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EXPERIMENTAL ARTICLES

Transport Systems for Carbonate in the Extremely Natronophilic Cyanobacterium *Euhalothece* sp.

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Abstract—The effect of carbonate concentration, pH of the medium, and illumination intensity on the major physiological characteristics (growth rate and the intensities of CO_2 assimilation and oxygen photoproduction) of the natronophilic cyanobacterium *Euhalothece* sp. Z-M001 have been studied. It was established that the investigated microorganism has at least two transport systems (TS) for CO_2, which differ in both the pH optimum and substrate affinity: TS I has a pH_{opt} 9.4–9.5 and a $K_{S\,0.5}$ of 13–17 mM, whereas TS II has a pH_{opt} 9.9–10.2 and a $K_{S\,0.5}$ of 600–800 mM. The substrate affinity of these transport systems is several orders of magnitude lower than the substrate affinity of the transport systems of freshwater cyanobacteria. It is suggested that they are unique for extremely alkaliphilic cyanobacteria and reflect their adaptation to the seasonal cycles of the lake hydrochemistry.

Key words: extremely alkaliphilic cyanobacterium *Euhalothece*, transport, carbonates, pH.

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Soda lakes are stable high-alkaline habitats where pH levels are high due to increased concentrations of carbonate minerals. Lake Magadi of the Eastern African Rift Valley, whose brine was the source of isolation of strain *Euhalothece* sp. Z-M001, is a classical example of the contemporary formation of soda evaporates. Its geology and hydrochemistry have been thoroughly studied [1–4]. The lake is situated in the hottest and most arid zone of Kenya and surrounded by arid savanna. High concentrations of NaCl and carbonates (Na_2CO_3 + $NaHCO_3$, up to 300 g/l), as well as a thick layer of trona sediments ($Na_2CO_3 \cdot NaHCO_3 \cdot 2H_2O$) at the bottom, high pH, and an almost total absence of Ca^{2+} and Mg^{2+} ions are the characteristic features of the lake.

At these pH values, beginning with an alkalinity of 0.5 N, natural communities do not include eukaryotic phototrophs, with the exception of the unicellular green algae *Chlorella minutissima* and *Dunaliella viridis*. However, these communities, in which cyanobacteria are the main producers, are highly productive systems (10 g C/(m^2 day) or more) due to high temperatures, intense illumination, and an unlimited amount of CO_2 [5, 6]. In such communities, cyanobacteria are the major primary producers.

Abundant development of cyanobacteria and intense production of organic matter is observed during the rainy season, when the lake becomes less saline and the mineralization of the water decreases. As the dry season advances, salination and precipitation of carbonate minerals occur, with the formation of saturated brines [7, 8].

In Lake Magadi, various haloalkaliphilic cyanobacteria were discovered, among them representatives of the genera *Synechocystis*, *Synechococcus*, *Chroococcus*, *Cyanospira*, *Phormidium*, *Oscillatoria*, *Spirulina*, etc. [6, 7]. At the same time, it was demonstrated that unicellular organisms were the most extremophilic. The optimal soda and NaCl concentrations for these microorganisms were 100 g/l and 70 g/l, respectively, whereas filamentous cyanobacteria preferred much lower concentrations [7].

Thus, during different periods of the annual cycle, changes in the concentrations of carbonates and sodium ions, as well as in the pH level, occur in Lake Magadi, the habitat of *Euhalothece* sp. Z-M001. One more peculiarity of the equatorial Lake Magadi is the high illumination intensity. The goal of this work was to elucidate the effect of these factors on the major physiological characteristics of *Euhalothece* sp. Z-M001 (the rates of growth, CO_2 assimilation, and oxygen photoproduction).

MATERIALS AND METHODS

This study used the cyanobacterial strain *Euhalothece* sp. Z-M001, isolated by G.A. Zavarzin from a microcosm with the sulfate-free imitation of the Lake

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Magadi brine inoculated in 1992 with a water sample collected from the lake [9]. The microcosm has been grown for 15 years in a 1-l plastic cylinder, at the bottom of which sodium carbonate precipitated. Distilled water is added at regular intervals to replace the evaporated water. The community is stable. The development of strain *Euhalothece* sp. Z-M001 manifested itself as a sudden blooming of water which resulted in the formation of a well-defined layer on the "liquid floor" over the mineral sediment one decimeter above the cylinder bottom, which occurred upon dissolution of the soda sediment. Subculturing on the same medium resulted in the development of a cyanobacterial monoculture which, according to data from 16S rRNA sequencing, was a member of the genus *Euhalothece* [10] with 97–98% homology with known representatives (unpublished data).

Euhalothece sp. Z-M001 is an extremely haloalkaliphilic unicellular planktonic cyanobacterium. Normally, its cells are spherical, single, 2.7–4 μm in diameter. A characteristic ecophysiological trait of this microorganism is its abundant planktonic development in saturated soda solutions at a total mineralization as high as 200 g/l.

The culture was grown on standard M medium containing the following (g/l): Na_2CO_3, 100; NaCl, 50; KCl, 2; Na_2SO_4, 1.4; KNO_3, 2.5; $K_2HPO_4 \cdot 3H_2O$, 0.5; $FeCl_3$, 0.0003; EDTA, 0.0005; and 1 ml of the A_5 trace element solution containing (g/l) H_3BO_3, 2.86; $MgCl_2 \cdot 6H_2O$, 1.81; $ZnSO_4 \cdot 7H_2O$, 0.222; $Na_2MoO_4 \cdot 2H_2O$, 0.39; $CuSO_4 \cdot 5H_2O$, 0.079; and $Co(NO_3)_2 \cdot 6H_2O$, 0.0494.

The culture was grown in 250-ml Erlenmeyer flasks containing 100 ml of medium at constant agitation on a shaker (130 rpm) at a temperature of 35°C and an illumination intensity of 2000 lx.

Optical density was measured at 683 nm (OD_{683}) on a Hitachi 200-20 spectrophotometer.

Protein concentration in cell suspensions was determined by measuring the optical density (OD_{683}) of the culture. The obtained results were evaluated in terms of the protein content (in μg/ml of the suspension) using an coefficient of 70.6, experimentally determined using the Lowry method.

To elucidate the effect of the carbonate concentration on the physiology of cyanobacteria, it was varied simultaneously with the NaCl concentration in the medium in order to maintain the total molarity at a level of 1.8 M to protect cells from osmotic shock. In the figures presented in this paper, only the concentrations of Na_2CO_3 are shown, implying that the total molarity remained unchanged.

The experiments were carried out with a 3-day early exponential phase culture of *Euhalothece* sp. Z-M001 grown on the standard medium. Cells were harvested by centrifugation (6000 *g*, 15 min) of the culture that was prediluted with water at a ratio of 1 : 2 to enhance precipitation.

Oxygen photoproduction was measured using a polarograph and a Clark-type electrode. Harvested cells were resuspended in the medium to a suspension density of 50–60 μg protein/ml. Then, the suspension was incubated for 15 min in the presence of light. After that, excessive dissolved oxygen was removed by vacuum degassing, and the suspension was transferred to a polarographic cell. An incandescent lamp served as the light source; the illumination intensity was measured with an luminometer. A water filter was installed between the light source and the polarographic cell in order to prevent heating of the suspension during the experiment.

For the experiments on $H^{14}CO_3^-$ fixation, a multiwell plate with 2-ml wells was used. Each well was supplemented with 500 μl of the medium with the required concentrations of $NaHCO_3$ and NaCl, as well as with 50 μl of the cell suspension resuspended in 10% (1.7 M) solution of NaCl (about 5 mg protein/ml). The cells were incubated in the dark for 15 min; then, the fixation was initiated by the addition of 10 μl of a $H^{14}CO_3^-$ solution (0.04 mBq). The cells were then incubated under certain illumination conditions for 30 min. A water filter was installed between the light source and the plate in order to prevent evaporation of the medium and overheating of the cells during the experiment. After incubation, 500 μl of the suspension from the well was passed through 0.45-μm nitrocellulose filter, washed with 10% NaCl solution, air-dried, placed into scintillation flasks with 7 ml of scintillation cocktail (Ecolum; United States), and analyzed in a RackBeta liquid scintillation counter (LKB, Sweden).

The pH of the medium was adjusted by changing the ratio of Na_2CO_3 to $NaHCO_3$.

The kinetic characteristics of the transport systems ($K_{S\ 0.5}$ and V_{max}) were determined using the Lineweaver–Burk equation.

RESULTS

Desalination of soda lakes during the rainy season is an important environmental factor. This process is accompanied by a decrease in the bicarbonate concentration and buffer capacity of the habitat, in which carbonate is the main buffer component. In open systems, this is also accompanied by a decrease in pH. After tenfold dilution of the standard M medium (modeling the water composition of a soda lake), a decrease in the pH level by 0.7 units (Fig. 1) occurs as atmospheric oxygen enters. This results from the hydration of atmospheric CO_2 ($CO_2 + H_2O \longrightarrow HCO_3^- + H^+$). In sealed vessels, such changes were not observed (Fig. 1). Under natural conditions, a decrease in pH by approximately 2.5 units was observed during the rainy season [11, 12].

Figure 2 demonstrates that *Euhalothece* sp. Z-M001 can easily adapt to the decrease in the pH level in the

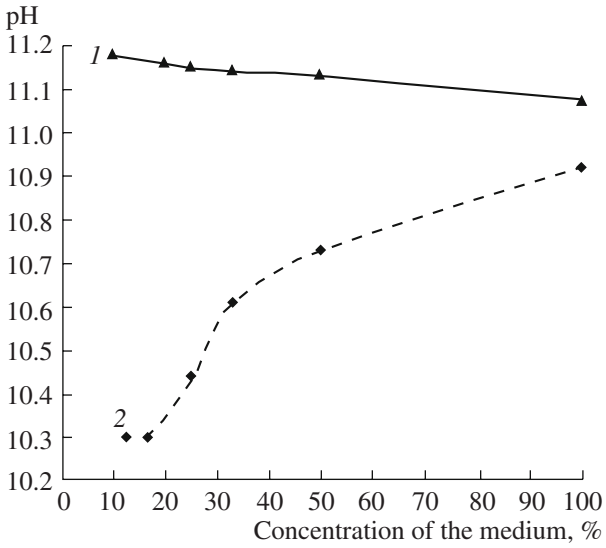

Fig. 1. Changes in the pH level after dilution of the M medium in sealed (*1*) and open (*2*) systems on the 2nd day of incubation. Standard M medium was taken as 100%.

habitat. At the standard carbonate concentration of 1 M, *Euhalothece* sp. Z-M001 grew well in a pH range of 8.0–10.0 (maximum growth was observed at pH 10.0). After tenfold dilution of the medium, the pH range for the culture growth shifts to acidic values with an optimum at pH 9.0.

The growth curves for cultures grown on media with various carbonate contents (but with the same total molarity) demonstrate that the decrease in the carbonate content in the medium results in a decrease in the biomass yield in the stationary phase (Fig. 3).

Good correlation between the biomass yield and the rate of CO_2 assimilation was observed as the carbonate content in the medium decreased (Fig. 4). This indicates that it is the decrease in the cell ability to fix CO_2 that is the chief cause of the decrease in the biomass yield.

The process of carbonate uptake by cyanobacterial cells is usually mediated by several transport systems (TS) with different pH optima [13]. Measurement of the rates of oxygen photoproduction and CO_2 assimilation by *Euhalothece* sp. Z-M001 as dependent on the pH level of the medium indicated that these two processes also had maxima that coincided with the pH maxima of growth in the standard and diluted media (Figs. 2 and 5).

It has been previously demonstrated that, in alkaliphilic cyanobacteria, the intracellular pH remains stable upon a significant shift in the pH of the medium. For example, in the unicellular alkaliphilic cyanobacterium *Rhabdoderma lineare* (also isolated from Lake Magadi), the intracellular pH remains neutral and varies within no more than half a unit (from 7.52 to 7.09) when the pH of the medium drops from 9.6 to 7.2 [14]. Thus, changes in the pH level of the medium do not affect significantly the intracellular metabolic processes, but primarily affect processes occurring in the near-cellular space and in cell membranes (e.g., carbonate transport). It may be assumed that the dependence patterns of oxygen photoproduction and CO_2 assimilation on the pH values indicates that *Euhalothece* sp. Z-M001 has at least two TS for carbonates. The first one (TS I) is

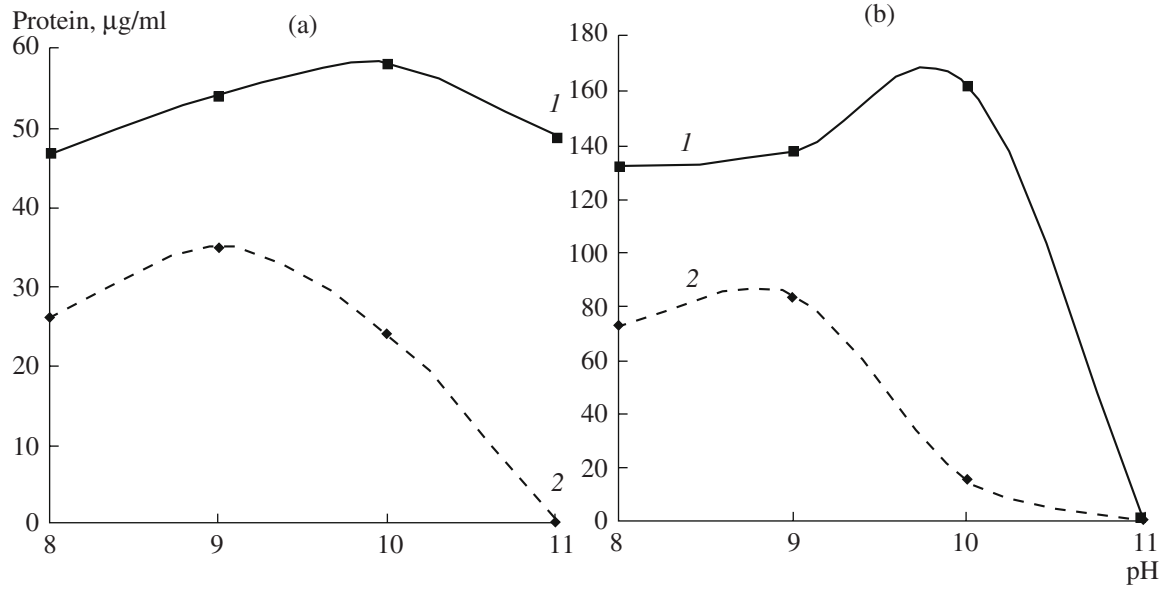

Fig. 2. Biomass yields after 3-day (a) and 7-day (b) cultivations of *Euhalothece* sp. Z-M001 on media with various carbonate concentrations and pH values: (*1*) 1M Na_2CO_3 + $NaHCO_3$; (*2*) 0.1 M Na_2CO_3 + $NaHCO_3$.

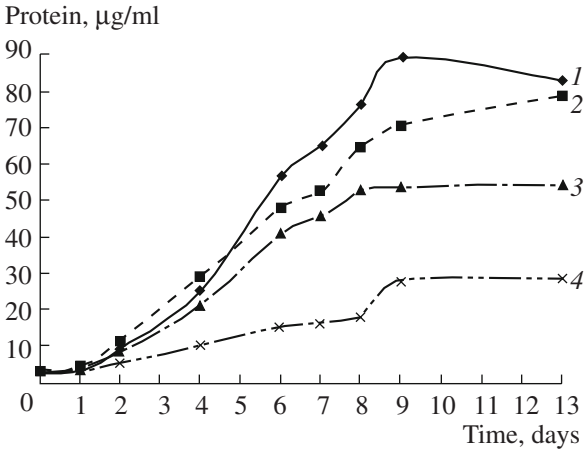

Fig. 3. Dependence of the growth of *Euhalothece* sp. Z-M001 on the carbonate content in the medium. The initial pH value in all experiments was adjusted to 10.5. The decrease in the Na_2CO_3 concentration was compensated for by the addition of NaCl so as to maintain the total molarity at a level of 1.8 M. (*1*) 1 M Na_2CO_3; (*2*) 0.7 M Na_2CO_3; (*3*) 0.4 M Na_2CO_3; and (*4*) 0.1 M Na_2CO_3.

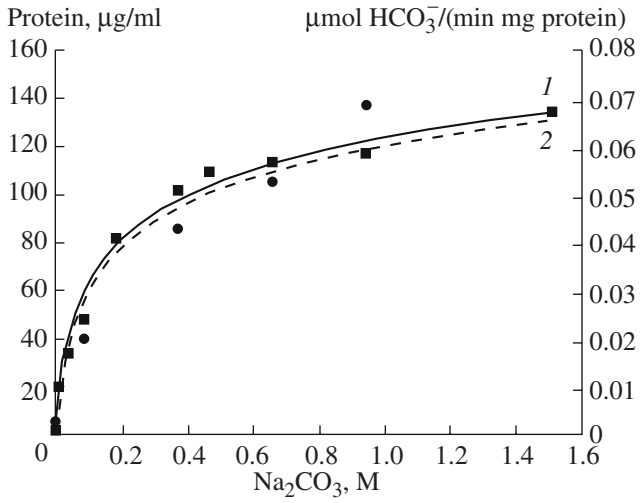

Fig. 4. (*1*) Rate of CO_2 assimilation and (*2*) the biomass yield of *Euhalothece* sp. Z-M001 cells on day 8 at various carbonate concentrations. The measurements were carried out at pH 10.

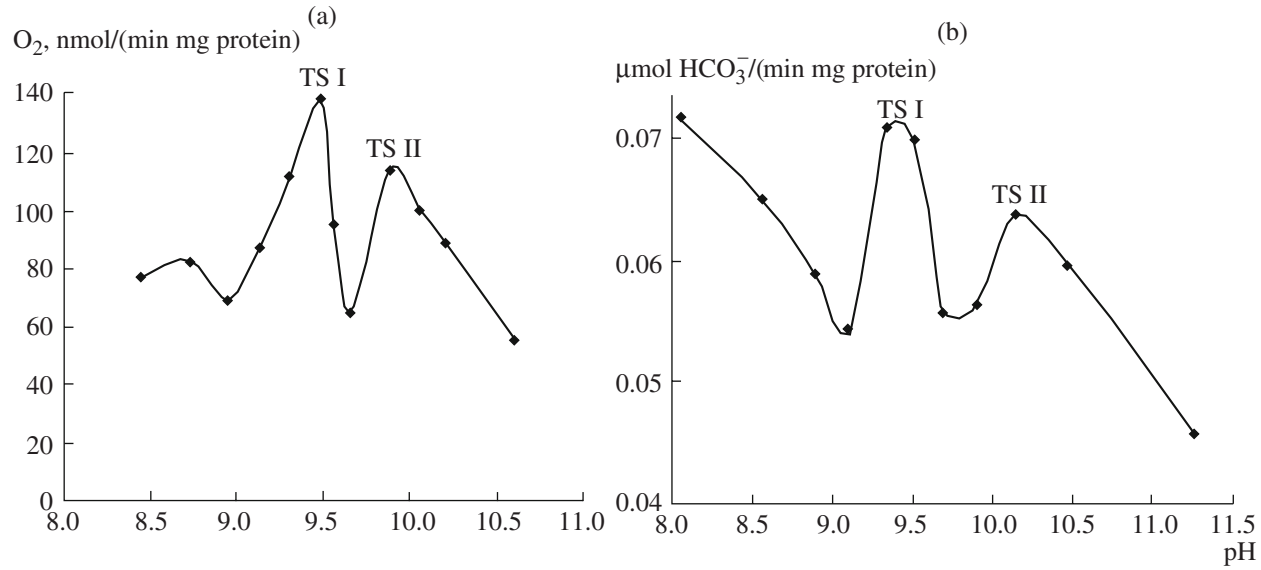

Fig. 5. Rates of (a) oxygen photoproduction and (b) CO_2 assimilation as dependent on the pH level at a carbonate concentration in the medium of 1 M. TS, transport system.

responsible for carbonate transport at low pH values (9.4–9.5), whereas the second one (TS II) operates at higher pH (9.9–10.2). The increase in the CO_2 assimilation rate with the pH change from 9 to 8 was probably due to a shift of the carbonate equilibrium toward an increase in the content in the medium of dissolved CO_2, which does not require a transport system.

The detected transport systems differed not only in the pH optimum, but also in substrate affinity and reaction to light. TS I has pH_{opt} 9.4–9.5 and has a high carbonate affinity ($K_{S\,0.5} \approx 13$–17 mM), whereas the carbonate affinity of TS II ($K_{S\,0.5}$) is low, 600–800 mM (Fig. 6). Actually, the $K_{S\,0.5}$ of TS II is lower (presumably about 450–500 mM), since the contribution of TS I in the transport of inorganic carbon cannot be completely excluded. Light saturation for TS II occurs at lower illumination intensities than for TS I (Fig. 7). A 15-fold increase in the illumination intensity (from 2 to 30 klx) results in an increase in the assimilation rate of labeled bicarbonate by 1.9 ± 0.2 times at pH 10.5 (TS II) and by 3.6 ± 0.7 times at pH 9.3 (TS I).

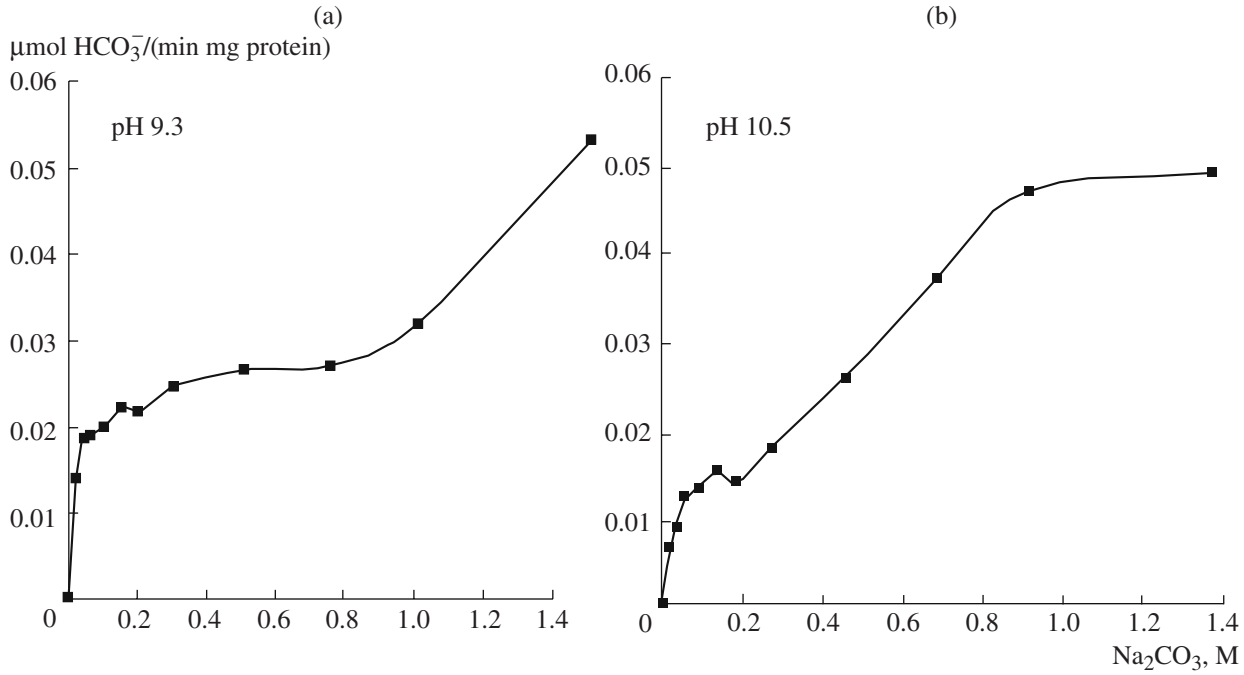

Fig. 6. Rate of bicarbonate assimilation by cells of *Euhalothece* sp. Z-M001 as dependent on bicarbonate concentration at the various pH values and at an illumination intensity of 9 klx.

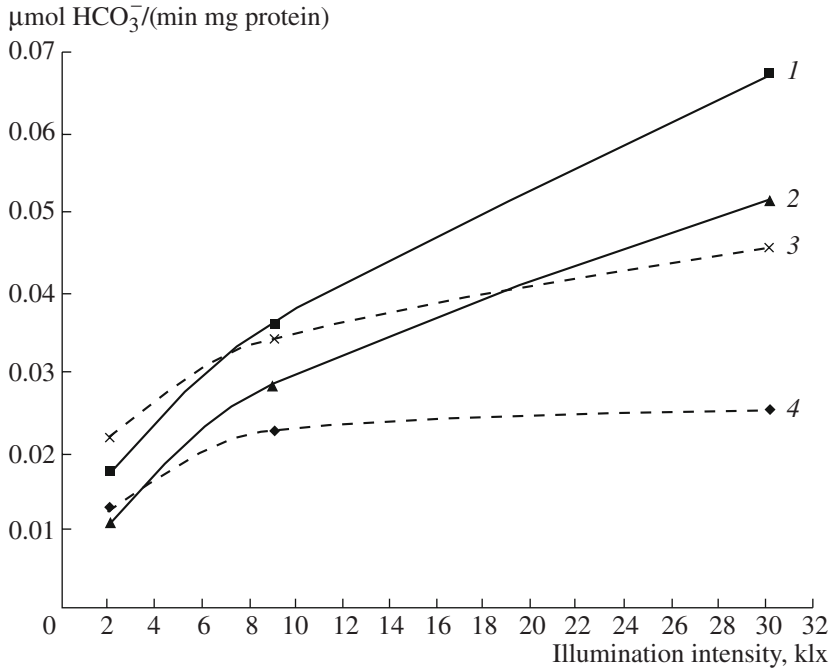

Fig. 7. Rate of bicarbonate assimilation by cells of *Euhalothece* sp. Z-M001 as dependent on illumination intensity, pH, and carbonate concentration: (*1*) pH 9.3, 1M Na_2CO_3; (*2*) pH 9.3, 0.15 M Na_2CO_3; (*3*) pH 10.5, 1 M Na_2CO_3; and (*4*) pH 10.5, 0.15 M Na_2CO_3.

DISCUSSION

The investigated cyanobacterium represents the natronophilic microflora of Lake Magadi, which is distinguished by high carbonate molar density (up to the point of saturation). In freshwater and marine ecosystems the carbonate content is no higher than 2 mM; therefore, in these ecosystems, cyanobacteria synthesize up to four transport systems with different CO_3^{2-} affinities (with $K_{S\,0.5}$ varying from 2 to 40 μm) to provide for efficient operation of ribulose bisphosphate

Kinetic characteristics of the CO_2 transport systems in the extremely alkaliphilic cyanobacterium *Euhalothece* sp. Z-M001

Characteristics	TS I	TS II
pH_{opt}	9.4–9.5	9.9–10.2
Saturation at an illumination intensity of	>10 klx	<10 klx
Carbonate concentration for V_{max}, M	0.15	≥1
V_{max}, μmol HCO_3^-/(min mg protein)	0.014–0.025	0.035–0.077
$K_{S\ 0.5}$, mM	13–17	600–800

carboxylase [13]. Usually, transport systems with high substrate affinity are inducible. They are synthesized when the carbonate concentration in the medium is below 40 μm in order to keep the rate of carbonate uptake at a level favorable for the efficient functioning of ribulose bisphosphate carboxylase [13]. The results shown in Figs. 4 and 5 suggest that at least two transport systems are involved in carbonate uptake by the cells of *Euhalothece* sp. Z-M001. They differ in both the pH optimum and substrate affinity. TS I has pH_{opt} 9.4–9.5, a $K_{S\ 0.5}$ of 13–17 mM, and V_{max} = 0.014–0.025 μmol HCO_3^-/(min mg protein), whereas TS II has pH_{opt} 9.9–10.2, a $K_{S\ 0.5}$ of 600–800 mM (for intact cells), and V_{max} = 0.035–0.077 μmol HCO_3^-/(min mg protein). It should be noted that the transport systems discovered in *Euhalothece* sp. Z-M001 differ radically in the substrate affinity from the previously described transport systems of freshwater and marine cyanobacteria. Even for TS I, the substrate affinity is three orders of magnitude lower than that of TS of freshwater and marine cyanobacteria. It is obvious that this phenomenon can be attributed to the fact that the amount of inorganic carbon in the water of soda lakes is several orders of magnitude higher than in freshwater and marine habitats, where dissolved carbon dioxide, which is in equilibrium with atmospheric CO_2, is the main source of bicarbonate. The cyanobacteria of soda lakes do not require transport systems with high carbonate affinity.

Within the framework of the "soda continent" hypothesis [15], epicontinental soda lakes are postulated as the centers of origination of the biodiversity of prokaryotes [8, 9]. In this connection, it may be assumed that the discovered transport systems with very low (as compared to freshwater TS) carbonate affinities are ancient systems. In the course of gradual decrease in the carbonate alkalinity of habitats, carbonate TS have increased their substrate affinity.

The presence of two transport systems can be attributed to the fact that organisms inhabiting soda ecosystems are faced with fluctuations in the carbonate contents in the course of desalination of their habitats during rainy seasons. For the same reason, the studied transport systems differ in their pH optima. During desalination, the pH may shift to lower values [11, 12]. During this period, TS I, whose pH optimum is lower and substrate affinity is higher, becomes the main transport system. During a drought period, the carbon content in the lake increases, which results in an increase in the pH level. Under these conditions, TS II becomes the most efficient system.

Thus, on the basis of the data obtained, we may distinguish at least two transport systems for CO_2 in the extremely alkaliphilic cyanobacterium *Euhalothece* sp. Z-M001. These systems differ in both the substrate affinities and operation optima (see table), which is important for the adaptation of the cyanobacterium during seasonal salination–desalination cycles.

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