcmM domain resembles γ-type CAs. It became obvious that CcmM-mediated CA activity seems to be more widespread among cyanobacteria than CcaA. In addition to its CA function, the CcmM protein plays a major role as a scaffold protein for the semicrystalline order of RubisCOs inside carboxysomes. Recently, the structural basis for CcmM and RubisCO interaction has been resolved, which showed unexpectedly that the RbcS-like domain of CcmM did not directly interact with RbcS in RubisCO, but CcmM rather binds to a region between RbcL dimers [54]. The RubisCO-CcmM structures are then anchored by CcmN to the inner carboxysomal shell [45].

The polyhedral protein shell of β-carboxysomes is mainly composed by CcmK proteins that form hexameric structures with a central pore, which is believed to facilitate the flux of bicarbonate and RuBP toward the carboxysome lumen and of 3PGA out of it [55]. CcmKs are the main constituents of the shell. In *Synechocystis*, four different CcmK proteins are present, which can be expressed to different extents under fluctuating C_i levels. The variable composition of carboxysome shell

by different CcmKs is anticipated to impact the function of the carboxysome because the CcmK homologs mostly differ in sequences around the pore-forming part, which influence their size and charge and possibly provide a range of selectivity toward RuBP and 3PGA as well as possibly bicarbonate, CO_2 and O_2 [45]. However, this assumption needs experimental support, which is hampered because of the difficulty to isolate intact, functional carboxysomes from cyanobacteria. Another hexameric shell protein is CcmP, which is found in lower abundances compared to CcmKs. The CcmP protein can form stacked dimers with a central chamber and gated pores, which are believed to allow a controlled permeation of larger molecules such as RuBP and 3PGA across the carboxysomal shell [45, 56]. Finally, the pentameric shell protein CcmL forms the vertices in the icosahedral carboxysomal shell [57].

As mentioned above, mutation of genes for structural carboxysomal proteins or for CA always resulted in the HCR phenotype, which shows that the compartmentalization of RubisCO and CA inside the carboxysome is an essential part of the cyanobacterial CCM (e.g. [58]). The *Synechocystis* Δ*ccmM* mutant accumulated high amounts of photorespiratory intermediates because RubisCO is exposed to much lower CO_2 and higher O_2 partial pressure than in intact carboxysomes [59].

1.3 Regulation of C_i Assimilation

Environmental fluctuations in the available C_i must be measured by cyanobacteria in order to coordinate the CCM activity and the downstream utilization of fixed carbon by the primary carbon metabolism. This regulation is achieved at different levels, including transcriptional but also posttranscriptional control. Using model cyanobacteria, the activity of the CCM and the carbon metabolism has been compared mainly under two conditions. Usually, cells grown under CO_2-supplemented conditions (1–5% CO_2, defined as high carbon, HC) have been challenged by shifts to ambient air, LC (0.04% CO_2) conditions. This shift scenario has been selected because many mutants with defined mutations in CCM components are characterized by the HCR phenotype and can be only cultivated and characterized at HC conditions. However, the HC to LC shift protocol usually needs to include a medium change, i.e. HC-grown cells are harvested by centrifugation or filtration to remove the C_i-enriched medium at time point zero before cells are then suspended in C_i-poor fresh medium. This treatment disturbs the steady-state light conditions and might be stressful for the cells, i.e. it is probably more physiological to shift from LC into HC conditions, which simply involves a change in the composition of the gas stream through the cultures.

1.3.1 Regulation of the CCM

The activity of the CCM is clearly higher in LC- than in HC-grown cyanobacterial cells, which is directly seen in the enhanced photosynthetic C_i affinity of LC cells [34]. This change is mainly regulated at the transcriptional level, influencing the differential expression of C_i uptake systems, while RubisCO and carboxysome