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EXPERIMENTAL ARTICLES

Changes in the Cell Ultrastructure of the Haloalkaliphilic Endoevaporite Cyanobacterium *'Euhalothece natronophila'* during Fossilization

O. I. Baulina[a], O. S. Samylina[b, 1], and L. M. Gerasimenko[b]

[a] *Faculty of Biology, Moscow State University, Moscow, Russia*

[b] *Winogradsky Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117312 Russia*

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Abstract—The ultrastructure of the haloalkaliphilic endoevaporite cyanobacterium *'Euhalothece natronophila'* Z-M001 from the soda Lake Magadi was investigated during the initial stages of fossilization in a model experimental system. The cyanobacterium was cultivated in concentrated carbonate solution supplemented with calcium chloride. It was revealed that the amorphous $CaCO_3$ formed under these conditions could interact with the cell wall during the first stages of *'E. natronophila'* calcification. Evidence is presented that the surface layer of the *'E. natronophila'* envelope, presumably containing polysaccharide and/or (glyco)protein components, can be involved in the adsorption and subsequent crystallization of $CaCO_3$ with the formation of a massive "shell" embedding the morphologically intact cells. It was established that the ultrastructure of the cell wall and the intrathylakoid space changed during $CaCO_3$ mineralization. During the later fossilization stages, cells covered by the calcium-containing "shell" were apparently mummified, and mostly retained their original shape. The encapsulation of cyanobacteria in the trona globule was characterized by a different pattern. It probably involved tight binding of the growing crystal to the glycocalyx components that are anchored in the outer membrane. This may result in its detachment from the underlying peptidoglycan layer. The peptidoglycan was retained, and the protoplasts were ultrastructurally similar to the intact ones. Cyanobacteria incorporated in large trona crystals underwent degradation, deformation, and destruction. This accounts for the fact that massive trona deposits of Lake Magadi lack cyanobacterial fossils that are abundant in calcium-containing strata.

Keywords: haloalkaliphilic endoevaporite cyanobacterium, *'Euhalothece natronophila'*, fossilization, ultrastructure, calcium carbonate, trona, cell surface, outer membrane, thylakoids.

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The shallow equatorial Lake Magadi is characterized by a copious growth of microbiota above the soda sediment layer; it is a classical example of a soda lake. The primary production of organic matter is due to mass development of phototrophic organisms, primarily of diverse cyanobacteria [1].

The ancient sedimentary rocks of the lake, the so-called Green Beds, contain massive microfossils of unicellular cyanobacteria. Based on the data of scanning electron microscopy, they were classified into the genera *Pleurocapsa*, *Gloeocapsa*, *Entophysalis*, *Chroococcus*, and *Synechococcus* [2]. Calcium carbonate precipitation probably proceeded in their mucilaginous capsules, preserving the morphology of *Pleurocapsa* and *Gloeocapsa* colonies. According to electron microscopic images, the content of the cells was not mineralized. The genus *Synechococcus* is characterized by a lack of developed mucilaginous surface structures, i.e., capsules or sheaths. The issue concerning the interaction of calcium carbonate with the cell surface was not addressed in the article cited. However, other works dealing with *Synechococcus* sp. GL24, which possesses (glyco)protein S-layers on the cell surface, provided evidence of their involvement in the formation of calcium-containing minerals [3, 4]. Moreover, it was established that the initial stages of calcium carbonate precipitation occur on the outer membrane surface of *S. leopolensis* PCC 7942, which is capable of producing an extracellular polymeric substance loosely bound to the cell [5, 6].

Recently, a detailed theoretical analysis of the cell surface of bacteria, including cyanobacteria, has been carried out, focusing on the possible involvement of macromolecules with different localization and chemical nature in cell mineralization [7]. Apart from the availability, structural organization, and chemical composition of the mucilaginous surface structures [8] and S-layers [3], calcium carbonate precipitation by cyanobacteria, with Ca^{2+} binding as the initial stage, depends on the physical and chemical properties of

[1] Corresponding author; e-mail: olga.samylina@gmail.com

the cell wall [9], pH [5], medium composition, and the growth phase of the microorganism involved [6].

'E. natronophila' is a representative of the natronophilic endoevaporite microbiota of Lake Magadi. The cyanobacterium develops in saturated NaCl and $NaHCO_3$ solutions in contact with the minerals halite and trona. It can be responsible for the primary production of the lake during the drought periods. The present work is a part of a complex research project dealing with *'E. natronophila'* fossilization. Our studies [10] revealed a structural and functional heterogeneity of the minerals formed in a model system, with carbonate calcium and trona as dominant components. In a medium simulating the water of the soda lake where free calcium cations are not present, carbonatization of alkaliphilic cyanobacteria was apparently due to adsorption of the amorphous $CaCO_3$ on the cell surface with its subsequent crystallization. The cells embedded in growing trona crystals were crushed, deformed, and destroyed by the pressure developing in the crystals [10].

The data presented in [10] indicate that only mineral-free *'E. natronophila'* cells actively carry out photosynthesis, which is impossible for the mineral-embedded cells. In all likelihood, the formation of a mineral "shell" around a cell results in a gradual loss of its viability. Accordingly, a part of the *'E. natronophila'* population, while existing in the endoevaporite system, may use protective mechanisms that block the interaction between the cell and the mineralizing agents. Recently, an analogous conclusion was drawn from the research on the cyanobacteria *Synechococcus* sp. and *Planktothrix* sp. that were grown at a pH of 8–10 with Ca^{2+} [11]. The results of this research testify to the operation of a mechanism based on the metabolic maintenance of a positive surface charge preventing Ca^{2+} adsorption and carbonate precipitation on the cell surface.

In addition to the chemical and structural properties of the cell surface, the feasibility of the calcification process may partly depend on adaptive cell modifications. However, the structural organization and chemical composition of the peripheral layer of the *'E. natronophila'* cell envelope and its involvement in mineral precipitation have not been studied yet. Of considerable importance for investigating the spatial organization of the boundary zone between the cell and the forming crystal are the new techniques of electron microscopy that do not involve chemical fixation. Nevertheless, they also have their limitations [12]. The conventional ultrathin sectioning technique makes it possible to monitor the alterations in the ultrastructural organization of the cyanobacterial envelope and protoplast during mineralization.

Hence the goal of this work was (i) to investigate the ultrastructural organization of *'E. natronophila'* Z-M001 cells under optimum growth conditions and (ii) to elucidate ultrastructural changes in the cells of this cyanobacterium during fossilization involving the interactions with various crystal types in our model system.

MATERIALS AND METHODS

The *research subject* was the unicellular haloalkaliphilic cyanobacterium *'Euhalothece natronophila'* strain Z-M001 that was isolated from the soda Lake Magadi (Kenya). The experimental system simulating the fossilization process was described in the previous paper [10].

Cultivation conditions. The cells were grown on a shaker at 30°C under permanent illumination by tungsten lamps. The total illumination was 2000 lx. The composition of medium "M" was as follows (mM): Na_2CO_3, 1000; NaCl, 800; KCl, 27; Na_2SO_4, 10; KNO_3, 20; $K_2HPO_4 \cdot 3H_2O$, 2; $FeCl_3$, 1.8×10^{-3}; and microelements A_5, 1 ml. The pH value was 10–10.5.

Studies on the calcification process. Modeling fossilization under natural conditions implied adding $CaCl_2$ solution to a final concentration of 17 mM into a 3-day culture. The experiment was continued for two days.

Transmission electron microscopy (TEM). After two days of incubation, the samples of the control (without $CaCl_2$) and experimental (with 17 mM $CaCl_2$) cells during the logarithmic growth phase were fixed with 2% or 0.5% glutaraldehyde in 0.1 M Na-cacodylate buffer or Millonig buffer [13], respectively, for 30 min. The postfixation procedure was carried out with 1% OsO_4 in the respective buffer for 4 h. Thereupon the samples were desiccated in a series of ethanol solutions with increasing concentrations, followed by uranyl acetate-saturated absolute ethanol. The samples were embedded in araldite. Ultrathin sections were obtained with an LKB-8800 ultramicrotome (Sweden). They were contrasted with lead citrate according to Reynolds [14] and examined in a JEM-100B microscope (Japan).

RESULTS

'E. natronophila' is a representative of the microbiota of a natural water body with an extremely high salt and mineral content. Growing the culture under favorable growth conditions on specialized medium "M" (the control variant of our model system) yielded *'E. natronophila'* cells that were spherical, 2.7–4 μm in diameter, and solitary or dividing [15]. During the logarithmic growth phase, the ultrastructural organization of the cells of this species was typical of cyanobacteria. However, it exhibits peculiar features (Fig. 1). The cell envelope includes a gram-negative cell wall and a thin surface layer (SL) with an unidentified chemical composition that is ultrastructurally different in different cells. Using our fixation techniques, we

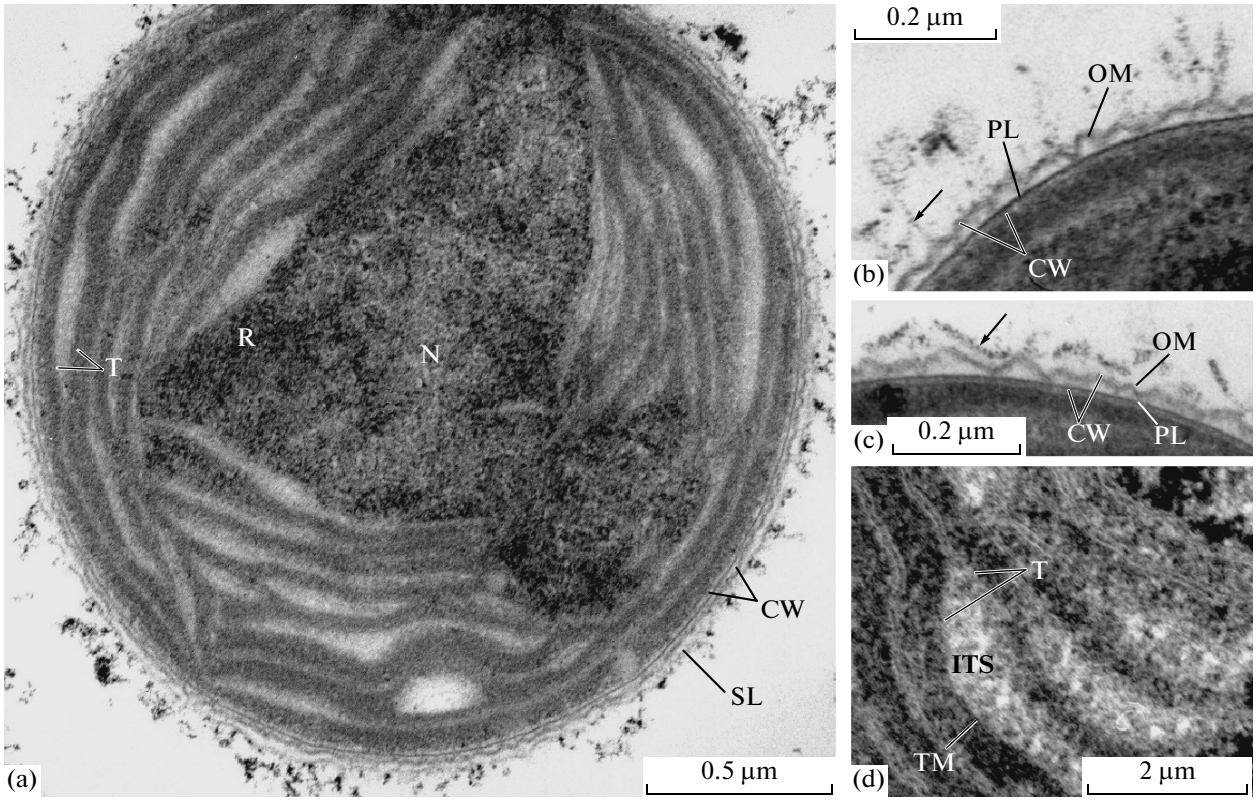

Fig. 1. Ultrastructural organization of the control cells of *'Euhalothece natronophila'* Z-M001 grown under optimum conditions on medium "M" containing 100 g/l Na_2CO_3 and 50 g/l NaCl: general image of the cell (a); cell envelope area with outer membrane-bound surface layer fibrils (b); cell envelope area with the surface layer fibrils arranged parallel to the outer membrane (c); and thylakoid organization (d). N, nucleoid; R, ribosomes; T, thylakoids; ITS, intrathylakoid space, TM, thylakoid membrane; CW, cell wall; SL, surface layer; PL, peptidoglycan layer; OM, outer membrane. Arrows indicate the fibrils that are perpendicular to the outer membrane and bound to it and the fibrils running parallel to the outer membrane.

revealed fine-grain deposits of an electron-dense substance (Fig. 1a), short fibrils arranged perpendicular to the outer membrane and attached to it (Fig. 1b), or solitary fibrils running parallel to the cell surface (Figs. 1a and 1c). The presence of each of these structural components and their combinations varied from cell to cell. Some ultrathin sections contained ordered subunit structures with both fibril types, which were particularly characteristic of the fibrils that are parallel to the cell surface (Fig. 1c). This pattern of ultrastructural organization of the SL suggests the presence of different components such as polysaccharides and (glyco)proteins. Both macromolecule types are known to participate in the mineralization of bacterial cells including various cyanobacterial species, providing a matrix for the formation of calcite crystals on the cell surface. The outer membrane in all studied cells was undulating, with a manifest periodicity of local contacts with the underlying peptidoglycan layer (Figs. 1b and 1c).

'E. natronophila' displayed an extraordinary thylakoid organization. The enlarged intrathylakoid space typically contained copious deposits of rounded granules of unidentified chemical nature with a diameter of approximately 18–24 nm (Fig. 1d). According to our data, massive accumulation of electron-dense spherical granules in the inner space of the thylakoids is characteristic of various cyanobacteria from soda lakes [16, 17].

'E. natronophila' cells contained sparse inclusions such as carboxysomes and polyphosphate-containing electron-transparent areas.

While investigating the internal ultrastructure of *'E. natronophila'* in the experimental variants of our model system, we paid special attention to the cells whose mineralization resulted directly from the interactions with calcium carbonate and trona crystals. These findings were interpreted taking into account the data obtained in [10] using scanning electron microscopy in conjunction with determination of the element composition of the minerals.

During mineralization, calcium carbonate coated the cell as an electron-dense "shell' (Figs. 2a–2c). The cells predominantly remained morphologically intact or became slightly deformed. Nevertheless, such cells structurally differed from the control cells fixed simultaneously according to the same procedure. No SL components were detectable, probably due to the

destruction of the SL during its interaction with the crystallizing calcium carbonate or fusion with the minerals. Detachment of the crystal from the cell surface probably resulted from the enlargement of the crystalline "shell" or from the fact that the treatment required for electron microscopy produced different effects on the solid crystal and the cell. The ultrastructure of the cell wall and the intrathylakoid space of the "shell"-coated cells changed visibly (Figs. 2b and 2c). The outer membrane of the cell wall lost its undulated pattern, and high electron-density areas prevailed in its structure. The peptidoglycan layer formed a dotted line that also included electron-dense zones (Fig. 2b). The electron density of the content of the intrathylakoid space increased, so that the contrast of its granular material became more prominent (Fig. 2c). Despite these alterations, the general pattern of the cell structure, including the organization of the thylakoid system, remained similar to that of the control sample. The protoplasts displayed no signs of destruction. Sporadically, the cells became deformed, and their cell walls lost integrity (Fig. 2d). This image demonstrates that the electron-dense substance (possibly the crystallizing calcium carbonate) penetrated into the cytoplasm in this system (Fig. 2d). The possibility that the cell wall and the intrathylakoid space have locally thickened zones resulting from the intrusion of small-size molecules of uncrystallized calcium carbonate, via the outer membrane, into the periplasmic space and apparently into the intrathylakoid space that is continuous with the periplasmic space (Figs. 2b, 2c) cannot be excluded. These findings, the fact that the morphology of the cells and the general pattern of their internal structure remained unchanged inside the "shell", together with the absence of photosynthetic activity [10], indicate that mineralization by calcium carbonate resulted in mummification of cyanobacterial cells. Deposition of an electron-dense substance in the sheath and on the periphery of the cell wall and the cytoplasm occurred in *Calothrix* cells incubated with Ca^{2+} for several weeks [18]. This phenomenon was attributed to the influx of calcium cations into live cyanobacterial cells. In conjunction with the data of Fig. 2, this supports our suggestion that calcium as an exogenous chemical element was present in the newly formed electron-dense cell structures.

Ultrathin sections revealed that electron-transparent, multi-faceted trona crystals ($Na_2(CO_3)$ $Na(HCO_3) \cdot 2H_2O$) contained cyanobacterial cells engulfed by them (Fig. 3). The electron microscopic images obtained indicate that trona precipitation on the cell surface apparently involved tight binding of the trona substance with outer membrane-anchored glycocalyx elements. Therefore, its fragments could detach during the crystal growth. This is depicted in Fig. 3b. It can be seen that the outer membrane was missing only in the cell wall part facing the trona crystal whose invagination is complementary to the cell surface. A zone of mostly uniform width (0.2–0.8 μm) was located between the cell surface and the concave face of the crystal. The peptidoglycan layer of the cell wall, like the whole protoplast, remained structurally intact (Fig. 3b). Since the ultrastructure was similar to that of the control system, cells overgrown by a trona crystal remained viable. This statement is not at variance with the fact that the outer membrane was lacking at certain points, so that this stage engulfing of the cell by the trona was, to some extent, similar to the initial phase of L-form generation. The possibility cannot be ruled out that the most active cells can still separate from the crystal at early stages of their inclusion. The less active remained crystal-engulfed, underwent further destruction, and perished (Fig. 3c).

Under our experimental conditions, a third type of crystals was formed in addition to calcite and trona crystals. These are electron-dense druses of needle-shaped potassium chloride crystals that were located on the external face of the outer membrane of the cell wall (Fig. 4a). Presumably, the formation of these crystals involved the glycocalyx components to which they were bound, at least during the initial stages of crystallization. Thereafter, they can fill intercellular spaces (Fig. 4b).

DISCUSSION

The current consensus is that the structural components of the cell surface (capsules, sheaths, and S-layers) are of paramount importance for the initial stages of the prolonged process of cyanobacterial cell mineralization, which ultimately results in the formation of a special biogenic group of geological deposits in water sediments. It has been established that calcium carbonate precipitation during the carbonatization of freshwater cyanobacterial cells initially involves adsorption of calcium cation via binding to the carboxyl groups of C_6 monomers of acidic exopolysaccharides, the bulk components of capsules and sheaths of most cyanobacteria. Accordingly, a higher calcium amount is accumulated in the mucilage than in the ambient solution [19]. An analogous calcification mechanism initiated by Ca^{2+} binding to the negatively-charged groups of the surface S-layers in the absence of exopolysaccharides was revealed in a representative of the *Synechococcus* genus [3].

Calcification of the cyanobacterial films from alkaline saline lakes was considered in [20]. This work dealt with three lakes located in different regions: the Pyramid Lake (USA), the Nuoertu Lake (China), and the Satonda Crater Lake (Indonesia). The alkalinity varied from 4 to 625 mg-eq/l; the pH values were 8.5–9.3. It was revealed that the calcification of cyanobacterial biofilms was not instantaneous under these conditions, in spite of high ambient concentrations of carbonate ions. This was due to the mucilage polysaccharides that initially stored adsorbed Ca^{2+} cations.

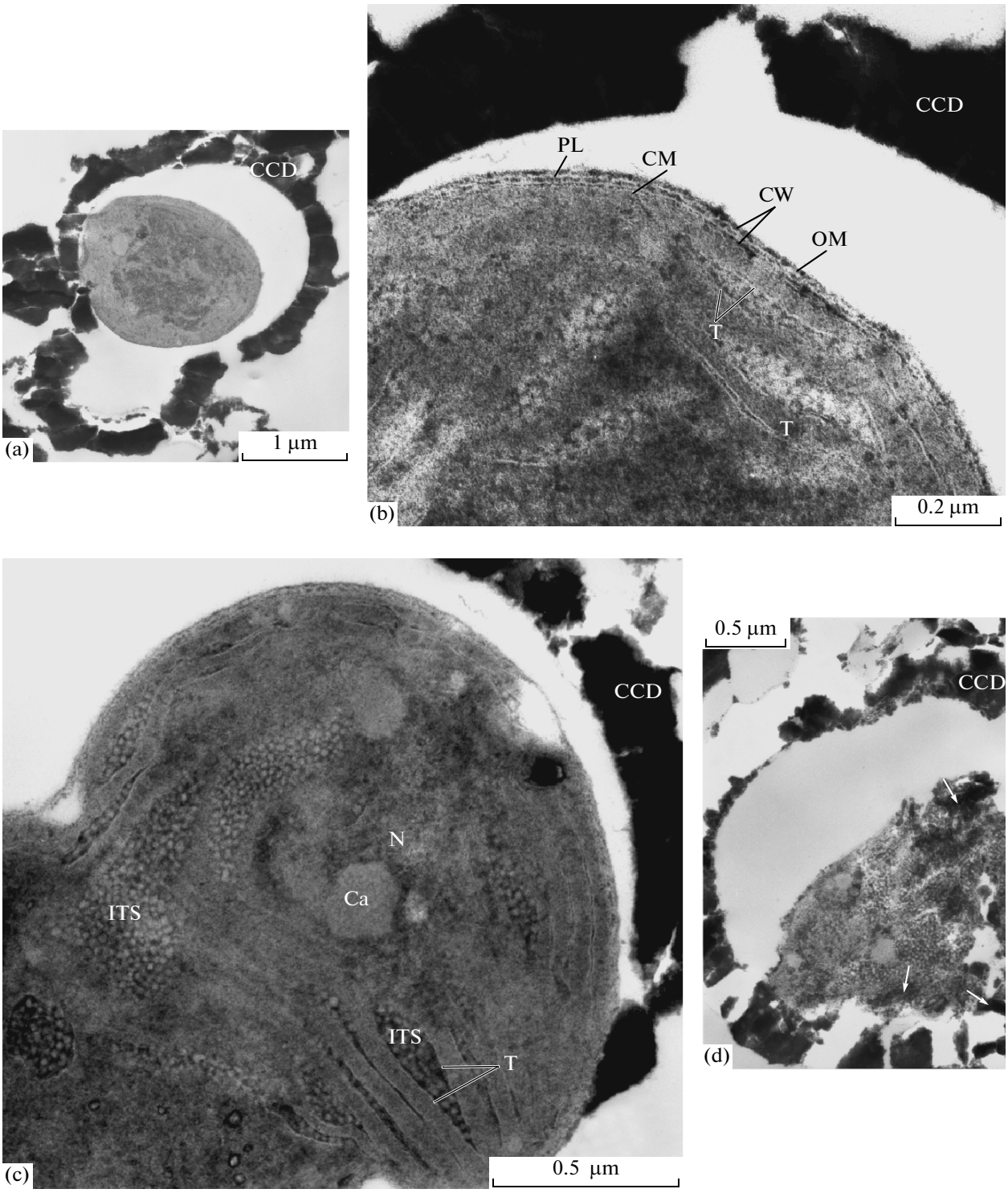

Fig. 2. Ultrastructure of $CaCO_3$ "shell"-enclosed *'Euhalothece natronophila'* Z-M001cells: general image of a calcium carbonate deposit-embedded cell (a); part of a cell with a changed cell wall (b); a cell with alterations in the intrathylakoid space (c); and a deformed cell with destructive changes (d). CM, cytoplasmic membrane; Ca, carboxysome; CCD, calcium carbonate deposits. See Fig. 1 for CW, PL, OM, T, N, and ITS. Arrows indicate deposits of an electron-dense substance, presumably $CaCO_3$, in the cytoplasm.

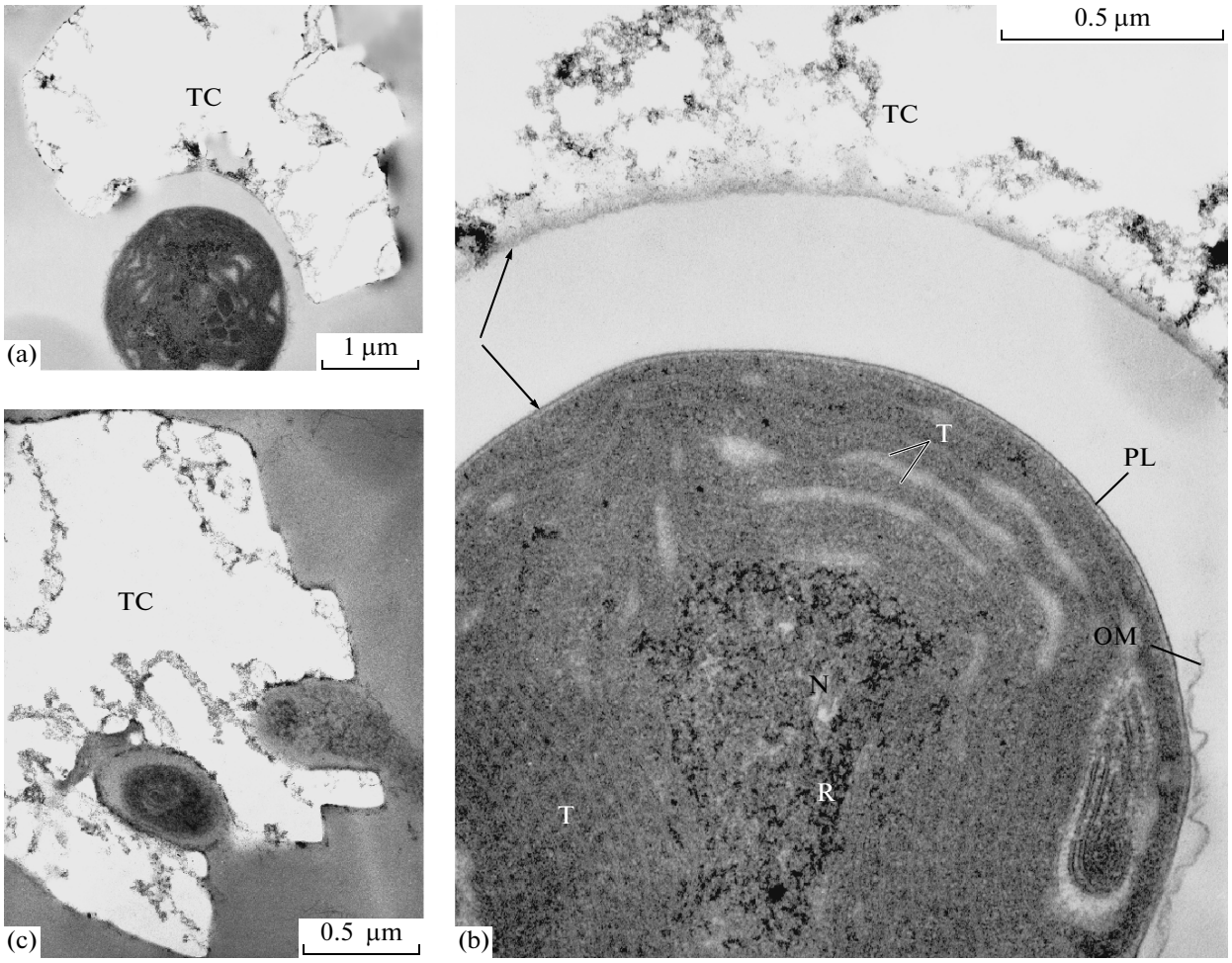

Fig. 3. Ultrastructure of *'Euhalothece natronophila'* Z-M001cells upon interaction with trona crystals (TC), $Na_2(CO_3)$ $Na(HCO_3) \cdot 2H_2O$: a cell at the initial stage of encapsulation in the crystal (a); part of a cell located close to the invagination zone of the crystal (b); and cell degradation upon complete encapsulation in the crystal (d). See the legend to Fig. 1 for PL, OM, T, N, and R. Arrows indicate the area between the cell surface and the concave face of the crystal.

$CaCO_3$ precipitation occurred only after the exopolysaccharides became calcium-saturated.

The data currently accumulated on carbonate precipitation processes in modern seas and lakes involving non-alkaliphilic cyanobacteria are summarized and analyzed in a number of review articles [5–9, 12, 20, 21]. It has been concluded that cyanobacteria can function as initial centers of structural reorganization of their micro-environment in two ways [21]: (i) via photosynthetic CO_2 consumption resulting in alkalinization and, accordingly, $CaCO_3$ precipitation and (ii) due to exopolysaccharides as described above. In both cases, organomineralization occurs according to [21], and the organic matrix determines the eventual morphology and composition of the crystals. Mineralization is active (biologically induced) in the first case and passive (biologically influenced) in the second one [21]. M. Obst et al. [5] demonstrated, however, that photosynthetic CO_2 adsorption did not directly influence $CaCO_3$ nucleation on the surface of *Synechococcus leopoliensis* cells, which is consistent with the passive mechanism.

Our studies [10, 17] revealed that carbonatization of the extremely halophilic cyanobacterium *'E. natronophila'* differed from the analogous process in freshwater and halophilic cyanobacteria investigated in earlier works. Calcium precipitated in the form of calcium carbonate due to a chemical reaction in the saturated carbonate solution and was attached to the cell surface as amorphous $CaCO_3$, not Ca^{2+} cations. During the later stages of this process, the substance crystallized on the cell surface, forming a cell-coating "shell". In terms of the classification suggested in [21], this is apparently passive bioorganically-mediated $CaCO_3$ precipitation formed under the conditions of soda lakes.

Under such physicochemical conditions, in the absence of developed polyanionic capsules or sheaths, the organization of the thin SL of *'E. natronophila'* and their macromolecular composition may be important

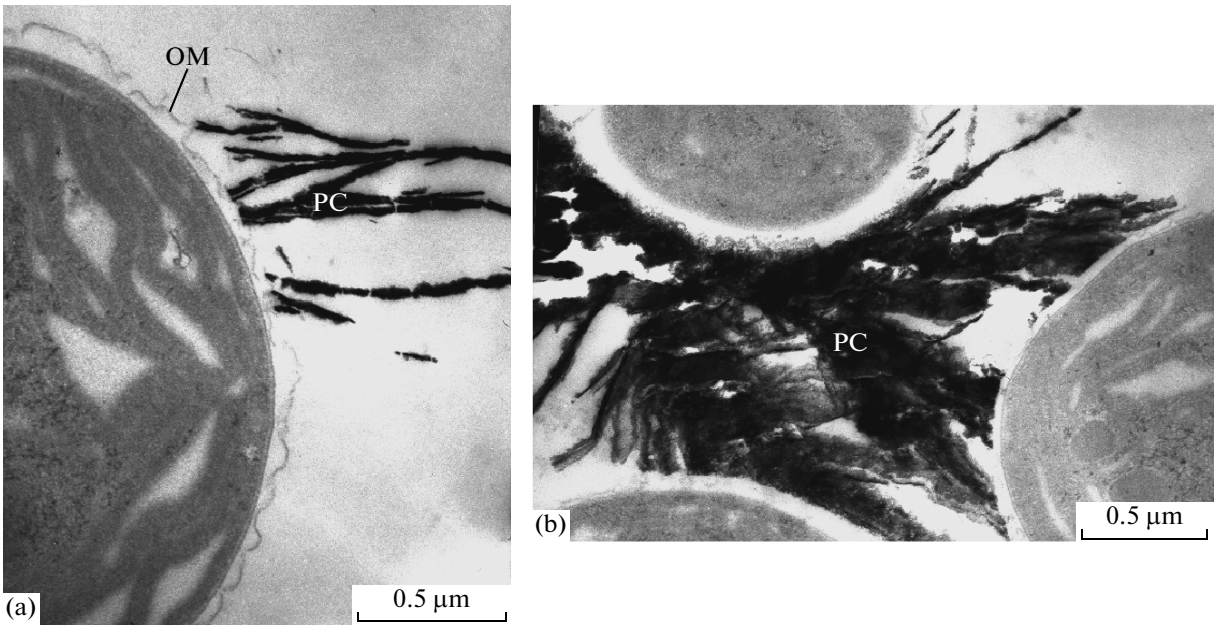

Fig. 4. Formation of potassium chloride (PC) druses in an *'Euhalothece natronophila'* Z-M001 culture: attachment to the OM (a); and accumulation of crystal druses in the intercellular space (b). OM, see legend to Fig. 1.

for the primary adsorption and subsequent crystallization of calcium carbonate.

Research on ultrathin cyanobacterial sections revealed that initial stages of carbonatization apparently involved the interaction of amorphous $CaCO_3$ with SL components, which was accompanied by changes in the ultrastructural organization of the cell wall and the intrathylakoid space. These pictures show that the cells inside the carbonate "shell" retained their morphology and the general structural pattern. However, as our previous work [10] demonstrates, they lose their photosynthetic capacity and, therefore, eventually die without visible destruction.

Of particular interest in this context is the fact that an analogous process of "shell" formation around morphologically intact cyanobacteria occurs with heat-killed cells [10]. This fact is consistent with the suggestion that calcium carbonate molecules penetrate into the cells via impaired outer membranes (Fig. 2d). This is feasible with heat-killed cells, or if the cell wall undergoes destructive changes during initial stages of mineralization. In both systems, cyanobacteria are mummified inside the "shell".

A somewhat different pattern occurs if trona crystals are generated around cyanobacterial cells. Trona precipitates form a solid phase upon the concentration of the solution. In our studies, this was observed after one day of incubation, and trona crystals with embedded cells appeared [10]. TEM enabled monitoring of the consecutive stages of cell encapsulation by a trona crystal. It was established that the mineral coated the cell during the crystal's growth, finally engulfing it. Initially, the process involved live cells whose internal ultrastructure did not change, even upon detaching the outer membrane. In contrast to calcite-induced mineralization, trona precipitation did not result in cell mummification. In most cells, nevertheless, impairing the outer membrane caused metabolic alteration or disruption that affected cell wall functions. The cells completely engulfed by a crystal are subject to degradation and destruction. Encapsulation in a trona crystal severely damages the cell, and this may result in the death of a cell accompanied by its serious or complete destruction.

Our studies demonstrated that the initial stages of *'E. natronophila'* mineralization involve interactions of various minerals with the cell wall (outer membrane) or the thin SL that presumably is of polysaccharide and/or (glyco)protein nature. The ultrastructural integrity of the outer membrane is retained upon overgrowing the cells with a $CaCO_3$ "shell". However, the electron density of this structure and the underlying peptidoglycan layer is changed. In contrast, if cyanobacteria contact with growing trona crystals, detaching and degrading the outer membrane results, although the structure of the peptidoglycan layer remains intact. The outer membrane of the cell wall also plays the decisive role in interacting with chloride minerals that are formed, like trona, upon concentrating the solution. Needle-shaped potassium chloride crystals tightly bind to the outer membrane, but this does not cause its detachment from the cell.

Hence, research on the ultrastructure of *'E. natronophila'* revealed that different minerals interact with the surface cell structures in different ways. These interactions implicate substantially differ-

ent degradation processes developing in the cell wall and the cytoplasm. The data obtained give grounds for the suggestion that these differences, i.e., cell mummification associated with carbonatization and their severe destruction upon encapsulation in trona, account for the lack of fossilized cyanobacteria in the massive trona deposits of Lake Magadi. However, they are abundant in the calcium-rich Green Beds of "High Magadi".

ACKNOWLEDGMENTS

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