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REVIEW PAPERS

Application of Molecular Genetic and Microbiological Techniques in Ecology and Biotechnology of Cyanobacteria

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Abstract—The review discusses the advances and problems in biotechnology and ecology of cyanobacteria and considers the possibilities of molecular genetic and microbiological techniques in this field. Due to the ease of cultivation, high growth rate, availability of synchronous cultures, and existence of numerous molecular genetic and microbiological techniques for various cyanobacterial strains, cyanobacteria—prokaryotic organisms that are ancient relatives of the chloroplasts—are model organisms in the studies of photosynthesis, dinitrogen fixation, cell division, hydrogen production, and in a number of other areas of basic and applied science. These techniques make possible deeper understanding of the role of cyanobacteria in various ecosystems and utilization of their potential in numerous applied projects, including production of molecular hydrogen, phycobiliproteins, and cyanophycin; formation of nanoparticles; removal of heavy metals from the environment; substrate biodegradation; manufacture of products for medicine and food industry; and solution of the problem of cyanobacterial toxins in freshwater and marine environments.

Keywords: cyanobacterial toxins, photoproduction of hydrogen, cyanobacterial nanoparticles, phycobiliproteins.

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Cyanobacteria are photoautotrophic bacteria containing chlorophyll *a* and carrying out photosynthesis similar to higher plants. These "modest" organisms need only sunlight, water, and air. In some cyanobacteria, a small portion of cells differentiates to heterocysts, specialized cells where fixation of atmospheric N_2 occurs. This is one of the few pathways involving dinitrogen in the biosphere. After the groundbreaking works carried out in the 1970s in Moscow State University, when the capacity of cyanobacteria for genetic transformation of exogenous DNA was demonstrated [1, 2], these organisms became attractive subjects of molecular genetic research. Various microbiological and genetic investigations of cyanobacteria are presently carried out in many laboratories worldwide. Molecular techniques have been developed for these organisms, and information on the complete genomic sequences of tens of cyanobacterial species and strains is available (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). Transformation, conjugation, and electroportation are used for transfer of cyanobacterial genes. Various techniques of mutagenesis make it possible to obtain numerous types of mutants. Reporter genes are used to assess the level of transcription of individual genes and investigate transcription in individual colonies or in the individual cells in a filament (chain of cyanobacterial cells) for filamentous strains [3]. Information on complete cyanobacterial genomes makes it possible to investigate gene expression on the levels of transcription and translation [4–6].

Attention to applied aspects of science is a result of the changing conditions of the development of our society. The present review deals with the major aspects of cyanobacterial biotechnology, including biohydrogen production, utilization of cyanobacteria-derived products in medicine and nutrition, formation of nanoparticles in cyanobacterial cells, etc.

The goal of this review is to outline the modern level of knowledge of cyanobacteria—unique phototrophic microorganisms—obtained by molecular genetic and microbiological techniques, as well as its application for various environmental and biotechnological purposes.

CYANOBACTERIA AND THE ENVIRONMENT

Cyanobacteria are widespread in marine, brackish, and freshwater aquatic environments; in soils and stone encrustations; and, especially, on moist surfaces. They occur in such extreme environments as hot springs and strongly alkaline lakes. Cyanobacteria form natural and artificial symbioses with algae, mosses, ferns, angiosperms, cycads [13], and invertebrates (corals, sponges, and hydroids) [7–16]. In associations with fungi, they form lichens that participate in the transformation of rocks into soil. Cyanobacteria are considered the first terrestrial organisms, which

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provided the chemical and physical substrate for the subsequent development of eukaryotic plants. Microorganisms developing as biofilms synthesize extracellular polymers, which enable existence of a biofilm as an integrated structure [17]. These biopolymers produced by algae and cyanobacteria may improve water retention in soil and counteract erosion [18]. Filamentous nitrogen-fixing cyanobacteria are used as biological fertilizers on rice paddy fields [19, 20].

Disposal of industrial, agricultural, and municipal liquid waste containing phosphates and nitrates causes contamination of lakes and ponds, resulting in massive cyanobacterial growth (blooms) [21]. The water surface becomes turbid, preventing penetration of light to the deeper water layers. The bacteria decomposing the dead cell within the cyanobacterial population exhaust available oxygen, causing fish death due to oxygen deficiency. Some cyanobacteria involved in water blooms produce toxins that make the water unusable [22, 23]. These toxins may accumulate in aquatic organisms and move upward along the trophic chains, presenting problems in human and animal health care [24]. Cyanobacteria synthesize hepatotoxins (microcystins and nodularins), hepato- and cytotoxins (cylindrospermopsins), neurotoxins (anatoxin-a, anatoxin-a(S), and saxitoxins), dermatotoxins, irritating toxins (lipopolysaccharides), and other marine biotoxins (aplysiatoxins, debromoaplysiatoxins, and lyngbyatoxin-a) [25–27].

In blooming freshwater reservoirs, cyanobacterial hepatotoxins are usually heptapeptides (microcystins), while a pentapeptide (nodularin) is widespread in saline environments. Microcystins were found in the members of such genera as *Anabaena, Anabaenopsis, Hapalosiphon, Microcystis, Nostoc, Planktothrix, Phormidium*, and *Synechococcus* [27]. Microcystins are cyclical heptapeptides with an unusual chemical structure, containing some amounts of nonproteinogenic amino acids [28]. They have three variable methyl groups. Methylation is a widespread modification of biologically active natural peptides, which is supposed to enhance their stability against proteolytic degradation [29, 30]. Microcystins are synthesized without participation of ribosomes by large enzyme complexes consisting of peptide synthetases and polyketide synthetases [31]. Peptide synthetases responsible for nonribosomal peptide synthesis have a conservative modular structure. Each module consists of catalytic domains responsible for adenylation, thioester formation, and condensation of specific amino acids [32]. Additional domains required for modification of amino acid residues (epimerization, heterocyclization, oxidation, formylation, reduction, or N-methylation) may be also included into the module [32–34]. The complex of these biosynthetic peculiarities results in high diversity of cyanobacterial microcystins. Over 60 isomorphs of microcystins are presently known. Complete nucleotide sequences of the biosynthetic gene clusters have been determined for *Microcystis, Planktothrix, Anabaena, Nodularia*, and *Nostoc* [27]. In *Anabaena*, this enzyme complex is encoded by the cluster containing 10 genes (*mcyA–J*) [35]. If the genes encoding a bioactive component are known, directed mutagenesis may be used to obtain the mutants with impaired biosynthesis of these compounds as a tool for better understanding of the functions of these metabolites. In the case of microcystins, such mutants were obtained by insertion or deletion of the *mcy* genes in *M. aeruginosa* and *P. agardhii* strains [31, 36–39]. Insertion mutagenesis revealed [31] that the genes encoding peptide synthetases were involved in microcystin production, with one gene cluster being responsible for production of all microcystin variants in *Microcystis aeruginosa* strain PCC 7806. The role of microcystins for cyanobacteria remains unclear. The loss of all microcystins in the *mcyB* mutant of *M. aeruginosa* PCC 7806 [31] resulted in no differences in growth under different laboratory conditions of illumination between the mutant cells and the wild type [39]. However, comparative two-dimensional protein electrophoresis demonstrated high levels of the MrpA protein in wild-type cells of PCC 7806, while this protein was not detected in the *mcyB* mutants [40]. Modern experimental approaches (transcriptomics and proteomics), together with genetic techniques, may prove useful for investigation of the key stages of biosynthesis of cyanobacterial toxins.

Cyanobacteria produce a nonprotein amino acid β-N-methylamine-L-alanine (BMAA) [41]. At critical concentrations, this amino acid may bind to the human glutamate receptor, resulting in such diseases as amyotrophic lateral sclerosis or Alzheimer's and Parkinson's diseases [41, 42]. Since glutamate receptors have been previously considered in relation to signal transduction in mammals only, discovery of glutamate receptors in plants [43] and cyanobacteria [44] was quite unexpected. What are the functions of these receptors in cyanobacteria? What is the role of BMAA in the cells of these phototrophic microorganisms? Molecular genetic investigation is required to answer these questions. Apart from its basic importance, this problem has an applied aspect, since BMAA accumulation in the trophic chains may affect the health of humans consuming fish grown on cyanobacterial phytoplankton or on zooplankton consuming cyanobacteria. In such cases, caution should be recommended in consumption of rice grown on the fields where cyanobacterial fertilizers were used.

Cyanobacteria are able to metabolize natural aromatic hydrocarbons [45–49] and xenobiotics [50, 51]. Strains with enhanced degrading capacity may be produced by molecular genetic techniques [51]. Since cyanobacteria utilize crude oil and individual *n*-alkanes as carbon and energy sources, microbial cyanobacterial mats participate in decontamination of oil-pol-

luted water [52, 53]. Moreover, cyanobacterial nitrogen fixation provides nitrogen compound for the heterotrophic microorganisms of the mat, which also participate in oil degradation. Free radicals resulting from cyanobacterial oxygenic photosynthesis may also indirectly participate in the photochemical degradation of oil [54]. Phototrophic biofilms are presently considered attractive for wastewater purification [55] and heavy metal removal [56–59]. Cyanobacteria are efficient biological sorbents for metals in aquatic environments. The mucilage from the envelopes (mucilage sheaths)of *Microcystis aeruginosa* and *Aphanothece halophytica* exhibits high affinity to heavy metal ions (copper, lead, and zinc) [59]. Apart from biosorption and bioaccumulation, elevated pH inside the actively photosynthesizing biofilms may promote precipitation of heavy metals [60]. Cyanobacteria carry out biotransformation of Hg(II) to β-HgS and may act as efficient mercury reducers [61]. Genetic techniques may be used for production of more efficient strains. For example, the envelope-free mutant of *Gloeothece* sp. PCC 6909 removed copper ions more actively than the wild type [62].

Since photoautotrophic cyanobacteria share the same ecological niches with mosquitoes and mosquito larvae feed on these microorganisms, cyanobacteria are attractive agents for mosquito control [63, 64]. Transgenic cyanobacterial strains were constructed that caused death of mosquitoes [65–73]. Recombinant clones of *Anabaena* 7120, carrying two genes encoding δ-endotoxin (*cryIVA* and *cryIVD*) and the *p20* gene from *Bacillus thuringiensis* subsp. *israelensis*, exhibited high toxicity [73]. Two-month tests demonstrated that *Anabaena* 7120 prevented development of *Culex* larvae in containers with natural water [72]. Importantly, the modified cyanobacterium expressing the proteins of *B. thuringiensis* subsp. *israelensis* δ-endotoxin protected the antimosquito toxins from inactivation by UV radiation [74], since cyanobacterial membrane pigment complexes act as screens protecting the cell from ultraviolet radiation.

Research on cyanobacterial acclimation provides useful information concerning their regulated adaptation to new habitats. Cyanobacteria accumulate diverse osmolites, depending on the nature of a stress. Trehalose, a well-known compound stabilizing membranes and proteins upon drying, is accumulated in cyanobacterial cells under salt or osmotic stress [75–80]. Molecular investigation on survival of cyanobacteria under conditions of extreme aridity were made possible by development of a genetic system for *Chroococcidiopsis*, which dominates in the natural communities of the most extreme hot and cold arid deserts [81]. Transfer of a single gene, *spsA,* encoding sucrose-6-phosphate synthase, from *Synechocystis* to the drying-sensitive *Escherichia coli* resulted in its 10^4 higher survival after freeze-drying, drying in air, or desiccation by phosphorus oxide, compared to the wild type cells [82]. DNA microchips combined with insertion mutagenesis made it possible to identify and investigate a number of stress-induced genes and regulatory systems in *Synechocystis* sp. PCC 6803, resulting in considerable progress in the understanding of the mechanisms of cyanobacterial reaction to environmental stress factors [83].

SYNTHESIS OF VARIOUS BIOLOGICALLY ACTIVE COMPOUNDS AND MOLECULAR HYDROGEN BY CYANOBACTERIA

Cyanobacteria are an efficient source of a number of biologically active compounds (BACs). The diversity of BACs synthesized by cyanobacteria, including those of biotechnological importance, is amazing. For example, they synthesize polyhydroxyalkanoates, thermoplastic biodegradable ethers, including polyhydroxybutyrate. They are used for storage of carbon and energy. Polyhydroxyalkanoates are accumulated as granules in the cytoplasm and are widespread in various cyanobacterial strains. At least 50 strains of four subsections were found to contain polyhydroxyalkanoates [84]. Such techniques as Southern DNA/DNA hybridization, hybridization with antibodies, and sequencing of specific PCR products are used for investigation of capacity of the strains for poly-3-hydroxybutyrate [poly(3HB)] synthesis and for detection of poly-β-hydroxyalkanoate (PHA) synthase [85]. Information on the nucleotide sequence of genomic DNA of *Synechocystis* sp. PCC 6803 was used to identify and characterize the gene encoding polyhydroxybutyrate synthase [86]. Subsequently, two genes encoding polyhydroxyalkanoate-specific β-ketothiolase and acetoacetyl-CoA reductase were also identified and characterized [87]. The transposon mutant of *Synechococcus* sp. MA19 exhibited enhanced polyhydroxybutyrate accumulation [88].

Cyanobacteria possess cyanophycin, a polymer unique for this group. This is a copolymer of arginine and aspartic acid, multi-L-arginine-polyaspartate [89, 90]. It is accumulated in the cells as structured granules [91]. This polypeptide is not synthesized on ribosomes and may be degraded to provide a source of intracellular nitrogen [92–94]. After isolation of cyanophycin synthase from *Anabaena variabilis* ATCC 29413 and determination of its amino acid sequence, the homologous gene was identified in the genome of *Synechocystis* PCC 6803 [95]. This, in turn, made it possible to isolate and sequence the corresponding gene of *A. variabilis* ATCC 29413, express the relevant protein, and analyze the mechanism of cyanophycin synthesis [96]. This gene is sufficient for cyanophycin synthesis in *E. coli* cells. The peptidase-encoding cyanophycinase gene from *Synechocystis* PCC 6803 was expressed in *E. coli* cells, and the purified enzyme hydrolyzed cyanophycin to the asparagine–arginine dipeptide [97]. Heterological expression of cyanophy-

cin makes it possible to obtain it in the quantities exceeding 26% of the dry cell mass [98].

From an applied viewpoint, capacity of cyanobacteria for fatty acid synthesis is attractive. Synthesis of eicosapentaenoic acid (20:5n–3, EPA), a polyunsaturated fatty acid that is significant for nutrition of the larvae of sea fish and important for human health, is an example. By conjugation, the gene cluster responsible for EPA biosynthesis from the EPA-producing *Shewanella* sp. SCRC-2738 was introduced into marine *Synechococcus* sp. NKBG15041c [99]. The transgenic cyanobacterium produced eicosapentaenoic acid and its precursor, 20:4n–3, in amounts depending on the cultivation conditions.

The blue light-gathering pigment, phycocyanin, is found in cyanobacteria and in eukaryotic algae (*Rhodophyta* and *Cryptophyta*). C-phycocyanin and allophycocyanin are phycobiliproteins, pigment components of the phycobilisome, an antenna structure of photosystem II [100]. Phycocyanin and related phycobiliproteins are used in food industry, cosmetics, biotechnology, diagnostics, and medicine. A patent search revealed 55 patents on phycobiliprotein production; 30 patents on their application in medicine, food industry, and other fields; and 236 patents on the use of phycobiliproteins based on their fluorescent properties [101]. Phycocyanin is a water-soluble, nontoxic fluorescent protein with pronounced antioxidant, antiinflammatory, and antitumor properties [102–104]. Phycocyanin may be purified directly from cyanobacteria [105, 106] or synthesized in *E. coli* cells using the expression vector containing all the necessary five genes (*hox1, pcyA, cpcA, cpcE,* and *cpcF*) and His-tag for convenient purification of the recombinant protein [107, 108].

Phycobiliproteins bound to monoclonal and polyclonal antibodies for formation of fluorescent derivative antibodies are valuable fluorescent markers for cell sorting, investigation of the surface cell antigens, and inspection of the chips with high density of applied material [109]. *Spirulina* is a cheap and convenient source of allophycocyanin and C-phycocyanin [110]. Expression of the α subunits of C-phycocyanin and holo-C-phycocyanin in the cells of *Anabaena* sp. and *E. coli* demonstrated the feasibility of protein engineering for production of the pigment with improved stability or new functions. For example, genetically modified *Anabaena* 7120 is capable of in vivo production of stable phycobiliprotein constructions carrying tags for affinity purification. They are convenient fluorescent markers requiring no subsequent chemical manipulations [111]. Successful application of C-phycocyanin in food and pharmaceutical industry requires production of C-PC under strictly controlled conditions.

Spirulina has been used as a natural source of protein and vitamins since ancient times [112, 113]. Cyanobacteria *Spirulina platensis* and *S. maxima* have unusually high protein content for photosynthetic organisms (up to 70% of dry cell mass) [114]. *Nostoc flagelliforme* is a Chinese delicacy [115, 116]. Other cyanobacteria are used as feedstuffs in India and the Philippines [117, 118]. Amino acid composition of *S. maxima* [119], which may be cultivated on livestock farming waste [120], make it one of the best photosynthetic organisms for feedstuff production. *Spirulina*, like other microalgae, is used as a source of natural dyes in food industry and as a dietary food additive [121]. Optimal physiological conditions (temperature and pH) were recently determined for the cultivation of a new *Spirulina* sp. isolate from oil-contaminated brackish water of the Niger delta as a producer of biomass and protein [122]. Other species of cyanobacteria, including *Aphanizomenon flos-aquae* and *Nostochopsis lobatus*, are also considered promising sources of microalgal biomass for food industry [123] and production of pigments and antioxidants [124].

Cyanobacteria produce large amounts of secondary metabolites, including toxic and bioactive peptides. Some of the toxins and other cyanobacterial metabolites may be applied in medicine [125–127]. Various cyanobacteria form microbial mats of the bottom layers of Antarctic lakes are used in the search for new antibiotics and antitumor compounds [128]. About 800 compounds of cyanobacterial origin are presently known, including pharmacologically promising ones, which exhibit antitumor and antimicrobial activity, as well as those decreasing blood pressure. Numerous metabolites produced by the "chemical factories" of the *Nostocaceae* cyanobacteria are discussed in review [129].

Recently, much attention has been paid to nanotechnologies, including manufacture and application of material particles less than a micrometer in size. Synthesis of nanoparticles by biological producers is of interest due to their unusual optical [130], chemical [131], photoelectrochemical [132], and electrical [133] characteristics. Cyanobacteria are among the potential producers of nanoparticles. The filamentous cyanobacterium *Plectonema boryanum* UTEX 485 was used for biosynthesis of gold, silver, and palladium nanoparticles [134–137]. *P. boryanum* UTEX 485 accumulates gold from gold (III) chloride solutions. Their interaction with aquatic gold (III) chloride solution results in precipitation of the nanoparticles of amorphous gold (I) sulfide on their cell walls and subsequently in deposition of metal gold as octahedral (III) plates close to the cell surface and in the solution [135]. Since the efficiency of production of chemical energy by a photosynthetic system increases significantly in the presence of metal nanoparticles, these objects combined with cyanobacterial photosynthetic molecular complexes are considered as a basis for energy-converting devices and sensors. For example, a hybrid photosystem including a photosynthetic reaction center bound to gold or silver nanocrystals was

shown to have a higher rate of generation of excited electrons inside the reaction center due to plasmon resonance and rapid separation of the electron–hole pairs [138].

Molecular hydrogen is among the promising energy resources to be used instead of the limited resources of the presently used fossil fuels. It is environmentally friendly, efficient, and renewable [139].

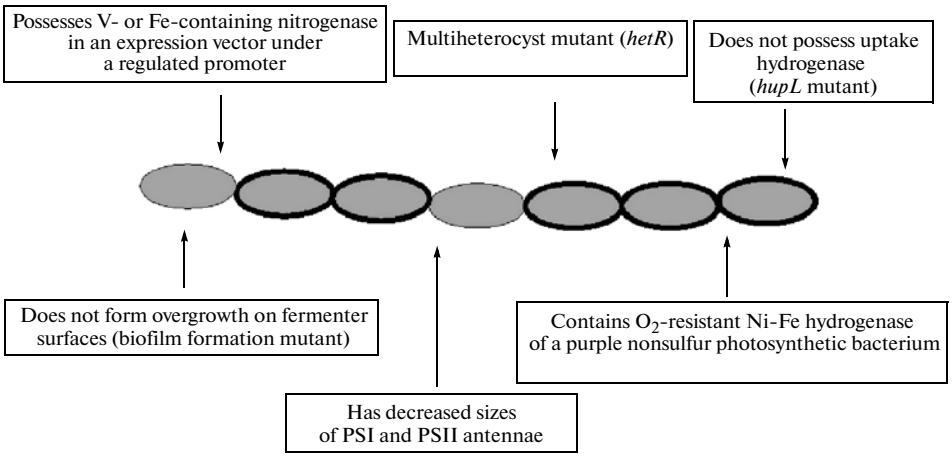
Many cyanobacteria are able to produce H_2. The genes encoding hydrogenases have been found in members of all the five major taxonomic groups. Over 100 cyanobacterial strains possessing hydrogenases have been described [140]. Comparative analysis of unicellular and filamentous cyanobacteria in order to select the most efficient strains for photobiological H_2 production revealed symbiotic, marine, and thermophilic cyanobacteria, as well as species producing hydrogen under aerobic conditions [141]. The promising systems for photoproduction of molecular hydrogen include immobilized cyanobacterial cells and liquid suspension cultures [142–145]. For example, immobilized culture of *Gloeocapsa alpicola* CALU 743 in a photobioreactor functioning in a two-stage "photosynthesis–endogenous fermentation" mode produced hydrogen reliably for over three months [145].

Cyanobacteria possess several enzyme complexes directly involved in hydrogen metabolism: (i) nitrogenase (encoded by *nif* genes), which produces H_2 as a side product in the course of N_2 reduction to NH_3; (ii) uptake hydrogenase (encoded by *hup* genes), catalyzing uptake of H_2 produced by nitrogenase; and (iii) bidirectional hydrogenase (encoded by *hox* genes), which may both uptake and evolve H_2 [146–150]. Production of hydrogen from water by bioconversion of the energy of photons is a multistage process involving the photosystems I and II, which are responsible for electron transfer to NADP via the electron transport chain. Electron transfer results in a transmembrane electrochemical proton gradient required for ATP synthesis. Cyanobacteria use the electrons transported via ferredoxin (or flavodoxin) in the nitrogenase reaction for the synthesis of ammonium and reduction of protons, resulting in production of molecular hydrogen. The latter is reutilized for the cellular needs by means of the uptake hydrogenase, rather than released to the environment. This Ni–Fe-containing enzyme provides additional energy for the cell and protects nitrogenase from inactivation by oxygen. The cells of all nitrogen-fixing cyanobacteria contain membrane-bound uptake hydrogenase. Bidirectional (reversible) cytoplasmic hydrogenase, another enzyme of hydrogen metabolism, catalyzes the reversible reaction: $2H^+ + 2e^- \cdot H_2$. Bidirectional hydrogenase is present in many, though not in all cyanobacteria. The functions of this enzyme are: (i) hydrogen oxidation in the periplasm and delivery of electrons to the respiratory electron transport chain, (ii) removal of excessive reducers under anaerobic conditions, and (iii) removal of excessive electrons produced in the light reactions of photosynthesis [148].

To construct producer strains with high rates of hydrogen evolution by genetic engineering techniques, thorough molecular genetic analysis of the systems of hydrogen metabolism is required. Up-to-date data on genetic control of hydrogen metabolism in cyanobacteria are presented in review [151]. Transcription analysis of hydrogenase genes in *Anacystis nidulans* and *Anabaena variabilis* was carried out by real-time polymerase chain reaction [152]. Transcription of the genes encoding bidirectional hydrogenase in *Nostoc* sp. PCC 7120 cells was investigated by Lindblad et al. (Sweden) [153].

Techniques of mutagenesis and genetic engineering techniques may be used for development of genetically modified strains suitable for commercial applications in photobiotechnology. The mutants of *A. variabilis* ATCC 29413 deficient in H_2 utilization, initially obtained at Moscow State University, exhibited enhanced H_2 production [154]. Two mutants with altered hydrogen metabolism were subsequently characterized [155, 156]. One of them, PK 84, was used for hydrogen production in photobioreactors [157, 158]. Insertionally inactivated Δ*hupL* mutants of *Anabaena* sp. PCC 7120 [159], *Nostoc punctiforme* ATCC 29133 [160, 161], and *Nostoc* sp. PCC 7422 [162] were also investigated. Masukawa et al. [163] demonstrated that a double mutant Δ*hupL* Δ*nifV1* grown in air produced hydrogen more actively than the initial Δ*hupL* strain of *Nostoc* sp. PCC 7120. It exhibited significantly longer H_2 production and higher nitrogenase activity. The mutants with blocked uptake hydrogenase, the structural *hup* genes inactivated by mutation, or with mutated *hyp* and *hupW* genes controlling nickel metabolism and assembly and functions of hydrogenase complexes and regulation of their activity are the most promising for enhanced efficiency of hydrogen production.

Semiartificial systems for biohydrogen production are developed, which include the integration of photosynthetic protein complexes and hydrogenases into a bioelectronic or bioelectrochemical device. In order to develop a device for biohydrogen production from photosynthetic oxidation of water, photosystem II (PSII) was immobilized on the surface of an electrode [164]. The PSII complex isolated from the cyanobacterium *Thermosynechococcus elongatus*, with six genetically attached histidine residues (His tag) was bound to the chemically modified gold electrodes. Another artificial system for hydrogen production consisted of hydrogenase and photosystem I. The artificially ligated protein complex of [NiFe] hydrogenase from the β-proteobacterium *Ralstonia eutropha* H16 and the PsaE peripheral subunit of the photosystem I (PSI) from the cyanobacterium *Thermosynechococcus elongatus*



Some properties of a potential mutant making it promising for production of molecular hydrogen.

was the key component. The hydrogenase–PSI complex produced hydrogen under illumination [165].

To enhance hydrogen production, various factors are used that affect this process, including the cyanobacterial strain used, gas phase composition, illumination, and the composition of the growth medium [166–170]. Some heterologous systems may be used for production of hydrogen. For example, inhibition of hydrogen uptake by both hydrogenase 1 and hydrogenase 2 resulted in enhanced hydrogen production by *E. coli* cells expressing the HoxEFUYH cyanobacterial enzyme (bidirectional hydrogenase from *Synechocystis* sp. PCC 6803) [171]. The system including the *Nostoc* and *Anabaena* mutants deprived of uptake hydrogenase and the heterotrophic bacterium *Rhodopseudomonas palustris* P_4 [172] is another example of a heterologous system for hydrogen production.

The major difficulties preventing high productivity of hydrogen production by cyanobacteria are the following:

1. Efficient hydrogen uptake by cyanobacterial cells.

2. Low efficiency of nitrogenase and/or hydrogenase.

3. Limited synthesis of active hydrogen-releasing enzymes.

4. High sensitivity of nitrogenase and/or hydrogenase to oxygen.

5. Inhibition of the electron flow due to ATP accumulation in the hydrogenase system.

6. Low quantum efficiency due to excessively large antenna both in PSI and PSII.

7. Electron-consuming metabolic pathways (respiration and the Calvin cycle) competing for electrons with hydrogenases.

The following ways to overcome these difficulties have been suggested.

1. Construction of mutants with impaired uptake hydrogenase.

2. Increased efficiency of hydrogen production by heterocysts of filamentous heterocyst-bearing cyanobacteria.

3. Development of the mutants with a higher number of heterocysts [151, 173].

4. Decreasing the size of antennae complexes of the photosystems in order to direct higher H^+ and e^- flows to hydrogenase [174].

5. Search for new cyanobacterial strains, including symbiotic, marine, and thermophilic ones, which are capable of hydrogen production under aerobic conditions.

Enhanced efficiency of H_2 production by heterocysts may be achieved either by genetic modification of *Anabaena* nitrogenase in order to produce mainly H_2, as was done with *Azotobacter vinelandii* [175], or by substitution of another enzyme for nitrogenase, e.g., an efficient reversible hydrogenase, which potentially produces more H_2. In *Anabaena* cells, this may be the reversible hydrogenase encoded by *hoxEFUYH* [147]. Other promising projects target development of the strains with blocked activity of uptake hydrogenase and Mo-containing nitrogenase (the *hif1* gene) replaced by V-containing nitrogenase (the *nifDGK* genes), which is more efficient in terms of utilization of the electron energy for proton reduction to molecular hydrogen [176].

The number of heterocysts may be increased by genetic engineering manipulations with the genes encoding heterocyst formation and with the genes responsible for nitrogen metabolism. One of these genes is *ntcA*. It encodes the DNA-binding protein that interacts with the promoters of the genes encoding uptake hydrogenase; the transcription of the latter is activated in heterocysts [153, 177, 178].

In theory, development of a cyanobacterial strain with numerous proton and electron flows redirected from the photosystems to hydrogenase could result in a significantly increased, stable H_2 production under

illumination [174]. For example, a constructed strain of *Synechocystis* sp. PCC 6803 obtained by deletion of the gene responsible for assembly of NADPH dehydrogenase (NDH-1) exhibited a fivefold increase in light-dependent H_2 production [179]. This mutation blocks the cyclic electron flow from PSI to the plastoquinone pool, resulting in the changed direction of the electron flow for $NADP^+$ reduction.

Some properties of the potential mutant making it promising for production of molecular hydrogen are presented on figure.

Photobiological production of hydrogen is certainly an important renewable source of energy. However, a prerequisite for optimization of an industrial process of photohydrogen production is improvement at the biochemical level of existing systems in order to achieve hydrogen production at higher rate and energy efficiency than existing photoelectric systems.

CONCLUSIONS

Cyanobacteria are convenient model organisms for basic investigation of molecular mechanisms of photosynthesis, the mechanisms of resistance to environmental stresses, cell differentiation and nitrogen fixation, carbon and hydrogen metabolism, cell division, and molecular evolution. Cyanobacteria are a significant, though still poorly exploited, resource for production of numerous compounds of biotechnological importance. Thus, the need for development of cyanobacteria-based applied processes is becoming clear. The existence of efficient genetic techniques makes it possible to find biotechnological applications for cyanobacteria in such fields as manufacture of specific products, including photosynthetic pigments, molecular hydrogen, and nanoparticles, for biodegradation of organic contaminants on the water surface and for many other applied tasks. Obtaining information on the genomic sequences of cyanobacteria, together with transcriptomics, proteomics, and metagenomics, will enable better understanding of the biology of these amazing organisms and their efficient application for environmental and biotechnological purposes.

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