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EXPERIMENTAL ARTICLES

Ecophysiology and Polymorphism of the Unicellular Extremely Natronophilic Cyanobacterium *Euhalothece* sp. Z-M001 from Lake Magadi

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Abstract—Strain Z-M001 of a unicellular cyanobacterium, assigned by analysis of the 16S rRNA gene sequence to the phylogenetic group of the generic level *Euhalothece*, was isolated from soda Lake Magadi. It was shown that strain Z-M001, unlike all other known cultured and uncultured organisms of the *Euhalothece* group, is extremely natronophilic, and it was named accordingly *"Euhalothece natronophila"*. In its ecophysiological characteristics, it is comparable to extremely alkaliphilic organotrophic natronobacteria, which is essential for soda ecosystems, because cyanobacteria belong to primary producers. *E. natronophila* exhibits considerable morphological variability depending on the concentration of carbonates in the medium. The polymorphism of "*E. natronophila"* is primarily connected to limitation by utilizable forms of carbon.

Key words: unicellular alkaliphilic cyanobacterium "*Euhalothece natronophila"*, polymorphism, carbonates, pH

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Soda lakes are typical natural alkaline habitats of athalassic origin, widespread in arid zones. They are unique ecosystems in which concentrated salt brines have high pH values. The main components of such brines are Na^+, K^+, Cl^-, HCO_3^-, and CO_3^{2-}, ions, while Mg^{2+} and Ca^{2+} ions are almost absent, since their content in the brine is limited by the solubility of their carbonate salts [1].

The communities of alkaliphilic microorganisms are interesting as analogues of ancient terrestrial ecosystems: soda lakes are regarded as possible centers of origin for prokaryotic diversity in the early history of the development of the biosphere; modern soda lakes are refugia for relict microorganisms [1–3].

By now, representatives of the main functional groups of microorganisms have been isolated from soda lakes and described, and this allows the autonomy of alkaliphilic microbial communities to be discussed [1]. The production phase in the functioning of these communities is provided for by cyanobacteria, whose abundant development is associated with rainy seasons, when freshening of soda lakes occurs [4]. In dry periods, the primary producers are inactive or weakly active, whereas extremely natronophilic organotrophic microorganisms develop intensely, driving the destructive phase of succession in these ecosystems. From equatorial Lake Magadi, we isolated a unicellular cyanobacterium that grew under extremely high mineralization conditions.

The aim of this work was to describe this organism phylogenetically, ecophysiologically, and morphologically.

MATERIALS AND METHODS

The material for culture isolation was a sample of water with abundant development of cyanobacteria taken by G.A. Zavarzin from Lake Magadi (Kenya) in 1992 at the end of the rainy season. It was used to inoculate a Winogradsky cylinder, from which, after development of abundant bloom, a unicellular cyanobacterium was isolated.

Cultivation was carried out on an M medium of the following composition (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; 1 ml of the A_5 trace

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element solution of the following composition: 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, $Co(NO_3)_2 \cdot 6H_2O$, 0.0494. The pH of the medium was 10.5.

To elucidate the dependence of the biomass yield on the osmotic characteristics and the salt ratio, the culture was grown in a gradient of concentrations of Na_2CO_3 (from 0 to 200 g/l with a step of 20 g/l) and NaCl (from 0 to 100 g/l with a step of 25 g/l). For all variants, the initial pH value was adjusted to 10.5.

To study how the concentration of sodium ions in the medium influenced the growth of cyanobacteria, equimolar amounts of K_2CO_3 were substituted for Na_2CO_3 for two concentration variants: 1 and 0.1 M. In each variant, the sodium ion content was changed in the 0–3.1 M range with a step of 0.34 M by adding NaCl. For all the variants, the initial pH value was adjusted to 10.5.

In order to reveal the relationship between cell morphology and the pH value of the medium, the culture was grown for 7 days on media with 1 and 0.1 M of total carbonate ($Na_2CO_3 + NaHCO_3$) at initial pH values 8, 9, 10, and 11. The pH was adjusted to required values by varying the ratio of equimolar solutions of Na_2CO_3 and $NaHCO_3$.

Depending on the objectives of the experiment, the culture was grown either in flat-bottomed 250- or 50-ml conical flasks or in 10-ml tubes on a shaker at 130–140 rpm, 35°C, and 2000 lx. The experiments were carried out in three replicates.

The optical density of the culture was determined at 683 nm (OD_{683}) on a PGN Type MK 6/6 spectrophotometer.

The protein content in cell suspensions was determined by measuring the optical density (OD_{683}) of the culture and converting it to the protein content (in μg/ml of suspension); for experimental determination of the conversion coefficient (70.6), the protein content was determined by the Lowry method.

Cell morphology and cell size were studied in specimens of living cells under a Carl Zeiss Axio Imager D1 light microscope.

The pH value was determined using a combined glass electrode on an Ekspert-001 pH-meter–ionometer directly in the medium, without dilution.

To determine the 16S rRNA gene nucleotide sequence, DNA was isolated from the biomass of strain Z-M001 by the phenol method [5]; the almost complete 16S rRNA gene was amplified with the universal bacterial primers 27f and 1492r on a GeneAmp PCR System 2700 device (Applied Biosystems, United States). The amplified 16S rRNA gene fragment was sequenced using an automatic CEQ2000 XL sequencer (Beckman Coulter, United States) according to the manufacturer's instructions. In order to find the microorganisms closely related to strain Z-M001, the NCBI GenBank (http://www.ncbi.nlm.nih.gov) was used. The phylogenetic trees were constructed using the TREECON software package [6].

RESULTS AND DISCUSSION

Isolation of an extremely alkaliphilic unicellular cyanobacterium. Soda lakes are environments with a high primary productivity, which results mainly from development of cyanobacteria. A classical example of a soda lake is shallow equatorial Lake Magadi, where an abundant development of microbiota occurs above the layer of soda precipitate. Water with abundant filamentous cyanobacteria was sampled in 1992. The cultures of these cyanobacteria were characterized in [4]. A 1-l Winogradsky cylinder containing a medium imitating the composition of the soda lake water was inoculated with this material. The medium did not contain any sulfates in order to avoid development of microorganisms of the sulfur cycle. The community was sustained for 15 years, with evaporating water replenished by distilled water. Over this period, the composition of the biocenosis changed, and a unicellular coccoid cyanobacterium became the dominant organism. Such a change in the composition of the biocenosis is consistent with Thienemann's rule that under extreme conditions, biodiversity decreases and one species develops abundantly.

A medium saturated with soda, which precipitated on the bottom of the vessels, was inoculated from the Winogradsky cylinder. Under these conditions, enrichment cultures of a unicellular alkaliphilic bacterium were obtained and maintained for 5 to 6 years.

The dissolution of soda sometimes produced the "liquid bottom" effect with a 10–20 g/l jump in density. In this case, the cyanobacteria developed as a clear-cut yellow-green layer above the "liquid bottom." Transfers of the material from the bloom zone into the same medium and isolation with the dilutions method resulted in the development of the cyanobacterial monoculture designated as strain Z-M001.

Determination of the taxonomic position of strain Z-M001 according to its morphological and phylogenetic characteristics. In liquid medium M, the culture of the cyanobacterial strain Z-M001 isolated from the bloom zone had an intense green color. Morphologically, it consisted of round cells, 2.7–4-μm in diameter, usually single or divided by a septum into two daughter cells of the same size. In the stationary phase, microcolonies consisting of a small number of cells were formed. Actively growing cells either do not produce mucus or have a very thin mucous layer around the cell, whereas in old cultures, the pericellular mucus is much more markedly pronounced, which also facilitates microcolony formation.

According to classical algological identification guides [7], the morphological characteristics of strain Z-M001 permit its identification as *Synechocystis salina.* According to the microbiological taxonomy [8], strain Z-M-001 could be assigned to the *Synechocystis* group of cultures.

Analysis of the 1240-bp nucleotide sequence of the 16S rRNA gene of strain Z-M001 and a GenBank search among both cultured and uncultured organisms (from clone libraries) revealed that the strain was most closely related to representatives of the not yet legitimate but widely studied genus of unicellular coccoid halophilic cyanobacteria *Euhalothece* [9–16] (97–98% homology levels) (Fig. 1). Depending on the group of microorganisms, this similarity level can be regarded as an intra- or interspecies one. However, since the genus *Euhalothece* presently contains no species or groups of strains of species status, interpretation of the similarity levels of the 16S rRNA gene sequences is difficult. Among the cultured representatives of the genus *Euhalothece*, strains MPI 95AH13 and MPI 95AH10 are the closest [9]. Among uncultured representatives of this genus, the organisms represented in the clone libraries [10, 15] are the most closely related.

Based of the phylogenetic, morphological, and physiological characteristics, a number of unicellular extremely halotolerant and halophilic cyanobacteria were assigned by Garcia-Pichel et al. [9] to the cluster *Halothece* and the subcluster *Euhalothece*. However, the taxonomic and nomenclatural position of the genus *Euhalothece* remains illegitimate, and the name is not included in the list of the cyanobacterial taxa [8] that correspond to the requirements of the Bacteriological Code.

At present, at least 45 clones and strains assigned to the subcluster *Euhalothece* are known. All known *Euhalothece* strains were isolated earlier from hypersaline reservoirs around the world [9–16 and a number of unpublished works (http://srs.ebi.ac.uk)]: Solar Lake and the Dead Sea (Israel) [14], Shark Bay (Australia) [13], hypersaline evaporative ponds in Guerrero Negro (Mexico) [9, 11, 16], Eilat (Israel) [9, 11, 12, 16], Salinas del Cabo de Gata (Spain) [9], and salt works in Italy, Greece, and Somalia [14]. Of these clones and strains, only four were isolated from the Wadi Al-Natrun alkaline hypersaline lakes (Egypt) with pH 8.5–9.8 [10]; in these lakes, however, NaCl is still the predominant salt (up to 30% wt/vol). Thus, these organisms are widespread in extremely mineralized waters and deserve special study.

Strain *Euhalothece* sp. Z-M001 differs from all the known *Euhalothece* strains in its ecological and physiological characteristics, because it was isolated from soda Lake Magadi with a high (up to saturation) concentrations of not only NaCl but also Na_2CO_3. To emphasize this distinction, we named strain Z-M001 "*Euhalothece natronophila"*.

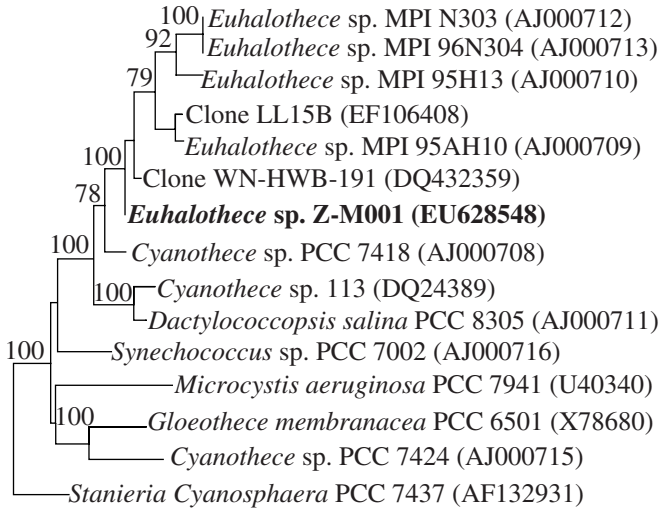

Fig. 1. Phylogenetic tree showing the position of strain Z-M001 among unicellular cyanobacteria. In the tree, uncultured cyanobacteria revealed in clone libraries of 16S rRNA genes isolated directly from natural samples (DQ432359 and EF106408) are also represented.

Ecophysiological characteristics of *E. natronophila* as an extreme natronophile. *"E. natronophila"* is an obligate natronophile. Experiments with the Na_2CO_3 (0–200 g/l) and NaCl (0–100 g/l) concentration matrix at pH 10.5 revealed that the best growth (highest biomass yield) was achieved within a vast region of concentrations with the total mineralization from 100 to 230 g/l Na_2CO_3 + NaCl (Fig. 2). However, with an increase in the Na_2CO_3 concentration, the NaCl concentration needs to be decreased. Thus, equally high biomass yields (80–100 μg protein/ml) were observed at 80–100 g/l Na_2CO_3 + 25–75 g/l NaCl and at 140–160 g/l Na_2CO_3 and 0–50 g/l NaCl; the maximum yield (100–140 μg protein/ml), at 180 g/l Na_2CO_3 and 0–25 g/l NaCl (Fig. 2). The culture did not grow in media to which no carbonates were added (i.e., at a carbonate content in equilibrium with atmospheric CO_2) even if the necessary pH and molarity values were maintained due to addition of NaCl.

The maximal yield obtained at 180 g/l $NaCO_3$ in the absence of NaCl shows that *"E. natronophila"* is an extremely natronophilic cyanobacterium, unlike haloalkaliphilic cyanobacteria, which depend on the Cl^- anion for development [1].

The increased requirement for NaCl at decreased carbonate concentrations results from NaCl being a source of sodium. Figure 3 shows the relationship between the biomass yield and the sodium concentra-

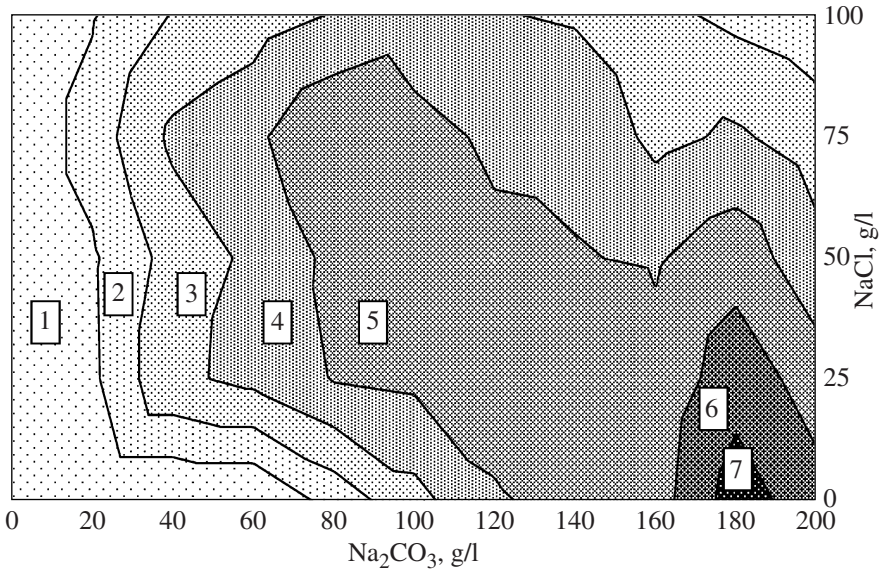

Fig. 2. Biomass yield (μg protein/ml) of "*E. natronophyla*" on the 7th day in the concentration matrix of NaCl and carbonate at an initial pH value of 10.5: 1, 20; 2, 20–40; 3, 40–60; 4, 60–80; 5, 80–100; 6, 100–120; 7, 120–130 μg protein/ml.

tion in the medium. The optimum and minimum sodium concentrations required for growth depend on the carbonate concentration: at $[CO_3]$ = 1 M in the medium, growth is possible at $[Na^+] > 1$ M, with $[Na^+]_{opt}$ = 1.5–2.4 M, while at $[CO_3]$ decreased to 0.1 M, growth is possible at $[Na^+] > 0.35$ M. This is probably due to the involvement of sodium in the process of energy conversion on the cytoplasmic membrane and/or sodium-dependent transport of inorganic carbon.

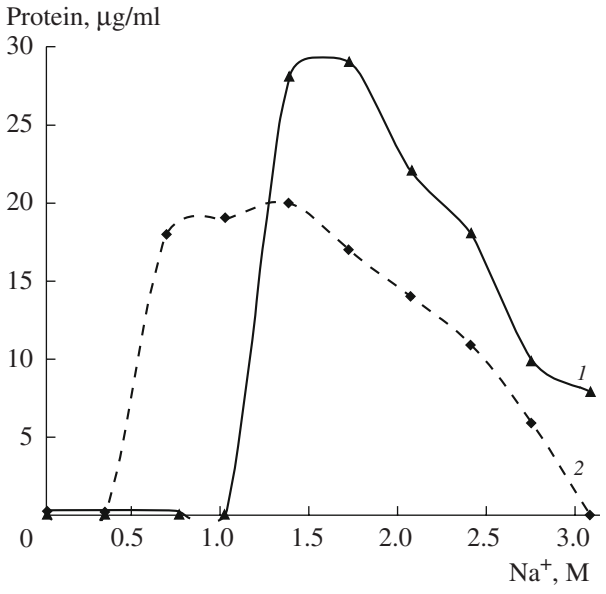

Fig. 3. Dependence of the biomass yield of "*E. natronophyla*" on the 7th day on the Na^+ ion concentration in the medium at (*1*) 1 M K_2CO_3 and (*2*) 0.1 M K_2CO_3.

The relationship between strain Z-M001 growth and its physiological characteristics and pH of the medium was considered by us earlier [19].

Soda lakes are ephemeral formations with a characteristic variable regimen with considerable seasonal fluctuations in the concentrations of inorganic salts. However, autonomous microbial communities with a closed carbon cycle develop in these environments. The typical model of development of microbial communities adopted in the literature is as follows (exemplified by Lake Magadi). Demineralization of the lake occurs during the rainy season and is accompanied by an abundant development of primary producers, namely, planktonic and benthonic cyanobacteria [4, 17], with which only several secondary settlers (such unicellular green algae as *Chlorella minutissima* and *Dunaliella viridis*), are able to compete.

Anoxygenic phototrophic bacteria (APB) are secondary producers in soda lakes; among them, *Ectothiorhodospira* species and representatives of the family *Chromatiaceae* predominate. In their relation to the salt composition and concentration, most APB isolated from soda lakes are assigned to halophiles and haloalkaliphiles; less often, to halotolerants and natronophiles [1].

With the onset of the dry season, salinization occurs, leading to the inhibition of cyanobacterial growth and to development of organotrophic microorganisms which mediate the destruction phase of the biota development [1, 2, 4]. These microorganisms are represented

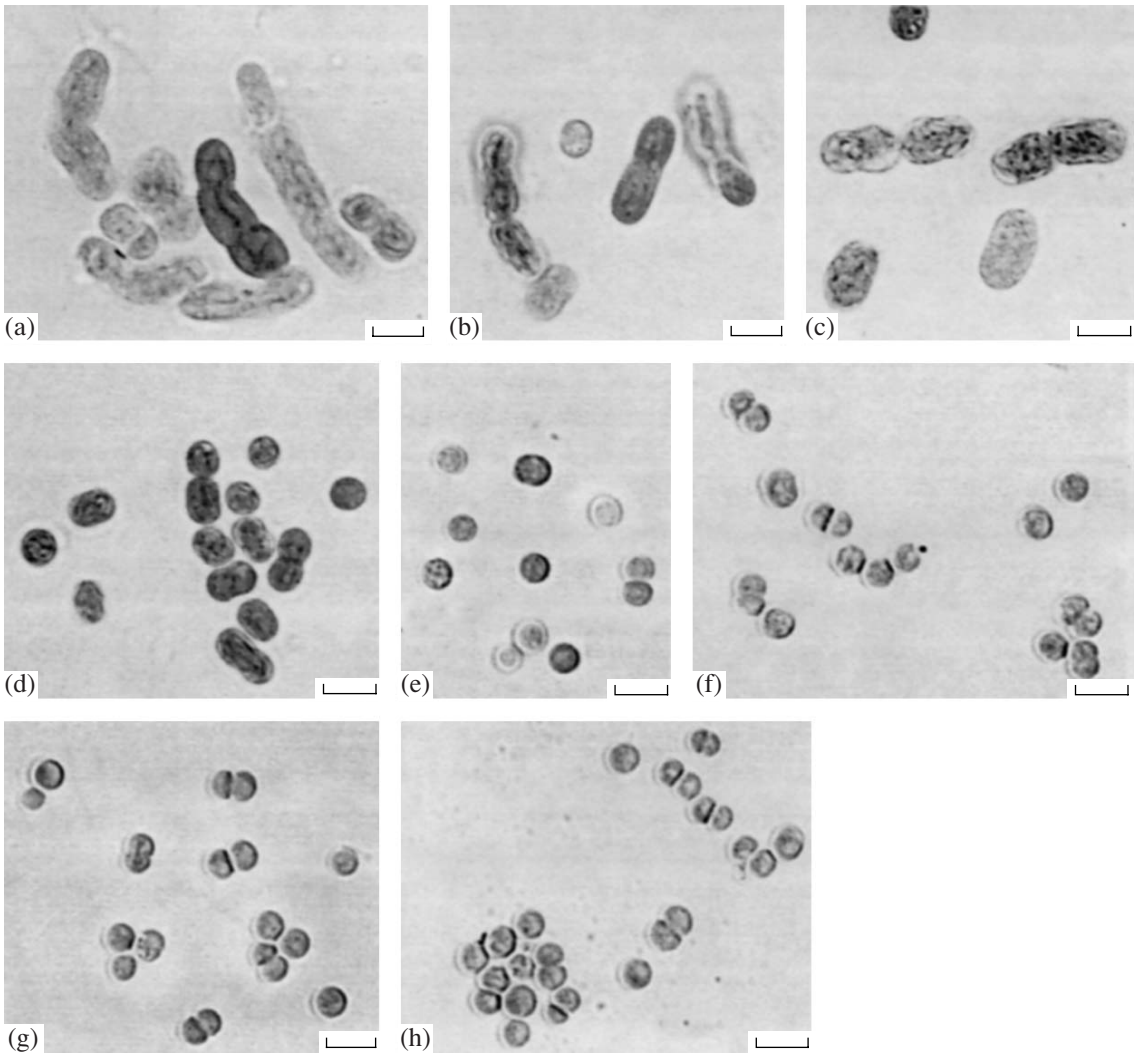

Fig. 4. Morphology of "*E. natronophyla"* cells grown on the media containing 75 g/l NaCl and Na_2CO_3 at concentrations (g/l) of (a) 20; (b) 40; (c) 60; (d) 80; (e) 100; (f) 120; (g) 140; and (h) 160. Bars, 5 μm.

by anaerobic, extremely natronophilic bacteria (cellulolytic and saccharolytic bacteria, as well as secondary anaerobes). Natronophilic and haloalkaliphilic saccharolytic anaerobes obligately dependent on the carbonate ions, capable of developing at 3 M Na^+ and higher, and thus adapted to exist in a drying-up brine (to the point of solid phase precipitation) were isolated from Lake Magadi [1]. It is seen from Fig. 3 that, at high concentrations of carbonate ions, strain *"E. natronophila"* is also capable of developing at 3M Na^+ in the medium, and $[Na^+]_{opt}$ = 1.5–2.4 M overlaps with the values for the extremophilic anaerobic saccharolytic bacteria isolated from Lake Magadi: *Halonatronum saccharophilum* ($[Na^+]_{opt}$ = 1.4–2.3 M), *Amphibacillus fermentum* ($[Na^+]_{opt}$ = 0.67–3.1 M), *A. tropicus* ($[Na^+]_{opt}$ = 1.0–1.87 M), and others [1].

Thus, *"E. natronophila"* is an obligate, extremely natronophilic cyanobacterium with optimum growth conditions corresponding to those in concentrated sodium carbonate brines. Under natural conditions, this may mean the development of cyanobacteria and active functioning of the production phase during the dry periods as well.

Polymorphism of *"E. natronophila".* The taxonomic identity of cyanobacteria is still usually determined using their morphological characteristics, especially in hydrobiological studies. In this respect, abundant material has been accumulated [7, 18]. Therefore, the dependence of morphology of the algologically

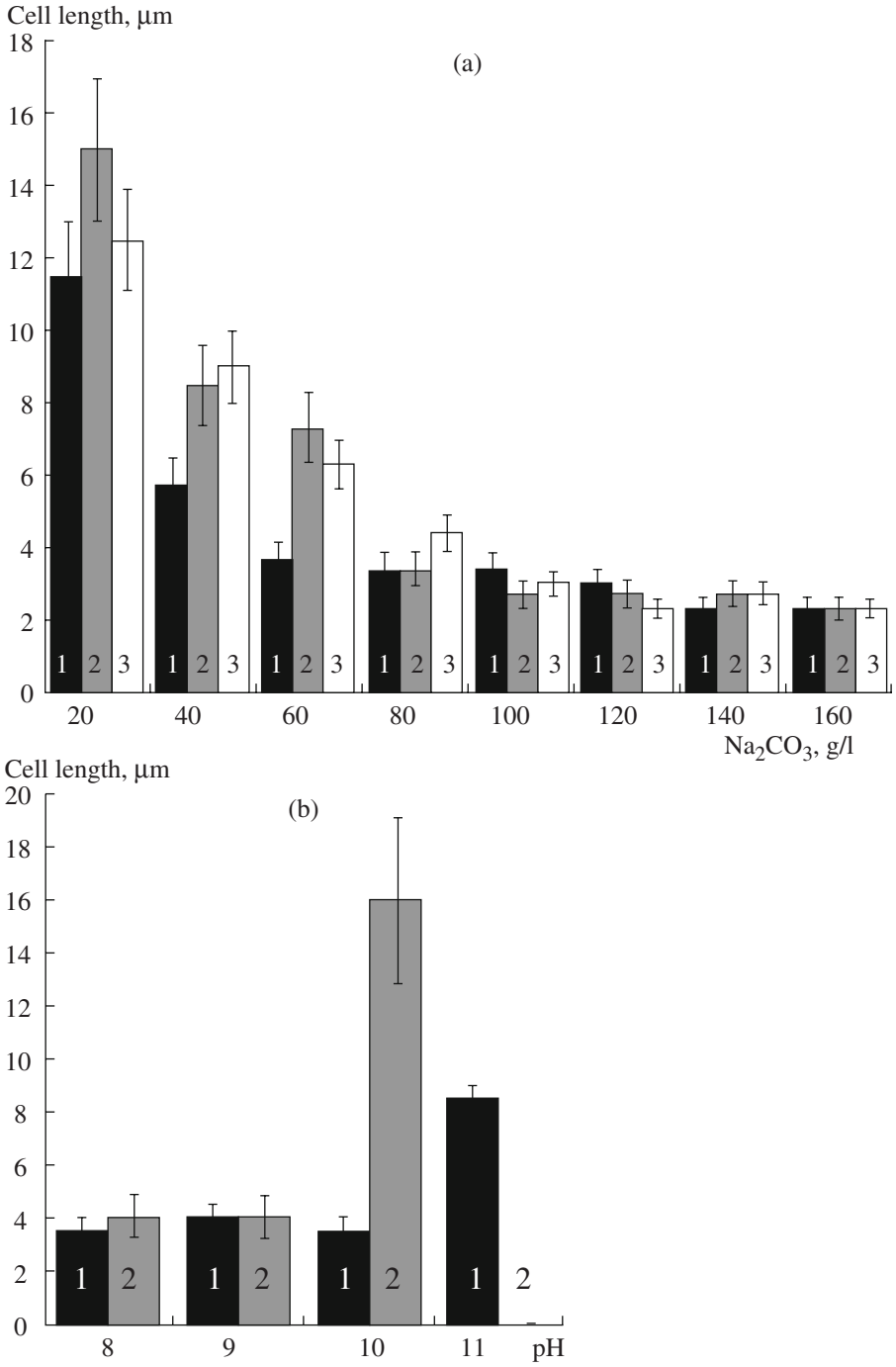

Fig. 5. Dependence of the length of "*E. natronophyla"* cells on the 7th day on (a) carbonate concentration at NaCl contents of (1) 50, (2) 75, and (3) 100 g/l and on (b) the pH values at (1) 1 and (2) 0.1 M Na_2CO_3 in the medium.

pure cyanobacterial cultures on the ecological conditions is of great value for interpreting the descriptions in situ.

For several cultured *Euhalothece* strains, marked morphological variability depending on water salinity was reported [9]. It is noteworthy that this variability was of a different character for different strains: in strain MPI 95AH13, the cell size at high salinity was smaller than at low salinity, whereas the cells of MPI 95AH10 and MPI 95AH11, on the contrary, became longer (and even pseudofilamentous) with increasing salinity [9].

The cells of *"E. natronophila"* are also characterized by marked polymorphism, which is definitely a

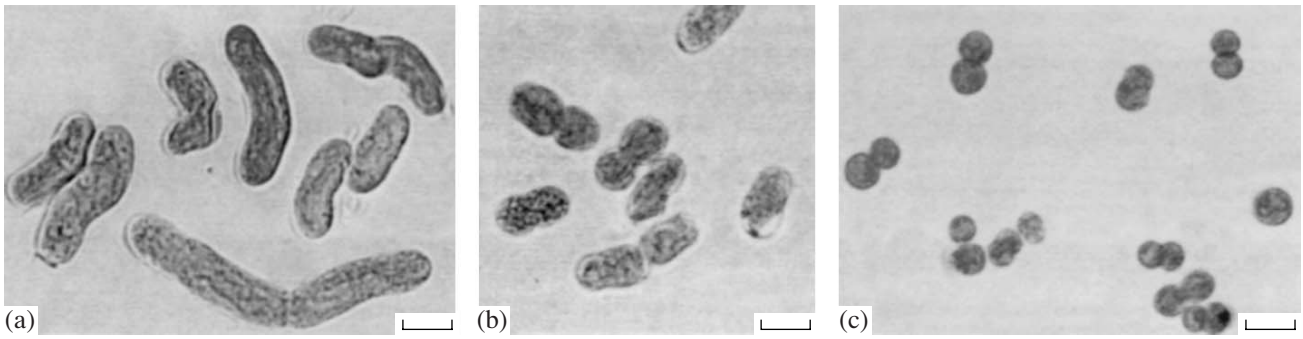

Fig. 6. Cell morphology at different salt ratios at a total molarity (Na_2CO_3 + $NaHCO_3$) of 1.86–1.95 mol/l. (a) 100 g/l NaCl + 20 g/l Na_2CO_3; (b) 75 g/l NaCl + 60 g/l Na_2CO_3; (c) 50 g/l NaCl + 120 g/l Na_2CO_3. Bars, 5 μm.

function of carbonate concentration in the medium: the cell shape varies from spherical at high carbonate concentrations (80–200 g/l) to elongated, thickened irregular rods at 20 g/l Na_2CO_3 (Fig. 4). The cell size increases from 2–3 μm in diameter to 10–17–(23) × 3.5–4 μm, respectively (Figs. 4, 5a). The microphotographs shown in Figs. 4 and 6 are the result of a combined experiment with the *"E. natronophila"* monoculture; the possibility of contamination by other cyanobacterial species having growth advantages at decreasing carbonate concentrations is therefore excluded.

The NaCl concentration has no significant effect on cell morphology. At varying NaCl concentrations in the medium (25, 50, 75, and 100 g/l), similar changes in cell morphology were observed upon changes in the Na_2CO_3 concentration. These changes are shown in Figs. 4 and 5a. Figure 4 shows the series only for 75 g/l NaCl. For other NaCl concentrations, the series were similar and therefore are not presented.

The change in cell morphology cannot be explained by changes in osmotic pressure. If the molecules of a substance dissociate, the value of osmotic pressure developed by a solution is known to depend on the number of ions dissolved in it but not on their chemical nature. Figure 6 demonstrates that, even if the total molarity of the medium is retained, the cell morphology changes, again following the changes in the Na_2CO_3 concentration.

The morphological variability of the unicellular alkaliphilic cyanobacteria isolated from Lake Magadi was noted earlier [4], although its distinct relation to the carbonate concentration was not revealed.

The morphology of "*E. natronophila*" cells also depends on the pH value of the medium. With pH increasing to 11, morphological changes were observed similar to those that occur upon a decrease in the carbonate concentration: coccoid cells turned into long (8–9 μm), thick, sometimes slightly curved rods (Fig. 5b).

This can possibly be explained by inorganic carbon (C_{in}) limitation. Earlier, we showed with intact *"E. natronophila"* cells that this strain has at least two transport systems (TS) for carbonate, differing in pH_{opt} and $K_{S\ 0.5}$ values [19]: TS I with pH_{opt} 9.4–9.5 and $K_{S\ 0.5}$ = 13–17 mM; and TS II with pH_{opt} 9.9–10.2 and $K_{S\ 0.5}$ within the range of 600–800 mM. Thus, at high pH values, only TS II operates, which requires high carbon concentrations. Under these conditions, C_{in} in the solution is represented by the CO_3^{2-} and HCO_3^-, anions, while C_{in} in the form of CO_2 is absent. HCO_3^- is therefore the only form of C_{in} available to the cells. The CO_3^{2-}/HCO_3^- ratio depends on the pH value, increasing with pH.

Based on our data, four situations can be singled out: (1) pH values 8–10 + high carbonate concentration; (2) pH values > 10 + high carbonate concentration; (3) pH values 8–9 + low carbonate concentration; and (4) pH values > 9 + low carbonate concentration.

(1) At pH 8–10 and the total carbonate concentration of 1 M and higher, the cells are typically spherical (Fig. 5b). This is the region of the optimum growth conditions;

(2) At pH 11 and 1 M of total carbonate, the cells are long irregular rods. Under these conditions, the bicarbonate concentration is insufficient for normal functioning of TS II (limitation by substrate). This is also confirmed by the fact that, at pH10.5, the rates of oxygen photoproduction and $H^{14}CO_3^-$ fixation decrease significantly at total carbonate concentrations of 1, 0.5, and 0.1 M [19 and unpublished data];

(3) pH values of about 9 and a carbonate content of 0.1 M are the optimum conditions for TS I functioning; at pH 8, the concentration of CO_2 capable of penetrating into the cell without carriers increases [19]. Under

Variants of classification of strain Z-M001 based on morphological criteria under different cultivation conditions

Cultivation conditions		Morphological peculiarities	Determination (according to [7])
Na_2CO_3, M	pH		
1	10	Green coccoid cells about 2 μm in diameter with homogeneous contents	*Synechococcus minuscula*
1	11	Long rods about 4 μm wide with homogeneous contents	*Synechococcus cedrorum*
0.1	8	Round or slightly elongated cells about 3.8 μm in diameter, pale green, with marked granularity inside	*Synechocystis salina*
0.2–0.4	10	Long (up to 20 μm and more), slightly curved rods, often forming small slightly mucous colonies	*Rhabdoderma lineare*

these conditions, the cells are spherical or slightly elongated and pale, but not increased in size;

(4) At a total carbonate concentration of 0.1 M and pH 10, the cells become very long (12–20 μm), pale, curved rods; at pH 11, no growth occurs. Under these conditions, TS I is substrate-limited and therefore cannot provide the cells with a sufficient amount of C_{in}.

Thus, the inability to utilize CO_3^{2-} ion for transporting C_{in} into the cell, the sharp decrease in the HCO_3^- anion concentration in the medium at pH > 10.5, the kinetic characteristics of the TS studied [19], as well as the presence of carboxysomes [unpublished data] in Z-M001 cells, give evidence that the polymorphism of "*E. natronophila"* is linked primarily to carbon limitation. In the final analysis, the cause of the morphological variability of the cells is a lack of an available form of C_{in}, irrespective of the reason for it (dilution of the medium or pH increase). The question as to how and why such a significant increase in cell size occurs when C_{in}, the main substrate for building the cell components, is limited requires further in-depth study.

As was shown for three monocultures of natronophilic cyanobacteria, their morphological diversity resulting from a change in the salt composition of the medium may go beyond the scope of recognized morphological genera [4]. The morphological variability of *"E. natronophila"* is so high that, under different conditions, it may lead to different variants of identification. The table demonstrates that the *"E. natronophila"* culture grown under different conditions may be identified as four different species assigned to three genera (*Synechocystis, Synechococcus*, and *Rhabdoderma*). Data supporting the possibility of such cardinal morphological changes in a cyanobacterial strain are also available in the literature [20]. Considering all these specific features, we should be very careful in identifying unicellular cyanobacteria in situ when it is not possible to perform genetic analysis; in any case, the conditions under which the cyanobacteria were detected should be precisely specified.

Thus, we have isolated the first alkaliphilic and natronophilic culture of the genus *Euhalothece*; all the representatives of this group have earlier been isolated from hypersaline habitats. The cyanobacterial strain *"E. natronophila"* Z-M001 proved to be as extremely alkaliphilic as the organotrophic natronobacteria identified as archaea and recognized as the *nec plus ultra* representatives of extremophiles. This is essential for soda ecosystems, since cyanobacteria belong to primary producers. Both the biomass yield and the cell morphology of unicellular alkaliphilic cyanobacteria depend directly on the carbonate concentration in the medium. The morphological variability of *"E. natronophila"*, which is beyond the recognized criteria for determination of accepted morphological genera, results, first and foremost, from the lack of the carbonate form available to the cells, irrespective of its causes (dilution of the medium or increased pH values).

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