Microbiology, Vol. 71, No. 5, 2002, pp. 563–569. Translated from Mikrobiologiya, Vol. 71, No. 5, 2002, pp. 654–661.

EXPERIMENTAL ARTICLES

Formation of Giant and Ultramicroscopic Forms of *Nostoc muscorum* CALU 304 during Cocultivation with *Rauwolfia* Tissues

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Received July 3, 2001

Abstract—The study of heteromorphic *Nostoc muscorum* CALU 304 cells, whose formation was induced by 6- to 7-week cocultivation with the *Rauwolfia* callus tissues under unfavorable conditions, revealed the occurrence of giant cell forms (GCFs) with a volume which was 35–210 times greater than that of standard cyanobacterial cells. Some GCFs had an impaired structure of the murein layer of the cell wall, which resulted in a degree of impairment of the cell wall ranging from the mere loss of its rigidity to its profound degeneration with the retention of only small peptidoglycan fragments. An analysis of thin sections showed that all GCFs had enlarged nucleoids. The photosynthetic membranes of spheroplast-like GCFs formed vesicles with contents analogous to that of nucleoids (DNA strands and ribosomes). About 60% of the vesicles had a size exceeding 300 nm. With the degradation of GCFs, the vesicles appeared in the intercellular slimy matrix. It is suggested that the vesicles are analogous to elementary bodies, which are the minimal and likely primary reproductive elements of L-forms. The data obtained in this study indicate that such L-forms may be produced in the populations of the cyanobionts of natural and model syncyanoses. Along with the other known cyanobacterial forms induced by macrosymbionts, L-forms may represent specific adaptive cell forms generated in response to the action of plant symbionts.

Key words: cyanobacteria, plant tissues, symbiosis, artificial associations, heteromorphism, *Nostoc*, L-forms.

Heteromorphism (or pleomorphism) is widely spread among prokaryotes, which change their "normal" morphological and physiological characteristics in response to almost any alteration in the environmental conditions. Heteromorphic cell variants may drastically differ from their parent forms in phenotypic characteristics. The development of advanced genetic methods of bacterial typing makes it possible to classify and phylogenetically study prokaryotes in communities even without isolating them in pure cultures [1]. It is difficult, however, to relate genetic data concerning particular (and often uninvestigated) species of a community to data on the structural integrity, metabolic activity, and viability of particular cell forms, the morpho-physiological heterogeneity of populations, and the ecological role of particular members of the community. From this standpoint, of interest is the study of abnormal forms of microorganisms and their role in the persistence of species and their adaptation to the variable environment during, for instance, reactivating and chronic infections, parasitism, and symbiosis. Among such forms, which typically have modified cell covers, there are the viable-but-nonculturable forms (VBNC-forms) of non-spore-forming bacteria [2, 3], cell forms with unbalanced growth [4], L-forms [5, 6], and likely nannobacteria [7].

The studies of the natural symbioses and artificial associations of higher plants with cyanobacteria (the so-called syncyanoses) revealed the presence of cyanobacterial forms with a reduced cell wall, namely, spheroplast and protoplasts [8–15]. However, the significance of cell wall modification was a subject of discussion only in few of the relevant papers. Thus, Baulina *et al.* [9], which found the spheric cells of different size and the structurally intact and lysed protoplasts and spheroplasts of *Anabaena variabilis* in an association with the tobacco callus tissue, suggested that they resulted from the transformation of a fraction of the cyanobacterial population into L-forms. The suggestion that the formation of cyanobacterial forms with the reduced cell wall in syncyanoses is an adaptive response of the cyanobacteria was confirmed by the detection of such forms in growing and functionally active cyanobiont populations [11–15]. Along with L-forms, those populations often contained giant cells and spheroplasts, minicells, microcells, and amorphous agglomerations of cell debris [11, 13, and Baulina's personal communication]. Using the artificial *Rauwolfia serpentina–Nostoc muscorum* CALU 304 syncyanosis as a model, it was shown that the increase in the amounts of heteromorphic *N. muscorum* cells, including those with the reduced cell wall (spheroplasts and

0026-2617/02/7105-0563$27.00

protoplasts), is induced by the plant partner and enhances the survival of the cyanobacterium in the association cultivated under conditions unfavorable for the microorganism. As is evident from the data on cell ultrastructure and cyanophycin accumulation, the cyanobacterial spheroplasts and protoplasts were structurally intact and metabolically active [13, 15].

The aim of the present work was to study the ultrastructure of the *N. muscorum* CALU 304 giant spheroplasts and cells found in a mixed culture with the rauwolfia callus tissue.

MATERIALS AND METHODS

The nitrogen-fixing cyanobacterium *Nostoc muscorum* Agardh CALU 304 was grown in a coculture with the callus of the nonsymbiotrophic *Rauwolfia serpentina* Benth. strain K-27 either in the form of mixed aggregates or on the agar medium surface without contacting the plant tissue. In the latter case, the partners interacted indirectly, through the exchange of their metabolites diffusing through the medium. The obtaining and cultivation of the axenic mono- and mixed cultures of the free-living cyanobacterium and the rauwolfia callus tissue are described in detail elsewhere [16, 17].

Electron microscopic studies were carried out using specimens fixed with 0.5% glutaraldehyde and 1% osmium tetroxide. The preparation of the specimens and the investigation of cyanophycin accumulation were described earlier [12, 16]. Electron microscopic images were digitally processed using a Videolab2.1A system (Uni-Export Instruments, United Kingdom). The size of cytoplasmic structures and the diameter of vesicles (d) were determined based on the analysis of the randomly taken 50–150 images of cells in each of the cultivation variants with the exclusion of serial sections. The vesicles were classified according to their diameters into the following ten classes: class 1 with $d \le 100$ nm, class 2 with 100 nm $< d \le 200$ nm, …, class 9 with 800 nm $< d \le 900$ nm, and class 10 with $d > 900$ nm. The occurrence frequency of vesicles in each class was expressed as a percent of all the vesicles tested (148 vesicles in cells and 20 vesicles in the intercellular matrix).

Twelve experiments were carried out in three series, from 3 to 9 replicated experiments in each of the variants. Electron microscopic analysis was conducted using 53 specimens.

RESULTS AND DISCUSSION

Earlier studies showed that the cocultivation of the cyanobacterium *N. muscorum* CALU 304 with the rauwolfia callus tissue either as mixed aggregates or as microcolonies without their direct contact with the plant partner gives rise to heteromorphic cells, whose formation increases the viability of the population of cyanobacterium as compared with its growth in a monoculture [13]. One of the groups of these heteromorphic cells is the group of giant cell forms (GCFs), which includes GCFs with a rigid cell wall, GCFs with an altered structure of the peptidoglycan layer of the cell wall, and spheroplasts. Unlike other heteromorphic cells, GCFs were produced in the mixed culture in the late terms of its incubation (6–7 weeks). The ultrastructure of GCFs was virtually the same in the microcolonies of mixed aggregates and when the partners were spatially separated.

GCFs were slightly oval in shape. The average dimension was $14.3 \pm 0.8 \times 12.8 \pm 0.7$ μm, although some GCFs had a diameter of up to 24.5 μm. Giant spheroplasts had an irregular, amoeboid shape and were larger than giant cells. The volume of the GCFs was 35–210 times greater than that of standard cyanobacterial cells. The specific effect of rauwolfia on the peptidoglycan metabolism of *N. muscorum* CALU 304 [15], as well as the structural similarity of various GCFs and the occurrence of GCFs with different degrees of impairment of the murein layer (Figs. 1a, 2, 3a), indicated that the giant spheroplasts were most likely formed from the giant cells.

The cytoplasmic membrane of GCFs retained its integrity and often produced profound invaginations and lomasome-like structures (Fig. 1b), whose formation is aimed at a compensatory increase in the contact area of the GCFs with the environment. The intracytoplasmic membrane system of GCFs represents multiple curved thylakoids, which form a lacy network (Figs. 1a, 2a) or concentric circles and parallel rows (Figs. 2b, 3a) around the nucleoid. The intrathylakoid space is enlarged, suggesting that the functional activity of the photosynthetic membranes of GCFs is not high.

The central part of GCFs is occupied by one or several distinct nucleoid zones with ribosome aggregates (Figs. 1a, 2, 3a). These aggregates tend to the periphery of the nucleoid zone, where they look like osmiophilic closely packed granules 17–20 nm in size. The area of the nucleoid zone on GCF sections reaches 25–40 μm^2, whereas the minimum area of GCFs is only 13 μm^2. On the sections of common vegetative *N. muscorum* CALU 304 cells, only some fragments of the nucleoid zone are visible. The area of the central longitudinal section of such cyanobacterial cells grown in both pure and mixed cultures did not exceed 13–16 μm^2. A comparison of these values shows that the nucleoid of GCFs is several times greater than that of common vegetative cyanobacterial cells. This suggests that the replication rate in GCFs is higher than that of cytokinesis and that the GCFs may contain genetic material in amounts sufficient for several common vegetative cells. The occurrence of large ribosome aggregates and polysomes in GCFs evidences for enhanced transcription–translation processes, which exhaust the material and energy resources of GCFs. As a result, unlike the vegetative

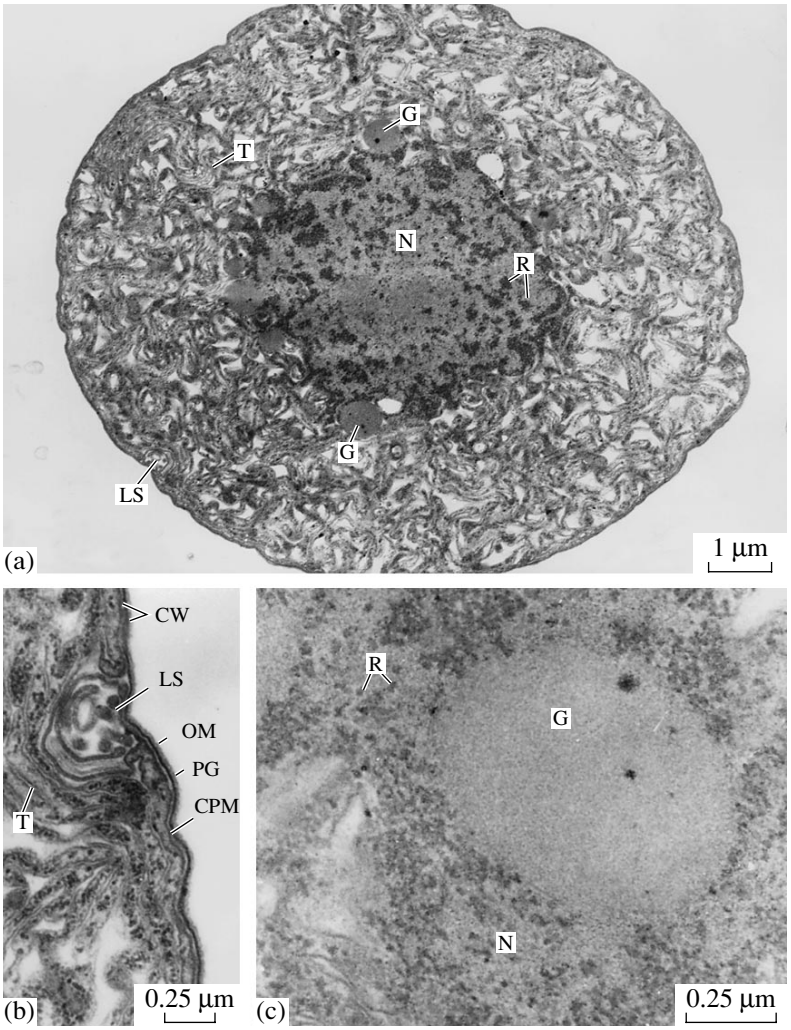

Fig. 1. (a) A giant *N. muscorum* CALU 304 cell and (b, c) its fragments at higher magnifications. G, globule of unknown origin; CW, cell wall; LS, lomasome-like structure; N, nucleoid; OM, outer membrane; PG, peptidoglycan; R, ribosome; T, thylakoid; CPM, cytoplasmic membrane.

cells of *N. muscorum* CALU 304, the GCFs of this cyanobacterium population contain small amounts, if any of intracellular inclusions (glycogen, polyphosphates, lipids, poly-β-hydroxybutyrate, and carboxysomes). The only storage material that is often observed in GCFs is cyanophycin granules (Figs. 2b, 3a). Although the mean total volume of cyanophycin granules in one GCF is the same as in one vegetative cyanobacterial cell (see

Accumulation of cyanophycin granules in *N. muscorum* CALU 304 cells cocultivated with the rauwolfia callus tissue

Cultivation conditions	Cell morphotype	Cyanophycin granules*		
		N	*D*, nm	$V, \times 10^6$ nm^3
Incubation of mixed aggregates for 58 days	Vegetative cells	0.50	357 ± 27	12.94
	GCFs	1.60	250 ± 27	13.09
Incubation on agar plates for 70 days	Vegetative cells	0.83	286 ± 23	10.17
	GCFs	1.66	231 ± 16	10.57

* The accumulation of cyanophycin granules was evaluated as their mean number visible on one cell section (*N*), the mean diameter of the granules (*D*), and the total volume of the granules per one cell, calculated by the formula ($V = 1/6\pi D^3 N$).

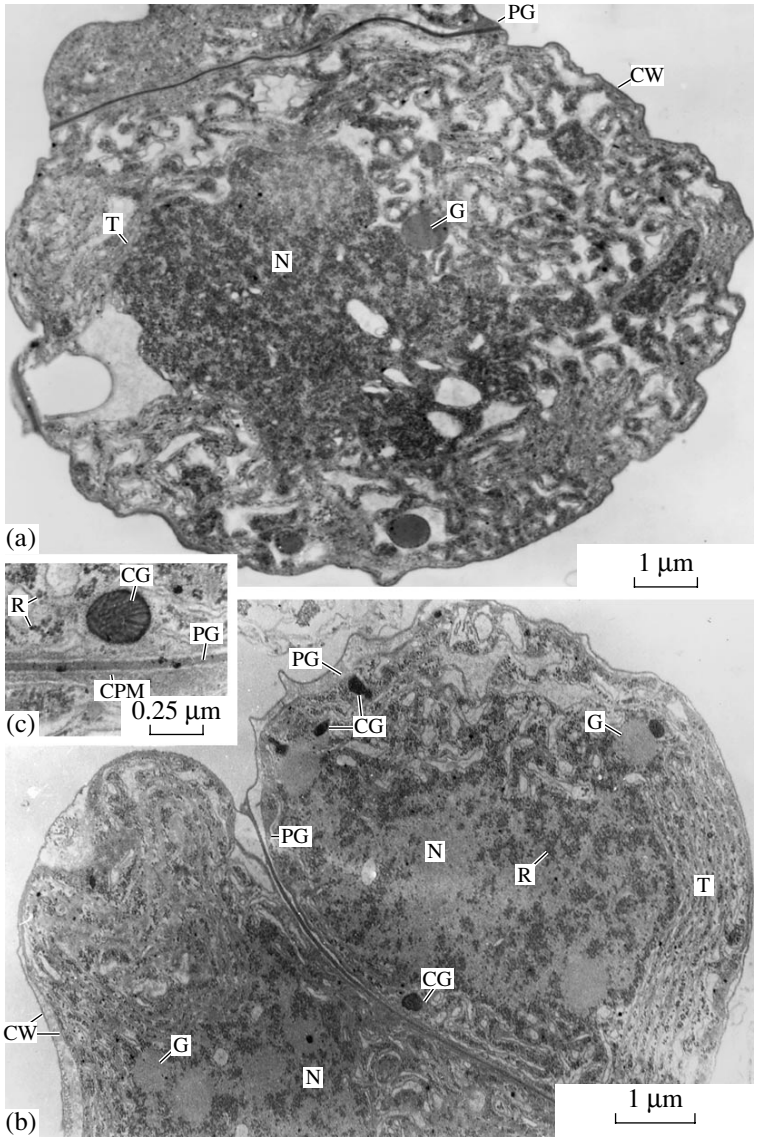

Fig. 2. Giant cell forms of *N. muscorum* CALU 304 with an altered peptidoglycan layer of the cell wall: (a) cell wall with an altered rigidity; (b) peptidoglycan layer in the form of a loose network in the swollen periplasm, septal peptidoglycan without visible alterations; (c) a magnified fragment of micrograph (b). CG, cyanophycin granule; other designations as in Fig. 1.

table and [16]), the relative amount of these granules in GCFs is considerably smaller than in vegetative cells, because of the great difference in the size of the GCFs and vegetative cells.

It should be noted that we observed some globules 50 to 650 nm in size, which were situated in the nucleoid zone or near it (between thylakoids) (Figs. 1–3). The globules were not enclosed in membranes and had a medium electron density. At a high magnification (Fig. 1c), one could see that these globules contain small (5 to 8 nm in size) subunits and thin threads, which were linearly joined and sometimes produced regular structures in the form of various loops, zigzags, and arcs. The boundary between the nucleoid and the globules was not always well distinguishable. The chemical nature of the globules is unknown. The location of the globules in, or close to, the nucleoid zone and their corpuscular structure suggest that they are analogous to carboxysomes, which consist of particles with a diameter of about 12 nm [18]. Unlike the carboxysomes, the globules were not enclosed in the monolayer membrane and did not exhibit the polyhedral profile.

The spheroplast-like GCFs with the remaining peptidoglycan fragments in the regions where photosynthetic membranes are in contact with the nucleoid could showed the presence of large local extensions in the intrathylakoid space, into which the thylakoid membrane invaginated with the formation of round vesicles (Fig. 3). On thin sections, the vesicles looked like single

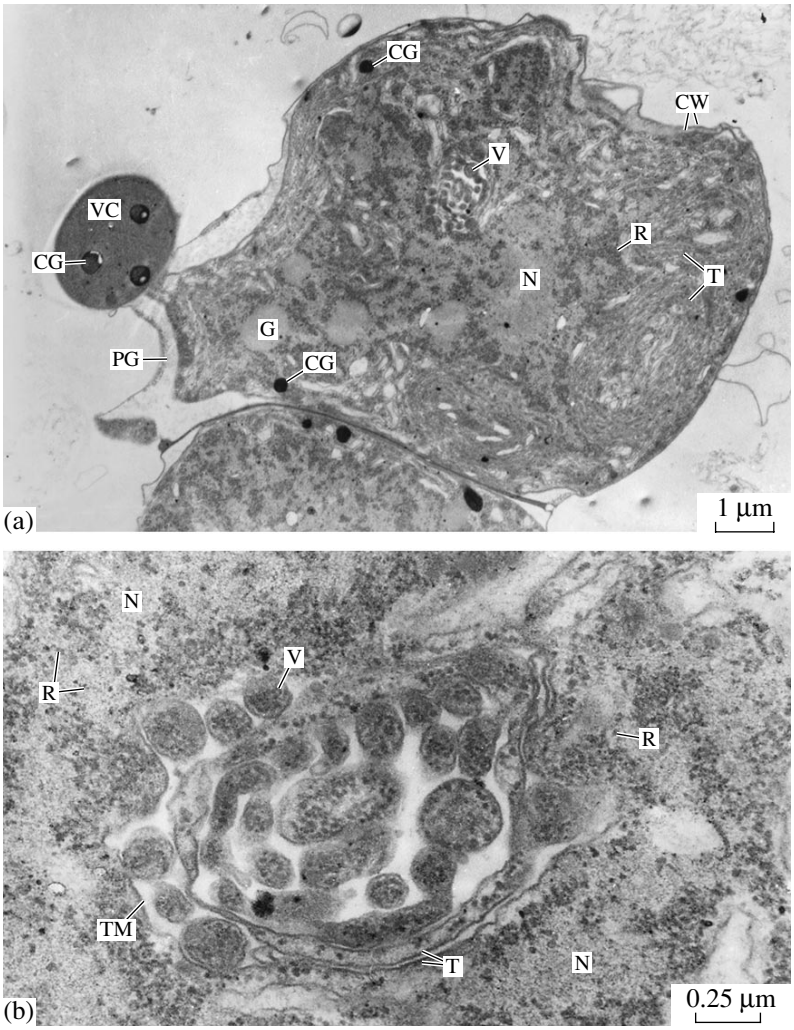

Fig. 3. (a) A spheroplast-like giant cell of *N. muscorum* CALU 304 with a fragmented peptidoglycan layer, contacting a normal vegetative cell, and (b) its fragment at a higher magnification with vesicles in the intrathylakoid space. TM, thylakoid membrane; V, vesicles; VC, vegetative cells; other designations as in Fig. 1.

or multiple structures, within which electron-dense granules and thin fibrils, most likely representing ribosomes and DNA threads, could easily be distinguished (Fig. 3b). During the formation of a vesicle, the invaginated thylakoid membrane may carry ribosomes and DNA. This follows from the fact that not only some ribosomes but also the chromosome are bound to the protoplasmic side of the photosynthetic membranes in cyanobacteria, whereas to the cytoplasmic membrane in other bacteria. Based on these data, Pinevich suggested that the intracytoplasmic membrane system of cyanobacteria is involved in DNA replication and in the segregation of daughter chromosomes [19].

The size of vesicles on ultrathin sections ranged from 80 to 1060 nm. About 87% of the vesicles had a diameter larger than the minimum size of viable cells (140 nm), whereas about 60% of the vesicles had a diameter larger than 300 nm. Taking into account the size distribution of the vesicles (Fig. 4a) and assuming that the spherical vesicles are cut during specimen preparation in a random manner, it would be natural to suggest that most vesicles had a diameter equal to or greater than 400 nm.

With the destruction of GCFs, the vesicles appear in the intercellular matrix. Since the preparation of specimens for microscopy involves multiple changes of incubation solutions, most such vesicles are lost, with the result that only vesicles with the most frequently encountered size can be seen in the intercellular matrix (Fig. 4b). In addition to the vesicles, the intercellular matrix also contains small (0.9 to 1.7 μm in size) binary dividing cells and protoplasts with poorly developed thylakoids (Fig. 5).

The vesicles are similar to elementary bodies, which are the minimal and likely primary reproductive elements of L-forms [5, 6]. The formation of elementary

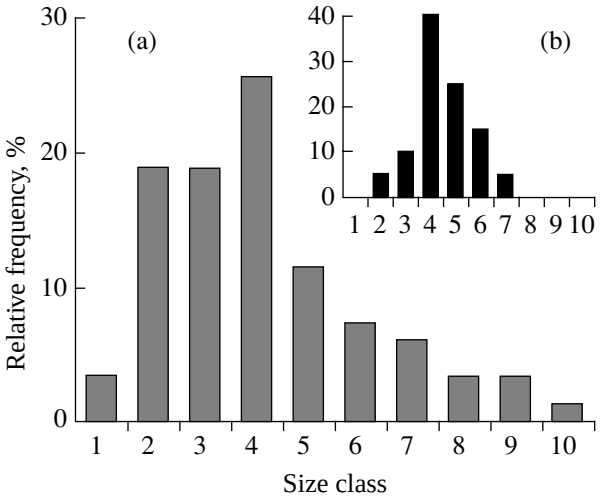

Fig. 4. The size distribution of vesicles in (a) the giant cell forms of *N. muscorum* CALU 304 and (b) the intercellular matrix.

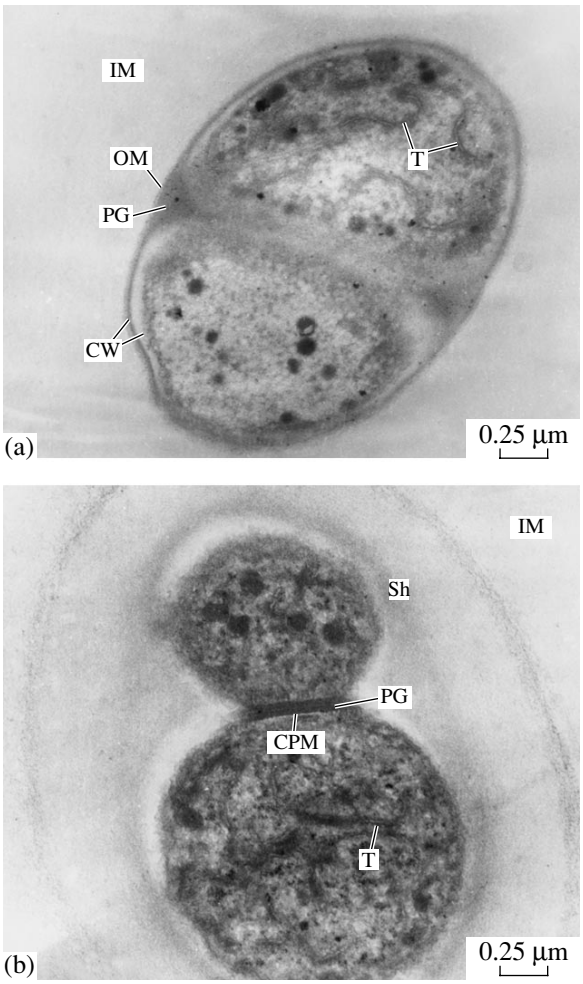

Fig. 5. Small cell forms of CALU 304 with poorly developed thylakoids. IM, intercellular matrix; Sh, sheath; other designations as in Fig. 1.

bodies by the parent L-forms was described for many heterotrophic bacteria, but not for the L-like colonies of the cyanobacterium *Chlorogloea* (*Chlorogloeopsis*) *fritschii* subcultured 8 times in the presence of lysozyme [20]. The formation of small spherical cells with a minimum diameter of 0.5 μm was also observed in the *A. variabilis* population cocultured with the tobacco callus tissue [9]. Although the ultrastructural organization of these cells was not investigated, it is clear that they represented either minicells lacking genome or nannoforms capable of reproduction (i.e., elementary bodies or microcells).

The elementary bodies of heterotrophic bacteria had sizes ranging from 50 to 1000 nm (the elementary bodies with sizes greater than 240 nm are considered to be viable). In the case of the heterocyst-forming cyanobacterium *N. muscorum* CALU 304, whose genome is considerably greater than that of other prokaryotes, the fully functionally active elementary bodies must obviously have greater sizes.

It is tempting to speculate that the above mentioned vesicles of cyanobacterial L-forms serve as elementary bodies and are formed with the involvement of the thylakoid membrane but not the cytoplasmic membrane, as in the L-forms of other bacteria. This speculation is based not only on the data of transmission microscopy, but also on the idea of the continuum of the membrane system of prokaryotes and on the unique properties of the thylakoid membranes of cyanobacteria. These membranes are characterized by high structural and functional flexibility, which extends to the photosynthetic and oxidative generation of energy and to the direct interaction with the genetic material of cells [19].

Thus, the investigation of the *N. muscorum* CALU 304 giant cell forms and their elementary body–like vesicles shows the possibility of the formation of L-forms in the populations of the cyanobionts of natural and model syncyanoses. Along with the known alternative differentiated cyanobacterial forms induced by macrosymbionts [21], cyanobacterial L-forms may represent specific adaptive cell forms generated in response to the action of plant symbionts.

ACKNOWLEDGMENTS

We are grateful to O. I. Baulina and M. V. Gusev for critical review of the manuscript and valuable discussion.

This work was supported by grant no. 00-04-48708 from the Russian Foundation for Basic Research.

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