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CCM in photosynthetic bacteria and marine alga

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Abstract
The evolution of organisms capable of oxygenic photosynthesis paralleled a long-term reduction in atmospheric CO_2 and the increase in O_2. Consequently, the competition between O_2 and CO_2 for the active sites of RUBISCO became more and more restrictive to the rate of photosynthesis. In coping with this situation, many algae and some higher plants acquired mechanisms that use energy to increase the CO_2 concentrations (CO_2 concentrating mechanisms, CCMs) in the proximity of RUBISCO. The CCM improves photosynthetic performance by raising the CO_2 concentration at the site of ribulose-1,5-bisphos-phate carboxylase/oxygenase (Rubisco), simultaneously enhancing carbon fixation and suppressing photo-respiration. Active inorganic carbon (Ci) uptake, Rubisco sequestration and inter-conversion between different Ci species catalyzed by carbonic anhydrases (CAs) are key components in the CCM, and an array of molecular regulatory elements is present to facilitate the sensing of CO_2 availability, to regulate the expression of the CCM and to coordinate interplay between photosynthetic carbon metabolism and other metabolic processes in response to limiting CO_2 conditions. Although the molecular components underpinning the above metabolics is very much clear, the related regulatory pathways still need to be elucidated.

Keywords: CO2 concentrating mechanisms, Pico planktons, Inorganic carbon uptake, Functional Genomics of CCM

Introduction
Carbon concentrating mechanism (CCM) systems, associated with evolutionarily diverse aquatic photosynthetic organisms, make a major contribution to global net primary productivity and marine carbon sequestration. Here, an overview of these global contributions is presented from their evolutionary origins, including a possible trigger for their diversification when the aqueous O2/CO2 ratio rose above parity, and a re-definition of the paradox of phytoplankton. The inorganic carbon substrate supply needed for photosynthesis in the aquatic milieu is limited by inorganic carbon solubility and diffusion across the boundary layer, cell wall and multiple membranes to the primary carboxylase Rubisco. Various biophysical carbon concentrating mechanism (CCM) systems are found in many aquatic phytoplankters and have overcome these chemical and physical limitations. Such CCMs deliver the appropriate inorganic carbon species demanded by Rubisco (CO2), at an enhanced concentration which compensates for the enzyme's low substrate affinity and competitive inhibition from oxygen.

Despite this adversity, aquatic organisms clearly punch above their weight of biomass relative to terrestrial plants. The instantaneous standing biomass crop of aquatic plants (primarily microorganisms) is 3 PgC (i.e. 1015 g carbon) relative to the 610 PgC usually quoted for terrestrial plant above-ground biomass. The paradox of how phytoplankton's deliver an annual net primary productivity of 47.5 PgC, relative to the 56.4 PgC of their terrestrial counterparts, has long intrigued researchers. In addition, the oceanic sink for net carbon sequestration is equal to that of land plants (2.3 PgC per year), such that marine organisms also facilitate the absorption of over 25% of annual anthropogenic CO2 emissions (Pan *et al*., 2011) [12]

The original paradox of the phytoplankton was thought to reflect phylogenetic diversity in competition for limiting light and inorganic resources. The high net primary productivity, could be explained by the interaction between ecological and environmental factors across space and time to prevent the dominance of any one phytoplankton group. However, the past few decades have seen several historical paradigms overturned – such as photosynthetic acclimation to light increasing the depth of the photic zone (Richardson *et al*., 1983; Raven *et al*., 2017) [16, 13], the breadth of productivity across oceanic gyres (Johnson *et al*., 2006; Partensky and Garczarek, 2010) [6, 13], and the molecular basis of niche differentiation found within cyanobacterial and eukarotic picoplankton populations in coastal and equatorial waters

(Not *et al*., 2012; Biller *et al*., 2015) [11, 1]. Additionally, we now recognize that more than 80% of marine primary productivity is facilitated by some form of CCM.

Evolutionary and biochemical perspective of origin of CCM

The evolution of organisms capable of oxygenic photosynthesis paralleled a long term reduction in atmospheric carbon dioxide and the increase in oxygen. Consequently the competition between oxygen and carbon dioxide for the active sites of the RUBISCO became more and more restrictive to rate of photosynthesis. In order to cope with this emerging situation CCM evolved. Following are certain biochemical challenges enhanced by the changing environment that led evolution in favor of CCM (Raven & Beardall, 2016) [15]

- Rubisco is an unusually slow enzyme with a low affinity for CO_2. At atmospheric levels of CO_2, Rubisco can function at only about 25% of its catalytic capacity because the concentration of dissolved CO_2 is less than the $K_m(CO_2)$ of Rubisco and due to the relatively high concentration of O_2 which competes with CO_2
- the diffusion of CO_2 in an aqueous solution is 10,000 times slower than the diffusion of CO_2 in air. Thus, the ability to scavenge CO_2 as quickly as it becomes available is highly advantageous to aquatic photosynthetic organisms.
- Aquatic environments oversee significant fluctuations in inorganic carbon (C_i -CO_2 HCO_3) levels and pH, which change the availability of CO_2 and HCO_3 for photosynthesis. At an acidic pH, the vast majority of C_i is in the form of CO_2, while at an alkaline pH, C_i is mostly in the form of HCO_3, with CO_2 making up only a small fraction of the available C_i

Different forms of CCM- convergence and divergence in function

Table 1 summarizes the mechanisms by which algae can accumulate CO2. These range from biochemical C4 and CAM mechanisms involv-ing additional DIC (Dissolved Inorganic Carbon) fixation prior to that by RUBISCO, to biophysical processes involving either localized enhancement of external CO2 concentration by acidification of the external medium, or the active transport of DIC across one or more cellular membranes. Most of the mechanisms have been confirmed by either pH drift experiments or isotope disequilibrium techniques, following the kinetics of assimila-tion of inorganic carbon following supply of radioactively labeled HCO−3 or CO2, have also been applied to this question**.**

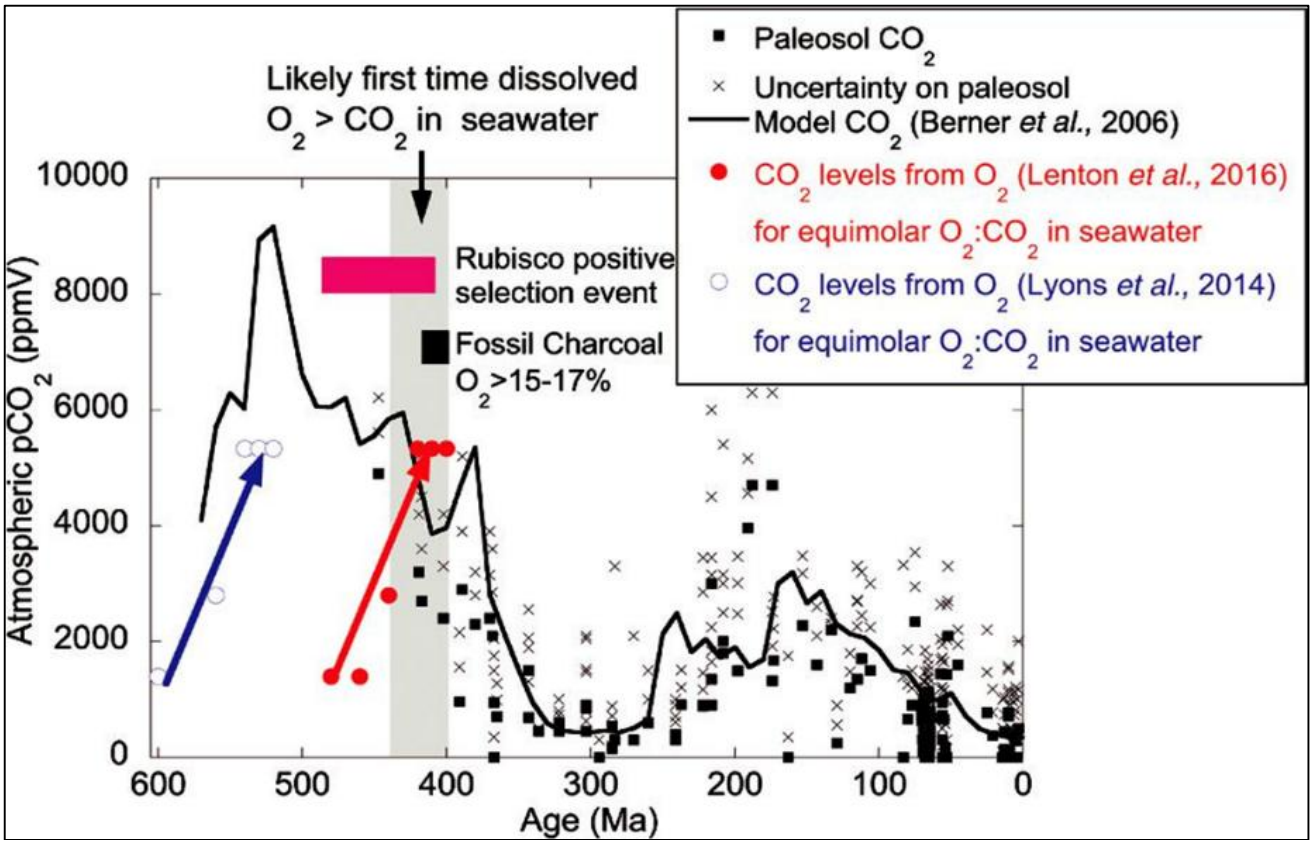

Fig 1: An estimate of the threshold when the concentration of dissolved aqueous O2 rose to a higher concentration than CO2 in seawater as a trigger for the emergence of CCMs (grey bar). Also shown are the first appearance of fossil charcoal evidence that O2>15–17% (black bar) & a Rubisco positive selection event. (Young *et al*., 2016) [19]

Convergence in CCM form and function-

The three pillars usually invoked to support a CCM

1. Biophysical inorganic transporters, operating in parallel across adjacent membranes, raising
2. the inorganic carbon pool by some 40-fold (Chlorophyte) to 400-fold (Cyanobacteria) and determining overall affinity and effectiveness of the CCM
3. A suite of strategically placed carbonic anhydrases (CA) and CA-like moieties, adjacent to the inorganic transporters, to assist in bicarbonate interconversion or regeneration (or recapture) of CO_2 close to Rubisco

A micro compartment within which Rubisco aggregates, and from which CO_2 leakage is minimized, such as the carboxysome in cyanobacteria and pyrenoid associated with most eukaryotic CCM systems

Table 1: The major categories of CCM in terrestrial and aquatic phototrophs, their need for an energy input, and their necessity to elevate intracellular or intracompartmental DIC above extracellular levels (Kaplan, 2017) [7].

Mechanism	Energy input	Necessity for mean DIC_i or CO_{2i} to exceed DIC_o or CO_{2o}
C_4: inorganic C + C_3 → C_4, dicarboxylate in the cytosol → C_3 + CO_2 in plastid containing RUBISCO	In generation of C_3 acceptor (PEP)	Depends on relative volume of RUBISCO containing high-CO_2 compartment
CAM: inorganic C + C_3 →C_4 dicarboxylate in the cytosol at night; C_4 stored in vacuole until next day, released and decarboxylated with minimal CO_2 leakage (stomata closed in land plants)	In generation of C_3 acceptor and its conversion during decarboxylation to stored products. Also in transport of C_4 dicarboxylate to vacuole	Yes, at least in terrestrial CAM in decarboxylation phase
HCO_3^- active influx, conversion to CO_2 by CA at RUBISCO site (often in carboxysome or pyrenoid)	In active influx of HCO_3^- at plasmalemma and/or plastid envelope	Yes for DIC_i, if active transport is at the plasmalemma
CO_2 active influx	In active influx of CO_2 at plasmalemma and/or plastid envelope	Yes, for CO_2, unless the compartment in which CO_2 is accumulated is relatively small
CO_2 passive influx at plasmalemma of cyanobacteria with conversion of CO_2 to HCO_3^- by NADHdh, then conversion to CO_2 by CA in carboxysome	In NADHdh, bringing about the unidirectional CA conversion of CO_2 to HCO_3^-	Yes for DIC_i
Acidified compartment to which HCO_3^- has access; conversion of HCO_3^- (using CA) to give high equilibrium level of CO_2, CO_2 diffusion to RUBISCO compartment	In producing and maintaining a low-pH compartment using H^+ pumps at plasmalemma, thylakoid, and/or other (?) membranes	Yes for CO_2 if the compartment generating CO_2 and adjacent compartments are relatively large

Divergence in CCM form and function

Cyanobacterial and microalgal CCMs exhibit some distinctly different features from C4 photosynthesis in higher plants (McGrath *et al.*, 2014) [14]:

1. The CCM is a single-cell-based CO2 enrichment mechanism relying on multiple energized inorganic carbon (Ci) uptake systems. The operation of these Ci uptake systems results in an intracellular Ci pool in the form of HCO3 which can represent a concentration increase up to 1000-fold from low-CO2 environments, possibly the most effective Ci uptake system thus far.
2. Unlike C4 photosynthesis, in which Ci enters the cells primarily by CO2 diffusion, microalgae and cyanobacteria possess both active CO2 and HCO3 uptake systems. This is very important because these organisms often live in aquatic environments where CO2 diffusion is much slower than in terrestrial environments, and where HCO3 frequently becomes the dominant Ci species over CO2; possessing both CO2 and HCO3 uptake systems enables them to switch between different modes of Ci acquisition so they can more quickly adapt to an environment with a constantly changing abundance of different Ci species. Note that multiple HCO3 transporters or CO2 uptake systems are typically present in an organism, especially in eukaryotic algae due to the presence of more complicated internal compartments and membrane systems
3. Since Rubisco can only use CO2 as a substrate for carboxylation, a carbonic anhydrase (CA) converts the accumulated HCO3 to CO2 at or near the site of Rubisco. Meanwhile, conversion between different Ci species by CAs at different intracellular locations also operates in concert with Ci uptake systems to facilitate the accumulation of Ci
4. Rubisco is sequestered in a specialized micro compartment (carboxysomes in cyano-bacteria or pyrenoids in eukaryotic algae). The highly localized Rubisco is considered as an important CCM function and allows the CO2 concentration proximal to Rubisco to be raised substantially
5. Since CO2 can easily dif-fuse from the Rubisco, a CO2 barrier or recapture system generally prevents leakage of internal CO2. (vi) While C4 photosynthesis is typically expressed constitutively, the microalgal/cyanobacteria CCM is generally an inducible mechanism. An array of molecular regulatory elements is present to facili-tate the sensing of CO2 availability and to regulate the expression of the microalgal/cyanobacteria CCM at multiple levels

Structural and functional components of CCM in cyanobacteria

The structural and functional components of CCM in cyanobacteria can be studied under three headings, the Ci transport systems, the carbonic anhydrases (CAs) and the carboxysomes. In water systems, Ci is represented by three forms, existing in an equilibrium:

$$CO2 + H2O \leftrightarrow HCO3^- + H^+ \leftrightarrow CO32^- + 2H^+.$$

The ratio of Ci forms depends on the ambient pH and is determined from the Henderson–Hassel Balch equation:

$pH = 6.3 + \log([HCO3^-]/[CO2])$,

$pH = 10.3 + \log([CO3^-]/[HCO3^-])$

Thus, $HCO3^-$, predominates in the solution at pH from 6.3 to 10.3, while at pH below 6.3 and above 10.3, CO2 and $CO32^-$, respectively, are the dominant forms.

Ci transport systems

Since the CO2 molecule is uncharged, it is well soluble in lipids and therefore may penetrate into the cell by passive diffusion through the cell membrane. These molecules may equally easily escape from the cell. To prevent this leakage, cyanobacterial cells employ the CO2 uptake systems, which convert CO2 to the charged bicarbon ate molecule, which is insoluble in lipids. Due to this insolubility, the inflow of exogenous $HCO3^-$ is possible only via active transport. Five transport systems (TS) for Ci are known in cyanobacteria, including three bicarbonate transport ers and two systems for CO2 uptake. Specificity of these TS for both Ci forms was demonstrated using the inhibitors that selectively suppress transport of CO2 (COS, H2S, Na2S, and ethoxyzolamide) or $HCO3^-$ (Li+ and monensin), as well as in the experiments

with the mutant strains with impaired systems of CO2 or HCO3− uptake (Biller *et al*., 2015) [1].

BCT1: It is an inducible, high affinity TS for HCO3− and first TS for Ci to be described in cyanobacteria BCT1 is a uniporter and belongs to the family of bacterial ABC (ATP binding cassette) transporters containing an ATP binding group for subsequent ATP hydrolysis and release of energy. BCT1 is encoded by the cmp ABCD operon, and its synthesis is induced by acute Ci limitation. The cmp ABCD genes encode four proteins of BCT1: CmpA (a periplasmic protein responsi ble for specific HCO3− binding), CmpB (a hydropho bic protein present in the membrane as a dimer capable of forming an ion channel CmpC and CmpD (large and small cytoplasmic proteins with ATP binding sites)

Sbt A: It is an inducible, high affinity Na+ dependent TS for HCO3−, which was originally described for Synechocystis PCC6803 in 2006 SbtA is supposed to act as a Na+/HCO3– symporter, although this has not been unequivocally confirmed. In Synechocystis, SbtA has molecular mass of ~40 kDa; however, in the cytoplasmic membrane, this protein occurs as a 160 kDa complex, which means that it is probably a tetramer.

Bic A: BicA is a constitutive low affinity Na+ dependent TS for HCO3− Bic A is the most recently discovered but probably the most common cyanobacteria HCO3− transporter Similar to SbtA, BicA probably carries out Na+/HCO3− symport While this TS has relatively low affinity to the substrate it is able to maintain high rates of transport and therefore the photosynthetic activity. BicA belongs to a big family of eukaryotic and prokaryotic transporters (the SulP/SLC26 family), which have been annotated in many bacteria as sulfate transporters or permeases.

NDH 1-3 and NDH 1-4 TS Complexes: These systems are based on NAD (P) H dehydrogenase type 1 (NDH-1) complexes comprising of NDH-13 and NDH-14 protein complexes. NDH-13 is the low-CO2 inducible high-affinity CO2 uptake system, encoded by ndhD3, ndhF3, and cupA (chpY). On the other hand, NDH-14 protein complex is the constitutive low-affinity CO2 uptake system encoded by ndhD4, ndhF4, and cup B (chpX) genes. These are multisubunit complexes. While protein subunits Ndh D and NdhF are responsible for CO2 uptake, CupA and CupB catalyze the hydration reaction of CO2 into HCO−3

Cyanobacterial carbonic anhydrases

Based upon amino acid sequence homology they may be divided into five independent classes: α, β, γ, δ, and ζ The only α CA characterized in cyanobacteria is the EcaA protein. Two important β CAs are CsoSCA in α cyanobacteria and CcaA in β cyanobacteria. The CcmM protein is an active γ CA in the species that have lost the homologues of ccaA gene. Based upon localization, they may be- A- internal Cas (present within carboxysomes), B- External CAs present outside carboxysomes EcaA and EcaB are important external α and β CAs (Kaplan, 2017) [7].

Carboxysomes

Carboxysomes are specialized sub-cellular compartments compos-ing of protein shells and two encapsulated enzymes, Rubisco and carbonic anhydrase (CA). In carboxysomes, CA catalyzes HCO−3 into CO2, which is a substrate for Rubisco. There are two types of carboxysomes, α- and β-. The cso-type of shell proteins, encoded by cso operon, is termed α-carboxysomes, while the ccm-type of shell polypeptides, encoded by ccm KLMNO operon, is termed β-carboxysomes. Based on this criterion, the cyanobacteria species carrying form 1A of Rubisco within α-carboxysomes are classified as α-cyanobacteria while the species containing form 1B of Rubisco within β-carboxysomes are classified as β-cyanobacteria.

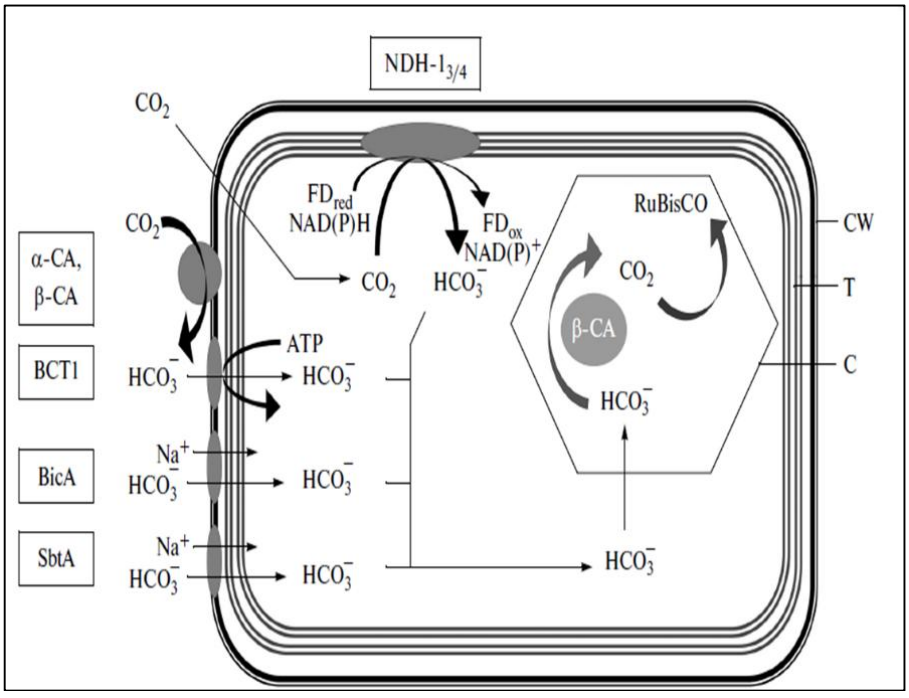

Fig 3: General scheme of cyanobacterial CCM operation. The scheme is based on the literature data obtained for the classical model organisms: freshwater and marine cyanobacteria *Synechococcus elongatus* PCC 7942, *Synechocystis* sp. PCC6803, and *Synechococcus* sp. PCC7002. Designations: BCT1, BicA, and SbtA, bicarbonate transporters; NDH $1_{3/4}$, CO_2 uptake systems; CW, cell wall; T, thylakoids; C, carboxysome; and CA, carbonic anhydrase

Most of the α-cyanobacteria such as Prochlorococcus and Synechococcus strains inhabit marine while β-cyanobacteria such as Synechocystis sp. PCC 6803, Anabaena variabilis, and *Synechococcus elongatus* PCC 7942 live mainly in freshwater. Although the two carboxysome types are different in gene organization, formation, and species distribution, they have similar functions which are to limit CO2 leaking, reduce the risk of photorespiration, and enhance the carboxylase activity of Rubisco (Heureux *et al*., 2017) [5]

Most recent insights into proteins associated with carboxysomes

Among the β-carboxysome proteins, which have been extensively studied, CcmK, Ccm L, and CcmO were proposed to be in the outer shell layer. Rbc X encoded by RBC X is a Rubisco assembly chaperone, which interacts with Rbc L to facilitate the assembly of Rbc L and Rbc S to form Rubisco holoenzyme. Cyanobacteria photosynthesis involves a flux of ribulose 1, 5-bisphosphate (Ru BP) into and of 3-phosphoglycerate (3PGA) out of the carboxysomes. This diffusion is facilitated by pores that are found in the carboxysomes shell proteins Ccm K and CcmO. These proteins form hexamers and build the carboxysomes surface, while the metameric Ccm L is found at the edges. The inner architecture of the carboxysomes is mostly determined by CcmM that is found in multiple forms. In addition to its potential CA function, the Ccm M is binding Rubis CO to form a semi crystalline internal order. Recently, it has been shown that newly translated Ccm M and Rubis CO form defined aggregates that serve as nucleation cores for the synthesis of novel carboxysomes (Kaplan, 2017) [7]

CCM in case of alkaliphile cyanobacteria

Some researchers have hypothesized that CCM might not be necessary in the alkaliphilic cyanobacteria because of unlimited supply of inorganic carbon in the form of HCO−3 and CO23− in the alkaline environments. However, the major function of the CCM in haloalkaliphilic cyanobacteria may consist of regulation of Ci amount arriving into a cell in order to saturate the RuBis CO carboxylation centers (the concentrating role in the induced CCM state at ambient Ci limita tion), and to protect the cell from excessive Ci, which may affect the intracellular homeostasis (protective role in the constitutive state of the CCM). Such regulation of Ci inflow into the cell implies that its rate is sufficient to maintain efficient photo synthesis. Excessive Ci input should, however, be pre vented in order to maintain a certain level of the intra cellular pH (Klanchui *et al*. 2017) [8].

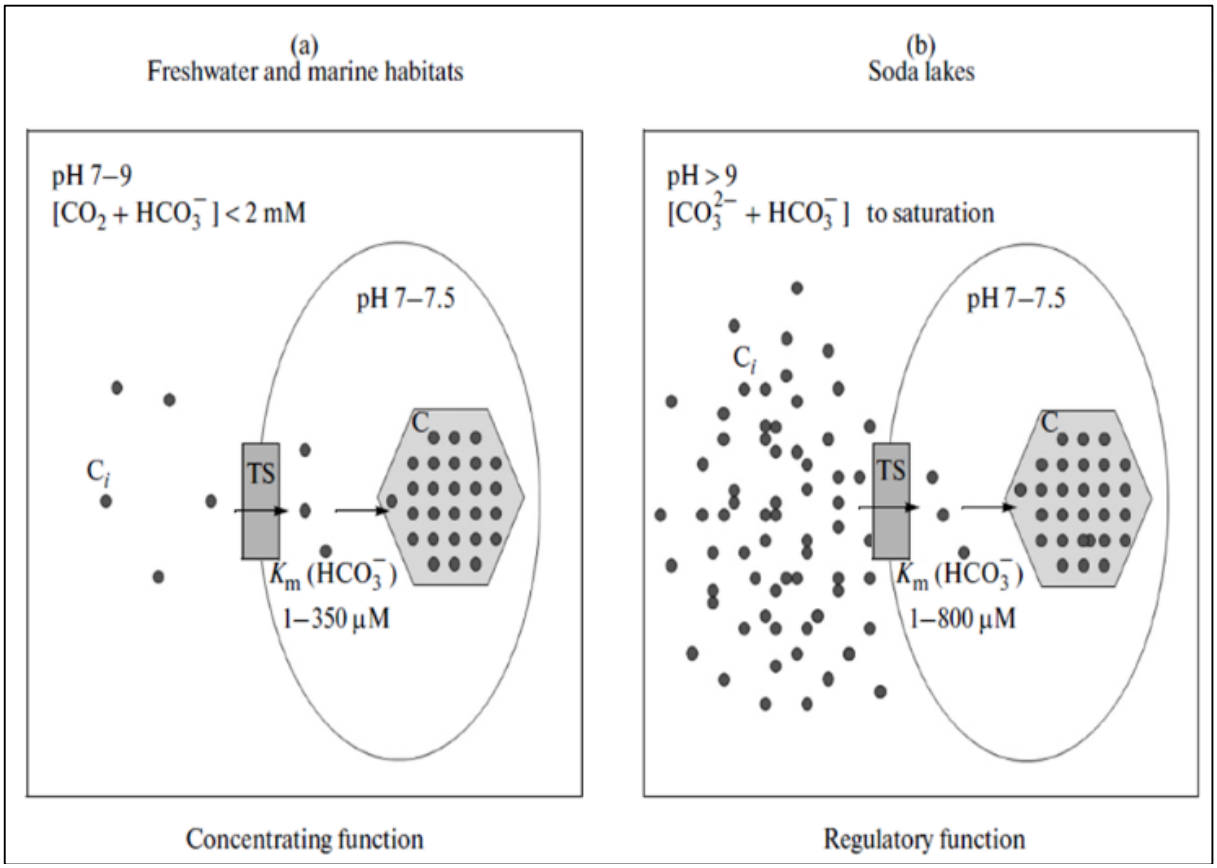

Fig 4: Functions of the CO2concentrating mechanism in freshwater and marine cyanobactaeria (a) and in haloalkaliphilic cyano bacteria from soda lakes (b). "C" indicates crboxysomes

The overall compositions of CCM components in alkaliphilic cyanobacteria are more similar to the freshwater than the marine groups. The cyanobacteria inhabiting freshwater and alkaline ecological niches possess both CO2 uptake systems, NDH-13 and NDH-14, while most strains inhabiting marine habitats seemed to lack the NDH-13. Focusing on the HCO−3 transport system, the results showed that marine and some alkaliphilic cyanobacteria consistently lacked the BCT1 type of the HCO−3 transporter. In addition, the freshwater β-cyanobacteria possessed the highest abundance of CAs, β-CA (CcaA and EcaB), α-CA (EcaA), and γ-CA (CcmM), while the alkaliphilic cyanobacteria were likely to possess only two conventional CAs, carboxysomal β-CA (CcaA) and γ-CA (CcmM). Freshwater β-cyanobacteria possess the highest abundance of CAs, β-CA (CcaA and EcaB), α-CA (EcaA), and γ-CA (CcmM), while the alkaliphilic cyanobacteria were likely to possess only two conventional CAs, carboxysomal β-CA (CcaA) and γ-CA (CcmM)

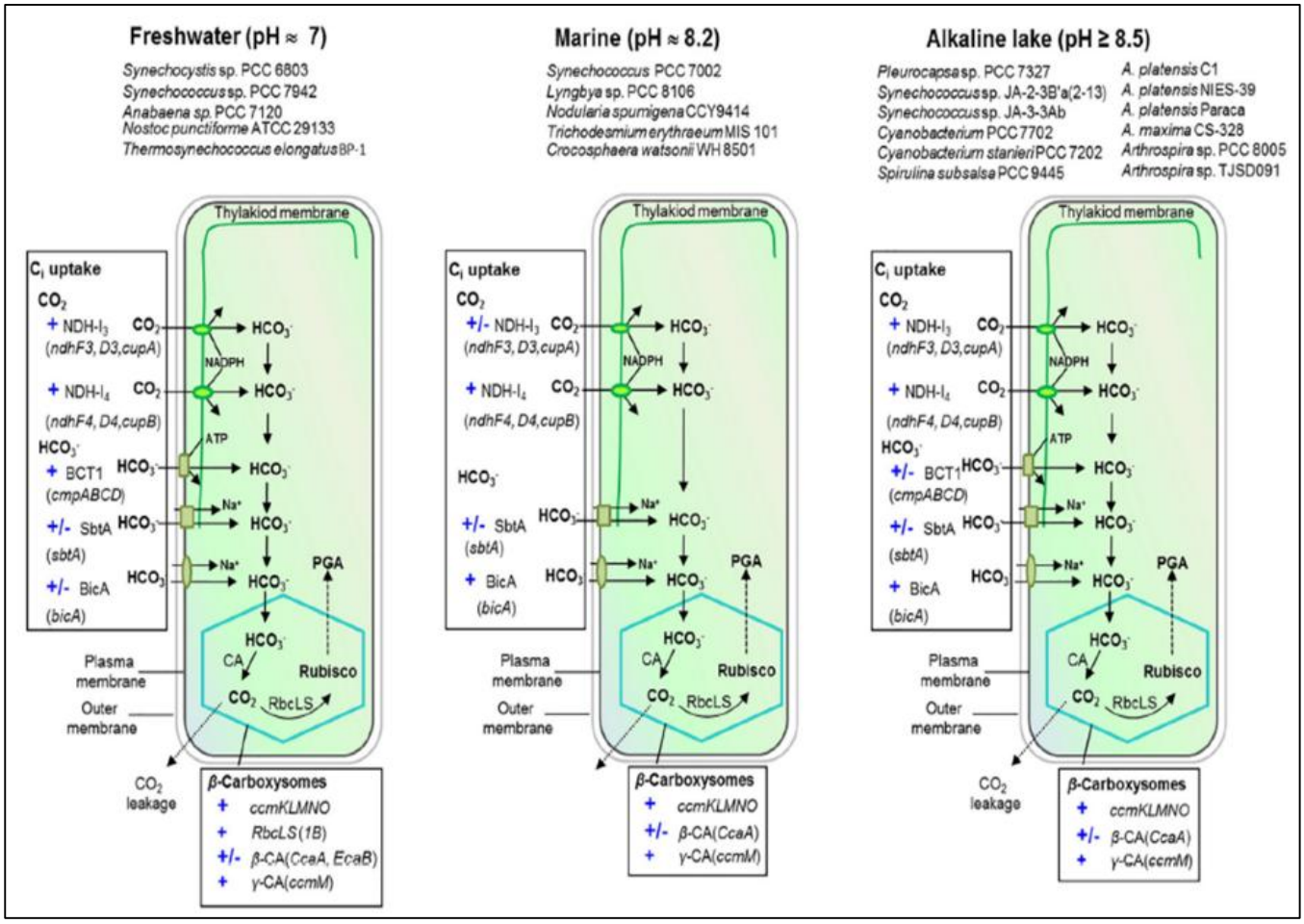

Fig 5: Diversity in characteristic components of the cyanobacterial CCM living in three different pH environments; freshwater (pH ~7), marine (pH ~8.2), and alkaline (pH N 8.5. + and ± indicate that the particular component is 'always present' and 'sometimes present', respectively (Klanchui *et al*. 2017) [8].

Transcriptional regulation of CCM in cyanobacteria

The structural components of the CCM are encoded by genes that are typically organized as operons, with some being constitutively expressed and others being inducible by exposure to conditions of limited Ci availability. A transition from HC to LC conditions results in an up-regulation of transcription of both inducible CO2 and $HCO3^{-}$ uptake systems. Most of the transcriptional regulators belong to the widely distributed protein family of regulators, the LysR transcriptional regulators (LTTR) (Table 2). The LTTRs include both repressors and activators and all known members of this family function through allosteric changes in their DNA binding affinity due to the binding of the small effector molecule. Consistent with this mode of operation, it was found that CmpR functions as a transcriptional activator that specifically bound to operator DNA sequences upstream of the RNA polymerase binding and initiation site of the *cmp* operon. During Ci-limitation, cells are anticipated to accumulate both RuBP and 2PG and, thus, these metabolites would be logical effectors of the CmpR activation of the *cmp* operon. According to this model, the accumulation of RuBP and 2PG under low Ci conditions would promote the binding of CmpR to its transcriptional activator site leading to the expression of the Cmp bicarbonate uptake system (Han *et al*., 2017) [12]

Table 2: LysR-type regulators in *Synechocystis* sp. PCC 6803

Gene Name	Synechocystis ORF	Function	Co-regulatory Metabolites
ndhR (*ccmR*)	sll1594	Repressor high affinity C_i uptake (genes for CupA, SbtA, Na^+-NDH-1)	α-KG, $NADP^+$
cmpR	sll0030	Activator of ABC-type bicarbonate transporter (*cmp* operon and *psbA* genes)	RuBP, 2PG
ycf30, *rbcR*	sll0998	Activation of CBB genes	NADPH, 3PGA, RuBP
ntcB	slr0395	Activation of nitrate assimilation genes	nitrite

Using surface plasmon resonance to study the interaction of NdhR with its cognate DNA binding regions of the NdhR regulon, it was shown that NADP+ and αKG act as co-repressors through their allosteric interactions with NdhR. In principle, intracellular concentrations of NADP+ and αKG are expected to decrease as photosynthesizing cells become starved of Ci. The decline of NADP+ is explained by the continuous action of the light reactions to reduce NADP+, while its regeneration due to the consumption of NADPH2 by the CBB cycle is slowed due to lack of substrate. Similarly, as carbon fixation by CBB cycle decreases, the flow of carbon into the cyanobacterial TCA cycle may also be expected to decrease, leading to a decrease in the concentration of αKG.

There is one additional identified member of the LTTR family that is performing an important function in Ci metabolism in cyanobacteria: RbcR. This protein is alternatively named CbbR in the annotation of some cyanobacterial genomes because of sequence similarities to the widely distributed LTTR that controls the expression of the enzymes of the CBB cycle in many members of the α-proteobacteria. CbbR in *Rhodobacter* spp. controls two major operons containing the genes for Rubis CO and other enzymes of the CBB cycle. RbcR in cyanobacteria is also very closely related to an LTTR, termed YCF30 that is found in the plastid genomes of glaucophytes, red algae, and affiliated algae

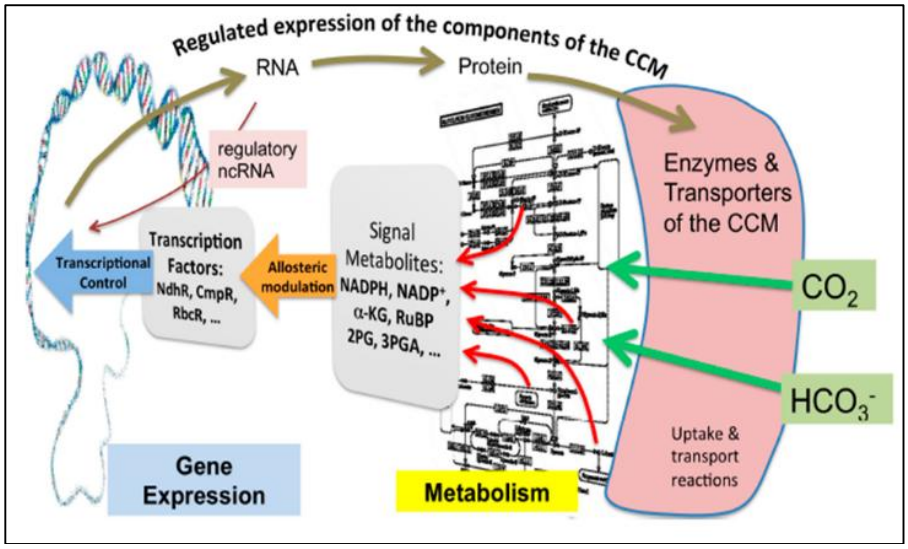

Fig 6: Overview of the different regulatory levels adjusting the activity of the CO2 concentrating mechanism (CCM) according to the ambient inorganic carbon levels (Han *et al.*, 2017) [4]

The 'Pump-Leak' CCM

Nannochloropsis spp. belong to the stramenopile (heterokont) clade and have a plastid of red algal origin that is separated from the cytoplasm by a total of four membranes, the outermost being contiguous with the endoplasmic reticulum (ER) and outer nuclear envelope However, the cells of these species lack pyrenoids or other CO2-concentrating structures, which are central features of known CCMs. Physiological studies have demonstrated that cells of Nannochloropsis gaditana primarily take up HCO3− and, strikingly, release CO2 into the surrounding media in excess of the chemical equilibrium. Energization of this phenomenon was dependent on mitochondrial respiration rather than on the chloroplastic light reactions that are thought to drive the more intensively studied CCMs. This indicates that Nannochloropsis spp. may operate what has been termed a "pump-leak" type of CCM, whereby bicarbonate transporter and carbonic anhydrase activity deliver CO2 in excess of what photosynthesis can use, resulting in a sizeable leakage back to the surroundings (Gee and Niyogi, 2017).

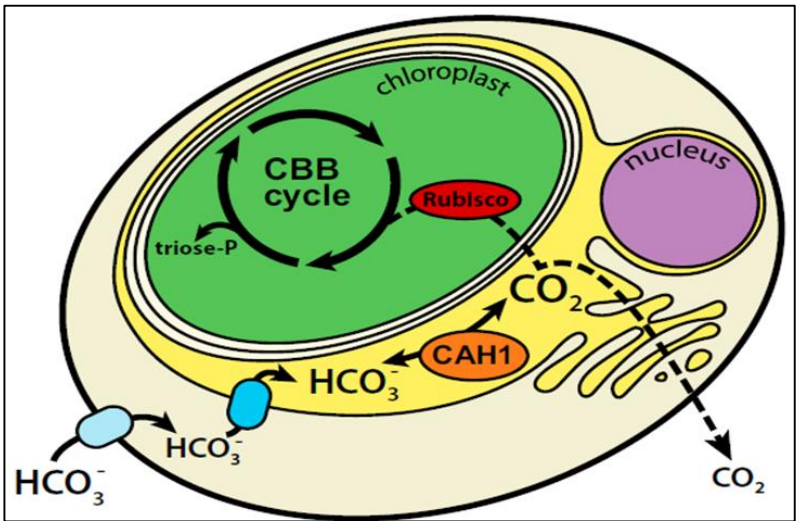

Fig 7: A proposed model for the CCM of N. oceanica. The plastid is separated from the cytoplasm by a total of four membranes, the outermost of which is contiguous with the ER and outer nuclear envelope (called the epiplastid ER) (Gee and Niyogi, 2017).

Operation of CCM in Microalgae

Can be studied under the following heads

1. At the cell surface
2. Crossing the plasma membrane
3. Cytosolic components
4. Crossing the chloroplast
5. Inside the chloroplast
6. Recapture of leaked CO2

At the cell surface: CAH1 is a periplasmic CA that has been exten-sively studied as a limiting-CO_2-inducible protein that may function in maintaining HCO_3^- for plasma membrane HCO_3^- transporters or CO_2 for active CO_2 uptake

Crossing the chloroplast envelope: So far only LCIA has been confirmed to function in active Ci uptake across this membrane, especially in very low CO2. The LCIA protein is located on the chloroplast envelope. LCIA belongs to a formate-nitrite trans-porter (FNT) family/NAR family (nitrate assimilation related). The possibility that LCIA functions as a channel to facilitate entry of Ci into the chloroplast in Chlamydomonas would require LCIA to function in concert with active plasma membrane Ci uptake systems, such as HLA3 or LCI1, to move HCO3– into chloroplasts against an opposing membrane potential Indeed, synergistic effects of LCIA and HLA3 on Ci uptake have been demonstrated by both co-knockdown and co-overexpression of HLA3 and LCIA. Two additional transporters CCP1/2 have also been identified.

Inside the chloroplast: LCIB is localized at the chloroplast stroma and appears to function in active CO_2 uptake that LCIB may facilitate accumulation of Ci by actively catalyzing unidirectional hydration of CO_2 to HCO_3^- in the stroma, a function analogous to the proposed function of cyanobacterial ChpX and ChpY LCIB forms a heteromultimeric complex with its close homolog LCIC. The peripyrenoid location of LCIB seems to be exclusively associated with acclimation to very low CO_2, whilst in low or high CO_2, LCIB is dispersed throughout the entire stroma. Similar to LCIB, CAH6, a putative stromal CA, has been proposed to convert CO_2 to HCO_3^- in the alkaline stroma, maintaining a high Ci concentration or recapturing leaked CO_2 in Chlamydomonas. Pyrenoids are proteinaceous bodies composed mainly of the large and small subunits of Rubisco Thy-lakoid membranes traverse pyrenoids to form net-like pyrenoid tubules Inside these pyrenoid thylakoid tubules, HCO_3 is believed to be dehydrated to CO_2 by a thylakoid CA, CAH3, then released to Rubisco in the pyrenoid to enter the CBB cycle. CAH3 is specifically concentrated in thylakoid tubules crossing the pyrenoid matrix in cells acclimated to limiting CO2. Similar to CAH3, dynamic distribution of Rubisco between chloroplast stroma and pyrenoids is also correlated with different CO2 conditions: almost all Rubisco is associated with the pyrenoid under limiting CO2 condi-tions, but only about 50% of Rubisco is located in the pyrenoid in high CO2. While both the Rubisco large subunit (LSU) and small subunit (SSU) are essential for pyrenoid formation it appears that small subunits (RBCS1 and RBCS2) contain the structural elements responsible for targeting Rubisco to the pyrenoid (Larkum *et. al.*, 2017) [9].

Recapture of leaked CO_2: Both CAH6 and the LCIB/LCIC complex have been proposed to
perform this function and this stems from three observations:

1. the LCIB/LCIC complex is located in the peripyrenoid space in very low CO_2, form-ing a sheath around the pyrenoid
2. The LCIB mutants pmp1 and ad1 accumulate little or no internal Ci in low CO_2
3. The cah3 mutation suppresses the airdier phenotype and defective Ci accumulation in LCIB mutants in low CO_2.The epistatic relationship of cah3 relative to lcib implies that LCIB functions downstream of CAH3, and thus is likely to capture CO_2 released by CAH3 and prevent CO_2 leakage. It should be noted, however, that this interaction of CAH3 and LCIB takes place under low-CO_2 conditions; in very low CO_2, LCIB mutants exhibit photosynthesis fairly comparable to that of wild type.

Air-Dier phenotype

What is most striking about LCIB mutants is their air-dier phenotype. It is obvious that other Ci uptake systems, such as LCIA-associated Ci uptake, function in very low CO2 to allow LCIB mutants to survive and perform nearly normal photosynthesis, but at the same time, although these same systems are still present, they cannot support Ci uptake and growth of LCIB mutants in low CO2. Photosynthetic activity in LCIB mutants driven by Ci uptake specific to very low CO2 appears to be quickly inhibited when the CO2 concentration is raised to near or above the air level (Yamano *et al.*, 2010) [18], and the contribution of Ci-dependent photosynthesis mediated by LCIA or HLA3 starts to decline as CO2 concentrations increase into the 'low CO2' range, where the apparent contribution of LCIB becomes substantial. It has therefore been proposed that post-translational or allosteric regulation may differentially regulate low- and very-low-CO2 acclimation. As shown in a hypothetical model illustrating the proposed functions and regulation of Ci uptake systems (Figure 2), Ci uptake associated with very low CO2, especially HLA3- and LCIA-based HCO3– uptake, is active in very low CO2 but inactive in low CO2, where LCIB-associated CO2 uptake has a more significant role (Wang and Spalding, 2014b; Gao *et al*., 2015) [17, 2]

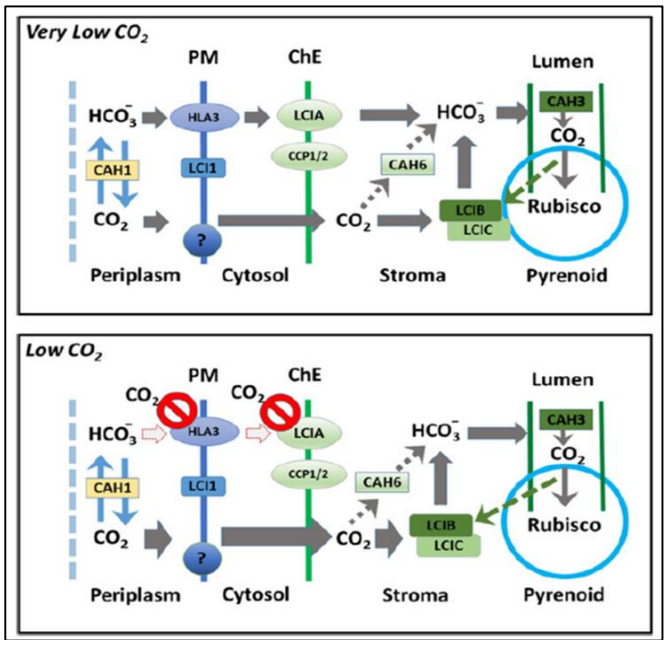

Fig 8: Figure 2. Schematic model of the Chlamydomonas CO_2 concentrating mechanism (CCM) in low CO_2 and very low CO_2 (Meyer *et al.*, 2017)

CCM in diurnal light/dark cycles- observations and mechanism

It has long been known that, in synchronized air-grown cells, the photosynthetic affinity for CO2 and Vmax increase and reach their peak during the light period, accompanied by increase in CA activity, but decline during the dark period. A recent study using synchronized air-grown cells with more detailed light–dark periods reported that photosynthetic Ci affinity increases even before the light period begins by 1 h before the end of the dark period (dawn), cells already exhibit a high CO2 affinity (low K½) similar to that of cells exposed to several hours of light and with a fully expressed CCM upregulated expression of LCIB and CAH3 begins and reaches nearly maximal levels toward the end of the dark period and before the light phase begins, while induc-tion of LCIA, HLA3, CCP1/2, CAH1 and LCI1 begins only after cells enter the light phase and reaches maximal expression levels after several hours of light. These diurnal changes in gene expression and protein localization may reflect an entrained regulation with different CO2 acclimation states. CO2 is probably more abundant in the culture during the dark period due to respiration, but the CO2 concentration should decline once photosynthesis is initiated by light, so cells probably experience a gradual decrease in CO2 con-centrations, from high CO2 to low CO2 and eventually to very low CO2 when photosynthetic activity reaches its peak. The synchronized cells appear to anticipate and 'pre-adapt' to this sequence by inducing early expression of 'low-CO2 acclimation' genes (LCIB, CAH3) and relocalizing Rubisco and CAH3 into pyrenoids before the light phase starts; Then, once cells are exposed to light and experience limiting CO2, they induce expression of genes responsible for very-low-CO2 acclimation (HLA3, LCIA, LCI1, etc.) (Meyer *et al.*, 2017).

Transcriptional and Post- Translational Regulation of CCM

Thus far, only two regulatory proteins, CIA5 (also known as CCM1) and LCR1, have been confirmed to regulate CCM-associated gene expression in Chlamydo-monas, and no proteins involved in post-translational reg-ulation of CCM function have yet been revealed. The CIA5 protein. The CIA5 protein is an extensively stud-ied master regulator controlling expression of the CCM. The amino acid sequence deduced from the identified CIA5 gene indicates that CIA5 is a hydrophilic protein with two predicted zinc-binding domains at its N-terminal region, a glycine repeat region characteristic of transcriptional activators and several putative phosphorylation sites near its C-terminus. Phosphorylation of specific CCM-associated proteins was only recently identified, such as phosphorylation of two thylakoid membrane proteins, LCI5 and UEP, during the transition from high CO2 to limiting CO2 Another example of post-trans-lational phosphorylation is that of CAH3, as mentioned ear-lier, CAH3 is phosphorylated and relocated into pyrenoids under conditions of limiting CO2 (Meyer *et al.*, 2017).

Summary and future perspectives

There have been major advances in our understanding, at the molecular, mechanistic, and regulatory level, of the CCMs of β - cyanobacteria. However, complete genome sequences for several α-cyanobacteria have shown that these organisms from the oligotrophic ocean lack many of the components of the β - cyanobacterial CCMs without flagging up alternatives. The β -cyanobacterial genomic data have also not helped significantly in establishing the molecular basis for eukaryote CCMs, e.g., in *Chlamydomonas reinhardtii*. Another recent significant advance is the revival, with better evidence, of the hypothesis that diatoms have a CCM resembling the C4 pathway of higher plants. This work on *Thalassiosira weissflogii* has not benefited as much as it might have from findings from the *T. pseudonana* genome project because we do not have a complete understand-ing of targeting sequences in diatoms to help establish the location of, for example, PEPck. Without playing down the advances that have been made, it is clear that much remains to be done to establish the mechanism(s), and regulation, of CCMs in many ecologically important groups of algae, such as the dino flagellates.

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