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Preface

The foundation of this monograph is laid by long-term research of the author together with her colleagues from the Professor Gusev school in microbial physiology at the Biology Faculty of M.V. Lomonosov Moscow State University. This monograph summarizes a considerable body of our pioneering investigations in the field of cell biology of microorganisms. The book deals primarily with cyanobacteria, a most ancient group of phototrophic microorganisms with unique physiology, versatile metabolism, and a vast interspecies morphological diversity. The high versatility of metabolism makes cyanobacteria, the primary producers in microbial communities, a key player determining the evolution pattern of biosphere. The monograph contains a detailed overview of the ultrastructure of the representatives of different subsections of the phylum Cyanobacteria under experimental and natural conditions causing dramatic changes of their mode of existence and behavior. The results of the analysis of ultrastructural changes in the cyanobacteria *Synechococcus* sp., *Anabaena variabilis, Chlorogloepsis fritschii* and *Nostoc* sp. comprise a large body of the material presented in this book. These species were deliberately chosen since they are, for a long time, a lovely object for scientists specializing in different fields of microbiology—physiology, biochemistry, genetics, cytologists, ecologists—and are thoroughly studied in many regards. The author truly hopes that the results of the ultrastructural research of their plasticity will give new exciting insights into these incredibly interesting microorganisms.

The work in the academic community of biological faculty of MSU and creative collaboration with other scientific institutes provided to the author as a specialist in the field of cytology a unique opportunity for investigation of numerous fundamentally different experimental systems revealing divers and often unanticipated adaptive capabilities. A detailed information is presented on various species of cyanobacteria (i) grown under optimum conditions, (ii) cultivated using different light regimens, (iii) undergoing enzyme-induced L-transformation, and (iv) existing in natural symbioses and model associations with plant partners. Diverse patterns of cyanobacterial ultrastructural reorganization are described. These patterns are interpreted as manifestations of phenotypic plasticity displayed

in response to environmental changes. The concept of bacterial ultrastructural plasticity as an indication of the operation of adaptation mechanisms functioning on the subcellular, cellular, and population level is discussed. This concept was developed by the author on the basis of her own extensive experimental data and present-day ideas concerning structural and functional adaptation mechanisms and their importance for prokaryotes. Since the experimental data and theoretical conclusions demonstrate that investigation of the ultrastructural plasticity is promising for the study of adaptive capabilities and intraspecific morphological variability in prokaryotes, this book may be of interest to the researcher specializing in various aspects of microbiology. The author believes that it would be useful for cell biologists, plant physiologists, symbiologists, and ecologists, as well as for undergraduate and graduate students of universities and colleges who focus on biology.

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Contents

Abbreviations

CCCP	Carbonyl cyanide *m*-chlorophenyl hydrazone
CM	Cytoplasmic membrane
CWDF	Cell wall-defective form(s)
CWRF	Cell wall-reduced form(s) of vegetative cell
CWRH	Cell wall-reduced heterocyst(s)
EM	Electron microscopy
MSS	Mucous surface structure
PSI	Photosystem I
PSII	Photosystem II
QS	Quorum-sensing
RuBisCo	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
UGF	Unbalanced growth forms

Chapter 1
Introduction

Abstract This introductory chapter demonstrates the topicality of the ultrastructural plasticity investigation—the direction this book is devoted to. It is warranted by the demand in new insights into the adaptation capabilities of species within microbial communities, primary determinants of the condition of microbial biosphere. The analysis of background for the advent and the development of this direction is carried out together with a modern-level discussion of the conception of 'phenotypic plasticity' of prokaryotes. The ultrastructural plasticity is considered as a component of the latter. Accordingly, the investigation of its manifestation diversity is suggested as a strategy of the elucidation of bacterial adaptive capabilities. The key role of the population approach in this methodology is emphasized taking into account the cell heterogeneity and basing on the conception of bacterial population as a cohesive self-regulated system. The choice of cyanobacteria as an object of investigation of the ultrastructural plasticity of prokaryotes is substantiated.

Investigation of the ultrastructural organization of microorganisms is a powerful means both for the understanding of the structural and functional fundamentals of their activity and for identification of the objects at various taxonomic ranks, including the previously unknown species. In contemporary microbiology, ultrastructural approach became an essential part of complex investigation, in row with molecular genetics-based techniques.

The structure and functioning of prokaryotic (mostly unicellular) microorganisms at the cellular and subcellular levels form the specific subject of microbial cytology, one of the major aspects of research and teaching in general microbiology. It is now evident that investigation in this field should consider the evolutionary development of the microbial characteristics which enhance the variety of their morphological and physiological forms (even within a single species). Among such forms, the only recently discovered, intensively investigated ultramicrobacteria (Trevors and Psenner 2001; Duda et al. 2012), uncultured bacteria (Colwell et al. 1985; Colwell 2000; McDougald et al. 1998; Golovlev 1998a), and

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mummified and cyst-like dormant cells (El-Registan 2005; Suzina et al. 2001; Mulyukin et al. 2009) are the most impressive ones. New cell types, such as unusual giant forms of cyanobacteria (Gorelova and Korzhenevskaya 2002), are still being discovered. Such diversity of cell forms may hinder species identification in situ, e.g., in the case of free-living (Palińska and Krumbein 1994) and symbiotic (Paulsrud and Lindblad 1998) cyanobacteria. The evolutionary and ecological aspects of prokaryotic biodiversity attract much attention (Schloter et al. 2000; Zavarzin 2002, 2008). Understanding the natural morphological heterogeneity in the populations of pathogenic microorganisms is essential for medical theory and practice (Vysotskii 2008).

Unlike most eukaryotic cells, the prokaryotic ones possess a less perfect system of homeostasis, together with higher capacity for rapid metabolic and structural rearrangements under changing environmental conditions. By the late 1980s, an array of experimental data was accumulated concerning the variability of molecular and ultrastructural organization of cell envelopes and other surface structures of pathogenic bacteria, promoting their survival in the process of infecting plant and animal tissues. Analysis of these data resulted in a conclusion that such rearrangements resulted from high phenotypic plasticity of prokaryotic cells, a complex of flexible systems for adaptive reaction, including the regulation of gene expression (Brown and Williams 1985; Costerton 1988). The notion of "phenotypic plasticity" is fundamentally similar to the well-known notion of "adaptive modifications" in genetics, which characterizes the phenomenon of nonheritable modification variability. This variability is understood as the capacity of an organism to react to environmental conditions by changes within the reaction norm (Inge-Vechtomov 1989). The reasons for modification variability, and especially for adaptive modifications, should lie in the mechanisms of the regulation of gene expression, which are relatively well investigated in prokaryotes. Active investigation of the molecular genetic mechanisms of bacterial response to various chemical and physical factors acting as signals for the subsequent reaction of a cell is presently under way (Golden 2003; Hellingwerf 2004; Los et al. 2008; Adams and Duggan 2012). The results of such modern disciplines as genomics and proteomics are the basis of significant progress in this field. Complete nucleotide sequences of a number of microorganisms have been determined. The technology of DNA microchips makes it possible to identify the genes (including the previously uncharacterized ones), which are induced or repressed by various environmental stress factors. It is also known that bacteria are characterized by high frequency of reversible recombinant variability of the genome, usually spontaneous and nonsynchronous in different cells of the population. This variability plays an adaptive role, together with the regulation at the epigenetic level (Prozorov 2001). It results in emergence of the cells with a set of different phenotypic characteristics, which is mostly similar to the one revealed by the previous investigation of the phenotypic plasticity of the surface structures of pathogenic bacteria. They include antigenic variations, changes in the synthesis of exopolysaccharides, including those of the capsule, the mechanism of periodic pili (fimbria) formation, etc. Thus, the modern interpretation of the phenotypic plasticity in

bacteria reflects the action of a number of regulatory processes both at the level of intragenomic adaptive rearrangements and at the epigenetic level. For example, a combination of these processes was revealed by investigation of the regulation of expression of the bacterial S-layers (Fernández and Berenguer 2000).

Application of various techniques of electron microscopy contributed greatly to the development of the notion of phenotypic plasticity. For continuity sake, it is therefore discussed by the author. According to the literature data, the phenotypic plasticity manifests itself at various levels of cellular organization, i.e., the metabolic (including chemical modification and alternative synthesis of the macromolecules), ultrastructural, and morphological ones, according to the specific characteristics of the complexes of adaptive processes involved at these levels. It is therefore acceptable and reasonable to interpret the individual manifestations of the metabolic, ultrastructural, and morphological plasticity as interrelated components of the phenotypic plasticity. The role of the metabolic plasticity in microbial activity is well known. Adaptation of *Escherichia coli* to lactose as a new substrate (a case of adaptive modifications) is a classical example. Since the intraspecific morphological plasticity of microbial cells may hinder in situ identification of bacteria, this phenomenon recently attracted much attention. The ultrastructural plasticity, which is both a manifestation of the metabolic plasticity and the factor responsible for the variations in cell morphology, is poorly studied. Specification of this notion against the background of the numerous currently used terms is important for clear formulation of the tasks related to investigation of the structural aspects of bacterial physiology on the basis of modern knowledge of the molecular genetic foundations of bacterial adaptation. The author suggests considering the ultrastructural plasticity as an indicator of the action of the mechanisms of phenotypic plasticity, which are understood according to the present level of knowledge, as a new strategy for exposure and investigation of adaptive capacity and intraspecific structural diversity in prokaryotes.

To solve these questions, an important feature of microorganisms should be kept in mind, namely, the morphological, physiological, and biochemical heterogeneity of developing microbial populations. In the course of evolution, unlike formation of the tissue structure by multicellular eukaryotes, the prokaryotes developed a populational mode of existence as colonies, biofilms, microcolonies, or suspensions. Depending on the species, the cells may be single individuals or participate in various complexes (aggregates, multicellular filaments, trichomes, etc). The species-specific structure of the population is determined by the topography of cell localization and intercellular structural relations, isolation of cell groups, and formation of multicellular individuals in some species. The vast experimental material accumulated in the course of years demonstrates that all prokaryotic populations possess the features determining their activity as a self-regulating system of a supercellular level (Shapiro and Dworkin 1997; El-Registan 2005; Vysotskii et al. 1991). The author considers specialization (task sharing) and cooperation (coordinated, regulated interaction) to be the most important of these features. In this respect, an analogy may be seen between the structural and functional organization of a microbial population and that of a

multicellular eukaryotic organism. The combination of these complementary features is associated with development of microbial populations in a constantly varying environment. In this respect, the role of cell heterogeneity is great. It results in increased resistance, flexibility, and adaptation of bacterial populations in response to changing environmental conditions (Pechurkin et al. 1990). As a result of cell heterogeneity, the plasticity of microbial populations, their capacity for adaptation to new ecological situations is known to be always higher than the individual plasticity of the cells (Ivanov and Ugodchikov 1984). Formation of specific cell types functioning in accordance with the changing conditions is mediated by the extracellular autoregulator molecules responsible both for the intercellular interactions and for those between the cells and the environment (El-Registan et al. 2006). According to Golovlev, the phenotype metastability developed by both pathogenic and saprophytic bacteria as a means for adaptation and stabilization of the species in an unstable environment is responsible for the dynamic heterogeneity of bacterial populations (Golovlev 1998b). At the population level, the phenotype metastability manifests itself in such processes as antigenic variations and other periodic changes designated as phase variations. Currently, an evolutionary significance of phenotypic heterogeneity in bacteria and molecular mechanisms underlying this phenomenon has intensively investigated (Ackermann 2011).

The foundation of this paradigm was laid by the classical works by outstanding microbiologists (Gamaleya 1894; Ravich-Birger 1936; Epstein et al. 1936; Peshkov 1955; Ierusalimskii 1952; Peshkov 1966) and other researchers, see review: (Baulina 2008). According to Ierusalimskii, a microbial culture develops as an integral system of the cell and environmental conditions. He wrote that a "culture is not a simple sum of the cells, but rather possesses the features and characteristics not present in the individual cells" Golovlev (1999).

The works of a Russian cytologist Vysotskii, from his mid 1960s investigations, developed the population approach to microbial ultrastructure (Vysotskii 2008). The author of this book considers the structural and functional characteristics and the ratio of various cell types formed in the course of the population development in its interaction with the environmental conditions, as well as the structural basis for the intercellular interactions, to be the subject of investigation in the population cytology of prokaryotes (Baulina 2005, 2006, 2008).

Investigation of the ultrastructural plasticity of the cells in heterogeneous microbial populations as an indicator of the causes and possible mechanisms of the morphological and functional rearrangements of the cells and populations in response to environmental conditions may be considered an independent topic. Its urgency comes from the necessity to improve our understanding of adaptive capacities of the species in microbial communities, since their activity determines the overall state of the microbial component of the biosphere.

In this respect, cyanobacteria, one of the most ancient groups of phototrophic microorganisms with unique physiology, flexible metabolism, and the morphologic diversity comparable to that of all other bacteria, are an interesting object. The complexity of their morphological organization is unique among

gram-negative bacteria (Gromov 1986). Many species of cyanobacteria is characterized by multicellularity that has evolved (Schirrmeister et al. 2011).

Members of various taxonomic groups of cyanobacteria differ significantly in their metabolic traits, capacity for cell differentiation, and other physiological properties, probably as a result of their high adaptive potential under various environmental conditions at the early stages of their evolution. The new biological characteristics emerging in ancient cyanobacteria (evolutionary "trials") were probably an important part of the evolutionary processes. Thus, development of oxygenic photosynthesis in these prokaryotes marked the turning point in the evolutionary history, i.e., transition from anaerobic conditions to the oxygen-containing atmosphere (Gusev 1966, 1968; Gusev and Nikitina 1979; Paumann et al. 2005). High-oxygen content in the present-day atmosphere results from the Proterozoic activity of this microbial group. Moreover, most cyanobacteria are efficient fixers of atmospheric nitrogen and supply other organisms with bound nitrogen. The metabolic capacities of cyanobacteria determine their leading role in the formation and development of the biosphere as primary producers of organic matter in microbial communities (Zavarzin 2004, 2008).

Investigation of the evolution of the biosphere requires direct studies of the paleontological samples, such as the ancient microbial communities (microfossils) by various methods, including the ultrastructural analysis (Gorlenko et al. 2000; Zavarzin 2004; Gerasimenko and Ushatinskaya 2002b). The characteristic morphological features of cyanobacteria preserved in the extant species are important for identification of certain groups of ancient microorganisms. Such research should, however, consider the structural diversity and plasticity typical of cyanobacteria.

Cyanobacteria presently inhabit water, soils, and rocks. They thrive in the deserts, ice, and thermal springs. These microorganisms are present in the autonomous structured cyanobacterial communities (biofilms or mats), which develop under various, often extreme conditions, such as hypersaline lakes and thermal springs (Zvyagintseva et al. 1995; Gerasimenko and Ushatinskaya 2002a; Zavarzin 2004). Many cyanobacteria form symbioses with eukaryotes in nature (Schenk 1992) and model associations with plant partners under experimental conditions (Gusev et al. 2002). Ability of cyanobacteria to colonize this diversity of the ecological niches results probably from their efficient adaptation within a broad range of environmental physicochemical parameters (Bazanova and Pinevich 2000).

The presently universally accepted concept of cyanobacteria as the evolutionary precursors of chloroplasts (Giovannoni et al. 1988; Pace 1997) is highly important for assessment of the global role of the adaptive potential of cyanobacteria.

Wide occurrence of cyanobacteria in nature, together with their resistance to unfavorable environmental factors has long attracted attention. The works of 1980s–1990s important from the point of view of the present work dealt with the physiological aspects of adaptation of cyanobacteria at the population level (Gapochka 1981), morphological heterogeneity of their populations (Kondratyeva 1989), and the morphological and functional lability of their membrane apparatus

(Pinevich 1992). Since the 1960s, application of electron microscopy and adequate techniques of sample preparation resulted in investigation of the ultrastructural organization of cyanobacteria under different conditions of growth. The results of the numerous early works have been summarized in a number of reviews and monographs (Gromov 1976, 1986; Drews 1973; Golecki and Drews 1982; Stanier(Cohen-Bazire) 1988; Stevens and Nierzwicki-Bauer 1991; Jensen 1993).

The present book is based on the results of our investigation of the ultrastructure of cyanobacteria carried out since the early 1970s. Variations of the structural and functional organization of metabolically and morphologically different cyanobacterial species under different conditions of growth and experimental impact are analyzed based on a range of classical and modern microbiological publications. The concept of ultrastructural plasticity as an indicator of the action of adaptive mechanisms at the level of subcellular structures, cells, and populations is discussed.

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Chapter 2
Ultrastructural Plasticity of Cyanobacteria Under Dark and High Light Intensity Conditions

Abstract This chapter deals with investigation of cyanobacteria species varying in their morphologic and metabolic properties during cultivation under different light regimes.The presented evidence suggests that the manifestations of ultrastructural plasticity in cyanobacteria grown under high light or in darkness are indicative of the build-up of certain mechanisms of adaptation to unfavorable illumination conditions: reversible thylakoid swelling; regulation of the synthesis, and degradation of glycogen; L-transformation, and heterogeneity of the responses of cells to similar stressor. The highest diversity of ultrastructural plasticity manifestations was found in species characterized by the highest versatility of metabolism. The pioneering works dedicated to the visualization of photooxidative destruction of thylakoids are included in this chapter. A special section is devoted to experiments on irradiation of the cell suspensions with visible high-intensity light.

2.1 The Structural and Functional Features of Cyanobacteria as Prokaryotic Phototrophs with Oxygenic Photosynthesis

The major property common to all cyanobacteria is their capacity for photosynthesis accompanied with the release of molecular oxygen (oxygenic photosynthesis). This process is related to the ability of cyanobacteria to use water as an electron donor for the electron transport chain to supply the cells with energy. While this property is also found in various eukaryotic organisms, such as algae and higher plants, it is untypical of other photosynthetic prokaryotes, which utilize the energy of sunlight without releasing oxygen. According to the concept formed on the basis of paleomicrobiological studies, the phylum Cyanobacteria is one of the most archaic branches of the Bacteria evolutionary tree (Paumann et al. 2005; Pinevich and Averina 2002; Pinevich 2006). However, the time of origin of cyanobacteria is

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being debated. Researchers assume that it could have happened rather early, i.e., about 2.45–2.22 billion years ago (Schirrmeister et al. 2011). The molecular mechanisms of photosynthesis as such evolved probably during the progenote stage, and the presence of a photosynthetic apparatus is among the most important phenotypic traits of archaic bacteria (Pinevich and Averina 2002). During such a long evolutionary process of cyanobacteria, along with the development of distinctive structural and functional features of their photosynthetic apparatus, a number of mechanisms operating at the molecular genetic level have been developed to promote the adaptation to changing illumination conditions (Golden 1995; Los et al. 2008). Modern cyanobacteria, many of which are obligatory phototrophs, are well adapted to existence both in the dark (dim caves and soil) and under conditions of overexposure to light (deserts and glaciers).

The arrangement of the photosynthetic apparatus determines the specificity of the ultrastructural anatomy of cyanobacteria compared to other prokaryotes. In the scheme, designed on the basis of the ultrastructural analysis of representatives of various taxonomic groups of cyanobacteria (excluding the genera *Prochloron, Prochlorococcus,* and *Prochlorothrix,* as well as *Acaryochloris marina*, which differ in the pigment composition and the structural organization of the intracytoplasmic membrane structures), the major structural components of a cyanobacteria vegetative cell are shown (Fig. 2.1). The cells belong to the gram-negative morphotype: they are surrounded by a cell wall comprising a peptidoglycan layer and the outer membrane. On the surface, sheaths, sometimes capsules, paracrystalline protein or glycoprotein S-layers, pili (also termed fimbriae), and spines may be found. The intracytoplasmic membrane structures responsible for energy supply (thylakoids) fill most of the cytoplasm. These membrane structures may form a single system, termed thylakoid networks (Nevo et al. 2009). The thylakoids are lamellae formed by paired membranes containing chlorophyll *a* as a component of the photosystems. Chlorophyll *a* is the major photosynthetic pigment in cyanobacteria, except for *A. marina*, which has chlorophyll *d* as the major pigment (Miyashita et al. 1996). On the outer side of the membrane elements of the photosynthetic apparatus, phycobilisomes are located (see also scheme in Fig. 2.2). The nucleoid with (poly)ribosome associates at its periphery is located between the thylakoids, preferentially in the central part of the cell. Various structures and inclusions are present in the cells, their set depending on the species and growth conditions of cyanobacteria. The main components include gas vesicles, which promote floatation of the cell within the water column; carboxysomes (polyhedral bodies), deposits of the key enzyme catalyzing CO_2 fixation, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO); cyanophycin granules, consisting of a unique polypeptide built of the L-arginine and L-aspartate residues and acting as an alternative nitrogen source; granules of glycogen (α-granules), lipid granules (β-granules), and granules of poly-β-hydroxybutyrate acting as sources of carbon and energy; and polyphosphate granules, sources of phosphorus. There are also other inclusions, as well as various microtubules and microfilaments (Jensen 1985; Bermudes et al. 1994; Baulina and Gorelova 1996; Baulina and Gusev 1977).

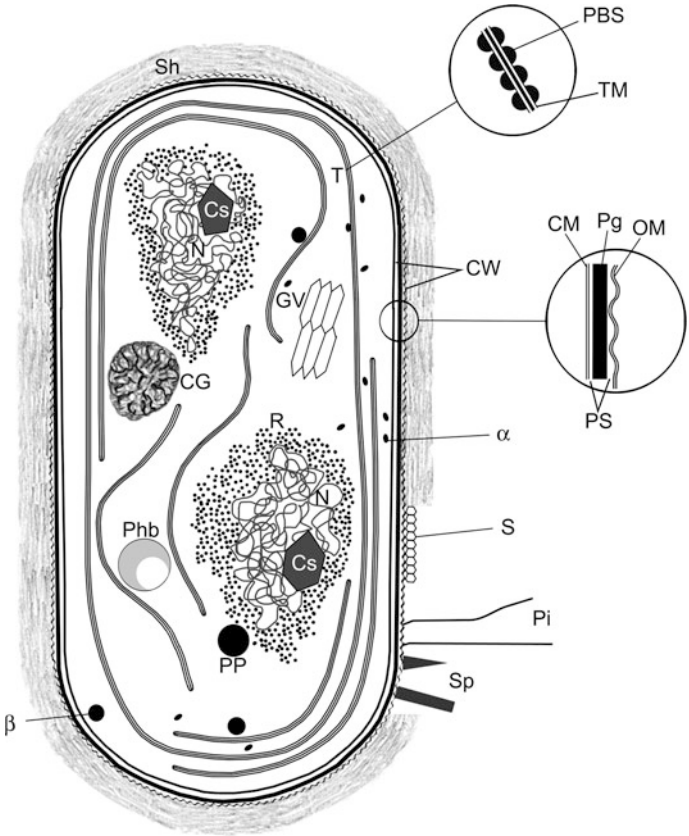

Fig. 2.1 General scheme of a cyanobacteria vegetative cell in section. α glycogen α-granules, *β* high electron density lipid *β*-granules, *CG* cyanophycin granule, *CM* cytoplasmic membrane, *Cs* carboxysome, *CW* cell wall, *GV* gas vesicles, *N* nucleoid, *OM* outer membrane, *Pg* peptidoglycan, *PBS* phycobilisome, *Phb* poly-*β*-hydroxybutyrate granules, *Pi* pili, *PP* polyphosphate granules, *PS* periplasmic space, *R* ribosomes, *S* S-layer, *Sh* sheath, *Sp* spines, *T* thylakoid(s), *TM* thylakoid membrane

The structural and functional organization of cyanobacteria is discussed in detail in many monographs and reviews (Drews 1973; Gromov 1976, 1986; Golecki and Drews 1982; Allen 1984; Stanier(Cohen-Bazire) 1988; Stevens and Nierzwicki-Bauer 1991; Jensen 1993; Hoiczyk and Hansel 2000; Herrero and Flores 2008).

Among cyanobacteria there are obligate and facultative photoautotrophs, as well as a few species capable of switching completely to the chemoheterotrophic mode. Obligate phototrophs *Synechococcus* sp. PCC 6301 and *Anabaena variabilis* CALU 458, as well as the facultative phototrophs *A. variabilis* ATCC 29413 and *Chlorogloeopsis fritschii* ATCC 27193 are traditionally used in research and are well studied in this respect. In obligate phototrophic cyanobacteria, the ability

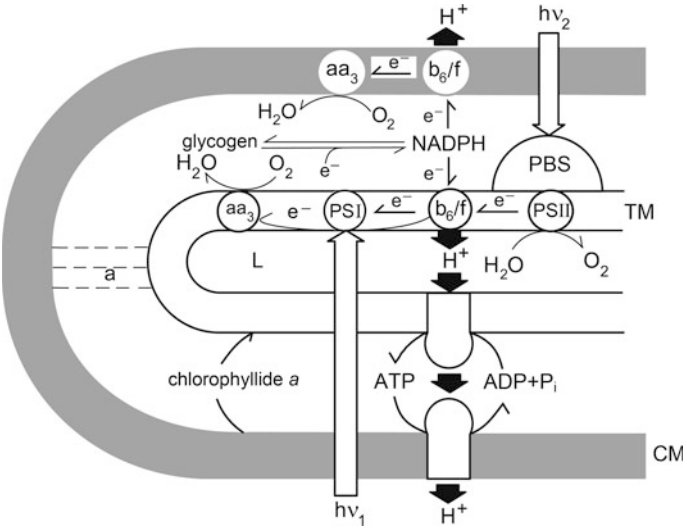

Fig. 2.2 Scheme of the major pathways of energy transfer in the membrane system of cyanobacteria [adopted from Pinevich (1997) with kind permission from author]. *CM* cytoplasmic membrane, *PBS* phycobilisome, *PSI* photosystem I, *PSII* photosystem II, P_i inorganic phosphate, *TM* thylakoid membrane, *L* lumen, *a* anastomosis between CM and TM, aa_2 cytochrome oxidase, b_6/f cytochrome b_6/f-complex. CM is *dark* that indicates its incompetence in light energy conversion. Outer membrane is omitted

to survive in the dark for over a month was discovered (Holm-Hansen 1968; Korzhenevskaya and Gusev 1973; Gusev and Nikitina 1974). Cyanobacteria survive the darkening periods owing to their capability to respire.

The energetic processes in cyanobacteria (photosynthesis and respiration), performed alternatively in the light and in the dark, are differentially associated with the cell membrane system including the cytoplasmic membrane (CM) and thylakoids. Numerous data on the structural and functional features of cyanobacterial membrane system are summarized in fundamental reviews and monographs (Golecki and Drews 1982; Stevens and Nierzwicki-Bauer 1991; Gantt 1994; Liberton and Pakrasi 2008; Mullineaux 2008; Nevo et al. 2009). Among them, the works of the scientific school of the St.-Petersburg State University are important, with their focusing at dynamics and adaptation of the membrane systems (Gromov 1976; Pinevich 1991, 1992, 1997; Pinevich and Averina 2002). The works using serial electron tomography combined with montaging to reconstruct large volumes of cyanobacterial cells in three dimensions show promise in the study of the whole-cell architecture developmental dynamics of the cyanobacterial membrane system (Liberton et al. 2011).

According to the modern concepts, the photosynthetic apparatus in cyanobacteria, excluding *Gloeobacter violaceus* with its photosystems incorporated in the CM, is associated with thylakoids. Paired membranes of thylakoid, appearing in ultrathin sections as two parallel elementary membranes, are either tightly packed

with their inner surfaces touching (internal adhesion) or separated, forming an intrathylakoid space (lumen). Typically, thylakoids do not touch each other, since, unlike the granal thylakoids of eukaryotic chloroplasts, thylakoids of cyanobacteria have no external adhesion factor, the chlorophyll *a/b*-binding protein of the light-harvesting complex II. Moreover, the major light-harvesting antennae of cyanobacteria are phycobilisomes, containing phycobilin pigments and transferring light excitation energy ($h\nu_2$) preferentially to photosystem II (PSII), are located at the outer surface of the thylakoids (Fig. 2.2). Phycobilisomes of most cyanobacteria are particles of semispherical shape located on the surface of the thylakoids in ordered tight rows. The spatial arrangement of thylakoids in many unicellular cyanobacteria (peripheral parallel lamellae) is more conservative than in the filamentous species and in those with complex life cycles (mostly unordered lamellae). A new model constructed using the larger-volume electron tomographic data showed a the unicellular cyanobacterium, *Cyanothece* sp. ATCC 51142 contained a band of radial thylakoids spirals around the cell periphery, forming an interconnected network derived from continuous branching and splitting of the membranes (Liberton et al. 2011). In this work, the thylakoid membranes were shown to form an extensive system enclosing a single space, the thylakoid lumen. The question of existence and organization of the structural and spatial connections between CM and the thylakoid membranes is still being debated (see Sect. 3.2). An anastomosis-like junction between these two structures was observed, albeit rarely. Specialized structures, namely, thylakoid centers, located immediately beneath the CM and connected to the thylakoids, participate in biogenesis of the intracytoplasmic membrane apparatus, particularly, in pigment transport and incorporation into the membranes (Nickelsen et al. 2011; Kunkel 1982). Functionally, cyanobacterial thylakoids are first of all energetic organelles capable of alternate photosynthetic, as well as respiratory, electron transport. On the contrary, CM contains no chlorophyll *a* and, most likely, participates only in respiration. The scheme (see Fig. 2.2) demonstrates localization of the photosynthetic apparatus and the major components of the electron transport chain in a thylakoid and in CM. Within the framework of this chapter, we should emphasize the following. First, in a thylakoid membrane, the cytochrome b_6/f-complex positioned between the two macromolecular complexes of photosystems PSI and PSII is a common unit of the two chains performing alternate photosynthetic (light-controlled, electrons provided by PSII) and respiratory (controlled by NADPH, an electron donor of the respiratory chain) proton transport into the lumen. Second, the reducing agent may be generated not only in the course of light-controlled electron transfer, but in the process of respiratory catabolism of storage polyglucosides (glycogen). Immobilization of the storage polyglucosides occurs exclusively through the oxidative pentose phosphate pathway yielding NADPH. In the dark, the aerobic respiratory chain completes the oxidation of polyglucosides converting the stored free energy into the proton-motive force. Third, a phycobilisome is located exactly above the PSII on the outer surface of thylakoid membrane facing the cytoplasmic matrix.

2.2 The Effects of Dark and High Light Intensity Conditions on Cyanobacterial Ultrastructure

The basic data on the influence of various illumination conditions including darkness and high light intensity, on the configuration and spatial organization of the thylakoids were obtained in the early days of investigation of cyanobacterial ultrastructure. Experiments were performed using the species differing in morphology and metabolic properties, including obligate phototrophs and those capable of chemoheterotrophic growth, such as *Symploca muscorum* (Bowen and Pankratz 1963); *Nostoc muscorum* (Wildon and Mercer 1963; Ginsburg and Lazaroff 1973); *Oscillatoria chalybia* (Giesy 1964); *Oscillatoria redekei* (Whitton and Peat 1969); *C. fritschii* (Peat and Whitton 1967; Findley et al. 1970); *Anacystis nidulans* (*Synechococcus* sp. PCC 6301) (Allen 1968; Nikitina et al. 1979); *Nostoc* sp., isolated from *Macrozamia lucia* roots (Hoare et al. 1971); *A. variabilis* (Nikitina et al. 1974); *Agmenellum quadraplicatum* (Kalley et al. 1977) and some other species(Gromov 1976). In these studies, various reorganizations of the thylakoids in the cytoplasm were reported. It was found that during dying off in the dark or when exposed to high light intensity, the intrathylakoid space increased and vesicles were formed of the thylakoid membranes; moreover, in the cells turned yellowish under high light intensity vesiculation could be reversible. The present chapter is focused on a series of consecutive experiments on comparative study of the effects of darkness and high light intensity on the cell ultrastructure of the species traditionally studied by microbiologists, namely:

Synechococcus sp. Näg. PCC 6301 (hereafter referred to as *Synechococcus* sp. 6301) previously known as *Anacystis nidulans* (Rippka 1972) from the collection of the Department of Physiology of Microorganisms (Faculty of Biology, Lomonosov Moscow State University); belongs to subsection I of the phylum Cyanobacteria[1] subsection I (previously known as order Chroococcales); it is a unicellular obligate phototroph incapable of photoheterotrophic growth, diazotrophy, or cell differentiation;

Synechococcus elongatus Näg. B-267 (hereafter referred to as *S. elongatus*) is a thermophilic strain provided by the Department of Biophysics (Faculty of Biology, Lomonosov Moscow State University);

Anabaena variabilis Kütz. CALU 458 (hereafter referred to as *A. variabilis* 458) is an obligate phototrophic strain, capable of photoheterotrophic growth in the presence of glucose and some other sugars (Suleimanova 1982) but not nitrogen-fixing and forming no differentiated cells to perform this process (heterocysts); the strain was obtained from the collection of the Department of Microbiology (Biology Research Institute of St.-Petersburg State University); belongs to subsection IV (previously, order Nostocales).

[1] Bergey's Manual of Systematic Bacteriology (Boone and Castenholz 2001).

Anabaena variabilis Kütz. ATCC 29413 (hereafter referred to as *A. variabilis* 29413) [strain of Dr. C. P. Wolk, United States] is a facultative phototrophic, diazotrophic strain, capable of chemoheterotrophic growth with fructose as a source of carbon and energy (Wolk and Shaffer 1976) and of heterocyst and akinete differentiation; the strain was kindly provided by Prof. S. V. Shestakov (Department of Genetics, Faculty of Biology, Lomonosov Moscow State University).

Chlorogloeopsis fritschii Mitra et Pandey ATCC 27193 (hereafter referred to as *C. fritschii*) is a strain characterized by the most flexible metabolism if compared to the first two species; it is a facultative phototroph capable of chemoheterotrophic growth on some sugars (Fay 1965), nitrogen fixation, and cell differentiation. The life cycle comprising several stages of progressive morphology metamorphosis is characteristic of these cyanobacteria; the culture was obtained from Dr. N. G. Carr, England; it belongs to subsection V (previously, order Stigonematales).

These organisms were chosen in order to study the ultrastructural plasticity of the representatives of various taxonomic groups of cyanobacteria differing in their physiological properties, which are mostly known and have been described in the literature.

2.2.1 *Ultrastructure of* Anabaena variabilis *CALU 458 Incubated in the Dark and Transferred to the Light*

For this strain, three successive stages of the culture incubated in the dark were established: (1) survival with the preservation of their capacity for proliferation upon transfer to the light; (2) preservation of the cells morphologically similar to the intact ones, yet incapable of proliferation in the light; and (3) culture lysis (Korzhenevskaya and Gusev 1976a). Study of the dynamics of the variations in the cellular ultrastructure during the first two stages revealed configurational changes in the thylakoids which could be reversed upon transfer to light after the first stage of incubation in dark (Baulina and Gusev 1978; Gusev and Baulina 1979). These ultrastructural changes may be interpreted as a result of the mechanism of reversible swelling of these organelles enabling the preservation of cell functionality in the dark, as will be confirmed below.

In these experiments, the ultrastructure of *A. variabilis* 458 during the phase of intense growth under optimal illumination conditions (1.5–2 klx) in the mineral medium was used as the control. Scanning electron microscopy demonstrated the cell morphology: ellipsoid cells joined into long chains (Fig. 2.3a). Transmission electron microscopy (TEM) revealed cell organelles, cellular structures, and various inclusions, characteristic of the species, which are depicted in the general scheme of a cyanobacterial vegetative cell (Fig. 2.1). It can be seen that *A. variabilis* 458 cells of the control culture possessed the cell wall structure

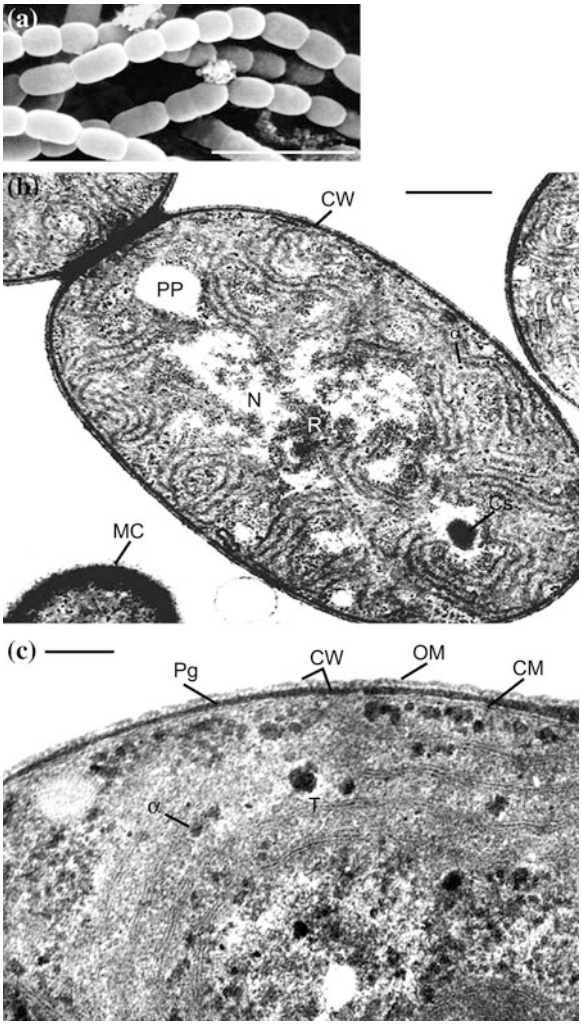

Fig. 2.3 Ultrastructure of *Anabaena variabilis* CALU 458 in the phase of intense growth in the light. General view of the cells in the culture: scanning (**a**) and transmission (**b**) electron microscopy; cell region in an ultrathin section (**c**). *α* glycogen *α*-granules, *CM* cytoplasmic membrane, *Cs* carboxysome, *CW* cell wall, *MC* capsular matter, *N* nucleoid, *OM* outer membrane, *Pg* peptidoglycan, *PP* electron-transparent region of polyphosphate granule localization, *R* ribosomes, *T* thylakoid. *Scale bar* **a** 10 μm, **b** 0.5 μm, **c** 0.1 μm

typical of cyanobacteria, containing the outer membrane and the peptidoglycan layer (Fig. 2.3b and c). As a rule, deposits of unstable capsular material were revealed on the surface of the cells. Thylakoids filled the cytoplasm, and among them, mostly in the central region of the cell, the nucleoid with peripheral accumulations of ribosomes was located. Within the section plane, the thylakoids were located in parallel to each other and bent together. The thylakoid-forming membranes were approximately 8 nm thick each. They were either pressed together or separated from each other with a distance of some 20 nm (Fig. 2.3b). The three-layer membrane profile characteristic of the ultrastructure image was revealed (Fig. 2.3c). The cytoplasmic matrix was of high electron density which probably resulted in poor resolution of phycobilisomes on the outer surface of the

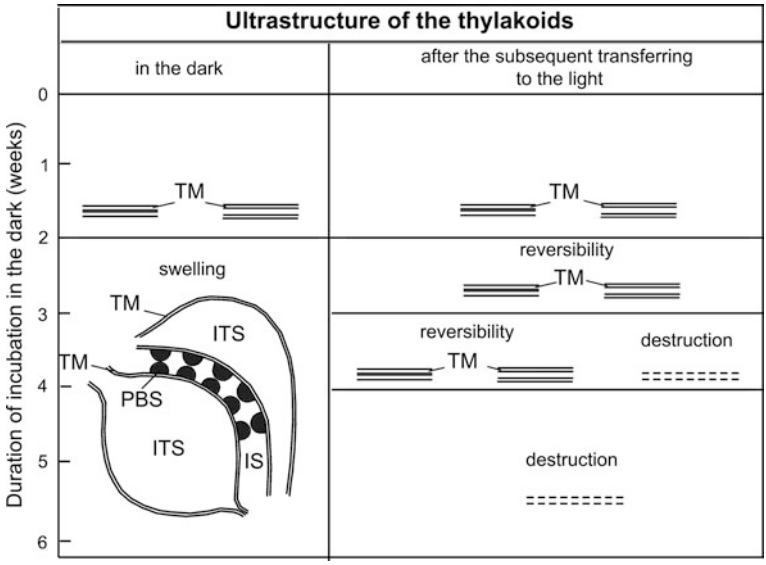

Fig. 2.4 Scheme of the ultrastructural organization of the thylakoids of *Anabaena variabilis* CALU 458 upon changes in illumination conditions. *ITS* intrathylakoid space, *IS* interthylakoid space, *PBS* phycobilisome, *TM* thylakoid membrane

thylakoids. The oval granules about 30 nm in diameter were present in the cytoplasm of all cyanobacterial species studied have been described in the literature as glycogen α-granules. The polyglucoside nature of the granules has been identified with various cytochemical techniques, as well as by biochemical analysis of the isolated granules (Fogg et al. 1973; Stanier(Cohen-Bazire) 1988). Carboxysomes, that is, deposits of the RuBisCO enzyme (Codd and Marsden 1984), were typically found in the region of the nucleoid.

After transfer of the culture to the dark and incubation for about 10 days (initial period of the first stage), the ultrastructure of the cells, including the thylakoids, did not change (Fig. 2.4). The cells retained the ability to proliferate upon return to light.

It is known from the literature data that the preservation of viability in the darkness occurs due to consumption of endogenous storage compounds, primarily glycogen, which is formed in abundance upon CO_2 photoassimilation. Catabolism of glycogen glucose occurs via the pentose phosphate cycle which is linked to the electron transport chain through NADPH (Kondrat'eva 1996) (see Fig. 2.2). The scheme of the respiratory chain was designed specifically for *A. variabilis* 458 (Pinevich 1991). In the experiments described in this chapter, at the initial stage of survival in the dark (first 2 weeks), glycogen α-granules could still be observed in the cells while the thylakoid structure was similar to that of the control cells.

It was also demonstrated that intense consumption of storage polysaccharide occurring during this first stage of the *A. variabilis* 458 culture incubated in the dark was associated with cell survival (Korzhenevskaya and Gusev 1976a). Moreover, it was demonstrated that during the initial stages of incubation in the dark, the cells of the strain under study retained high levels of ATP synthesis (Sentzova et al. 1975). Therefore, there is firm ground for the assumption that thylakoid ultrastructure (closely connivent membranes) during the period reflects the presence of membrane-coupled energetic process of oxidative phosphorylation which occurs apparently within a chain of transformation of endogenous glycogen reserves. Similar to the energetic organelles of eukaryotic cells, this state may be termed an energized one. In the classical studies, it was demonstrated that isolated plant chloroplasts condensed upon addition of sufficient amounts of ATP into the incubation medium (Molotkovsky and Dzubenko 1969). Therefore, closely connivent thylakoid membranes and the presence of glycogen during the initial stage of incubation in the dark corresponded to a switch in the electron transport chain functioning under these conditions.

This reported interpretation of the results is in accordance with the concept of interrelation of the thylakoid configuration with formation and direction of the transmembrane proton flow in the course of cyanobacterial thylakoid electron transport chain functioning during both respiration and photosynthesis (Albertsson 1982; Pinevich and Topchieva 1991; Pinevich 1997, 2006). Membrane surfaces of the organelles are presumably brought together by adhesion via hydrophobic interactions, van der Waals bonds, and hydrogen bonds upon compensation of the negative charge with the protons released into the intrathylakoid space.

Proton entry into the thylakoid lumen of cyanobacteria is an established fact (Barsky et al. 1981; Paumann et al. 2005). The observed difference in the thylakoid ultrastructure both in a single cell and in multiple cells of the culture, in the darkness and in the light, namely, that the membranes were either tightly connivent or separated by a distance of $\sim$20 nm (see Figs. 2.3b, c, and 2.4), is probably associated with the changes in adhesive properties of the membranes in accordance with the periodic functioning of the electron transport chain.

Prolonged incubation of the culture in the dark resulted in a period (within the first stage) when the cells retained their ability to proliferate upon transfer to light, although in most of them the ultrastructure changed. This was manifested mainly in the several-fold increase in the distance between the thylakoid membranes, on average, up to 100 nm or more (Figs. 2.4 and 2.5). Upon bending and possible stretching of the membranes, their typical three-layer profile was retained, indicating the absence of destructive changes and the ultrastructural integrity of the membrane lipid bilayer. Curving and stretching of the membranes may be due to the changes in the level of saturation of fatty acid residues of the membrane phospholipids determining the viscosity and fluidity of the lipid bilayer, which was observed upon transfer to the dark of a number of cyanobacterial species (Al-Hasan et al. 1989). The presence of the enzymes involved in membrane fluidity modulation (fatty acid desaturases) was demonstrated in cyanobacteria (Tasaka et al. 1996). The function of desaturases is to form double bonds between the

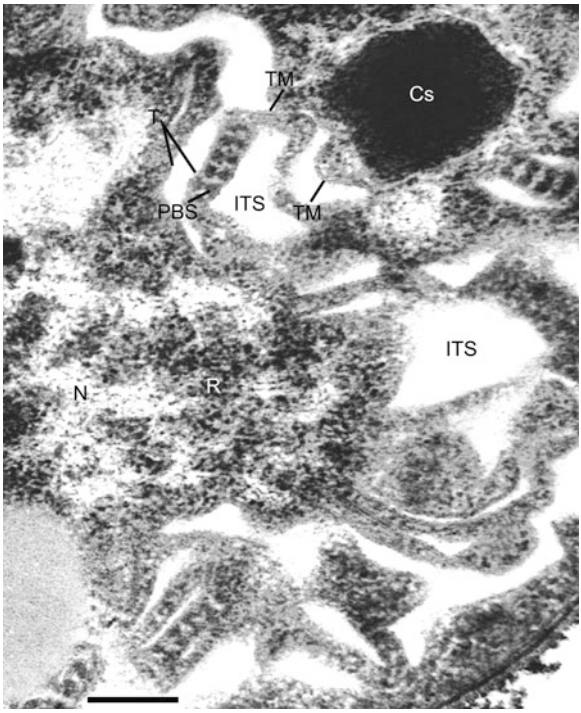

Fig. 2.5 Region of the *Anabaena variabilis* CALU 458 cell after incubation in the dark for 17 days (TEM). Adopted from Baulina and Gusev (1978). *Cs* carboxysome, *ITS* intrathylakoid space, *IS* interthylakoid space, *N* nucleoid, *PBS* phycobilisome, *R* ribosomes, *TM* thylakoid membrane. *Scale bar* 0.2 μm

carbon atoms of the fatty acid chains of the membrane lipids. It was demonstrated that in cyanobacteria illumination conditions were among the factors affecting the fatty acid composition, and therefore the fluidity of the thylakoid membrane through desaturase activity (Sciuto et al. 2008).

In our experiments, the preservation of the ultrastructural integrity of the thylakoid membranes upon stretching was also confirmed by the fact that on the membrane surface ordered phycobilisomes approximately 28 nm in diameter were visualized. These structures apparently remained connected to the membrane. In parallel experiments, it was demonstrated that phycocyanin content in the cells during this period was practically the same as in the cells of the original culture (Korzhenevskaya and Gusev 1973). At the same time, more distinct appearance of phycobilisomes if compared to the control indicated reorganization and probably even a loss of some electron-dense components of the cytoplasm in the interthylakoid space. The size of the interthylakoid space was relatively stable and corresponded to the thickness of the double layer of phycobilisomes (see Fig. 2.5).

During 3 weeks of incubation in darkness, the observed change in configuration of the thylakoids was reversible upon transfer to optimal illumination conditions in all studied cells in the population (see Fig. 2.4). After 2 days of growth in the light, which corresponds to the period of slow transformation of the culture from the lag

phase to the exponential growth phase under these conditions (Korzhenevskaya 1975), the thylakoid membranes approached each other again in accordance with their return to the energized state apparently due to resumed photosynthesis.

Taking into account the theoretical background presented in the Introduction, the ultrastructural plasticity of prokaryotic cells should be considered as a constituent of the general phenotypic plasticity, a complex of adaptive structural adjustments of the cell in response to the changes in the environment. Adaptive nature of the configurational changes in the thylakoids was indicated by the fact that they were coupled to the preservation of viability of the culture in the dark. Reversibility of the ultrastructural rearrangements of the thylakoids indicated the preservation of their structural and functional integrity. Consequently, the observed configurational changes of the thylakoids may be considered a manifestation of their ultrastructural plasticity. Apparently, reversible stretching is a form of ultrastructural plasticity of thylakoid membranes while reversible increase of the intrathylakoid space is a form of the ultrastructural plasticity of these organelles. Similar results evidencing reversibility of the degradation processes associated with iron deficiency, which include swelling of the thylakoids, were obtained for a unicellular cyanobacteria *Agmenellum quadruplicatum* in the course of investigation of their ultrastructure during cultivation (Hardie et al. 1983).

In the reported series of experiments, it was demonstrated that in those cells where a considerable increase in intrathylakoid space occurred, glycogen granules were absent (see Fig. 2.5). The correlation between thylakoid configuration and the presence of glycogen granules was observed throughout the period of incubation in the dark (up to 1.5 months). No α-granules were observed upon increase of the intrathylakoid space. In the case when the thylakoids still preserved their structure (connivent membranes), if only in sporadic cells, α-granules were typically present in the cytoplasm. The revealed pattern is in accordance with the idea that endogenous glucan is used as a source of carbon and energy during the initial period of persistence under unfavorable conditions in the dark. If the period of incubation in the dark was prolonged, the total ATP level in the *A. variabilis* 458 culture decreased significantly (Sentzova et al. 1975). Thus, these observations indicate that considerable separation of the thylakoid membranes is associated with the ceased or decreased activity of the respiratory electron transport chain and thus with the transfer of the membranes to the non-energized state. In this case, the proton flow into the lumen probably decreases (stops) and the negatively charged membranes loose their adhesive properties. A variety of interrelationships between the structural state of thylakoid membranes and proton transport has long been known for isolated chloroplasts (Packer et al. 1970; Murakami and Packer 1970). It is assumed that the changes in the configuration of the chloroplast thylakoid (swelling) are related to the movement of osmotically active ions resulting from the changes in permeability of the thylakoid membranes upon disappearance of the proton gradient required for their active transport, which may lead to water influx into the intrathylakoid space. Phosphorylation is considered the major process responsible for the changes of the chloroplast structure: swelling of the chloroplasts occurring in the dark is reversible upon transfer to light or changes in the

medium pH. Modern research confirmed and significantly augmented our understanding of the nature and mechanism of thylakoid swelling in the chloroplasts of higher plants in the dark (Johnson et al. 2011).

Comparison of the literature data on the mechanisms of light-dependent changes in the structure of chloroplasts and the facts on the behavior of the obligate phototroph *A. variabilis* 458 in the dark listed above suggest that the hypertrophy of the intrathylakoid space in this cyanobacterium reflects the process of thylakoid swelling in the dark due to slowing down of electron transport and the associated proton transport. The swelling process is apparently associated with the ability of the membranes to stretch. Indirectly, this is supported by the fact that in the cells of a chemoheterotrophic culture *C. fritschii* grown in the dark where the electron transport chain most probably functions, no thylakoid swelling was observed (Baulina et al. 1978). Therefore, the reversible configurational changes in the thylakoids of *A. variabilis* 458 revealed upon its long-term incubation in the dark, may be explained by the osmotic mechanism linked to the light-dependent changes in the intensity of electron transport. This hypothesis is in agreement with the interpretation of the morphological and functional dynamics of the membrane apparatus of cyanobacteria (Pinevich 1991, 1997), while the results obtained in the model system described above indicated, as was expected, that the ultrastructure plasticity of thylakoids and constituent membranes was associated with the metabolic rearrangements under varied illumination conditions.

The leading role in various types of structural and functional reorganizations in the thylakoid system of cyanobacteria is often assigned to the redox status of the electron transport chain, which transmits external signals, such as changes in the intensity and quality of light, to various epigenetic-level regulatory systems (Pinevich 1991). Moreover, evidence exists suggesting that illumination conditions regulate expression of the genes coding for the components of PS II complex and phycobilisomes via the photoreceptors specific for the light of different wave lengths and involving the electron transport chain and the photosynthesis apparatus as such (Golden 1995). The state of cyanobacterial photoreceptors, including at least one putative rhodopsin-like chromoprotein (Hoff et al. 1995), may be in connection with the activity of specific transcription regulators (Bazanova and Pinevich 2000). In purple photosynthesizing bacteria *Rhodobacter,* a protein sensitive to blue light and redox-dependent signal and involved in the initiation of regulatory processes, inducing gene expression was detected (Braatsch et al. 2002, 2004). In recent years, the process of light-dependent regulation of the physiological state of cyanobacteria has been studied intensely (Montgomery 2007).

The recent data support a conclusion that redox signals formed in a bacterial cell, that is, the signals evolving upon the changes in the redox status, are received by various regulatory systems able to control numerous metabolic functions including photosynthesis, carbon and nitrogen fixation, as well as aerobic and anaerobic respiration, to maintain the homeostasis and adaptation of cell (Kaplan 2004). Cyanobacteria possess also the regulatory systems triggered by specific sensors, e.g., the membrane proteins reacting to changes in such parameters of the membrane physical state as fluidity, independently of the nature of the stress signal

(Los and Murata 2004). Basing on these general concepts, light-dependent reversible changes in the configuration of thylakoids may be considered as a process involving the regulatory systems at various levels, including osmoregulation and expression or modification of the enzymes involved in reorganization of the membrane phospholipids. Thus, the manifestation of ultrastructural plasticity of the thylakoids (reversible divergence of their membranes and considerable increase in the intrathylakoid space) in the obligate phototroph *A. variabilis* 458 at the stage of survival in the dark indicates a complex adaptive mechanism of reversible swelling of these organelles. Deciphering of the molecular genetic basics of this mechanism is far from complete.

As is known form the literature, the absence of light, if only for a short time, induces reorganization of the pigment–protein complexes of PSII in obligate phototrophic cyanobacteria (Lebedev et al. 1989). Duration of the swelling adaptive mechanism functioning during the period of the preservation of cell viability in the dark probably correlates with the intensity of destructive changes in the photosynthesis apparatus, primarily in PSII, or, in other words, with the ability to repair the darkness-induced damage and turn on the photosynthetic electron transport chain upon transfer back to light. It was demonstrated for *Synechocystis* sp. PCC 6803 that cyanobacterial cells were able to repair PSII defects (caused by ultraviolet light) by means of a reparation mechanism based on de novo synthesis of D1 and D2 polypeptides (Friso et al. 1994). However, it should be taken into account that molecular mechanisms of the adaptation of the photosynthetic apparatus to stress in cyanobacteria and plants are multifactor and complex [see review (Kreslavski et al. 2007)].

Further studies of the behavior of the model described in this chapter during the period designated as the second stage demonstrated that adaptive abilities of obligatory phototrophic cyanobacteria may be limited by the damaging effects of light on the cells weakened by incubation in the dark. Starting from week 4, transfer of the cyanobacterial culture back to the illumination conditions optimal for growth resulted in a loss of viability of most of the cells and development of the degradation processes (Korzhenevskaya 1975). After five and more days of incubation under the light of moderate intensity, bleaching of the culture was observed. Deep destructive changes in the thylakoids were observed by TEM: the membranes lost their three-layer profile and appeared as vague outlines (see Figs. 2.4 and 2.6) (Baulina et al. 1976, 1977; Gusev and Baulina 1979). In these cells, both intra- and interthylakoid space was filled with homogenous fine-grained cytoplasm content, while the phycobilisomes were destroyed. All the cytoplasm was apparently in a homogenized state, no α-granules or ribosomes could be revealed. The nucleoid was detected only in rare sections in the central part of the cell (Fig. 2.6a). As a rule, carboxysomes were preserved in the nucleoid zone. Biochemical analysis revealed considerable destruction of DNA, RNA, proteins, and pigments (Korzhenevskaya and Gusev 1976b). On the basis of these data, the authors of this work proposed that the reason for destructive changes in the cellular components accompanied by bleaching of the culture was photooxidation induced by light of moderate intensity after long-term incubation in the dark. The outer

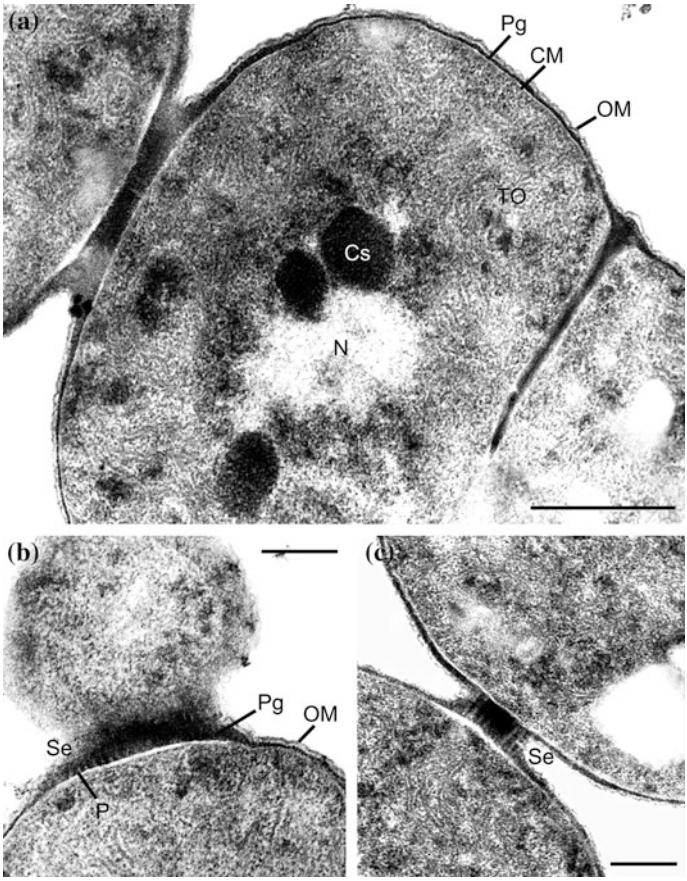

Fig. 2.6 Ultrastructure of *Anabaena variabilis* CALU 458 cells in the culture after 1.5 months of incubation in the dark followed by 5 days of cultivation in the light: general view (**a**) and regions adjacent to the septum (**b**, **c**) (Baulina et al. 1977). *CM* cytoplasmic membrane, *Cs* carboxysome, *N* nucleoid, *OM* outer membrane, *P* pore-like structures in the cell wall peptidoglycan layer, *Pg* peptidoglycan, *Se* septum, *TO* outlines of the thylakoids. *Scale bar* **a** 0.5 μm, **b** 0.2 μm, **c** 0.2 μm

membrane of the cell wall and the CM turned out to be the relatively stable membrane structures. At the same time, in most cells reorganization of the structure of the septum-forming peptidoglycan layer were evident. Numerous evenly alternating electron-transparent pore-like regions were revealed. Depending on the direction of the ultrathin section, these structures may be revealed at the periphery of the septum (Fig. 2.6b); in this case they resemble the junctional pores involved in mucus secretion in gliding cyanobacteria including another *A. variabilis* strain (see Sect. 5.1.2). In the sections of the central part of the septum, pore-like formations were revealed distinctly as canaliculi in the thick peptidoglycan layer (Fig. 2.6c). It should be noted that occasionally the intercellular channels were observed in the control *A. variabilis* 458 culture grown under optimal

conditions. No connection between these structures and CM was detected; they therefore cannot be identified as microplasmodesmata, which are considered to be present in the septa between the vegetative cyanobacterial cells, as well as in the region of their contact with heterocysts (Lang and Fay 1971; Merino et al. 1994; Flores et al. 2006). Electron-transparent channels similar to microplasmodesmata were revealed in the septum peptidoglycan between the vegetative cells, as well as between the vegetative cells and heterocysts in trichomes of a symbiotic cyanobacterium *Nostoc* sp. f. *Blasia* upon long-term storage in the dark at low temperature (Gorelova and Baulina 2009). Ultrastructural plasticity of this cyanobacterium is described in Chap. 5. Study of a symbiotic cyanobacterium in *Azolla caroliniana* ferns also revealed the structures identified as microplasmodesmata between the vegetative cells and heterocysts (Braun-Howland and Nierzwicki-Bauer 1990). Internal organization of the channels similar to microplasmodesmata remains poorly studied, although occasionally strands of electron-dense matter contacting with CM were revealed in them (Flores et al. 2006; Gorelova and Baulina 2009). The role of the latter in channel formation is, however, not strictly proved.

An ultrastructural manifestation of destruction of the thylakoids and other cytoplasm components similar to the one described above was revealed in an *Anacystis nidulans* L 1402-1 (SAG) (*Synechococcus* sp.) culture in the light in the presence of O_2 and under some additional conditions enhancing the photooxidation processes (Peschek and Schmetterer 1978; Schmetterer et al. 1983).

Transfer of a 3–4-week-old *A. variabilis* 458 culture from the darkness to the light exhibited cell heterogeneity in the population since only some of the cells underwent destruction in the light, while reversible changes occurred in the remaining ones. At the same time, the studied samples of the culture transferred to the light after incubation in the dark for 1.5 months contained only the cells described above, in which photooxidative destruction apparently occurred. In the described system, such cells lost their ability to proliferate but did not undergo autolysis during a long period of time (up to 4 months of incubation in the light); in other words, they were preserved in this state. The absence of lysis in most *Anacystis nidulans* (*Synechococcus* sp.) cells during photooxidation in the case of total experiment duration up to 5 days was also observed in the work cited above (Schmetterer et al. 1983). In our opinion, rather interesting and important is the ultrastructural similarity between the cyanobacterial cells with the described type of destruction and the mummified cells of other microorganisms, in particular *Micrococcus luteus*, which were formed under the influence of a chemical analog of an anabiosis autoinducer (Suzina et al. 2001). In both cases, the cells retained their shape changes in the cell wall structure occurred, which, however, did not lead to its destruction; on the contrary, cell membranes were totally destroyed, DNA and RNA degraded, and the cytoplasm became homogenous and fine grained. All these destruction processes were not accompanied by autolysis. It is assumed that the absence of autolysis in mummified cells results from enzyme inactivation with anabiosis-inducing agents, alkylhydroxybenzenes, or chaotropic salts known to damage the three-dimensional structure of the macromolecules, i.e., proteins, DNA, and RNA, and denature them, also

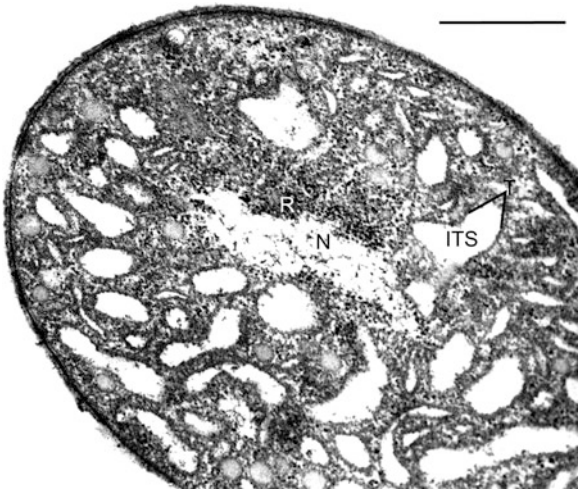

Fig. 2.7 Ultrastructure of an *Anabaena variabilis* CALU 458 cell in the first transfer of the culture from a flask where secondary growth was detected (see text for explanation). *ITS* intrathylakoid space, *N* nucleoid, *R* ribosomes, *T* thylakoid. *Scale bar* 0.5 μm

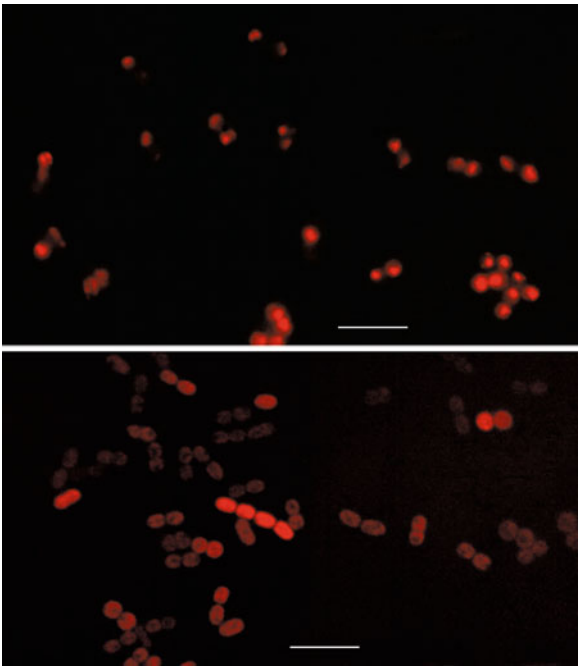

Fig. 2.8 The fluorescence of chlorophyll in *Anabaena variabilis* CALU 458 cells under a fluorescence microscope upon irradiation with ultraviolet light. In the first transfer of the culture from a flask where secondary growth was detected (see text for explanation) (**a**) and in the culture grown under optimal light intensity (**b**). *Scale bar* **a**, **b** 10 μm

causing similar changes in microorganisms (Duda et al. 2004). Similarly to this, conservation of *A. variabilis* 458 in the experiments described above may be due to inactivation of hydrolytic enzymes accompanied by degradation of other macromolecules caused by photooxidative damage.

Upon complete bleaching of the *A. variabilis* 458 culture, after a period of 6–42 days, in some experimental flasks, singular, very small blue–green colonies

were observed, consisting of viable cells initiating the secondary population growth. It is known that the individuals in bacterial populations differ by the resistance to external stimuli. Prior to complete death of a culture, viable cells are present, which may revive the population under favorable conditions (Rabotnova 1980). In experiments with *A. variabilis* 458 in the flasks where secondary growth was observed, cells with swollen thylakoids were found after 2–4 weeks and upon the first transfer of the culture. In serial sections of these cells, a distinct tendency to the closure of the thylakoid membranes was revealed indicating their ability to form vesicles (vacuoles) (Fig. 2.7). These changes could not be revealed after the following transfers, which probably indicates their reversible nature. The phenomenon of swelling and vesiculation of the thylakoids correlated with the changes in the fluorescence of chlorophyll under a fluorescence microscope upon irradiation with ultraviolet light (Fig. 2.8a) if compared to the control (Fig. 2.8b). In the control cells, the red emission of chlorophyll was of moderate intensity and rather evenly spread, while in the experimental samples brighter local fluorescence was observed.

Thus, the reported results of ultrastructural research made it possible to visualize the consequences of oxidative destructive processes. Their initial stages are probably linked to irreversible degradation of the photosynthetic apparatus upon cell long-term growth in the dark accompanied by damage (bleaching) of the pigments after transfer to light of moderate intensity. To confirm that the interpretation of the described ultrastructural picture of thylakoid destruction as a result of photooxidative damage of the photosynthetic apparatus, it seemed reasonable to study the same cyanobacterial strain upon growth under photooxidative conditions, that is, under light of extremely high intensity in the presence of air oxygen.

2.2.2 *Ultrastructure of* Anabaena variabilis *CALU 458,* Synechococcus *sp. PCC 6301 and* Chlorogloeopsis fritschii *ATCC 27193 Grown Under High Light Intensity*

Cyanobacteria, similar to eukaryotes, developed various systems of protection against oxygen toxicity caused by formation of its reactive species, obviously expected under high flows of sunlight (Abeliovich et al. 1974; Merzlyak 1989; Obinger et al. 1998). However, despite the efficient mechanisms of photoadaptation, in many situations cyanobacteria are subjected to damages which are often irreversible (Abeliovich and Shilo 1972; Abeliovich et al. 1974; Eloff et al. 1976; Korzhenevskaya and Gusev 1976b; Sinha et al. 2002) this phenomenon was termed photooxidative death (Abeliovich and Shilo 1972). Molecular mechanisms of photodamage to the photosynthesis apparatus and the membranes are relatively well studied in eukaryotic cells (Merzlyak 1989), and to a lesser extent, in prokaryotes (Pinevich and Averina 2002). On the basis of these works, visualization of the destructive changes of the photosynthetic membranes in cyanobacteria

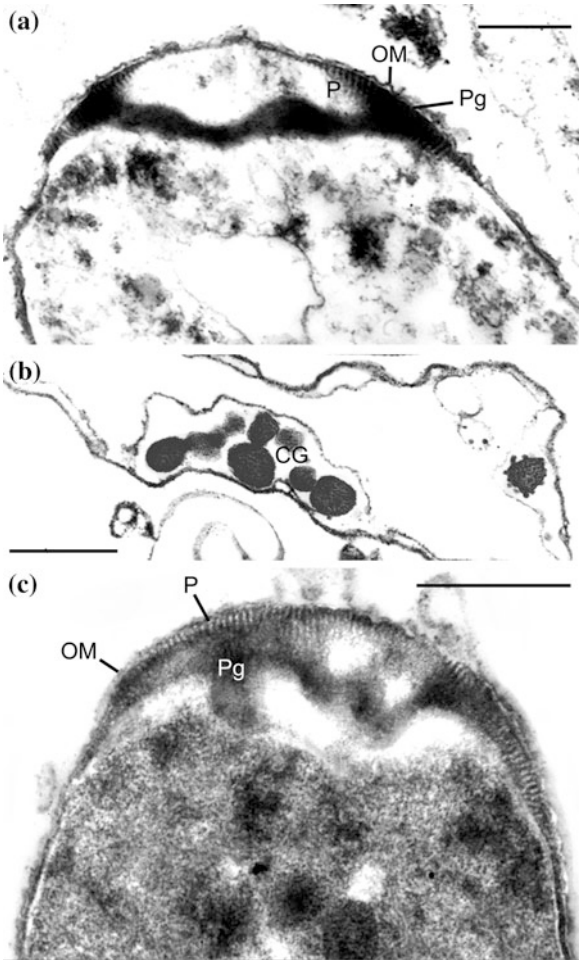

Fig. 2.9 Ultrastructure of *Anabaena variabilis* CALU 458 at late stages of destruction in a 12-day culture incubated under high light intensity (**a**, **b**) and upon transfer to light of optimal intensity after preliminary growth in the dark for 1.5 months (**c**): cell regions close to the septa with pore-like structures in the peptidoglycan layer (**a**, **c**) and cyanophycin granules remaining after cell autolysis (**b**), [**a**, **b** adopted from Baulina et al. (1981a)]. *CG* cyanophycin granule, *OM* outer membrane, *P* pore-like structures in the cell wall peptidoglycan layer, *Pg* peptidoglycan. *Scale bar* **a**, **b**, **c** 0.5 μm

under conditions of photooxidative stress is of particular importance for further detection of this process not under the controlled experimental conditions but in situ as well. Investigation of cyanobacteria of different taxonomic position, metabolic abilities, and, most importantly, level of dependence on light energy, is necessary to identify the specifics of manifestation of the photooxidative destruction, as well as of the cellular protective mechanisms. That is why in addition to the filamentous *A. variabilis* 458 capable of photoheterotrophic growth, unicellular obligate photoautotrophic cyanobacterium *Synechococcus* sp. 6301 and *C. fritschii*, which is capable of photoheterotrophic growth and possesses a complex life cycle, were also used in the comparative study of the effects of high-intensity light. The cells grown under light intensities of 9–10 klx in a mineral medium (Baulina et al. 1981b) or in the presence of glucose and ribose as additional carbon sources (Baulina et al. 1982) were studied by electron microscopy.

High light intensity suppressed considerably the growth of *A. variabilis* 458 in mineral medium (Suleimanova and Mineeva 1981). Electron microscopy showed that after 12 days of cultivation most cells underwent autolysis. To elucidate the primary changes in the ultrastructure, cell samples collected at days 2, 4, 7, and 12 of cultivation under high light intensity were studied. On day 2, in most cells thylakoid vesiculation was observed and lipid β-granules appeared (the latter are shown on the scheme in Fig. 2.1). The deposition of lipid granules could be caused by destruction of the thylakoid membranes (de Vasconcelos and Fay 1974). Cells at various stages of considerable destructive changes were observed rarely in 2- and 4-day cultures, while they predominated in the cell population of older cultures. At the ultrastructural level, these changes were observed as degradation of the thylakoid membranes and CM, formation of the characteristic pore-like structures described above in the peptidoglycan layer of the septa, as well as further destruction of the cytoplasm content, including the nucleoid, and cytoplasm autolysis with the relative intact cell wall (Fig. 2.9a). Cyanophycin granules, a form of deposition of a nitrogen-containing storage polypeptide multi-L-arginyl-poly-L-aspartic acid (Simon 1971), turned out to be the most stable structures (Fig. 2.9b). Parallel study of the effect of high light intensity on the state of the pigments demonstrated that under the experimental conditions chlorophyll and phycocyanin degraded rapidly (more than a threefold decrease in content) while carotenoid content decreased much less (1.8 times) (Suleimanova 1982).

As was demonstrated in the two models described above, the ultrastructural manifestations of the *A. variabilis* 458 cell destruction induced by moderate light after long-term incubation in the dark or by high-intensity light had both similar and different features. The picture of thylakoid membrane destruction accompanied by the loss of cell viability was similar in both cases. The major differences were the absence of autolysis in the cells subjected to photodestruction upon transfer from darkness to the optimal illumination conditions (Fig. 2.9c) during the whole period of observations and, on the contrary, deep autolysis of damaged cells upon cultivation under high light intensity (see Fig. 2.9a).

When grown in mineral medium under high light intensity, *Synechococcus* differed from *A. variabilis* 458 in the character of the ultrastructural changes. General view of the cells of a unicellular rod-shaped *Synechococcus* sp. 6301 grown under optimal illumination conditions of 1.5–2 klx (control) is presented in Fig. 2.10a. On day 20 of cultivation under 9–10 klx (extreme illumination conditions for the culture), a decrease in the growth rate was distinct (Suleimanova and Mineeva 1981), although no sings of cell autolysis were present. The culture contained two types of cells different in the ultrastructure of their cytoplasm. Cells of the first type were prevailing and analogous to those grown under optimal illumination conditions. The ultrastructure of the latter was considered as the control (Fig. 2.10b). In ultrathin sections of the cells of the first type, as well as of the control ones, the thylakoids (three to five) appeared as concentric circles organized in parallel with each other and with CM at the periphery of the cell. The thylakoid membranes touched each other tightly; the ultrastructure of each one of them (two electron-dense layers and one electron-transparent one between them)

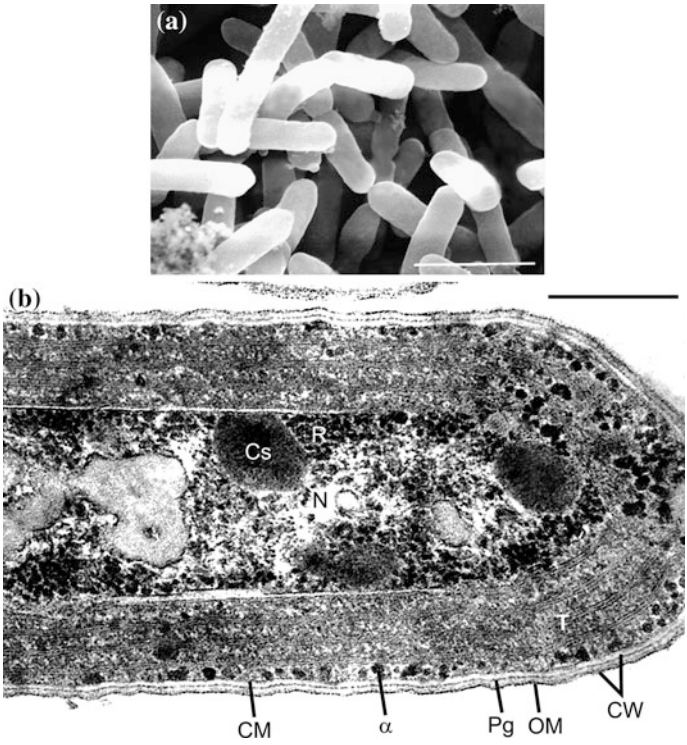

Fig. 2.10 Ultrastructure of *Synechococcus* sp. PCC 6301 grown under optimal illumination conditions: general view of the cells in the culture, SEM (**a**) (Baulina et al. 1994) and cell region in an ultrathin section (**b**) (Baulina et al. 1981a). α glycogen α-granules, *CM* cytoplasmic membrane, *Cs* carboxysome, *CW* cell wall, *N* nucleoid, *OM* outer membrane, *Pg* peptidoglycan, *R* ribosomes, *T* thylakoid. *Scale bar* **a** 3 μm, **b** 0.2 μm

corresponded to the molecular organization of a typical elementary membrane. No increase in the intrathylakoid space was observed. The cytoplasm matrix of the interthylakoid space was granular, containing α-granules of glycogen, although phycobilisomes could not be revealed distinctly on the outer surface of the thylakoids. Ribosomes filled all the space between the innermost thylakoid and the thin-fibrillar nucleoid. Carboxysomes were localized in the region of the nucleoid in the central part of the cell. Distinct separation between the central and peripheral parts of the *Synechococcus* sp. 6301 cells was typical for the species, whose ultrastructure has been studied by many researchers at the early stage of electron microscopic investigation of cyanobacteria [(Allen 1968; Fedorov and Tomina 1969) etc.].

Certain cells in the culture (cells of the second type) differed dramatically from the control ones (Fig. 2.11). The thylakoid membranes were destroyed and practically indistinguishable from the homogenous fine-grain cytoplasm. Glycogen granules were not revealed in most of these cells, no ribosomes accumulated at the

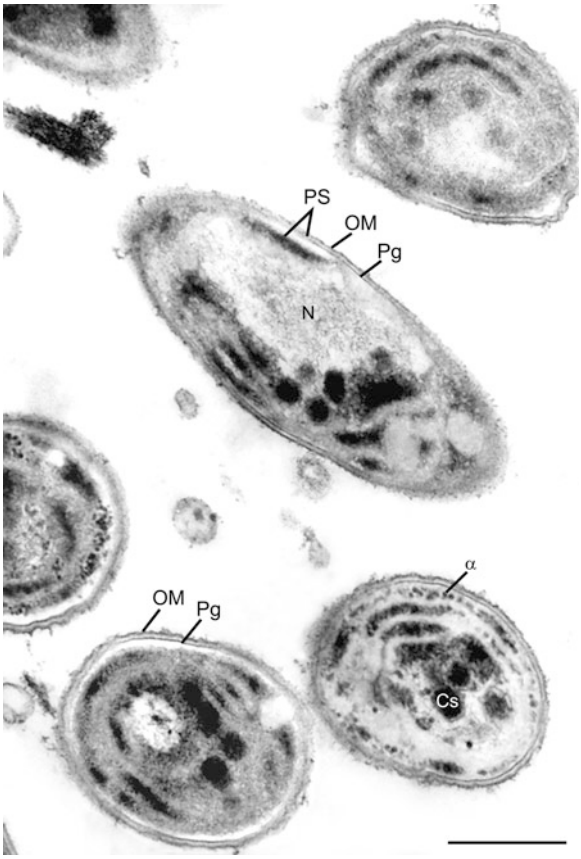

Fig. 2.11 Degrading *Synechococcus* sp. PCC 6301 cell in a 20-day culture incubated under high light intensity (TEM, data obtained by Baulina, Suleimanova and Mineeva). α glycogen α-granules, *Cs* carboxysome, *N* nucleoid, *OM* outer membrane, *Pg* peptidoglycan, *PS* periplasmic space. *Scale bar* 0.5 μm

periphery of nucleoid were observed. Demarcation of the nucleoid and the peripheral cytoplasmic zones was disrupted; fusion of the nucleoid zone and the periplasmic space was observed in some cells. CM was not revealed. At the same time, the nucleoid retained its fibrillar structure and, as is typical of cyanobacteria, was associated with carboxysomes whose granal surfaces were somewhat flattened and the surrounding proteinaceous outer monolayer shell was not revealed. The ultrastructure of the cell wall did not change. No cells with electron-transparent lysed cytoplasm were observed during the experimental period of growth under high light intensity. Parallel spectrophotometry of the culture revealed a decrease in the levels of chlorophyll and phycocyanin by 1.7 and 1.9 times, respectively, while carotenoid content during the phase of growth retardation practically did not change (Suleimanova and Mineeva 1981). These data, together with the induction curves, decay kinetics, and light curves recorded for delayed luminescence, characterizing the functional state of the photosynthetic apparatus (Suleimanova and Markarova 1980), definitely indicated the changes in its functioning in *A. variabilis* 458, *Synechococcus* sp. 6301 and *C. fritschii*. According to other

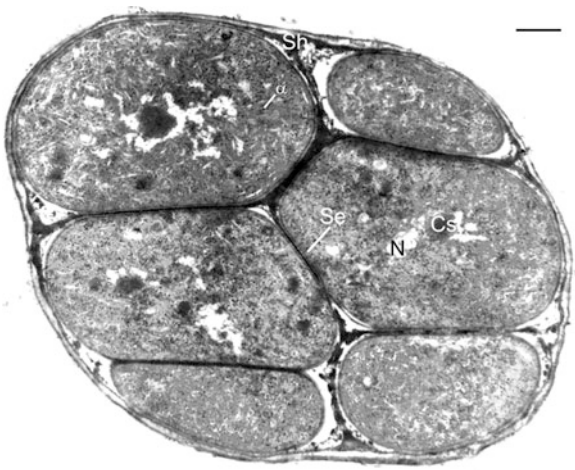

Fig. 2.12 Dividing *Chlprpgloeopsis fritschii* ATTC 27193 cells joined by a common sheath at the stage of intense culture growth under optimal illumination conditions (TEM). α glycogen α-granules, *Cs* carboxysome, *N* nucleoid, *Se* septum, *Sh* sheath. *Scale bar* 0.5 μm

authors, *A. variabilis* B 337 cultivation under conditions of intense illumination resulted in pigment bleaching accompanied by a significant decrease in the amount of the intramembrane macromolecular complexes corresponding to PS II and inhibition of the photosynthetic activity (Sidirelli-Wolf et al. 1992). Upon modeling of photooxidative conditions in *Anacystis nidulans* L 1402-1 (SAG) (*Synechococcus* sp.) with various metabolic inhibitors, CO_2 limitation, and elevated temperature, the degradation of the thylakoid membrane was coupled to the loss of PS II activity and chlorophyll bleaching (Schmetterer et al. 1983).

In contrast to *A. variabilis* 458 and *Synechococcus* sp. 6301, in *C. fritschii* growth inhibition under high light intensity in the mineral medium was insignificant and occurred only by the end of the phase of intense growth (Suleimanova and Mineeva 1981). No destruction of thylakoids, CM, or other structural components of the cell similar to the one described above was observed during this period (20 days). However, if compared to the control culture grown under moderate light (Fig. 2.12), the cells with swollen and vesiculated thylakoids dominated (Fig. 2.13). It should be noted, however, that *C. fritschii* cells grown under various illumination conditions could differ considerably in their thylakoid ultrastructure even in the case of the cells of similar size surrounded by a common sheath. Thylakoid membranes in some cells may be pressed to each other, while in others they were separated by a more or less wide intrathylakoid space. Moreover, the configuration of thylakoids on ultrathin sections indicated possible closure of the membranes of these organelles with formation of vesicles, or vacuoles. Such picture is presented in Fig. 2.7. These changes in the thylakoids happen in some *C. fritschii* cells under both moderate and intense illumination. It should be emphasized that thylakoid swelling and vesiculation probably reflect a nonspecific reaction of cyanobacteria not only toward unfavorable conditions of illumination but also to nutrient shortage (e.g. lack of nitrogen or iron), or may result from the degradation changes in the cells upon long-term incubation under stationary

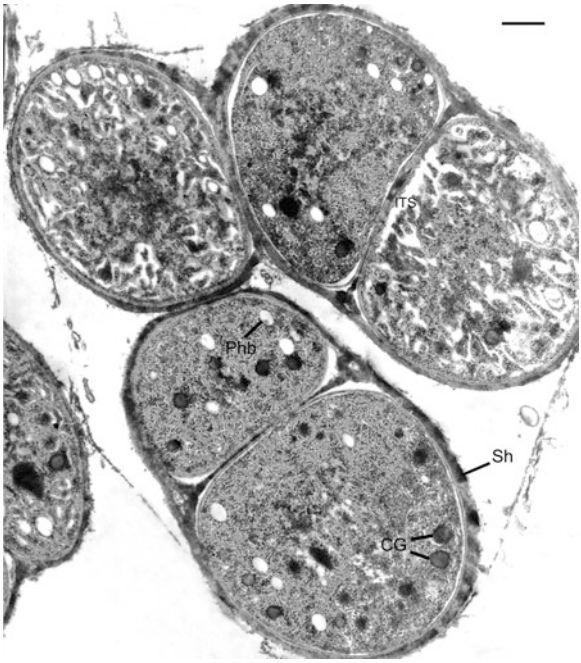

Fig. 2.13 Ultrastructure of the cells of a photoautotrophic *Chlorogloeopsis fritschii* ATCC 27193 culture grown under high light intensity (Baulina et al. 1981a). *CG* cyanophycin granule, *ITS* intrathylakoid space, *Phb* poly-*β*-hydroxybutyrate granules, *Sh* sheath. *Scale bar* 0.5 μm

conditions (Nikitina et al. 1974); (Ermakova et al. 1977; Hardie et al. 1983; Balkwill et al. 1984; Stevens and Nierzwicki-Bauer 1991). Some researchers reported reparation of the indicated changes in the thylakoid structure upon transfers (Hardie et al. 1983) or introduction of the deficient nutrient to the growth medium (Balkwill et al. 1984). At the same time, apparently, swelling and vesiculation is a non-obligatory stage of destructive changes in the thylakoids (Stevens et al. 1981).

Thus, comparative study of the ultrastructure of three cyanobacterial species during the initial period of the inhibition of culture growth by high-intensity light demonstrated that they differed significantly in their response to the same stimuli. High light intensity had the smallest effect on the ultrastructure of *C. fritschii.* No damage visible as destruction of the thylakoid membrane, CM, and other cellular components, which could be attributed to photooxidative reactions, was detected at the ultrastructural level. Decrease in chlorophyll and phycocyanin content was practically the same as in *Synechococcus* sp. 6301, carotenoid content did not change (Suleimanova 1982). The presence of the cells with significant vesiculation of the thylakoids in the culture was probably associated with the initial stages of the functional impairment of the photosynthetic apparatus. The important peculiarity of *C. fritschii* was the presence of cells with various thylakoid structure (compressed, swollen, or vesiculated) independently of the culture age and illumination condition. These data are in accordance with the results of the studies on the effect of illumination intensity on the ultrastructure of *C. fritschii* grown at high

temperature (45 °C) (Findley et al. 1970). In the cited work, it was also demonstrated that under high light intensity thylakoids swelled, lost their parallel orientation, and formed vesicles. The authors suggested that there should be a correlation between the organization of the thylakoid system, chlorophyll content, and photosynthesis rate, as well as between the ultrastructure and chemical composition of the thylakoids under various illumination conditions. The experiments described in the current chapter demonstrated that, on the contrary, in *Synechococcus* sp. 6301, thylakoid membranes were either connivent (under illumination intensity of 2 and 9 klx) or destroyed (9 klx). Swelling and vesiculation were not observed. In general, the *Synechococcus* sp. 6301 population was relatively stable. Most cells did not change ultrastructurally; the number of concentric thylakoids decreased in some cells (from 4–5 to 3). A similar observation, that is, a decrease in the number of thylakoids and lowered chlorophyll content under increased light intensity, was made in a classical study of the ultrastructure of the same species (strain) (Allen 1968). At the same time, in the described experiments deep destructive changes could be revealed in the thylakoids, CM, and other components of the cytoplasm of some cells, although they were not associated with rapid autolysis of the cytoplasmic content, at least during the period of observation. No intermediate forms with changes in spatial arrangement, swelling, or vesiculation of the thylakoids was observed. In *A. variabilis* 458, similar deep destruction of thylakoids, CM, and other intracellular components occurred. However, in contrast to *Synechococcus* sp. 6301, it was most likely preceded by thylakoid vesiculation, which occurred already on days 2–4 of cultivation, and was accompanied by the changes in the peptidoglycan layer ultrastructure characteristic of *A. variabilis* 458. Moreover, destruction of most of the cells occurred earlier and was coupled to cell autolysis revealed during the earlier period of observations.

The phenomenon of photooxidative death of cyanobacteria had been known for a long time (Abeliovich and Shilo 1972). PS II is the primary target under illumination stress. The occurring sharp suppression of photosynthesis, or photoinhibition, is a combined process of photoinactivation of non-cyclic transport of electrons and degradation of the D1 subunit of the PS II macromolecular complex (Pinevich and Averina 2002). Photoinhibition is determined by the length of exposure to light and includes a reversible stage when reparation is possible, which is followed by the irreversible phase of photodamage. Subsequently, accumulation of degradative oxidative processes may occur, such as photooxidative destruction, caused by formation of excessive reactive oxygen species and the associated processes of lipid peroxidation, resulting in significant changes in the characteristics of the membranes (Merzlyak 1989). It is assumed that the capacity of the pigment–protein complexes for reparation provides for the reversible nature of the damage. D1 is the most actively renewed membrane polypeptide under normal illumination conditions. Expression of the *psbA* gene coding for D1 depends on light at all levels of protein synthesis regulation. In cyanobacteria, the control is carried out at the level of transcription, while in algae and higher plants, at the level of translation. D1 renewal is stimulated by inhibiting light (Aro et al. 1993).

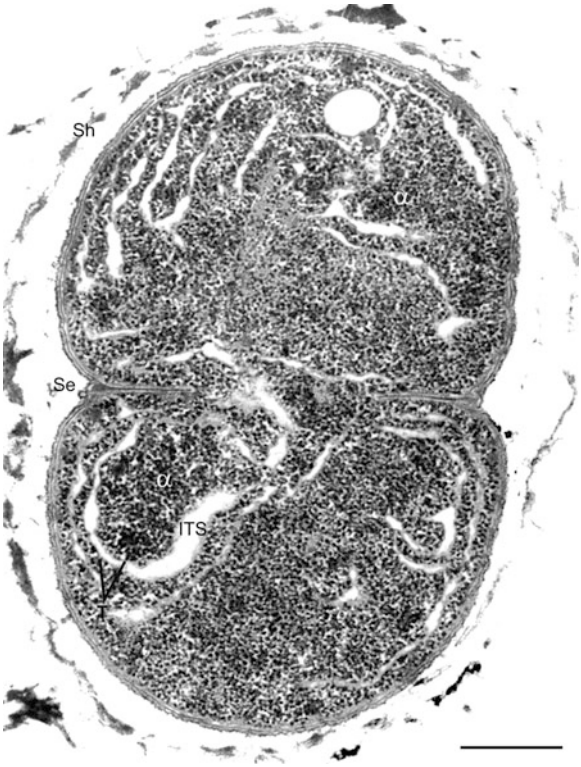

Fig. 2.14 Ultrastructure of a dividing *Chlorogloeopsis fritschii* ATCC 27193 cell in the culture grown in the glucose-supplemented medium under high light intensity (Baulina et al. 1982). α glycogen α-granules, *ITS* intrathylakoid space, *Se* septum, *Sh* sheath, *T* thylakoid. *Scale bar* 0.5 μm

Study of the mutant strains demonstrated that protease FtsH, the key enzyme involved in the degradation of light-damaged D1 subunits, is localized in cyanobacterial thylakoid membranes, and not in CM. Thus, the enzyme plays a key role in renewal of the damaged photosynthetic apparatus of cyanobacteria (Mullineaux 2008; Komenda et al. 2006).

It may be assumed that the mechanisms of damage reparation under high light intensity function longer in *C. fritschii* than in the other two species. One more possibility to avoid total photodestruction of the cell components is probably the ability of the species to switch over to photo- or chemoheterotrophic growth. This assumption is based on the fact that in the presence of glucose under high light intensity *C. fritschii* proliferated actively while possessing a reduced thylakoid system, often swollen, and mass depositions of glycogen (Fig. 2.14). Cultivation of *A. variabilis* 458 in the presence of glucose and ribose contributed to resistance of the population to high-intensity light. Increased growth rate and elevated levels of all pigments were noted in the cells of such cultures (Suleimanova and Mineeva 1981). Under these conditions (in the presence of glucose), the cells containing larger amounts of storage substances, including glycogen, dominated in the population (Fig. 2.15). Most of the cells were dividing. In these cells, the cytoplasmic matrix was diluted, the thylakoids were highly curved and spread chaotically over

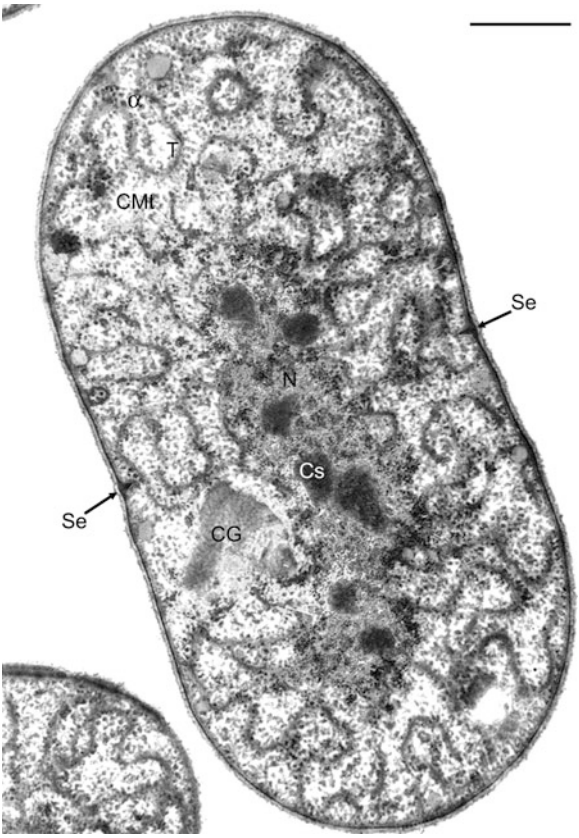

Fig. 2.15 Ultrastructure of a dividing *Anabaena variabilis* CALU 458 cell in culture grown in the glucose-supplemented medium under high light intensity (data obtained by Baulina, Suleimanova and Mineeva). α glycogen α-granules, *CG* cyanophycin granule, *CMt* cytoplasmic matrix, *Cs* carboxysome, *N* nucleoid, *Se* septum, *T* thylakoid. *Scale bar* 0.5 μm

the cytoplasm, and the nucleoid was compact and located typically in the center of the cell. The photoheterotrophic *A. variabilis* 458 culture under high light intensity was heterogeneous in terms of resistance to photodamage: in some cells the typical picture of photooxidative destruction was observed. The response of the *Synechococcus* sp. 6301 culture to high light intensity in the presence of sugars was the same as when grown in the mineral medium.

2.2.3 Photodestruction of Cyanobacteria In Vitro

To reveal the differences in the levels of light sensitivity of the photosynthetic apparatus in various cyanobacteria species, experiments on irradiation of the cell suspensions with visible high-intensity light [5.5 mmol quanta/(m^2 s), that is, ~200 klx] were performed directly in spectrophotometric cuvettes without subsequent cultivation, that is, under conditions of limited opportunities for adaptive cell response through the regulatory reparation mechanisms (Baulina et al. 2001,

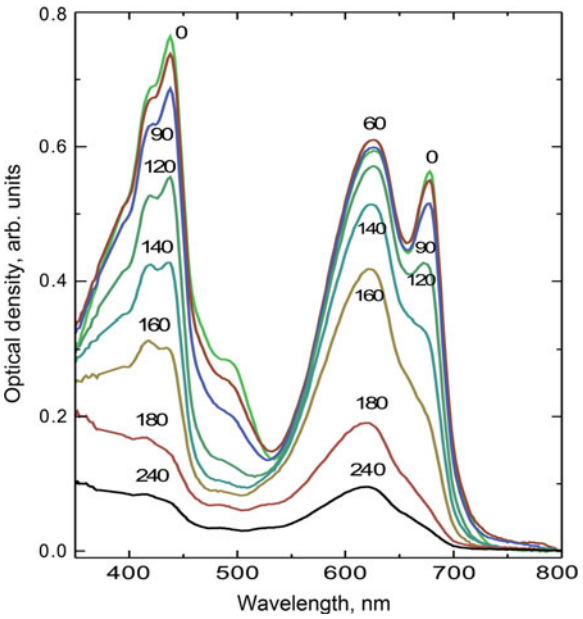

Fig. 2.16 Changes in the absorption spectra of *Synechococcus elongatus* B-267 cell suspension during irradiation with high-intensity light (reprinted from Baulina et al. (2004) with kind permission from Pleiades Publishing, Ltd.) *The numbers* the exposure time (in minute)

2004). The state of pigments and the ultrastructure determined immediately after irradiation were the criteria for damage evaluation. The same species were studied using strains *A. variabilis* 29413 and *S. elongatus*. The plan was to follow progressive stages of degradation of chlorophyll, carotenoids, and phycobilins in real time controlling the possible destructive changes in cells by electron microscopy. The experiments of this kind are necessary to investigate to which extent the changes induced by high light intensity correlate with the picture of the degradation of the cells of these cyanobacteria cultured in photooxidative conditions. Adequate description of the ultrastructural picture of photooxidative destruction should also contribute to the evaluation of the state of the population by the presence of irreversibly damaged and resistant cells under conditions of extreme changes in illumination conditions both under laboratory cultivation and in natural environment.

Presented below, result of our experiments are cited mainly from (Baulina et al. 2004).

Absorption spectra of the cell suspensions of *A. variabilis* 29413, *S. elongatus*, and *Synechococcus* sp. 6301 contained distinctive absorption bands of chlorophyll, phycobilins, and carotenoids, with respective maxima at 680, 626, and 480 nm (Figs. 2.16 and 2.17). The band with a maximum of ~420 nm caused by the presence of the products of chlorophyll photooxidation (Merzlyak et al. 1996) was also present. This figure also shows the spectral changes occurring during irradiation (absorption spectra for *Synechococcus* sp. 6301 are not shown because these changes were generally similar to those of *S. elongatus*). At the early stages of irradiation (10–30 min), an insignificant rise of phycobilins absorption maximum

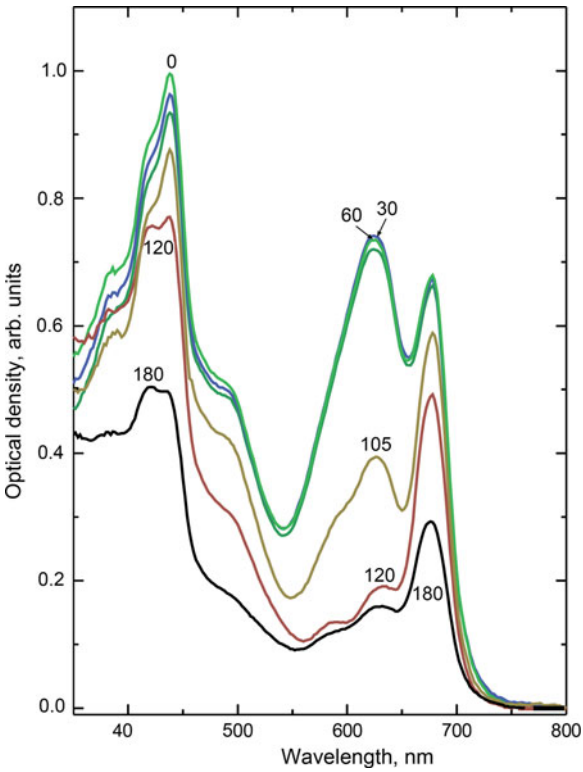

Fig. 2.17 Changes in the absorption spectra of *Anabaena variabilis* ATCC 29413 cell suspension during irradiation with high-intensity light (reprinted from Baulina et al. (2004) with kind permission from Pleiades Publishing, Ltd.) *The numbers* the exposure time (in minute)

was observed. As seen from figures, bleaching of the pigments in irradiated samples commenced after a certain lag period. Exposures to longer irradiation periods resulted in decreased optical density at the absorption bands of all pigments. Judging from the changes in optical density at 480 nm, the early stages of photodestruction of carotenoids in cyanobacteria occurred synchronously with chlorophyll degradation. At the terminal stages, only small quantities of carotenoids were retained in *S. elongatus.* In *A. variabilis* cells, the destruction of phycobilins proceeded faster than that of chlorophyll, and this trend persisted up to the final disappearance of phycobilins upon prolonged exposures. In *S. elongates* (as well as in *Synechococcus* sp. 6301), the presence of phycobilins was observed even after complete bleaching of chlorophyll.

Morphology and ultrastructure of *S. elongates* were typical for this genus (Fig. 2.18a). As a rule, in the plane of the section, five to six thylakoids with a structure typical of cyanobacteria—two closely appressed membranes with three-layer profile—surrounded the central part of the cell (Fig. 2.18b). The cytoplasm was dense, so that the phycobilisomes located at the outer surface of the thylakoids were not resolved. The spaces between thylakoids contained occasional α-granules of glycogen and moderately dense granules of presumably lipid origin. The ribosomes occupied all the space between the internal thylakoid and a thin-fibrillar

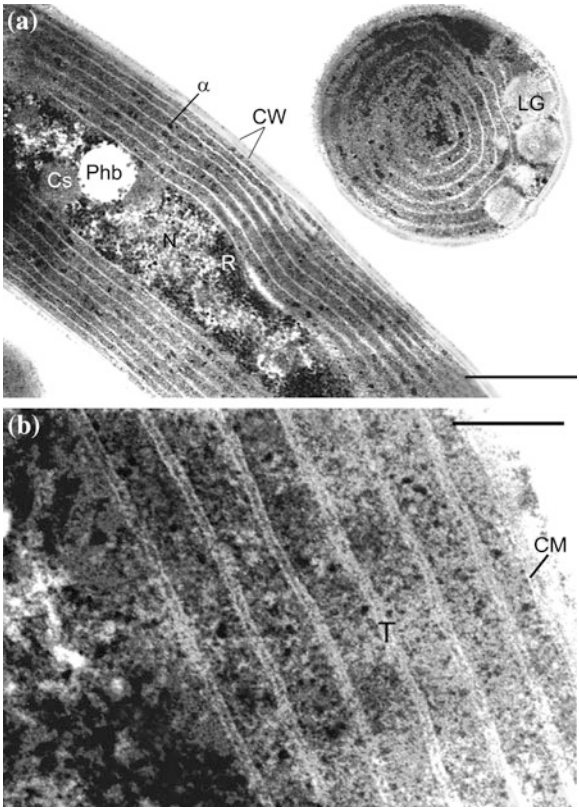

Fig. 2.18 Ultrastructure of *Synechococcus elongatus* B-267: general view (**a**) and the thylakoid region (**b**) (reprinted from Baulina et al. (2004) with kind permission from Pleiades Publishing, Ltd.) α glycogen α-granules, *Cs* carboxysome, *CW* cell wall, *LG* lipid granules of moderate electron density, *N* nucleoid, *Phb* poly-*β*-hydroxybutyrate granules, *R* ribosomes, *T* thylakoid. *Scale bar* **a** 0.5 μm, **b** 0.1 μm

nucleoid. In the nucleoid area, there were carboxysomes and structures similar to granules of poly-*β*-hydroxybutyrate (see Fig. 2.18a).

During the lag period preceding the pigment bleaching (60 min), the ultrastructure of the majority of the *S. elongatus* cells was fully similar to that of the intact cells. At the same time, during this period, some cells already exhibited destructive changes in the thylakoids, cytoplasmic matrix, and nucleoid. Upon irradiation for 180 min, modified cells were abundant in the suspension (Fig. 2.19). The photosynthetic membranes in such cells lost their three-layer profile. The thylakoids were seen as fragmented parallel lines of moderate electron density. At the same time, destructive changes of the CM were usually not discernible in these cells. The cytoplasmic matrix became homogeneous, with fine grains uniformly distributed between poorly visible contours of the thylakoid membranes. The nucleoid zone visible on the section (with peripheral ribosomes) was significantly reduced, and the DNA strands adhered to each other. The carboxysomes and α-granules did not show conspicuous changes. After irradiation for 240 min, no undamaged cells were observed in the suspensions. Nevertheless, throughout the period of observations, we did not reveal the cells with pronounced signs of autolysis. In addition to the alterations described above, upon long

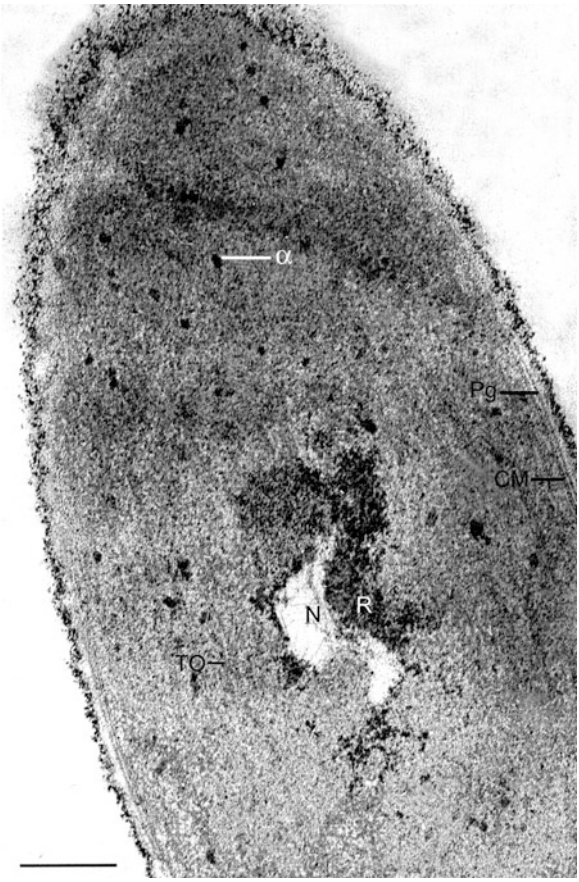

Fig. 2.19 Ultrastructure of *Synechococcus elongatus* B-267 after 180 min irradiation of cell suspension with high-intensity light (reprinted from Baulina et al. (2004) with kind permission from Pleiades Publishing, Ltd.) α glycogen α-granules, *CM* cytoplasmic membrane, *N* nucleoid, *Pg* peptidoglycan, *R* ribosomes, *TO* outlines of the thylakoids. *Scale bar* 0.2 μm

irradiation we observed ultrastructural changes in the outer membrane of the cell wall. In most cases, this membrane lost its contact with peptidoglycan and acquired an atypical nonuniform wave-curved profile. The contours of this membrane were not clearly visualized, because its surface was masked by electron-dense depositions similar to the glycocalix material. The examination of ultrathin sections did not reveal release of the degradation products into the periplasmic space.

In the case of both *Synechococcus* sp. 6301 and *S. elongatus*, the cells with evident signs of destruction were observed in the initial period of pigment degradation. The ultrastructural changes in both species were generally similar. Similar to *S. elongatus*, the thylakoid membranes lost their three-layer profile but were seen as lines of low electron density (Fig. 2.20) or two parallel lines of moderate electron density. In *Synechococcus* sp. 6301, other ultrastructural changes were as follows. At the late stages of irradiation, CM was usually not observed, while the cell wall looked undamaged. In the central part of the cell, where the destruction signs were most evident, there were ribosome aggregates

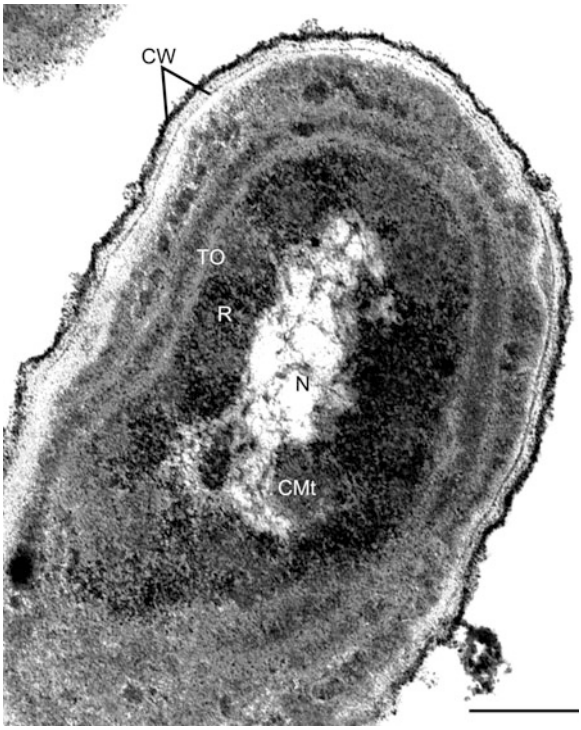

Fig. 2.20 Ultrastructure of *Synechococcus* sp. PCC 6301 after 360 min irradiation of cell suspension with high-intensity light (reprinted from Baulina et al. (2004) with kind permission from Pleiades Publishing, Ltd.) *CMt* cytoplasmic matrix, *CW* cell wall, *N* nucleoid, *R* ribosomes, *TO* outlines of the thylakoids. *Scale bar* 0.2 μm

separated by regions of homogenous cytoplasmic matrix similar in electron density to that of the thylakoids. Neither *Synechococcus* sp. 6301 nor *S. elongatus* showed any sign of cell autolysis. Thus, the impact of irradiation on *Synechococcus* sp. 6301 involved not only thylakoids, but also the cytoplasmic matrix and nucleoid, similar to the previously described species, and affected also the CM. It should be noted that, even after prolonged irradiation of *Synechococcus* sp. 6301, apart from the cells with destructive changes, intact-looking cells occurred frequently in the suspensions.

The ultrastructure of *A. variabilis* 29413 was typical of this species grown under optimal conditions and similar to that of *A. variabilis* 458 (see Fig. 2.3). The thylakoids were seen as two closely appressed membranes with three-layer profile, although in some cells they were located at a distance of ∼20 nm. They occupied the major part of the cytoplasm and formed numerous bendings and concentric circles. In many cases, the neighboring thylakoids were positioned in parallel; occasionally, there were aggregates of closely connivent thylakoids. Within the cells there were distinguished zones of the nucleoid with peripheral aggregates of ribosomes, the carboxysomes, and glycogen depositions (α-granules). The surface of the outer membrane contained depositions of finely dispersed capsular material.

Already after 30 min of irradiation, no structurally intact thylakoids were found on ultrathin sections (Fig. 2.21a). The thylakoid membranes lost their distinct

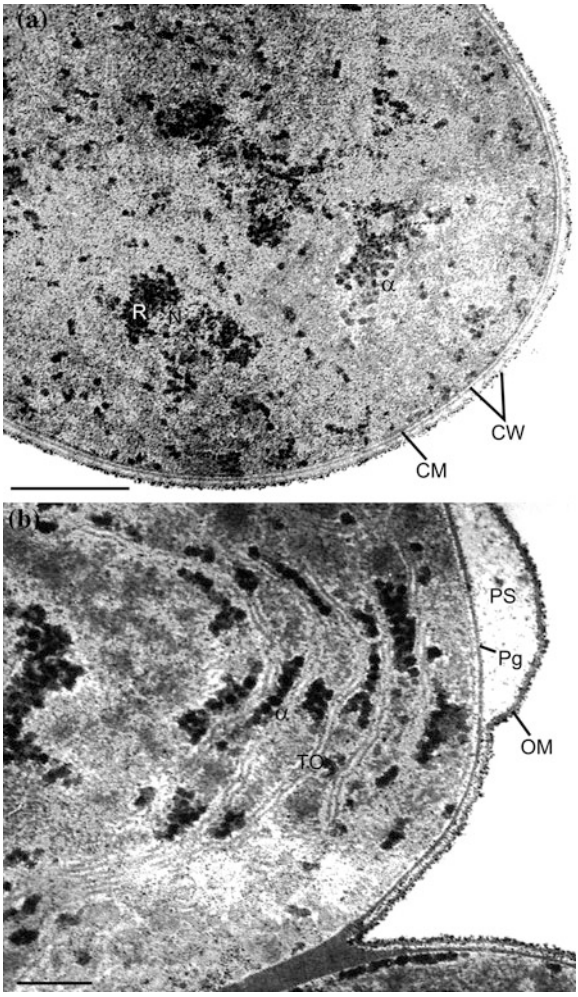

Fig. 2.21 Ultrastructure of *Anabaena variabilis* ATCC 29413 after 30 min (**a**) and 180 min (**b**) irradiation of cell suspension with high-intensity light (reprinted from Baulina et al. (2004) with kind permission from Pleiades Publishing, Ltd.) α glycogen α-granules, *CM* cytoplasmic membrane, *CW* cell wall, *N* nucleoid, *OM* outer membrane, *Pg* peptidoglycan, *PS* periplasmic space, *R* ribosomes, *TO* outlines of the thylakoids. *Scale bar* **a** 0.5 μm, **b** 0.2 μm

three-layer ultrastructure; the thylakoid contours became almost indistinguishable from the adjacent cytoplasm. The observed picture was similar to the one presented in Fig. 2.6. Occasional cells (data not shown) on the same sections contained a granular background of the cytoplasmic matrix and clearly visible electron-transparent winding lines that corresponded in thickness and position to the thylakoids of the intact cells. In the immediate proximity to these lines, low-electron-density granules of presumably lipid origin were located; these granules probably resulted from the degradation of thylakoids (de Vasconcelos and Fay 1974). Similar to the intact cells, the spaces between destroyed thylakoids accommodated a compact nucleoid zone, ribosomes, and α-granules (Fig. 2.21a). The CM and the cell wall also appeared intact. Upon prolongation of irradiation exposure to 60 min, we did not observe additional changes in cell ultrastructure.

Thus, the disturbance of the structural organization of the thylakoid membranes occurred in parallel with retention of the spectral characteristics of all types of pigments at the same level as in the intact cells.

Dramatic destructive changes in the ultrastructure of *A. variabilis* were observed after prolonged irradiation (Fig. 2.21b): the content of chlorophyll and carotenoids was drastically reduced, and phycobilins were almost fully destroyed (Fig. 2.17). The ultrastructural changes after exposures for 120 and 180 min were principally similar. The cytoplasm appeared as irregularly spaced clots of various electron densities. The nucleoid zones, the ribosome clusters, and the carboxysomes were not revealed. The "ghosts" of the thylakoid membranes were distinctly seen within the cytoplasmic matrix of nonuniform density. On the sections, these ghosts had an appearance of irregular bended lines, often organized in pairs. Granules similar to glycogen particles occupied the interstices between the cytoplasmic clots and degrading thylakoids. In such cells, CM was destroyed, and the peptidoglycan layer acquired a finely grained structure. The outer membrane retained its ultrastructural integrity and three-layer profile, but it produced protuberances at small regions of the cell surface. Further on, these protrusions apparently transformed to separated vesicles. The capsular material had the same appearance as in the intact cells. In many cases, the three-layer profile of the outer membrane was quite obvious in the separated vesicles. Thus, the prolongation of light exposure promoted the alteration of the outer membrane binding to the peptidoglycan layer; this tendency became evident already after 30 min of light exposure. The peptidoglycan layer turned loose, while retaining its rigidity. The space between this layer and the protrusions of the outer membrane, as well as the rest of the periplasmic space were filled with a finely dispersed granular material containing presumably the destructed components of the cytoplasm (see Fig. 2.21b). Even after long irradiation, we observed neither disruptions of the outer membrane nor the leakage of electron-dense products of lysis into the medium. At these stages, the populations occasionally contained cell remnants with fully lysed content and still unbroken bounding layer that was formed by the structurally modified cell wall. In the structure of this bounding surface layer, the outer membrane and the peptidoglycan layer were indistinguishable.

It should be noted that ultrastructural changes in the cell population of *A. variabilis* 29413 at different stages of irradiation (30, 60, 120, and 180 min) were comparatively uniform. However, even after long exposure to light, we occasionally observed the cells retaining the ultrastructure characteristic of short irradiation periods. At the same time, after long irradiation we could not observe cells with structurally intact thylakoids.

In contrast to *A. variabilis* 29413, the original (control) *C. fritschii* culture, as it is typical of the species, was heterogeneous in cell morphology and ultrastructure. Irradiation during 60 min did not affect the ultrastructure; this result correlated with the practically undamaged state of the pigments during this period (Fig. 2.22). Moreover, cells similar in organization to the control ones, predominated after 120–150 min exposure and were present in the population after 210–240 min irradiation. After 120–150 min of irradiation, together with

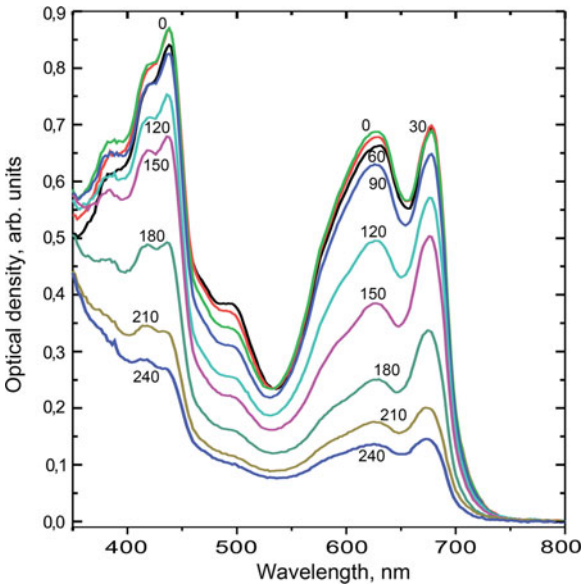

Fig. 2.22 Changes in the absorption spectra of *Chlorogloeopsis fritschii* ATCC 27193 cell suspension during irradiation with high-intensity light (data obtained by Chivkunova, Merzlyak and Baulina). *The numbers* the exposure time (in minute)

increasing bleaching of pigments (Fig. 2.22), the cells with destructive changes in the thylakoid membranes were revealed in the population, although the cell wall, CM, and nucleoid retained their integrity (Fig. 2.23a). In the case of 210–240 min exposure, destructive changes in these cells increased: CM was being destroyed and granules similar to the lipid ones appeared. In some cells, the peptidoglycan layer was lost, thylakoids turned to disordered accumulations of membrane debris, the cytoplasm matrix underwent autolysis, the nucleoid, carboxysomes, and granules of poly-β-hydroxybutyrate could not be revealed, while the granules of glycogen were present in abundance (Fig. 2.23b). In the case of these cells, similarity between the ultrastructural pictures of cell destruction in two species, *C. fritschii* and *A. variabilis* 29413, should be noted, with the modifications being revealed earlier and developing to a greater extent in the latter strain.

The results presented in this section (Baulina et al. 2004) suggest a conclusion that the pattern of photodestructive processes in the examined cyanobacterial species is complicated; this is clearly manifested in diverse distortions of the membrane structure and cell integrity. A characteristic feature of these distortions is dramatic changes in the ultrastructure of thylakoids. The lack of a three-layer profile of the thylakoid membranes, which is typical of the elementary membrane, which was revealed by TEM, points to the impairment of the phospholipid bilayer in consistency with the conclusions reached by other authors (Schmetterer et al. 1983). It is noteworthy that the destructive alterations in the ultrastructure of thylakoid membranes in *A. variabilis* 29413 were observed already during the lag period preceding bleaching of chlorophyll, carotenoid, and phycobilins. On the other hand, in *Synechococcus* species and *C. fritschii*, the ultrastructural changes occurred simultaneously with the destruction of all pigments. In the case of

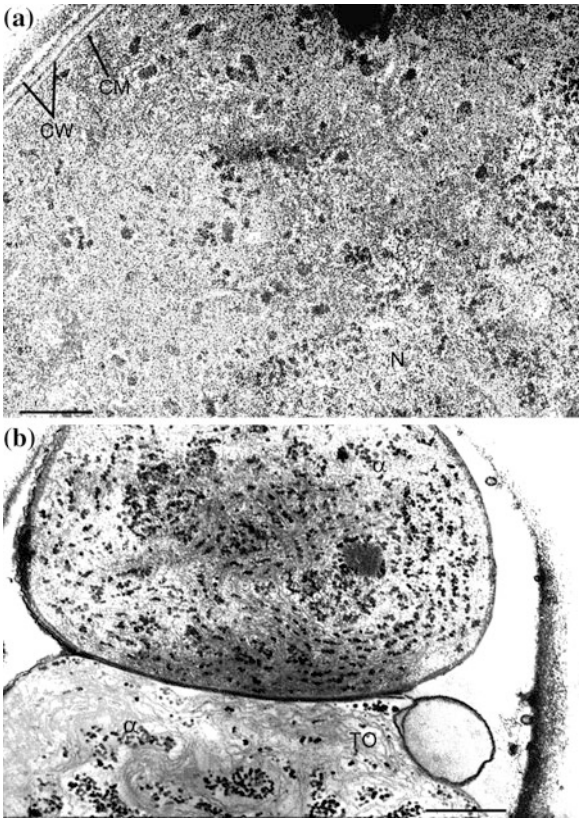

Fig. 2.23 Ultrastructure of *Chlorogloeopsis fritschii* ATCC 27193 after 150 min (**a**) and 120 min (**b**) irradiation of cell suspension with high-intensity light (data obtained by Baulina, Chivkunova, Merzlyak). α glycogen α-granules, *CM* cytoplasmic membrane, *CW* cell wall, *N* nucleoid, *TO* outlines of the thylakoids. *Scale bar* **a** 0.2 μm, **b** 0.5 μm

A. variabilis 29413, the initial damage to the thylakoid membranes was followed by a cascaded amplification of destructive changes in other cell structures.

Apart from destruction of the thylakoids, in all species under study, homogenization of the cytoplasm, and degradation of the nucleoid were observed, although carboxysomes still persisted in the nucleoid zone. In *A. variabilis* 29413 and *Synechococcus* sp. 6301, the disturbances of CM ultrastructure progressed up to full degradation of this membrane. The outer membrane of *A. variabilis* 29413 retained for a long time the ultrastructure typical of an elementary membrane and showed extensibility in local regions. The capacity for extension was also observed in *S. elongatus*, although to a much lesser extent. By the end of the irradiation period, destructive processes in the cytoplasm apparently occurred in all the cells in the population of these two species, so that undamaged cells were not revealed. In *A. variabilis* 29413 some cells completely lost their cytoplasmic content. It is conceivable that destruction of the cytoplasm in this case could be caused not only by direct action of irradiation but also by the activity of hydrolytic enzymes released from the periplasmic space due to impaired integrity of the CM. The products of cytoplasm degradation apparently penetrated through the

peptidoglycan gel which, judging from its altered ultrastructure, became more permeable to macromolecules.

The comparison of responses of cyanobacteria to the short-term action of extremely high-intensity light (200 klx) and the behavior of the same organisms during cultivation under high light intensity (9–10 klx) revealed a certain similarity in the ultrastructural manifestations of cell destruction. Exposure of *Synechococcus* sp. 6301 to both treatments resulted in destruction of the thylakoids and CM, homogenization of the cytoplasm, and degradation of the nucleoid, with the retention of carboxysomes in the nucleoid zone. At the same time, the cell wall remained unchanged, and cell lysis did not occur; at late stages, many cells in the population were similar to intact cells in terms of their ultrastructure. When *A. variabilis* 458 was grown at a high light intensity, similar destructive changes took place at shorter exposures to irradiation, and many cells were lysed at later stages. Upon culturing of this cyanobacterial species under extreme illumination conditions, the thylakoids became vesiculated at early stages of irradiation and were destroyed at late stages. The outer membrane was found to be the most resistant cell structure. On the other hand, the peptidoglycan layer of the cell wall lost its rigidity and became perforated with numerous pores and narrow canaliculi. Similar changes were also characteristic of the cells of *A. variabilis* 29413 irradiated with high-intensity light for short periods. The most significant distinction was that no vesiculation of the thylakoids was observed in *A. variabilis* 29413. Both under physiological conditions and during irradiation of cell suspensions with high-intensity light, cyanobacteria of different genera exhibited destructive changes of variable degrees: both strains of *A. variabilis* were more sensitive to light treatment than *Synechococcus* species and *C. fritschii*. As for the latter species, only irradiation of the suspension with light of high intensity demonstrated that this cyanobacterium was also susceptible to photooxidative destruction, although no destructive processes could be revealed upon cultivation under high light intensity during the period of observation.

It appears that the pattern of ultrastructural changes occurring in cyanobacteria under photooxidative conditions are affected by the regulatory mechanisms of cell response and by species-specific differences in the chemical composition and macromolecular organization of the thylakoid membranes, CM, cell wall, and other cell components accounting for the differences in their structural stability (Baulina et al. 2004). In general, the described picture of destructive changes in cyanobacteria indicates a more complex nature of photooxidative damage than it was considered previously.

2.2.4 *Ultrastructure of* Chlorogloeopsis fritschii *ATCC 27193 Grown in the Dark*

Genetically determined metabolic flexibility of *C. fritschii* is responsible for its capacity for chemoheterotrophic growth in the dark in the presence of exogenous sources of carbon and energy (Fay 1965). In this section, the results of studies on

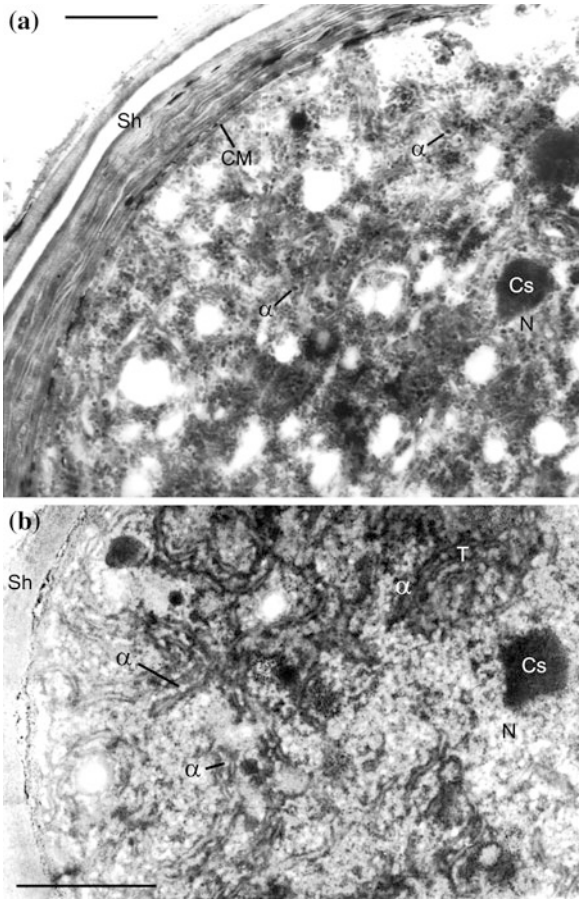

Fig. 2.24 Ultrastructure of *Chlorogloeopsis fritschii* ATCC 27193 in the culture growing in the dark in the presence of glucose: ultrathin sections contrasted with lead citrate (**a**) and uranyl acetate (**b**). α glycogen α-granules, *CM* cytoplasmic membrane, *Cs* carboxysome, *N* nucleoid, *Sh* sheath, *T* thylakoid. *Scale bar* **a**, **b** 0.5 μm

the ultrastructure of this cyanobacterium grown in the presence of glucose are presented (Baulina et al. 1978). It should be noted that even in the presence of sugars growth in the dark was slow, the cells were typically individual and surrounded by thick multilayer sheaths (Figs. 2.24 and 2.25).

As it will be demonstrated in this and the following chapters, cyanobacterial sheaths and other external surface structures of similar chemical nature and functions, i.e., capsules, depositions of amorphous mucus, and mucous thin covers over groups of cells, all play an important role in the adaptation processes developing in the cells and populations of these microorganisms. In the case of comprehensive analysis of experimental data, it seems appropriate to group all these structures under a common term mucous surface structures (MSS) in contrast to the widely used term glycocalix. The latter term was initially used in description of eukaryotic organisms and, to our mind, is not accurate in description of prokaryotes, whose cell envelope organization is totally different comprising a

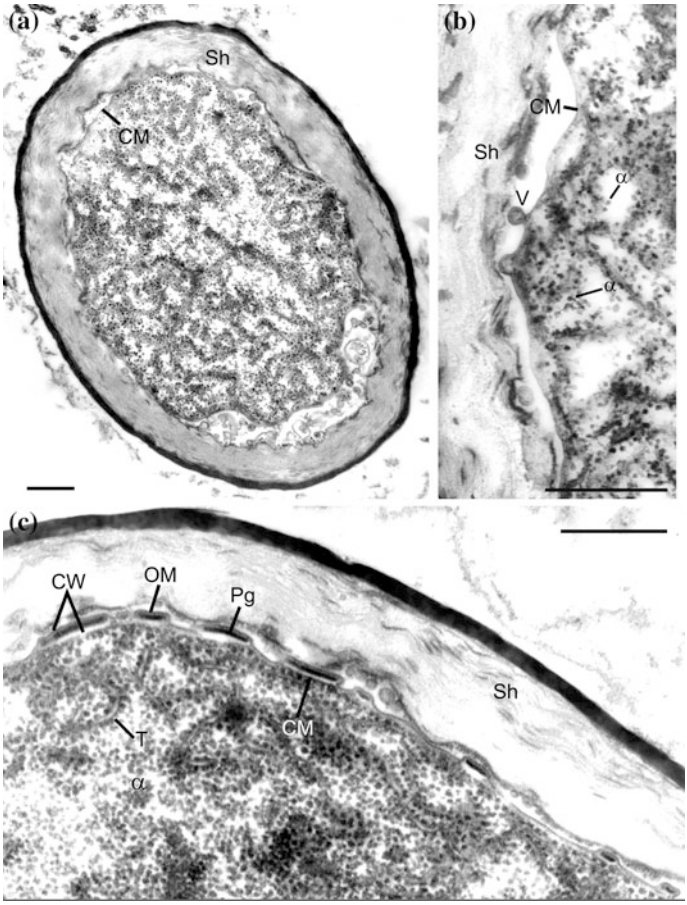

Fig. 2.25 Ultrastructure of *Chlorogloeopsis fritschii* ATCC 27193 in the culture growing in the dark in the presence of glucose: a protoplast surrounded with a sheath (**a**), formation of CM vesicles in the protoplasts (**b**), changes in structure of the cell wall (**c**) (Baulina et al. 1978). α glycogen α-granules, *CM* cytoplasmic membrane, *CW* cell wall, *OM* outer membrane, *Pg* peptidoglycan, *Sh* sheath, *T* thylakoid, *V* vesicle. *Scale bar* **a**, **b**, **c** 0.5 μm

peptidoglycan layer, a periplasmic space, and an outer membrane. The only exception is prokaryotes lacking cell wall, that is mycoplasmas and some archaea.

Laminated MSS of cyanobacteria, often of complex structure, are typically called sheaths. They may differ in the number and volume of the layers, as well as in their chemical features, packing and orientation of the polymers comprising each layer. To define the individual layers with polymeric fibril orientation perpendicular to the cell wall, a number of researchers use the term capsule, common for all bacteria. Multilayered sheaths, including those comprising homogenous electron-dense layers, in addition to the fibrillar ones, and lack covalent bonds with the cell wall outer membrane, are common in *C. fritschii* and members of the

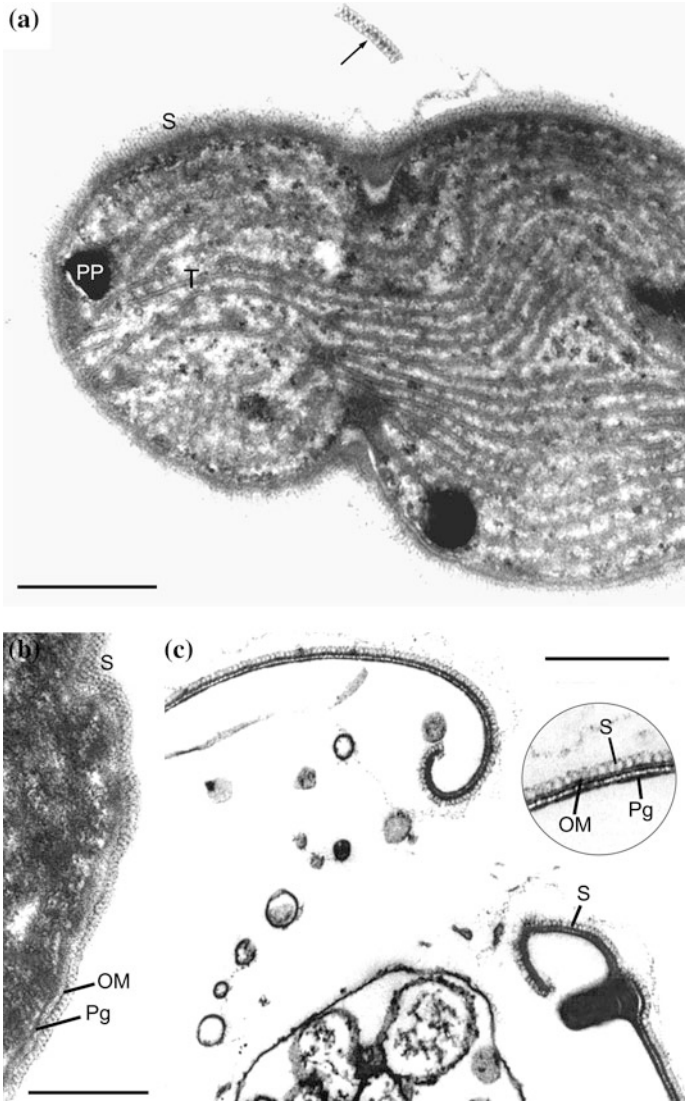

Fig. 2.26 Ultrastructure of *Synechocystis aquatilis* CALU 428 general view of a dividing cell (**a**), cell region with the envelope (**b**), envelope of an autolysed cell (**c**). *OM* outer membrane, *Pg* peptidoglycan, *PP* polyphosphate granules, *S* S-layer, *T* thylakoid. The *arrow* indicates an peculiar paracrystalline structure. *Scale bar* **a**, **b**, **c** 0.5 μm

genus *Gloeocapsa*, *Gloeothece*, *Gloeobacter* (Rippka et al. 1979), *Fischerella* (Pritzer et al. 1989), *Nostoc* (Hill et al. 1994). For *Nostoc commune*, the outer layer of the sheath was shown to be enriched with calcium, silicon, and magnesium (Hill et al. 1994). Electron-dense layers may contain proteinaceous components

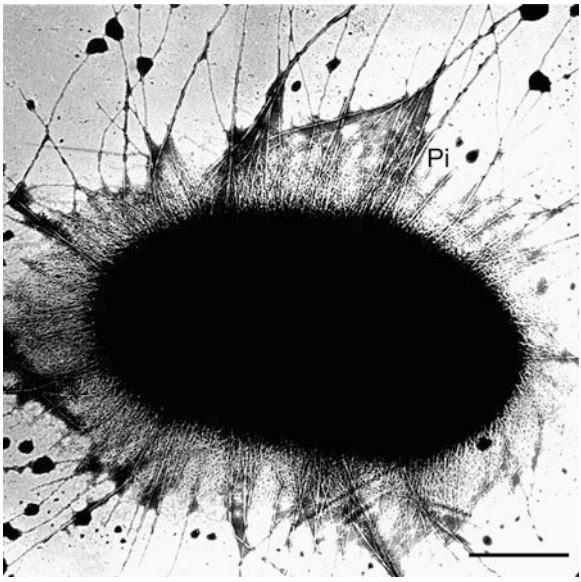

Fig. 2.27 *Synechocystis aquatilis* CALU 428 with peritrichous pili. Negative staining with phosphotungstic acid, TEM. *Pi* pili. *Scale bar* 1 μm

(Jürgens and Weckesser 1985; Baulina et al. 2008). Data on the chemical composition of cyanobacterial MSS are presented in greater detail in Sect. 5.1.1.

For a reader getting the first acquaintance with the diverse world of cyanobacteria, it should be specified that MSS, such as capsules or sheath, are characteristics of most, although not all species of these microorganisms. In many, other structures called S-layers, typical of most prokaryotes and comprising protein or glycoprotein surface layers (see Figs. 2.1 and 2.26), are located above the cell wall outer membrane (Jensen and Sicko 1972; Lounatmaa et al. 1980; Vaara 1982; Gromov 1986; Mamkaeva 1986; Sleytr et al. 1994; Hoiczyk and Hansel 2000; Šmarda et al. 2002). In cyanobacteria with capsules and sheath, S-layers may also be present. Bacterial S-layers possess numerous functions such as adhesive, protective, and barrier ones, including those of adaptive importance (Sára et al. 1990). While the functions of these structures in cyanobacteria are studied less, their role in the processes responsible for gliding in a number of filamentous cyanobacterial species (Hoiczyk and Baumeister 1995) and swimming of a unicellular *Synechococcus* sp. WH8102 (McCarren et al. 2005) has been established. Besides, cyanobacterial S-layers act as a matrix for formation and deposition of calcium, magnesium, and strontium minerals (Schultze-Lam and Beveridge 1994a).

In our study of the cyanobacterial species *Synechocystis aquatilis* Sauv. CALU 428 (referred to as *Synechocystis aquatilis* hereinafter), S-layers were found to consist of hexagonally packing subunits, often of uneven thickness (Fig. 2.26a and b) and may be peeled off cell surface. The latter fact indicates the lability of these structures. At the same time, upon autolysis these layers retain their intact ultrastructure and connection to the cell wall outer membrane (Fig. 2.26c). This interesting phenomenon was revealed in other bacteria, for example, in *Flexibacter*

polymorphus (Ridgway et al. 1975). For cyanobacteria of genus *Synechococcus* (strain GL24), high resistance of the S-layer complex and the underlying cell wall layers in the absence of cell lysis upon treatment with the structure-breaking chemical agents is considered to be connected with the extreme conditions of the natural habitat of the microorganism (Schultze-Lam and Beveridge 1994b).

On the surface of a number of unicellular and filamentous cyanobacteria, pili (fimbriae) are localized (Ratner et al. 1976; Dick and Stewart 1980; Vaara 1982; Johansson and Bergman 1994; Adams and Duggan 2008) (Fig. 2.27; see also Fig. 2.1). These are fibrillar accessory structures characteristic of prokaryotes built of protein subunits packed in cylinders. Various types of spines also occur on the surface of cyanobacterial cells (see Fig. 2.1; see also Fig. 4.8d and references in Chap. 4). Their function is not studied yet. Concerning the pili, which are present in *Synechocystis aquatilis* (Fig. 2.27), as well as the S-layers, it should be noted that for *Synechocystis* sp. PCC 6803, another representative of this genus, involvement of these structures, particularly the pili of type IV, in the twitching motility has been established (Bhaya et al. 2000). In general, bacterial pili of various types (I–IV) are involved in cellular adhesion, motility, and conjugation process (Pinevich 2006).

Going back to description of *C. fritschii* growing in the dark, it should be noted that the fact of predomination of unicellular forms is in accordance with the data on suppressed formation of filamentous forms under conditions of photoheterotrophic and chemoheterotrophic growth (Evans et al. 1976; Evans and Britton 1983). On the basis of these data, the authors concluded that emergence of various morphological cell types in a *C. fritschii* culture was a consequence of external factors.

Upon switching to chemoheterotrophic growth, the ultrastructural plasticity of the species was revealed to its full extent at both subcellular and cellular levels. The thylakoid system was reorganized, resulting in the changes of thylakoid spatial arrangement in the cytoplasm matrix, shortening of the membrane contours in the section plane, and inability to reveal their three-layer ultrastructure (see Fig. 2.24). Different changes in the thylakoid system were observed in *A. variabilis* 29413 growing in the dark in the presence of fructose (Pinevich and Volkov 1988). In this latter case, curved, but not shortened thylakoids filled the whole cytoplasm. Analysis of the pigment composition and a number of other parameters suggested that the changes in the photosynthesis apparatus were of adaptive nature. At the same time, low electron density of the cytoplasmic matrix is typical of this species as well as of the chemoheterotrophic *C. fritschii* culture under study. In the cells of the latter one, the cytoplasm was filled with α-granules of glycogen of atypical spherical shape (they are typically ellipsoid in cyanobacteria). The fact that these granules were filled with neutral polysaccharides was confirmed by differential staining of ultrathin sections with lead citrate according to Reynolds (Reynolds 1963) (Fig. 2.24a) and uranyl acetate (Fig. 2.24b) upon fixation with glutaraldehyde and osmium tetroxide. In the first case, glycogen granules appeared electron dense, while in the second case, they remained electron transparent since uranyl acetate does not interact with neutral polysaccharides (Geyer 1973).

In the early studies, similar data on *C. fritschii* ultrastructure upon long-term cultivation in the dark in the medium with sucrose, including the data on spatial arrangement of the thylakoid system and accumulation of numerous glycogen granules, were obtained using the preparations fixed with $KMnO_4$ which does not preserve proteins and nucleic acids (Peat and Whitton 1967). The connection between glycogen deposition in the cells of various cyanobacterial species and the heterotrophic type of their constructive metabolism was noted by a number of researchers (Rippka 1972; Raboy et al. 1976). The direction of biosynthetic processes in *Aphanocapsa* sp. 6714 has been studied in detail depending on the type of constructive and energy metabolism (Pelroy et al. 1976). Determination of the activity of the synthesis of major cell polymers (proteins, lipids, RNA, and glycogen) revealed that growth of this cyanobacterium in glucose-containing medium in the dark (chemoheterotrophic growth) or in the light (photoheterotrophic) in the presence of a photosynthesis inhibitor Diuron led to significant decrease in the rates of lipid, RNA, and protein syntheses. The main substance synthesized under these conditions was glycogen. In the experiments on *C. fritschii* growth in the dark reported in this section, as well as in the experiments with this species and *A. variabilis* 458 grown under high light intensity in the presence of sugars (see Sect. 2.2.2), similar processes probably occurred. This is confirmed by a decrease in the electron density of the cytoplasmic matrix, the absence of ribosome aggregations, inability to detect the characteristic nucleoid zones in many of thin sections, and, on the contrary, abundance of glycogen α-granules.

The spectacular feature of most of the cells in a population at the stage of growth (day 15) was reorganization of the cell wall up to complete reduction of its components revealed by electron microscopy, that is, destruction of the outer membrane and the peptidoglycan layer and formation of protoplasts (see Fig. 2.25a). At the same time, the cells in the studied preparations always contained a complex sheath. CM of the protoplasts formed characteristic protrusions which subsequently formed vesicles localized between the protoplast surface and the sheath (see Fig. 2.25b). In the above-cited literature (Peat and Whitton 1967), the absence of the cell wall could be seen in some images, although this fact was not discussed in the text. Moreover, while there are indications by other authors that protoplasts enclosed in sheaths are present in *C. fritschii* culture upon growth under low-intensity illumination and at 45 °C (above the optimal temperature), the phenomenon of the absence of the cell wall in cyanobacteria is not discussed (Findley et al. 1970). It should be noted that *C. fritschii* protoplasts are sometimes revealed in the stationary-phase culture growing under optimal light and temperature conditions (Gusev et al. 1982). Cyanobacteria *N. muscorum* CALU 304 was also able to form the protoplasts producing surface vesicles upon growth under optimal conditions. In this case, however, the cells tended to form constrictions and divide; chains of 2–5 protoplasts were detected (Gorelova 2001). To the best of our knowledge, there is no other data on spontaneously formed protoplasts or spheroplasts in laboratory cultures of free-living cyanobacteria.

Observation of numerous protoplasts in *C. fritschii* culture suggested a hypothesis that at a definite stage of growth in the dark, cells of this cyanobacterium are

L-forms which are characterized by the presence of a multilayer sheath probably performing some of the cell wall functions and facilitating protoplast protection against osmotic shock (Baulina et al. 1978; Gusev et al. 1982). It is interesting, that protective function of the colanic acid capsule established in the study of L-form-like growth *Escherichia coli* K-12 (Joseleau-Petit et al. 2007).

In microbiological literature, the term L-forms is used to define bacteria capable of growth in the absence of a rigid cell wall (Prozorovsky et al. 1981; Domingue and Woody 1997). In cyanobacteria, the ability for L-transformation and induction of L-form-like cells was experimentally demonstrated only in the early 1980s by the researchers of the group headed by Professor Mighael V. Gusev, at the Faculty of Biology, Moscow State University (Gusev et al. 1981, 1983). These studies are reported in Chap. 3.

Determination of the ability of darkness-induced *C. fritschii* protoplasts to reverse to original cells is an unsolvable problem for an individual cell form. It is known from the literature that the capacity for reversion depends on the ability to reconstruct the cell wall with the obligatory presence of macromolecular precursors of peptidoglycan and components of the outer membrane. As a rule, the protoplasts of gram-positive bacteria, spheroplasts of gram-negative bacteria, and unstable L-forms can be reversed easily (see Chap. 3). Therefore, to determine the possibility of reversion for at least some L-form-like variants of *C. fritschii*, ultrastructural evidence of synthesis of peptidoglycan and cell wall outer membrane should be collected. This issue is presently far from resolution. However, there is evidence that many cell forms in dark-growing *C. fritschii* cultures were at the stage of cell wall resynthesis: discontinuous peptidoglycan layer and alternating regions with complete cell wall, including the outer membrane, and those with no cell wall, are characteristic of these cells (Fig. 2.25c). Systematic studies performed on pathogenic bacteria at the Gamaleya Research Institute of Epidemiology and Microbiology (Russian Academy of Medical Sciences) demonstrated that discontinuous cell wall was only observed during the early stage of reversion (Prozorovsky et al. 1981). This conclusion, as well as our data (Fig. 2.25c), agree with the results of the later study of *E. coli,* which established that L-form-like growth required ongoing peptidoglycan synthesis and hypothesized that all L-forms have possibly residual peptidoglycan synthesis (Joseleau-Petit et al. 2007).

Thus, formation of protoplasts in *C. fritschii* culture upon changes in the growth conditions is a manifestation of its ultrastructural plasticity at the cellular level indicating the possible L-transformation under conditions of growth in the absence of light.

2.3 Concluding Remarks

Results reported in this chapter allow for the following conclusions. Among the representatives of three cyanobacterial genera, *Synechococcus*, *Anabaena* and *Chlorogloeopsis*, differing by the degree of obligate phototrophy upon cultivation

under varying light regimes, various forms of ultrastructural plasticity were revealed at subcellular, cellular, and population levels. These include:

Subcellular level

(1) Changes in configuration of the thylakoids reflected in a considerable separation of the constituent membranes with the preservation of their ultrastructural integrity in the obligate phototroph *A. variabilis* 458 upon long-term cultivation in the dark. In the case of transfer to optimal illumination conditions after a limited term of incubation in the dark, this phenomenon is reversible and is accompanied by the preservation of cell viability.
(2) Formation of vesicles by CM of the *C. fritschii* protoplasts upon growth in the dark.
(3) Ultrastructural changes of the cell wall peptidoglycan layer in dark-growing *C. fritschii*.

Cellular level

(1) The absence of glycogen in *A. variabilis* 458 cells due to its consumption as a carbon and energy source in the dark-incubated culture and, on the contrary, its presence in the culture returned into the phototrophic conditions. Predominant (compared to other intracellular structures) glycogen accumulation in photoheterotrophic cells of *A. variabilis* 458 and *C. fritschii* and in chemoheterotrophic cells of *C. fritschii.*
(2) Complete reduction of of the cell wall in *C. fritschii* growing in the dark (formation of protoplasts).

Population level

Changes in the population structure. Heterogeneity of the cells in populations of all studied species by (a) ultrastructural manifestation of photooxidative destruction of the thylakoids and other cellular components and (b) the degree of changes in the cell wall ultrastructure.

Experiments on the damaging effects of high-intensity light on the ultrastructure of cyanobacteria demonstrated that in *A. variabilis* strains, all cell structures (except for cyanophycin granules) including thylakoids, CM, peptidoglycan layer, and cell wall outer membrane, are subjected to destruction much faster than in *C. fritschii*, *Synechococcus* sp. 6301, and *S. elongatus*. In the two latter species, ultrastructural changes which could be attributed to manifestations of the ultrastructural plasticity, such as increased intrathylakoid space, were not registered. On the contrary, at least in *Synechococcus* sp. 6301, manifestation of ultrastructure "fragility", that is the absence (or transiency) of reversible changes, may be stated. This observation correlates with the known conservatism of both the metabolism of this cyanobacterium and its ultrastructural organization and cell morphology.

The most versatile forms of ultrastructural plasticity were characteristics of *C. fritschii*, possessing the most flexible metabolism and heterogeneity of the organization intracellular membrane structures and cell morphology among species under study.

The revealed forms of manifestation of ultrastructural plasticity of various species of cyanobacteria indicate the following possible adaptive mechanisms under unfavorable changes in the illumination regime: reversible thylakoid swelling, regulation of synthesis and degradation of glycogen, L-transformation, and population heterogeneity in terms of cell susceptibility to the same stimuli.

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Chapter 3
Ultrastructural Plasticity of Cyanobacteria During the Enzymatic Induction of L-Transformation

Abstract In this chapter, the results of electron microscopy investigation of the dynamics of changes in cyanobacterial cells during enzymatic induction of their L-transformation in model systems in vitro are presented. These data are evident of the correspondence between metabolic versatility and ultrastructural complexity of different cyanobacterial species and their ability to exist as cell wall-defective forms. The unique data on the *Chlorogloeopsis fritschii* capability of L-transformation during cultivation in the presence of lysozyme are emphasized as an important contribution to current understanding of cyanobacterial physiology.

3.1 Bacterial L-Transformation

The study of light-dependent changes in the ultrastructure of *C. fritschii* showed that cell forms of the protoplast type, i.e., those without the murein layer and the cell wall outer membrane but with a well-developed multilayer sheath, may be present in the populations of cyanobacteria (see Chap. 2). This observation was essential to reveal the capacity of cyanobacteria for spontaneous formation of protoplasts, which indicates the possibility of their existence without cell wall, i.e., as L-forms.

The phenomenon of L-transformation has been described for many species of gram-positive and gram-negative heterotrophic bacteria, mostly pathogenic to humans. Transition to the L-form is viewed as a way to survive unfavorable conditions, such as persistence in the host body or in the environment, in particular, in water, under prolonged starvation, i.e., as a phenomenon of adaptive character (Pavlova 1999; Prozorovsky et al. 1981) Bacteria without a cell wall were recently shown to be among the stable forms of bacterial life (Dell'Era et al. 2009) The capacity of L-forms for reproduction in the absence of cell wall (by dividing with constriction or by forming elementary bodies) affords the population

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with long-term existence in the modified form, but with the preservation of the main traits of the species. Apart from the structural level, molecular genetic aspects of division of L-forms have been recently investigated. A remarkable extrusion-resolution mechanism was discovered (Leaver et al. 2009; Dominguez-Cuevas et al. 2011). These authors believe that investigation of this novel form of propagation may improve our understanding of the possible forms of proliferation in early forms of cellular life

At the same time, certain metabolic properties, the surface antigenic structure, and the macromolecular organization and lipid composition of the CM, which becomes more rigid, are changed in the L-forms (Prozorovsky et al. 1981; Nishiyama and Yamaguchi 1990; Dominque 1995; Hoischen et al. 1997; Pinevich 2006). The L-forms differ in their ability to reverse upon removal of the factor that induced their formation, depending on the markedness of the metabolic and structural rearrangements occurring in the cells in the course of transformation. Each population (colony) of L-forms is heterogeneous, with the individual cells differing in morphology and ultrastructure, as well as in their capacity for reversion. The latter is determined not only by the possibility of expression of the relevant genes, but also by the spatial organization of the biosynthetic processes, which includes the presence of the peptidoglycan priming elements, such as the undecaprenyl-disaccharide-pentapeptide [besides see Sect. 2.2.4 about possibly residual peptidoglycan synthesis in L-forms (Joseleau-Petit et al. 2007)]. It is also considered that the so-called unstable L-forms, retaining the cellular components of the cell wall (for example, the elements of the outer membrane of gram-negative bacteria) and the possibility of their expression, reverse relatively easily to the cells of the initial species. Stable L-forms of the protoplast type, some of which seem to emerge as a result of mutation of the genes responsible for the cell wall synthesis, may lose the capacity for reversion and be maintained for a long time after the action of the inductor has ceased (Allan 1991; Hoischen et al. 1997). At the same time, the data are available that the CM of stable L-forms of the protoplast type of *E. coli* is capable of synthesizing the lipopolysaccharide (O-antigen), which is the main component of the outer membrane (Michailova et al. 1986; Ivanova et al. 1997). The stable L-forms of the protoplast type of gram-negative bacteria may be suitable for use in biotechnology as more effective protein producers compared to intact cells (Sieben et al. 1998).

The initial stage of L-transformation is the emergence in the population of the so-called unbalanced growth forms (UGF) and its transition to the unbalanced (Prozorovsky et al. 1981), or heteromorphous, growth phase according to M.A. Peshkov (Peshkov 1955). He proposed to treat L-transformation as one of the manifestations of heteromorphous growth and to consider the cells enlarged 3- to 10-fold, often with an irregular shape and a modified cell wall, as heteromorphous forms (or UGF).

The outstanding Russian microbiologist N.F. Gamaleya initiated the study of cellular heteromorphism of bacteria. In 1984, he was the first to describe the morphologically variable *Vibrio cholerae* cells emerging in the culture treated with lithium salts and to introduce the concept of heteromorphism (Gamaleya 1894).

This happened in the period of intensive development of research into the effect of different physicochemical factors (including the salts added to the nutrient media) on bacteria. The cells with altered shape and size, abnormal division, and impaired ultrastructure of the peptidoglycan layer of the cell wall are considered UGF. In natural biocenoses, these forms, resulting from easily reversible phenotypic changes in the cells, are common (Prozorovsky et al. 1987; Pavlova 1999). Cell forms with more profound cell wall changes, namely, protoplasts and spheroplasts, may appear during the phase of unbalanced growth. Protoplasts are predominantly formed by the gram-positive bacteria that completely lost their cell wall. Spheroplasts are usually formed by gram-negative bacteria, which lose the cell wall peptidoglycan layer but preserve a modified outer membrane (Fig. 3.1). We found that gram-negative cyanobacteria were capable of forming both protoplasts and spheroplasts. We assigned cyanobacteria with an impaired structure of the peptidoglycan layer or with its reduction, including spheroplasts and protoplasts to the group of cell wall-defective forms (CWDF) of bacteria. In the opinion of microbial cytologists, this group includes the cells in bacterial populations, which have with various abnormalities of the cell wall structure (Vysotskii et al. 1984; Kats 1980). This is in agreement with the opinion of (Domingue and Woody 1997) who stated that the term "cell wall-deficient/defective bacteria (CWDB)" to describe the aberrant bacterial forms present in clinical specimens was both the most broadly inclusive and the most descriptive. In out opinion, it will be more precise to say that diverse cell forms, from cells with impaired rigidity of the peptidoglycan layer to spheroplasts and protoplasts, should be attributed to bacterial CWDF. Moreover, it is expedient to designate the latter two cell types (spheroplasts and protoplasts) differing between each other in the presence of the outer membrane (Fig. 3.1) as cell wall-reduced forms (CWRF) (Gusev et al. 2002; Gorelova and Baulina 2000). This is, in fact, a synonym to the well-known term cell wall-deficient (CWD) bacteria, which is also applied to L-forms (Allan et al. 2009). The term CWRF is preferred in the present work, since it may be used in a broader sense, to designate both cyanobacterial L-forms and the spheroplasts and protoplasts formed by the vegetative cells or heterocysts in plant–cyanobacterial symbioses and specializing in overproduction of extracellular polymers (see Chap. 5).

The cell forms of the phase of unbalanced growth (also includes spheroplasts and protoplasts) may reverse to the initial vegetative cells, be destroyed, or be converted to the L-forms capable of reproduction (Prozorovsky et al. 1981). The L-forms differ from the unbalanced growth phase spheroplasts and protoplasts in the duration of transfers, the rapidity and quality of reversion, the number of the elementary bodies formed, and the role of the latter as a mode of reproduction (Prozorovsky et al. 1987). Elementary bodies are considered to be the minimal and possibly the main reproductive elements of the L-forms (Dominque 1995).

The researchers of the Faculty of Biology, Moscow State University, and the Gamaleya Research Institute of Epidemiology and Microbiology, Russian Academy of Medical Sciences, have developed a method for obtaining subcultured of cyanobacteria using the enzyme lysozyme known to hydrolyze the cell wall peptidoglycan (Gusev et al. 1981). The experiments were carried out with the same

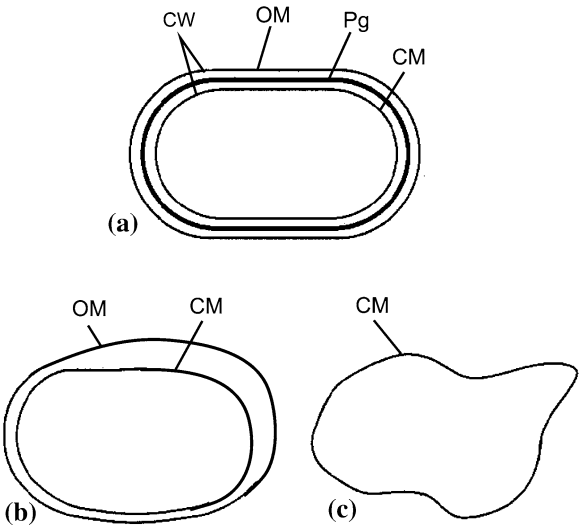

Fig. 3.1 Surface structures of intact vegetative cells **a** and the cell wall-reduced forms of spheroplast type **b** and protoplast type **c** of gram-negative bacteria. *CM* cytoplasmic membrane, *OM* outer membrane, *Pg* peptidoglycan

strains which have been studied earlier under different illumination conditions: *Synechococcus* sp. 6301, *A. variabilis* 458, and *C. fritschii* 27193. The colonies formed from the morphological elements characteristic of chemoheterotrophic bacteria in the process of L-transformation were revealed during subculturing in the presence of lysozyme of *A. variabilis* 458, which is partially capable for photoheterotrophic growth, and *C. fritschii,* which is capable for chemoheterotrophic growth. These colonies were designated as L-form-like colonies. We succeeded in sustaining the growth of L-form-like *A. variabilis* 458 colonies only during two transfers. In the case of *C. fritschii,* the duration of subculturing was limited to the eighth transfer by the experimenters. For the obligate phototroph *Synechococcus* sp. 6301, the attempts to induce L-transformation proved to be unsuccessful (Gusev et al. 1981). In all three cases, the suspensions of the cells treated with lysozyme under specially adjusted conditions (Mineeva et al. 1979) with a view to obtaining CWDF (among which spheroplasts and protoplasts were supposed to be present) were initially introduced into the culture, which was a necessary prerequisite for in vitro simulation of the possible similar processes under natural conditions.

3.2 Ultrastructure of Cyanobacteria After the Lysozyme Treatment

The electron microscopic methods using adequate techniques of fixation of the preparations of lysozyme-treated cyanobacteria, containing, as was preliminarily estimated with light microscopy, up to 70 % of CWDF, gave evidence of the

preservation of the structural integrity of the cytoplasm of most of the spheroplasts contained in the suspensions of the three strains of cyanobacteria (Mineeva et al. 1980; Gusev et al. 1982; Agafodorova et al. 1982; Baulina et al. 1984).

Synechococcus sp. 6301 quickly responded to the treatment with lysozyme forming spheroplasts with varied morphology (Fig. 3.2). A distinctive feature of *Synechococcus* sp. 6301, compared to the other two strains, was the formation of bean-shaped spheroplasts, along with the spherical ones (Fig. 3.2a), as was confirmed by the light microscopic control of the preparations. The ultrathin sections of bean-shaped and irregularly shaped spheroplasts revealed vast areas of the surface preserving the relative arrangement of the cell wall layers: the incompletely degraded peptidoglycan layer and the outer membrane. At the other sites, where the peptidoglycan layer was probably completely destroyed, the outer membrane lost its rigidity, extended, and stretched far away from the protoplast surface, losing its distinct outlines. The described state of the outer membrane was observed irrespective of the presence of EDTA and magnesium cations in the incubation medium (according to Semenova's technique) (Semenova et al. 1982). These reagents have a destabilizing and stabilizing effect, respectively, on the outer membrane. No ruptures were revealed in the preparations studied. A change in the relative position of the nucleoid zone and thylakoids due to invaginations of the latter from the periphery to the central part occurred in the cytoplasm (Fig. 3.2b). Thylakoids in many spheroplasts looked intact (Fig. 3.2b, insert). However, in the presence of EDTA in the preparations, the cells where the thylakoids were swollen were observed more often. Importantly, the periplasmic space in such spheroplasts was connected with the intrathylakoid space with a visible link between the CM and the thylakoid membrane (Fig. 3.3). This fact for the same strain has been also noted in the work of Cosner and Troxler (Cosner and Troxler 1978). In addition, using labeled leucine, these authors showed that spheroplasts were metabolically active and capable of protein synthesis. In our opinion, the existence of a common periplasmic and intrathylakoid space in *Synechococcus* sp. 6301 cells is supported by the possibility of simultaneous enlargement (swelling) of the intermembranous regions of the cell envelope and the thylakoids, which was observed in the cells of the culture dying at increased cultivation temperatures (Nikitina et al. 1979). The connection between the thylakoids and the CM was also observed in another representative of the genus *Synechococcus* (strain 715) (Gromov and Mamkaeva 1976).

The connection between the thylakoid membrane and the CM and the formation of a single membrane system in cyanobacteria have been repeatedly discussed by the researchers who demonstrated various experimental data, including those obtained with the use of the modern method of electron tomography (Pinevich and Topchieva 1991; Pinevich 2006; Liberton and Pakrasi 2008; Gromov et al. 1983; Liberton et al. 2011). In recent years, evidence was presented in favor of the absence of obvious structural relationships between the CM and the thylakoid membrane, i.e., against the formation of the so-called membrane continuum and the integration of the periplasmic and intrathylakoid space (Liberton et al. 2006; van de Meene et al. 2006; Schneider et al. 2007, 2008). However, this evidence

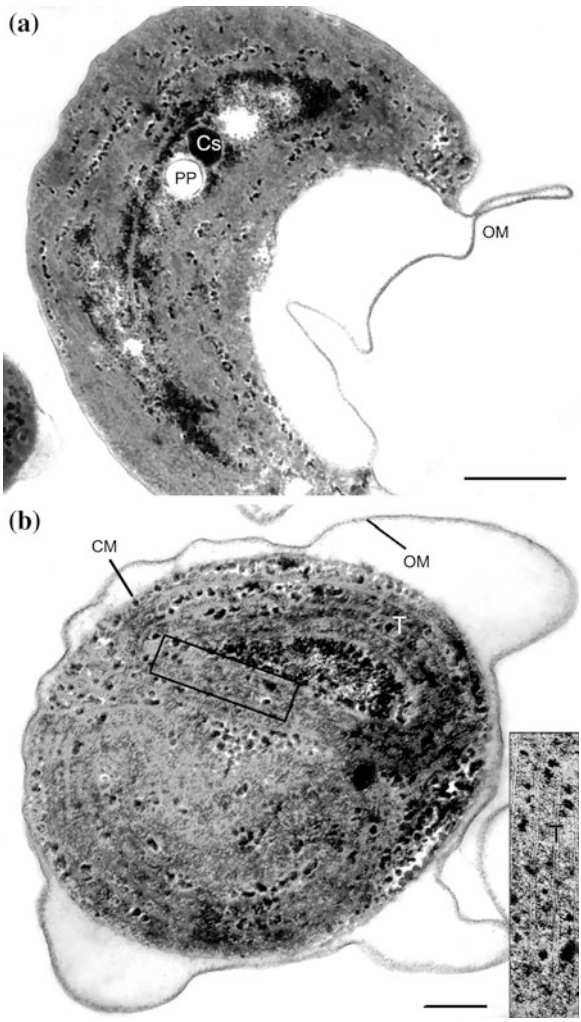

Fig. 3.2 *Synechococcus* sp. PCC 6301 spheroplasts obtained as a result of exposure to lysozyme (**a**, **b**) (TEM, data obtained by Baulina, Semenova and Mineeva). *Bottom right* magnified fragment of **b**. *Cs* carboxysome, *OM* outer membrane, *PP* electron-transparent region of polyphosphate granule localization, *T* thylakoid. *Scale bar* **a** 0.5 μm, **b** 0.2 μm

was obtained only using the unicellular cyanobacterium *Synechocystis* sp. 6803, currently the best studied in all respects. The results described in this chapter (see Fig. 3.3) showed the continuity of the CM and the thylakoid membrane, at least in *Synechococcus* sp. 6301. That is why this issue remains controversial.

Anabaena variabilis 458 grown in mineral medium or in the presence of sucrose formed spheroplasts (Fig. 3.4) similar in principle to the *Synechococcus* sp. 6301 spheroplasts. The spatial organization of the thylakoids and other intracellular components in different experimental variants was similar to that in intact cells. When mannitol or sucrose was selected as an osmotic stabilizer, the swelling thylakoids were not observed in the preparations. It is interesting to note that single cells with the canaliculi piercing the septum, which were similar to those of the

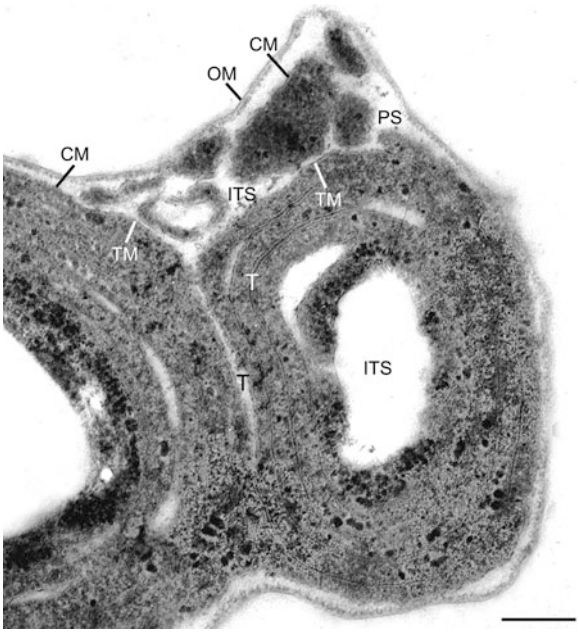

Fig. 3.3 Ultrastructure of the *Synechococcus* sp. PCC 6301 spheroplast where the swelling of thylakoids occurred. Obtained treatment with lysozyme in the presence of EDTA (data obtained by Baulina, Semenova and Mineeva). *ITS* intrathylakoid space, *OM* outer membrane, *PS* periplasmic space, *T*, thylakoid, *TM* thylakoid membrane. *Scale bar* 0.2 μm

same strain subjected to photooxidative destruction, occurred in the spheroplast preparations (see Chap. 2). In the cells of the initial culture, as was mentioned above, similar structures were also observed, although very rarely, e.g., in the case of autolysis of one of the neighboring cells in the filament (Fig. 3.5).

It is known from the literature and was noted in the previous chapter that *C. fritschii* is able to form populations with considerable morphological and ultrastructural heterogeneity under different growth conditions. Evidently, this is mainly with a result of a relatively complex life cycle of this cyanobacterium. Different cells in the population at the stage of intense growth are not equally responsive to lysozyme exposure. A certain part of them does not undergo any ultrastructural changes at all, neither do the two strains described above. It is important to note that the destruction of the cell wall by lysozyme does not necessarily entail the breakdown of the aggregates into single cells owing to the reinforcing sheath, which may also undergo destructive changes (Fig. 3.6). The size and shape of CWDF, as well as the density of the cytoplasm in aggregated cells, may vary, which is likely to be related to the varying permeability of the modified surface structures for osmotically active substances.

At the initial stages of peptidoglycan degradation, the periplasmic space was increased in volume unevenly and quite significantly. Peptidoglycan is always adjacent to the outer membrane, even in the cell wall fragmented due to ruptures (Fig. 3.7a), which was most markedly pronounced in the presence of EDTA in the incubation medium in the absence of magnesium cations. The distance between the outer membrane with a typical three-layer profile and the peptidoglycan layer

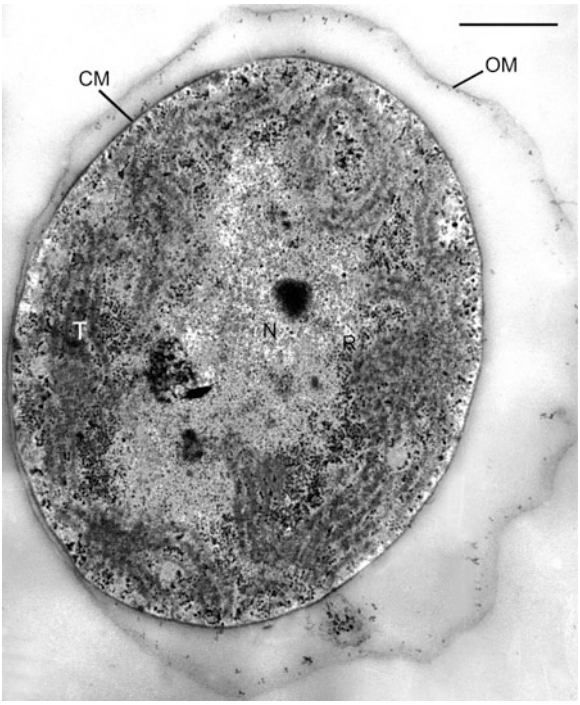

Fig. 3.4 Ultrastructure of the *Anabaena variabilis* CALU 458 spheroplast obtained as a result of lysozyme treatment (Agafodorova et al. 1982). *CM* cytoplasmic membrane, *N* nucleoid, *OM* outer membrane, *R* ribosomes, *T* thylakoid. *Scale bar* 0.5 μm

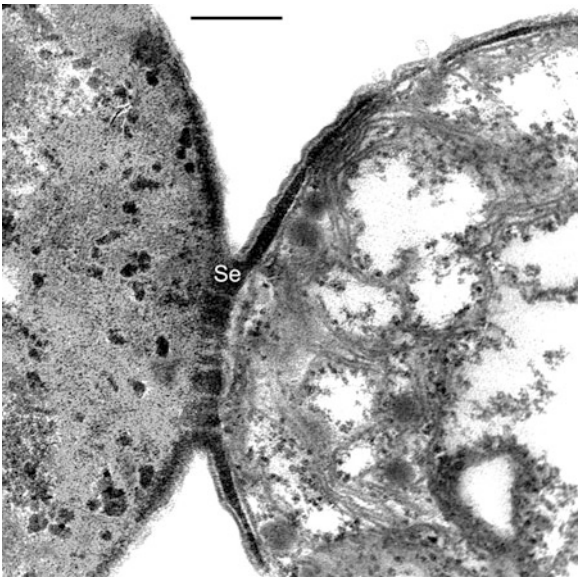

Fig. 3.5 Area in the region of the septum pierced by the canaliculi between the intact and lysed cells of the initial (before exposure to lysozyme) *Anabaena variabilis* CALU 458 culture (TEM). *Se* septum. *Scale bar* 0.2 μm

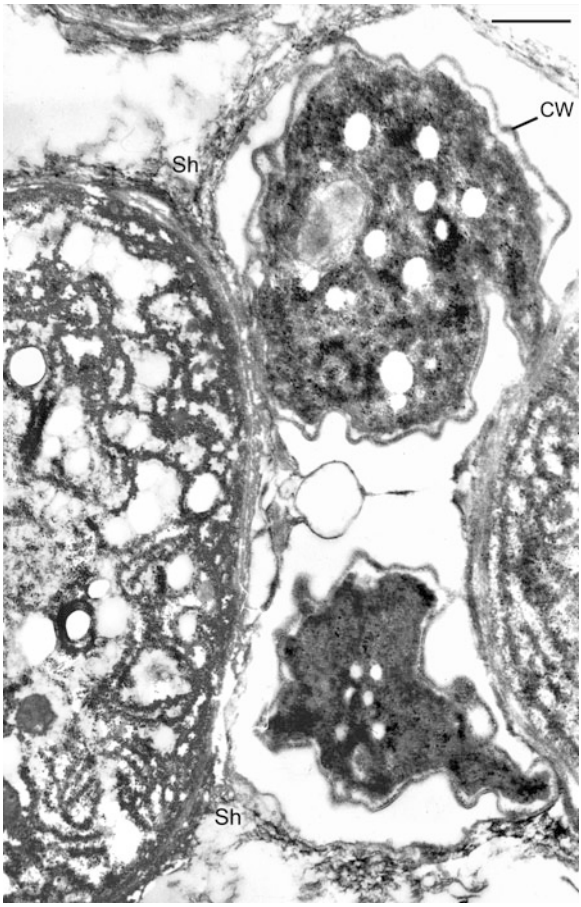

Fig. 3.6 Aggregated *Chlorogloeopsis fritschii* ATCC 27193 cells after exposure to lysozyme on the ultrathin sections (Gusev et al. 1982). *Sh* sheath, *CW* cell wall. *Scale bar* 0.5 μm

remained uniform (about 15 nm), as in the intact cells. The outer membrane with the peptidoglycan remnants sometimes flaked off from cell surface, resulting in the formation of protoplasts (Figs. 3.7a, b). This is a peculiarity of *C. fritschii* when compared with *Synechococcus* sp. 6301 and *A. variabilis* 458. Another distinguishing feature is that, in many cells with virtually complete peptidoglycan degradation, the outer membrane did not stretch and was in parallel to the CM at a distance corresponding to the thickness of the cell wall intact rigid layer (Fig. 3.8a). The sheaths could be lost due to the degradation, but in most cases, in the presence of magnesium cations the formation of CWRF—spheroplasts and protoplasts, occurred inside these structures. Both CWRF types were characterized by vesicle formation with the involvement of the outer membrane and the CM, respectively (Fig. 3.8b). An additional treatment with ultrasound (22 kHz for 30 s) resulted in a significant increase in the number of structurally integrity protoplasts with removed sheaths in the preparations (Baulina et al. 1984).

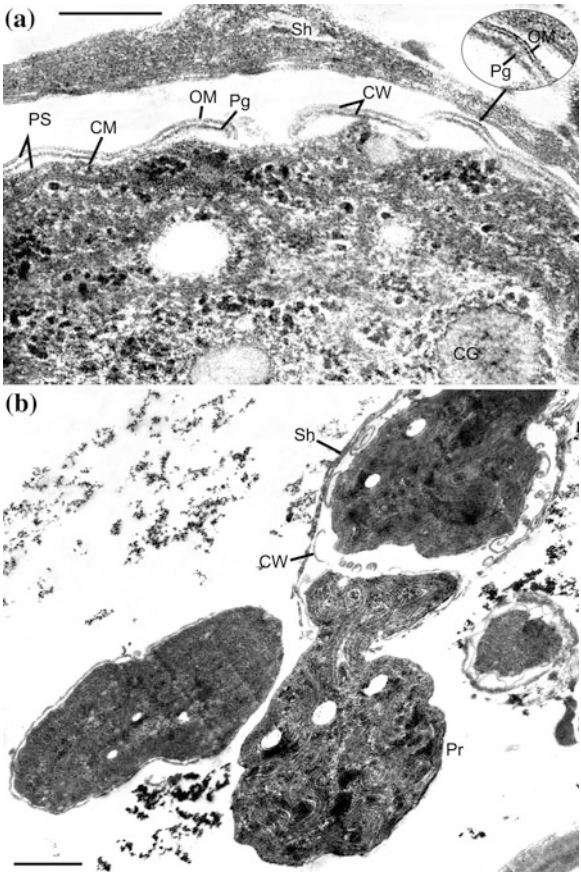

Fig. 3.7 Ultrastructure of *Chlorogloeopsis fritschii* ATCC 27193 after exposure to lysozyme: the cell area with destructive cell wall changes (**a**) and protoplast formation (**b**) (**a**, **b** data obtained by Baulina, Semenova and Mineeva). *CG* cyanophycin granule, *CM* cytoplasmic membrane, *CW* cell wall, *OM* outer membrane, *Pg* peptidoglycan, *Pr* protoplast, *PS* periplasmic space, *Sh* sheath. *Scale bar* **a**, 0.2 μm, **b** 1 μm

Thus, the ultrastructural changes in the cell wall of *C. fritschii* on exposure to lysozyme differed substantially from those of *Synechococcus* sp. 6301 and *A. variabilis* 458. The fundamentally different character of damage to the outer membrane of *C. fritschii* determined the possibility of obtaining the ultrastructurally intact protoplasts from the cells of this species. It was shown in the work of Pinevich that lysozyme treatment on addition of 5 mM of EDTA in *Tris* buffer resulted in the formation of true *A. variabilis* protoplasts. However, their internal structure was disrupted (Pinevich 1977). Evidently, complete degradation and the loss of the outer membrane under the conditions of experimental exposure to lysozyme (i.e., with a view to obtaining both protoplasts and spheroplasts) can lead to striking destructive changes of the latter in this species. A similar picture was also observed in the early works studying the possibility of obtaining *A. variabilis* 458 spheroplasts, in which the author of this book participated (Baulina et al. 1975). According to the data of other researchers published a decade later, over 75 % of viable *A. variabilis* 29413 spheroplasts were obtained (Berliner et al. 1987). Ultrastructurally, they were similar to the spheroplasts described in this

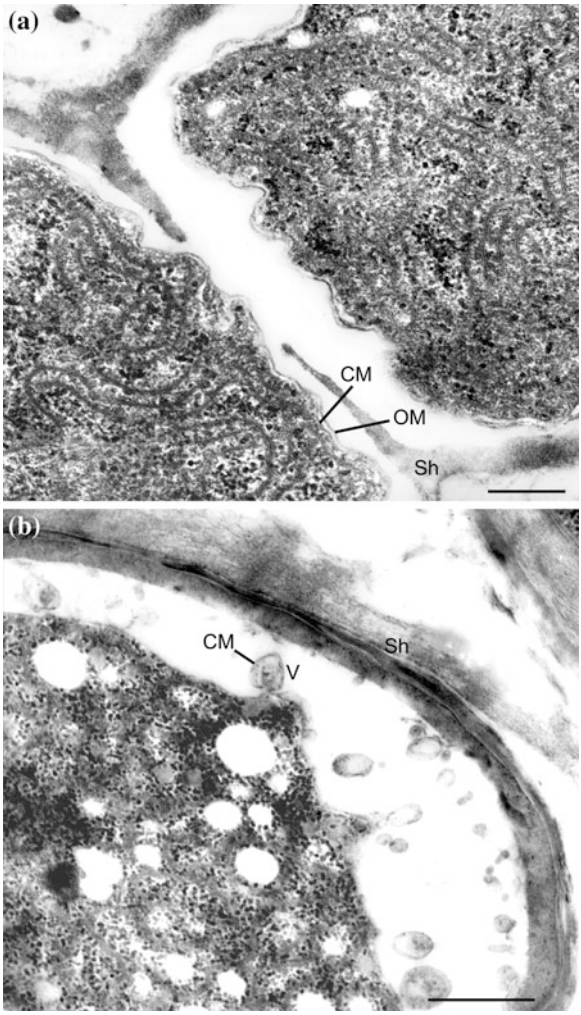

Fig. 3.8 Ultrastructure of the *Chlorogloeopsis fritschii* ATCC 27193 cell wall-reduced forms obtained treatment with lysozyme: the spheroplast region (**a**) and the protoplast region (**b**) (**a**, **b** data obtained by Baulina, Semenova and Mineeva). *CM* cytoplasmic membrane, *OM* outer membrane, *Sh* sheath, *V* vesicle. *Scale bar* **a** 0.2 μm, **b** 0.5 μm

chapter. The outer membrane was also retained when *Anacystis nidulans* (*Synechococcus* sp.) cells were treated with lysozyme (Lindsey et al. 1971; Gabriel 1977).

The ultrastructurally integral CWRF of all the three species of cyanobacteria characterized in this section were probably metabolically active. The rate of photosynthesis and oxygen uptake in the dark (indicators of energy processes), as well as the rate of incorporation of organic ^{14}C-compounds (an indicator of biosynthetic processes) of *Synechococcus* sp. 6301 and *A. variabilis* 458 cell forms in the preparations with a high spheroplast percentage were retained at the level of the intact cells (Semenova et al. 1982; Semenova 1983). At least some of the CWRF of all the three species were capable of reverting to the original state and

germinating on removal of lysozyme and transfer into the nutrient medium. At the same time, at the subsequent stages of induction of L-transformation, *Synechococcus* sp. 6301 spheroplasts were subjected to complete lysis, and the lysozyme-treated cell suspensions of the other two species were suitable for subsequent isolation (on agarized medium in the presence of lysozyme) of the colonies similar to those formed by bacterial L-forms (L-form-like colonies) (Gusev et al. 1981).

3.3 Ultrastructure of Cell Types in the L-Form-Like Colonies of Cyanobacteria

The L-form-like colonies of *A. variabilis* 458 (passage II), along with unbroken cells, contained the CWDF, often plasmolysed and usually surrounded by deposits of a homogeneous substance, which resembled pressed sawdust or villi. In some cases, the presence of such deposits, unusual for cyanobacteria, correlated with formation of the outer membrane surface protrusions limiting the clumps of electron-dense substance merging with peptidoglycan (Fig. 3.9). For comparison, it is interesting to demonstrate in this context the similar formations we revealed in the *C. fritschii* cell wall under the optimal growth conditions (Fig. 3.10) (Gusev et al. 1982). Thickening of the peptidoglycan layer and the protrusion of the outer membrane in this region are the data which should be used to confirm the recently developed molecular models of the mechanism of formation of the membrane vesicles in gram-negative bacteria(Mashburn-Warren and Whiteley 2006). The scheme for model 2 from the cited paper, which implies accumulation of peptidoglycan in the local regions of the periplasmic space, absolutely corresponds to the state of peptidoglycan and the outer membrane shown in Fig. 3.10. In *C. fritschii,* formation of such kind of membrane vesicles and their localization in the sheaths, especially in the region of contacts between the neighboring cells, is observed very often. It can not be excluded that both patterns shown in Figs 3.9 and 3.10 demonstrate the initial states of transport of the extracellular substances which are polymerized in the periplasmic space with the involvement of the CM. In the series of works pertinent to the early period of the electron microscopic studies of cyanobacteria, the data indicating secretion of the MSS material (sheath, capsule, or amorphous mucus) by means of the vesicles formed with the participation of the outer membrane were obtained [(Lamont 1969; Butler and Allsopp 1972); etc.] The vesicles migrating into the forming sheath, especially in the vicinity of the cell septum, were observed in cyanobacteria *Hammatoidea simplex* (Gromov 1976) and *Mastigocladopsis repens* (Merino et al. 1994). Among the cyanobacteria studied in these works, there are representatives of the family *Stigonemataceae* (subsection V) to which *C. fritschii* is also assigned. The possibility of secretion of the components of the intercellular mucous matrix by producing the vesicles formed by the cell wall outer membrane was also established for the CWDF of the symbiotic cyanobacteria *Nostoc* sp. f. *Blasia* forming

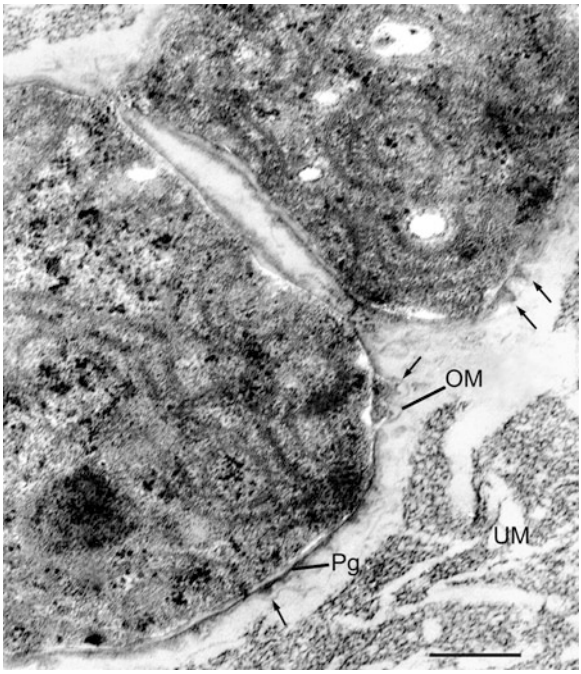

Fig. 3.9 *Anabaena variabilis* CALU 458 cells in an L-form-like colony. The cell wall ultrastructure indicates the formation of transport vesicles. *OM* outer membrane, *Pg* peptidoglycan, *UM* unidentified matter. The *arrows* indicate the forming vesicles. *Scale bar* 0.2 μm

in the culture on long storage in the dark at low temperatures (Gorelova and Baulina 2009). In the case of other gram-negative bacteria, it was confirmed that the outer membrane was capable of forming the vesicles, which act as a vehicle between bacterial cells, for toxins, various enzymes, and other substances, including the peptidoglycan fragments—muramyl peptides (Mayrand and Grenier 1989; Pinevich 2006), as well as DNA, and are components of the intercellular matrix in biofilms (Mashburn-Warren and Whiteley 2006; Mashburn-Warren et al. 2008). The data have been accumulated suggesting that such vesicles are formed by virtually all gram-negative bacteria. As a rule, the vesicles are filled with the products accumulating in the periplasm. They are transported to the extracellular space can reach other cells, both prokaryotic and eukaryotic, and merge with their surface membranes (Beveridge 1999). Investigation of the mechanism of formation and transport of the vesicles formed by the outer membrane as a mode of secretion of high-molecular weight substances from the cells of gram-negative bacteria (along with other known modes) is, undoubtedly, important for the understanding of their physiology. In particular, this issue is crucial for the study of cyanobacteria, since they are producers of various high-molecular weight compounds, including those that affect other organisms (Fedorov and Karaush 1974; Bateman et al. 1999; Sivonen and Börner 2008).

We did not succeed in revealing CWRF when studying the L-forms-like *A. variabilis* 458 colonies. Occasionally, we observed protoplast-like forms with thylakoids and a homogeneous, dense, fine-grained cytoplasm, but no CM was

Fig. 3.10 Ultrastructure of *Chlorogloeopsis fritschii* ATCC 27193 under the optimal conditions of growth in the light (Gusev et al. 1982). α glycogen α-granules, *CM* cytoplasmic membrane, *N* nucleoid, *OM* outer membrane, *Pg* peptidoglycan, *R* ribosomes, *Sh* sheath, *T* thylakoid. *Scale bar* 0.5 μm

revealed in them. Many cell forms in such *A. variabilis* 458 colonies were lysed. When lytic processes involved the cells with peripheral deposits of the above-mentioned substance (villi), it was also revealed inside the destroyed cells between the thylakoids remnants (Fig. 3.11).

Thus, the ultrastructural studies did not identify the typical protoplasts or spheroplasts, which could be attributed to the L-forms at the early stages of stabilization in the *A. variabilis* 458 culture transferred in the presence of lysozyme.

On the contrary, such CWRF were found when the *C. fritschii* L-form-like colonies of the 1st, 2nd, and 8th passages were studied (Gusev et al. 1983). In the first passage, we found the spheroplasts surrounded by thick sheaths consisting of two components, fibrillar, and homogeneous, as is characteristic of *C. fritschii.* A distinctive feature of the spheroplasts was that the outer membrane and the CM were close together and their outlines coincided exactly (Fig. 3.12). At the same time, only one membrane was revealed at certain areas of their surface. An important property of these CWRF was abundance of ribosomes, while the nucleoid zones were not markedly pronounced. It is possible that these cell forms are unstable L-forms of the spheroplast type, similar to those observed in other gram-negative bacteria under L-transformation conditions in vitro (Prozorovsky et al. 1981). However, such CWRF were in the minority in the first passage preparations, and the lysis-undergoing spheroplasts and protoplasts forming

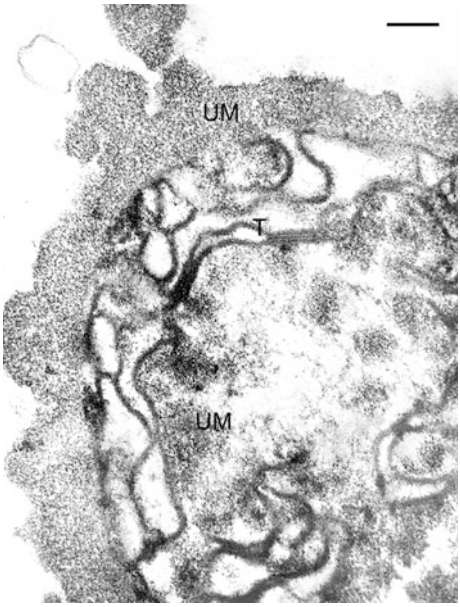

Fig. 3.11 Lysis-subjected cellular form with deposits of material of unidentified nature in an L-form-like *Anabaena variabilis* CALU 458 colony (TEM). *T* thylakoid, *UM* unidentified matter. *Scale bar* 0.2 μm

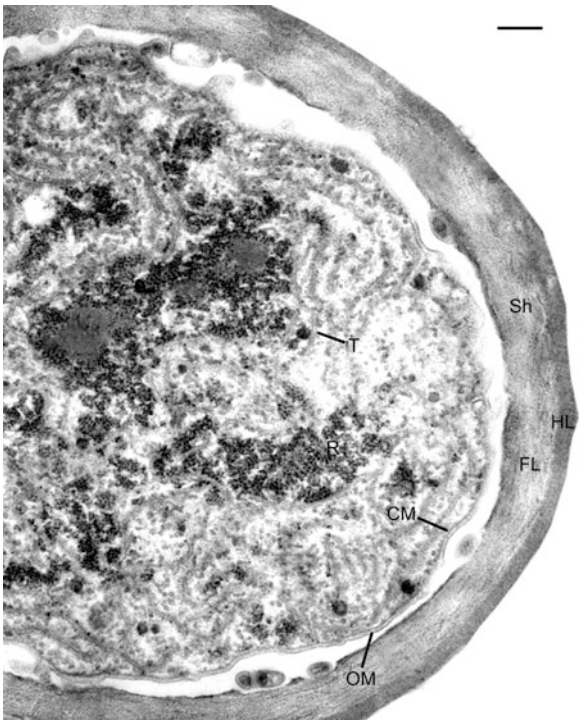

Fig. 3.12 Ultrastructure of the *Chlorogloeopsis fritschii* ATCC 27193 spheroplast in a first-passage L-form-like colony (Gusev et al. 1983). *CM* cytoplasmic membrane, *FL* fibrillar layer, *HL* homogeneous layer, *OM* outer membrane, *R* ribosomes, *Sh* sheath, *T* thylakoid. *Scale bar* 0.1 μm

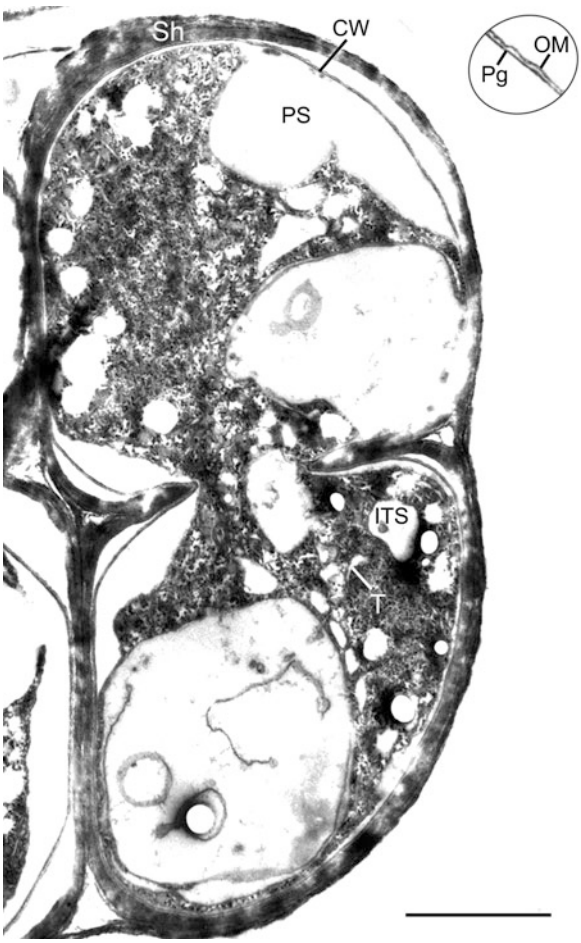

Fig. 3.13 Cell forms of *Chlorogloeopsis fritschii* ATCC 27193 with a changed ultrastructure in a second-passage L-form-like colony (TEM) (Gusev et al. 1983). *CW* cell wall, *ITS* intrathylakoid space, *OM* outer membrane, *Pg* peptidoglycan, *PS* periplasmic space, *Sh* sheath, *T* thylakoid. *Scale bar* 0.5 μm

numerous 80-nm vesicles filled with material predominated. In some of these disintegrated CWRF, the thylakoids consisting of the closely located membranes with a three-layer profile, as in the intact cells, were well-preserved.

In the second passage, along with the spheroplasts, unbroken and lysed cells, we succeeded in observing the forms which did not occur earlier. Those were the cells with the wall consisting of the outer membrane and the peptidoglycan layer, but with a substantially restructured cytoplasm (Fig. 3.13).

The periplasmic space in the region between peptidoglycan and the CM expanded, probably due to plasmolysis, forming extensive pseudovacuoles. The latter also were probably formed with the participation of the intrathylakoid space. Such cells resembled the finger ring-shaped cells observed in the L-form-like *C. fritschii* colonies by phase-contrast microscopy (Gusev et al. 1981), and were characteristic elements of bacterial colonies in the process of L-transformation.

On subsequent cultivation (passages 5–8), there were almost no colonies of *C. fritschii* formed by the cells characteristic of the initial culture in the dishes, whereas growth of the L-form-like colonies was observed with a naked eye after 5–6 days (Gusev et al. 1981). As a rule, such colonies contained aggregates of CWDF with a varying degree of cell wall degradation up to its complete reduction), which shared a common sheath (Figs. 3.14 and 3.9). The aggregated CWDF were fundamentally different in morphology from the spheroplasts of the first and second passages. Due to the loss of rigidity by peptidoglycan, which was thinner and looser than in the intact cells, the CWDF acquired the stellate or amoeboid shape (Fig. 3.14a). The cytoplasm contained all the main components typical of the intact cells. The CWDF were characterized by active formation of the transport vesicles formed by the outer membrane, which penetrated into the sheath and further into the intercellular matrix of the colony (Fig. 3.14b).

Some of the CWDF aggregates, along with the CWDF described, contained protoplasts, which were also surrounded by sheaths (Fig. 3.15). This fact, being fundamentally important confirmation of the possibility to obtain cyanobacteria as L-forms of the protoplast type, also confirmed the earlier idea of the probability of spontaneous emergence of the latter, for example, on transition to chemoheterotrophic metabolism [see Chap. 2; (Baulina et al. 1978)]. In such protoplasts, the CM was revealed more distinctly than in the intact cells. This might be associated with a change in its chemical composition in the absence of the cell wall. In this case, we may suggest activity of the adaptation mechanisms, which change the CM macromolecular organization in a way similar to those in L-transformation of bacteria (see Sect. 3.1).

One of the characteristic properties of bacterial L-forms is the reorganization of the intracellular membrane apparatus (Kats 1980). Typical thylakoids were no longer revealed in the *C. fritschii* protoplasts described in this chapter (see Fig. 3.15). In our observation, vesicles are likely to form in the protoplast cytoplasm during thylakoid degradation. This process is, in principle, reversible as it is known that, in cyanobacteria, thylakoids may be resynthesized from the membrane remnants and pigments (Schmetterer et al. 1983). It is difficult to reach an unambiguous conclusion concerning the reproductive capacity of L-form-like cyanobacteria. At the same time, the viability of L-form-like colonies is confirmed by the fact of their regular formation when they are transferred under the conditions of increasing lysozyme concentration, as well as by the possibility of their reversal to the colonies formed by the original cells upon transfer onto the lysozyme-free nutrient media (Gusev et al. 1981).

3.4 Concluding Remarks

It was established for three species of cyanobacteria: *Synechococcus* sp. (strain PCC 6301), *A. variabilis* (strain CALU 458), and *C. fritschii* that the ultrastructural plasticity under the conditions of enzymatic removal of peptidoglycan manifested

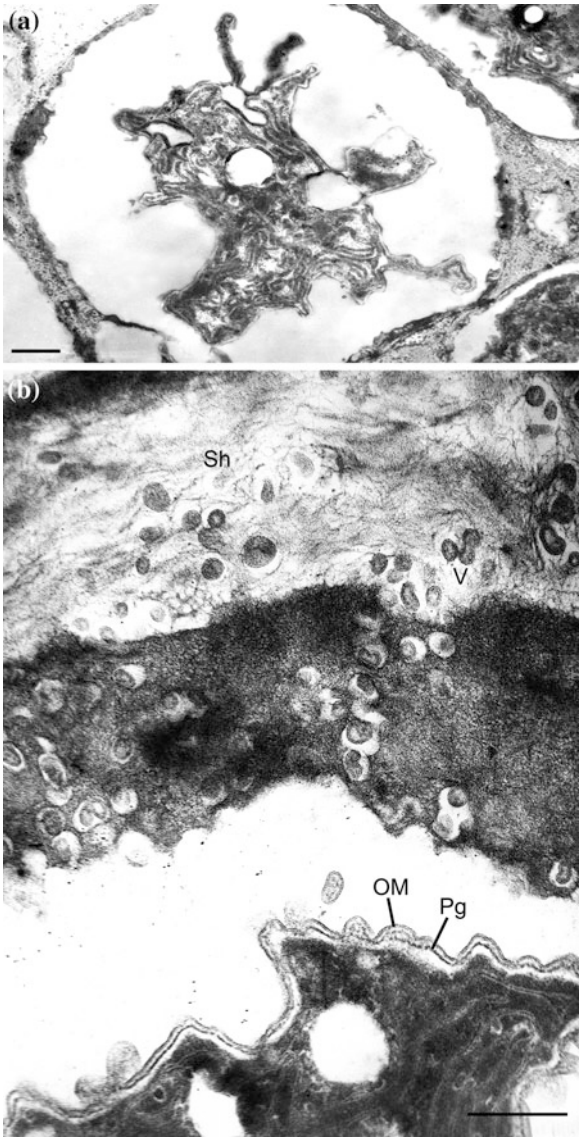

Fig. 3.14 Cell wall-defective forms of *Chlorogloeopsis fritschii* ATCC 27193 in an L-form-like colony, 8th passage: general view of CWDF as part of the aggregate on an ultrathin section (**a**) and the area of a CWDF aggregate with transport vesicles (**b**) (**a**, **b**, data obtained by Baulina, Semenova and Mineeva). *OM* outer membrane, *Pg* peptidoglycan, *Sh* sheath, *V* vesicle. *Scale bar* **a** 0.5 μm, **b** 0.2 μm

itself as the property of the cells to retain their structural integrity in the partial or complete absence of the cell wall. At the subcellular level, the ultrastructural plasticity is manifested by formation of vesicles (which presumably have a transport function) by the outer membrane of the *A. variabilis* 458 and *C. fritschii* CWDF. The marked conservatism of the structural organization of *Synechococcus* sp. 6301 cells when the spheroplast preparation was obtained should be noted. Considering the possibility of the ultrathin sections passing both longitudinally and transversely in the cell, it may be concluded that the initially rod-shaped *S.* cells, at

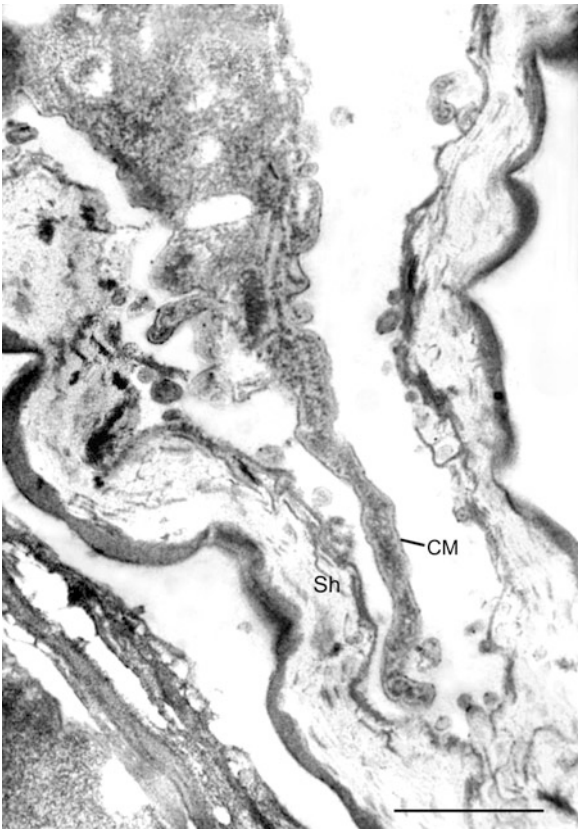

Fig. 3.15 Region of a sheath-surrounded *Chlorogloeopsis fritschii* ATCC 27193 protoplast in an L-form-like colony, 8th passage (TEM, data obtained by Baulina, Semenova and Mineeva). *CM* cytoplasmic membrane, *Sh* sheath. *Scale bar* 0.5 μm

least, many of them, did not become rounded, but assumed a curved or bean-like shape. In so doing, the architecture and the relative arrangement of the nucleoid, ribosomes, and thylakoids remained virtually unchanged, with the only exception that the latter invaginated into the central part of the cell. The outer membranes of *Synechococcus* sp. 6301 and *A. variabilis* 458 stretched considerably without rupture. The ultrastructural changes in the *C. fritschii* cell wall on exposure to lysozyme differed from those of these two species mainly, because the outer membrane virtually did not stretch and ruptured in some of the cells. The latter determines the possibility of obtaining the ultrastructurally integral protoplasts from the cells of this species.

These data indicate the action of the mechanism of L-transformation when transferred culture of *C. fritschii* developed for a long time under continuous presence of lysozyme. Formation of CWDF, including spheroplasts and protoplasts, occurring inside the complexly organized sheaths is a peculiar feature of L-transformation in this species. Dynamic interactions involving the vesicles which transport the material synthesized by CWDF seem to occur between CWDF and their sheaths. The ultrastructural reorganization of those cell forms which

remained unbroken and were not subjected to lysis correlated with the survivability of *C. fritschii* under the conditions of continuous lysozyme exposure during eight passages and the possibility of reversion of the L-form-like colonies to the initial state. Since the fact of reversion was established at the population level, regeneration of the cell wall in the protoplasts remains an open issue. The ultrastructural changes of the cell wall described in Sect. 2.2.4 and manifesting themselves in the discontinuity of the peptidoglycan layer and in the alternation of the sites where the cell wall (including the outer membrane) is formed and the sites where it is absent (see Fig. 2.25c) are the only data confirming this possibility. However, no similar picture was observed in the experiments with lysozyme exposure. The literature data suggest that the reversion occurs most probably in the spheroplasts. At the same time, the importance of the discovery of protoplasts in the transferred *C. fritschii* colonies is due to the fact that this phenomenon indicates the ability of cyanobacteria to produce the L-forms of the protoplast type, presumably, and stable ones.

Thus, electron microscopic study of the dynamics of cell changes upon the enzymatic induction of L-transformation in the model systems in vitro using three species of cyanobacteria with a different set of metabolic possibilities showed that, in accordance with diverse metabolism and complex structural organization, it is *C. fritschii* that exhibited the most adequate reaction and marked ultrastructural plasticity. Under the same conditions, *A. variabilis* 458 revealed only the signs of transition to the phase of unbalanced growth. *Synechococcus* sp. 6301, despite the metabolic activity of the initially obtained spheroplasts, appeared to be unable to reorganize for long-standing existence as the CWDF.

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Chapter 4
Ultrastructural Plasticity of Cyanobacteria in Model Associations with Plant Partners

Abstract This chapter contains the description of ultrastructural plasticity manifestations in laboratory maintained strains of cyanobacteria with different metabolic traits during formation and functioning of model associations with cells, tissues, regenerated plantlets, and whole plants non-symbiotrophic species. A wide array of examples supporting the tight coupling of the diversity of ultrastructural plasticity manifestations of sub-cellular, single-cell, and cell population levels with metabolic potential of the cyanobacteria studied and their capability of forming association with plants. Special attention is paid to the description of the structure of stable model association of plant partners with free living *Nostoc muscorum* strains. These systems, as well as natural symbioses of *Nostoc* sp. with higher plants, are characterized by the maximum manifestation of ultrastructural plasticity of cyanobacteria. The extent of the manifestations correlates with the capability of a given species to form symbiotic associations with plants.

4.1 Model Associations of Cyanobacteria with Plant Partners

Investigation of both natural cyanobacterial–plant symbioses and the model associations of these microorganisms with plant partners provided new possibilities for the study of adaptive capacities of cyanobacteria, especially within the framework of the concept of the evolutionary origin of the chloroplasts. Research on such model systems is a high-priority field in symbiology. Available data demonstrate that both symbiotic and free-living species of cyanobacteria are capable of associated growth with plant tissues of various taxonomic position and level of organization (see reviews: (Korzhenevskaya et al. 1986a, 1993; Gusev and Korzhenevskaya 1990; Rai et al. 2000; Gusev et al. 2002). Creation of new symbioses between plants and nitrogen-fixing cyanobacteria is important for investigation of the mechanisms of formation of the natural cyanobacterial–plant symbioses. This work is related to the problem of increasing the share of the

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economically advantageous and environmentally pure biological nitrogen in plant nutrition. The methods for obtaining the artificial associations, many of which proved stable (see examples in Table 4.1) use isolated protoplasts, suspensions of dedifferentiated cells, callus tissues, organs, regenerated plantlets, or whole plants as initial plant partners (Gusev et al. 1980, 1982, 1983, 1986; Butenko et al. 1982; Agafodorova et al. 1982a, b; Korzhenevskaya et al. 1984, 1993, 1985a, b, 1986a; Gorelova et al. 1984; 1995; Lobakova et al. 1984 ; Pivovarova et al. 1985; Yagodina et al. 1990; Skripnikov 1987; Korzhenevskaya 1989, 1990). The species and strains for which the physiology and ultrastructural plasticity under various conditions have been previously studied, namely, *Synechococcus* sp. 6301, *A. variabilis* 458, and *C. fritschii* (see Chaps 2, 3) were the first to be tested as cyanobacterial partners. They were used in order to determine whether nonsymbiotic laboratory strains of different metabolic properties could form stable symbiotic associations with plant partners. For example, the obligate phototroph *Synechococcus* sp. 6301 was used in a model of the autotroph–heterotroph relationships (Gusev et al. 1982). Moreover, members of the genera *Synechococcus* and *Anabaena* are components of natural symbioses [see review (Schenk 1992)], while *Chlorogloeopsis* strains isolated from soil were able to reconstitute the symbioses with mosses (West and Adams 1997).

Formation and activity of artificial associations require spatial integration of the partners into an anatomic system of a new level, with compartmentation of cyanobacteria inside or on the surface of a plant partner [see reviews (Gusev and Korzhenevskaya 1990; Gusev et al. 2002; Gorelova 2005)]. The cell walls and membranes of the plant and the intercellular or surface mucous matrix which is usually produced in the course of formation of the integrated system are responsible for compartmentation. The plant partner plays the major role in the limitation of available space (habitat) for associated cyanobacteria. Apart from the surface areas specific for each system, in associations with dedifferentiated plant cells (suspension and callus cultures), regenerated plantlets, and whole plants, cyanobacteria also colonize intercellular spaces, cell wall-delimited volumes of the dead cells, vessels, and the air-bearing stoma cavities. Model associations of cyanobacteria with plant partners listed in Table 4.1 are an example of such systems.

Importantly, in suspension cultures, the cyanobacteria localized on the surfaces and inside the plant aggregates may contact directly with the metabolically active plant cells. For example, such biologically active compounds of plant origin as panaxosides (Butenko et al. 1979; Lobakova et al. 1984) and diosgenin and yamogenin (Yagodina et al. 1990) were detected in mixed cultures with ginseng and dioscorea, respectively. The author and other researchers observed that, unlike suspension associations, in mixed calluses and infected plants cyanobacterial microcolonies within plant tissues are surrounded by the walls of dead plant cells, which form harbor in intact plant tissues (Pivovarova et al. 1986; Gorelova 2000b). The conditions created by plant cells and tissues are evidently quite different from those of laboratory cyanobacterial cultures. Since successful formation of associations required cultivation using the media for the plant partners, which were modified depending on the specific characteristics of the systems, these differences became

Table 4.1 Model associations of cyanobacteria with plant partners

Cyanobacterial species and strains	Plant species and strains	Duration of association growth (see references, p. 90)
Mixed suspension cultures		
Synechococcus sp. Näg. PCC 6301 (*Anacystis nidulans*)	*Nicotiana tabacum* L. Wisconsin-38 variety	1–1.5 months with transfers (2–3 transfers[a])
Synechococcus sp. PCC 6301	*Dioscorea deltoidea* Wall. IFR D1	2 weeks—1 month with transfers (1–2 transfers)
Synechococcus sp. PCC 6301 (mutant bio-2 1142)[b]	*Nicotiana tabacum* Wisconsin-38 variety	2 months with transfers (4 transfers)
Chlorogloeopsis fritschii Mitra et Pandy ATCC 27193	*Panax ginseng* C. A. Mey IFR ZH1 (ginseng)	2 years with transfers
Mixed callus cultures		
Anabaena variabilis Kütz. CALU 458	*N. tabacum* Samsun variety[c] (from protoplasts)	2 years with transfers
A. variabilis Kütz. ATCC 29413	*N. tabacum* Samsun variety (from leaf mesophyll)	2 years with transfers
A. variabilis ATCC 29413	*Medicago sativa* L. (alfalfa)	5.5 months with transfers
Nostoc muscorum Agardh. VKM 16	*M. sativa*	18 months with transfers
Regenerated plants from mixed callus cultures		
A. variabilis CALU 458	*N. tabacum* Samsun vaiety (from protoplasts)	1,5–2 months
A. variabilis ATCC 29413	*N. tabacum* Samsun vaiety (from leaf mesophyll)	2–3 months
A. variabilis ATCC 29413	*M. sativa*	9 months
Inoculated rooted cuttings of whole plants		
N. muscorum VKM 16	*M. sativa*	1,5 months

[a] Further transfers of associations with *Synechococcus* sp. strains were impossible
[b] Biotin-dependent mutant bio-2 strain 1142 (Herdman and Carr 1972) obtained from S.V. Shestakov (Moscow State University)
[c] Here and further on the poecilophyllous plastomic mutant (von Wettstein and Eriksson 1965) used to facilitate the visualization of blue-green cyanobacterial colonies

even more significant. In vitro cultivated plants, as well as plant cells and tissues, are known to require both the nutrients (e.g., sucrose, potassium, and sodium) and the physicochemical conditions of cultivation differ from those preferred by cyanobacteria. The media used to obtain associations do not favor cyanobacterial growth without the plant partner, i.e., in monocultures. In a series of works on formation and functioning of the model associations of plants and cyanobacteria, changes in cyanobacterial physiology in these systems were demonstrated [see review (Gusev and Korzhenevskaya 1990; Gusev et al. 2002; Gorelova 2005)].

The application of model associations is therefore a new and promising approach for comparative investigation of the ultrastructural plasticity of various cyanobacterial species in a broader range of their adaptive abilities under unique conditions. In this respect, model associations of plant objects with nonsymbiotic laboratory strains of cyanobacteria are preferable to the naturally occurring symbioses, since they make it possible to investigate, apart from the evolutionally

formed behavior of symbiotic cyanobacteria during infection and subsequent development, also the phenotypic plasticity of bacterial partners in response to the changed conditions.

4.2 Ultrastructure of Cyanobacteria in Model Associations

Investigation of a range of model associations revealed that in these systems cyanobacteria suffered structural and functional transformations, which in some cases were significant (Gusev et al. 2002). Depending on the species used to obtained associations, various options of cell differentiation may be realized, such as formation of motile hormogonia (Gorelova et al. 1995), nitrogen-fixing heterocysts (Korzhenevskaya et al. 1984; 1985a, b; Gorelova and Kleimenov 2003), and, less often, of dormant akinetes (Gorelova 2000b). Moreover, some of the vegetative cells may exhibit the ultrastructural changes of various macromolecular complexes, structural components, and organelles, including MSS, cell wall, and thylakoids. In this respect, as well as in cell morphology, associated cyanobacterial populations are heterogeneous. Investigation of diverse model systems (see Table 4.1) makes it possible to analyze these changes in various species and strains of cyanobacteria in the dynamics of development of associated populations.

4.2.1 Synechococcus *sp. PCC 6301 in Associations with Tobacco and Dioscorea Cells*

The morphological and ultrastructural uniformity of most of the cells in laboratory pure cultures of *Synechococcus* sp. 6301 makes this species a good choice for investigation of the structural manifestations of phenotypic plasticity at the subcellular, cellular, and population levels under associated growth with plant cells. Morphology and ultrastructure of the wild-type S and its biotin-dependent mutant[1] *Synechococcus* sp. 6301 in mixed suspension cultures with tobacco and dioscorea cells was investigated (Baulina et al. 1988b, 1989b, 1994).

Both strains grown in mixed suspension cultures with tobacco cells exhibited morphological differences from the cyanobacteria in pure cultures (Fig. 4.10a). The morphological plasticity was more pronounced in the biotin-dependent mutant. The increase in cell length was especially pronounced (10 times), from 3–4 μm in pure cultures to ~37 μm in associations. After 5 days of cultivation, changes in cell length were observed in 73 % of the single cyanobacterial cells not adsorbed on tobacco cells; on the 16th day, they constituted 94 %. Moreover, cell

[1] All experiments with the mutant were carried out in the medium supplemented with biotin, since in its absence this biotin-dependent cyanobacterium did not form associations.

shape also changed. The cells became sinuous with characteristic bent ends, the surface became uneven and wavy (Fig. 4.1a). In the monoculture of the mutant grown in the medium for associations, which contained sucrose as a component required by the plant partner, lengthening of the cells (up to 20 and 32 % of the total cell number on the 5th and 16th day of cultivation, respectively) was the only morphological change observed, while the cells themselves usually remained straight rods with smooth surface. Wild-type cyanobacteria grown in association with tobacco cells also developed bends in the surface resulting in the changed cell shape. The cells also became elongated, although to a lesser degree. No elongation to the extent of the mutant cells, accompanied by characteristic curling of the cell ends, was observed.

Cell elongation was probably associated with discoordination of the processes of growth (biomass increase), DNA replication, and cell division proper (nucleoid segregation and formation of the septa). In this case, the morphological plasticity was the result of unbalanced growth. Amino acids present in the medium or those excreted by plant cells were the possible factors inducing unbalanced growth. Sucrose, a necessary component of the media for associated cultivation, was probably another factor with the same effect. Significant cell elongation with a decrease in the number of dividing cells was observed in the culture of the wild-type strain supplemented with ribose (Baulina et al. 1981) or after prolonged adaptation to growth in the presence of sucrose (Semenova et al. 1984). Moreover, cell elongation (2.5–3 fold) was observed in *Synechococcus* sp. 6301 at elevated or decreased temperature of cultivation (Nikitina et al. 1979). A similar change in morphology observed in bacillary forms of bacteria transferred into fresh nutrient medium is interpreted as an active process of phenotypic adaptation (Waisman 1984).

The mutants of *Synechococcus* sp. PCC 7942 were obtained by transposon mutagenesis, which were 20–100 times longer than the cells of the wild type (Koksharova and Wolk 2000, 2002). Sequencing of the mutant genes *ftn2* and *ftn6* revealed no homology with the known *fts* genes responsible for cytokinesis in various bacteria (mutations in the latter genes result in filamentous phenotypes *Escherichia coli*) (Bramhill 1997). Although the mutants of *Anabaena* PCC 7120 (Kasuza) in the genes homologous to *ftn2* and *ftn6* obtained by Koksharova and Wolk were also capable of cell elongation, it was insignificant (seldom up to twofold) compared to that of the wild type. It may be therefore concluded that capacity for significant cell elongation is a characteristic feature of cyanobacteria of the genus *Synechococcus*.

Monocultures of the wild-type strain of *Synechococcus* sp. 6301 on media for associations with tobacco and dioscorea cells developed numerous appendages (Fig 4.1b–d) similar to the sharpened spines of other bacteria (Easterbrook et al. 1973; Gromov 1985). In *Synechococcus* sp. 6301, the spines were different from the straight tubular appendages typical of marine unicellular cyanobacteria of the genus *Synechococcus*, which were termed spines (Perkins et al. 1981; Sarokin and Carpenter 1981). Spine-like surface structures were found in the epiphytic *Synechococcus* sp.strain, which, together with filamentous cyanobacteria, formed epibioses on the thallus of marine brown algae (Moskvina 2000). Importantly, in

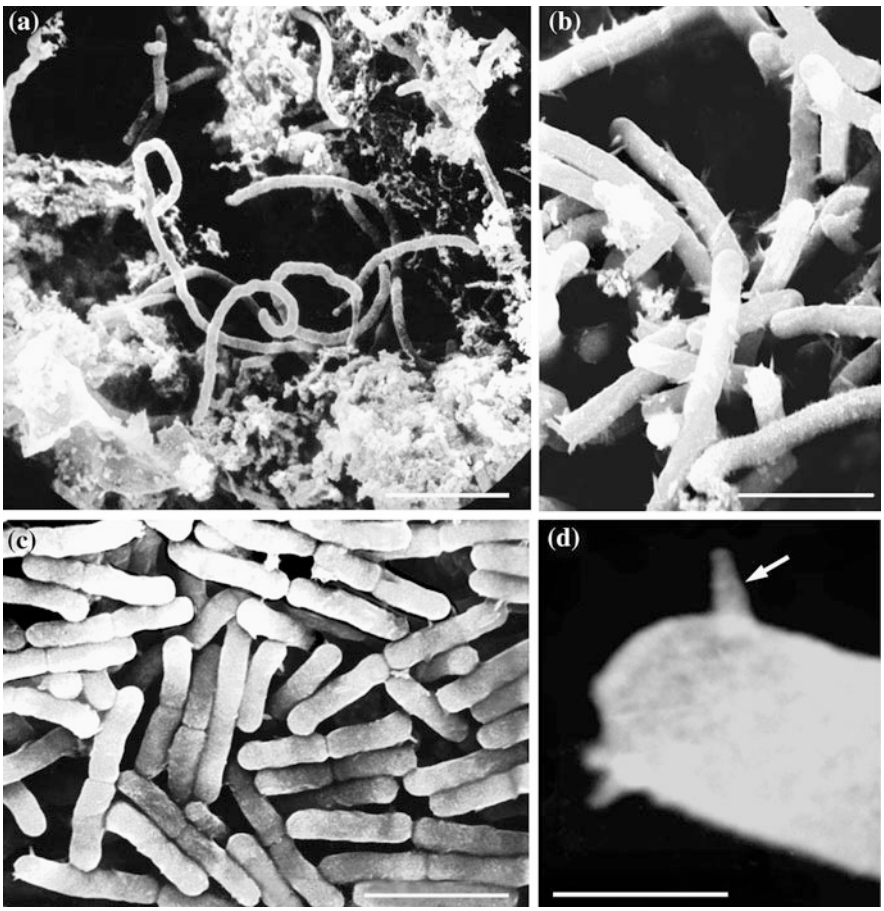

Fig. 4.1 Morphology of *Synechococcus* sp. PCC 6301 in experiments on creating associations with plant cells, SEM: biotin-dependent mutant in association with tobacco cells (**a**), wild-type strain in monoculture in the medium for associations with tobacco cells (**b**) (Baulina et al. 1994), wild-type strain in monoculture in the medium for associations with *dioscorea* cells (**c**, **d**, data obtained by Baulina, Yagodina and Korzhenevskaya). *Arrow* points to spine. *Scale bar* a, 10 μm; b, c, 5 μm; d, 1 μm

these associations cyanobacteria were closely attached to the thallus tissue and were not washed off by water.

Investigated by SEM, the spines in the *Synechococcus* sp. 6301 monoculture on the medium used for associations were 0.5–0.5 μm long, with a broadened base. At the base, the diameter was ∼180 nm, while nearer to the tip, ∼80 nm. Spines in bacteria are considered a manifestation of unfavorable growth conditions (Gromov 1985). This is the case when cyanobacteria are grown in specialized media for associations, which are not optimal for components of the system. No spines were found in mixed cultures with plant cells, where conditions were more

favorable for cyanobacteria than in monocultures, or in the control pure cultures of *Synechococcus* sp. 6301 (see Fig. 4.10a). Thus, formation of spines on the cell surface may be considered a manifestation of ultrastructural plasticity at the cellular level. There is, however, no direct evidence of participation of these structures in the maintenance of cyanobacterial activity under unfavorable conditions.

Electron microscopy of freeze-fractioning preparations of the same strain revealed the presence of pili (Ratner et al. 1976). These surface structures have been presently found in a limited number of cyanobacterial species (see Sect. 2.2.4). The presence of pili in pure cultures of *Synechococcus* sp. 6301 and in associations with plant cells remains an open issue. Evidence exists in the literature of detection of pili in symbiotic *Nostoc* strains: in lichens *Peltigera canina* (Dick and Stewart 1980), in the cultures infective and noninfective against *Gunnera* plants (Johansson and Bergman 1994), and in strains isolated from the cycads of the genera *Cycas* and *Zamia* (Lindblad and Bergman 1990). The presence of pili and of the genes involved in their functioning was observed in *N. punctiforme* hormogonia (motile trichomes) infecting *Blasia* mosses (Adams and Duggan 2005, 2008). Proteomic and transcription analyses suggested classification of these surface organelles as type IV pili, which are responsible for bacterial motility (Klint et al. 2006; Bergman et al. 2008).

Analysis of the ultrastructural organization of the cells of the wild and mutant *Synechococcus* sp. 6301 strains in associations with tobacco cells obtained either by simultaneous inoculation or by addition of the wild-type inoculum to the tobacco culture indicated significant differences in the physiological state of cyanobacteria existing within mixed aggregates and as individual cells in the medium. The very fact that some cyanobacteria were not sorbed on the surface or inside the aggregates of tobacco cells (Fig. 4.2a), remaining as free cells in the medium, indicate cell heterogeneity in associated populations of both strains. Some individuals adhered to the cell walls of ultrastructurally unimpaired and probably living tobacco cells. Electron micrographs (Fig. 4.2b) suggest that the outer membrane lipopolysaccharide was quite probably responsible for adhesion. The difference between the cells within a population in their capacity for adhesion and tight binding may be considered to result from the state of the cell surface of both components at the specific moment of development of a mixed culture, as is the case for other symbiotic bacteria.

Cyanobacterial cells not adsorbed within the aggregates usually degraded and died off quicker than the adsorbed ones. The number of free cells in the associations with tobacco cells obtained by simultaneous inoculation decreased by the 16th day of cultivation. Throughout this period, the unadsorbed individuals occurred with such destructive changes as plasmolysis, thylakoid degradation, the absence of glycogen (a storage compound), and agglomeration of the DNA strands, although relative rigidity of the cell wall structural components and the rod-shaped cells persisted. Transfer of a 70 day association into the medium for cyanobacteria revealed that, unlike adsorbed cells, unassociated cyanobacteria were nonviable. Such ultrastructural changes are therefore pathological. Neither at the cellular or the population level are they adaptive. Thus, in monocultures

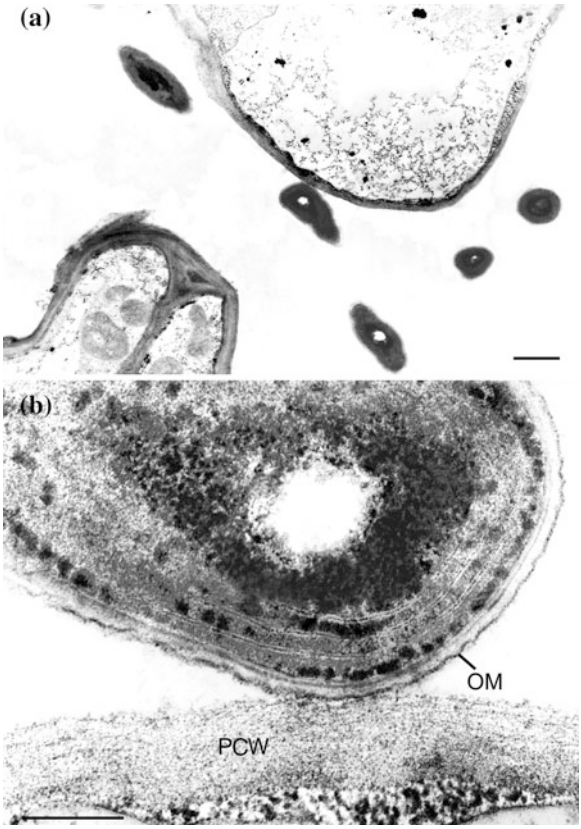

Fig. 4.2 Cells of the wild *Synechococcus* sp. PCC 6301 strain in association with tobacco cells in a suspension culture, TEM (Baulina et al. 1994): between plant cells (**a**) and adhered to the surface of plant cells (**b**). *OM* outer membrane, *PCW* plant cell wall. *Scale bar* a, 1 μm; b, 0.2 μm

without plant partners, the medium for associations, which was decidedly unfavorable for cyanobacteria, caused analogous (although with certain variations) destructive chains in cyanobacterial cells.

Investigation of ultrathin sections showed that the morphological plasticity of cyanobacteria in the presence of tobacco cells, including that of the viable cyanobacteria within compound aggregates, probably resulted from decreased rigidity of the cell wall peptidoglycan layer. The thickness of this layer varies within the same cell, so that it is poorly pronounced at certain sites (Fig. 4.3). The cells of both *Synechococcus* sp. 6301 strains developing in the course of associated growth with tobacco cells should be therefore classified as CWDF. In the composition of the structural components of the cell wall exhibited, however, no significant differences was revealed between cyanobacteria growing in mixed aggregates and the original pure cultures grown in optimal media for cyanobacteria. This statement applies to thylakoids as well: they were formed by closely placed membranes and located at the cell periphery parallel to the cell wall. Glycogen was deposited between the thylakoids, and the intrathylakoid space was not broadened. No signs of degradation were observed in the ultrastructure of the systems of reproduction and protein synthesis. Usual inclusions (carboxysomes and polyphosphate

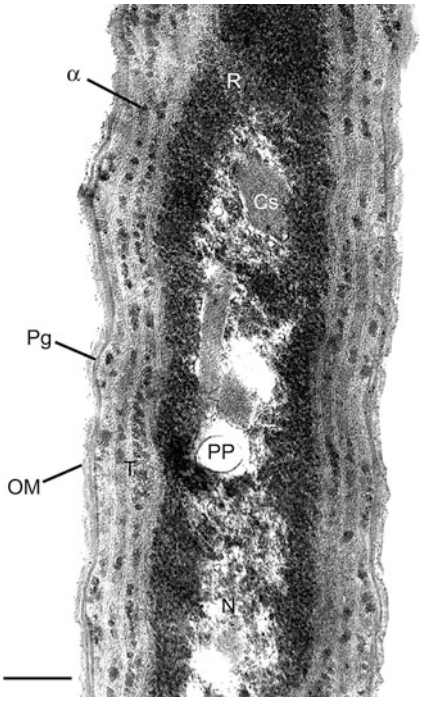

Fig. 4.3 Ultrastructure of the mutant *Synechococcus* sp. PCC 6301 strain within a mixed aggregate with tobacco cells, 1st transfer (Baulina et al. 1994) α, glycogen α-granules; *Cs* carboxysome, *N* nucleoid, *OM* outer membrane, *PP* electron-transparent region of polyphosphate granule localization, *R* ribosomes, *T* thylakoid. *Scale bar* 0.2 μm

granules) were present in the nucleoid zone (Fig. 4.3). Thus, the intracellular components of both *Synechococcus* sp. strains did not exhibit the ultrastructural plasticity found in peptidoglycan of the cell wall. Comparison of the ultrastructural organization of adsorbed and unadsorbed cyanobacteria suggests that a contact with plant cells alleviates the unfavorable effect of the media for associations on cyanobacteria as observed in the previous research of developmental dynamics of mixed cultures and monocultures (Gusev et al. 1982). Capacity for passaging was higher when the wild-type strain was inoculated into the 5 day suspension culture of tobacco cells than in the case of simultaneous inoculation. In the former case, on the 11th day after inoculation of the wild-type cells, their ultrastructure in the free-living fraction (subpopulation) and inside the mixed aggregates was identical (elongated cells with wavy surface).

In the works on development of the model associations, exometabolites of plant cells are considered a factor facilitating cyanobacterial growth in the media for associations. Tobacco cells either excrete compounds favorable for cyanobacterial growth or modify the medium, so that it becomes more favorable for the operation of the mechanisms of phenotypic plasticity (Baulina et al. 1994). In cyanobacteria *Synechococcus* sp. 6301 (wild type) grown in suspension mixed cultures with dioscorea cells, significant morphological alterations were, however, not observed, while adsorption of cyanobacteria on plant aggregates occurred but seldom. This result correlated with the lower capacity of the partners for formation of

subculturable associations than in the variants with tobacco cells (see Table 4.1). It should be noted that the medium for dioscorea cells differs from that for tobacco cells in the composition of vitamins, phytohormones, and amino acids.

The morphological and ultrastructural plasticity associated with decreased rigidity of the peptidoglycan layer in the presence of tobacco cells, together with the relative stability of cell shape and surface in the monoculture indicated that the loss of peptidoglycan rigidity was induced by the exometabolites of the plant partner. Induction of CWDF formation by the plant partner was investigated in a stable association of *N. muscorum* CALU 304 (hereafter referred to as *N. muscorum* 304) with rauwolfia callus (Gorelova 2000b, 2001). The data obtained in the cited works suggest that some factors of plant origin act as inducers of heteromorphic variations in *N. muscorum* 304, which have an adaptive character. Survival of the CWDF of the wild and mutant *Synechococcus* sp. 6301 strains within mixed aggregates with tobacco cells also indicates the adaptive character of the changes in the cell wall. A correlation may be traced between the life duration of an association and the more pronounced morphological plasticity of the mutant (longer and more curved cells) compared to the wild-type strain. Subcultivation with the mutant was possible for four transfers, compared to two transfers for the wild type (see Table 4.1). It is difficult to determine the causal relations between the changes in the cell wall structure and the influencing factors in associations formed by both strains. It is, however, possible to conclude that the ultrastructural plasticity of the cell wall, which causes the morphological plasticity, favors the interaction of the partners. No pronounced heteromorphism, i.e., diverse morphology of the individual cells, was observed within cyanobacterial associations of this species.

Invariability of the general plan of the cellular internal structure, especially of the thylakoid organization, correlates with the metabolic conservatism of *Synechococcus* sp. 6301 as an obligate phototroph, which was observed in mixed cultures, since illumination is required for the growth of associations. The structural and functional conservatism at the cellular and population levels was probably among the causes for the incapacity of these strains for modifications in order to develop stable symbiotic relationships: mixed cultures could be maintained only for 2–4 transfers.

Analysis of the results reported in this section and in Chaps 2, 3 suggests that cyanobacteria of the genus *Synechococcus* have low capacity for either metabolic or structural modifications under changing cultivation conditions, i.e., a low level of phenotypic plasticity.

4.2.2 Anabaena variabilis *CALU 458 in Associations Constructed Using Tobacco ProtoplastsF*

The series of our works was devoted to this field (Agafodorova et al. 1982a, b; Gusev et al. 1983; Baulina et al. 1984, 1988b; Baulina and Agafodorova 1983).

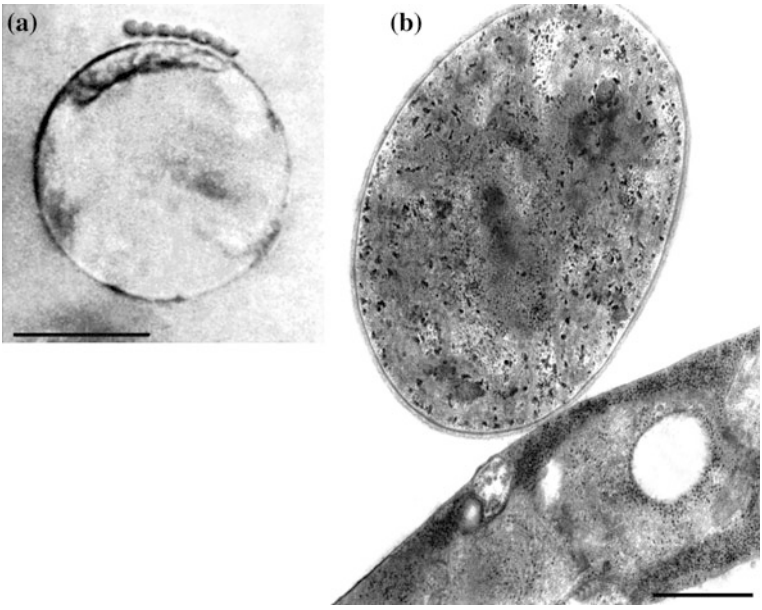

Fig. 4.4 Adhesion of *Anabaena variabilis* CALU 458 on the surface of tobacco protoplasts in the medium supplemented Ca^{2+} at pH 7.5, light microscopy (**a**) (Agafodorova et al. 1982a) and polyethylene glycol, TEM (**b**) (Agafodorova et al. 1982b). *Scale bar* a, 20 μm; b, 5 μm

The stages of formation of such associations were as follows:

(1) Incubation of cyanobacterial cell suspensions (in some cases, of lysozyme-treated suspensions containing spheroplasts) together with tobacco protoplasts under conditions favorable for adsorption and incorporation of cyanobacteria by plant protoplasts;

(2) Cultivation of the suspensions to obtain the mixed cell aggregates;

(3) Induction of callus formation with subsequent transfers of the mixed callus;

(4) Induction of organogenesis to obtain regenerant plant from the mixed callus.

At the first stage, under specially adjusted conditions (in media supplemented with PEG or calcium cations at high pH values), efficient adhesion of the *A. variabilis* 458 cells on the surface of plant protoplasts was achieved (Fig. 4.4a, b). In PEG-treated suspensions, tobacco protoplasts containing the ultrastructurally unimpaired *A. variabilis* 458 cells; ultrathin sectioning suggested their localization within vacuoles (Fig. 4.5a, b). Formation of cyanobacteria-containing vacuoles within plant cells probably resulted from their engulfment via endocytosis. In some protoplasts, cyanobacteria were found in the central vacuoles. PEG had, however, a negative effect on tobacco protoplasts (Gorelova et al. 1981), causing in many cases degradation processes in the cytoplasm (Fig. 4.5b). PEG-treated mixed suspensions proved unviable upon subsequent cultivation.

Application of 0.05 M $CaCl_2$ at pH 7.5 promoted intense adhesion of cyanobacteria on the protoplast surface (on average, up to 60 % of the protoplasts

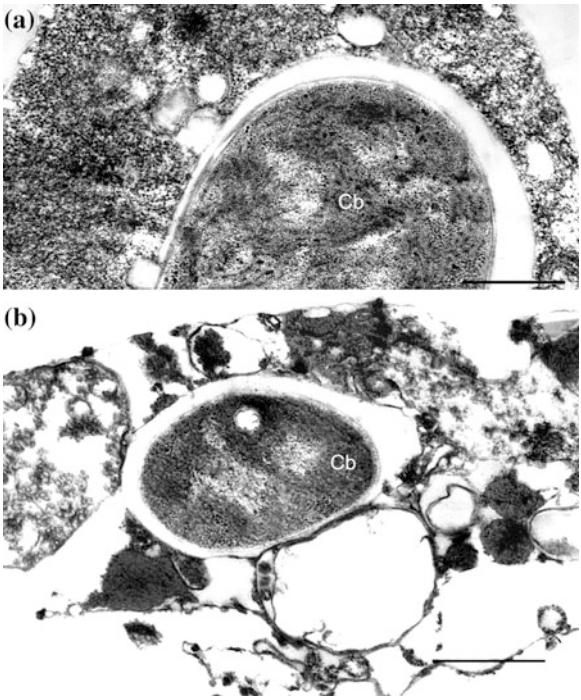

Fig. 4.5 Ultrathin sections demonstrating localization of the *Anabaena variabilis* CALU 458 cells within tobacco protoplasts: with unimpaired ultrastructure (**a**) (data obtained by Gorelova, Agafodorova and Baulina) and with destructive changes (**b**) (Agafodorova et al. 1982b) *Cb* cyanobacterium. *Scale bar* a, 0.5 μm; b, 1 μm

adsorbed cyanobacteria) and did not affect the viability of the components of the suspension. Subsequent cultivation and induction of callus formation (stages 2 and 3 of association formation) were successful and resulted in development of a mixed callus (Fig. 4.6). The association of tobacco callus with *A. variabilis* 458 was maintained for 2.5 years with transfers every 1–1.5 months (see Table 4.1). Electron microscopic investigation of the callus tissue at the first transfer revealed cyanobacteria localized both on the surface and inside the callus, as well as in the central vacuoles of the plant cells (Fig. 4.7). This was an indication of engulfment of cyanobacteria by tobacco protoplasts at the early stages of formation of the association, since their penetration into the cell would have been impossible after the regeneration of the plant cell wall. In the vacuolar populations of cyanobacteria, CWDF were present (Fig. 4.7), which is important for discussion below on L-transformation of *A. variabilis* 458 developing within the callus. The parietal cytoplasm layer in the regions where the major cell organelles, including the nucleus, were not located was very thin, which is typical of dedifferentiated tobacco cells at the stage of growth by distention. However, cyanobacterial microcolonies were rarely observed in the central vacuoles. Investigation of three subsequent transfers revealed a tendency characteristic of various cyanobacterial–plant model associations: inside the tissue, cyanobacterial microcolonies are localized in the cavities, or harbor formed by the cell walls of the dead plant cells decomposing within the viable tissues of the callus.

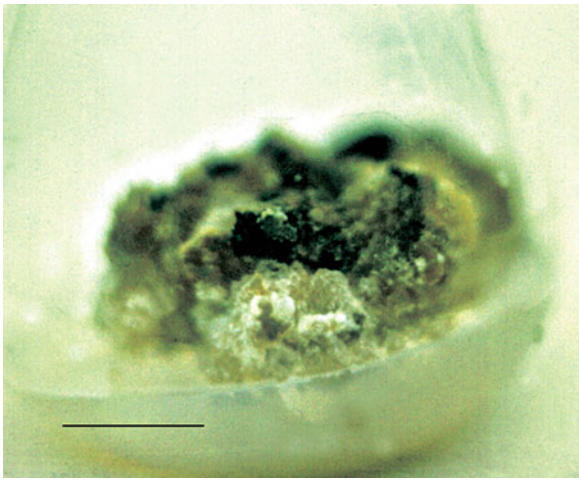

Fig. 4.6 Model association of a tobacco callus tissue and *Anabaena variabilis* CALU 458 (data obtained by Agafodorova, Korzhenevskaya and Baulina). *Scale bar* 1 cm

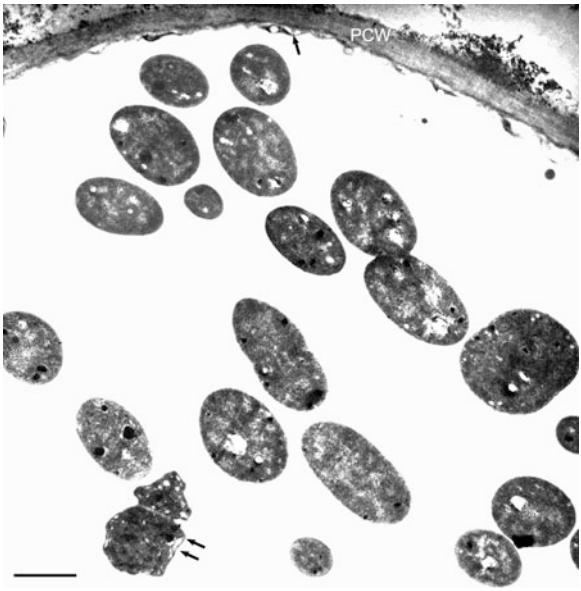

Fig. 4.7 *Anabaena variabilis* CALU 458 localized in a vacuole in a tobacco callus cell, TEM (Baulina et al. 1984) *PCW* plant cell wall. *Single arrow* points to the parietal layer of the cytoplasm, a *double arrow* points to cell wall-defective form. *Scale bar* 2 μm

A cyanobacterial population growing in association with callus tissue is heterogeneous in morphology and ultrastructure of its constituent cells. Inside the callus, this diversity is so great that it deserves the term "extremely possible". Scanning electron microscopy revealed the cells of ovoid, spherical, or often of irregular shape, single or in chains, 0.5–4 μm in diameter, with smooth or uneven surface (Fig. 4.8). Division may occur simultaneously in several planes. Small cells about 0.5 μm in diameter may be formed by non-uniform division (Fig. 4.9a) and probably by budding (Fig. 4.9b). In the latter case, the cells have uneven

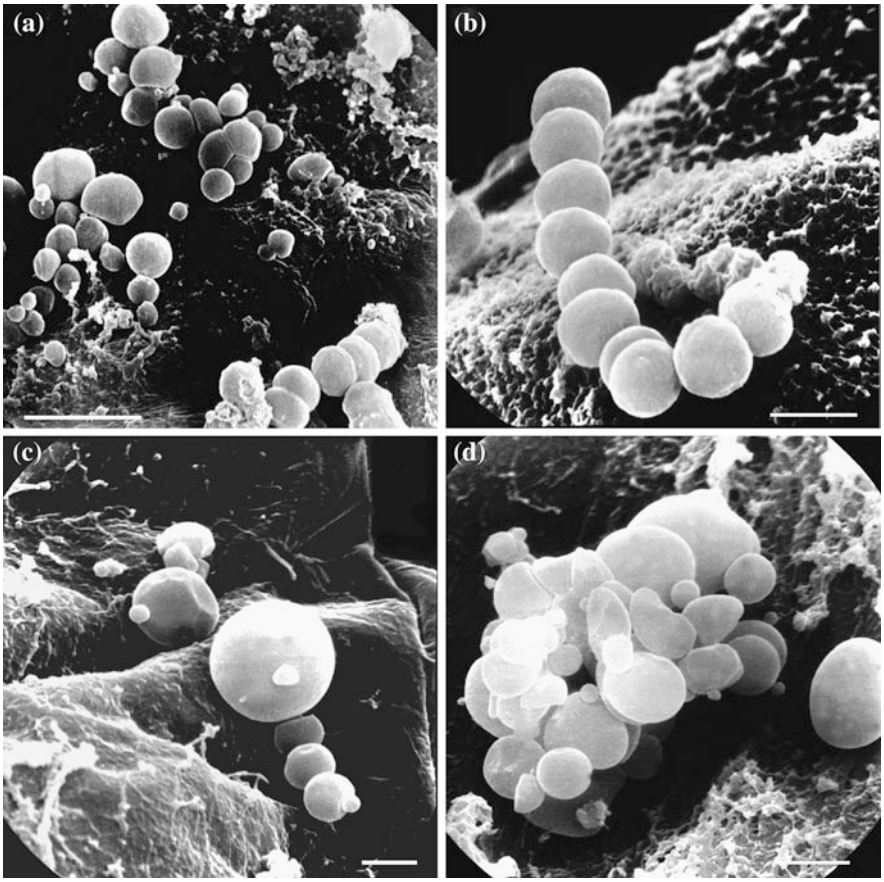

Fig. 4.8 Variety in the morphology of the *Anabaena variabilis* CALU 458 cell forms in the population localized within the tobacco callus tissue; **a**, **b**, **c**, (Baulina et al. 1984); **d**, (data obtained by Baulina and Agafodorova). *Scale bar* a, 5 μm; b, c, d, 2 μm

surface, being therefore CWDF. Varieties of small cell forms have been identified on ultrathin sections. (1) Small cells 1–1.5 μm in diameter, retaining the cell wall and ultrastructure similar to those of the vegetative cells of normal size ($\sim$2 μm in diameter). Small forms, often sharing a septum with larger *Anabaena variabilis* cells, i.e., resulting from non-uniform division (Fig. 4.10a), are usual in this strain. Their viability and genomic adequacy have not been sufficiently studied. (2) Single, apparently independently located, oval cells with transversal section of 0.7–1.0 μm, with reduced cell wall, in which the nucleoid and ribosomes are revealed and thylakoids are not (Fig. 4.10b). Irregular cell shape, close to a polyhedron, developed probably as a result of impaired cell division (see Fig. 4.8). Asymmetric formation of the septum in several planes simultaneously, sometimes in the daughter cells of changed size and shape, which were not separated, was

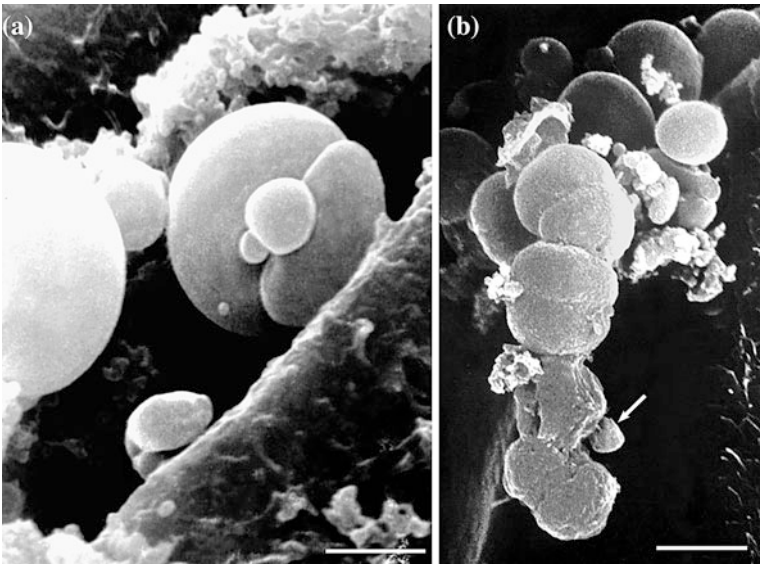

Fig. 4.9 Non-uniform division of the cells (**a**) and forms, probably having a defective cell wall (**b**), resulting in emergence of small cell forms in the *Anabaena variabilis* CALU 458 population within tobacco callus tissue, SEM (**a**, **b**, data obtained by Baulina and Agafodorova). *Arrow* points to a form tentatively identified as a budding CWDF. *Scale bar* a, b, 2 μm

revealed on ultrathin sections (Fig. 4.11a). This type of division is not usual in the pure culture of *A. variabilis* 458 grown under optimal conditions. Anomalous division was reported in another *A. variabilis* 29413 strain grown as a monoculture in the medium for alfalfa callus formation (Gorelova 2005). Such anomalous division is an indication of impaired coordination of the regulation of the position of the division plane, which is active in the prokaryotic cell cycle (Margolin 2000). Moreover, peripheral accumulation of peptidoglycan may occur at the site of the septum formation in *A. variabilis* 458 cells (Fig. 4.11b). These findings indicate that the *A. variabilis* 458 population inside the tissues was in the phase of unbalanced growth. Formation of heteromorpic cell forms or UGF, including those with anomalous division, as well as emergence of the cells with impaired peptidoglycan synthesis, was observed and investigated in the model systems with *N. muscorum* 304 and calluses of nightshade and rauwolfia, as well as in monocultures of this cyanobacterium grown in the media for the cultivation of these systems (Gorelova et al. 1999, 2000; Gorelova 2000b, 2001).

Apart from the anomalously dividing cells, which are typical UGF, CWRF were detected in cyanobacterial colonies inside the tissues. Among them, apart from the above-mentioned small forms, giant protoplasts occur, albeit seldom (Fig. 4.12). True protoplasts with the size comparable to that of the vegetative cells, forming compact groups together with the cells with unimpaired cell walls and amorphous masses of decomposing cell residues within the harbor limited by the cell wall of

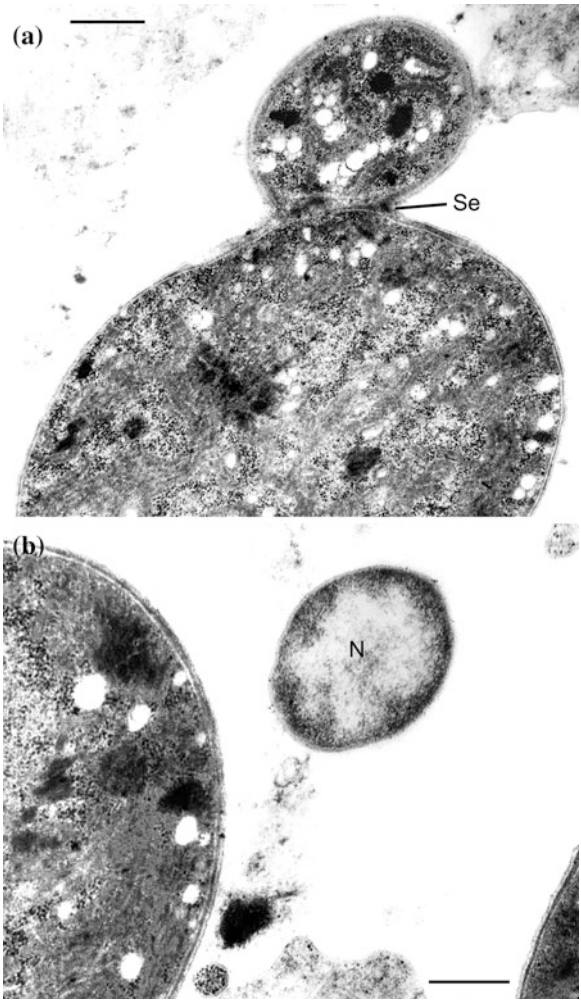

Fig. 4.10 Small cell forms in the *Anabaena variabilis* CALU 458 population localized within tobacco callus tissue, ultrathin sections: cells (mini-cells) (**a**) and a cell wall-reduced form (**b**) *N* nucleoid, *Se* septum. *Scale bar* a, b, 0.5 μm

the host plant deserve special attention (Fig. 4.13a). Such cell groups (microcolonies) exhibit a pronounced tissue-like organization. In this case, however, an important component of "pseudotissues" was not revealed, namely, the fibrillar intercellular matrix, which is typical of bacterial colonies. The fibrillar matrix, similar to the mucous one, was not found in this association. The intercellular space in these tissue-like microcolonies was filled with the fragments of the structures and organelles of decomposed cells, which formed amorphous masses. Together with the filamentous structures and CWRF of various sizes, these amorphous [or structureless, according to (Prozorovsky et al. 1981)] are the components of the L-form colonies.

The *A. variabilis* 458 protoplasts in such microcolonies are characterized by their amoeboid shape. The protoplasts often form projections similar to

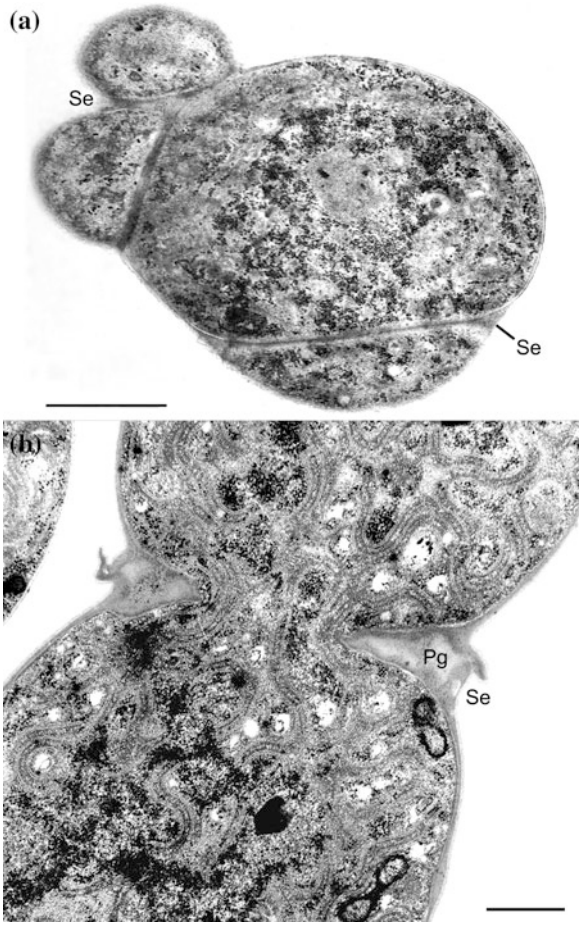

Fig. 4.11 Cell ultrastructure in the *Anabaena variabilis* CALU 458 population localized within tobacco callus tissue: anomalously dividing cell (**a**) (Baulina et al. 1984) and a cell with anomalous septum formation (**b**) *Se* septum, *Pg* peptidoglycan. *Scale bar* a, 1 μm; b, 0.5 μm

pseudopodia and "squeeze" between the intact cells. Amoeboid protoplasts which look like dividing ones may be found on ultrathin sections (Fig. 4.13b). Some of them form chains. In some cases, SEM revealed the budding CWDF which could have also been protoplasts (see Fig. 4.9b). Capacity for division and thus the viability of these protoplasts is confirmed by the fact that these cell forms maintained their ultrastructural integrity. Compact zones of the nucleoid with peripherally located ribosomes (Fig. 4.13a) and numerous regularly located thylakoids formed by closely connivent membranes with the typical three-layer profile were revealed (Fig. 4.13b). The hyaloplasm was of uniform electron density without signs of autolysis, although it usually contained few inclusions of storage compounds. Membranous vesicles separating into the intercellular space were formed on the protoplast surface. Although uniform division and budding of the protoplasts in compact microcolonies inside the tissues were difficult to confirm reliably, our results suggest the possibility of L-transformation in the *A. variabilis* 458

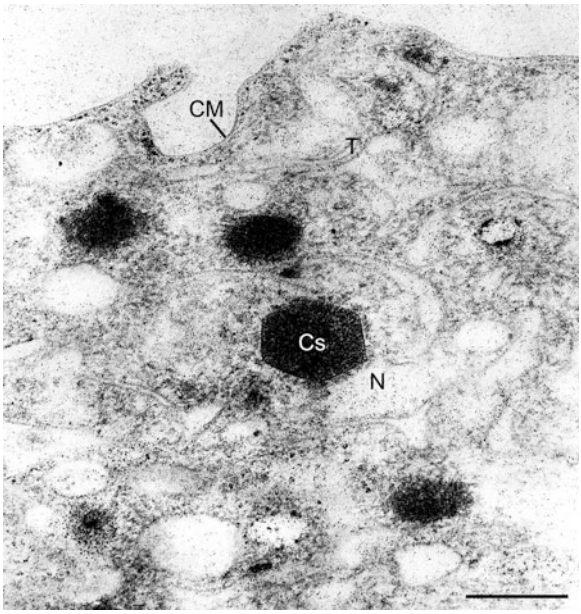

Fig. 4.12 Part of a giant protoplast in the *Anabaena variabilis* CALU 458 population localized within tobacco callus tissue (TEM) *CM* cytoplasmic membrane, *Cs* carboxysome, *N* nucleoid, *T* thylakoid. *Scale bar* 0.5 μm

populations resulting in formation of the protoplast-like L-forms. Due to their rare occurrence on the ultrathin sections, interpretation of the functional status of small protoplasts as elementary bodies is difficult. The possibility of de novo formation of thylakoids in small CWRF (Fig. 4.10b) from the minimal membrane fragments and chlorophyll molecules should not be ruled out. This mechanism of thylakoid regeneration probably operates in cyanobacterial cells after photooxidative destruction (Schmetterer et al. 1983). Putative mechanisms of biogenesis of thylakoid membranes in cyanobacteria are currently under discussion (Liberton et al. 2011; Nickelsen et al. 2011).

In intact, *A. variabilis* 458 cells and probably in viable UGF, the ultrastructural patterns of thylakoid organization were observed, which correlated with the manifestations of the ultrastructural plasticity of these organelles described in Chap. 2. These include swelling and vesiculation, which is reversible at certain stages. The cells with such alterations of the thylakoids were at the stage of division. Compared to pure cultures, many cells in associated populations have a less developed thylakoid system and the cytoplasm, including the interthylakoid space containing mostly ribosomes and nucleoid areas "scattered" within the cell volume. Several carboxysomes are usually located in the zones of more compact DNA placement (Fig. 4.14). Localization of carboxysomes in the central part of the nucleoid is known as a characteristic feature of cyanobacteria, which facilitates its identification on ultrathin sections. Cyanophycin granules were revealed, while the glycogen α-granules were absent (Fig. 4.15a). Unusual fibrillar inclusions looking as groups of electron-dense strands were observed (Fig. 4.14). These inclusions are characteristic of the cell of this cyanobacterial strain not only under

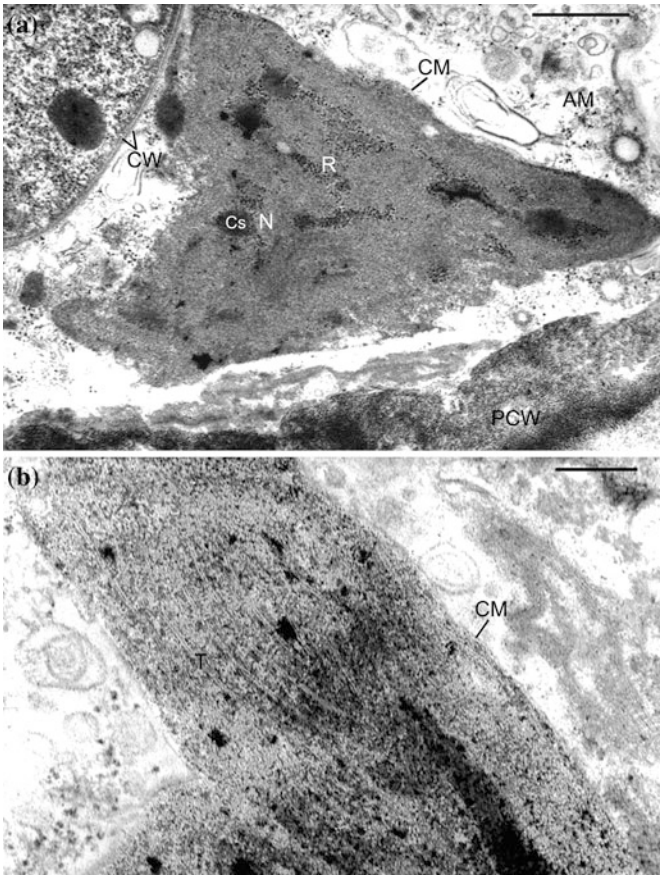

Fig. 4.13 Ultrastructure of the protoplasts in the *Anabaena variabilis* CALU 458 population localized in an intercellular space of tobacco callus tissue: protoplast near a vegetative cell, with surrounding amorphous masses (**a**) (Reprinted from Gusev et al. (2002) with kind permission Springer Science + Business Media B.V.) and tentatively dividing protoplasts (**b**) *AM* amorphous masses, *CM* cytoplasmic membrane, *Cs* carboxysome, *CW* cell wall, *N* nucleoid, *PCW* plant cell wall, *R* ribosomes. *Scale bar* a, 0.5 μm; b, 0,2 μm

optimal growth conditions, but also during incubation in the dark (Baulina and Gusev 1977).

A unique manifestation of the thylakoid ultrastructural plasticity which was observed in *A. variabilis* 458 developing within the tissues is their fragmentation, so that in the plane of the section the thylakoids appear as spindles of a regular shape, with one or both pointed ends or, less often, as cylinders (Fig. 4.15a, b). The intrathylakoid space is electron transparent, with an insignificant admixture of granular materials. The membranes retained the three-layer profile. On the outer membrane surface, small granules were clearly revealed. In all "spindle-shaped" and "cylindrical" thylakoids, the granules were of the same size and were orderly

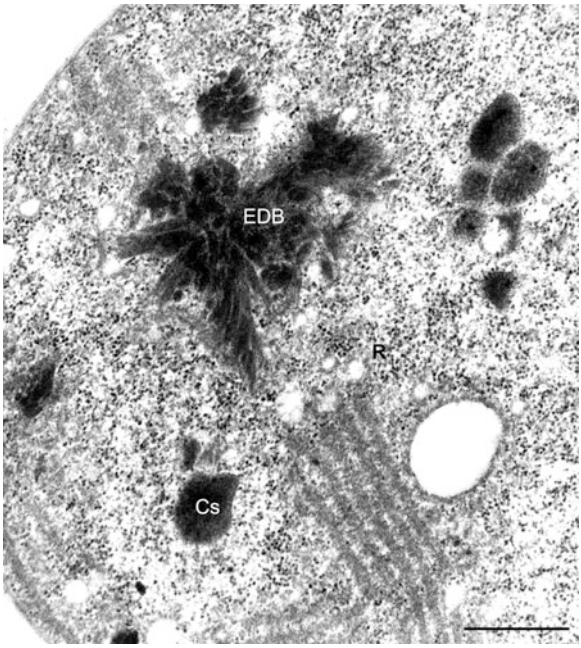

Fig. 4.14 Ultrastructure of an *Anabaena variabilis* CALU 458 cell localized within tobacco callus tissue *EDB* electron-dense bands of unknown nature, *Cs* carboxysome, *R* ribosomes. *Scale bar* 0.5 μm

located. Analogous structures termed tubules by the authors and formed by the thylakoid membranes were observed in heterotrophic, dark-grown *A. variabilis* 29413 cells using the freeze–fracture technique (Peschek and Sleytr 1983). This approach made it possible to reveal the regular rows of intramembrane particles which were not present on the fractures surfaces of the thylakoid membranes of the photoautotrophically grown cells. Unfortunately, it is now difficult to guess the function of such structures.

Thus, the different manifestations of the ultrastructural plasticity of the cells and organelles within the *A. variabilis* 458 population transferred in the association with tobacco callus tissue make it possible to identify several cell types. In this case, the ultrastructural plasticity of the population as a whole correlates with the viability of the system and with the balanced growth of both components of the mixed callus. During both formation and transfers of the association, diauxic growth occurs, so that on transfer of the callus tissue with cyanobacteria into fresh medium, tobacco tissue starts intensive growth, while cyanobacteria are not visible in the newly formed tissue. However, by the end of the stationary growth phase of the callus, intensive growth of these microorganisms occurs. The system, therefore, exhibits a lag phase in cyanobacterial development. It is logical to assume that the protoplasts similar to bacterial L-forms are the persisting cell forms in the *A. variabilis* 458 population. Persistence of symbiotic cyanobacteria has been recently investigated using a vast array of experimental data (Gorelova 2005; Gorelova and Baulina 2009; Lobakova 2004).

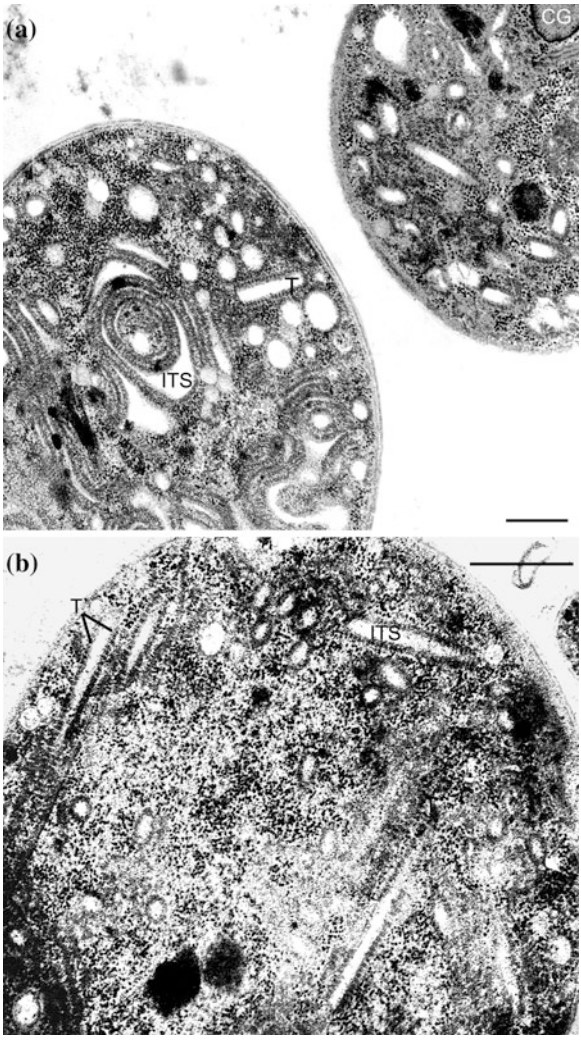

Fig. 4.15 Ultrastructure of an *Anabaena variabilis* CALU 458 cells localized within tobacco callus tissue: various changes in the thylakoids (**a**, **b**) *CG* cyanophycin granule, *ITS* intrathylakoid space, *R* ribosomes, *T* thylakoid. *Scale bar* a, b, 0.5 μm

Although as was shown above, formation of specific cyanobacterial cell forms probably depends on the conditions in the microenvironment, the dynamics of development of the individual, ultrastructurally homogeneous cell groups are impossible to monitor. However, the experimental approaches used for the development of artificial symbioses, specifically, induction of organogenesis in the mixed callus, made it possible to obtain isolated microcolonies within the plant tissue, which mainly consisted of the ultrastructurally similar cells of significantly modified cyanobacteria. The pieces of mixed callus from the 3rd and 15th transfers were placed on the surface of solid medium containing the relevant plant hormones. After approximately 2 weeks of growth under illumination, organogenesis was observed, with formation of the visible plantlets with leaves by the end of the

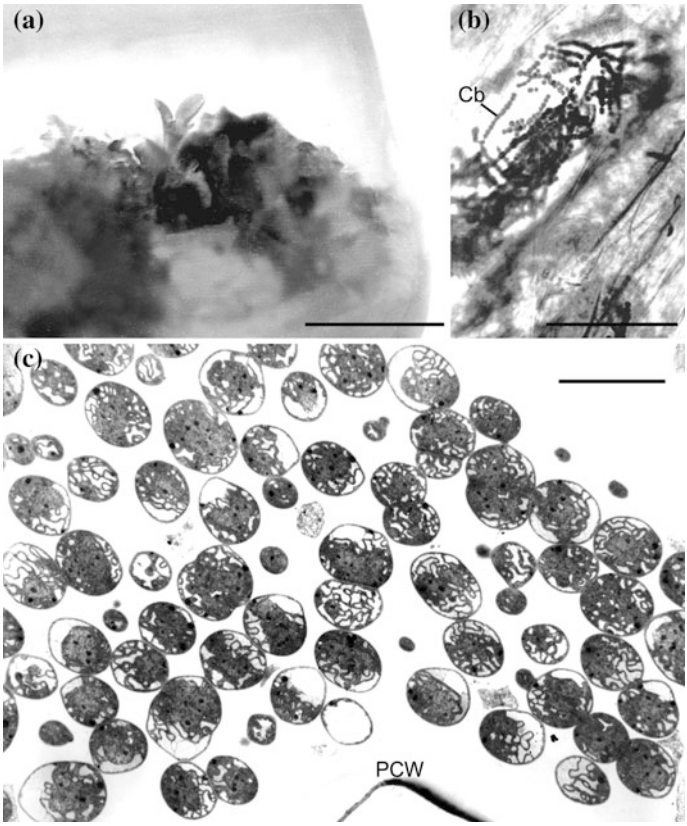

Fig. 4.16 Model association of *Anabaena variabilis* CALU 458 and tobacco tissues at the stage of regenerated plant formation: mixed callus culture of tobacco and the cyanobacterium with the regenerating plantlet (cyanobacterial colonies are dark) (**a**) (data obtained by Agafodorova, Korzhenevskaya and Baulina), local aggregation of cyanobacterial filaments (a microcolony) in the leaf tissue of a regenerating plantlet (**b**) (Gusev et al. 1983), and ultrathin section of the microcolony (**c**) *Cb* cyanobacterium, *PCW* plant cell wall. *Scale bar* a, 1 cm; b, 100 μm; c, 5 μm

4th week (4th stage of association formation) (Fig. 4.16a) (Gusev et al. 1983). After two more weeks, blue-green streaks appeared on some of the leaves, revealing the aggregations of cyanobacteria. Ultrathin sections of the zone of such aggregations (microcolonies) revealed that, similar to the callus tissues, they were localized in extended intercellular spaces between degrading plant cells or within a carcass of the dead cells' cell walls. Importantly, the degradation occurred only in the cells directly involved in formation of the zone of cyanobacterial localization and surrounding it. The regenerating plantlet remained in the state of growth and development.

Electron microscopic investigation of the cells from an isolated microcolony within the leaf tissue of a regenerated plantlet obtained from the third transfer of the mixed callus provided further insight into the ultrastructural plasticity of the

thylakoids and whole cells of *A. variabilis* 458. Light microscopic control of the TEM preparation revealed that all cells were organized in long chains (Fig. 4.16b), unlike the cells in the microcolonies within the callus. All cells were rounded in shape. As can be seen from ultrathin sections, the cells in the chains were joined by surface contacts (probably adhesion) of the outer membranes at relatively small areas, while the septa were completely formed (Figs. 4.16c, 4.17). Peculiarly, the neighboring chains were joined together in the same way due to the presence of additional lateral sites of septa formation in the cells. Formation of new septa perpendicular to the already formed ones may began. In the latter case, incomplete division usually occurred. Moreover, small cells were formed at the ends of the chains (Fig. 4.16c). Small cells may probably be singular. Importantly, all the cells, including the small ones, were completely uniform in their ultrastructure. This finding suggests a certain "synchronization" of the physiological state of the cells in such a microcolony. The structure and mutual position of the branching thylakoids with pronounced hypertrophy of the intrathylakoid space and the relatively constant interthylakoid distance are the main peculiarity of these cells (Fig. 4.16c). On a section, the intrathylakoid space may occupy about half of the cell area. In all cells it was filled with fine-grained material grouped in short, irregularly located fibrils, with a tendency to condense close to the surface of the thylakoid membranes, where these fibrils lie usually perpendicular to the membranes and are possibly bound to them (Fig. 4.16c). Accumulation of this material in the enlarged intrathylakoid space probably results from its "overproduction" due to the shifts in the metabolic pathways. Thylakoids in these *A. variabilis* 458 cells probably act as intracytoplasmic membrane compartments, where the products of cell metabolism are deposited or isolated from the rest of the cytoplasm. The so-called keritomic vacuoles (from Greek *keratos*, horn and *tomein*, to cut) are considered the possible compartments isolating deficient metabolic products from the cytoplasm (Pinevich 2006). On ultrathin sections they have spherical shape and are probably derivatives of the thylakoids. Accumulation of matter in expanded intrathylakoid space was observed in various cyanobacteria (Jensen 1985; Zhu et al. 1998). In particular, accumulation of fibrillar material in this region occurred in *C. fritschii* cells grown in the medium for associations with ginseng cells, which is unfavorable for cyanobacteria (Baulina and Lobakova, unpublished data). In the haloalkaliphilic *Euhalothece* sp. isolated from the Magadi soda lake, Kenya, unusual profuse aggregation of electron-dense spherical granules in the intermembranous space of the thylakoids are common (Baulina et al. 2011). The publications on molecular mechanisms for the translocation of proteins into the intrathylakoid space (lumen) should be mentioned as additional information for discussion of the problem of material accumulation in the intrathylakoid space (Spence et al. 2003; Mullineaux 2008). Analysis of this problem is, however, beyond the scope of this book.

In tobacco tissues, *A. variabilis* 458 cells exhibited the morphological and ultrastructural evidence of active growth and division (long chains of cells, forming septa, and nucleoid with peripheral ribosomes) (see Fig. 4.16b, c). It may be therefore concluded that they were in the exponential growth phase. In this

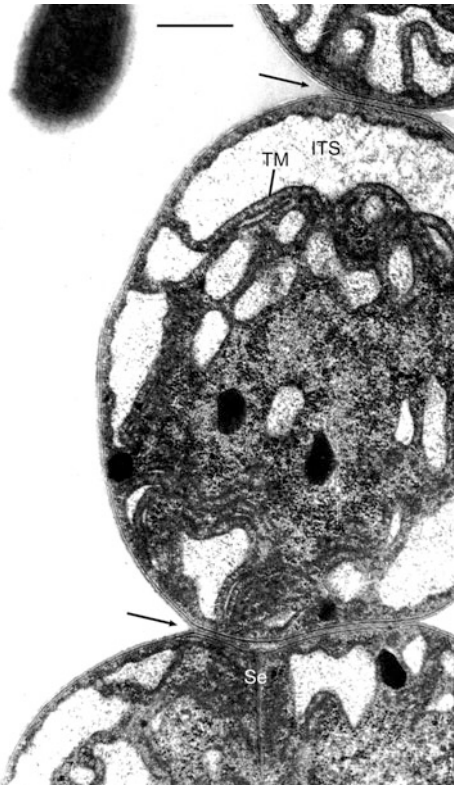

Fig. 4.17 Cell ultrastructure in an *Anabaena variabilis* CALU 458 microcolony within the leaf tissue of a regenerating tobacco plantlet (shown of Fig. 4.16**c**) *ITS* intrathylakoid space, *TM* thylakoid membrane, *Se* septum. *Arrows* points to the sites of intercellular contacts. *Scale bar* 0.5 μm

case, their physiological state should be adequate to the conditions inside the leaf tissue of regenerating plant. During the latent period of cyanobacterial development associated with organogenesis in plant tissues selection of the cells adapted to further development under changed conditions probably occurred. The cells with certain new physiological properties manifested primarily at the level of the ultrastructural plasticity of the thylakoids probably gain advantage for growth within the tissues of regenerating plant.

4.2.3 Anabaena variabilis ATCC 29413 in Associations with Callus Tissues and Regenerated Plants of Tobacco and Alfalfa

Cyanobacteria capable of dinitrogen fixation were used to develop a number of model systems (Korzhenevskaya et al. 1986b; Korzhenevskaya 1990). This ability determines the biological sense of formation of cyanobacterial–plant symbioses in nature, since cyanobionts provide nitrogen not only for themselves, but also for the

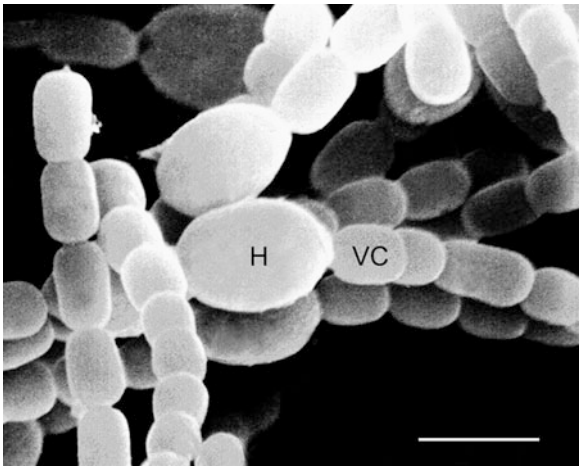

Fig. 4.18 Morphology of the dinitrogen-fixing, heterocyst-forming cyanobacterium *Anabaena variabilis* ATCC 29413 (SEM) (the photo was kindly provided by Pivovarova) *H* heterocyst, *VC* vegetative cell. *Scale bar* 5 μm

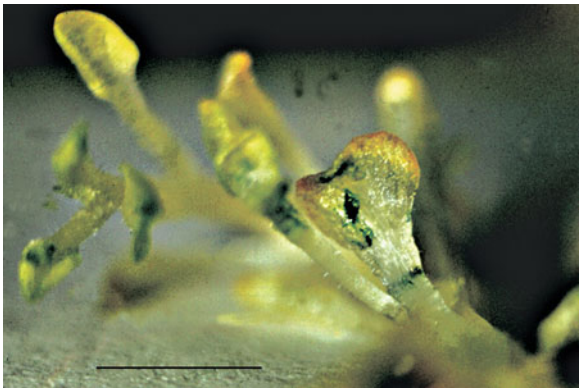

Fig. 4.19 Model association of a regenerated tobacco plantlet and *Anabaena variabilis* ATCC 29413 (data obtained by Pivovarova, Korzhenevskaya and Baulina). *Scale bar* 1 cm

plant partner, i.e., for the system in general. Cyanobacteria of subsection IV (previously, order Nostocales, to which the species *A. variabilis* and *N. muscorum*), which will be discussed in the next Sect. 4.2.4 carry out dinitrogen fixation in specialized cell, heterocysts (Fig. 4.18).

Formation of a stable association of *A. variabilis* 29413 with tobacco callus tissue and the regenerated plantlet obtained by organogenesis induction in this tissue (Table 4.1, Fig. 4.19) started after the introduction of cell suspensions of cyanobacteria as drops on the callus surface or its injection within the callus (Korzhenevskaya et al. 1984; Pivovarova et al. 1985; Gusev et al. 1986). In these works, cyanobacteria were shown to colonize the intercellular spaces and surface of the callus tissue, as well as the intercellular spaces, vascular system, air-bearing cavities of the stomata, the zones below the leaf cuticle, and local areas on the surface of regenerant. In all these zones, active growth of cyanobacteria usually occurred, with formation of long chains of the vegetative cells of equal size (Fig. 4.20a, b). The chains could contain heterocysts. A tendency for the

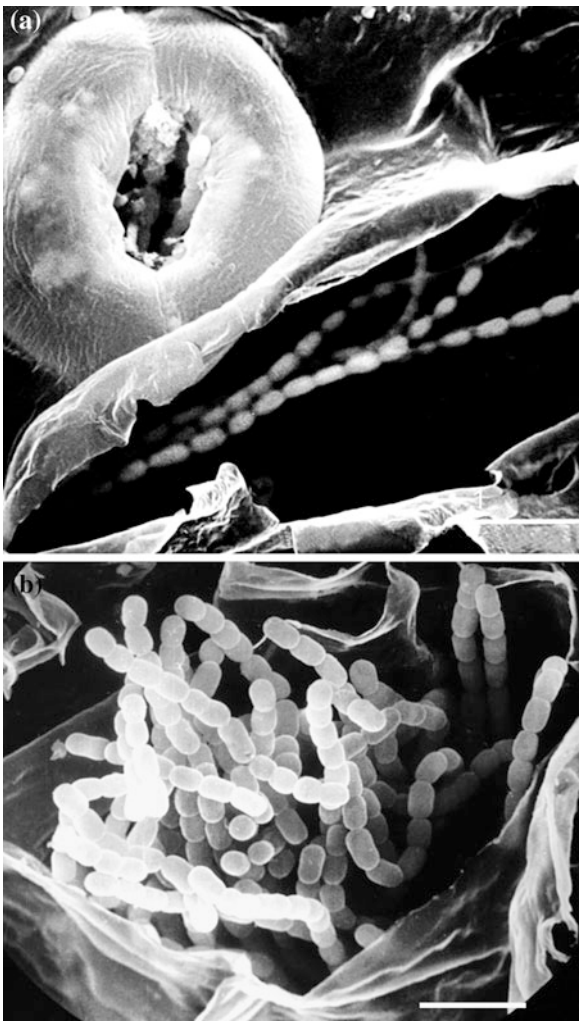

Fig. 4.20 *Anabaena variabilis* ATCC 29413 localized inside the stomata and below the leaf cuticle (**a**) and in the intercellular space (**b**) of the tobacco regenerated plantlet, SEM (a, b, data obtained by Pivovarova, Baulina and Korzhenevskaya). *Scale bar* a, 15 μm; b, 10 μm

fragmentation of long chains into shorter ones or into single cells, sometimes irregular, and heterocysts was observed sometimes (Pivovarova et al. 1986) (Fig. 4.21a). In the surface zones of localization, cell size remained constant within a chain, while varying significantly between different chains. In these samples, no spheroplasts and protoplasts similar to those of *A. variabilis* 458 in model associations obtained with tobacco protoplasts, i.e., the cell forms with pronounced characteristics of L-transformation, were detected. In different zones of the surface and interstitial localization, the ultrastructure of the vegetative cells was usually similar to that observed in *A. variabilis* 29413 cells grown in pure culture. The cells from different parts of a regenerating plant differed, however, in their assortment of the granules of storage material (glycogen, cyanophycin, and

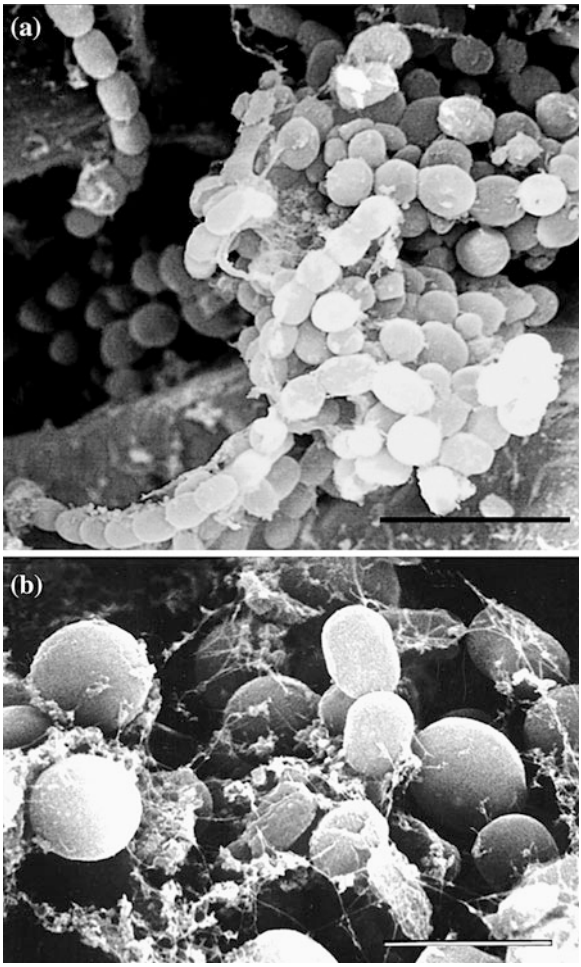

Fig. 4.21 *Anabaena variabilis* ATCC 29413 localized inside the tobacco callus tissue (**a**) (data obtained by Pivovarova, Baulina and Korzhenevskaya) and on the surface of the regenerated plantlet (**b**) (Baulina et al. 1989b) (SEM). *Scale bar* a, 15 μm; b, 5 μm

lipids), which reflected the ratio of carbon and nitrogen sources in specific microenvironments. In some cells, the peptidoglycan layer became thinner, with "pores" in some local areas. Thus, the tendency for CWDF formation, even if exists in this system, is not dominant in the development of this population.

In the cases of interstitial localization, cyanobacteria were usually associated with the regions well provided with nutrient compounds, such as the vascular system and the air-bearing cavities of the stomata. The morphological similarity of *A. variabilis* 29413 cells in such harbor to the actively growing original culture is an indication of conditions favorable to cyanobacterial growth and development. Typical aggregates of cyanobacterial chains are shown in Fig. 4.20a, b. Thin cover layers formed by reticulate-fibrillar mucous matter were often observed at the periphery of the *A. variabilis* 29413 microcolonies localized at the surface of regenerated tobacco plantlets (Pivovarova et al. 1986; Baulina et al. 1989b).

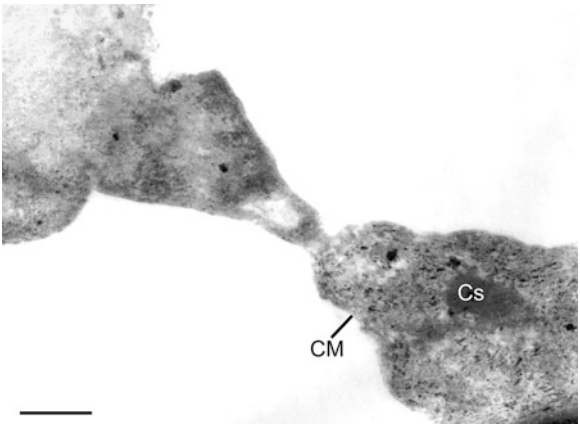

Fig. 4.22 Possibly a dividing protoplast in the L-form-like *Anabaena variabilis* ATCC 29413 colony growing on the surface of solid medium for associations close to a tobacco callus, TEM (data obtained by Baulina and Pivovarova) *CM* cytoplasmic membrane, *Cs* carboxysome. *Scale bar* 0.5 μm

In some cases of surface growth, the mucous matter enveloped heteromorphic cells (Fig. 4.21b). The conditions in these local zones were probably less favorable for cyanobacteria growth. This suggestion is supported by the fact that complete rejection of cyanobacteria occurred in the experiments when they were applied to the stem and leaf surfaces of regenerated tobacco plants in order to obtain associations. Morphological characteristics alone are, however, insufficient to determine reliably whether large rounded cells are vegetative ones, heterocysts, or akinetes (Fig. 4.21b). Formation of mucus by *A. variabilis* 29413 cells localized on the callus surface was not a universal phenomenon and was rather observed at certain sites. Mucus formation was not observed in the zones of active growth mentioned above.

The absence of significant ultrastructural changes in the cells and organelles of cyanobacteria developing in this model system indicates that the metabolic capacities of the strain, specifically, its capacity for photo- and chemoheterotrophic growth and for cell differentiation, make it possible to maintain its activity within the range of homeostasis corresponding to the optimal growth conditions. This ability of the strain was to some degree observed in associations with alfalfa callus tissues and regenerated plants (see below). Importantly, planting of leaf-bearing cuttings on the medium with cyanobacteria, application of cyanobacteria to intact leaves or on a damaged surface, as well as injection of cyanobacteria into leaves or stems resulted in dying off of cyanobacteria, which did not penetrate into the plant (Pivovarova et al. 1985). The absence of pronounced morphological and ultrastructural changes in *A. variabilis* 29413 may result from the characteristics of the strain, the relatively favorable conditions within plant tissue, and the experimental procedure for induction of cyanobacterial growth in associations. In the variant with *A. variabilis* 29413, introduction of cyanobacteria directly into the callus tissue probably favored selection already at the level of the injected suspension (population) favoring the cells in the most adequate physiological state. In the case of *A. variabilis* 458, however, a period of adaptation probably occurs, first during incubation as a suspension with tobacco protoplasts in the medium for plant tissues

supplemented with an osmotic stabilizer, then during formation of the callus from regenerating protoplasts, and finally, during the long lag period within plant tissues, prior to formation of a "mixed callus" (see Sect. 4.2.3).

The finding that CWRF, including protoplasts, were formed in the *A. variabilis* 29413 colonies growing for 2 months close to the tobacco callus on the medium for organogenesis (Pivovarova 1985; Baulina et al. 1988b) (Fig. 4.22) is important for comparative analysis of the ultrastructural plasticity of two *A. variabilis* strains, which manifests itself differently in associations with plant tissues. The photograph shows that *A. variabilis* 29413 protoplasts are probably capable of division, so that the mechanism of L-transformation is possibly active in these systems. Moreover, the structural elements characteristic of the colonies of bacterial L-forms (amorphous masses and filamentous structures) were found in cyanobacterial colonies. The L-form-like colonies of *A. variabilis* 29413, the strain capable of developing differentiated forms, are characterized by the presence of akinetes. In relation to the possibility of L-transformation in *A. variabilis* 29413, the data are of interest concerning development of L-form-like cells in a mutant of this cyanobacterium, an overproducer of amino acids and auxin (Selyakh et al. 1996).

Thus, in principle, both *A. variabilis* strains are probably able to produce L-forms of the protoplast type. However, no ultrastructural plasticity resulting in CWRF formation was found in model associations of *A. variabilis* 29413 with plant tissues. Apart from tobacco, this applies to associations with alfalfa tissues. For example, in the association of *A. variabilis* 29413 with alfalfa callus tissues obtained by application of cyanobacteria on the surfaces of the medium and the leaf explant and subsequent transfers of the mixed callus, heteromorphic variations observed on the 17th day of associated growth manifested themselves in the different size of the neighboring cells within the filaments (Gorelova et al. 1988; Korzhenevskaya et al. 1993). No CWRF were found on ultrathin sections. In the control monoculture on the 17th day of growth, the cells also varied in size, although single cells or short chain predominated. Another system investigated was regenerated alfalfa plants formed by application of *A. variabilis* 29413 to the callus surface with plantlets-like structures at transition from the medium for organogenesis induction to the medium for plant regeneration. In these plants, cyanobacteria originally appeared at the stem and leaf surface and in the intercellular spaces of the leaf mesophyll and epidermis, but were eliminated in the course of time. During regenerant development, active growth of cyanobacteria occurred at the surface of differentiating roots, where they persisted up to 9 months of the experiment (Skripnikov 1987; Korzhenevskaya 1990). The rhizoplane and root and leaf surfaces were investigated in parallel by SEM and TEM. Ruthenium red was used as a contrasting agent for detection of acidic polysaccharides of the mucus (Baulina et al. 1986, 1989b; Korzhenevskaya et al. 1993). Numerous long cyanobacterial filaments were localized at the root surface, including the root hairs (Fig. 4.23a), as well as close to the root tip (Fig. 4.23b). Cyanobacteria with the morphology and ultrastructure typical of this species were also found in the dead cells of the root epidermis and primary cortex.

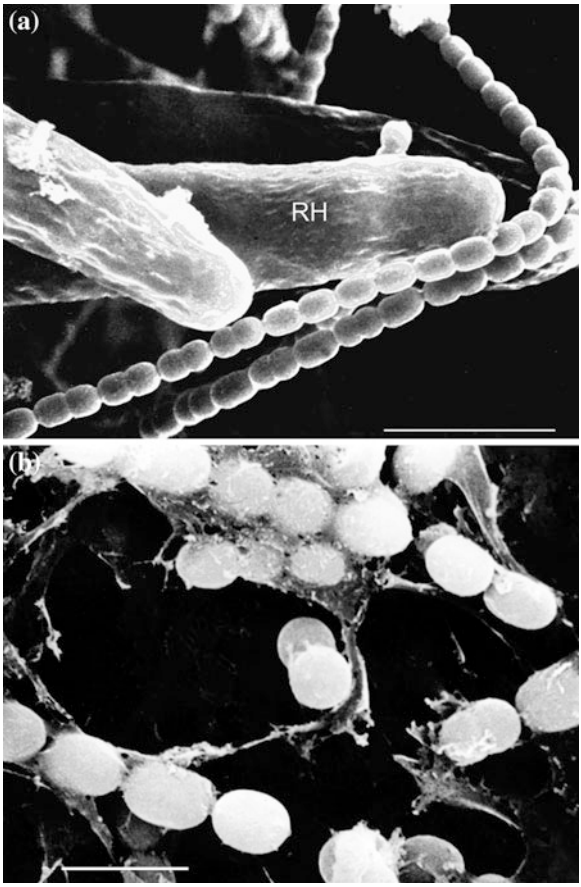

Fig. 4.23 *Anabaena variabilis* ATCC 29413 localized on the surface of an alfalfa regenerant in the zone of root hairs (**a**) (data obtained by Skripnikov and Baulina) and near the root tip (**b**) (Baulina et al. 1989b) (SEM) *RH* root hair. *Scale bar* a, 30 μm; b, 5 μm

The ultrastructural plasticity of *A. variabilis* 29413 in association with plant partners was apparent in the presence of strands of mucous material in the sites less favorable for growth, such as the surfaces of alfalfa leaves and stems, form which cyanobacteria were subsequently eliminated. Growth within a mucous sheath probably protects the cells from unfavorable factors of plant origin. Growth of cyanobacteria in the root zone of suction of regenerated plant was not accompanied by mucus formation (Fig. 4.23a), while it gradually became more pronounced closer to the root tip, where growth and differentiation of the root tissue occurred (Fig. 4.23b). Cyanobacterial chains had a thin cover. In the zone of the root cap, mucus was produced by both partners. The cytochemical reaction (contrasting with ruthenium red during fixation) revealed the presence of acidic polysaccharides in the mucus between the desquamated cells of the root cap and at the surface of cyanobacterial cells (Fig. 4.24a). The fibrils of the mucus with regularly located subunits detected in their structure were perpendicular to the cell surface, their bases blending with the outer membrane. After contrasting with ruthenium red, the electron density of the fibrils and outer membrane was

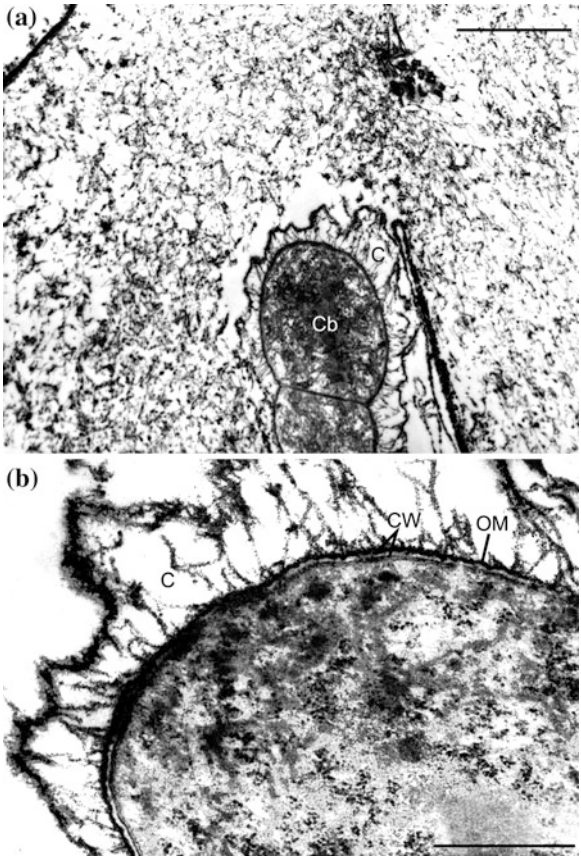

Fig. 4.24 Ultrathin sections of the mucous zone of the root cap of an alfalfa regenerant: localization of *Anabaena variabilis* ATCC 29413 cells in the mucous material (**a**) (data obtained by Baulina and Skripnikov) and fibrils of acidic polysaccharides forming a capsule at the surface of the cyanobacterial cell (**b**) (Reprinted from Korzhenevskaya et al. (1993) with kind permission from Balaban, Philadelphia/ Rehovot) Contrasting with ruthenium red during fixation *C* capsule, *Cb* cyanobacterium, *CW* cell wall, *OM* outer membrane. *Scale bar* a, 2 μm; b, 0.5 μm

significantly higher than that of the poorly determined fibrillar material on the surface of cyanobacterial and plant cells and between the cells in the unstained control. Cyanobacteria grown on the medium for plant morphogenesis, where mucus formation was not detected visually and by SEM, where another control used for identification of the nature of the mucous material. In this case, the fibrils on the surface and enhanced contrast of the outer membrane were not revealed even after contrasting with ruthenium red. Thus, in *A. variabilis* 29413 cells at the root tip of the regenerated alfalfa plant, formation of MSS was established. According to Mamkaeva and Gavrilova, who studied these structures in various *A. variabilis* strains, these structures should be defined as "capsules" (Mamkaeva and Gavrilova 1987). They are simpler in structure than the *C. fritschii* sheaths, being single-layered, and relatively homogeneous, with their fibrillar elements attached to the outer membrane. These features are characteristic of bacterial capsules in general (Gromov 1985). Importantly, similar to other bacterial MSS, the capsules are characterized by the variability of their ultrastructure and chemical composition. They may be present or absent depending on the stage of the life cycle, strain properties, the growth phase of a culture, and conditions of the microenvironment.

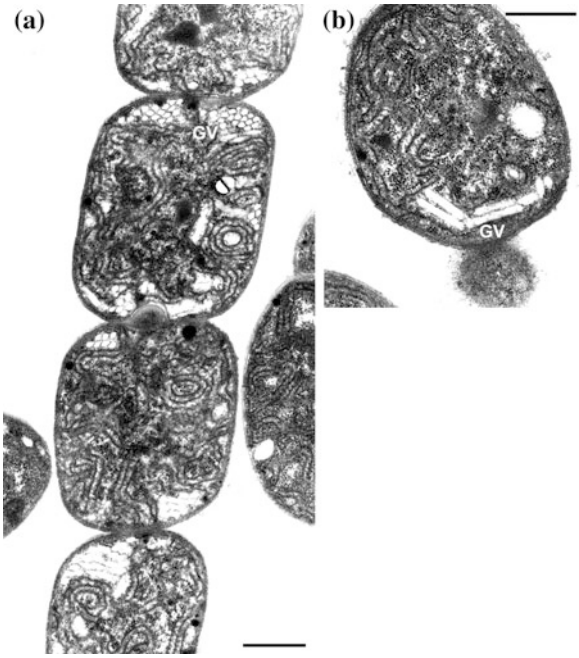

Fig. 4.25 Ultrastructure of *Anabaena variabilis* ATCC 29413 with gas vesicles: GV localized in every cell of the filament (**a**) and longitudal section showing the characteristic GV structure (**b**) (**a**, **b**, data obtained by Skripnikov and Baulina) *GV* gas vesicles. *Scale bar* a, b, 0.5 μm

Contrasting of acidic polysaccharides forming the capsule in *Anabaena* species with ruthenium red and alcian blue was originally carried out in the classical work of Leak for *Anabaena* sp. from the collection of the Indiana State University, United States (Leak 1967). The photographs presented in the article demonstrate that, similar to Fig. 4.24b, the fibrils forming the capsule (or "sheath", according to the author of the article) are of about the same length and are located perpendicular to the cell surface, forming a "brush bristle"-like layer. Detailed investigation of the capsule structure in 10 *A. variabilis* strains demonstrated the diversity of their organization depending on the strain (Mamkaeva and Gavrilova 1987). The fibrils at the periphery of the capsule may stick together forming an electron-dense peripheral layer (Fig. 4.24a, b). This layer separates the mucus of cyanobacteria from the surrounding mucus of the root cap. The mucus of the root cap stained with ruthenium red looks as a fine fibrillar, fine-grained reticulate material. An important conclusion may be therefore made, that the mucus produced by cyanobacteria separates them from the mucus of plant origin, and thus participates in formation of the border zone between the partners at the sites which are less favorable for cyanobacterial growth. It is generally accepted that the synthesis of extracellular polysaccharides is one of the metabolic strategies for survival and growth of cyanobacteria, especially under unfavorable conditions (Navarini et al. 1992).

In one of the control variants used for investigation of the ultrastructural changes in *A. variabilis* 29413 cells associated with alfalfa callus and regenerated plants, formation of gas vesicles was observed, which is not characteristic of this species. Cyanobacteria were grown on agarized Gamborg' B-5 medium for alfalfa

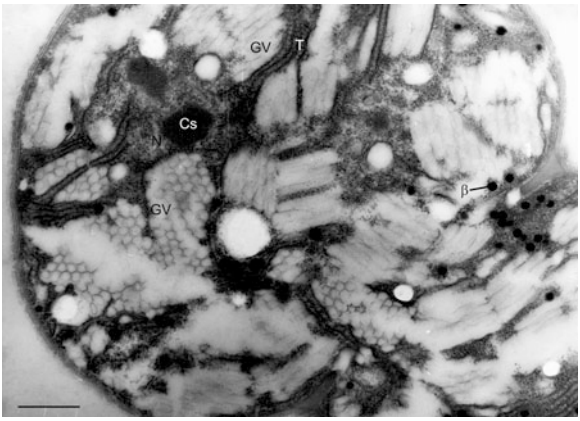

Fig. 4.26 Ultrastructure of the planktonic cyanobacteria *Microcystis aeruginosa* Kütz. emend. Elenk. Cell with gas vesicles (data obtained by Baulina and Dmitrieva) *β* high electron density lipid *β*-granules, *Cs* carboxysome, *GV* gas vesicles, *T* thylakoid. *Scale bar* 0.5 μm

cells without mineral nitrogen (Skripnikov and Korzhenevskaya 1986). Cyanobacterial growth did not occur under these conditions, since the medium for plant cells, as was already stated, is not favorable for these organisms. According to Skripnikov, transition to the Allen–Arnon nitrogen-free medium for cyanobacteria (Allen and Arnon 1955) resulted in renewal of active growth. On the 7th day of cultivation under these conditions, all *A. variabilis* ATCC 29413 cells were found to contain gas vesicles with the typical cyanobacterial organization (Fig. 4.25a, b). They were regularly packed cylinders with tapered ends, surrounded by a membrane 2–3 nm thick. The gas vesicle membranes of cyanobacteria and other prokaryotes have been shown to be a protein monolayer (Walker and Walsby 1983). Gas vesicles are common among *Anabaena* species. They have been well studied in planktonic *A. flos-aquae* (Smith et al. 1969), but have not been observed previously in *A. variabilis*. In planktonic species of cyanobacteria, both marine and freshwater ones, these structures are of high adaptive importance, determining the buoyancy and distribution of the microorganisms at different depths (Walsby 1994). A *Microcystis aeruginosa* Kütz. emend. Elenk. strain from the collection of the Institute of Hydrobiology, Natl. Acad. Sci. Ukraine, causing water blooms with unfavorable environmental consequences in the Kremenchug reservoir, is a good example. The strain was provided for investigation by A. G. Dmitrieva (Biological Faculty of Lomonosov Moscow State University). Even in laboratory culture, many cells of this microorganism contained gas vesicles and are literally "filled up" with them (Avakyan and Baulina 1972) (Fig. 4.26). The morphology and ultrastructure of *M. aeruginosa* Kütz. emend. Elenk has been studied in detail (Reynolds et al. 1981). A similar picture of the cytoplasm filled with gas vacuoles was observed in *Nodularia spumigena*, which is responsible for large-scale water blooms in the Baltic Sea (Gumpert et al. 1987). These structures may also be involved in protection of cyanobacteria from the damaging action of high-intensity light, which causes photooxidative cell decomposition. This statement, however, is open to discussion.

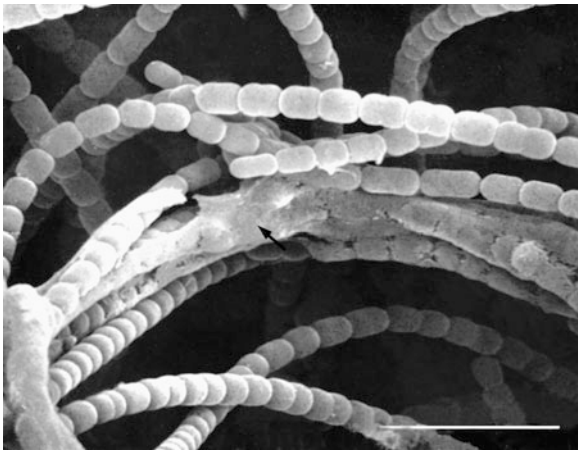

Fig. 4.27 General appearance of the cells in the *Nostoc muscorum* VKM 16 culture grown under optimal conditions, SEM. *Arrow* points to the mucus on the surface of the trichomes. *Scale bar* 12 μm

4.2.4 Nostoc muscorum *VKM 16 in Associations with Callus and Rooted Cuttings of Alfalfa*

The cyanobacterial culture was obtained from the All-Russian Collection of Microorganisms (VKM). Its overall appearance under optimal growth conditions is shown in Fig. 4.27. The species belongs to subsection IV (previously, order Nostocales). This is a filamentous nitrogen-fixing cyanobacterium capable of cell differentiation. Members of the genus *Nostoc* are characterized by complex cycles of development of their populations, with formation of heterocysts, motile trichomes (hormogonia), and dormant akinetes. A member of the genus *Nostoc* was used in the experiments for obtaining model associations with plants, since the microsymbionts of the natural cyanobacterial–plant symbioses belong mainly to this genus (Rai 1990; Vagnoli et al. 1992; Bergman et al. 2008). Ability to function in a symbiosis correlates not only with the capacity for dinitrogen fixation by heterocysts, but also with the high metabolic plasticity of the vegetative cells of these cyanobacteria, which may switch from the photoautotrophic mode of existence to the photoheterotrophic or chemoheterotrophic one and back again. This was, for example, demonstrated for the cyanobionts of the cycad plants (Tredici et al. 1989). Under experimental conditions, stable associations of *Nostoc* sp. (strains 2S3B and 2S9B) with wheat roots (Gantar et al. 1991), *N. muscorum* 304 with nightshade callus tissues (Gorelova et al. 1995), and *N. muscorum* VKM 16 (hereafter referred to as *N. muscorum* 16) with alfalfa cells, tissues, and plants (Gorelova et al. 1988; Korzhenevskaya et al. 1993) were obtained.

The ultrastructural plasticity of *Nostoc* in model associations was studied in the mixed callus culture of alfalfa tissue and *N. muscorum* 16. This system was obtained in the same experimental series in which *A. variabilis* 29413 was investigated (see Sect. 4.2.3, p. 117), also by the application of cyanobacterial suspension on the surfaces of the medium and of the leaf explant, with subsequent transfers of the formed mixed callus. The model system obtained by infecting

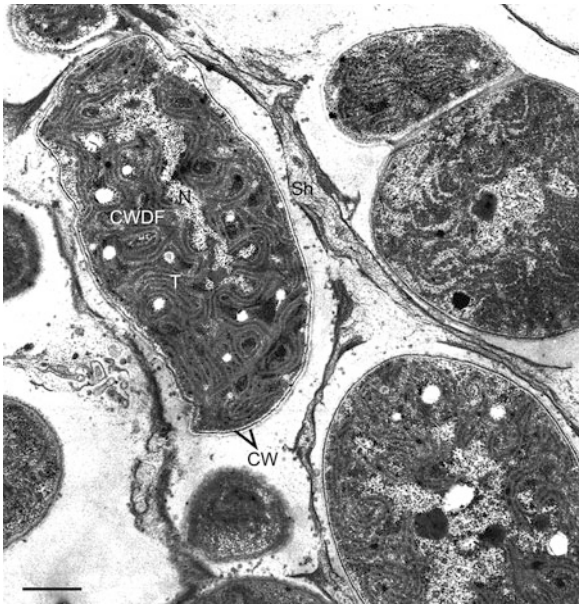

Fig. 4.28 Ultrathin section of a *Nostoc muscorum* VKM 16 aggregate on the surface of a petiole of a rooted alfalfa cutting (data obtained by Gorelova and Baulina) *CW* cell wall, *CWDF* cell wall-defective form, *N* nucleoid, *Sh* sheath, *T* thylakoid. *Scale bar* 0.5 μm

alfalfa plants with *N. muscorum* 16 during the rooting of alfalfa cuttings on solid medium was also used (Baulina et al. 1990; Gorelova 2005; Gorelova et al. 1990; Korzhenevskaya et al. 1993).

During 2 weeks after infection, cyanobacterial growth was not detected visually in alfalfa cuttings. Since the 4th week of cultivation, both the surface growth of *N. muscorum* 16 on the stem and lower leaves and its interstitial growth as microcolonies in the intercellular spaces of the leaf mesophyll were observed. Cyanobacteria growing on the surface of the plant or callus were covered with mucus, which formed an uninterrupted cover over significant areas. Ultrathin sections of the surface cyanobacterial aggregates revealed that individual cells or short filaments shared the sheaths, which formed the peripheral mucous cover (Fig. 4.28). Production of significant amounts of mucus and development of the sheaths surrounding long filaments or grouping together the folded and coiled filaments forming at certain stages of the life cycle is a characteristic feature of the genus *Nostoc* (Boone and Castenholz 2001).

Cyanobacteria growing interstitially in the callus or in the leaf mesophyll were also surrounded by a fibrous, often multilayered material. In the mesophyll of alfalfa leaves, *N. muscorum* 16 formed microcolonies in expanded intercellular spaces between degrading plant cells (Fig. 4.29) similar to both *Anabaena variabilis* strains in regenerated tobacco plantlets. Importantly, in this case degradation also occurred only in the plant cells directly surrounding the microcolonies. As was stated above, dying off of the plant cells immediately adjacent to cyanobacterial aggregates and formation of the specialized harbor was observed in most stable associations at the callus or plant level studied by us and by other authors.

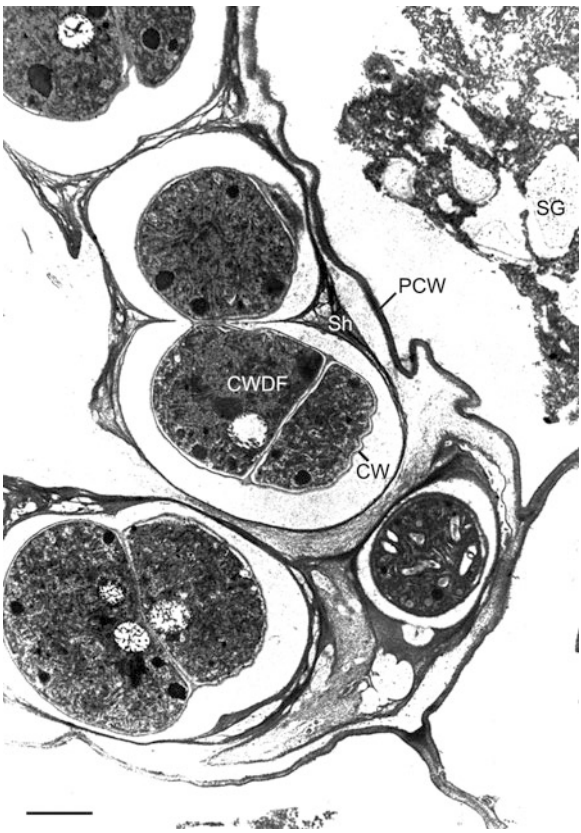

Fig. 4.29 Ultrathin section of a part of a *Nostoc muscorum* VKM 16 microcolony within the leaf mesophyll of a rooted alfalfa cutting (data obtained by Gorelova and Baulina) *CW* cell wall, *CWDF* cell wall-defective form, *PCW* plant cell wall, *Sh* sheath, *SG* starch grain. *Scale bar* 1 μm

The interstitial microcolonies of *N. muscorum* 16 were compact groups of cells with thick multilayered sheaths, packed into the harbor. On ultrathin sections, the fibrillar elements forming the sheath were seen as concentric circles around the cell surface. Contacts between the sheaths bind together the short filaments consisting of the cell forms differing in morphology and ultrastructure (see Fig. 4.29), including spheroplasts and protoplasts (Fig. 4.30). The sheaths often blend together with the surfaces of the plant cell walls, so that the harbor becomes filled with the mucous fibrillar matrix, which, together with the plant cell wall, forms the structure of the boundary zone between the partners. Low-molecular weight products of starch hydrolysis, which may diffuse through the boundary zone of the lysed plant cells surrounding the harbor, are probably additional substrates for mucus formation. The numerous vesicles, which separate from the CM of the protoplasts (Fig. 4.30a), outer membranes of the spheroplasts, and sometimes from the outer membrane of the intact cells (Fig. 4.30b), probably participate in the transport of material in the border zone. The function of transport between the symbionts was originally suggested for similar structures by Peveling, who observed vesicle formation by the cell wall outer membrane in the lichen cyanobionts: *Calothrix* sp., the cyanobiont of *Lichina pygmea*, and *Nostoc* sp., the cyanobiont of *Peltigera canina*,

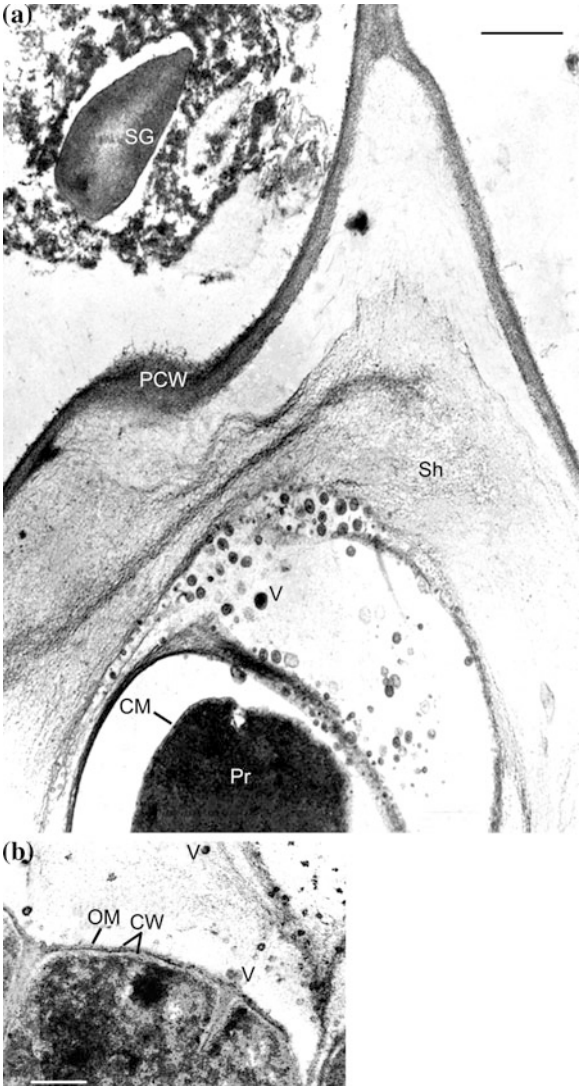

Fig. 4.30 Parts of a *Nostoc muscorum* VKM 16 microcolony within the leaf mesophyll of a rooted alfalfa cutting with a protoplast (**a**) and a vegetative cell (**b**) TEM (reprinted from Korzhenevskaya et al. (1993) with kind permission from Balaban, Philadelphia/ Rehovot) *CM* cytoplasmic membrane, *CW* cell wall, *OM* outer membrane, *PCW* plant cell wall, *Pr* protoplast, *Sh* sheath, *V* vesicle. *Scale bar* a, 0.5 μm; b, 0.2 μm

P. aphthosa and *Collema lingerinum* (Peveling 1973). Participation of the outer membrane vesicles in communications between the prokaryotic and eukaryotic partners within bacteria–plant symbioses has been recently hypothesized (Mashburn-Warren and Whiteley 2006). Immunocytochemical data were obtained on DNA location in such vesicles produced y the symbiotic cyanobacteria of *Azolla microphylla* ferns (Zheng et al. 2009). As was shown in this chapter for the <Nostoc–alfalfa> system, in the model cyanobacterial–plant symbioses, both the outer cell membrane and the CM of cyanobacterial protoplasts are involved in formation of the transport vesicles (Baulina et al. 1990, 2000; Gusev et al. 2002;

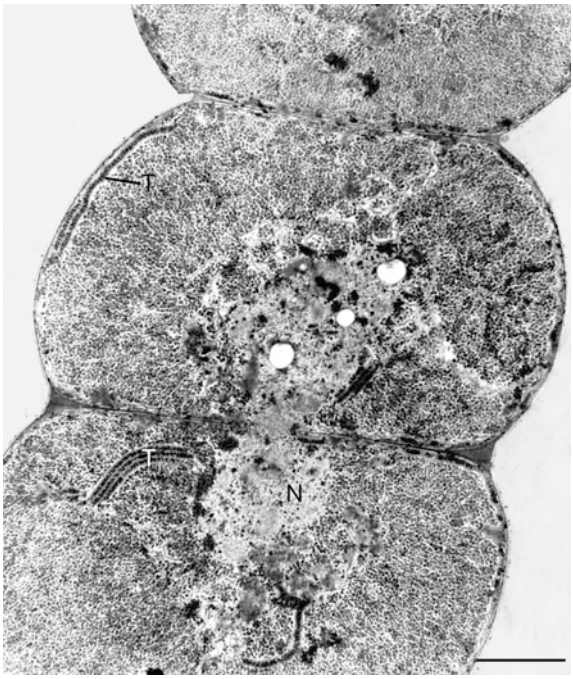

Fig. 4.31 Ultrastructure of *Nostoc muscorum* VKM 16 associated with alfalfa callus (data obtained by Gorelova and Baulina) *N* nucleoid, *T* thylakoid(s). *Scale bar* 1 μm

Gorelova 2005; Korzhenevskaya et al. 1993). We have obtained similar data for natural symbioses as well (see Chap. 5).

The presence of single cell forms and short filaments consisting of anomalously dividing vegetative cells and CWDF, including spheroplasts and protoplasts, was a characteristic feature of *N. muscorum* 16 in the microcolonies of alfalfa leaf mesophyll. Many of them are considered to be transition forms typical of the process of L-transformation, with some protoplasts being L-form-like variants (Baulina et al. 1990; Gorelova et al. 1990). CWDF, specifically, the cells with significantly impaired rigidity of the cell wall peptidoglycan layer, are also characteristic of *N. muscorum* 16 inhabiting plant surfaces, e.g., leaf butt (see Fig. 4.28), and localized on the surface or inside alfalfa callus tissue (Gorelova et al. 1988).

Internal organization of the cells of *N. muscorum* 16 associated with alfalfa plants and callus was also diverse. In many cases, they contained massive accumulations of glycogen, together with reduction of the thylakoid system and its less ordered organization, which is typical of the photo- or chemoheterotrophic cyanobacterial cultures (see Chap. 2). An extreme manifestation of the reorganization of the thylakoid system is its decrease to isolated fragments, as can be seen on the ultrathin sections of callus-associated cyanobacteria (Fig. 4.31). The outlines of the thylakoids were located strictly in parallel, two to four in each stack, with small, closely packed electron-dense granules between them, so that the thylakoid system looks quite unusual. Characteristically, this type of cells has no sheaths.

It should be noted that, unlike *Nostoc* strains, no CWRF were found in *A. variabilis* 29413 associated with plant tissues, including alfalfa callus. While the internal ultrastructure of the *A. variabilis* 29413 cells also corresponded to the heterotrophic mode of existence, the cells remained relatively uniform, without unusually organized thylakoids. Thus, the ultrastructural plasticity of the cells of this strain growing with the tissues of the same plant species was less pronounced than in the case of *N. muscorum* 16. Comparative investigation of the model associations of these two species with alfalfa callus revealed that the system with *A. variabilis* 29413 died off after several months of subculturing, while the system with *N. muscorum* 16 exhibited no tendencies for dying off and could be transferred throughout the long period of the experiment (Gorelova 2005). Thus, in this case the variety of forms of manifestation of the ultrastructural plasticity in different cyanobacterial species correlated with their viability as components of a mixed callus.

Formation of CWDF, including protoplasts and spheroplasts, was observed not only in *N. muscorum* 16 in the systems with alfalfa, but also in *N. muscorum* 304 associated with rauwolfia (Gorelova 2000a, 2001; Gorelova and Korzhenevskaya 2002) and nightshade *Solanum dulcamara* L. (Gorelova 2005) callus tissues. Among the CWDF revealed in the *N. muscorum* 304 population associated with the rauwolfia callus were giant spheroplasts producing the vesicles similar to the elementary bodies formed in the course of L-transformation and amorphous masses typical of bacterial L-form colonies. These data form a solid ground for a concept of the possible L-form production and the adaptive role of this process in cyanobacterial populations within both the model associations and natural symbioses with plant partners.

4.2.5 Chlorogloeopsis fritschii *ATCC 27193 in Associations with Ginseng Cells*

According to the available data, unlike members of subsection IV (previously, order Nostocales), including the genera *Nostoc* and *Anabaena*, cyanobacteria of the genus *Chlorogloeopsis* (subsection V, previously, order Stigonematales) do not occur in symbioses with higher plants (Schenk 1992). Within the order Stigonematales, *Stigonema* and *Mastigocoleus* were identified in lichens (where the species composition of cyanobionts is rather diverse), but not in symbioses with higher plants (Schenk 1992). However, West and Adams successfully used free-living *Chlorogloeopsis* spp. strains, as well as cyanobacteria of the genera *Nostoc* and *Calothrix*, in laboratory reconstructions of symbioses with the mosses of the genera *Phaeoceros* and *Blasia*. In nature, only the *Nostoc* cyanobacteria form symbioses with these mosses (West and Adams 1997).

In the experiments with *C. fritschii,* it was found to form associations with the cells of ginseng (see Table 4.1) (Butenko et al. 1982) and nightshade *Solanum laciniatum* (Gorelova et al. 1984) in suspension cultures, as well as with the rauwolfia callus (Gorelova and Kleimenov 2003).

In natural symbioses with higher plants, cyanobacteria are known to develop mostly under darkening, in specialized tissues and organs, e.g., in the cortical parenchyma of the apogeotropic roots of the cycad plants (see Chap. 5). In the experiments modeling the symbiotic interactions, some associations were therefore grown both under illumination and in the dark, including the *C. fritschii*—ginseng cells system. Importantly, under mixed cultivation in the medium unfavorable for cyanobacteria,[2] growth was observed, unlike the control variant (monoculture in the same medium), which resulted in cell lysis at the early stationary phase (Butenko et al. 1982). In mixed aggregates with ginseng cells, cyanobacteria retained their blue-green coloration throughout the time of combined cultivation (up to 5 months), while unadsorbed cells lost their color by this time, similar to the monoculture. In mixed aggregates, cyanobacteria were in proximity to or in direct contact with the plant cells (Fig. 4.32a, b). The ultrastructure of the latter (Baulina and Lobakova 1986; Lobakova and Baulina 1986; Baulina et al. 1995), together with the results of biochemical analysis (Lobakova et al. 1984), suggested synthesis of the biologically active compounds, namely, panaxosides. As a rule, contacting cyanobacteria were surrounded by sheaths, usually more massive in the case of growth on the surface of plant aggregates in the dark (Fig. 4.32a). In associations growing under illumination, cyanobacteria adsorbed on the surface of the aggregated ginseng cells developed, apart from the sheaths, also mucous cover layers over the groups of cells (Fig. 4.32b, c), which were similar to those occurring in the model associations with *A. variabilis* 29413 and *N. muscorum* 16. The mucus between the cells of *C. fritschii* and ginseng also contained acidic polysaccharides revealed in the preparations fixed with ruthenium red (Baulina et al. 1988a). Formation of the capsules, sheaths, or additional mucous cover layers by various cyanobacterial species during associated growth probably plays an adaptive role as a way of compartmentation within the plant tissues producing biologically active compounds.

In this context, a certain generalization is required. Spatial integration of plant partners and cyanobacteria in model associations is often accompanied by formation of sheaths, capsules, amorphous mucous depositions, or mucous thin covers over the groups of microbial cells, which in (Sect. 2.2.4) were designated as mucous surface structures (MSS). These outermost layers of cyanobacterial envelopes exhibit plasticity of their chemical composition, macromolecular organization, and ultrastructure under varying conditions of the microenvironment. Thus, various factors, including nutrition, probably control alternative production of different types of exopolysaccharides in *Phormidium uncinatum* (Hoiczyk 1998). The ultrastructure and chemical composition of the sheath depends also on the stage of the life cycle and the age of the population. This applies to laboratory cultures as well. The cells in old cultures usually have better developed sheaths than in the young ones (Gantt and Conti 1969). Cyanobacterial species forming individual sheaths for each cell, apart from the general cover of microcolony, may also alternatively produce amorphous

[2] Mixed cultures were grown in diluted medium for plant cells with varying concentrations of sucrose.

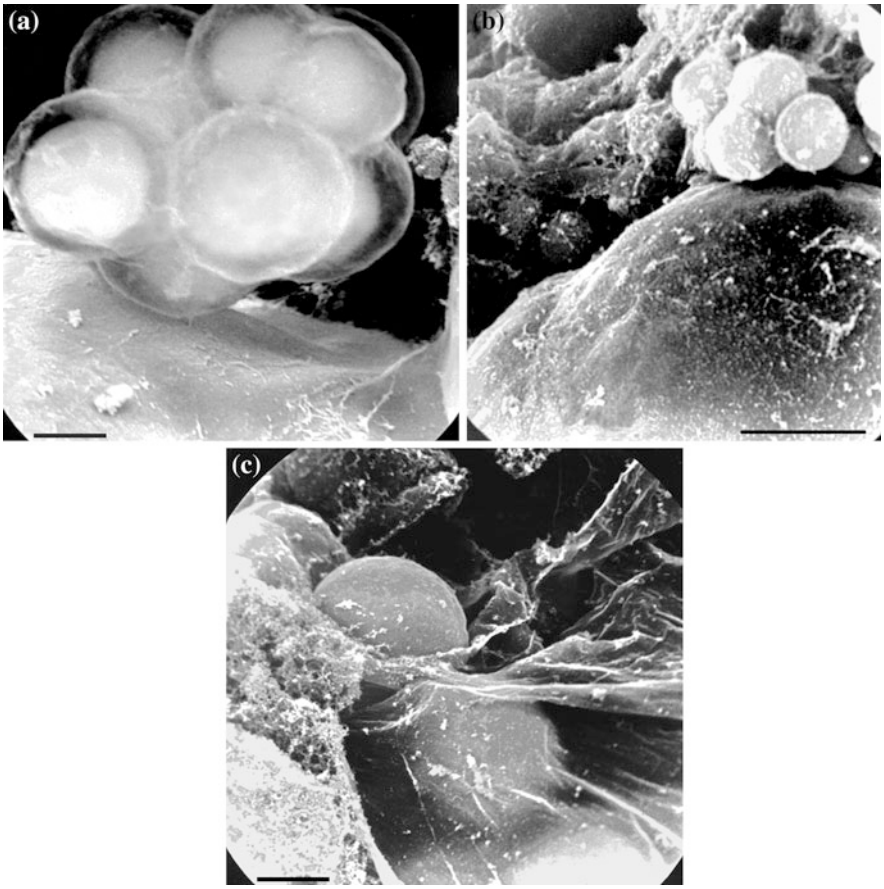

Fig. 4.32 SEM image of *Chlorogloeopsis fritschii* ATCC 27193 associated with ginseng cells in suspension cultures grown in the dark (**a**) and under illumination (**b**, **c**) (Baulina and Lobakova 1986). *Scale bar* a, 2 μm; b, 10 μm; c, 2 μm

mucus, depending on the stage of the culture development. This feature is, for example, pronounced in *Nostoc* species (Bazzichelli et al. 1986). The presence of MSS, as well as of the intercellular matrix, is a form of manifestation of the ultrastructural plasticity both at the cellular and population levels. Their formation may be considered an adaptive modification of the cyanobacterial partner associated with its integration and simultaneous compartmentation in associations with plant cells and tissues, many of which produce biologically active compounds. Formation of MSS in the interstitially developing cyanobacteria is associated with formation of the intercellular matrix, which, together with the plant cell walls, most probably acts as a border zone between the partners (Gusev et al. 2002). Although the presence of mucus in the regions of localization of cyanobacteria is also a characteristic trait of the natural cyanobacterial–plant symbioses, the role of mucus production in the infection process is very poorly studied (Rai et al. 2000). It is, however, evident that

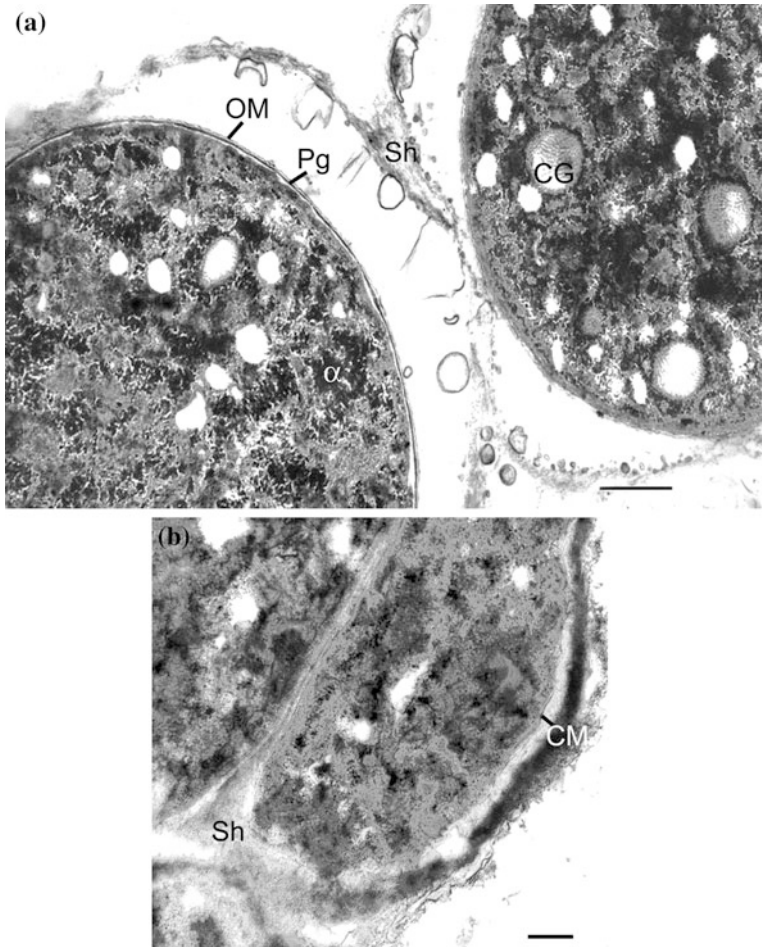

Fig. 4.33 Ultrastructure of *Chlorogloeopsis fritschii* ATCC 27193 growing in association with ginseng cells in suspension culture in the dark: vegetative cells (**a**) and protoplasts surrounded by a sheath (**b**) (**a**, **b**, data obtained by Baulina and Lobakova) α glycogen α-granules, *CG* cyanophycin granule, *CM* cytoplasmic membrane, *OM* outer membrane, *Pg* peptidoglycan, *Sh* sheath. *Scale bar* a, b, 0.5 μm

the functioning of MSS and the intercellular matrix in both model and natural symbioses is related to the permeability of these structures for metabolites, signal molecules, and bactericidal or toxic agents. High-molecular weight arabinogalactan proteins produced by the symbiotically competent *Nostoc* sp. PCC 9229 are an example of such compounds, since they may probably act as signal molecules during formation of symbioses in nature (Bergman et al. 1996; Bateman et al. 1999).

During joint cultivation of *C. fritschii* with ginseng cells, at the late exponential and early stationary growth phases of the first transfer, the ultrastructure of cyanobacterial cells changed, in many respects in accordance with the illumination

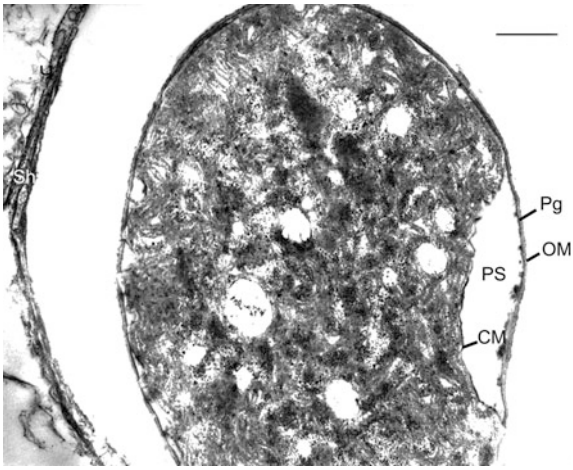

Fig. 4.34 Ultrastructure of *Chlorogloeopsis fritschii* ATCC 27193 cells in a mixed suspension culture with ginseng cells grown in the light (data obtained by Baulina and Lobakova) *CM* cytoplasmic membrane, *OM* outer membrane, *Pg* peptidoglycan, *PS* periplasmic space, *Sh* sheath. *Scale bar* 0.5 μm

conditions (light or darkness), but exhibited no signs of irreversible decomposition (Baulina and Lobakova 1986; Baulina et al. 1989a, b, 1995). Cultivation of *C. fritschii* in the dark in the medium for associations with sucrose resulted in the organization of the thylakoid system similar to that in the chemoheterotrophic cells of the pure culture. Under these conditions, the thylakoids did not form parallel rows, had shortened profiles in the section plane, and were often branching. The cytoplasm usually contained numerous glycogen α-granules, which were extensively formed in the dark due to consumption of the exogenous sucrose (Peat and Whitton 1967) contained in the medium. This picture corresponds to switching of the cell metabolism to the chemoheterotrophic mode (metabolic plasticity). In many cells, glycogen granules were the major structural components of the cytoplasm, often masking the thylakoid profiles (Fig. 4.33a). Endogenous processes of utilization of the synthesized polysaccharide were probably impaired in these cells, similar to hyaline degeneration in eukaryotes. Apart from glycogen, nitrogen is deposited in the cells of such type as cyanophycin granules. This also confirms an imbalance between the processes of substrate accumulation and utilization for constructive metabolism. In associations grown in the light in the medium with significantly lower sucrose content, accumulation of glycogen and cyanophycin was minimal. The ultrastructure of the cell content, including the nucleoid, was typical of the cells actively growing under optimal conditions (including illumination conditions) in pure culture in mineral media. Both in the light and in the dark, the organization of the surface cell structures changed, with, for example, partial degradation of the cell wall peptidoglycan layer resulting in its irregular thinning (Fig. 4.33a) and development of pore-like perforations. After numerous transfers in the dark, peptidoglycan was not revealed at certain areas of the surface and in the case of some cells it was probably absent (Fig. 4.33b). In the same cells, the outer membrane was also not revealed, i.e., they were protoplasts surrounded by sheaths, as is typical of *C. fritschii* (and as was described in Chaps 2, 3). In this case, however, formation of protoplasts should not be considered a specific trait of an associated culture, since

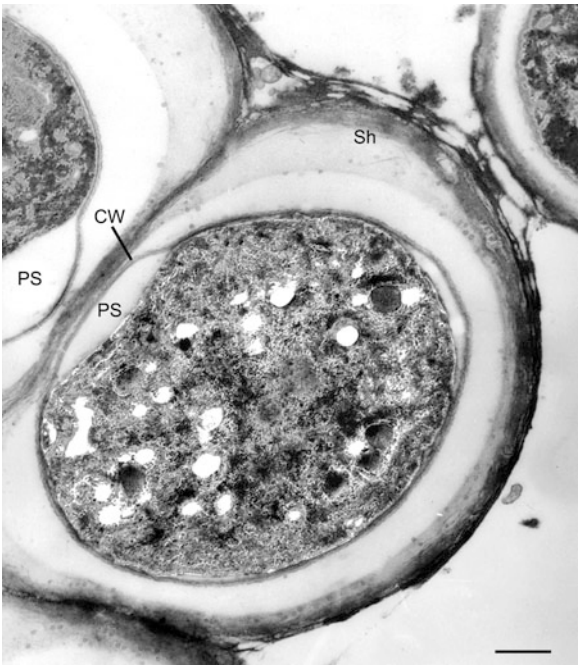

Fig. 4.35 Ultrastructure of *Chlorogloeopsis fritschii* ATCC 27193 cells in the 9th transfer in the medium with penicillin (data obtained by Baulina, Semenova and Mineeva) *CW* cell wall, *PS* periplasmic space, *Sh* sheath. *Scale bar* 0.5 μm

they were also found in the degrading monoculture of this cyanobacterium grown under illumination. Moreover, CWRF were found in *C. fritschii* grown in a mixed suspension culture with nightshade *S. laciniatum* cells (Gorelova 2005). These observations confirm the conclusion made in the previous chapters concerning the high ultrastructural plasticity of *C. fritschii*, which is probably able to exist as protoplasts or protoplast-like L-forms under various conditions.

C. fritschii grown in the light in association with ginseng cells is characterized by development of plasmolysis in many cells, which retain the relative position of the cell wall layers (peptidoglycan and the outer membrane) over the whole cell surface (Fig. 4.34). The sheaths of the plasmolyzed cells usually show signs of degradation: uneven thickness, disorganized fibrillar packing, and ruptures. In some cells of the dark-grown association, insignificant plasmolysis also occurred. Since this cyanobacterium is able to grow both photoheterotrophically and chemoheterotrophically in the presence of sucrose, the latter is most probably not responsible for plasmolysis. The factors causing plasmolysis may be diverse. In the light-grown association of *C. fritschii* with ginseng cells, it may be the ionic composition of the medium unfavorable for cyanobacteria, i.e., the presence of potassium ions and sodium deficiency. Special investigation is required to answer the question. Importantly, plasmolysis occurred in *C. fritschii* cells after 9 transfers in the medium with penicillin,[3] an efficient inducer of L-form development in a

[3] In these experiments, L-form-like colonies were not obtained.

Table 4.2 Manifestations of the ultrastructural plasticity of cyanobacteria in model associations with plant partners

Species and strains	Manifestations of the ultrastructural plasticity				
	Subcellular level				
	Diversity in the organization of the mucous surface structures	Changed ultrastructure of the cell wall peptidoglycan	Vesicle formation by the cytoplasmic membrane	Vesicle formation by the outer membrane	Changes in the thylakoid configuration
Synechococcus sp. PCC 6301					
Synechococcus sp. PCC 6301 (mutant bio-2 1142)					
Anabaena variabilis CALU 458					
A. variabilis ATCC 29413					
Chlorogloeopsis fritschii ATCC 27193					
Nostoc muscorum VKM 16					
	Cellular level				
	Presence or absence of the mucous surface structures	Absence of the peptidoglycan layer	Absence of the outer membrane	Anomalous position of the septum	Changed amount of glycogen α-granules
Synechococcus sp. PCC 6301					
Synechococcus sp. PCC 6301 (mutant bio-2 1142)					
Anabaena variabilis CALU 458					
A. variabilis ATCC 29413					
Chlorogloeopsis fritschii ATCC 27193					
Nostoc muscorum VKM 16					
	Population level				
	Presence of the intercellular mucous matrix or cover layers	Presence of the forms with impaired structure of the peptidoglycan layer	Presence of the cells with anomalous size, shape, or location of the septa	Presence of the cell wall- reduced forms (spheroplasts and protoplasts)	Presence of the cells with the changed organization of the intracellular structures
Synechococcus sp. PCC 6301					
Synechococcus sp. PCC 6301 (mutant bio-2 1142)					
Anabaena variabilis CALU 458					
A. variabilis ATCC 29413					
Chlorogloeopsis fritschii ATCC 27193					
Nostoc muscorum VKM 16					

number of bacteria (Fig. 4.35), as well as in *A. variabilis* 458 transferred in the medium with lysozyme in the course of experiments of induction of L-transformation. Moreover, plasmolysis was seldom observed in *A. variabilis* 458 cells inside the vacuoles of tobacco callus cells.

4.3 Concluding Remarks

Various manifestations of the ultrastructural plasticity were revealed in laboratory cyanobacterial strains of different metabolic capacities in model associations with the cells, tissues, and organisms of non-symbiotrophic plant species. The variety of the forms manifestation of the ultrastructural plasticity at the subcellular, cellular, and population levels corresponds to the metabolic potential of the cyanobacterial species studied (Table 4.2, marked by different color). Analysis of the results presented in this chapter and summarized in Tables 4.1 and 4.2 suggests that the variety of the forms of manifestation of the ultrastructural plasticity is related to the different capacity of these cyanobacteria for formation of the model cyanobacterial–plant associations. The lowest degree of the ultrastructural plasticity in associations with plant tissues was found in the wild and mutant strains of *Synechococcus* sp. 6301, which formed associations incapable of prolonged subcultivation. In other cyanobacteria, the variety of the forms of manifestation of the ultrastructural plasticity in associations was determined, apart from the physiological characteristics of the species and strain, also by the specific cultivation conditions, microenvironment in the zones of their localization, and the interactions between partners in every specific system. A clear tendency can be seen to the domination of the *N. muscorum* strains in the degree of correspondence between their ultrastructural plasticity and ability to form associations with plant partners. It is important in this respect that the highest diversity of cell forms, including giant cells, mini-cells, and CWDF (including the giant spheroplasts and the ultramicroscopic forms produced by them and corresponding to the elementary bodies formed during L-transformation), was found in *N. muscorum* CALU 304 associated with the rauwolfia cultured tissues (Gorelova and Korzhenevskaya 2002; Gorelova 2005). It was shown that the cellular heteromorphism of cyanobacteria increased with increased duration of the interaction with the plant partner, with the ratio of spheroplasts and protoplasts reaching up to 30 % of the whole population (Gorelova 2001).

Detection of the ultrastructurally integral protoplasts of two *A. variabilis* strains (CALU 458 and ATCC 29413) with an indication of division by constriction is of significant interest. In the first case, they were revealed in the microcolonies within tobacco callus tissue, in the second one—in the colonies growing on the medium for associations unfavorable for cyanobacteria, close to the "mixed" callus of the same sort of tobacco (see Table 4.1). Importantly, such forms were not detected in the special experiments on the enzymatic induction of L-transformation in strain CALU 458 (see Chap. 3). Thus, while cyanobacteria of these *A. variabilis* strains are probably capable of transformation into the L-form-like protoplasts, the special conditions required for induction of this process were not created or were impossible to create in the experiments with lysozyme as an inducer of L-transformation. The limited applicability of the ultrathin sectioning method for investigation of large volumes of material should be, however, considered for the sake of correctness.

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Chapter 5
Ultrastructural Plasticity of Cyanobacteria in Natural Symbioses with Plants

Abstract This chapter is dedicated to investigation of ultrastructural plasticity cyanobacteria in natural symbioses with liverwort *Blasia pusilla* and several species of cycad plants. According to the result presented therein, morphological and structural features of cyanobacteria in symbioses with higher plants suggest that these microorganisms possess previously unstudied or unknown physiological traits which appear within symbiosis. The evidence obtained in studies of *Nostoc* in natural symbioses as well as in model associations with higher plants together with the data on formation of L-form-like cells in other species reviewed in the book outlined a new field of symbiology dealing with the occurrence of L-transformation of cyanobiont in symbioses. Furthermore, diverse functional specificity of the CWDF within cyanobiont populations was determined. Special attention is paid to the novel phenomenon of the mass deposition in cytoplasm and transport to vesicles formed by cytoplasmic membrane of exopolysaccharides of symbiotic cyanobacteria featuring defective cell wall. Results presented in this chapter suggest that specialized cells including vegetative forms and heterocysts in populations of cyanobacteria adapted to the conditions of life in symbiosis with plants presumably have modified mechanisms of the synthesis and export of these compounds.

5.1 Cyanobacteria in Natural Symbioses with Plants

Cyanobacteria in symbioses with eukaryotic organisms are an interesting and promising object for investigation of the range of the possible structural and functional modifications and the morphological diversity of prokaryotes under specific conditions of the microenvironments within the cells and/or tissues of macrosymbionts. Due to their prokaryotic nature and therefore higher plasticity,

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cyanobacteria undergo structural and functional modifications, which are quite considerable in a number of symbiotic systems.

Cyanobacteria are known to be able to form symbioses with taxonomically very diverse higher plants, namely, bryophytes (liverworts and hornworts), the *water* ferns of the genus *Azolla*, the gymnospermous plants of the order *Cycadales*, and the angiospermous plants of the genus *Gunnera.* Each of these groups of symbioses possesses individual characteristics of the functioning and anatomical organization of the interstitial symbiotic zones. Taxonomy of cyanobacteria forming symbioses with higher plants is, however, rather conservative: as a rule, they belong to the genus *Nostoc*. Excluding the case of the fern *Azolla*, these symbioses are not strictly specific and are formed de novo in every new generation of the macrosymbiont (Rai 1990; Rai et al. 2002).

The metabolic plasticity of symbiotic cyanobacteria manifests itself mainly in significant enhancement of dinitrogen fixation and a significant decrease or complete suppression of the photosynthetic activity (Rai et al. 2000). In the course of formation and functioning of the symbioses, various options for cyanobacterial cell differentiation are realized, so that motile forms (hormogonia), nitrogen-fixing cells (heterocysts), and in rarer cases dormant akinetes are formed. In the vegetative cells, reproduction is usually suppressed and morphology is changed: unicellular individuals predominate, cell size is increased, cell shape is different from that of free-living cyanobacteria, and in some cases thinning of the cell wall peptidoglycan layer occurs (Meeks 1998; Rai et al. 2000). Due to the traits of their structure and growth, as well as, in some cases, due to their increasing numbers in the aging symbiotic tissues, most researchers consider unusually shaped vegetative cells as an indication of degeneration of the cyanobiont (Duckett et al. 1977; Obukowicz et al. 1981; Towata 1985; Söderbäck et al. 1990; Johansson 1994; Johansson and Bergman 1992). For example, *Nostoc* sp. cells of unusual shape, with curved cell walls predominate at the late stages of infection of the *Gunnera magellanica* stem glands (Söderbäck et al. 1990). These cells possess electron-dense cytoplasm with numerous densely packed membranes and do not contain granules of storage materials. Degenerating protoplasts of cyanobionts were observed in the cycads (Grilli Caiola 1980; Grobbelaar et al. 1988; Joubert et al. 1989). Moreover, Towata observed numerous osmiophilic *Nostoc* sp. protoplasts in the apical parts of *Gunnera kaalensis*, i.e., in the young symbiotic tissues(Towata 1985). Curved cyanobacterial cells within the narrow intercellular spaced were also reported for *G. chilensis* at the initial stages of the infection (Johansson and Bergman 1992). Our investigation demonstrated that development of L-forms and unusual morphotypes of heterocysts and the vegetative cells was possible in cyanobiont populations (Baulina et al. 2011; Baulina et al. 2000a; Baulina and Lobakova 2003a, b; Gorelova et al. 1992, 1996; Gusev et al. 2002; Korzhenevskaya et al. 1993). These data are reported in detail in the present chapter, where they are interpreted from the point-of-view of the modern knowledge of the ultrastructural plasticity of cyanobionts and its adaptive role.

Fig. 5.1 The liverwort thallus on the soil (the photo was kindly provided by Gorelova). *Scale bar* 2 cm

5.1.1 *Ultrastructure of Cyanobacteria in Symbiosis with the Liverwort* Blasia pusilla

Fine structure of *Nostoc* sp. in symbiosis with the liverwort *Blasia pusilla* L. has been investigated by few researchers (Duckett et al. 1977), although many aspects of the physiology of this system have been thoroughly studied (Adams and Duggan 2008). For our work (Gorelova et al. 1996), the liverwort thalli (Fig. 5.1) were collected from the soil in the open moistened sites of a mixed forest in the Moscow region (Zvenigorod Biological Station, Moscow State University). In *B. pusilla*, most of cyanobacteria were localized within the specialized cavities of the thallus, as is typical of this symbiosis (Duckett et al. 1977; Kimura and Nakano 1990) (Fig. 5.2a–c). Cyanobacteria filled the volume almost completely, forming a compact microcolony (Fig. 5.2c). The vegetative cells of *Nostoc* sp. were single or formed short chains. Small cells 1–1.7 μm in diameter were not numerous. The cyanobiont population usually contained numerous heterocysts (see Sect. 5.1.2). Cyanobacteria inhabiting the intracavitary microcolony were characterized by the presence of protoplasts with the intact cell structure (Fig. 5.3a, b). All cell forms were immersed in the mucous intercellular matrix. Importantly, the ultrastructure of the protoplasts indicated their interaction with the intercellular matrix. While the fibrils of the matrix were in some sites in close contact with the surface, the CM was involved in formation of the vesicles with electron-dense content, 150–200 nm in diameter. Similar vesicles were also localized in the adjacent intercellular matrix (Fig. 5.3b).

Internal structure of the protoplasts was similar to that of the vegetative cells. The system of thylakoids, grouped in stacks with numerous ribosomes and compact nucleoid zones between them, was well developed (Fig. 5.3b). No deposition of the metabolic products, which is typical of cyanobacteria, was usually found in the protoplasts.

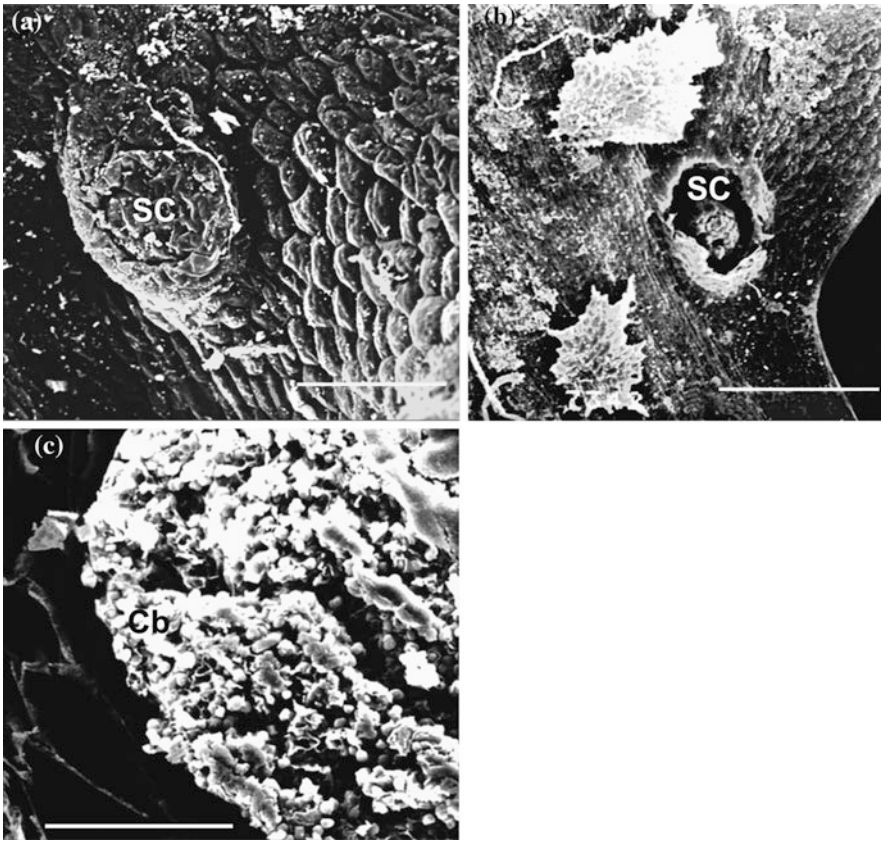

Fig. 5.2 SEM image of the surface of the liverwort *Blasia pusilla* with specialized cavities: overall view of a cavity (**a**) and sections of cavities with the microcolonies of the symbiotic cyanobacteria *Nostoc* sp. (**b**, **c**) (**a–c**, data obtained by Gorelova and Baulina) *Cb* cyanobacteria, *SC* specialized cavity in the liverwort thallus. *Scale bar* a, 0.15; b, 0.8; c, 60 μm

The ultrastructurally intact protoplasts were often found in the microcolonies (clusters) characteristic of *Nostoc* sp. f. *Blasia* in the first two transfers after isolation on solid media (Gorelova et al. 1996). They occurred in almost every cluster in the microscope field. The clusters (6–15 μm in diameter) were formed by densely packed cells and short filaments, often curved due to anomalous position of the septa, which were covered with a common mucous sheath with a pronounced peripheral layer (Fig. 5.4a). Within the clusters, many protoplasts became elongated, as if pressed between the neighboring vegetative cells (Fig. 5.4b). Importantly, both in the clusters and in the intracavitary microcolonies, the cyanobacterial cells were immersed in the fibrillar matrix of the colony sheaths, which was ultrastructurally similar to the intercellular matrix of the thallus cavities. All protoplasts produced numerous vesicles into the surrounding matrix

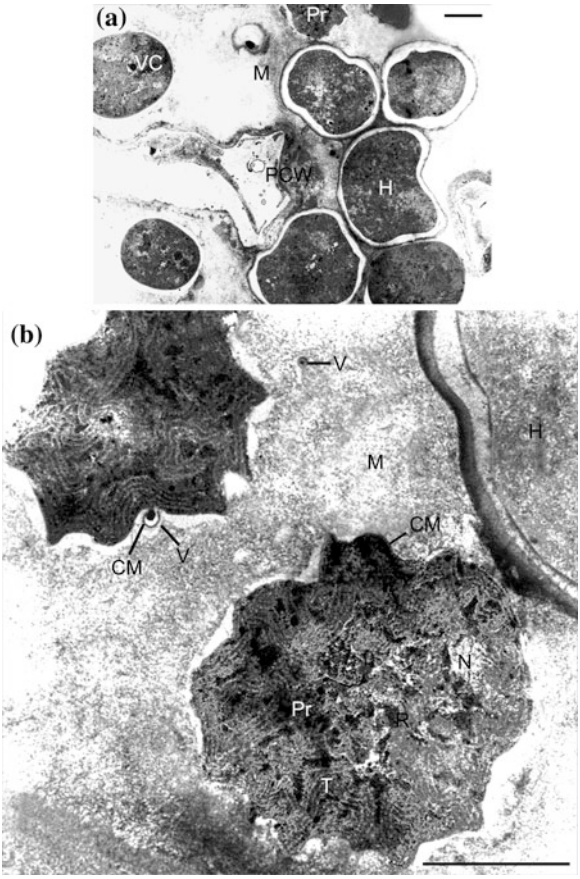

Fig. 5.3 Ultrathin sections of the *Nostoc* sp. intracavitary colony in the *Blasia pusilla* thallus: cell diversity within the cyanobiont population (**a**) (reprinted from Korzhenevskaya et al. (1993) with kind permission from Balaban, Philadelphia/ Rehovot) and cyanobiont protoplasts (**b**) (data obtained by Gorelova and Baulina) *CM* cytoplasmic membrane, *H* heterocyst, *M* intercellular mucous matrix, *N* nucleoid, *PCW* plant cell wall, *Pr* protoplast, *R* ribosomes, *T* thylakoids, *V* vesicle, *VC* vegetative cell. *Scale bar* a, b, 1 μm

(Fig. 5.4c). The peripheral layer of the microcolony sheath had a denser package of the fibrils, as did the fibrillar layers separating cyanobacterial chains, cells identified on the sections as single ones, and the nearby protoplasts (Fig. 5.4b). Acidic polysaccharides, which were specifically stained with ruthenium red during fixation by Luft (1971; Geyer 1973), were abundant at the surface of the peripheral layer. Since ruthenium red poorly penetrates within tissues, the underlying matrix was stained to a lesser degree. However, in the clusters where the peripheral compacted layer was absent, the entire sheath was revealed as an accumulation of the fibrils of acidic polysaccharides (Gorelova et al. 1996).

Extensive experimental material has been accumulated which indicates that exopolysaccharides are the major components of cyanobacterial MSS, namely, the capsules, sheaths, depositions of amorphous mucus, etc. (see Sect. 2.2.4). These exopolysaccharides (glycans) include neutral sugars (such as glucose, galactose, and mannose) and uronic acids (mainly glucuronic and galacturonic), as well as

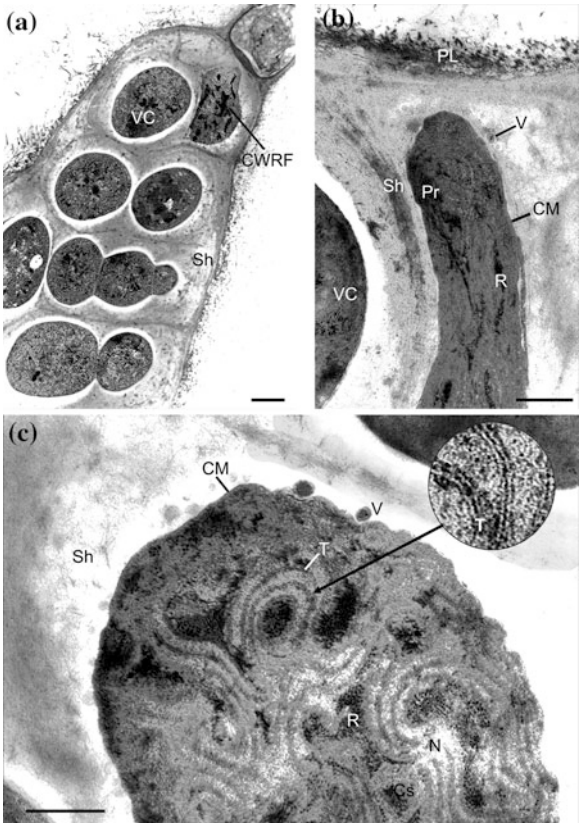

Fig. 5.4 Ultrastructure of *Nostoc* sp. cells isolated from the cavities of the *Blasia pusilla* thallus: overall view of a microcolony (cluster) (**a**) (reprinted from Gorelova et al. (1996) with kind permission from Pleiades Publishing, Ltd.,) part of the microcolony with a vegetative cell and a protoplast, staining with ruthenium red (**b**) (data obtained by Baulina and Gorelova), and a protoplast in the microcolony (**c**) (Reprinted from Korzhenevskaya et al. (1993) with kind permission from Balaban, Philadelphia/ Rehovot) *CM* cytoplasmic membrane, *Cs* carboxysome, *CWRF* cell wall-reduced form of vegetative cell, *N* nucleoid, *PL* peripheral layer of the sheath, *Pr* protoplast, *R* ribosomes, *Sh* sheath, *T* thylakoid, *V* vesicle, *VC* vegetative cell. *Scale bar* a, 1 μm; b, c, 0.5 μm

their derivatives containing amine, methyl, and sulfate groups (e.g., *N*-acetylglucosamine) (Dunn and Wolk 1970; Wang and Tischer 1973; Jürgens and Weckesser 1985; Weckesser et al. 1988; Hill et al. 1994; Reddy et al. 1996; Shah et al. 2000; Vicente-García et al. 2004; Kanekiyo et al. 2005). Due to the presence of negatively charged groups, primarily carboxyl groups, MSS polysaccharides are polyanions, which can form hydrated gels. Particular attention is paid scientists gelling property of exopolysaccharides marine cyanobacteria, from the perspective of their application for the absorption of metal cations from industrial wastewaters (Shah et al. 2000)

The gel matrix of the sheaths contains the so-called water stress proteins (Wsp), which participate in carbohydrate modification during drying, as well as the pigments protecting the cells from ultraviolet radiation, both the lipophilic (scytonemin) and hydrophilic ones (Hill et al. 1994).

The protein components found in the sheaths of many cyanobacteria may be the components of proteoglycans (Painter 1995). The gels formed by proteoglycans are thought to play a regulatory role, controlling the movement of the molecules, since they act as filters with different pore sizes and charge densities.

Proteoglycans perform this function in the basal membrane of the renal glomerulus, which filter the molecules from the blood flow into the urine (Alberts et al. 1994). Significant effect of the outer gel-forming polysaccharide layers on molecular diffusion into and out of the cell has been previously suggested for bacteria (Dudman 1977).

The presence of sulfated sugars in some acidic polysaccharides of the microcolony sheaths of free-living *N. muscorum* suggested the similarity between cyanobacterial sheaths and the cartilage matrix of the animals, where a glucosaminoglycan chondroitin sulfate is the major component (Abdelahad et al. 1984). This conclusion is important for investigation of the structural organization and the intercellular interactions in microbial populations. Similarity in the chemical nature of bacterial extracellular polymers and animal extracellular matrix indicates the analogous functions of these polymer structures binding the individual cells, which, apart from their other functions, are involved in the regulation of the transport of metabolites between the symbionts.

The presence of galacturonic acid and capacity for gel formation likens the glycans of cyanobacterial MSS to acidic pectins (rhamnogalacturonans) of the plant cell wall (Parker et al. 1996; Hoiczyk and Hansel 2000; Baulina et al. 2008; Baulina et al. 2000b). Functioning of the MSS and of the intercellular matrix in the model and natural cyanobacterial-plant symbioses is evidently associated with the permeability of these structures for metabolites, signal molecules, and bactericidal or toxic agents, some of which belong to macromolecules (proteins or glycoproteins). Polysaccharides of cyanobacterial sheaths, like other hydrated gels, should not prevent free diffusion of hydrophilic macromolecules of a size allowing penetration through the gel lattice. Since the permeability of plant cell walls for macromolecules is believed to be determined by the porosity of the pectin matrix (Baron-Epel et al. 1988), there is a solid ground to apply the procedures developed for plant objects in the study of the permeability of cyanobacterial MSS. Analysis of the existing methods showed that the technique for determination of the permeability range by fractionation of polydisperse dextrans developed by Woehlecke and Ehwald and used for plant and yeast cell walls could provide some information (Woehlecke and Ehwald 1995). The possibility of free diffusion of the neutral hydrophilic dextran molecules with the size (Stokes radius r_s) from 1.5 to 9 nm in the MSS was established using this method for the cyanobacteria described in this book (Baulina et al. 2000b; Baulina et al. 2008). Such molecular size corresponds to proteins with molecular masses from 12.4 kDa (cytochrome c with $r_s = 1.64$ nm) to 669 kDa (thyreoglobulin with $r_s = 8.5$ nm), according to (Woehlecke 1996).

The similarity of the MSS polymers of some cyanobacterial species both to the components of the animal connective tissue and to the plant cell walls is increased by the fact that, apart from the major components, oxyproline was found in the sheaths, e.g., of *Scytonema myochrous* status *petalonemoides* (Fjerdingstad et al. 1979). This unique amino acid is a component of the specific glycoproteins of plant cell walls (extensins) and of collagen, which is the basis of the animal connective tissue. Hydroxyproline is known as a marker molecule of some plant lectins (Korolev 1984). In early publications, the presence of cellulose in the

sheaths of *Nostoc* was suggested (Leak 1967; Drews 1973). By now, this suggestion has been experimentally confirmed for nine species (including *Anabaena* sp. PCC 7120 and *N. punctiforme* ATCC 29133), which belong to three of the known five subsections of cyanobacteria (Nobles et al. 2001). In *Phormidium uncinatum*, neutral polysaccharides were found, which, however, differed from cellulose in their sugar composition (Hoiczyk 1998). Uronic acids were not identified in the sheaths of these cyanobacteria.

Detection of the ultrastructurally intact protoplasts in the cyanobiont populations inhabiting the specialized symbiotic structures (thallus cavities) of *B. pusilla* and in the cell clusters of the cyanobacterium after isolation indicates that under unusual conditions of existence within plant tissues some vegetative cells may transform into the L-form-like variants. Emergence of protoplasts among cyanobionts inhabiting the symbiotic plant structures probably improves the possibility for the intercellular exchange of metabolites, including macromolecules, for the population as a whole, probably via the mechanism of vesicular transport, which is alternative to free diffusion through the intercellular gel matrix (Gusev et al. 2002). It should be noted that protoplasts, rather than the vegetative cells, form cytoplasmic projections and produce numerous vesicles into the matrix.

After transfer of *Nostoc* sp. f. *Blasia* cultures from solid media into liquid ones and cultivation under the conditions optimal for cyanobacteria, formation of clusters ceased and the colony sheaths were therefore lost. Filamentous forms without protoplasts became predominant (Gorelova et al. 1996). This is an indication of the reversible nature of the ultrastructural changes (at least at the population level), and therefore of an adaptive character of the cellular and population reorganizations of cyanobacteria within a symbiosis, including the possible reversion of unstable L-form-like variants to vegetative cells. Moreover, this is in accordance with the above-mentioned suggestion (see Chaps. 2 and 3) that the fibrillar matrix creates favorable osmotic conditions for the preservation of integrity of the protoplasts.

Thus, the presence of protoplasts, together with the vegetative cells and heterocysts, within the symbiosis and shortly after isolation is both a manifestation of the ultrastructural plasticity of *Nostoc* sp. f. *Blasia* cells and populations and a characteristic trait of this particular strain, specifically, its ability to exist as a heterogeneous population in which some cells have a reduced cell wall. Moreover, we consider a cell cluster containing the vegetative cells and L-form-like variants bound with the extracellular matrix as a persistent structural unit of the supracellular level (Gorelova and Baulina 2009). This conclusion was based on the studies of persisting populations of this strain during prolonged storage in the dark at decreased temperature.

Blasia pusilla was not the only natural symbioses with protoplasts in cyanobiont colonies. Ultrastructurally diverse CWRF were also found among the cyanobionts of the cycads.

5.1.2 The Diversity of Cyanobacterial Cell Types in Symbioses with Cycads

Prior to our studies, the ultrastructure of the cyanobionts of the plants of the order Cycadales has been investigated in a series of works. The species studied were *Macrozamia communis* (Nathanielsz and Staff 1975; Grilli Caiola 1974, 1980), *Cycas revoluta* (Obukowicz et al. 1981; Grilli Caiola 1980), *Cyc. circinalis* (Grilli Caiola 1980), *Zamia shinneri* (Lindblad et al. 1985), *Encephalartos altensteinii* (Grilli Caiola 1975a), *E. arenarius, E. transvenosus, E. woodii* (Joubert et al. 1989), *E. villosus* (Grilli Caiola 1980), *Dioon edule* (Grilli Caiola 1975b, 1980) and some other *Encephalartos* species (Grilli Caiola 1980; Grobbelaar et al. 1988). The ultrastructure of the cyanobionts of the cycads was studied in most detail and for the greatest number of species in the works of Grilli Caiola.

Cycads are the relic angiosperms of the class Cycadopsida, which emerged at the same time as seed tree ferns (Fig. 5.5). The class Cycadopsida presently contains the only order Cycadales with about 150 species. All known members of this order form symbioses with cyanobacteria (Rai et al. 2000). This chapter presents the new data obtained by electron microscopic investigation of the cyanobionts *Cyc. circinalis* L., *Cyc. revoluta* Thunb., *Cyc. micholitzii* Dyer, *Ceratozamia mexicana* Brongn., and *E. villosus* Lehm. (Baulina et al. 2000a; Lobakova and Baulina 2001; Baulina and Lobakova 2002, 2003a, b, c; Lobakova 2004; Baulina et al. 2009; Baulina et al. 2011).

The plants grown under the conditions of tropical and subtropical climate in the greenhouses of the Tsitsin Main Moscow Botanical Garden, Russ. Acad. Sci., were kindly provided by the workers of the Department of Tropical and Subtropical Plants in 1995–2004.

Cycads like other plants capable of forming symbioses with cyanobacteria are characterized by the development of dichotomously branched, apogeotropic roots with limited growth and characteristic organization also called coralloid roots for the appearance similar to corals (Fig. 5.6). The symbiotic cyanobacteria which belong mostly to the genus *Nostoc* are localized mainly in the cortical parenchyma apoplast. The cortical parenchyma colonized by symbiotic cyanobacteria is characterized by the development of a specific mucilage zone shaped as a cylinder spanning the entire coralloid root except for its apex (Fig. 5.7). The cyanobiont population developed in the intercellular spaces of the specialized parenchymal zone as microcolonies (Fig. 5.8). In all parts of the root—the apical (1–3 mm from the tip), central (4–10 mm from the apex), and basal ones,—the microcolonies were heterogeneous in cell morphology and ultrastructure. They contained vegetative cells and heterocysts organized in chains, single cells, and ultrastructurally diverse CWDF, including spheroplasts and protoplasts (CWRF). Ultrastructurally intact CWRF were found in the microcolonies of the *Cyc. circinalis*, *Cyc. revoluta*, *Cyc. micholitzii*, and *E. villosus* symbiotic organs (Fig. 5.9a, b). Thin structure of these cell forms differs in different species of cyanobionts. For example, in CWRF of the cyanobiont of *Cyc. revoluta* contains numerous cyanophycin granules,

Fig. 5.5 Relic angiosperm cycad plant *Encephalartos villosus* (photo obtained by Lobakova and Baulina)

which is not typical of cyanobacterial protoplasts. In the cyanobionts of *Cyc. circinalis* (Fig. 5.9a) and *Cyc. micholitzii*, many CWRF were probably at the stage of protoplast formation, since one or two membranes were revealed at different areas of their surface. In the CWRF of the *Cyc. circinalis* cyanobiont, the indication of intensified protein synthesis is especially pronounced extensive zones of the nucleoid and numerous clusters of orderly located ribosomes at its periphery and between the thylakoids (Fig. 5.9a). The ultrastructural evidence of intensified transcription and translation (significantly increased nucleoid area and massive aggregation of the ribosomes) was also typical of the *N. muscorum* 304 spheroplasts in the model association of this strain with the rauwolfia callus (Gorelova 2001). In some of these cell forms, reorganization of the thylakoid system was also observed as close abutment of the neighboring thylakoids (Fig. 5.9a). Due to the absence of phycobilisomes, these structures probably lost their capacity for photosynthesis. In some places where the interthylakoid space in the thylakoid stacks widened, rows of ribosomes appeared at the surface of the membranes. Localization of ribosomes on the thylakoid membranes has been observed by other researchers, who suggested an analogy between the thylakoids of chloroplasts and cyanobacteria in this respect (Jagendorf and Michaels 1990; Zabalueva et al.

Fig. 5.6 Cycad *Cycas revoluta* coralloid root (reprinted from Lobakova (2004) with kind permission from author). *Scale bar* 1 cm

1993). A new methodic approach involving electronic tomography is presently used to investigate the spatial structural organization of cyanobacterial thylakoids in order to determine the possible functional heterogeneity of these organelles (van de Meene et al. 2006; Liberton et al. 2006; Liberton et al. 2011). This technique made it possible to reveal the ribosomes localized on the surfaces of thylakoids and associated intracytoplasmic membranes (not double ones) of the unicellular cyanobacterium *Synechocystis* sp. 6803. The role of the specialized regions of the membrane system in the synthesis of thylakoid proteins is therefore discussed (van de Meene et al. 2006; Mullineaux 2008).

Some CWRF of the *Cyc. circinalis* cyanobiont had an intact ultrastructure, numerous ribosomes, and extensive nucleoid zones, being probably at the stage of division (Fig. 5.9b). This is an indication of their viability by the time of observation.

Diverse cell types of the cyanobiont were embedded in an intercellular matrix with the ultrastructure typical of the gel-forming polysaccharides of the mucus (Fig. 5.10). While the vegetative cells within the trichomes differed in shape, their ultrastructure was similar to that of free-living cyanobacteria grown in the light under optimal conditions (Fig. 5.10). This is an indication of active growth of the trichome individuals. The mucous sheaths typical of *Nostoc* species were not found around the vegetative cells in the samples. CWDF were present in all zones of the coralloid roots (apical, central, and basal). In the coralloids of *E. villosus*, *Cer. mexicana* and *Cyc. circinalis*, CWDF of the vegetative cells were found, which differed in their ultrastructure from those described above. Most of them were protoplasts (Fig. 5.11a, b) and less often, visible spheroplasts (Figs. 5.12 and 5.13a). Both types were characterized by the ultrastructural features of production

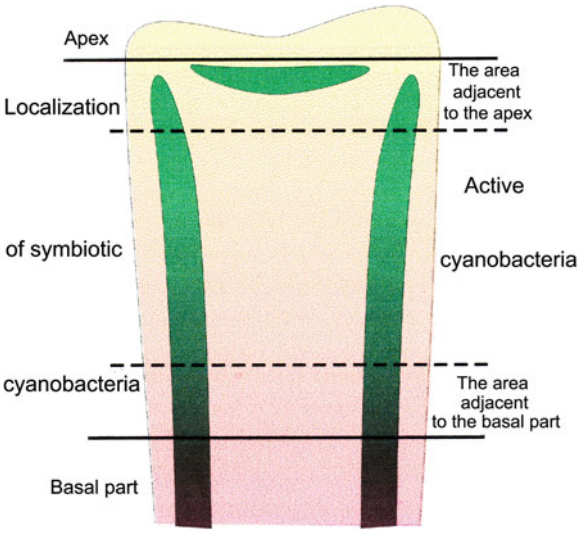

Fig. 5.7 Scheme of cyanobacterial localization in the cycad coralloid root (created Lobakova and Baulina). Green indicates the specialized zone of the cortical parenchyma with cyanobacteria, pink-violet indicates the presence of phenolic compounds in the root parenchyma

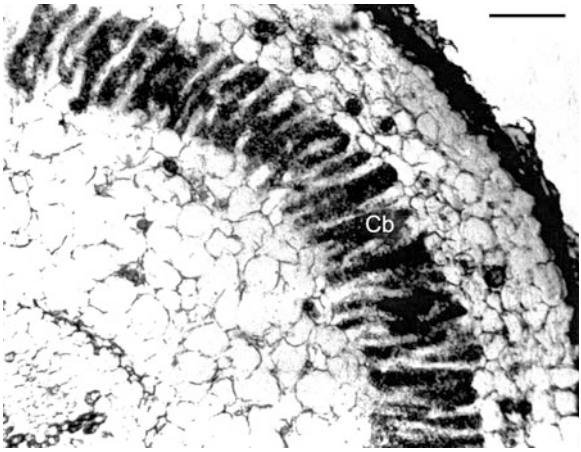

Fig. 5.8 The transverse section of the *Cycas revoluta* coralloid root with cyanobacterial microcolonies in the intercellular spaces of the specialized zone of the cortical parenchyma (reprinted from Lobakova et al. (2004) with kind permission from Pleiades Publishing, Ltd.) *Cb* cyanobacteria. *Scale bar* 50 μm

and intense secretion of the interstitial mucous material, which is not typical of bacteria. These cell forms were sometimes localized near to the intact vegetative trichomes (see Fig. 5.1). Large CWRF of the spheroplast type was found in the apical part of the coralloid roots of *Cer. mexicana*. Characteristically, not one, but two membranes separated by the material of the same structure as the cytoplasm were revealed at extensive areas of their wavy surface (Fig. 5.12). In this case, the outer membrane exhibited no ultrastructural characteristics differentiating it from the CM.

CWDF with the ultrastructural indication of production of mucous intercellular material do not belong to another strain, since in some cases they were sharing the septum with intact vegetative cells. The few works on strain diversity of the cycad

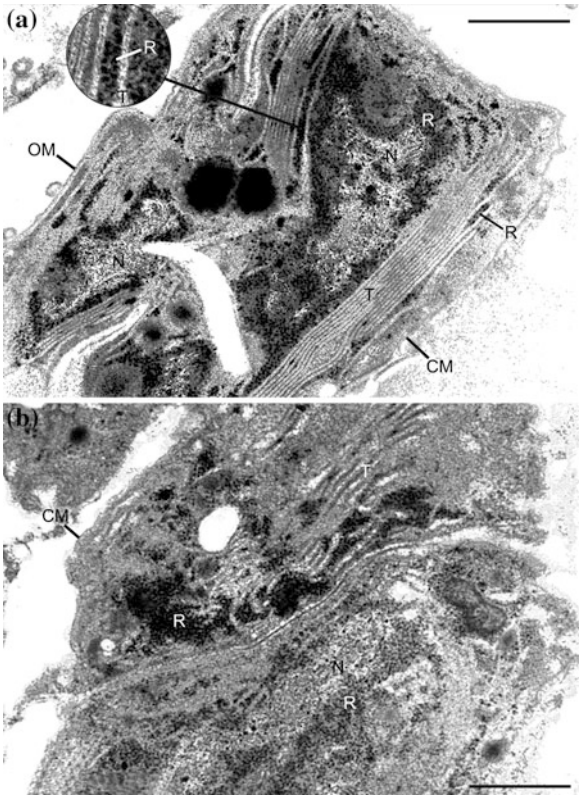

Fig. 5.9 Ultrastructure of *Nostoc* sp. in a symbiotic microcolony of *Cycas circinalis*: a cell wall-reduced form with extensive nucleoid and numerous ribosomes (**a**) and tentatively, a dividing cell wall-reduced form (**b**) (**a**, **b**, data obtained by Baulina and Lobakova) *CM* cytoplasmic membrane, *N* nucleoid, *OM* outer membrane, *R* ribosomes, *T* thylakoid(s). *Scale bar* a, b, 0.5 μm

cyanobionts gave evidence to the existence of several variants of infection of individual plants and of the different coralloid roots of the same plant by one or several strains (Bergman et al. 2008; Costa et al. 2004). To the best of our knowledge, a single report exists on occurrence of different cyanobiont strains within one coralloid root of a cycad plant growing in China (Zheng et al. 2002). The phenomenon of CWDF overproducing mucus was observed in a number of cycad genera and species, and therefore in different strains and possibly different species of the symbiotic cyanobacteria *Nostoc*.

In the investigated microcolonies, most CWRF of the protoplast and spheroplast types were at the final stages of intensive production and excretion of the extracellular matter, and therefore experienced major organizational rearrangements. Most of the cytoplasmic content of such CWRF was an aggregation of fine-grain and thin-fibrillar material packed in a network similar to the material of the intercellular mucous matrix, although probably denser than in the latter case (Fig. 5.13a, b). The fibrils in the cytoplasm and in the microcolony matrix were about 5 nm thick. The thin-fibrillar and fine-grain material are revealed in both the cytoplasm and the microcolony matrix by different traditional fixation techniques involving glutaraldehyde and osmium tetroxide, and by staining the ultrathin

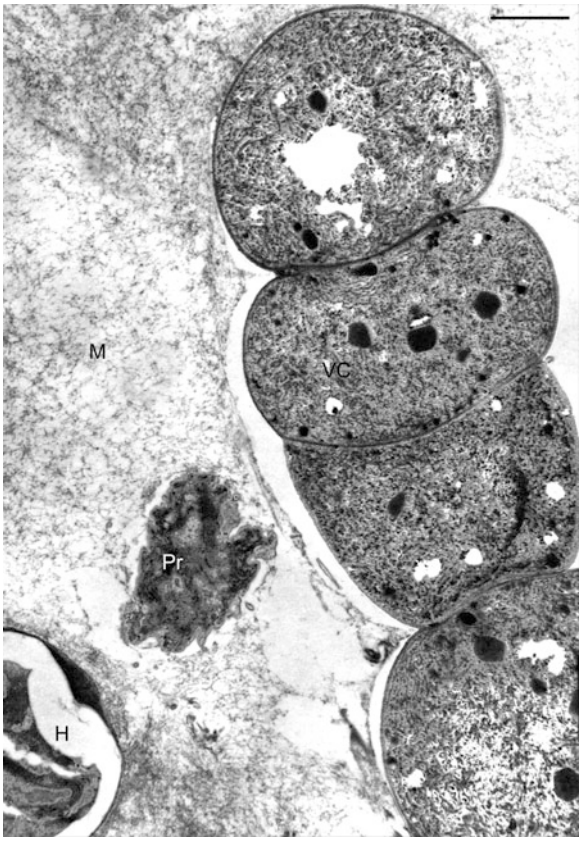

Fig. 5.10 Ultrathin section of *Nostoc* sp. microcolony in the intercellular space of cortical parenchyma specialized zone near the apex of the *Cycas circinalis* coralloid root (reprinted from Baulina and Lobakova (2003a) with kind permission from Pleiades Publishing, Ltd.) *H* heterocyst, *M* intercellular mucous matrix, *Pr* protoplast, *VC* vegetative cell. *Scale bar* 1 μm

sections with uranyl acetate or lead citrate. The staining reagents may interact with the negatively charged carboxyl groups, which are responsible for the weakly acidic properties of the cellular materials (Geyer 1973). According to the above literature references on the chemical composition of cyanobacterial exopolysaccharides, they are mostly acidic polysaccharides. For specific detection of acidic polysaccharides in the thin-fibrillar material within cyanobacterial cells and the intercellular mucous matrix, vital staining of the sections of the cycad coralloid roots with ruthenium red was carried out, as well as the cytochemical reaction with this dye during fixation for electron microscopy. Ruthenium red is used in light microscopic and ultrastructural cytochemistry as a reagent for specific detection of acidic polysaccharides (Luft 1971; Geyer 1973). Light microscopy of the native or fixed in Carnoy's solution sections of the *E. villosus* and *Cer. mexicana* coralloid roots showed pink staining of the cells and the intercellular matrix (Fig. 5.14a, b), indicating the presence of acidic polysaccharides (Baulina and Lobakova 2005). Interestingly, different procedures of staining revealed also the fibrillar structure of the middle lamella, the specific structure between the plant cells surrounding the cyanobiont

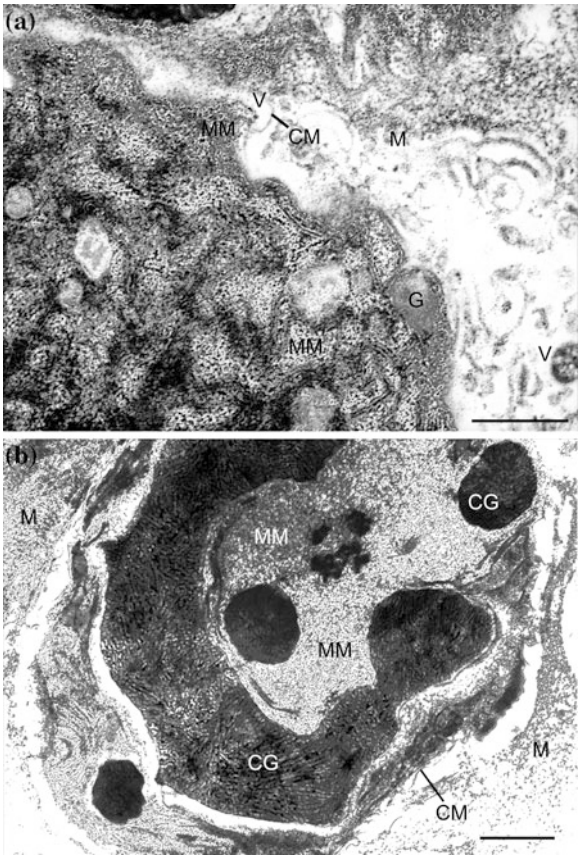

Fig. 5.11 Cyanobiont protoplasts from the basal part of the coralloid roots of *Encephalartos villosus* (**a**) and *Ceratozamia mexicana* (**b**) with the ultrastructural indication of overproduction of the intercellular matrix material (**a**, **b**, reprinted from Baulina and Lobakova (2003a) with kind permission from Pleiades Publishing, Ltd.) *CG* cyanophycin granule, *CM* cytoplasmic membrane, *G* granules of unidentified composition, *M* intercellular mucous matrix, *MM* cytoplasmic material similar to the mucous intercellular material, *V* vesicle. *Scale bar* a, 0.2; b, 0.5 μm

microcolony. This structure is known to be formed by acidic exopolysaccharides (pectins). Acidic pectins of the matrix of the plant cell wall were also stained pink.

After the cytochemical reaction, the contrast of both the extracellular and intracellular thin-fibrillar material observed on ultrathin sections was higher to that in the control samples fixed without the dye (Fig. 5.13) (Baulina et al. 2009).

Thus, microscopy confirmed the presence of acidic polysaccharides in the fine-grain and thin-fibrillar material in the cytoplasm and the intercellular matrix.

In the few CWRF of this type which were at the initial stages of formation, such material could be found not only in the cytoplasm, but in the periplasmic space as well (Baulina et al. 2009). Importantly, agglomerates of the material containing acidic polysaccharides were revealed in the cytoplasm of these cells, in which destructive processes were as yet not very pronounced. The fibrils in the periplasmic space were bound to the cytoplasmic membrane and could form bridges between CM and the inner surface of the outer membrane (Fig. 5.15a). In many CWDF, surface localization of the fibrils was observed, which were oriented perpendicular or at an angle to the outer membrane. Interestingly, similar

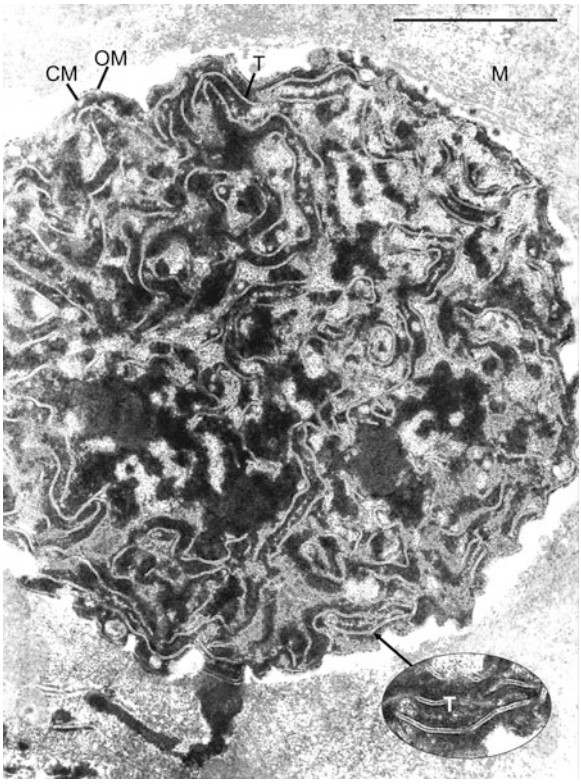

Fig. 5.12 The spheroplast-type *Nostoc* sp. cell wall-reduced form in the zone near the apex of the *Ceratozamia mexicana* coralloid root, TEM (reprinted from Baulina and Lobakova (2003a) with kind permission from Pleiades Publishing, Ltd.) *CM* cytoplasmic membrane, *M* intercellular mucous matrix, *OM* outer membrane, *T* thylakoid. *Scale bar* 0.5 μm

orientation of the fibrillar material of the intercellular matrix has often been observed locally at the protoplast surface of the cyanobionts of the liverwort *Blasia pusilla* (Gorelova et al. 1996)

We suggest that detection in the periplasmic space of the acidic polysaccharide-containing fibrils associated with CM and/or closely contacting with the outer membrane is in accordance with the modern notions on synthetic mechanisms, which are coupled in time and space to the export of the extracellular and capsule polysaccharides in gram-negative bacteria. However, the capacity of cyanobionts for exopolysaccharide accumulation in the cytoplasm contradicts these notions. The main parameters characterizing the functioning of these mechanisms and the interpretation of these data will be presented below.

Characteristically, in most CWRF investigated, the material containing acidic polysaccharides was accumulated in the interthylakoid space, with the fibrils sometimes bound to the cytoplasmic surface of the thylakoid membranes (Fig. 5.15b). Typical phycobilisomes were not detected in these cell forms. Thylakoid membranes were close to each other, their internal layers were apparent as distinct contours. It should be noted that the traditional fixation method in combination with ruthenium red contrasting used in this work allowed much more

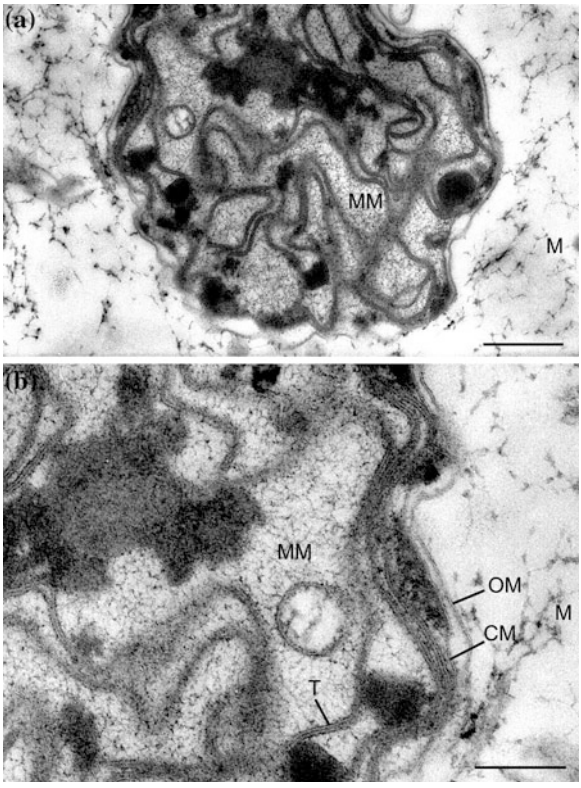

Fig. 5.13 Ultrastructure of the spheroplast-type *Nostoc* sp. cell wall-reduced form in *Encephalartos villosus* coralloid root; cytochemical reaction with ruthenium red during fixation for electron microscopy: overall view (**a**) (data obtained by Baulina and Lobakova) and enlarged fragment (**b**) (Baulina et al. 2009) *CM* cytoplasmic membrane, *M* intercellular mucous matrix, *MM* cytoplasmic material similar to the mucous intercellular material, *OM* outer membrane, *T* thylakoid. *Scale bar* a, 0.5; b, 0.2 μm

distinct revealing of the thylakoids in comparison with the majority of previously studied symbiotic and free-living cyanobacteria. The absorption spectra of the freshly isolated thylakoids of the cyanobionts from all the cycads studied contained the peaks of chlorophyll, phycocyanin, and carotenoid absorption typical of cyanobacteria (Baulina and Lobakova 2003a). The ratio between the peak amplitude of the photosynthetic pigments was compatible to that of rapidly growing free-living cyanobacteria (Merzlyak and Razi Naqvi 2000). The data on the pigment composition provide an indirect evidence of the preserved structural integrity of the thylakoid membranes in cyanobionts. Analysis of localization of the fibrils localized on the surface of the thylakoid membranes suggests that they probably participate in the polymerization of polysaccharide chains together with the CM, for which this role has been proved by molecular genetic research on bacteria (see below in this section). It should be noted, therefore, that the possible functional heterogeneity of cyanobacterial intracytoplasmic membranes is presently an important issue, at least considering their role in the synthesis of the thylakoid proteins (van de Meene et al. 2006; Liberton et al. 2006; Mullineaux 2008).

Thorough analysis of the ultrastructural changes in the CWRF associated with filling up most of the cell volume with the newly synthesized mucoid material

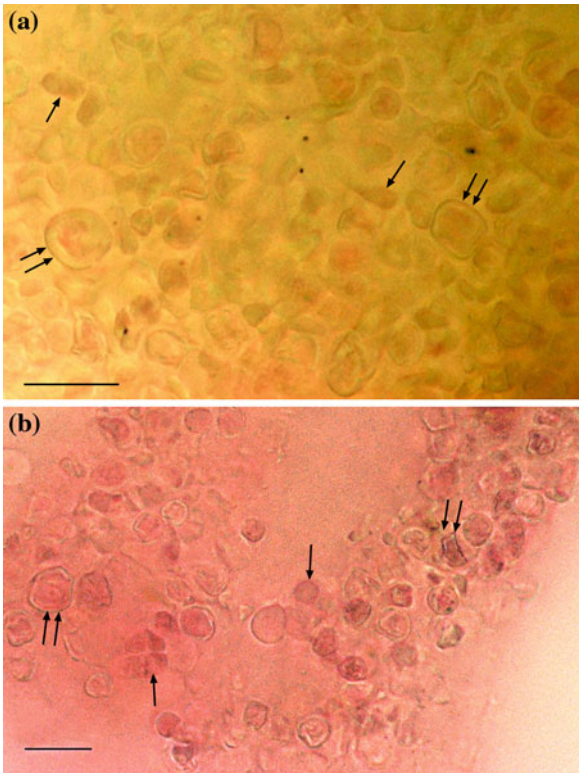

Fig. 5.14 Specific staining (with ruthenium red, pink) of cellular and intercellular substance containing acidic polysaccharides in *Encephalartos villosus* cyanobiont on sections of coralloid roots: native root (**a**) and (**b**) the root fixed in Carnoy's solution (**a**, **b**, data obtained by Baulina and Lobakova). *Arrow* points to vegetative cell forms, *double arrow*—modified heterocysts. *Scale bar* a, b, 10 μm

showed that the ribosomes and the nucleoid zone were no longer revealed in the sections of such cells. This phenomenon was observed in the preparation stained with lead citrate by Reynolds (Reynolds 1963) (Fig. 5.11) or with uranyl acetate. In the practice of electron microscopy, uranyl acetate is known as a good staining agent for nucleic acids, especially for bacterial nucleoids (Kellenberger et al. 1958; Geyer 1973). Interestingly, among cyanobionts of the *Cer. mexicana* and *E. villosus* coralloid roots, very large cyanophycin granules of intricate shape were observed in some of the protoplasts undergoing this degradation (see Fig. 5.11b).

The material similar to the intercellular mucus filled the cytoplasm and was accumulated between the CM and peripheral thylakoids; it was incorporated into the forming surface vesicles, which are excreted into the intercellular matrix [Figs. 5.11a and 5.16; see also the scheme (Fig. 5.17)]. In some protoplasts, the vesicles were formed all over the surface. The vesicles were observed close to the CWRF. A presently unstudied mechanism of vesicular transport involving CM is probably one of the mechanisms of exopolysaccharide secretion in the protoplasts of symbiotic cyanobacteria.

Further degradation accompanied by CM fractures reveals another unusual feature of these cell forms: the cytoplasmic material passes through the ruptures as characteristic fibrillar strands and combines with the surrounding intercellular

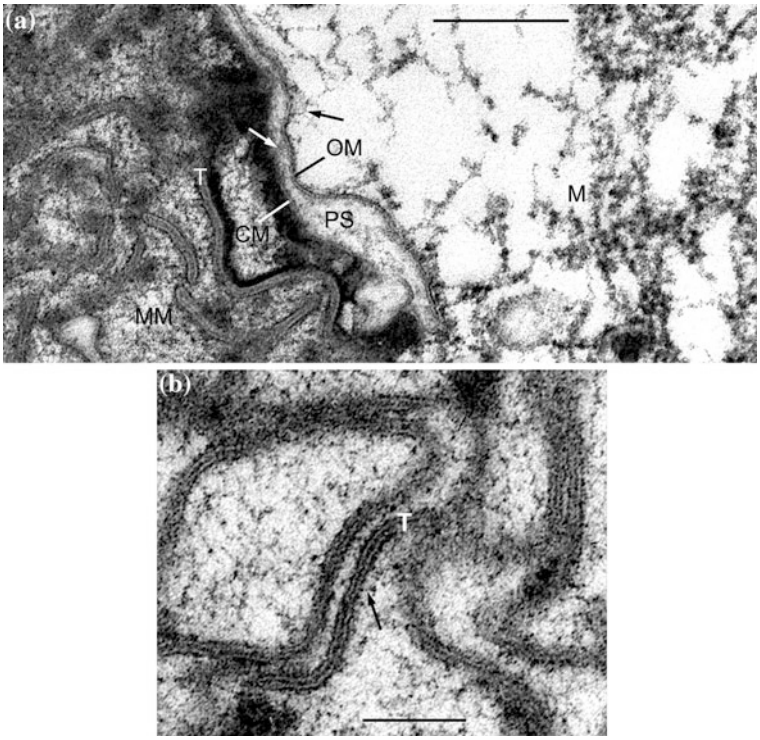

Fig. 5.15 The *Nostoc* sp. spheroplasts in the coralloid root of *Encephalartos villosus*; cytochemical reaction with ruthenium red during fixation (data obtained by Baulina and Lobakova): peripheral area (**a**) and central area (enlarged fragment of Fig. 5.13a) (**b**) *CM* cytoplasmic membrane, *M* intercellular mucous matrix, *MM* cytoplasmic material similar to the mucous intercellular material, *OM* outer membrane, *PS* periplasmic space, *T* thylakoid. *Arrows* point to the fibrils of the acidic polysaccharides-containing material. *Scale bar* a, 0.2; b, 0.1 μm

material (Figs. 5.16 and 5.18). In the spheroplast-like forms, both membranes were ruptured at the same sites (Fig. 5.19). This is an indication of the relation and affinity of the chemical nature of the intra- and extracellular polymers, as well as of another way of their excretion, associated with cell death. Thus, the ultrastructural organization of these CWRF of the vegetative cells of cycad cyanobionts suggests overproduction of the material involved in formation of the intercellular matrix of the mucous zone of the coralloid roots. At the late stages of CWRF development, this process is accompanied by the degradation of the reproductive and protein synthesizing systems and the impairment of the CM, eventually causing the death of the CWRF. The CM undergoes complete lysis, the cytoplasm merges with the extracellular substance, and the thylakoids prove to be embedded in the mucilaginous matrix (Fig. 5.20). These degradative changes in the CWRF are analogous to those observed in the mucilage-producing cells of the root cap (Danilova and Barmicheva 1980). In both cases, cells excrete intracellularly

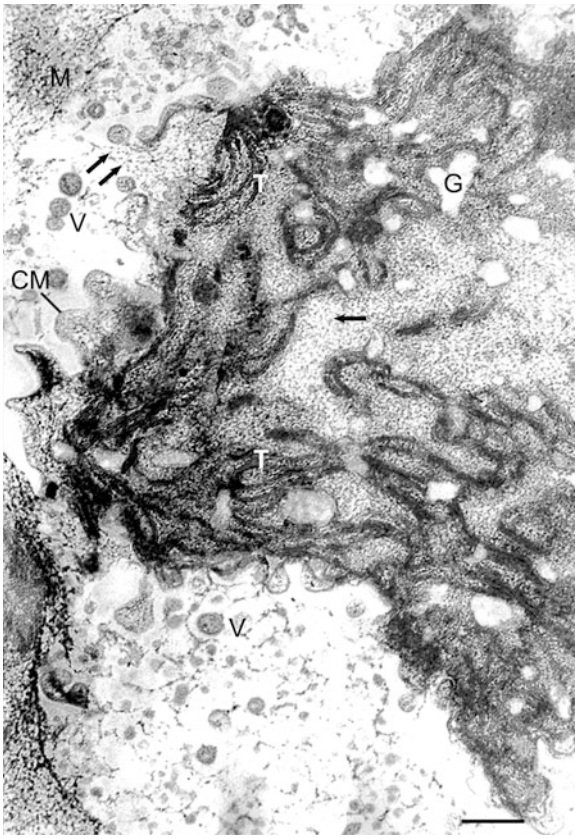

Fig. 5.16 The protoplast of the *Encephalartos villosus* cyanobiont with the ultrastructural indication of overproduction of the intercellular matrix material at the stage of its extensive excretion; ultrathin section, staining with lead citrate (reprinted from Baulina and Lobakova (2003a) with kind permission from Pleiades Publishing, Ltd.) *CM* cytoplasmic membrane, *G* granules of unidentified composition, *M* intercellular mucous matrix, *T* thylakoid, *V* vesicle. *Arrow* points to the aggregations of fine-grain and thin-fibrillar material in cytoplasm, *double arrow*—site of this material passing through the CM rupture. *Scale bar* 0.2 μm

synthesized substances with the aid of vesicles, then undergoing progressive degradation and becoming eventually part of the mucilaginous matrix. The most degraded CWRF were observed in the basal zones of the coralloid roots (Baulina and Lobakova 2003a).

Local CM ruptures and passage of the fibrillar material through them were also observed in some protoplasts of *B. pusilla* cyanobionts, but deeper destructive changes were not found (Gorelova et al. 1996). A similar process of excretion of the mucoid fibrillar material is therefore probably possible in other symbiotic systems.

The presented data on the structure of unusual CWRF in the cycad cyanobionts (similar ultrathin organization of the intra- and extracellular material, its characteristic fibrillar structure revealed by various techniques of fixation and staining, positive cytochemical reaction with ruthenium red, and fusion of the extra- and intracellular fibrillar strands during deep degradation) indicate that the substance filling the cytoplasm of the protoplasts and spheroplasts of the cycad cyanobionts is a cell-produced mucus of the intercellular matrix containing acidic polysaccharides.

Bacteria, including cyanobacteria, synthesize a variety of extracellular polysaccharides, which are primarily the capsular exopolysaccharides and the

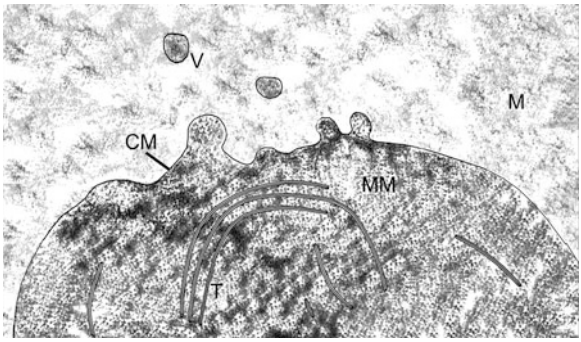

Fig. 5.17 Scheme of the structure of the cell wall-reduced forms of cycad cyanobionts' vegetative cells with the ultrastructural evidence of the intercellular matrix material overproduction at the stage of its intensive secretion involving the surface vesicles of CM origin *CM* cytoplasmic membrane, *M* intercellular mucous matrix, *MM* cytoplasmic material similar to the mucous intercellular material, *T* thylakoid, *V* vesicle

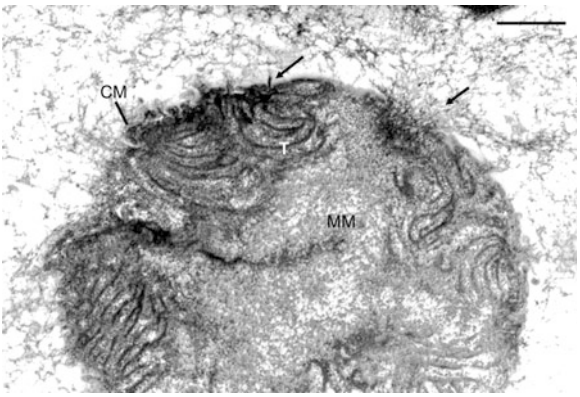

Fig. 5.18 Ultrastructure of a cyanobiont protoplast close to the apex of the *Ceratozamia mexicana* coralloid root, producing the intercellular matrix material, at the stage of degradation (reprinted from Baulina and Lobakova (2003a) with kind permission from Pleiades Publishing, Ltd.) *CM* cytoplasmic membrane, *MM* cytoplasmic material similar to the mucous intercellular material, *T* thylakoid. *Arrows* point to the fibrillar strands continuously connecting the intracellular and extracellular substances. *Scale bar* 0.5 μm

exopolysaccharides of labile mucus (usually acidic gel-forming polysaccharides); some authors include into this group also the O-antigenic oligosaccharide chains of the outer membrane lipopolysaccharide of gram-negative bacteria. Hundreds of capsular polysaccharides (K-antigens), thousands of O-antigen variants, and a vast variety of the polysaccharides produced into the medium have developed in the course of evolution (Vimr and Steenbergen 2009). The patterns of accumulation in the cytoplasm and excretion of exopolysaccharides reported here have not,

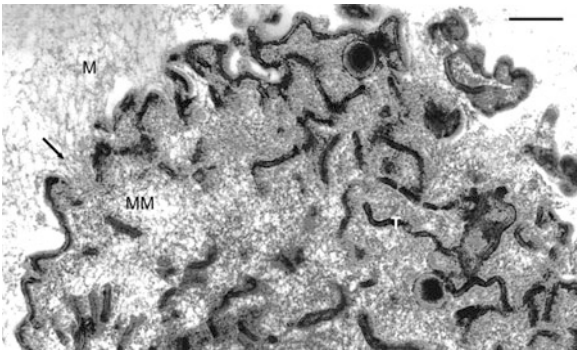

Fig. 5.19 Ultrastructure of a cell wall-reduced form of cyanobiont from the area adjacent to the apex of the *Ceratozamia mexicana* coralloid root, producing the intercellular matrix matter, at the stage of degradation (reprinted from Baulina and Lobakova (2003a) with kind permission from Pleiades Publishing, Ltd.) *M* intercellular mucous matrix, *MM* cytoplasmic material similar to the mucous intercellular material, *T* thylakoid. *Arrow* points to the fibrillar strands continuously connecting the intracellular and extracellular substances. *Scale bar* 0.2 μm

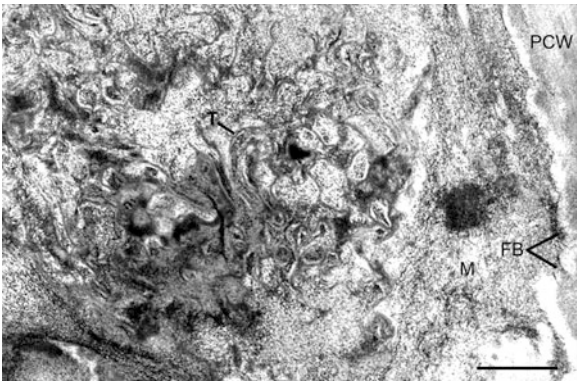

Fig. 5.20 Part of intercellular space of the specialized zone of the cortical parenchyma close to the apex of the *Encephalartos villosus* coralloid root with embedded thylakoids of the degraded form of the cyanobiont, TEM (reprinted from Baulina and Lobakova (2003a) with kind permission from Pleiades Publishing, Ltd.) *FB* fibrous bands of intercellular mucus, *M* intercellular mucous matrix, *PCW* plant cell wall, *T* thylakoid. *Scale bar* 0.5 μm

however, been described previously for cyanobacteria and other bacteria. Thus, an interpretation of the above data is required considering the modern concepts of the pathways of exopolysaccharide synthesis and export by prokaryotic cells of the gram-negative type, which comprises cyanobacteria.

In these microorganisms, the molecules exported from the cell (exopolysaccharides and proteins) should penetrate a complex structure of the cell wall, with the lipid bilayer of the outer membrane as the external barrier. Coupling of the

processes of synthesis and export of polysaccharides of various types is presently an accepted concept, which has been recently confirmed by new data, including the results of proteomic analysis (Boulnois and Jann 1989; Whitfield 2006; Vimr and Steenbergen 2009; Cuthbertson et al. 2009; Cuthbertson et al. 2010). These data were mostly obtained for enterobacteria, including *Escherichia coli*. Variants of a molecular "bridge" exist, which facilitates the transportation of polymers, coupled to assembly, through the cell wall barriers. In spite of the variations in assembly pathways, the output of the product through the outer membrane is carried out through the channels formed by members of the family of outer membrane auxiliary proteins. For some systems, strict evidence was obtained that formation of the bridge involved in the export of polymers through the cell wall requires the interaction of the outer membrane auxiliary proteins with members of the polysaccharide copolymerase family, which are responsible for assembly of the polysaccharide chains.

In spite of the enormous number of the structural variations of the exopolysaccharides (forming groups 1–4), only two basic mechanisms (two models) exist of the polysaccharide export from the site where their assembly commences to the cell surface (Vimr and Steenbergen 2009).

The model for the one and four of polysaccharide groups and lipopolysaccharides includes assembly of monosaccharides as building blocks on a lipid carrier (usually undecaprenyl phosphate), which carries out the transport of the assembled oligosaccharide block through the CM and either polymerization on its periplasmic surface or direct export to the outer membrane (if polymerization occurred at the CM surface adjacent to the cytoplasm). This model is highly variable and is applicable, for example, to colanic acid (M-antigen) of most *E. coli* strains) and to various O-antigens.

The model for the two and three groups of the capsular polysaccharides, such as polysialic acid of *E. coli* K1 capsule and K5-antigens, includes polymer synthesis (with phosphatidic acid at the membrane-bound reducing end) at the inner side of CM adjacent to the cytoplasm, shortly before export or directly during their export to the outer membrane through the channels formed by multiprotein complexes. Elongation of the polymer occurs at the end of the macromolecule which is not bound to the membrane and is in fact located within a cytoplasmic compartment.

The mechanisms of exopolysaccharide export and secretion of mucous material by cyanobacteria are very poorly known. Recent application of modern techniques of proteome analysis revealed the GumB protein in the outer membrane of the cyanobacterium *Synechocystis* sp. This protein was tentatively classified as a member of the family of auxiliary proteins involved in the export of the acidic polysaccharide xanthane in a plant pathogen *Xanthomonas campestris* (Huang et al. 2004). Assembly of xanthane from oligosaccharide subunits in this microorganism by the Wzy O-polysaccharide polymerase occurs at the periplasmic side of CM, where they are transported by undecaprenyl phosphate. This is the mechanism involved in the synthesis of group 1 exopolysaccharides. These polysaccharides are extracted from the cell at the sites where CM contacts with the outer membrane, traditionally named Bayer bridges. It is therefore interesting that

proteomic analysis of the proteins of the outer membrane and CM in *Synechocystis* sp. PCC 6803 suggested the presence of proteins responsible for the contact between these two organelles (Huang et al. 2002; Huang et al. 2004). However, in *Synechocystis* sp. PCC 6803 were identified components of ABC transporter (ABC—ATP-binding cassette), involved in the export of group 2 exopolysaccharides according the ABC transporter-dependent pathway. These components are nucleotide-binding domains (NBDs) that associate with the TMDs (transmembrane domains) on the cytoplasmic face of the membrane. NBDs were identified also in cyanobacteria *Arthrospira maxima* CS-328, *Lyngbya* sp. PCC 8106, and *Microcoleus chthonoplastes* PCC 7420 (Cuthbertson et al. 2010)

It was found that in motile filamentous cyanobacteria of the order Oscillatoriales (subsection III) secretion of the mucus involved in their gliding movement occurs through the junctional pores, which are localized symmetrically at both sides of the transversal cell walls of the trichome (Lamont 1969; Hoiczyk and Baumeister 1995, 1998). Each pore is a complex structure penetrating the cell wall and containing a tubule and a terminal site. Similar pores were found in *A. variabilis* B1403-4b, which, while differing from subsection III members in the character of gliding movement, also uses mucus secretion for the purpose. In the vegetative cells of *Nostoc* sp. f. *Blasia* from a pure culture after prolonged storage in the dark at low temperature, we observed by electron microscopy that the mucous material of the intercellular matrix was excreted through the channels similar to the junctional pores in their localization, regular placement, and size (Gorelova and Baulina 2009). Secretion of the extracellular material, including polysaccharides, by the outer membrane vesicles is also a possible mechanism (see Sect. 3.3).

Thus, as was stated above, accumulation of exopolysaccharides in the cytoplasm and their secretion by CM vesicles in the original members of various species (wild type) of cyanobacteria and other bacteria has not been previously described.

In the course of molecular genetic research aimed at the identification of the genes responsible for various stages of biosynthesis and export of group II capsular polysaccharides, *E. coli* strains were obtained, which carried mutations in the sites of the chromosomal gene clusters responsible for the translocation of these polymers. Electron microscopic immunocytochemistry revealed accumulation of the polysaccharide corresponding to the K-5 antigen unmodified into the transport form (shortened polymers and the absence of phosphatidic acid) in the local cytoplasmic areas of *E. coli* K5 mutants deficient in the capsule formation (Kröncke et al. 1990a). Similar results were obtained in the model systems for investigation of the synthesis and transport of the capsular polysaccharides in the mutant strains of *E. coli* K1 (Pavelka et al. 1994), as well as by inhibition of the synthesis of proteins involved in translocation by chloramphenicol, and by suppression of the energy electron transport in the CM by carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) (Kröncke et al. 1990b). A series of works on capsuleless *E. coli* K1 mutant strains confirmed the coupling between transportation and synthesis of the capsular polysaccharides (Steenbergen and Vimr 2008).

In strains deficient in the genes responsible for the synthesis of polysialic acid, a capsular component and electron-transparent areas in the cytoplasm were observed, which were not present in the wild type strain. The authors interpreted them as the sites of accumulation of the untransported exopolysaccharide. In the prototrophic hybrid strain EV36, polysialic acid in its transport form is deposited in the intracellular compartments named sialisomes.

The above-described massive accumulation of the exopolysaccharides of symbiotic cyanobacteria in the cytoplasm and in the vesicles formed by CM or the outer membrane was observed only in CWDF, mainly spheroplasts and protoplasts. The cells of this type are probably at the stage of comprehensive reorganization of the processes of synthesis and transport. The specialization of these cell forms involved in their transformation to CWDF is associated with a modification of the mechanisms of polysaccharide synthesis and export resulting in uncoupling of these processes and emergence of new possibilities for polysaccharide excretion when cell wall barriers are degraded or absent. These possibilities include excretion inside the vesicles of CM or the outer membrane and through the local ruptures of these membranes.

Thus, our work demonstrated existence of the modified (compared to the presently known ones) mechanisms for exopolysaccharide synthesis and export in the specialized cells of cyanobacterial populations adapted to development in a cyanobacterial-plant symbiosis (Baulina et al. 2009, 2011).

Investigation of the intracytoplasmic synthesis and accumulation of the mucus polysaccharides by CWDF of the cycad cyanobionts encounters many problems, including that of the sources of carbon and low molecular weight precursors. Production of polymers in massive amounts requires significant volumes of the substrates, nucleotide sugars in the case of polysaccharide synthesis (Shibaev 1982). Visible evidence for the degradation of the ribosomes and nucleoid in CWRF is an indirect indication of the possible utilization of nucleotides. In the absence of stored glycogen, products of the degradation of starch from the plant cells of the cortical parenchyma, which form the intercellular cavities colonized by cyanobacteria, may act as sugar sources. In spite of existence in the dark, CO_2 fixation continues in cyanobacteria inhabiting the cycad coralloid roots, although at a very low level compared to growth in the light (Costa and Lindblad 2002). This is, however, probably true only for the intact vegetative cells of the heterogeneous cell populations of the cyanobiont. Sources of energy for extensive biosynthesis under microaerobic conditions in the dark inside the cycad tissues are also a problem. Utilization of sugars by fermentation may be one of the pathways used to obtain the required energy. This suggestion was prompted by capacity of *Nostoc* sp. isolated from *Macrozamia lucia* roots for heterotrophic growth under anaerobic conditions in the dark (Hoare et al. 1971). Later, the dark anaerobic metabolism of *Nostoc* sp. strain Cc, isolated from coralloid roots of *Cycas circinalis*, was investigated (Margheri and Allotta 1993; Moezelaar and Stal 1997). It was found that the homoacetic fermentation using an exogenous glucose allowed the strain to survive, but not to grow. The nonsymbiotic cyanobacterium *Microcystis* PCC7806, grown under continuous light, fermented endogenously stored

glycogen when incubated anaerobically in the dark (Margheri and Allotta 1993; Moezelaar and Stal 1997). Theoretically, cyanobacteria possess, apart from oxidative (respiration) and photosynthetic phosphorylation, also the glycolytic pathway of energy metabolism, i.e., substrate phosphorylation (Koksharova et al. 1998). For the cyanobionts of *Cer. mexicana* and *E. althenshteinii*, evidence was obtained by us in the preliminary experiments indicative of the absence of PSII activity and O_2 absorption in darkness (Fedorenko et al. 2003) These data support the opinion that the cycads' cyanobionts possess functional glycolytic pathway.

The mucilage-like substance synthesized by the CWDF did not form sheaths around them but filled the entire intercellular space more or less uniformly. Near the plant cell wall, this substance occurred as fibrous bands attached to the internal surface of the wall (Fig. 5.20). The package of fibrils in the bands differed from that observed on the periphery of the plant cell wall. The plant cells themselves exhibited no manifestations of the active production of mucilage. In particular, they did not have the developed Golgi apparatus, exocytosis-specific vesicles, and slimy depositions between the plasmalemma and the cell wall. It can be suggested that mucilage in the intercellular space of cycads plants is of cyanobacterial origin, at least partially. This suggestion is supported by the literature data that the intercellular mucilage in the coralloid roots of the *Mac. communis* seedlings appeared only after they had been infected with cyanobacteria (Ahern and Staff 1994). The 3-year observations of mature cyanobacterium-free *Cyc. circinalis* plants also showed that the coralloid roots of these plants did not contain mucilage (in these experiments, more than 100 samples of the coralloid roots were analyzed) (Baulina and Lobakova 2003a). Moreover, investigation of the symbiosis of cyanobacteria with the *Azolla* fern suggested a hypothesis concerning the role of both components in production of mucus (Forni et al. 1998).

Discovery of the relation between deep rearrangements in exopolysaccharide-producing cells and reduction (evidently to complete loss) of the nucleoid and other structures, apart from the thylakoids and cyanophycin granules, and cell death is important for the understanding of the physiology of symbiotic cyanobacteria. Reduction of these cellular components may serve as an indicator of the engagement of the programmed cell death (apoptosis) mechanisms. This assumption is based on comparative analysis of the described structural changes in cyanobacterial CWRF and in the degraded in the course of apoptosis cells of eukaryotic organisms in this section. In the latter case, the action of specialized protease (caspase) cascades brings about a rapid destruction of cell structures, including the nuclear membrane and DNA (Raff 1998). Among prokaryotes, myxobacteria could serve as the most striking example of programed death of certain cells in population. At the stage of fruiting body formation in the life cycle of myxobacteria, dead cells serve as a substrate for the construction of mucous extracellular matrix of the fruiting body (Yarmolinsky 1995). In the trichome-forming cyanobacteria, the phenomenon of 'appropriate' ('programed,' according to the modern terminology) cell death is characteristic of nekridial cells. The formation of such cells and their separation facilitated by peripheral perforations in the septa ultrastructurally similar to the above described junctional pores, is the

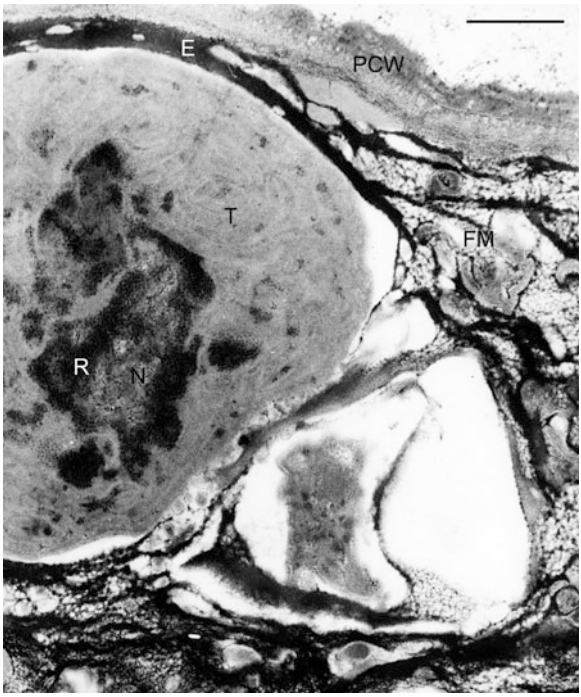

Fig. 5.21 Part of the intercellular space in the specialized zone of the cortical parenchyma close to the apex of the *Cycas circinalis* coralloid root with a cyanobiont protoplast, presumably involved in the synthesis of extracellular proteinaceous material, TEM (reprinted from Baulina and Lobakova (2003a) with kind permission from Pleiades Publishing, Ltd.) *E* electron-dense envelope, *FM* high electron density fibrillar matter, *N* nucleoid, *PCW* plant cell wall, *R* ribosomes, *T* thylakoid. *Scale bar* 0.5 μm

mechanism of trichome break-up into smaller child fragments (Lamont 1969). Lamont (1969) calls this death "sacrificial". However, in contrast to the CWRF described by us, a characteristic ultrastructural rearrangements of the nekridial cells is not accompanied by mucilage formation.

The intercellular space of the cortical parenchyma of the *Cyc. circinalis* coralloids contained not only the mucilage-producing CWRF but also other type of CWRF (both protoplasts and spheroplasts) with the ultrastructural changes indicating the intense synthesis and excretion of proteins (Figs. 5.21 and 5.22). These cell forms had enlarged nucleoid zones and clusters of regularly arranged ribosomes which were larger and were clearly seen than in the typical vegetative cyanobacterial cells. Among the ribosome clusters located on the periphery of the nucleoid zone were high electron density fibrils of a material that could be identified as protein-like based on its cellular location and electron density (Fig. 5.22). Cyanophycin granules, which represent the depositions of the reserve nitrogen source of cyanobacteria (a polypeptide containing arginine and aspartic acid), were not detected. This observation is consistent with the ultrastructural manifestations of enhanced protein synthesis (such as enlarged nucleoid and a considerably increased number of ribosomes in the cell) observed by us in this study and by Gorelova in a model association of the cyanobacterium *Nostoc muscorum* CALU 304 with the rauwolfia callus (Gorelova 2001). In spite of the enhanced biosynthetic activity and the intact structure of various cytoplasmic components,

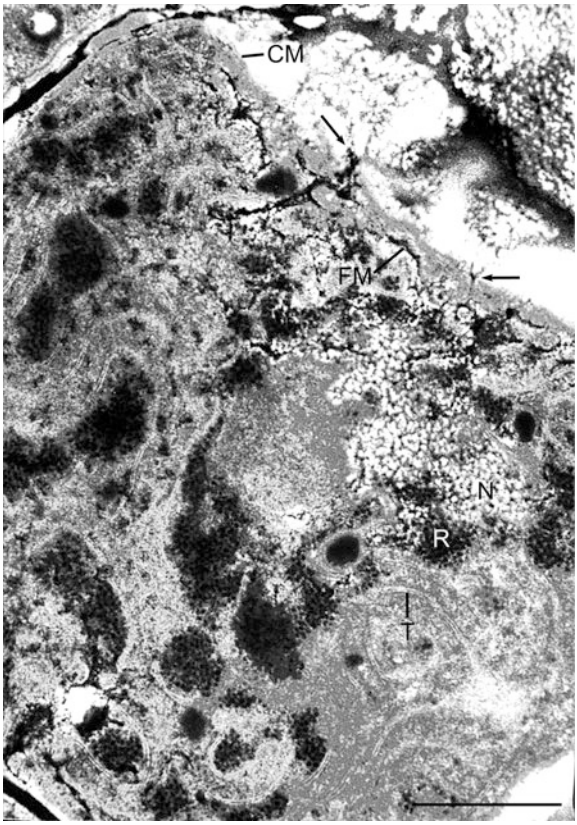

Fig. 5.22 Ultrastructure of a cyanobiont protoplast, presumably synthesizing the extracellular proteinaceous material close to the apex of the *Cycas circinalis* coralloid root (reprinted from Baulina and Lobakova (2003a) with kind permission from Pleiades Publishing, Ltd.) *CM* cytoplasmic membrane, *FM* high electron density fibrillar matter, *N* nucleoid, *R* ribosomes, *T* thylakoid. *Arrows* point to the site of the proteinaceous material extension into extracellular space. *Scale bar* 0.5 μm

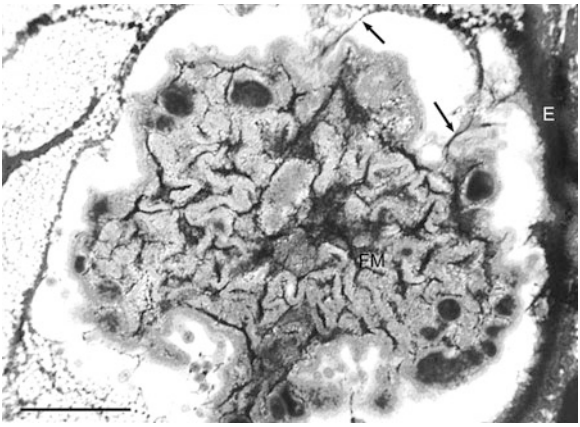

Fig. 5.23 The degraded cell wall-reduced form of cyanobiont in the intercellular space of the cortical parenchyma specialized zone close to the apex of the *Cycas circinalis* coralloid root, TEM (reprinted from Baulina and Lobakova (2003a) with kind permission from Pleiades Publishing, Ltd.) *E* electron-dense envelope, *FM* high electron density fibrillar matter. *Arrows* point to the site of fusion of high electron density fibrillar material with an electron-dense envelope surrounding CWRF. *Scale bar* 0.5 μm

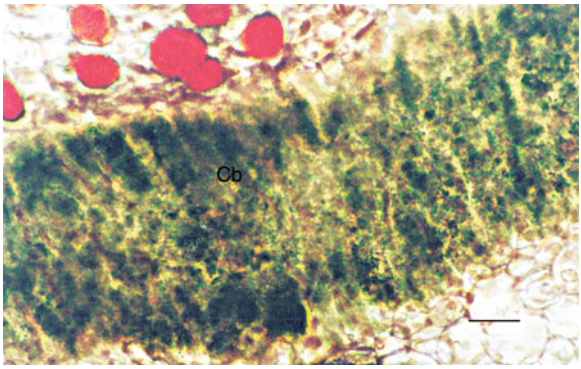

Fig. 5.24 Specific staining (with the vanillin reagent, *red* color) of flavanes in the parenchymal cells surrounding the cyanobacteria microcolonies in the sections of native coralloid root of *Encephalartos villosus* (data obtained by Lobakova and Baulina) *Cb* cyanobacteria. *Scale bar* 50 μm

including thylakoids, some CWRF had rupture in their CM, by which the protein-like substance could be released into the intercellular space (Fig. 5.22). Around the intact cells and the CWRF of the cyanobiont, there were electron-dense envelopes and/or conglomerates of a fibrillar substance (Fig. 5.21). The cytoplasm of some degraded cells was filled with an electron-opaque fibrillar substance, whereas ribosomes, nucleoid, and thylakoids were not detected (Fig. 5.22). After these cells had been completely degraded, their contents were merged with the intercellular matrix, as in the case of the mucilage-producing CWRF described above. Figure 5.23 shows a local region where the protein-like cytoplasmic substance releasing by the CM rupture merge with the electron-dense envelope surrounding the CWRF (Baulina and Lobakova 2003a).

Thus, a new unusual CWRF type was found characteristic of vegetative cells of the cyanobacteria displaying the ultrastructural features of specialization on the synthesis of extracellular matter. On the other hand, these forms could be considered as a sort of experimental control substantiating the essential difference in the organization of two CWRF types (overproducing a protein-like matter or acidic polysaccharides of the intercellular matrix).

A single mechanism of CWDF formation probably exists, with the subsequent differences between them resulting from their different specialization, e.g., as persisting L-forms or producers of the material of the mucous matrix and extracellular proteins. Investigation of this problem using diverse cyanobacterial-plant symbioses shows some promise.

The production of extracellular polymers, mainly exopolysaccharides, combined with the formation of extracellular matrix is characteristic of prokaryotes forming the colonies or biofilms. The intercellular matrix of pathogenic bacteria colonies is considered as a structural integration component providing for the vitality and normal functioning of the populations representing polymorphic multicellular systems including CWDF (Vysotskii et al. 1984). The colonies and biofilms formed both by pathogenic bacteria within macroorganisms and by free-living microorganisms in various natural habitats have a similar structural and functional organization (Costerton et al. 1987; Pavlova 1999; Davey and O'Toole

2000; Romanova et al. 2006). Development of bacterial communities as biofilms is presently considered among the major survival strategies of microorganisms in the environment. As was mentioned above, the mucous intercellular matrix is involved in microcolony organization, uniting the vegetative cells, protoplasts, heterocysts, and microcells within the population of *B. pusilla* cyanobionts. It probably plays a regulatory role in the intercellular transport of metabolites. In the interstitial microcolonies of the cycad cyanobionts, the intercellular matrix probably plays the same role.

Protection of the population against bactericidal phenolic compounds is probably one of the functions of the mucus produced by these microorganisms. A diverse spectrum of these compounds was found in the parenchymal cells forming the intercellular harbors for cyanobacteria and close to the specialized zone (Lobakova et al. 2004; Lobakova 2004). This localization of flavanes on the sections of native roots stained with the vanillin reagent is shown on Fig. 5.24). Bacterial cells inside biofilms (in natural environments and in the course of an infection process) are known to acquire higher resistance to antibacterial agents, including disinfectants and antibiotics (Costerton et al. 1987).

Complex rearrangements leading to the formation of mucilage-overproducing CWRF in accord with the demand of the cyanobiont for the protection from bactericidal phenolics formed by the plant is evident of the functioning of regulation mechanisms in the course of the initiation and the development of these processes. Obviously, it is the manifestation of well-known bacterial capability of acclimation via occurring under multilevel control regulation of the behavior in response to extracellular physical and chemical factors (signals). Coordinated cell behavior may become more pronounced due to modification of the microenvironment by bacteria by nutrient consumption or release of toxic metabolites, enzymes, and the extracellular matrix, e.g., the capsular material (Surette 2002). Investigation of exopolysaccharide secretion in motile cyanobacteria revealed variations in the polysaccharide type in response to environmental stimuli (Hoiczyk 1998). Cyanobacteria in natural symbioses and in vitro model symbiotic associations are affected by the plant partners. The conditions of the microenvironment (limited space and the presence of exocellular agents, i.e., plant metabolites, including those acting as signal molecules) (Gorelova et al. 1995; Rai et al. 2000a; Meeks and Elhai 2002); (Gorelova 2006; Adams and Duggan 2012) are not optimal for microorganisms. In model systems, formation of heteromorphic cyanobacterial cells with modified or completely reduced peptidoglycan is one of the effects of the plant extracellular agents (Gorelova 2001). We agree with the authors of the cited work that this phenomenon is associated with the adaptive character of modifications of the microsymbionts. Investigation of new proteins and the previously unknown metabolic pathways probably affecting cyanobacteria within plant symbioses commenced recently (Bergman et al. 2008). Preliminary evidence was obtained concerning both the preservation of most processes in cyanobacterial cells in their symbiotic state and their possible modifications.

The notion that development and behavior of microbial populations under varying environmental conditions is determined by cooperative interaction

between the cells coordinated by the intercellular signal communication has been established in the recent 15 years (Oleskin et al. 2000; Demuth and Lamont 2006; Williams 2007). It should be mentioned that this problem is historically related to the works of Gamaleya in 1894, who was the first to describe heteromorphism in bacteria [see review: Baulina (2008)]. The modern knowledge on the structural and molecular genetic basis of microbial intercellular interactions is immense. These issues are discussed at specialized conferences worldwide (Kaprelyants and Antonyuk 2006; Golden 2003; Hellingwerf 2004). Cooperation as a result of the intrapopulation regulatory processes in response to external stimuli has been proved in many cases. The sensor-signal strategy of the intercellular interactions was, for example, revealed for various types of spore morphogenesis in myxobacteria, bacilli, and streptomycetes, as well as for the cell cycle-coordinated differentiation in prosthecate bacteria. In many bacteria, communication is based on the quorum-sensing signals, with their effect depending on cell density (Kaplan and Plamann 1996; Whitehead et al. 2001; Williams 2007). The quorum-sensing (QS) systems are considered as a global factor in the regulation of gene expression in microorganisms (Khmel 2006). These systems are involved in the regulation of a variety of cell characteristics in biofilms formed by *Pseudomonas aeruginosa* (Sutherland 2002) or pathogenic bacteria (Romanova et al. 2006). In gram-negative bacteria, homoserine lactone derivatives act as autoinducers of QS systems. Existence of the QS regulation system in cyanobacteria has not been confirmed as yet, although recent works provide an indirect indication of such a possibility, primarily due to detection of homoserine lactone in axenic *Gloeothece* PCC 6909 culture (Sharif et al. 2008) and in specific lake populations of *Planktothrix rubescens* and *Microcystis wesenbergii* producing the microcystin toxin (Braun and Bachofen 2004). In the latter work, the possible role of homoserine lactone in the regulation of microcystin production is discussed. Other researchers suggest that macromolecules, such as the cyclic heptapeptides microcystins (probably of lectin nature) may act as species-specific signals for the intercellular interactions (recognition and contact) in the course of *M. aeruginosa* cell aggregation during colony formation (Dittmann et al. 2002; Kehr et al. 2006; Zilliges et al. 2008), as well as act as autoinducers of the biosynthesis of these toxic peptides (Schatz et al. 2007). The PatS peptide, diffusing between the cells of *A. cylindrica* filaments during heterocyst differentiation, is another example of cyanobacterial signal molecules (Sarcina et al. 2000).

Thus, the possible action of intrapopulation signals and cooperative behavior of cyanobacterial cells in the symbiotic microcolonies can not be ruled out. First of all, their architectonics is in principle similar to that of various bacterial colonies and biofilms, for which intercellular signal communications have been confirmed. Moreover, specialized mucus-producing cell forms are present in the microcolonies of the cycad cyanobionts. It is logical to conclude that both the formation and death of such forms are related to protection of the population as a whole against bactericidal phenolic compounds. Finally, various cell types are formed within a microcolony, including intact cells and heterocysts, CWRF of the vegetative cells with overproduction of the mucus polysaccharides, and cell wall-reduced

heterocysts (CWRH). The structural and functional characteristics of the latter also indicate rearrangements related to exopolysaccharide production (see next Sect. 5.1.3). Thus, an intrapopulation sensor-signal system coordinating formation of the cells with different functional specialization may be switched on as a response the actions of a plant partner. Moreover, emergence of mucus-producing CWRF with specific structure and function within populations of the cycad cyanobionts supports the suggestion of an action of a previously unknown mechanism of cell differentiation. The fact that CWRF with exopolysaccharide overproduction are "kamikaze" is an important evidence of the regulated induction of their formation. Cell rearrangements resulting in overproduction are probably similar to apoptosis or associated with it. The formation of such CWRF is likely to be a result of the long-term evolution f cyanobacterial species capable of symbiotic relations.

Collectively, the evidence presented above suggest that the CWRF that produce the mucilaginous substance can serve as a criterion of the adequacy of the heteromorphic alterations of cells and their cooperative interactions in response to the action of environmental factors. The functional significance of the CWRF that are specialized in the production of the protein-like substance is difficult to estimate, as there is no evident correlation between the formation of such CWRF in a cyanobiont population and its development (Baulina and Lobakova 2003a).

5.1.3 *Ultrastructure of Cyanobacterial Heterocysts in Symbioses with Cycads*

Dinitrogen fixation from the atmosphere in order to supply the whole system with bound nitrogen is the main role of the cyanobacterial component in plant symbiosis. This function is carried out by specialized cells (heterocysts), which become significantly more numerous on transition of cyanobacteria into the symbiotic state (Rai et al. 2000). Heterocysts differ from the vegetative cells in a number of specific morphological and ultrastructural features relevant to their specialization (Lang and Fay 1971). The presence of an envelope consisting of three layers (a lamellar glycolipid layer, as well as the homogeneous and fibrillar polysaccharide layers) is one of these features (Fig. 5.25; see also the scheme (Fig. 5.26)). The envelope protects the cytoplasm from oxygen of the air, which is able to inactivate nitrogenase, the enzymatic system reducing N_2 to NH_3. The envelope is not sealed at the sites of contact with the vegetative cells, forming a heterocyst pore through which the intercellular exchange of metabolites is carried out. The structure of heterocysts in the cycad coralloid roots, which is typical of these differentiated cells in organization, is schematically presented on Fig. 5.26. It is known from the literature that heterocysts with some ultrastructural features different from the typical ones are present within cyanobiont populations of higher plants, including cycads (Grilli Caiola 1980; Lindblad et al. 1985; Joubert et al. 1989). For example, the heterocysts featuring an irregular shape and a highly

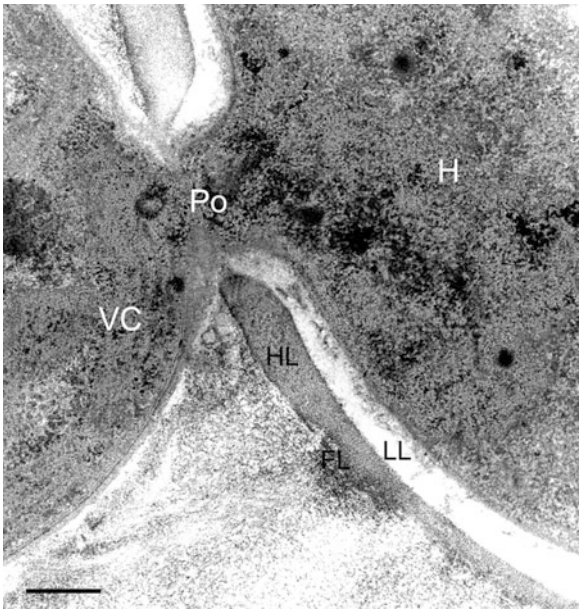

Fig. 5.25 Ultrastructure of a heterocyst envelope of *Nostoc* sp., a *Blasia pusilla* cyanobiont, in the pore region (the photo was kindly provided by Gorelova); heterocyst envelope is composed of fibrillar, homogeneous and lamellar layers *FL* fibrillar layer, *H* heterocyst, *HL* homogeneous layer, *LL* lamellar layer, *Po* heterocyst pore, *VC* vegetative cell. *Scale bar* 0.2 μm

osmiophilic cytoplasm were detected in the population of *Nostoc* existing in symbiosis with the flowering plant *Gunnera kaalensis* (Towata 1985). The heterocysts with such features were referred to as degenerated. They were numerous in both aging and in young symbiotic tissues. The heterocysts of *N. punctiforme* associated with the hornwort *Anthoceros punctatus* also possessed an irregular shape and a significantly increased size (Meeks 1998). In the older parts of cycad coralloid roots, the heterocysts displaying a significant signs of the ultrastructure degradation dominated (Grilli Caiola 1980). Grilli Caiola (1980) suggested that the modified heterocyst ultrastructure indicates that they play an additional role in coralloid roots besides nitrogen fixation. Therefore, a more thorough study of the ultrastructure and degenerative changes in the heterocysts of the cyanobacteria living in symbioses with plants is necessary.

In this section, the data on the ultrastructure of heterocysts localized in close vicinity of the CWRF of the vegetative cells, in the same microcolonies of *E. villosus, Cer. mexicana*, and *Cyc. circinalis* which were described above.

Heterocysts with typical ultrastructure differed from each other in some structural characteristics of their cytoplasm, envelope, and especially thylakoids (Figs. 5.27 and 5.28). The ultrastructural characteristics of the thylakoids were similar to those observed for the cyanobionts of *E. transvenosus, E. woodii,* and *E. arenarius* plants (Joubert et al. 1989). For instance, the cyanobionts of *E. villosus* usually had heterocysts with shortened or convoluted thylakoids and a narrow, electron-transparent interthylakoid space (Fig. 5.27). At the same time, some heterocysts of the same coralloid roots were found to contain thylakoids with a

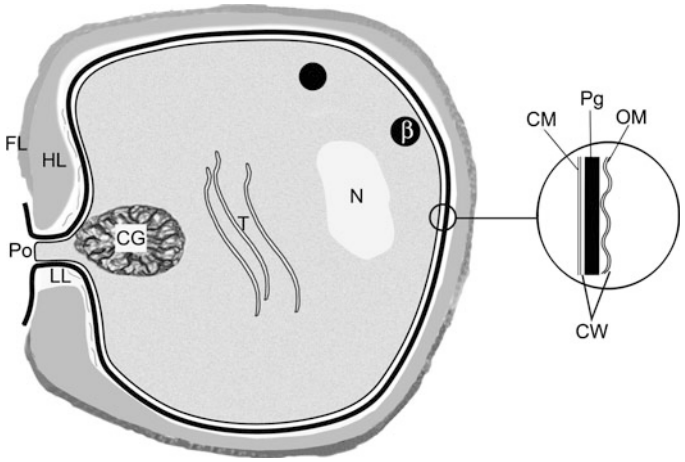

Fig. 5.26 Scheme of the *Nostoc* sp. heterocyst structure in the cycads' coralloid roots *β*, high electron density lipid *β*-granules; *CG* cyanophycin granule, *CM* cytoplasmic membrane, *CW* cell wall, *N* nucleoid, *OM* outer membrane, *Pg* peptidoglycan, *Po* heterocyst pore, *T* thylakoids, *FL* fibrillar, *HL* homogeneous and *LL* lamellar layers of heterocyst envelope. The structure of the septum between the heterocyst and the vegetative cell is not shown

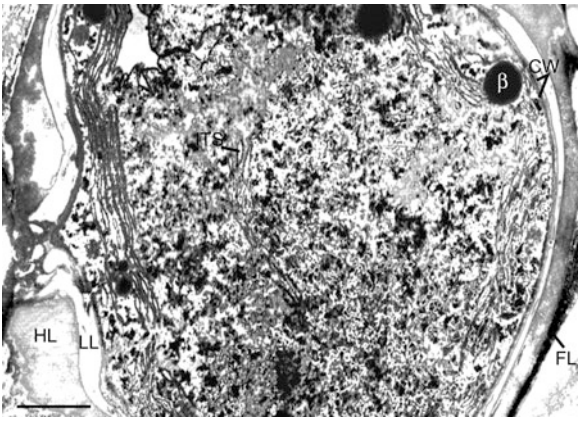

Fig. 5.27 Ultrastructure of the *Nostoc* sp. heterocyst in the central area of an *Encephalartos villosus* coralloid root (Reprinted from Baulina and Lobakova (2003b) with kind permission from Pleiades Publishing, Ltd.) *β*, high electron density lipid *β*-granules; *CW* cell wall, *ITS* intrathylakoid space, *FL* fibrillar, *HL* homogeneous and *LL* lamellar layers of heterocyst envelope. *Scale bar* 0.5 μm

considerably enlarged interthylakoid space filled with a fibrillar substance. Thin fibrils, composed of subunits, were located rarefied and often close to the thylakoid membranes (Fig. 5.28), suggesting that the latter may be involved in the synthesis of fibrillar polymers. A similar fibrillar substance was observed in the CM invaginations below the peptidoglycan layer (Fig. 5.28), indicating a functional relationship between the CM and thylakoids. The fibrillar substance formed a distinct layer immediately outside the outer membrane of the cell wall, producing compact depositions of electron-opaque globules in some regions. The

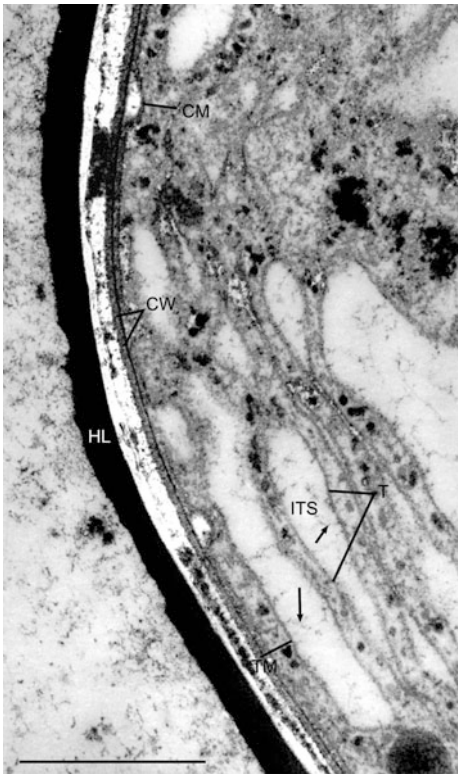

Fig. 5.28 Ultrastructure of a heterocyst of a cyanobiont in the central area of an *Encephalartos villosus* coralloid root (Reprinted from Baulina and Lobakova (2003b) with kind permission from Pleiades Publishing, Ltd.) *CM* cytoplasmic membrane, *CW* cell wall, *HL* homogeneous layer of a heterocyst envelope, *ITS* intrathylakoid space, *T* thylakoid, *TM* thylakoid membrane. *Arrows* point to the fibrils in the intrathylakoid space. *Scale bar* 0.5 μm

homogeneous layer of the heterocyst envelope was more electron-opaque than it usually is. The outer fibrillar layer and the intercellular matrix were not separated by a boundary (Baulina and Lobakova 2003b).

In the microcolonies of *E. villosus*, *Cer. mexicana* and *Cyc. circinalis* cyanobionts, apart from heterocysts with the ultrastructure of the cytoplasm, cell wall, and envelope typical of these cells, numerous modified heterocysts were found, in which the cell wall was reduced (CWRH) (Baulina and Lobakova 2003b). They were sometimes the dominant cell type in cyanobiont microcolonies along the length of the coralloid roots (Fig. 5.29). CWRH were found in the apical, central, and basal zones of the root in different years, both in spring–summer and winter period. They may be generally characterized as protoplasts within heterocyst envelopes, which exhibited the signs of overproduction of the extracellular compounds of the polysaccharide or protein nature, similar to the specialized CWRF forms of the vegetative cells described in the previous Sect. 5.1.2. Similar to the latter ones, modified heterocysts formed two groups with significant ultrastructural differences depending on the supposed nature of excreted high-molecular compounds. The structure of CWRH of the first type, with the ultrastructural characteristics of intracytoplasmic synthesis, accumulation, and export of the material similar to the intercellular mucus is schematically presented on Fig. 5.30. Their

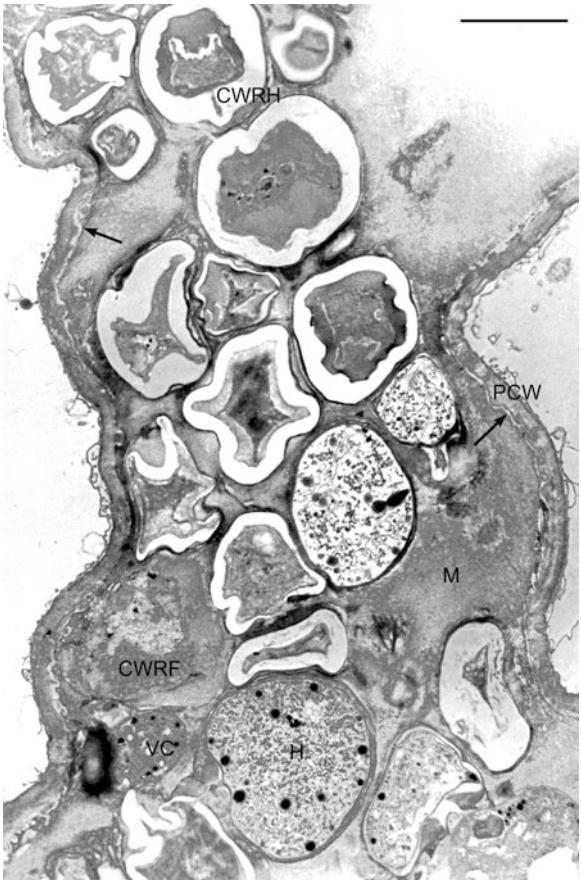

Fig. 5.29 Ultrathin section of a cyanobiont microcolony in the intercellular space of the cortical parenchyma specialized zone close to the apex of an *Encephalartos villosus* coralloid root (Reprinted from Baulina and Lobakova (2003b) with kind permission from Pleiades Publishing, Ltd.) *CWRH* cell wall-reduced heterocyst, *CWRF* cell wall-reduced form of vegetative cell, *H* heterocyst, *M* intercellular mucous matrix, *PCW* plant cell wall, *VC* vegetative cell. *Arrows* point to the boundary between the mucus of the cyanobiont microcolony and the plant cell wall. *Scale bar* 2 μm

morphology was determined by the configuration of the homogeneous layer of the envelope, which was usually preserved in spite of the loss of the cell wall and the deep internal reorganization of the protoplast. The CWRH protoplasts often acquired irregular shape (amoeboid, stellar, often with long cytoplasmic projections). The ultrastructure of CWRH of the first group was characterized by formation of exoprotoplast sheaths, apart from the fibrillar, homogeneous, and lamellar envelope layers typical of heterocysts (Fig. 5.31a, b). The exoprotoplast sheath was probably formed from the material excreted from the cytoplasm by the vesicles separating from the CWRH protoplast (see scheme Figs. 5.30 and 5.32), and in cases of deeper degradation, through the local ruptures of CM (see scheme Figs. 5.30 and 5.33). In the latter instance, it is evident from the picture that the cytoplasmic material is continuously bound to the exoprotoplast sheath. The exoprotoplast sheath material is probably unable to penetrate through the heterocyst envelope and is excreted through pores into the neighboring CWRH and intact heterocysts, or into the mucous intercellular matrix, as can be seen from Fig. 5.34a, b. The latter finding suggests CWRH involvement in formation of the

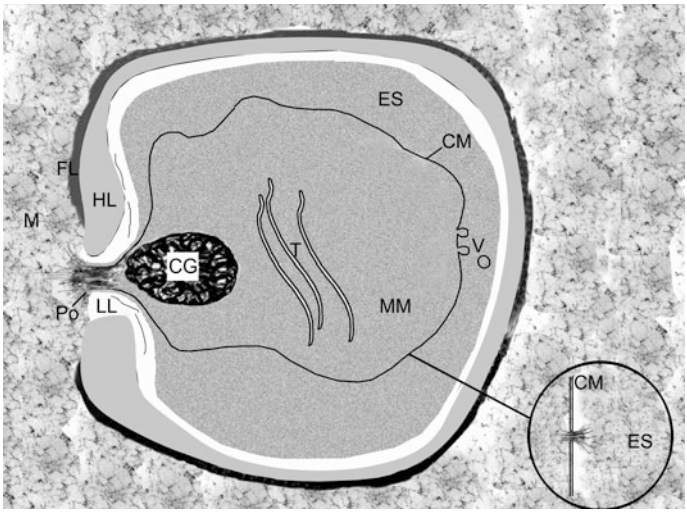

Fig. 5.30 Scheme of the cell wall-reduced heterocyst structure of *Nostoc* sp., a cycads' cyanobiont, with the ultrastructural indication of overproduction of the material similar to the intercellular mucus *CG* cyanophycin granule, *CM* cytoplasmic membrane, *ES* exoprotplast sheath, *M* intercellular mucous matrix, *MM* cytoplasmic material similar to the mucous intercellular material, *Po* heterocyst pore, *T* thylakoids, *V* vesicle, *FL* fibrillar, *HL* homogeneous and *LL* lamellar layers of heterocyst envelope

intercellular mucus in symbiotic microcolonies, together with the CWRF of the vegetative cells described in Sect. 5.1.2. A pronounced boundary exists between the mucus of a cyanobiont microcolony and the plant cell wall (see Fig. 5.29), suggesting microbial origin of most of the material forming this intercellular matrix.

The thylakoid membranes within the cell forms with additional exoprotoplast sheath were usually strongly compressed. The fine-grain and thin-fibrillar material, ultrastructurally similar to the intercellular mucous material and to the material forming the exoprotoplast sheath, was located between the thylakoids. This similarity was observed in EM preparations fixed by various methods, including ruthenium red staining (Baulina and Lobakova, unpublished data), as well as on ultrathin sections contrasted with lead citrate or uranyl acetate. Comparative analysis of these data suggests the presence of acidic polysaccharides in the cytoplasm, exoprotoplast sheath, and the intercellular matrix. Moreover, pink staining of the heterocyst content was revealed by light microscopy of the sections of the coralloid roots (intact of fixed with the Carnoy's solution) stained with ruthenium red (Fig. 5.14 a, b) (Baulina and Lobakova 2005). All these techniques have been used for investigation of production of the mucous material by the CWDF of the vegetative cells (see Sect. 5.1.2).

In the CWRH of the first group, the ribosomes and nucleoid zones typical of cyanobacteria were usually not detected. The cytoplasm contained lipid β-granules (see Fig. 3.33), granules of unknown nature with low electron density (see

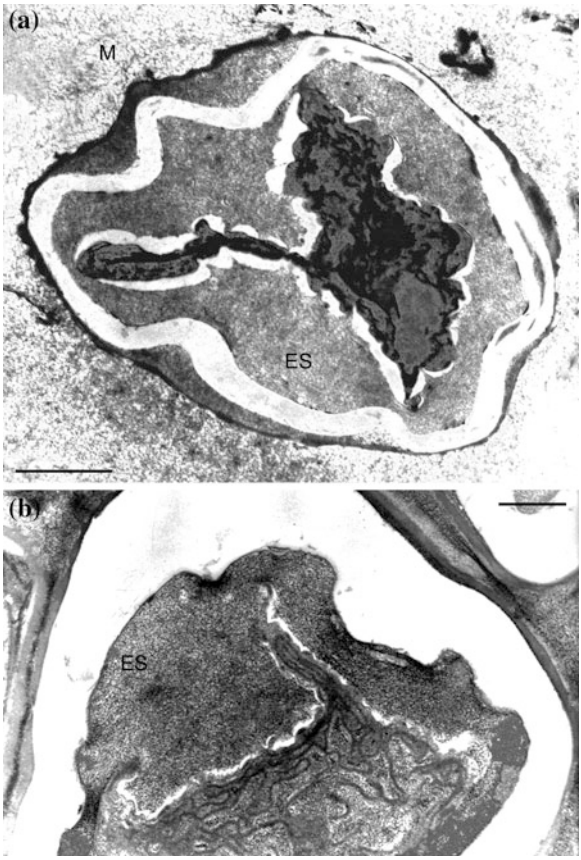

Fig. 5.31 Ultrastructure of the *Nostoc* sp. heterocysts with reduced cell wall and exoprotoplast sheaths close to the apex of the coralloid roots of *Cycas circinalis* (**a**) (reprinted from Baulina and Lobakova (2003b) with kind permission from Pleiades Publishing, Ltd.) and *Encephalartos villosus* (**b**) (data obtained by Lobakova and Baulina) *ES* exoprotplast sheath, *M* intercellular mucous matrix. *Scale bar* a, 1; b, 0.5 μm

Fig. 5.32) similar to those of the CWRF of the vegetative cells (see Fig. 5.11a), and large cyanophycin granules close to the pore, which are characteristic of intact heterocysts (see Fig. 5.34 and schemes (Figs. 5.26 and 5.30)).

In *Cyc. circinalis* cyanobionts, the ultrastructure of CWRH with indications of overproduction of extracellular proteins (second group) is characterized by the nucleoid/s' zones, numerous orderly located ribosomes, and accumulation of electron-dense material in the cytoplasm, above CM, and at the periphery of the protoplast invagination area of the pore (Fig. 5.35). In the latter case, this proteinaceous material was blended with the additional electron-dense layer, which surrounds CWRH, as well as the intact heterocysts and other cell forms in the microcolonies of the same intercellular spaces.

Ultrastructural investigation of the cyanobionts from various genera and species of the cycads suggests that the direction of the action of the regulatory mechanisms may change during heterocysts differentiation, resulting in beginning or increasing of the intracytoplasmic synthesis of the extracellular material unusual for this cell type. The possible involvement of CWRH in formation of the intercellular mucus

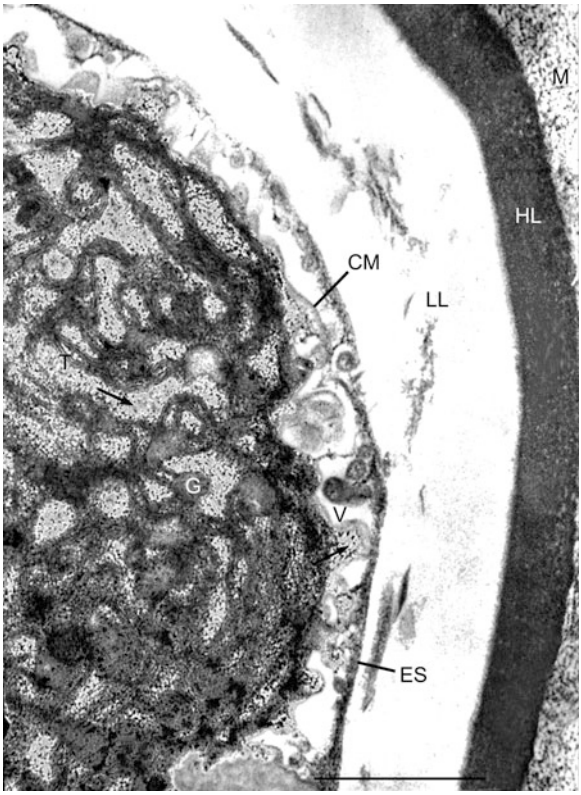

Fig. 5.32 Ultrastructure of the cell wall-reduced heterocyst of cyanobiont in the basal part of the *Encephalartos villosus* coralloid root (reprinted from Baulina and Lobakova (2003b) with kind permission from Pleiades Publishing, Ltd.) *CM* cytoplasmic membrane, *ES* exoprotplast sheath, *G* granules of unidentified composition, *HL* homogeneous and *LL* lamellar layers of the heterocyst envelope, *M* intercellular mucous matrix, *T* thylakoid, *V* vesicle. *Arrow* points to the aggregations of fine-grain and thin-fibrillar material in cytoplasm and in surface formed vesicles. *Scale bar* 0.5 μm

is an indication of the adaptive nature of such changes (see the previous Sect. 5.1.2). Thus, CWRH of this type, although the deep degradation processes associated with their rearrangement are incompatible with further life activity, probably function at the population level as active components, together with the specialized CWDF of the vegetative cells, in a complex with intact vegetative cells and nitrogen-fixing heterocysts.

5.2 Concluding Remarks

In natural symbioses with plants (liverwort *B. pusilla* and cycads), the symbiotic cyanobacteria of the genus *Nostoc* are able to produce CWRF of the vegetative cells, which may be grouped into three types, according to their structural features. These are, first of all, the ultrastructurally integral protoplasts and spheroplasts; then, degrading protoplasts and spheroplasts with evidence for overproduction of the extracellular material similar to the mucus polysaccharides; and finally,

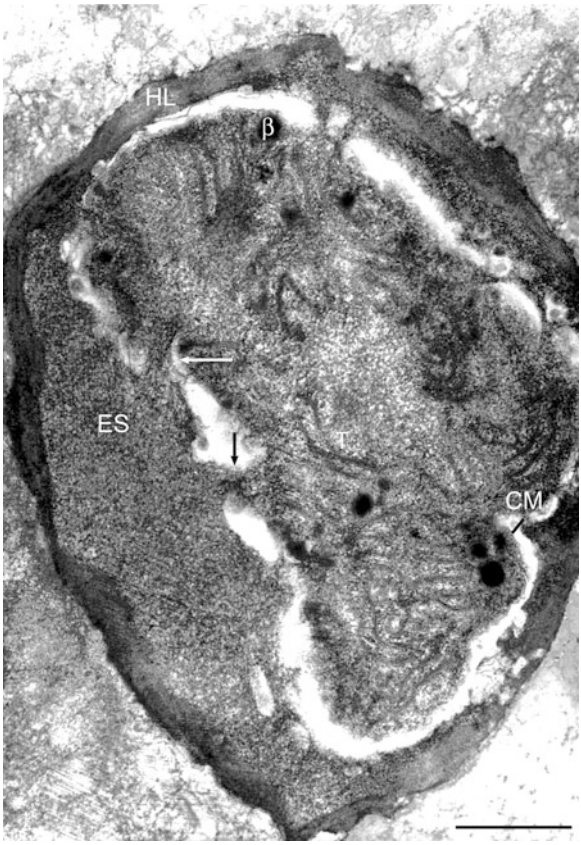

Fig. 5.33 Ultrastructure of the cell wall-reduced heterocyst of cyanobiont close to the apex of the *Ceratozamia mexicana* coralloid root (reprinted from Baulina and Lobakova (2003b) with kind permission from Pleiades Publishing, Ltd.) *β*, high electron density lipid *β*-granules; *CM* cytoplasmic membrane, *ES* exoprotplast sheath, *HL* homogeneous layer of the heterocyst envelope. *Arrow* points to the strands of fine-grain and thin-fibrillar material of cytoplasm continuously bound to the exoprotoplast sheath. *Scale bar* 0.5 μm

degrading protoplasts with evidence of overproduction of the extracellular proteinaceous material. Occurrence of the CWRF of the first type is an indication of the ultrastructural plasticity of CM, as well as cyanobiont cells and populations, and of the possibility of L-transformation of cyanobacteria in natural symbioses. The presence of the second type of CWRF indicates the existence of a previously unknown mechanism of intracytoplasmic synthesis and accumulation of acidic polysaccharides of the mucus participating in formation of the intercellular matrix within the specialized symbiotic zone of the cycad coralloid roots. In this case, the structural and functional plasticity of the thylakoids may be involved, which preserved their integrity even at the late stages of degradation and dying off of the mucus-producing CWRF. Due to their multifunctionality, they are probably involved in polymerization of the acidic polysaccharides via some presently unknown mechanism. The ultrastructural plasticity at the cellular level can not be confirmed, since the CWRF of the second type undergo irreversible degradation associated with the loss of DNA and ribosomes, as well as CM ruptures. This is in agreement with the incompatibility of the intracytoplasmic synthesis of acidic

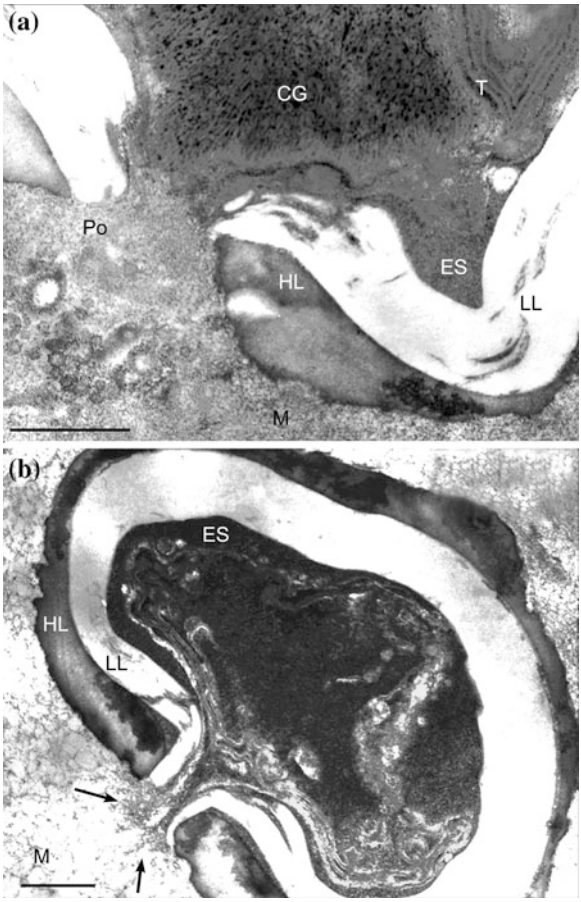

Fig. 5.34 Ultrastructure of the cell wall-reduced heterocyst of the *Encephalartos villosus* cyanobiont: in the central area of coralloid root (the pore region) (**a**) and close to the apex (**b**) (**a**, **b**, reprinted from Baulina and Lobakova (2003b) with kind permission from Pleiades Publishing, Ltd.) *CG* cyanophycin granule, *ES* exoprotplast sheath, *HL* homogeneous and *LL* lamellar layers of the heterocyst envelope, *M* intercellular mucous matrix, *Po* heterocyst pore, *T* thylakoids. *Arrow* points to the strands of fine-grain and thin-fibrillar material continuously bound to the intercellular mucous matrix. *Scale bar* a, b, 0.5 μm

polysaccharides with the maintenance of the cellular homeostasis. Sequential stages of development of these cell forms from the vegetative cells are the subject of our ongoing research.

Simultaneous detection of intact vegetative cells and heterocysts together with the mucus-producing CWRF and CWRH in the same *Nostoc* sp. microcolonies in the intercellular spaces of the parenchymal specialized zone in the cycads suggest the ultrastructural plasticity at the population level. Investigation of other bacteria resulted in a conclusion that adaptive changes in a population may include dying off of the individual cells or cell groups (Waisman 1984). The results presented in this chapter suggest that emergence of the moribund cells with specialized functions has an adaptive character, since the forming intercellular matrix protects the population as a whole from bactericidal action of the plant phenolic compounds. Consideration of this process from the point-of-view of the modern concepts of cooperative interactions between the cells (coordinated by extracellular signals) in immobilized populations, such as biofilms, may be important for the future

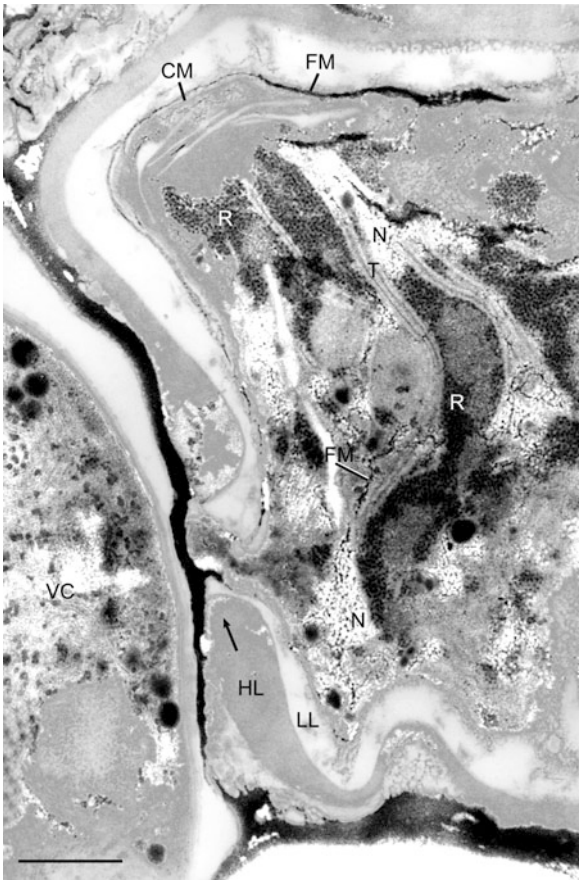

Fig. 5.35 Ultrastructure of the cell wall-reduced heterocyst producing extracellular material (probably of protein nature) from the *Cycas circinalis* cyanobiont population close to the apex of a coralloid root (reprinted from Baulina and Lobakova (2003b) with kind permission from Pleiades Publishing, Ltd.) *CM* cytoplasmic membrane, *FM* high electron density fibrillar matter, *HL* homogeneous and *LL* lamellar layers of the heterocyst envelope, *N* nucleoid, *R* ribosomes, *T* thylakoids, *VC* vegetative cell. *Arrow* points to the pore region. *Scale bar* 0.5 μm

decoding of the molecular genetic mechanisms of development (probably by differentiation) of unusual cell forms in cyanobiont populations.

The morphological and ultrastructural diversity of the cell forms in the populations of the cycad cyanobionts is a regular phenomenon observed in different plant species and in different years or seasons. In *Cyc. circinalis*, apart from the cyanobiont microcolonies containing the cell forms of the first two types, the microcolonies formed by the protoplasts of the third type were found, as well as CWRH with an indication of overproduction of extracellular proteins. Although it is difficult to suggest the biological role of these cell forms within a symbiosis, their existence indicates an unusually broad spectrum of manifestations of the ultrastructural plasticity in the symbiotic cyanobacteria at the population level.

Thus, the physiological diversity of the CWRF within bacterial populations was determined, with these forms having diverse functional specificity as L-forms or overproducers of the extracellular material (polysaccharides or proteinaceous).

The data presented in this chapter suggest the following conclusion. The morphological and ultrastructural features of cyanobacteria forming symbioses

with higher plants indicate that a broader range of the physiological characteristics of these microorganisms is developed or intensified in symbioses than was known previously. These obvious cases of cyanobacterial ultrastructural plasticity show that their potential (genetically determined) adaptive capacities are much more diverse than it was previously supposed.

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Chapter 6
Conclusion

Abstract The concluding chapter is devoted to the comparative analysis of ultrastructural plasticity of diverse in metabolic capabilities cyanobacterial species in experimental and natural systems described in the book. The concept of ultrastructural plasticity is defined, its basic manifestations on the subcellular, cell, and population levels are summarized. The characteristic relationship between metabolic and ultrastructural plasticity in cyanobacteria is emphasized; the extent of the plasticity is a determinant of the interstrain and interspecies difference in the adaptive potential. As the key conclusion, the list of cyanobacterial adaptation mechanisms is presented including those described in the pioneering publications by the author and her colleagues, the revealing of the novel mechanisms made possible by investigation of the ultrastructural plasticity of these microorganisms.

We have thoroughly considered the ultrastructure of cyanobacteria of different taxonomic groups under experimental and natural conditions causing cardinal changes in the mode of existence and behavior of these organisms. The presented data demonstrate that a number of the discovered modifications in the fine structure of cellular organelles, individual cells, and cell groups are associated with the preservation of the structural and functional integrity of both cells and cell populations and with the maintenance of the viability of cyanobacteria under unfavorable conditions. Consequently, these modifications may be regarded as forms of manifestation of the ultrastructural plasticity, which is considered here as a component of the phenotypic plasticity inherent in prokaryotes. This plasticity is a complex of reactions of adaptive cellular reorganization adequate to the changes in environmental conditions. Cyanobacteria belong to the bacterial world and have the typical organization of gram-negative cell wall, the reproductive and protein-synthesizing systems, and a variety of cell structures and organelles. Analysis of specific patterns of the ultrastructural modifications, of the presence or absence of the mucous surface structures, the outer membrane and cell wall peptidoglycan layer, CM, thylakoids, nucleoid, ribosomes, and glycogen, as well as comparison of these characteristics in different cyanobacterial cells within the populations

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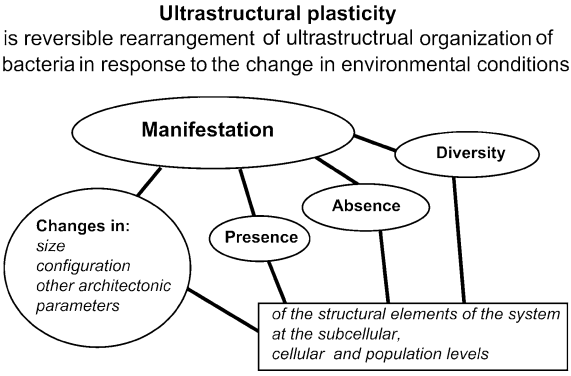

Fig. 6.1 Bacterial ultrastructural plasticity and its manifestation

studied under both optimal and unfavorable conditions, makes it possible to define the term "ultrastructural plasticity" for cyanobacteria and bacteria in general. Ultrastructural plasticity is reversible rearrangement of the ultrastructural organization of bacteria in response to the change in environmental conditions. It manifests itself in the changes in size, configuration, and other architectonic parameters, as well as in the presence, absence, or diversity of the structural elements at the subcellular, cellular, and population levels to the extent of conservation of the structural and functional integrity of a system (Fig. 6.1) (Baulina 2005, 2008). The introduction of this concept makes it possible to consider the ultrastructural criteria as indicators of the causes and potential mechanisms of the interdependent morphological and functional modifications of subcellular structures, cells, and populations adequate to the modified habitat conditions. Moreover, investigation of the ultrastructural plasticity at the population level facilitates determination of the role of the intrapopulation intercellular interactions in the processes of adaptation, in particular, such as emergence of specialized cells within a population.

The basic forms and interconnected levels of expression of the ultrastructural plasticity in cyanobacteria grown under different conditions of illumination, enzymatic induction of L-transformation, or as components of the model associations and natural symbioses with plants are summarized in the scheme (Fig. 6.2). In addition to this scheme, for the sake of generalization, the types of cells with different morphology and ultrastructure reflecting their physiological state and functional specificity should be enumerated, which in certain combinations, depending on the conditions, impart adaptive cellular heterogeneity to the populations:

(1) Intact vegetative cells;
(2) Specialized cells (heterocysts and akinetes in the experiments described);
(3) CWDF, including spheroplasts and protoplasts, which may have different functional specificity, viz. UGF, L-forms, and (over)producers of extracellular substances;
(4) CWRH with (over)production of extracellular substances;

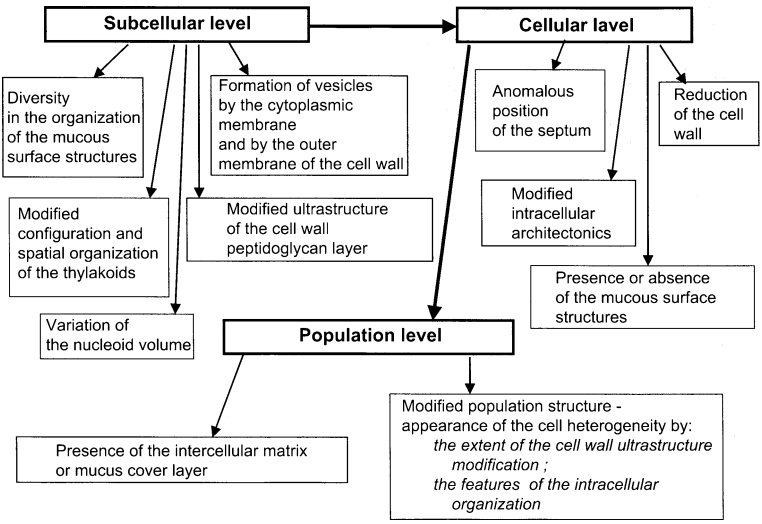

Fig. 6.2 Diverse manifestations of cyanobacterial ultrastructural plasticity (adopted from Baulina (2008) with kind permission from Allerton Press, Ltd.)

(5) Cells at reversible stages of degradation: plasmolyzed, with the swelling of thylakoids;
(6) Cells with modified arrangement of the intracellular structures, particularly thylakoids;
(7) Cells in the terminal state.

It should be emphasized that the character and diversity of the forms of manifestation of the ultrastructural plasticity are associated with the physiological properties of the species, including its metabolic potential and capacity for cell differentiation. This conclusion results from analysis of the ultrastructural plasticity in the specially selected species (*Synechococcus* sp., *A. variabilis,* and *C. fritschii*) investigated under conditions of cultivation in the dark and under high light intensity, induction of L-transformation, growth as part of the model associations with plant partners, and exposure of cell suspensions to extremely high-intensity light.

Synechococcus sp. 6301, the organism with the lowest metabolic plasticity, showed the highest conservatism of its ultrastructural organization and cell morphology. The ultrastructural plasticity was not revealed during growth under high light intensity and irradiation of the suspensions to extremely high-intensity light: some cells of the population underwent rapid photodestruction, while other cells demonstrated high stability. An analogous situation was observed in these experiments for *S. elongatus*. Under the conditions inducing L-transformation, the *Synechococcus* sp. 6301 spheroplasts obtained at the first stages of experiments were incapable of cultivation. Two strains of this species in the model associations with plant partners exhibited only the changes in the ultrastructure of the cell

wall peptidoglycan layer, i.e., ultrastructural plasticity was limited in this case. This finding correlated with the relatively low lifetime of subcultured associations. Formation of spines during the growth in monoculture on the medium for associations unfavorable for cyanobacteria may probably be considered as a form of manifestation of the ultrastructural plasticity of *Synechococcus* sp.

It is presently considered that unicellular cyanobacteria, particularly those assigned to subsection I (order Chroococcales), do not form symbioses with higher plants under natural conditions, although they possess symbiotic potential toward some animals, protozoa, fungi, and diatoms (Schenk 1992; Rai et al. 2000; Webb and Maas 2002; Steindler et al. 2005; Bergman et al. 2008). Members of some genera of unicellular cyanobacteria from subsections I and II (orders Chroococcales and Pleurocapsales) are components of lichens (Rikkinen 2002). At the same time, cyanobacteria of the genus *Synechococcus* are found quite seldom, even among non-plant symbioses. For example, the unicellular cyanobacterium described as *Anacystis montana* exists in a symbiosis with ascomycete lichens (Bubrick and Galun 1984). Phylogenetic analysis of the 16S rRNA nucleotide gene sequence of cyanobacteria associated with sponges shows the presence of members of the genus *Synechococcus* as part of this symbiosis (Steindler et al. 2005). We have recently found a unicellular cyanobacterium with the ultrastructure typical of this genus as a component of an epibiotic microbial biofilm developing on the exoskeleton of the White Sea hydroid polyp *Dynamena pumila* (L.,1758) (Gorelova et al. 2009). Phylogenetic analysis of the nucleotide sequences of the ribosomal cluster demonstrated high probability of classification of this unicellular cyanobacterium within the order Chroococcales (Gorelova et al. 2012).

The greatest diversity of the forms of manifestation of the ultrastructural plasticity in the line of *Synechococcus* sp., *A. variabilis* and *C. fritschii* was observed in the latter species, which is characterized by the highest metabolic plasticity. First and foremost, ultrastructural rearrangements associated with the preservation of the culture viability occurred under all of the above experimental cultivation conditions for this cyanobacterium. Moreover, only *C. fritschii,* unlike the tested *Synechococcus* sp. 6301 and *A. variabilis* 458, can exist as CWRF (including protoplasts) during cultivation in the presence of lysozyme (conditions for induction of L-transformation). This is presently the only species (strain) of cyanobacteria, for which the possibility of L-transformation has been shown in special in vitro experiments. To the best of our knowledge, there are no other reports, and the descriptions of the ultrastructure of the CWRF obtained under short-term treatment with lysozyme are few (see Chap. 3). *C. fritschii* is also capable of spontaneous formation of protoplasts in the dark in the presence of glucose and in the model associations with plant cells in suspension culture. Moreover, although *C. fritschii* could undergo photooxidative destruction, like other cyanobacteria tested in these experiments, it could grow for a long time under high light intensity (9–10 klx) with significant modifications in the fine structure and quantity of cellular structures and organelles, particularly thylakoids, under both photoauto- and photoheterotrophic conditions, while the members of other species gradually degraded and died off.

Although both *A. variabilis* strains occupied an intermediate position in some or other experiments as regards the diversity of the forms of manifestation of the ultrastructural plasticity, they were altogether comparable with *C. fritschii* and distant from *Synechococcus* sp. 6301. The whole complex of experiments has revealed a great variety of ultrastructural changes in the two *A. variabilis* strains including, e.g., the abnormal (for this species) formation of gas vesicles under unusual growth conditions and a peculiar form of photooxidative destruction coupled with the "conservation" of unviable cells. The thylakoids of the *A. variabilis* 458 incubated in the dark underwent configuration changes, expressed in enlargement of the intrathylakoid space, which was reversible when the culture was transferred to the light. In this case, this manifestation of ultrastructural plasticity indicated the action of an adaptive mechanism of reversible thylakoid swelling, which has been studied only in the chloroplasts of higher plants (Johnson et al. 2011). The theoretically possible forms of ultrastructural plasticity of the *A. variabilis* strains can be realized regardless of anticipated experimental results. For example, no formation of L-form-like CWRF was observed under deliberate induction of L-transformation, although these cell forms were found in the experiments on creation of model associations with plant partners.

Inclusion of the stable model associations of plant partners with free-living *N. muscorum* 16 and 304, as well as the natural symbioses of *Nostoc* sp. and higher plants, into the scope of investigated objects is of particular importance for investigation of the ultrastructural plasticity of cyanobacteria. *Nostoc* sp., forming a symbiotic association with the cycad *Cyc. circinalis.,* exhibited considerable metabolic plasticity, namely, the capacity for chemoheterotrophic growth in symbiotic tissues and rapid resumption of photosynthesis after isolation (Tredici et al. 1989). The members of the genus *Nostoc,* in contrast to *Synechococcus* sp., are widespread in symbioses. The results of research on cyanobacteria of the genus *Nostoc* in model associations and natural symbioses with higher plants, together with the data on L-form-like CWRF formation in other species listed above, made it possible to formulate a new problem in the field of microbiology and symbiology concerning the existence and significance of the process of L-transformation of cyanobionts in the symbioses. Table 6.1 shows the model associations and natural symbioses with ultrastructurally integral protoplasts and spheroplasts of cyanobacteria interpreted as developing or completely developed L-forms. The results of research on these symbiotic systems correspond to the concepts of the role of bacterial L-transformation as a peculiar form of adaptation to the modified habitat and of persistence within a macroorganism. Moreover, the revealed possibility of existence cell wall-reduced forms of cyanobacteria is in agreement with the present concept of the evolutionary role of these microorganisms as chloroplast precursors, and our findings are the new experimental material for its development.

The study of the ultrastructural plasticity of *Nostoc* sp. in the natural symbioses considerably extends the concept of adaptive capacities of cyanobacteria. The interstitial populations of cycad cyanobionts contained CWRF of the vegetative cells and CWRH apparently specializing in (over)production of the mucilaginous polysaccharides of the intercellular matrix, which is supposedly associated with the protective function under enhanced synthesis of bactericidal phenolic

Table 6.1 The model association and the natural symbioses containing protoplasts and spheroplasts of cyanobacteria

Cyanobacteria	Plant partner	References
Anabaena variabilis CALU 458	*Nicotiana tabacum* Samsun variety (callus)	Baulina et al. (1984)
Chlorogloeopsis fritschii ATCC 27193	*Panax ginseng* IFR ZH1 (suspension culture)	Gusev et al. (2002); Baulina (2005)
C. fritschii ATCC 27193	*Solanum laciniatum* (suspension culture)	Gorelova (2005)
Nostoc muscorum VKM 16	*Medicago sativa* (rooted cuttings, callus, suspension culture)	Baulina et al. (1990), Gorelova (2005), Korzhenevskaya et al. (1993)
N. muscorum CALU 304	*S. dulcamara* (callus)	Gorelova (2005)
N. muscorum CALU 304	*Rauwolfia serpentina* K-27 (callus)	Gorelova (2001), Gorelova and Korzhenevskaya (2002)
Nostoc sp.	Liverwort *Blasia pusilla*	Gorelova et al. (1992), Korzhenevskaya et al. (1993), Gorelova et al. (1996)
Nostoc sp.	Cycads: *Cycas circinalis, Cyc. revoluta, Cyc. micholitzii, Encephalartos villosus*	Baulina et al. (2000), Gusev et al. (2002), Lobakova (2004), Baulina (2005)

compounds by plant cells. We have every reason to consider that bulk synthesis and accumulation of acidic polysaccharides in these cell forms occurs within the cytoplasm, which has not been shown for other bacteria, except for the mutant *E. coli* strains and wild type *E. coli* with inhibited exopolysaccharide export (see Chap. 5). It is supposed that the formation of CWRF and CWRH with the specialized function of exopolysaccharide overproduction associated with the dying away of these forms is an adaptation mechanism with its action implemented at the population level. The results of research on the ultrastructural plasticity of the cells and the structure of interstitial cyanobacterial populations as part of symbioses with cycads, as well as the analysis of modern literature data on the functioning of regulatory sensory/signaling systems in bacterial populations, suggest the existence of cooperative interaction between the cells in the symbiotic microcolonies of cyanobacteria.

In general, the experimental and theoretical data considered in the book lead to a conclusion that the analysis of the forms of manifestation of the ultrastructural plasticity permits indication of the adaptive mechanisms realized at the levels of subcellular structures, cells, and populations (Table 6.2). Interpretation of the molecular genetic processes underlying some or other specific mechanisms is a problem for research in the future. Our work offers a clue to the possibility of revealing such adaptation mechanisms in prokaryotes. Prior to our research, some of the important mechanisms were unknown in cyanobacteria (e.g., reversible swelling of thylakoids and L-transformation) or did not attract researchers'

attention (e.g., spontaneous formation of protoplasts in *C. fritschii*). At the same time, it would be fair to note that the results of electron microscopy indicative of the mechanism such as vesicular transport with involvement of the outer membrane in cyanobacteria were obtained long ago. This mechanism is considered as one of the pathways of secretion of material from the cells of gram-negative bacteria (Beveridge 1999). The research on cyanobacteria described in this book demonstrates the possibility of vesicular transport in prokaryotes with involvement of not only the outer membrane but also CM. The latter is typical of the protoplasts of these microorganisms. As regards the mechanism of regulation of glycogen synthesis and degradation, its possible existence in cyanobacteria is certainly confirmed not only by the results described. The dependence of glycogen synthesis and degradation on the cultivation conditions and the carbon turnover in the granules formed by this glucan has been described earlier by many authors (Gromov 1986). The molecular genetic mechanisms of regulation of the switching of carbohydrate anabolic and catabolic processes are investigated as a part of the general problem of carbon metabolism and mutual conversions between photoautotrophy and photomixotrophy in cyanobacteria; however, they have not been sufficiently studied as yet (Haimovich et al. 2008; Kaplan et al. 2008). Moreover, as regards the adaptive mechanism of the changes in the macromolecular organization of peptidoglycan (regulation of synthesis and the effects of endogenous or exogenous lytic enzymes), it may be stated that no particular experimental research on the possibility of its action have been performed in cyanobacteria, according to the data available to the author of this book. For other bacteria, at least for the gram-positive *Lactococcus lactus*, the flexibility of cell wall biosynthesis machinery toward a new substrate was investigated (Deghorain et al. 2010). Investigation of the ultrastructural changes of CWDF peptidoglycan in cyanobacterium *N. muscorum* 304 in association with the rauwolfia callus yielded the data showing that a plant partner can induce the activation of cyanobacterial autolytic enzymes, which not only causes direct lysis of peptidoglycan but also impairs its synthesis (Gorelova 2001). In opinion of the author of the cited work, the presented results visualize not only the unbalance of the lytic and synthetic components of the enzyme complexes but also the shift in the ratio of enzyme activities: penicillin-binding proteins PBP2 and PBP3. These proteins catalyze the assembly of peptidoglycan chains at the last stages of its biosynthesis and are necessary for growth and division of bacterial cells (Macheboeuf et al. 2006; Legaree et al. 2007). Gorelova's paper (2001) presents also the electron microscopy data demonstrating the intensified activity of the protein-synthesizing system in the *N. muscorum* 304 spheroplasts.

The adaptation mechanisms summarized in Table 6.2 include those which, to the best of our knowledge, have not been found previously in cyanobacteria or in other bacteria. What is meant here is a complex of mechanisms functioning at the subcellular, cellular, and population levels, which is associated with the synthesis and bulk accumulation of acid exopolysaccharides in the cytoplasm of symbiotic *Nostoc* sp. by the specialized cells with reduced cell wall.

Table 6.2 Adaptation mechanisms revealed in cyanobacteria by investigation of the ultrastructural plasticity

Mechanisms	Interrelated levels of functioning
1. Vesicular transport with involvement of the outer membrane	**Subcellular**
2. Vesicular transport with involvement of the cytoplasmic membrane	
3. Change in the macromolecular organization of the cytoplasmic membrane in the absence of cell wall	
4. Changes in macromolecular organization of peptidoglycan via the regulation of synthesis or the influence of endogenous or exogenous lytic enzymes	
5. Reversible swelling of thylakoids	
6. Intracytoplasmic synthesis and accumulation of acid exopolysaccharides	
7. Intensification of activity of the protein-synthesizing system	
1. (Over)production of extracellular polysaccharides by specialized cell forms	**Cellular**
2. Changed localization of the initiation sites of septum formation (generation of unbalanced growth forms)	
3. Formation of spheroplasts	
4. Formation of protoplasts	
5. Changes in the differentiation of heterocysts with the modification of their functional specialization	
6. Regulation of glycogen synthesis and degradation	
1. Transition of the population into the unbalanced growth phase	**Population**
2. L-transformation	
3. Formation of intercellular matrix	
4. Formation of unusual cell forms of vegetative cells and heterocysts with specialized function	
5. Heterogeneity of the population by physiological state and functional specialization of cells	

Thus, with cyanobacteria as an example, it was shown that the discovery of new forms of manifestation of the ultrastructural plasticity and the resultant possibility of indicating different adaptation mechanisms, including the previously unknown ones, considerably extend our concept of the diversity of adaptive responses of microorganisms.

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