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REVIEWS

CO_2-Concentrating Mechanism and Its Traits in Haloalkaliphilic Cyanobacteria

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Abstract—Cyanobacteria are a group of oxygenic phototrophs that have existed for at least 3.5 Ga. Photosynthetic CO_2 assimilation by cyanobacteria occurs via the Calvin cycle, with RuBisCO, its key enzyme, having very low affinity to CO_2. This is due to the fact that atmospheric CO_2 concentration in Archaean, when the photosynthetic apparatus evolved, was several orders higher than now. Later, in the epoch of Precambrian microbial communities, CO_2 content in the atmosphere decreased drastically. Thus, present-day phototrophs, including cyanobacteria, require adaptive mechanisms for efficient photosynthesis. In cyanobacterial cells, this function is performed by the CO_2-concentrating mechanism (CCM), which creates elevated CO_2 concentrations in the vicinity of RuBisCO active centers, thus significantly increasing the rate of CO_2 fixation in the Calvin cycle. CCM has been previously studied only for freshwater and marine cyanobacteria. We were the first to investigate CCM in haloalkaliphilic cyanobacteria from soda lakes. Extremophilic haloalkaliphilic cyanobacteria were shown to possess a well-developed CCM with the structure and functional principles similar to those of freshwater and marine strains. Analysis of available data suggests that regulation of the amount of inorganic carbon transported into the cell is probably the general CCM function under these conditions.

Keywords: CO_2-concentrating mechanism (CCM), cyanobacteria, transport systems, carboxysomes, carbonic anhydrase, soda lakes

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CO_2 concentration in the immediate vicinity of the active centers of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is one of the adaptive mechanisms for efficient photosynthesis at low atmospheric CO_2 concentration in the present geological epoch [1]. For the mechanism of CO_2 concentration functioning in the cells of microalgae and cyanobacteria (CO_2-concentrating mechanism), abbreviation CCM is traditionally used.

Development of adaptive photosynthetic processes, including CCM, was necessary because the early photosynthetic apparatus, which was adapted to conditions of the ancient atmosphere with a high $[CO_2]/[O_2]$ ratio, became inefficient when the modern-type oxidative atmosphere with low $[CO_2]/[O_2]$ ratio was formed ca. 2 Ga ago.

In recent decades, studies on model cyanobacterial strains revealed that CCM function is based on combined operation of the systems for inorganic carbon (C_i) uptake and carbonic anhydrase (CA), the enzyme catalyzing interconversion of CO_2 and HCO_3^- [2–5]. CCM also requires co-localization of CA and RuBisCO in carboxysomes, resulting in decreased CO_2 leakage from the cells. CCM provides elevated CO_2 concentrations in the vicinity of RuBisCO active centers, which significantly increases the efficiency of C_i fixation in the Calvin cycle.

CCM operation in haloalkaliphilic cyanobacteria from soda lakes is of special interest. The environment in which these photosynthetic prokaryotes live contain high concentrations of bicarbonate. Theoretically, CO_2 concentration is not required under such conditions. Thus, detection of the functionally active CCM components in the cells of haloalkaliphilic cyanobacteria may contribute to our knowledge on various aspects of the functioning of this mechanism.

CYANOBACTERIA—OXYGENIC PHOTOSYNTHETICS. RUBISCO-BASED CLASSIFICATION OF CYANOBACTERIA

The structure of cyanobacterial photosynthetic apparatus and the principles of its operation are well-studied [6].

The electron donor for oxygenic phototrophy is H_2O, which is oxidized to O_2. Electron transport from photosystem (PS) II to the PS I reaction center results in generation of a proton gradient, which is used for

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ATP synthesis by ATPase. Via the system of electron transporters, electrons are transferred from PS I reaction center to $NADP^+$. Thus, the outcome of two photochemical reactions is the generation of $NADPH_2$ and ATP, which are then used for CO_2 fixation.

Photoassimilation of CO_2 by cyanobacteria is carried out via the Calvin cycle, in which three ribulose-1,5-bisphosphate molecules are carboxylated by RuBisCO to form six molecules of 3-phosphoglycerate. This is followed by a series of enzymatic conversions, including formation of six molecules of glyceraldehyde-3-phosphate, one of which is consumed for intracellular biosynthesis and the other five are used for regeneration of three acceptor molecules. Thus, oxygen evolution and CO_2 fixation are stoichiometric.

RuBisCO may also bind O_2, acting as an oxygenase and catalyzing ribulose-1,5-bisphosphate oxidation at the first stage of photorespiration. CO_2 and O_2 are therefore competing substrates for RuBisCO. The balance between photosynthesis and photorespiration is determined by two factors: (1) RuBisCO specificity factor ($S_{C/O}$), which accounts for affinity of the enzyme to both substrates and the maximal rates of carboxylation and oxidation, and (2) the ratio of CO_2 and O_2 concentrations in the vicinity of an operating enzyme [5, 7].

All known RuBisCO and RuBisCO-like proteins fall into four forms, of which only forms I and II are involved in the Calvin cycle [8]. Cyanobacteria contain form I of RuBisCO, which consists of eight large (L) and eight small (S) subunits (~55 and ~15 kDa, respectively) [9]. Molecular mass of the native enzyme is ~500–570 kDa.

Form I of RuBisCO, depending on the primary structure of its large subunits, may be subdivided into four subforms: IA–ID [9, 10], of which only two (IA and IB) have been found in cyanobacteria. All cyanobacteria therefore form two major groups: α-cyanobacteria with IA RuBisCO (mainly marine species) and β-cyanobacteria with IB RuBisCO (freshwater and estuarine species) [11].

In cyanobacterial cells, RuBisCO is localized in specialized microcompartments, carboxysomes [12]. These are polyhedral bodies up to 400 nm in diameter and covered by a thin proteinaceous shell. Besides the RuBisCO, carboxysomes contain carbonic anhydrase (CA), which catalyzes the CO_2/HCO_3^- mutual conversion. CA is important for the functioning of the Calvin cycle, since it supplies CO_2, the substrate for ribulose-1,5-bisphosphate carboxylation [13].

Cyanobacteria with different RuBisCO forms were found to possess different sets of genes encoding carboxysome proteins, which are structurally or functionally analogous [14]. Carboxysomes of α- and β-cyanobacteria are termed α- and β-carboxysomes, respectively. The type of RuBisCO and carboxysomes does not provide any physiological advantage; their parallel existence is considered an instance of convergent evolution [15].

EVOLUTION OF THE COMPOSITION OF THE ATMOSPHERE. BACKGROUND FOR DEVELOPMENT OF THE CO_2-CONCENTRATING MECHANISM

It is generally accepted that in the Archean Eon, CO_2 content in the atmosphere was several orders higher than now, while oxygen was practically absent [16]. These were the conditions for which the early model of the photosynthetic apparatus was well adapted.

Global changes in the composition of the atmosphere occurred in the Precambrian Supereon. Activity of cyanobacterial communities resulted in a drastic decrease in CO_2 concentration, accompanied by an increase in oxygen concentration [17]. These changes resulted in formation of the modern oxidative type of the atmosphere approximately 2 Ga ago.

Under these conditions, cyanobacteria had to maintain the efficiency of their photosynthesis. Due to low affinity of RuBisCO to CO_2, decreased carbon dioxide concentration in the atmosphere limited photosynthetic production dramatically [18]. Moreover, increased O_2 partial pressure resulted in suppression of the carboxylase function of RuBisCO, while enhancing its oxygenase function. Apart from this, cyanobacteria, being aquatic photosynthetic organisms, experienced difficulties due to decreased CO_2/O_2 diffusion rates in their environment.

Two strategies employed by oxygenic photosynthetic organisms under CO_2 limitation are presently known. The first one implies increased content of RuBisCO, the key enzyme of the Calvin cycle, and its increased affinity to CO_2. This strategy is used by C_3 higher plants, in which K_m (CO_2) for RuBisCO is about 8–14 μM [18, 19].

The second strategy implies increased intracellular CO_2 concentration in the vicinity of RuBisCO active centers. C_4 and CAM plants with K_m (CO_2) for RuBisCO of 16–30 and 9.5–10.5 μM, respectively, use different mechanisms of CO_2 concentration [19, 20]. CCM, the third variant of this strategy, is used by microalgae and cyanobacteria (C_3 type of metabolism) [4, 21].

In most studied cyanobacteria K_m (CO_2) for RuBisCO varies from 200 to 300 μM [18, 19]. This is much higher than the CO_2 concentration achieved by its passive diffusion from the environment. Thus, in most lakes and oceans CO_2 concentration does not exceed 15 μM [2]. Moreover, compared to other photosynthetic organisms, cyanobacterial RuBisCO has low rates of enzymatic activity not exceeding 11–13 carboxylation acts per second [19]. CCM, however, makes it possible for them to carry out productive pho-

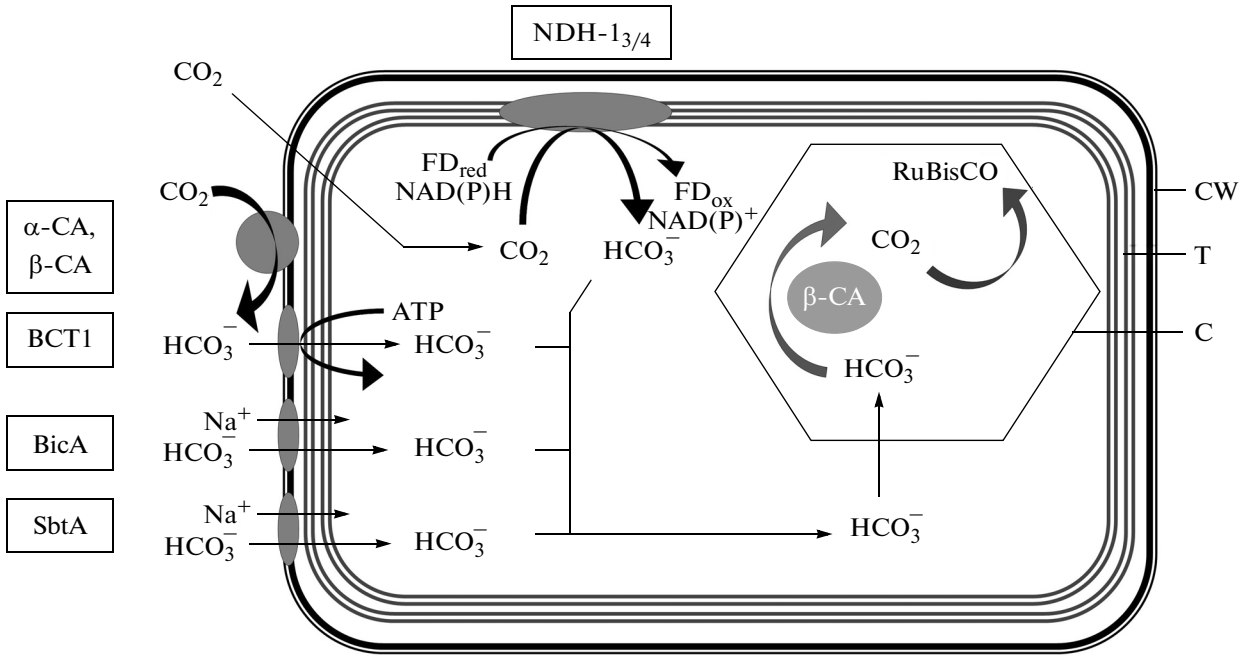

Fig. 1. General scheme of cyanobacterial CCM operation. The scheme is based on the literature data obtained for the classical model organisms: freshwater and marine cyanobacteria *Synechococcus elongatus* PCC7942, *Synechocystis* sp. PCC6803, and *Synechococcus* sp. PCC7002. Designations: BCT1, BicA, and SbtA, bicarbonate transporters; NDH-$1_{3/4}$, CO_2 uptake systems; CW, cell wall; T, thylakoids; C, carboxysome; and CA, carbonic anhydrase.

tosynthesis even more efficiently than in C_4 plants [22].

CO_2-CONCENTRATING MECHANISM OF CYANOBACTERIA: COMPONENTS AND PRINCIPLE OF OPERATION

Nowadays, the principle of CCM operation has been well-studied on model cyanobacterial strains [2]. These studies revealed cyanobacterial CCM comprise the following structural and functional components (Fig. 1): (1) systems for inorganic carbon (C_i, CO_2 + HCO_3^-) uptake; (2) the carbonic anhydrase system, which is responsible for conversion of C_i forms and thus providing the substrate (CO_2) for carboxylation of ribulose-1,5-bisphosphate by RuBisCO; and (3) carboxysomes, specialized microcompartments increasing the efficiency of CO_2 fixation in the Calvin cycle.

Unlike the mechanisms for CO_2 concentration in C_4 and CAM plants, CCM is inducible and is activated in response to a decrease in ambient C_i content. At the same time, basic level of CCM activity supporting the photosynthesis is present even in the absence of C_i limitation. This basic CCM activity is carried out by its constitutive components. Thus, in this case C_i transport into the cells is carried out only by low-affinity transporters.

Decreased intracellular C_i pool (presumably below 5 mM) is a signal for CCM induction. This process is light-dependent [3]. An increase in intracellular oxygen level, as well as in photorespiration activity, can also participate in CCM induction. This hypothesis, however, has not been strictly confirmed.

The CCM acts as follows. After transfer of the cells to photosynthesis-limiting conditions, C_i uptake by newly synthesized high-affinity transporters is activated. An intracellular C_i is accumulated as HCO_3^-, due to slightly alkaline pH in the cytoplasm. The concentrations of C_i accumulated in the cells may reach 20–40 mM, i.e., up to 1000 times higher than in the medium [3]. Bicarbonate is then converted to CO_2, the substrate for RuBisCO, in the immediate vicinity of its active centers. In cyanobacteria, RuBisCO is localized inside carboxysomes. CA, the enzyme catalyzing conversion of HCO_3^- to CO_2, is located in the same microcompartment. Co-localization of RuBisCO and CA has physiological importance, since it prevents spontaneous CO_2 leakage [4]. Recently, C_i limitation was shown to result in "maturation" of carboxysomes. The latter become covered with a proteinaceous shell 3–6 nm thick, which also decreases CO_2 loss [23]. Moreover, since carboxysome shell is impenetrable to oxygen, this also promotes carboxylation of ribulose-1,5-bisphosphate by preventing photorespiration [24].

The structural and functional components of CCM found and characterized in model cyanobacterial strains are described below.

Transport of Inorganic Carbon into Cyanobacterial Cells

C_i sources for transport into the cell. In water systems, C_i is represented by three forms, existing in an equilibrium:

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+.$$

The ratio of C_i forms depends on the ambient pH and is determined from the Henderson–Hasselbalch equation:

$$pH = 6.3 + \log([HCO_3^-]/[CO_2]),$$

$$pH = 10.3 + \log([CO_3^{2-}]/[HCO_3^-]).$$

Thus, HCO_3^-, predominates in the solution at pH from 6.3 to 10.3, while at pH below 6.3 and above 10.3, CO_2 and CO_3^{2-}, respectively, are the dominant forms.

In general, the Henderson–Hasselbalch equation is adequate for freshwater environments. It can not be used directly for marine, hypersaline, and soda water systems, since under these conditions the total salinity of the environment affects pH and the ratio of C_i forms [25].

It is presently universally accepted that cyanobacterial cells consume C_i as CO_2 and HCO_3^- [2, 3]. The possibility of direct transport of CO_3^{2-} into the cell has been considered previously [26]. This suggestion was based on the correlation between C_i consumption at different pH values and the CO_3^{2-} calculated from the ratio of C_i forms at specific pH values for *Synechocystis* PCC 6803. Existence of the systems for active CO_3^{2-} uptake into cyanobacterial cells has not, however, been confirmed.

Systems for C_i transport. Since the CO_2 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 CO_2 uptake systems, which convert CO_2 to the charged bicarbonate molecule, which is insoluble in lipids. Due to this insolubility, the inflow of exogenous HCO_3^- is possible only via active transport.

Five transport systems (TS) for C_i are known in cyanobacteria, including three bicarbonate transporters and two systems for CO_2 uptake [3]. Specificity of these TS for both C_i forms was demonstrated using the inhibitors that selectively suppress transport of CO_2 (COS, H_2S, Na_2S, and ethoxyzolamide) or HCO_3^- (Li^+ and monensin), as well as in the experiments with the mutant strains with impaired systems of CO_2 or HCO_3^- uptake [27, 28].

The functional features of these TS are described below (Fig. 1):

(1) BCT1 is an inducible, high-affinity TS for HCO_3^-, the first TS for C_i described in cyanobacteria [29]. In *Synechococcus* PCC7942, its affinity to the substrate (K_m (HCO_3^-)) is ~15 μM. 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. This transporter family is also known as traffic ATPases. BCT1 is encoded by the *cmpABCD* operon, and its synthesis is induced by acute C_i limitation. The *cmpABCD* genes encode four proteins of BCT1: CmpA (a periplasmic protein responsible for specific HCO_3^- binding), CmpB (a hydrophobic protein present in the membrane as a dimer capable of forming an ion channel), as well as CmpC and CmpD (large and small cytoplasmic proteins with ATP-binding sites). Operation of the BCT1 transporter was described in detail in the review [3].

BCT1 is present mostly in freshwater β-cyanobacterial strains. The few species of marine α-cyanobacteria possessing the *cmpA–D* probably acquired them via horizontal gene transfer [3].

(2) SbtA is an inducible, high-affinity Na^+-dependent TS for HCO_3^-, with K_m (HCO_3^-) ~5 μM, which was originally described for *Synechocystis* PCC6803 in 2002 [30]. SbtA was supposed to act as a Na^+/HCO_3^- 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 [3, 31]. Most of β-cyanobacteria possess proteins exhibiting high homology to SbtA of *Synechocystis*. The genomes of α-cyanobacteria contain the genes encoding proteins with low homology to SbtA; their function as bicarbonate transporters has not been confirmed.

(3) BicA is a constitutive low-affinity Na^+-dependent TS for HCO_3^-. BicA is the most recently discovered [32] but probably the most common cyanobacterial HCO_3^- transporter. Similar to SbtA, BicA probably carries out Na^+/HCO_3^- symport. While this TS has relatively low affinity to the substrate (from 74 μM in *Synechococcus* WH8102 to 353 μM in *Synechocystis* PCC6803), 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. The BicA homologues are present in most α- and β-cyanobacteria, which often contain more than one gene encoding the relevant protein [2].

(4, 5) NDH-1_4 is a constitutive low-affinity system for facilitated CO_2 uptake; NDH-1_3 is an inducible high-affinity system for facilitated CO_2 uptake [33, 34]. These are not true transporters, since their opera-

tion does not result in CO_2 transfer through the membrane (Fig. 1). They are, however, described among the transport systems in the literature.

The NDH-$1_{4/3}$ systems represent a modified NADPH dehydrogenase multienzyme complex located in the thylakoid membranes. According to some data, NDH-1_4 may be also located in the cytoplasmic membrane, at least in the case of *Gloeobacter*, which has no thylakoids [3, 35]. These systems are common in both α- and β-cyanobacteria. In *Synechococcus* PCC7942, their affinity to the substrate (CO_2) is ~10 μM for NDH-1_4 and 1–2 μM for NDH-1_3.

Importantly, the operation of NDH-$1_{4/3}$ is supported by the functioning of their constituent CA-like proteins (ChpX/Y), which are probably responsible for unidirectional CO_2 hydratation. Their role in prevention of CO_2 leakage from the cell has also been considered [3]. Conversion of CO_2 to HCO_3^- is probably associated with the operation of the photosynthetic electron transport chain and with proton translocation via the membrane into the thylakoid lumen. The detailed hypothetical scheme of NDH-$1_{4/3}$ operation is presented in the review [36].

Cyanobacterial Carbonic Anhydrases

The CA system (EC 4.2.1.1) is another component of cyanobacterial CCM. CAs are metal-containing (Zn/Cd/Fe) enzymes catalyzing mutual conversion of two C_i forms: $CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$. The $[CO_2]/[HCO_3^-]$ ratio obeys Henderson–Hasselbalch equation; the equilibrium of these forms occurs at pH 6.3.

Based on mutual homology of their amino acid sequences, all presently known CAs may be subdivided into five independent classes: α, β, γ, δ, and ζ [37]. These classes are evidently a case of convergent evolution of the catalytic function. The catalytic mechanisms of most CAs are absolutely identical and occur in two stages [37]. The first one is nucleophilic CO_2 attack on the hydroxide ion bound to a metal (Me) in the active center of the enzyme (E) and its conversion to bicarbonate:

$$E{-}Me^{2+}{-}OH^- + CO_2 \leftrightarrow E{-}Me^{2+}{-}HCO_3^-,$$

$$E{-}Me^{2+}{-}HCO_3^- + H_2O \leftrightarrow E{-}Me^{2+}{-}H_2O + HCO_3^-.$$

At the second limiting stage, the active form of the enzyme is restored with proton transfer to an external buffer (B):

$$E{-}Me^{2+}{-}H_2O \leftrightarrow H^+{-}E{-}Me^{2+}{-}OH^-,$$

$$H^+{-}E{-}Me^{2+}{-}OH^- + B \leftrightarrow E{-}Me^{2+}{-}OH^- + BH^+.$$

Cyanobacteria possess α-, β-, and γ-CAs with Zn^{2+} in the active center of the enzyme [37].

The only α-CA characterized in cyanobacteria is the EcaA protein. Localization of this protein in the outer cell layers was shown for *Anabaena*. While EcaA contains all amino acids characteristic of the α-CAs active site, its functional activity has not been confirmed. Moreover, enhanced *ecaA* expression at high ambient C_i levels imply that this CA does not have a role in the CCM.

Several β-CAs have been identified in cyanobacteria. Similar to the α-CA EcaA, the EcaB protein is an external β-CA with unconfirmed catalytic activity. The *Synechocystis* PCC6803 mutant with a deletion at the *ecaB* gene exhibited the growth rates practically identical to those of the wild type under both elevated and decreased C_i content in the medium. Similar to EcaA, this CA is probably not involved in CCM.

Two other β-CAs are associated with carboxysomes, where they are involved in bicarbonate conversion to CO_2 for RuBisCO. These are CsoSCA in α-cyanobacteria and CcaA in β-cyanobacteria. CcaA is bound to the internal side of the β-carboxysome shell and it is a part of a multiprotein complex responsible for bicarbonate conversion to CO_2 and its fixation [38]. Apart from CcaA, this complex includes RuBisCO, CcmN, and CcmM proteins.

The CcmM protein is an active γ-CA in the species that have lost the homologues of *ccaA* gene [39]. Unlike CcaA, CcmM is present in all β-cyanobacteria. CcmM is encoded by the relevant gene of the *ccmKLMN* operon located in immediate vicinity of the *rbcLS* operon encoding RuBisCO subunits. Other *ccm* genes code for the proteins for β-carboxysome shells. CcmM is a bifunctional protein with the N-terminal domain homologous to the Cam protein (γ-CA) from the archaeon *Methanosarcina thermophila*. The C-terminal domain of CcmM contains three to five repeats of the RuBisCO small subunit [38]. In the case when CcmM is not a catalytically active CA, this protein is considered responsible for regulation of the activity of carboxysome multiprotein complex by binding the substrate HCO_3^-.

Thus, the physiological role of bacterial CAs depends on their localization in the cell. The function of carboxysomal CAs was discussed above. The role of active external cyanobacterial CAs (which will be mentioned below) may be related to conversion of CO_2 to HCO_3^- for TS or to prevention of CO_2 leakage from the cell, similar to the ChpX/Y proteins of the NDH-$1_{4/3}$ systems.

Carboxysomes

Carboxysomes are proteinaceous cytoplasmic structures 100–200 nm in diameter [12]. They have been found in all known cyanobacteria. Carboxysome proteins may be encoded by two different groups of genes. The same applies to the RuBisCO subform and

carboxysome CA, associated with a specific type of carboxysomes. Different types of carboxysomes are termed α and β. Similarly, cyanobacteria with α- and β-carboxysomes are termed α- and β-cyanobacteria, respectively (see above).

The proteins of two types of carboxysomes are structural or functional analogues. Molecular structure of both carboxysome types is presently relatively well-studied. Details may be found in the review [14].

As was already stated above, the main role of carboxysomes in CCM is to enhance the efficiency of CO_2 fixation in the Calvin cycle, which can be achieved due to specific structural features of these microcompartments [40].

Carboxysomes are covered with a shell 3–6 nm thick, containing a number of proteins which form a generally polyhedral structure. Some of the protein globules form pores, which are capable of selective influx of bicarbonate molecules. CAs converting HCO_3^- to CO_2 (the substrate for RuBisCO) are associated with the proteinaceous shell. Internal matrix of carboxysomes is filled with RuBisCO. Unlike α-carboxysomes, in β-carboxysomes RuBisCO is associated with one of the structural proteins.

Carboxysomes contain almost all cellular RuBisCO; the content of this enzyme in these microcompartments is up to 60% of the total protein content [41]. Since carboxysome shell is impenetrable for uncharged CO_2 and O_2 molecules, carbon dioxide is concentrated close to RuBisCO active centers, while oxygen does not interfere with the carboxylase activity of this enzyme. Such organization of carboxysomes provides for most efficient CO_2 fixation.

The structure and number of carboxysomes in the cell depends on the ambient C_i concentration. The cells grown at high C_i concentrations have loose, irregularly shaped carboxysomes, while at C_i limitation carboxysomes become denser and larger, and their number increases. Changes in carboxysome structure after transfer of the cells to photosynthesis-limiting conditions are associated with their "maturation" [23]. The latter implies synthesis of the shell proteins and their assembly around procarboxysomes (the internal content of these microcompartments).

Variability in the Composition of CCM Components

As a rule, a cyanobacterial cell does not possess all the CCM components listed above. This applies primarily to the type of carboxysomes (α- or β-) and of associated RuBisCO form (IA or IB) and carboxysomal CA type (CsoSCA or CcaA/CcmM, respectively). It was recently shown that a specific carboxysome type, as well as specific content, provides no physiological advantages [15]. This is not the case for the TS present in the cell. Their composition may vary depending on environmental conditions in order to ensure the maximum efficiency of photosynthesis [35].

In general, the following pattern may be observed: freshwater and estuarine cyanobacteria usually have the highest number of TS types, while marine and oceanic species have the minimal required number. Thus, analysis of available full-size cyanobacterial genomes revealed that the BCT1 (TS for bicarbonate) was present mainly in freshwater β-cyanobacteria [2]. This was probably due to the fact that application of the Na^+ electrochemical gradient, rather than of ATP required for BCT1 operation, is preferable for C_i consumption in marine environments. Moreover, since HCO_3^- concentration in marine environments is relatively high, the cell does not require highly efficient, energy-consuming TS. Indeed, analysis of the genomes of a number of marine α-cyanobacteria confirmed that practically no strains possessed inducible, high-affinity systems for C_i uptake [2].

Cyanobacteria apply the strategy of mutually complementary TS to achieve more efficient C_i uptake. The cells may have several transporters for each C_i species, using TS pairs with complementary kinetic characteristics [35]. For instance, *Synechococcus* PCC7002 has two TS for HCO_3^-: BicA with relatively low affinity supporting high transport rate and high-affinity SbtA supporting a lower rate [2]. Together these TS provide the affinity of about 6.5 μM and a high rate of transport. This applies for the systems of CO_2 uptake: NDH-1_4 and NDH-1_3 of *Synechococcus* PCC7942 have different kinetic characteristics, providing the overall affinity to CO_2 of ~0.8 μM and a high transport rate.

CO_2-CONCENTRATING MECHANISM IN HALOALKALIPHILIC CYANOBACTERIA

The CCM structure and function described above have been established in the studies on freshwater and marine strains [2]. However, continental alkaline lakes, including soda lakes, are natural habitats for numerous groups of cyanobacteria. These environments are of special interest, since they are considered to be analogous to ancient continental lakes—putative centers of prokaryotic diversity which emerged during the early stages of biosphere development. Modern soda lakes may act as refugiums (centers of preservation) for the relict microorganisms [42]. In this type of lakes, haloalkaliphilic cyanobacteria are the major primary producers.

Hydrochemical Properties of Soda Lakes and Ecophysiology of Soda Lake Cyanobacteria

Hydrochemical characteristics of alkaline lakes are the primary factors determining the specific conditions of these environments. Stable alkaline pH values

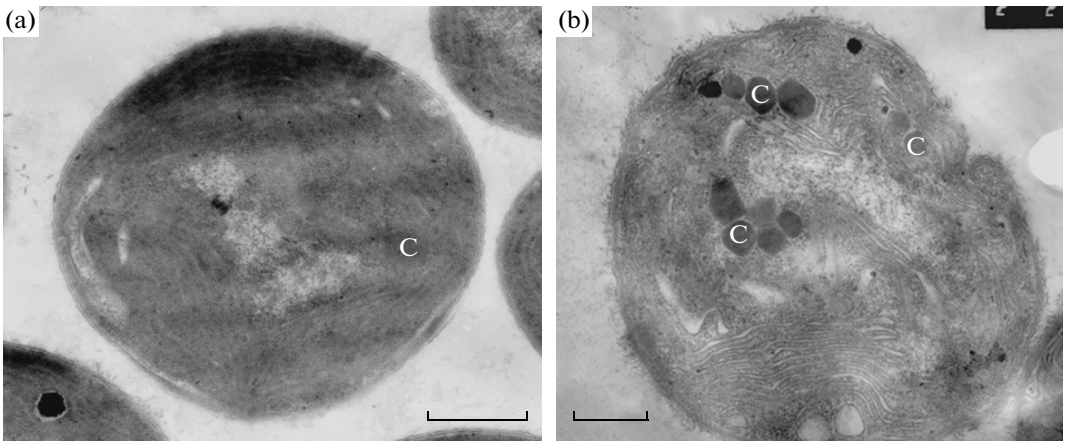

Fig. 2. Carboxysomes in *'E. natronophila'* (transmission electron micrographs were obtained by A.G. Markelova, Timiryazev Institute of Plant Physiology, Russian Academy of Sciences) grown in the standard medium with 1 M Na_2CO_3 (a) and on the third day after transfer into the medium with 0.5 M Na_2CO_3 (b). "C" indicates carboxysomes. Bar—0.5 mkm.

usually result from high concentration of C_i in two major forms: HCO_3^- and CO_3^{2-}. The lakes with soda type water, with HCO_3^- and CO_3^{2-} as the dominant anions at concentrations which may reach saturation, are the extreme example.

Total salinity, alkalinity, and pH are interrelated hydrochemical parameters, which undergo periodic variations, e.g., in the course of seasonal salinization/desalination cycles.

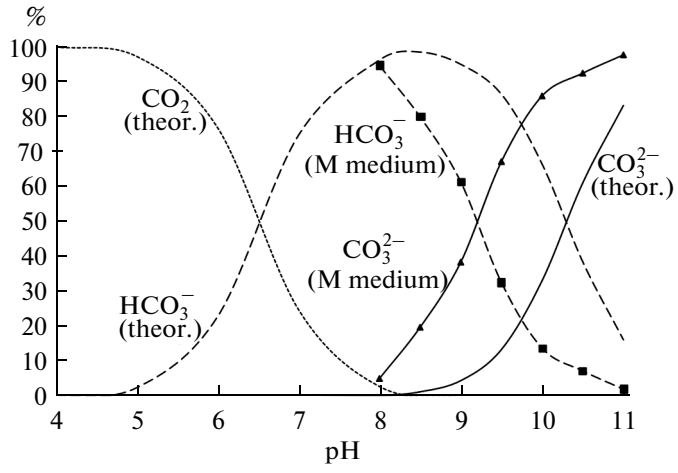

Fig. 3. Concentrations of C_i forms depending on pH: theoretical distribution according to the Henderson–Hasselbalch equation for water solutions compared to the distribution in the brine simulating the water composition of a soda lake; theoretically calculated curves (theor., indicating the values correct for distilled water and applicable for freshwater) and titrimetric determination of CO_3^{2-} and HCO_3^- concentrations in a soda solution containing 1 M (CO_3^{2-} + HCO_3^-) and 0.86 M NaCl (M medium).

In carbonate solutions, pH depends on the ratio of CO_3^{2-} and HCO_3^-, ions, as well as on the total salinity [25], and do not directly comply with the Henderson–Hasselbalch equation. At a given pH, HCO_3^- concentration in a concentrated solution is considerably lower than the calculated value (Fig. 3).

Despite the seasonal changes in alkaline lakes, CO_3^{2-} and HCO_3^- remain the dominant anions in these environments. In soda lakes, these anions prevail. Thus, it seems unlikely that cyanobacteria developing under such conditions require CO_2 concentration. Such organisms are therefore of interest for investigation of the presence and functioning of the CCM components.

CCM was studied in three strains of haloalkaliphilic cyanobacteria: planktonic unicellular *Rhabdoderma lineare* Z-9404 (IPPAS B-354) and *'Euhalothece natronophila'* Z-M001 from Lake Magadi [43; 44], as well as benthic filamentous cyanobacteria *Microcoleus chthonoplastes* IPPAS B-353 from the alkaline Lake Khilganta [45].

Lake Magadi (Kenya) is a classical ultrasaline soda lake with stable high pH (10–11) resulting from high carbonate alkalinity. Carbonates (with predominance of the CO_3^{2-} form) constitute up to 80% of the anions. During the rainy season, salinity of the lake decreases to some degree, and massive bloom of cyanobacteria occurs. The ecophysiological properties of *'Euhalothece natronophila'*, which grows in a saturated Na_2CO_3 + NaCl solution (an obligate extreme haloalkaliphile and natronophile), are comparable to those of organotrophic natronobacteria—the archaea considered the *nec plus ultra* extremophiles [44]. *R. lineare* is a moderate haloalkaliphile and natronophile [43].

The alkaline soda Lake Khilganta (Southeastern Transbaikalia) has water of the chloride–sulfate sodium type with elevated C_i content. The lake is

Table 1. Properties of bicarbonate transport systems (TS) of an extremely natronophilic cyanobacterium *'E. natronophila'*

Parameter	TS I	TS II	TS III
pH_{opt}	8.5	9.4–9.5	9.9–10.2
Saturation at illumination	ND	>10 klx	<10 klx
The concentration of carbonates for V_{max}, M	~0.002	0.15	≥1
V_{max}, μmol HCO_3^-/(min mg protein)	ND	0.014–0.025	0.035–0.077
K_m (HCO_3^-), mM	0.8–1	13–17	600–800
Na^+ requirement	ND	++	+/–

Designations: pH_{opt} is the pH at which operation optima of TS is observed; V_{max} is the maximum rate of bicarbonate assimilation. "++" and "+/–" stand for pronounced dependence and weak or absent dependence, respectively. ND stands for no data.

characterized by periodic humidification and drying up. Observation for over ten years revealed considerable variations in the chemical composition of lake water, with total salinity from 40 to 260 g/L, pH from 9.9 to 7.1, CO_3^{2-} concentration from 0 to 0.33 g/L, and HCO_3^- concentration from 0.14 to 1.16 g/L [46]. During the humidification period, a *M. chthonoplastes*-dominated cyanobacterial mat develops. This organism belongs to moderate haloalkaliphiles [45].

Thus, these three cultures of haloalkaliphilic cyanobacteria represent the transition from moderately haloalkaliphilic to extremely natronophilic cyanobacteria. These organisms were found to contain the functionally active CCM components. For *R. lineare*, accumulation of the intracellular bicarbonate pool at low C_i concentration in the medium was demonstrated by direct measurements. These findings indicate that haloalkaliphilic cyanobacteria possess a functional mechanism for CO_2 concentration.

Bicarbonate Transport Systems in the Natronophilic Cyanobacterium 'E. natronophila'

TS of haloalkaliphilic cyanobacteria have been studied only in the natronophilic strain *'E. natronophila'* [47–49].

In this organism, at least three transport systems are responsible for bicarbonate transport (TS I, II, and III); their properties are summarized in Table 1. The kinetic characteristics of these TS determined using the Lineweaver–Burk equation were as follows: TS I with the optimum at pH 8.5 has K_m (HCO_3^-) ≈ 0.8–1 mM, i.e., the highest affinity to bicarbonate; TS II with the optimum at pH 9.4–9.5 has intermediate affinity to bicarbonate (K_m (HCO_3^-) ≈ 13–17 mM), and TS III with the optimum at pH 9.9–10.2 was characterized by low affinity to the substrate (K_m (HCO_3^-) ≈ 600–800 mM).

The bicarbonate TS found in '*E. natronophila*' have the properties radically different from those of freshwater and marine cyanobacteria, for which affinity to the substrate varies from 5 to 170 μM [3]. This is evidently the result of relatively high C_i concentration in soda lakes, which in Lake Magadi may reach 300 g/L Na_2CO_3 + $NaHCO_3$. In marine ecosystems, C_i concentration does not exceed 2 mM [2]; this value for freshwater environments is even lower.

C_i transport is a light-dependent process. TS II and TS III differ in their relation to light intensity. For TS III, light saturation occurs at lower illumination than for TS II [47]. At pH 10.5 (TS III) and 9.3 (TS II), a 15-fold increase in illumination (from 2 to 30 klx) results in the 1.9 ± 0.2 and 3.6 ± 0.7 times increase of bicarbonate assimilation, respectively [47].

'*E. natronophila*' requires Na^+ ions (Table 1), particularly for the functioning of Na^+/H^+ antiporter NhaS3, which is involved in maintenance of the intracellular pH. The functioning of NhaS3, together with the specialized Mnh NDH-1 complex, results in formation of Na^+ electrochemical gradient, which is used for bicarbonate transport into the cell [3]. The TS of *'E. natronophila'* differ in their Na^+ requirements. Thus, the activity of TS II increases with the increase of sodium concentration. On the contrary, TS III activity might be Na^+-independent, since it retained activity in the absence of ambient Na^+ [49].

Measurement of the rates of bicarbonate assimilation by intact '*E. natronophila*' cells under selective conditions revealed inhibition of TS II activity by high substrate concentrations, exceeding 20 mM CO_3^{2-} + HCO_3^-. The kinetics of this process indicates the inhibition mechanism with partial preservation of the transport activity [48]. This effect points to regulation of the rate of C_i transport into the cell.

The presence of three TS with different properties in '*E. natronophila*' may be explained by the fact that inhabitants of soda environments, especially ephemeral ones, encounter fluctuations of pH and C_i concentrations during periodic desalination over the rainy periods. During the period of maximum desalination, C_i concentration decreases significantly, and pH may

consequently shift to more acidic values. Under these conditions, TS I and TS II, which have lower pH optima and higher affinity to the substrate than TS III, become the major transport systems. During the dry period, bicarbonate concentration in the lake increases, which is accompanied by an increase in pH. TS II and TS III (in conditions of maximal salinization) become more efficient (Table 1).

Carbonic Anhydrases and Carboxysomes of Haloalkaliphilic Cyanobacteria

Electrometric measurements of the rates of proton concentrations change in the reaction of CO_2 hydration revealed the presence of active CAs in haloalkaliphilic cyanobacteria *M. chthonoplastes* [50, 51], *R. lineare* [52], and '*E. natronophila*' [48, 49]. Specific activity of the enzyme in *R. lineare* was observed in intact cells (0.24 WAU/mg protein, Wilbur-Anderson units/mg protein) as well as in the cell homogenate (1.71 WAU/mg protein). In *M. chthonoplastes* CA activity was observed only in intact cells (0.238 WAU/mg protein) and in the cell envelope fraction (0.318 WAU/mg protein). Since CA inhibition by cell metabolites could occur in the cell homogenate, the absence of its activity determined under such conditions require careful consideration.

The intact cells of '*E. natronophila*' exhibited negligible CA activity; thus the presence of active enzyme could not be confirmed unequivocally.

Immunological analysis revealed that the investigated haloalkaliphilic cyanobacteria possess of at least two CA classes, α and β, with different subcellular localization.

The cells of *M. chthonoplastes* contain α- and β-CAs located in the cell envelope [50]. The α-enzyme was located in the glycocalix (exopolysaccharide sheath) of the *M. chthonoplastes*. The *cahB1* gene encoding one of the external β-CA was recently cloned [51], and high specific CA activity (~53.5 WAU/mg protein) was confirmed for the relevant recombinant protein. This is presently the first and only detailed characterized CA from a haloalkaliphilic cyanobacterium.

Similar to *M. chthonoplastes,* α-CA of *R. lineare* is localized in the glycocalix of the cell envelope. Apart from this external CA, the cells of *R. lineare* contain two intracellular enzymes of the β class: a constitutive CA associated with PS II of the thylakoid membranes and soluble CA induced by low C_i concentrations and probably located in carboxysomes [52]. Since emergence of this inducible enzyme correlated with accumulation of the intracellular bicarbonate pool in *R. lineare*, this β-CA is probably involved in CCM functioning.

In '*E. natronophila*' only the β-CA induced by decreased ambient C_i concentration was detected. Its intracellular localization is presently unknown. The presence of α-CA in this cyanobacterium was not revealed by immunological techniques (Western blot analysis) [48, 49]. It should be noted, however, that immunological identification alone is often insufficient and requires confirmation at the genetic level. Thus, these results should not be considered as final.

Prevention of CO_2 leakage from cells and its conversion to HCO_3^-, may represent the physiological role of the active external CAs, similar to the function attributed to CA-like proteins ChpX/Y of the NDH-$1_{4/3}$ systems [2, 3]. CAs do not require energy equivalents and therefore may support the function of the NDH-$1_{4/3}$ systems "free of charge." The presence of the enzyme at the internal side of the cytoplasmic membrane seems quite rational, since CO_2 leakage into the periplasmic space leads to additional energy expenditure for C_i uptake.

Existence of an additional (CA-dependent) mechanism preventing CO_2 leakage may seem relevant only for inhabitants of freshwater and marine environments with low concentrations of exogenous C_i, while the need for such mechanism in soda lakes with naturally high C_i concentrations is not self-evident. It may, however, be preferable for haloalkaliphiles to maintain several intracellular systems for CO_2/HCO_3^-, interconversion, rather than to spend energy for C_i uptake to compensate the leakage. This economy may be explained by the low K_m (HCO_3^-) values of their TS, resulting in the paradoxical C_i limitation.

The functional importance of external CAs in haloalkaliphilic organisms is confirmed by their high activity, determined on the example of recombinant β-CA CahB1 from *M. chthonoplastes* [51]. Importantly, the highest CahB1 activity was observed at high pH (similar to the ambient values). For freshwater and marine cyanobacteria, specific activity of external CAs has not been confirmed.

The external CAs located at the outer side of the periplasmic membrane (including the CA located in the glycocalix) may function as a secondary trap for leaked CO_2, converting it to bicarbonate for a new round of uptake by transporters. Since TS operation requires energy, this mechanism may be important for benthic cyanobacteria or cyanobacteria with a thick exopolysaccharide layer. Such species may encounter a deficiency of exogenous C_i due to its hampered diffusion. Accumulation of photosynthetic O_2 in the immediate vicinity of the cell, resulting in enhanced RuBisCO oxygenase activity, may result in further complications, as well as mineralization of the cells often occurring in alkaline lakes.

Participation in stabilization of the intracellular pH, which is highly important under extreme conditions in soda lakes, may be another possible function of external CAs. All the versions concerning the physiological role of external CAs in haloalkaliphilic

cyanobacteria are, however, hypothetical and require experimental confirmation.

The role of the potential carboxysomal CA of *R. lineare* might be in conversion of intracellular bicarbonate to CO_2 for RuBisCO. The role of the thylakoid β-CA in this cyanobacterium remains unclear. The role of inducible intracellular β-CA of '*E. natronophila*' is probably determined by its localization in cells and also requires specification.

The correlation between the number of carboxysomes in cells and their structure depending on C_i concentration in the growth medium was studied for '*E. natronophila'* [48, 49]. Decrease in C_i concentration from 1 to 0.5 M resulted in formation of additional carboxysomes on the second day. This phenomenon was pronounced on the third day, i.e., at the beginning of the exponential growth phase (Fig. 2, Table 2). At the same time, an increase in the contrast of these microcompartments, observed by transmission electron microscopy, is probably the result of their "maturation."

Table 2. Carboxysome number in the cells of '*E. natronophila*' depending on the ambient Na_2CO_3 concentration

Na_2CO_3 in the medium, M	Time, days	Number of cell sections containing carboxysomes, %	Average carboxysome number per section
0.5	1	0	0
0.5	2	60	1.6
0.5	3	74.4	3.4
0.5	5	83.4	2.5
1	5	28	0.7

FUNCTIONS OF THE CO_2-CONCENTRATING MECHANISM IN HALOALKALIPHILIC CYANOBACTERIA FROM SODA LAKES

As was stated above, CCM activity in cyanobacterial cells results in accumulation of the cytoplasmic bicarbonate pool, which is subsequently used to maintain elevated CO_2 concentrations in the vicinity of RuBisCO reaction centers. This mechanism is essential for high efficiency of photosynthesis by freshwater and marine cyanobacteria. Considering extremely high ambient C_i concentrations in soda lakes, this mechanism seems superfluous in cyanobacteria from these environments. However, detection of the functionally active CCM components in haloalkaliphilic cyanobacteria may indicate that CO_2 concentration may be necessary under such conditions. There may be several explanations to this fact.

First of all, cyanobacteria may require operational CCM due to periodically unfavorable conditions caused by the seasonal desalination cycles. At these periods, the total concentration of salts, including C_i concentration, decrease considerably. At this time, the cells may require CO_2 concentration and activation of the inducible CCM components. Moreover, TS of haloalkaliphilic cyanobacteria have much lower affinity to the substrate (K_m (HCO_3^-)) than those of freshwater and marine species. Thus, desalination may be a dramatic event for these organisms, and the relatively high resulting C_i concentrations in the environment can be sensed by a cell as the limiting conditions.

This suggestion is supported by the presence of several TS with different optima in '*E. natronophila*' (Table 1), suggesting their involvement in the adaptation to changing conditions [47, 48]. Under conditions of these limiting (although rather high) C_i concentrations, the genes encoding higher-affinity TS may be induced. Together with increased number of carboxysomes, emergence of inducible CA forms, and elevated total CA activity, this results in accumulation of the intracellular HCO_3^- pool [49, 52], thereby increasing the cell affinity to C_i in general. Thus, intact cells of '*E. natronophila*' grown at 1 M carbonate concentration and pH 10 have affinity to bicarbonate of ~250–270 mM. Simultaneously, in the cells grown in 0.15 M carbonate and pH 9, affinity to the substrate increases to ~7 mM [Samylina and Ivanovsky, unpublished data]. Similar to marine and freshwater forms, this is an indication of CCM induction in cells of '*E. natronophila*' and of its concentrating function. The values of affinity to C_i determined for '*E. natronophila*' were 1000 times lower than those for *Synechococcus elongatus* PCC7942 under conditions of CO_2 excess and limitation (200 and 10–15 μM, respectively) [2]. Capacity of the cells for efficient photosynthetic C_i assimilation is probably determined mostly by the properties of their TS.

The limitation in acceptable forms of exogenous C_i may be another reason for the preservation of active CCM in cyanobacteria of soda lakes. According to the Henderson–Hasselbalch equation, carbonate ions predominate in the medium at high pH. At the same time, existence of cyanobacterial TS for CO_3^{2-} has not been confirmed. This suggestion is supported by the data on accumulation of an intracellular C_i pool by *R. lineare* cells at elevated pH and on the correlation of this process with an increased total activity of CAs [52].

Moreover, other environmental factors may affect photosynthesis in soda lakes. Thus, high salinity results in HCO_3^- concentrations considerably lower than the calculated values (Fig. 3). High summer temperatures (up to 55°C in the brine of Lake Magadi) inhibit RuBisCO activity and facilitate its binding to O_2 [35]. CO_2 solubility in water also decreases at elevated temperature. All these factors make CO_2 concentration essential.

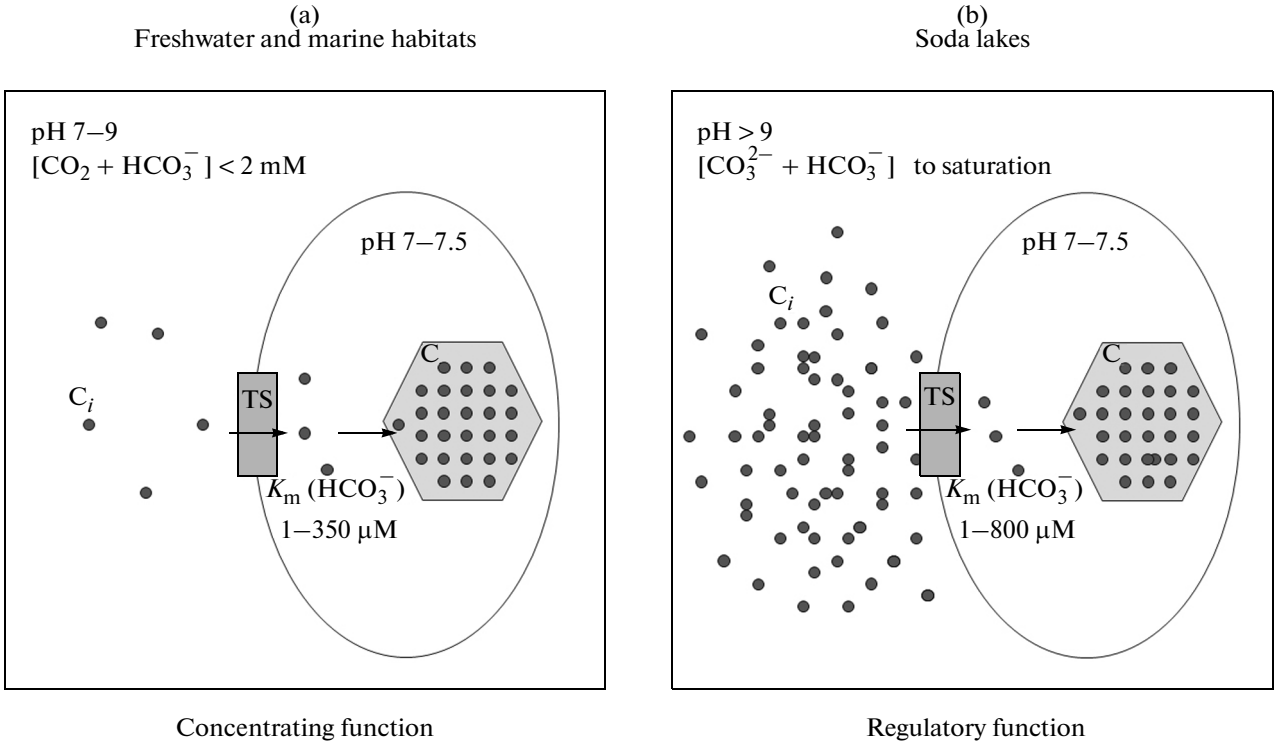

Fig. 4. Functions of the CO_2-concentrating mechanism in freshwater and marine cyanobacteria (a) and in haloalkaliphilic cyanobacteria from soda lakes (b). "C" indicates carboxysomes.

Low rate of CO_2/O_2 diffusion between the medium and the cells in dense cyanobacterial mats is one more factor favoring the preservation of an active CCM in cyanobacteria of soda lakes. The species with a pronounced exopolysaccharide sheath may encounter similar problems.

Thus, the presence in haloalkaliphilic cyanobacteria from soda lakes (which grow in carbonate solutions at alkaline pH) of the functionally active CCM components, as well as intracellular C_i accumulation [47, 50–52] may indicate that these extremophiles possess a full-fledged CCM, with the structure and principles of functioning similar to those of the studied freshwater and marine strains.

However, extremely low affinity of '*E. natronophila*' bicarbonate transporters to the substrate may indicate another important function of these TS. It was shown for *R. lineare* that soda lake cyanobacteria maintain close-to-neutral intracellular pH, in spite of the high ambient pH values [53]. The CO_2 and HCO_3^-, arriving into the cell may, however, affect the cytoplasmic pH. Thus, in soda lakes with high concentration of exogenous C_i , low TS affinity to the substrate (K_m (HCO_3^-) ~ 0.8–800 mM) may be required to protect the cell from excessive HCO_3^-, which could potentially be pumped into the cell by a high-affinity TS typical of freshwater and marine cyanobacteria (K_m (HCO_3^-) ~ 5–170 μM) (Fig. 4).

Such regulation of C_i inflow into the cell implies that its rate is sufficient to maintain efficient photosynthesis. Excessive C_i input should, however, be prevented in order to maintain a certain level of the intracellular pH. Limited C_i transport into the cell is indirectly confirmed by the substrate inhibition of TS II activity in '*E. natronophila*' [48].

Thus, the major function of the CCM in haloalkaliphilic cyanobacteria may consist of *regulation of* C_i *amount arriving into a cell* in order to saturate the RuBisCO carboxylation centers (the concentrating role in the induced CCM state at ambient C_i limitation), and to protect the cell from excessive C_i, which may affect the intracellular homeostasis (protective role in the constitutive state of the CCM).

The specific features of CCM functioning in haloalkaliphilic cyanobacteria may result from the preservation of the ancient model of this mechanism in the relic microbial communities. In Precambrian times, in spite of high concentration of CO_2 and trace amount of O_2 in the environment, cells still required a basic set of the CCM components to support photosynthesis, similarly to modern cyanobacteria, which preserve a constitutive level of the CO_2-concentrating function in the presence of elevated ambient concentrations of C_i.

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