# Hydrogenases and Hydrogen Metabolism of Cyanobacteria

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## INTRODUCTION

### Cyanobacteria

Cyanobacteria (also called blue-green bacteria, blue-green algae, cyanophyceae, or cyanophytes) are a large and widespread group of photoautotrophic microorganisms, which originated, evolved, and diversified early in Earth's history. The earliest forms attributed to this group were found in sedimentary rocks formed 3.5 billion years ago, and it is commonly accepted that cyanobacteria played a crucial role in the Precambrian phase by contributing oxygen to the atmosphere (175).

At present, cyanobacteria are found in a wide range of habitats including aquatic (saltwater and freshwater), terrestrial, and extreme environments (like frigid lakes of the Antarctic or hot springs). Although called blue-green, cyanobacteria may display a variety of colors due to different combinations of the photosynthetic pigments chlorophyll *a*, carotenoids, and phycobiliproteins. All cyanobacteria combine the ability to perform an oxygenic photosynthesis (resembling that of chloroplasts) with typical prokaryotic features. The possession of chlorophyll *a* and the use of oxygenic photosynthesis distinguishes cyanobacteria from other photosynthetic bacteria, such as purple and green bacteria. Nevertheless, some cyanobacterial strains can perform anoxygenic photosynthesis by using hydrogen sulfide ($H_2S$) as the electron donor. Many cyanobacteria can fix atmospheric dinitrogen ($N_2$) (a capacity not possessed by any eukaryote) into ammonia ($NH_3$), a form in which the nitrogen is further available for biological reactions. Although quite uniform in nutritional and metabolic respects, cyanobacteria are a morphologically diverse group with unicellular, filamentous, and colonial forms. Among certain filamentous cyanobacteria, there is some degree of cellular differentiation. Within the filament, vegetative cells may develop into structurally modified and functionally specialized cells: the akinetes (resting cells) or the heterocysts (cells specialized in nitrogen fixation). For more detailed information on cyanobacteria, see reference 218.

Unicellular and filamentous cyanobacteria can form symbioses with a wide diversity of hosts. In symbiosis, some cyanobionts perform both photosynthesis and nitrogen fixation while others exhibit only one of these properties (3, 22, 133, 161, 184). It is believed that ancestors of cyanobacteria evolved to become plastids after a long period of endosymbiosis. In biochemical and structural detail, cyanobacteria are especially similar to the chloroplasts of red algae (47, 104).

The taxonomy of cyanobacteria is still a controversial sub-

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ject, with two prevailing approaches, the botanical approach (8–10, 102, 103) and the bacterial approach (167, 168, 190). Despite the differences, both botanists and bacteriologists divide the cyanobacteria into four or five major subgroups. In fact, the five sections recognized by Rippka et al. (167) coincide broadly with orders of other classifications: *Chroococcales*, *Pleurocapsales*, *Oscillatoriales*, *Nostocales*, and *Stigonematales* (for reviews on this subject, see references 218 and 219). At present, different molecular methods are being used and developed to infer phylogenetic relationships. However, the data available for cyanobacteria are still scarce (207, 219). Analysis of 16S rRNA sequences has given the most detailed hypothesis of the evolution of cyanobacteria (218, 219). A polyphasic taxonomy of cyanobacteria, integrating both phenotypic and genotypic characters is currently being assembled. A milestone in the study of cyanobacteria was the publication of the entire genome (3,573,470 bp) of the non-nitrogen-fixing unicellular cyanobacterium *Synechocystis* strain PCC 6803 (95, 144). A number of other cyanobacterial genome projects are being completed (see below).

Cyanobacteria may possess several enzymes directly involved in hydrogen metabolism: nitrogenase(s) catalyzing the production of hydrogen ($H_2$) concomitantly with the reduction of nitrogen to ammonia, an uptake hydrogenase catalyzing the consumption of hydrogen produced by the nitrogenase, and a bidirectional hydrogenase which has the capacity to both take up and produce hydrogen (Fig. 1) (13, 21, 58, 75, 87, 113, 115, 117).

This review summarizes our knowledge about cyanobacterial hydrogenases. It is based on scientific publications and sequence information from the genome projects of the unicellular non-nitrogen-fixing *Synechocystis* strain PCC 6803 (95, 144) and the filamentous heterocystous strains *Anabaena* strain PCC 7120 (The Kazusa DNA Research Institute [http://www.kazusa.or.jp/cyano/anabaena/]) and *Nostoc punctiforme* (ATCC 29133, PCC 73102) (for preliminary sequence data, see the DOE Joint Genome Institute website [http://www.jgi.doe.gov/JGI_microbial/html/nostoc/nostoc_homepage.html]) (Fig. 2). In addition, genome projects are currently being performed using, e.g., unicellular cyanobacteria such as several *Synechococcus* strains (e.g., *S. elongatus* [http://www.kazusa.or.jp/en/] and *Synechococcus* strain WH8102 [http://spider.jgi-psf.org/JGI_microbial/html/synechococcus/synech_homepage.html]), *Gloeobacter* strain PCC 7421 (http://www.kazusa.or.jp/en/), and *Microcystis aeruginosa* (http://www.pasteur.fr/externe.html); the filamentous, nonheterocystous *Spirulina platensis* (http://hgc.igtp.ac.cn/); as well as the prochlorophyte *Prochlorococcus marinus* (http://www.jgi.doe.gov/JGI_microbial/html/prochlorococcus/prochlo_pickastrain.html). After introducing nitrogenases and hydrogenases, we focus on the two cyanobacterial hydrogenases, the uptake and the bidirectional enzymes, their regulation, and their accessory genes before finishing with an applied aspect—the use of cyanobacteria in the photoproduction of molecular hydrogen.

## Nitrogenases

Although nitrogenase is unquestionably a key enzyme for cyanobacterial hydrogen production, it is not subject of this review and so its properties will be discussed only briefly here.

The activity of nitrogenase, the enzymatic multiprotein complex for nitrogen fixation, is essential for the maintenance of
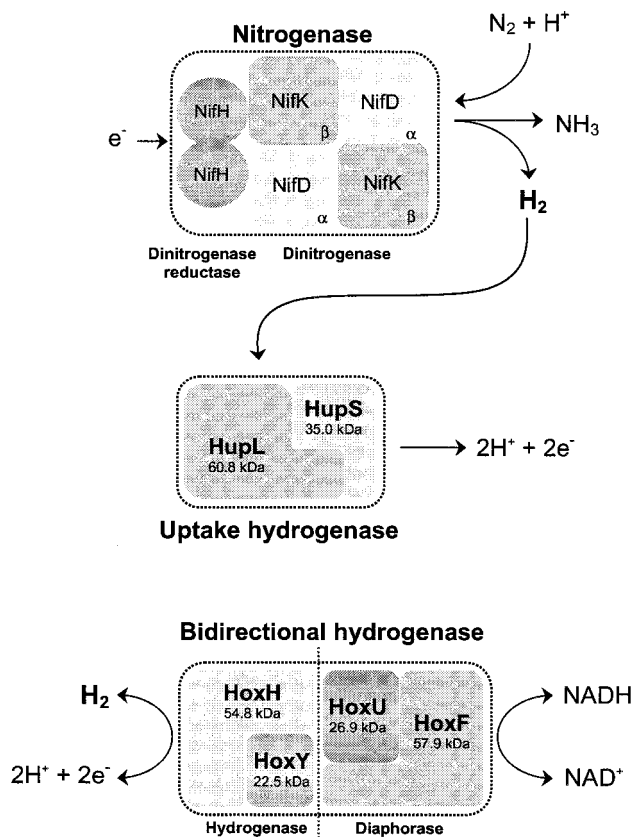


FIG. 1. Enzymes directly involved in hydrogen metabolism in cyanobacteria. While the uptake hydrogenase is present in all nitrogen-fixing strains tested so far, the bidirectional enzyme is distributed among both nitrogen-fixing and non-nitrogen-fixing cyanobacteria (although it is not a universal cyanobacterial enzyme) (192). The molecular masses indicated for the uptake hydrogenase subunits are mean values calculated from the deduced amino acid sequences of *Anabaena* strain PCC 7120 (39), *Nostoc* strain PCC 73102 (150), and *A. variabilis* ATCC 29413 (79), while the values for the subunits of the bidirectional enzyme are based on data exclusively from *A. variabilis* ATCC 29413 (173).

the nitrogen cycle, since this element often limits the growth of organisms (35, 65, 158, 187). The diversity of prokaryotic microorganisms with the ability to fix nitrogen is in contrast to the remarkable conservation of the nitrogenase itself (36, 58, 80).

The nitrogenase complex consists of two proteins: the dinitrogenase (MoFe protein or protein I) and the dinitrogenase reductase (Fe protein or protein II), (Fig. 1). The dinitrogenase is an $\alpha_2\beta_2$ heterotetramer of about 220 to 240 kDa, and the $\alpha$ and $\beta$ subunits are encoded by *nifD* and *nifK*, respectively. The dinitrogenase reductase, encoded by *nifH*, is a homodimer of about 60 to 70 kDa and plays the specific role of mediating the transfer of electrons from the external electron donor (a ferredoxin or a flavodoxin) to the dinitrogenase (58, 127, 149). In addition to the three structural *nif* genes, many other genes are involved in the nitrogen fixation process and its regulation (for a review, see reference 26). Recently, following the completion of several cyanobacterial genome projects, it became evident that some strains contain multiple copies of certain *nif* genes; for example *N. punctiforme* (*Nostoc* strain
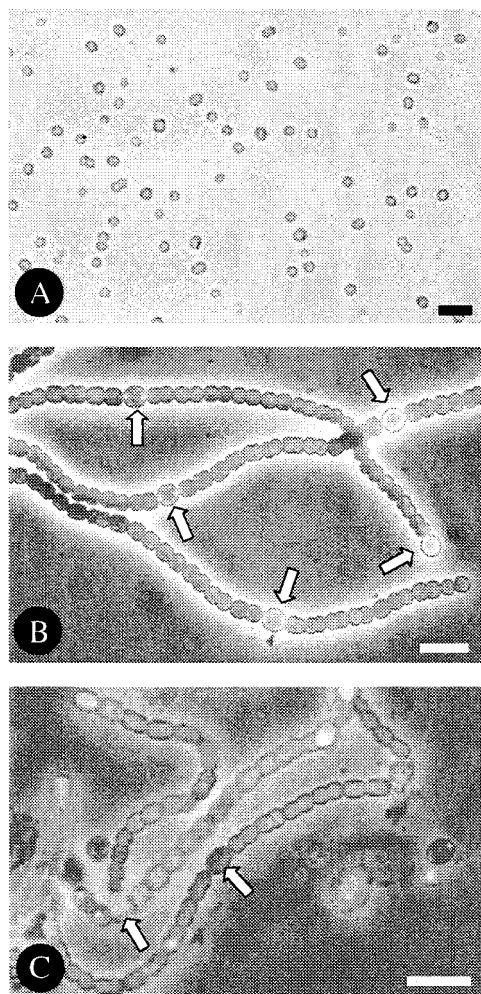
FIG. 2. Microphotographs of the three cyanobacteria from which genome sequence information is included in the present review. (A) *Synechocystis* strain PCC 6803; (B) *Anabaena* strain PCC 7120; (C) *Nostoc punctiforme* PCC 73102/ATCC 29133. Note the presence of heterocysts (arrows) in the nitrogen-fixing cultures of *Anabaena* strain PCC 7120 and *Nostoc* strain PCC 73102. Bar, 10 μm.

PCC 73102) appears to have three copies of *nifH* (199; http://www.jgi.doe.gov/JGI_microbial/html/nostoc/nostoc_homepage.html).

The reduction of nitrogen to ammonia, catalyzed by nitrogenase, is a highly endergonic reaction requiring metabolic energy in the form of ATP. Moreover, this reaction is accompanied by an obligatory reduction of protons ($H^+$) to hydrogen. Apparently, with a pure nitrogen atmosphere, at most 75% of the electrons ($e^-$) would be allocated for nitrogen reduction and at least 25% would be allocated for proton reduction. Since two ATP molecules are required for each electron transferred from dinitrogenase reductase to dinitrogenase, the reaction requires a minimum of 16 ATP molecules until the dinitrogenase has accumulated enough electrons to reduce nitrogen to ammonia. The overall reaction can be written as follows:

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$$
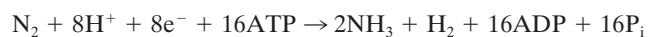
Nitrogenase is very oxygen labile; hence, all diazotrophs must protect the enzymatic complex from the deleterious effects of oxygen ($O_2$). Cyanobacteria and prochlorophytes are the only prokaryotic organisms known to perform oxygenic photosynthesis (58, 129). Cyanobacteria have evolved different mechanisms and strategies, ranging from fixing nitrogen only under anoxic conditions to temporal or even spatial separation of nitrogen fixation and oxygen evolution, to protect their nitrogen-fixing machinery not only from atmospheric oxygen but also from the intracellularly generated oxygen (for reviews, see references 22, 26, 56, 140, 188, 220, and 221). Temporal separation between photosynthetic oxygen evolution and nitrogen fixation seems to be the most common strategy adopted by nonheterocystous cyanobacteria (92, 137, 165). Spatial separation of the two processes is achieved in many filamentous cyanobacteria by differentiation of vegetative cells into cells specialized in nitrogen fixation, i.e., the heterocysts. In the marine filamentous nonheterocystous *Trichodesmium*, a spatial separation probably occurs between the two processes without any obvious cellular differentiation; only a small fraction of cells within a colony or along the filament are capable of nitrogen fixation. In contrast to the irreversible changes occurring during heterocyst differentiation, those occurring in nonheterocystous cyanobacteria can be reversed (21, 44, 61, 140).

Some filamentous cyanobacteria are able to differentiate 5 to 10% of their vegetative cells into heterocysts (Fig 2). Heterocyst formation does not take place randomly within the filament. Recently, it was proposed that this process in *Anabaena variabilis* is not controlled by individual cells but by the entire filament (199). The filament senses the nitrogen status and responds by producing heterocyst differentiation signals. However, this does not exclude cell-to-cell signaling in the maintenance of heterocyst pattern (26, 199, 220), and it has been shown that the small diffusible peptide PatS and products of nitrogen fixation control heterocyst differentiation in *Anabaena* strain PCC 7120 (226, 227). The heterocyst provides a microanaerobic environment suitable for the functioning of nitrogenase since (i) it lacks photosystem II activity, (ii) it has a higher rate of respiratory oxygen consumption, and (iii) it is surrounded by a thick envelope that limits the diffusion of oxygen through the cell wall (56, 221). Heterocysts import carbohydrates and in return export glutamine to the vegetative cells. An interesting feature of heterocyst differentiation in cyanobacteria is the occurrence of developmentally regulated genome rearrangements (39–41, 67–69, 130, 134, 141, 142). These rearrangements occur late in heterocyst differentiation, at about the same time as the nitrogenase genes are transcribed. In vegetative cells of *Anabaena* strain PCC 7120, the *fdxN*, *nifD*, and *hupL* genes are interrupted by DNA elements that are excised, during heterocyst differentiation, by site-specific recombinases encoded by *xisF*, *xisA*, and *xisC*, respectively. In addition, the excision of the *fdxN* element requires the products of the two genes *xisH* and *xisI* (162).

**Alternative nitrogenases.** In the heterocystous cyanobacterium *A. variabilis* ATCC 29413 several different nitrogenases have been identified and characterized (for details, see references 98, 99, 195, and 197 to 199).

**(i) Mo-containing nitrogenases.** Two of the *A. variabilis* nitrogenases are molybdenum-dependent enzymes. The first one (the so-called conventional nitrogenase, encoded by the *nif1*

gene cluster) functions in heterocysts. The second enzyme (encoded by the *nif2* gene cluster) functions strictly under anaerobic conditions in vegetative cells only (199).

**(ii) V-containing nitrogenase.** The occurrence of a vanadium-containing nitrogenase was first reported by Kentemich et al. (98) and subsequently confirmed by Thiel (195). This enzyme is encoded by the *vnfDGK* gene cluster, which is transcribed in the absence of molybdenum and in which *vnfDG* are fused into a single gene (195).

**(iii) Fe-containing nitrogenase.** A fourth nitrogenase is believed to be an iron enzyme similar to the one encoded by the *anf* gene cluster in *Azotobacter vinelandii* (23, 99).

Alternative nitrogenases have also been found in other cyanobacterial strains (23), and it is known that they differ from the conventional enzyme physically, chemically, and by their catalytic properties. One must bear in mind that all of the alternative enzymes investigated so far seem to allocate a higher proportion of electrons to the reduction of protons to hydrogen than does the conventional Mo enzyme complex. However, hydrogen produced by nitrogenase is generally taken up by an uptake hydrogenase, so that net hydrogen evolution by nitrogen-fixing cyanobacteria is barely observed, at least under aerobic conditions (7).

## HYDROGENASES

The capacity of certain microorganisms to metabolize molecular hydrogen was discovered at the end of the 19th century (85) and later identified to be catalyzed by a hydrogenase (191). Since then hydrogenases have been observed and characterized in many microorganisms, including some algae, trichomonads, anaerobic ciliates, and chytrid fungi (4, 5, 59, 77, 86, 94, 100, 176, 222, 223, 225). The enzyme catalyzes the simplest of chemical reactions, the reversible reductive formation of hydrogen from protons and electrons:

$$2H^+ + 2e^- \leftrightarrow H_2$$
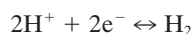
According to the metal composition of the active site, hydrogenases are classified into three major groups: NiFe, Fe, and metal-free hydrogenases (209).

The majority of hydrogenases are NiFe-containing enzymes, occurring in all bacterial classes (38, 159, 209). The first crystal structure of a NiFe hydrogenase, the representative heterodimeric soluble enzyme of *Desulfovibrio gigas* (6), was published in 1995 (212). This hydrogenase is involved in periplasmic $H_2$ oxidation and is phylogentically related to other bacterial uptake hydrogenases (209). The binuclear Ni-containing active site in the large subunit contains an additional Fe ion (37, 60, 212). The nickel ion is ligated to four conserved cysteine residues (RxCGxCxxxH and DPCxxCxxH) at the N- and C-terminal regions, respectively, two of which are bridging the Fe and Ni ions. The Fe is also coordinated by two cyanide ions and one carbon monoxide molecule, two very uncommon biological ligands (18, 78). The hydrogen molecule is believed to access the active site through an identified hydrophobic channel (139), while conserved histidine and glutamate residues spanning from the active site to the molecule surface are responsible for the proton transfer (212). The transfer of electrons between the active site and the redox partner involves the participation of [FeS] clusters in the small subunit of NiFe

hydrogenases. Sequence comparisons demonstrated that they possess several conserved cysteines, shown to participate in the formation of the [FeS] clusters. The heterodimeric NiFe hydrogenases are often directly linked to a variable diaphorase part containing additional prosthetic groups, e.g., [FeS] clusters, flavins, and cytochromes (6).

Cyanobacteria possess two functionally different NiFe hydrogenases, an uptake enzyme and a bidirectional enzyme. In 1995, structural genes encoding the latter enzyme were sequenced and characterized (173). These authors, to avoid confusion with the tritium exchange 42-kDa enzyme previously cloned (51–54), chose to call it a bidirectional hydrogenase, a name that is used throughout this review. The 42-kDa enzyme is commonly accepted to be an aminotransferase (228).

### Cyanobacterial Uptake Hydrogenases

An uptake hydrogenase, with the evident function of catalyzing the consumption of the hydrogen produced by nitrogenase, has been found in all nitrogen-fixing cyanobacteria examined so far (87, 107, 192). Some physiological data, together with analysis of a mutant of the bidirectional hydrogenase gene *hoxH*, indicate that the unicellular non-nitrogen-fixing *Synechococcus* strain PCC 6301 (*Anacystis nidulans*) may also possess a hydrogen uptake enzyme (29, 48, 154–156). However, the presence of an uptake hydrogenase in non-nitrogen-fixing cyanobacteria is still a controversial subject, but the increasing availability of molecular data, particularly from genome projects, will help to clarify this question.

Since the small subunits of all known cyanobacterial uptake hydrogenases lack any N-terminal signal peptide (39, 79, 150) it has been suggested that the enzyme is localized on the cytoplasmic side of either the cytoplasmic or the thylakoid membrane (13, 209). In some filamentous strains, it is particularly expressed in the nitrogen-fixing heterocysts with little or no activity in the photosynthetic vegetative cells (see "Transcription of *hupSL*" below) (39, 87, 88, 152). The recycling of hydrogen produced by nitrogenase has been suggested to have at least three beneficial functions for the organism: (i) it provides the organism with ATP via the oxyhydrogen (Knallgas) reaction; (ii) it removes oxygen from nitrogenase, thereby protecting it from inactivation; and (iii) it supplies reducing equivalents (electrons) to nitrogenase and for other cell functions (32, 33, 90, 183, 216).

The uptake hydrogenase activity versus the bidirectional hydrogenase activity was characterized by Houchins and Burris (89) for *Anabaena* strain PCC 7120. In filaments, the uptake hydrogenase is resistant to atmospheric oxygen levels, but an inactivation that increased with the disruption of the filament was observed. The two hydrogenases differ in their thermal stability. Specifically, the uptake enzyme is more sensitive to high temperature, with a half-life of 12 min at 70°C, but less sensitive to competitive inhibition by carbon monoxide than is the bidirectional enzyme. Both enzymes have a low $K_m$ for hydrogen, but only the uptake hydrogenase activity was shown to be elicited by addition of hydrogen to the gas phase (48, 87, 116, 151, 169).

Biochemical and molecular data concerning filamentous heterocystous cyanobacteria point to the existence of at least two dissimilar subunits of about 60 and 35 kDa (39, 79, 88, 89,

112, 116, 150, 193). However, the exact subunit composition of cyanobacterial uptake hydrogenases and the molecular mass of the active holoenzyme are still unknown. It is premature to exclude the presence of additional subunits in the active form of the enzyme, since no active cyanobacterial uptake hydrogenase has yet been purified.

The cellular and subcellular localization of hydrogenases in cyanobacteria is unclear. It has been suggested that the enzyme is located in the thylakoid membranes of heterocysts (48), while others reported its presence in both the vegetative cells and heterocysts and a particular association with the cytoplasmic membrane (88, 116, 160). There is a diversity in the number of nitrogenases in a single nitrogen-fixing cyanobacterial strain, ranging from one in, e.g., *Anabaena* strain PCC 7120 and *Nostoc* strain PCC 73102 to at least three different nitrogenases in *A. variabilis* (98, 99, 195, 197, 198). However, since no study has addressed the question of putatively several uptake hydrogenases in a single strain, the pattern(s) of uptake hydrogenase cellular and subcellular localization might be different for different strains under different environmental conditions.

A strong correlation between the activity of an uptake hydrogenase and nitrogen fixation has been demonstrated in filamentous cyanobacteria (87, 107, 151, 221). In *A. variabilis*, exogenous hydrogen produces only a slight stimulatory effect on the in vivo hydrogen uptake during the early stages of nitrogenase induction (203). The authors suggest that the level of hydrogen evolved by nitrogenase is enough for the induction of hydrogenase synthesis. The temporary stimulation of hydrogen uptake in *Nostoc* strain PCC 73102 cultures incubated in darkness (i.e., cells with a much lower in vivo nitrogenase activity [126]) implies a strong correlation between the hydrogen uptake and nitrogenase activities and indicates that the addition of exogenous hydrogen alone is not enough to maintain the hydrogen uptake activity (151).

The biosynthesis of NiFe hydrogenases is closely related to Ni metabolism, both to form an active enzyme and at the transcriptional level (101, 135, 211, 212). Cyanobacterial hydrogenases seem to be no exception. In several strains the hydrogen uptake activity is dependent on the concentration of nickel in the growth medium (45, 46, 151, 152, 182, 202, 224).

Addition of organic carbon to the growth medium demonstrated that cells of *Nostoc* strain PCC 73102, grown either photo- or chemoheterotrophically, reach both higher nitrogenase and hydrogen uptake activities than do photoautotrophically grown cells (151). The maximal in vivo light-dependent hydrogen uptake activity occurred in cells grown heterotrophically in darkness, a condition that mimics symbiosis. This finding is in agreement with the suggested coupling between hydrogen consumption and an efficient nitrogen fixation in *Nostoc* sp. strain Cc and other symbiotic microorganisms (122, 124, 202). In contrast, autotrophically grown cells of a cyanobacterium isolated from *Macrozamia communis* have a higher level of both nitrogenase and hydrogenase activity than do heterotrophically grown cells (46). A rapid decrease in hydrogen uptake activity in chemoheterotrophically grown cultures of *Anabaena cycadeae* has been reported (105). Moreover, an inhibition of the uptake hydrogenase activity by the addition of glucose, with a consequent increase in hydrogen production by nitrogenase, was observed in the *Gunnera-Nos-*

*toc* symbiosis (123). This set of conflicting data is not easily explained. As already pointed out (124), the omission of nickel from the growth medium in some of the experiments might have influenced their results. Moreover, symbiotic cyanobacteria within coralloid roots of the cycads *Cycas revoluta* and *Zamia furfuracea* showed a significant in vivo hydrogen uptake (124).
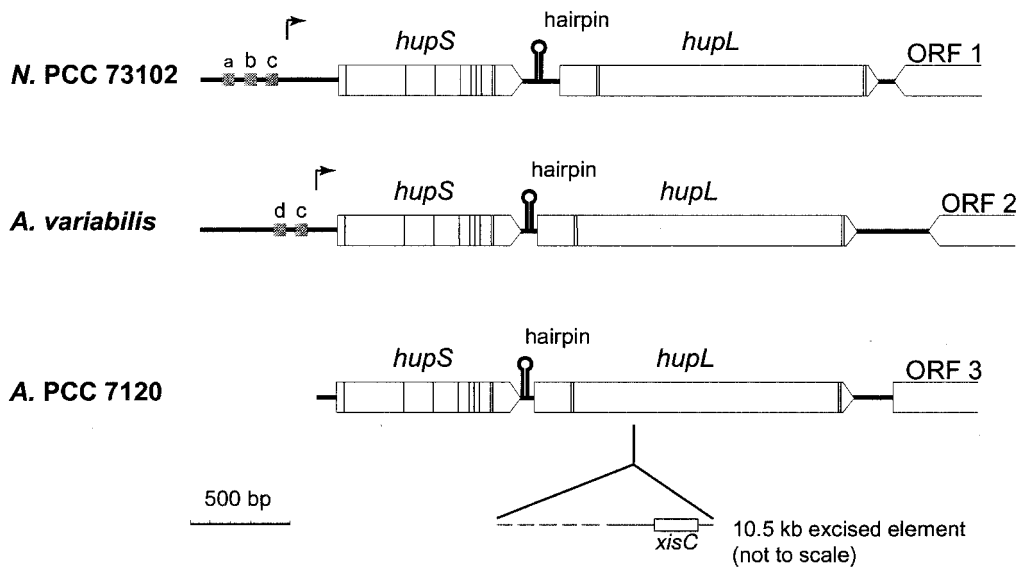
Addition of the protein biosynthesis inhibitor chloramphenicol to *Nostoc* strain PCC 73102 cultures (151) inhibited the stimulation observed by nickel and organic carbon, suggesting a regulation at the synthesis and transcriptional level and not regulation of the activity of preexisting protein(s). A similar phenomenon has been reported for the filamentous nonheterocystous cyanobacterium *Oscillatoria subbrevis* (182). Based on midpoint potentials and inhibitor studies, the probable candidate for the initial electron acceptor of the uptake hydrogenase is plastoquinone (87). In *A. variabilis*, in vitro the uptake hydrogenase is activated by a reduced thioredoxin, leading to the proposal that in vivo the enzyme may be subjected to a form of redox control that also involves a thioredoxin (152, 185).

**hupSL and the deduced proteins.** The first molecular data concerning cyanobacterial hydrogenases appeared in 1995. Carrasco et al. (39) described a novel developmental genome rearrangement for *Anabaena* strain PCC 7120, occurring in addition to the known *nifD* and *fdxN* rearrangements (see above), during the differentiation of a vegetative cell into a heterocyst. This third rearrangement occurs within a gene (*hupL*) encoding the large subunit of the uptake hydrogenase. The excision of a 10.5-kb DNA element occurs late during the heterocyst differentiation process, indicating that HupL in *Anabaena* strain PCC 7120 is expressed in heterocysts only. The excision occurs by a site-specific recombination, and the recombinase gene, *xisC*, was found 115 bp inside the right border of the excised element (Fig. 3A). Subsequently, the structural genes encoding both the small (*hupS*) and the large (*hupL*) subunits of the uptake hydrogenase have been sequenced and characterized in *Anabaena* strain PCC 7120 (www.kazusa.or.jp/cyano/anabaena), *Nostoc* strain PCC 73102 (150), and *A. variabilis* (79) (Fig. 3A).

All cyanobacterial *hupSL* genes sequenced so far are highly conserved, ranging from 83.8 to 95.1% nucleotide identities (Table 1), except that the occurrence of the *hupL* rearrangement/presence of *xisC* is not ubiquitous (17, 79, 192). They do, however, collectively differ significantly from the corresponding genes in other microorganisms, such as *D. gigas* (110), *Bradyrhizobium japonicum* (172), or *Rhodobacter capsulatus* (79, 108, 150). The deduced amino acid sequences of the cyanobacterial uptake hydrogenases all have >93% similarities to each other (Table 1; note the 99.7 and 99.6% amino acid similarity for HupS and HupL from *Anabaena* strain PCC 7120 and *A. variabilis*, respectively), whereas the corresponding percentage for *Nostoc* strain PCC 73102 HupL compared to *D. gigas* HupL is only 43%.

HupS of cyanobacteria contains eight cysteine residues that clearly correspond to those involved in the formation of [FeS] clusters in *D. gigas* and a ninth cysteine whose position is only slightly different from that in other bacterial sequences (Fig. 3A; Table 2) (39, 79, 150, 212). The cyanobacterial small subunit also lacks the N-terminal twin-arginine signal peptide pro-
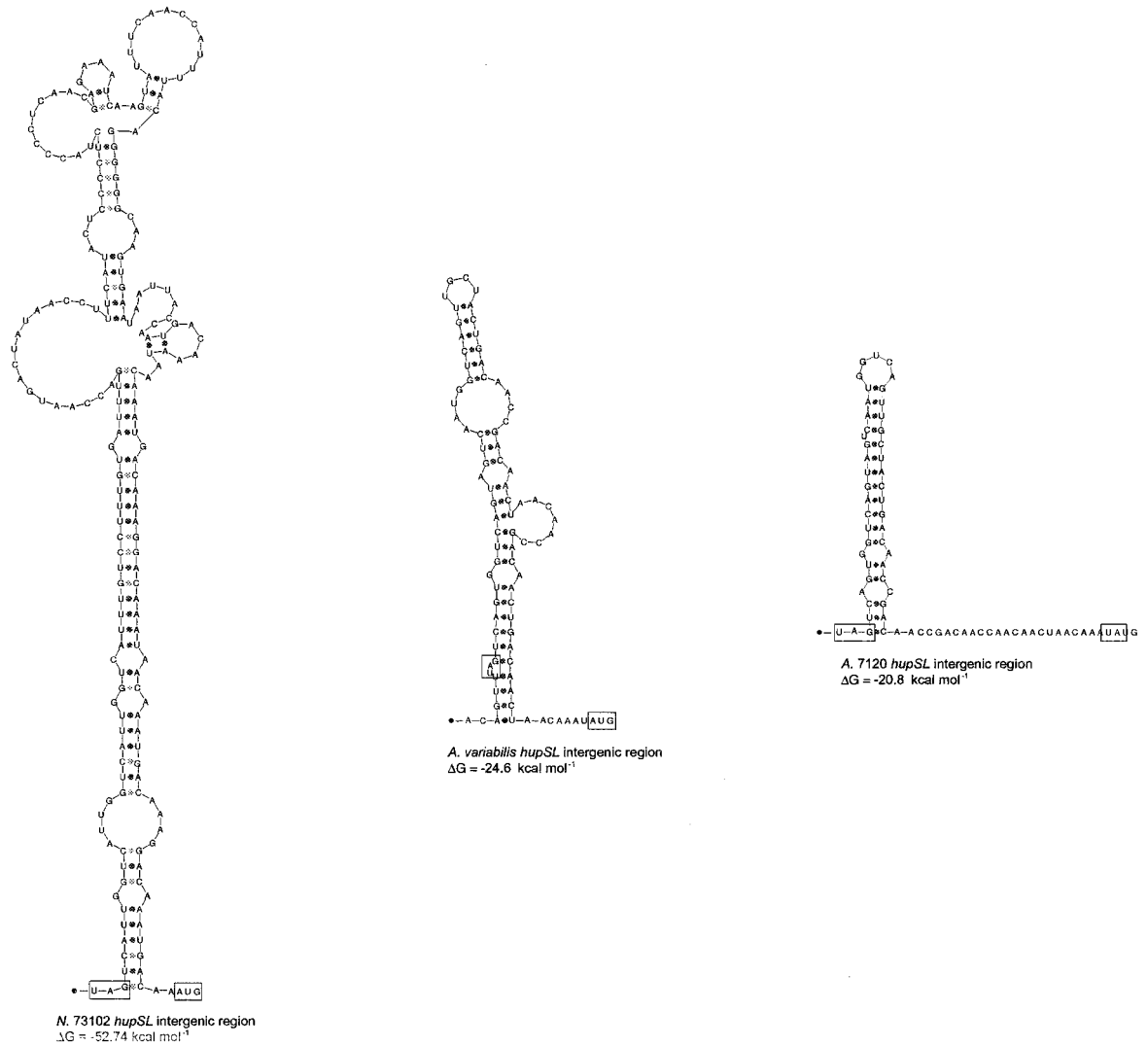
A



B



N. 73102 hupSL intergenic region
ΔG = -52.74 kcal mol⁻¹

A. variabilis hupSL intergenic region
ΔG = -24.6 kcal mol⁻¹

A. 7120 hupSL intergenic region
ΔG = -20.8 kcal mol⁻¹

TABLE 1. Sequence comparison of cyanobacterial *hupSL* and deduced amino acid sequences[a]

| Strains | Nucleotide identity (%) | | Amino acid identity/ similarity (%/%) | |
|---|---|---|---|---|
| | *hupS* | *hupL* | HupS | HupL |
| *Nostoc* strain PCC 73102 vs *A. variabilis* | 84.4 | 83.8 | 88.8/93.8 | 91.1/95.1 |
| *Anabaena* strain PCC 7120 vs *A. variabilis* | 95.1 | 94.9 | 98.1/99.7 | 98.7/99.6 |
| *Anabaena* strain PCC 7120 vs *Nostoc* strain PCC 73102 | 84.2 | 85.0 | 88.8/93.8 | 90.6/95.1 |

[a] Based on references 39, 79, and 150.

TABLE 2. Positions and putative functions of conserved cysteine residues (Cys) in cyanobacterial uptake hydrogenases compared to those in *Desulfovibrio gigas* uptake hydrogenase[a]

| Protein | Conserved residues | Function |
|---|---|---|
| HupS | Cys12, Cys113, Cys161 | Binding of putative [4Fe4S] cluster[b] |
| | Cys203, Cys224, Cys231 | Binding of putative [4Fe4S] cluster[c] |
| | Cys240, Cys258, Cys261 | Binding of putative [3Fe4S] cluster |
| HupL | Cys62, Cys509 | Ni binding |
| | Cys65, Cys512 | Ni-Fe bridging ligands |

[a] Based on references 39, 79, 150, and 212.
[b] Second ligand (cysteine) in *D. gigas* HupS missing in cyanobacterial HupS.
[c] First ligand (histidine) missing and third ligand (Cys224) differently positioned compared to the corresponding residues in *D. gigas* HupS.

posed to be involved in translocation of the protein, suggesting that the enzyme is not transported by the same machinery as in other organisms. Also missing in cyanobacterial HupS is the hydrophobic motif at the C terminus, but the role of this region in membrane anchoring is not clear since it is not present in all membrane-bound hydrogenases (122). The cyanobacterial HupL sequences contain putative Ni-binding sites characteristic for uptake hydrogenase large subunits at their N-terminal (FRGFE[I/V]ILRGKDPQAGLIVTPRIC$^{62}$GIC$^{65}$G[A/G]SH) and C-terminal (DPVEVGHVARSFDSC$^{509}$LVC$^{512}$TVHAH) ends (Table 2; Fig. 3A) (209), although at the C-terminal end the proline at position 508 is exchanged for a serine.

It has been noted that some features of the cyanobacterial *hupSL* make it very similar to *hupUV* (*hoxBC*) involved in the regulation of the uptake hydrogenase in other microorganisms (*R. capsulatus* [50, 210], *Ralstonia eutropha* [109], and *B. japonicum* [24]), and as a consequence they may actually be encoding a regulatory hydrogenase rather than the uptake hydrogenase (150). One can point out that cyanobacterial HupS lacks the N-terminal signal peptide that is present in the small subunit of uptake hydrogenases from other microorganisms. However, the cyanobacterial HupL contains the C-terminal region, which is cleaved off during the maturation process of the protein, and an amino acid extension that is not present in HupV (150, 211); the *N. punctiforme* genome (http://www.jgi.doe.gov/JGI_microbia/html/nostoc/nostoc_homepage.html) does not contain any open reading frames (ORFs) similar to *hupSL* or *hupUV*, except for the already known *hupSL*. Low-stringency Southern hybridizations using *Nostoc* strain PCC 73102 and cloned *hupSL* (150) as the probe resulted in the identification of additional DNA fragments (115). However, recent experiments demonstrated that the noncoding region between *hupS* and *hupL* was the reason for the additional hybridization signal (112). Additionally, a *hupL* mutant of *A. variabilis*, cultivated under nitrogen-fixing conditions, exhibits significantly increased rates in hydrogen accumulation and

produces three times more hydrogen than the wild type does (79). These results clearly demonstrate that the characterized cyanobacterial *hupSL* encode an uptake hydrogenase.

Generally, the structural genes encoding NiFe uptake hydrogenases are clustered in a similar physical organization forming a transcript unit, with *hupS* being located upstream of *hupL*. In several microorganisms, a third ORF, *hupC*, has been identified and is located directly downstream of the *hupSL* genes (83, 208, 211). It has been proposed that HupC, a *b*-type cytochrome, could play a role in mediating the electron transport to the terminal acceptor oxygen (43, 211). The cyanobacterial *hupSL* genes follow the general pattern (Fig. 3A) and are transcribed as a single transcript in *A. variabilis* ATCC 29413 (79) and in *Nostoc* strain PCC 73102 (112). In both organisms, as well as in *Anabaena* strain PCC 7120, ORFs have been detected downstream of the respective *hupL* genes, but none of them show any similarity to *hupC* and they are different in the three strains examined (Fig. 3A).

**Transcription of *hupSL*.** The first transcriptional study concerning cyanobacterial *hupSL* genes was performed with *Anabaena* strain PCC 7120, where non-nitrogen-fixing filaments of vegetative cells were transferred to nitrogen-fixing conditions. Reverse transcription-PCR (RT-PCR) demonstrated that *hupL* transcription coincides with heterocyst formation (39). Subsequent studies of *Nostoc muscorum* and *A. variabilis* ATCC 29413 (cyanobacteria without rearrangement of *hupL*; see "Genetic diversity of cyanobacterial hydrogenases" below) using RT-PCR and Northern blot analysis, respectively, confirmed the induction of a *hupL* transcript under nitrogen-fixing conditions only (17, 79). In *N. muscorum*, the induction of the transcript was followed by the appearance of an in vivo hydrogen uptake activity (Fig. 4) (17). In contrast to these results, a low level of *hupL* expression (detected by RT-PCR) has been found in *A. variabilis* ATCC 29413 vegetative cells grown with the addition of ammonia (28). Interest-

FIG. 3. *hupSL* in the cyanobacteria *Nostoc* strain PCC 73102 (*N.* PCC 73102) (150), *A. variabilis* ATCC 29413 (79), and *Anabaena* strain PCC 7120 (*A.* PCC 7120) (39). (A) Thin vertical lines correspond to the nucleotide triplets encoding the conserved cysteine residues compared to HupSL in other organisms. Arrows show the positions of transcriptional start in *Nostoc* strain PCC 73102 and *A. variabilis* ATCC 29413. Grey boxes (a, b, c, and d) indicate the positions of putative promoter elements; a, NtcA-binding site; b, IHF-binding site; c, −10 promoter sequence; d, part of an FNR-binding site. Intergenic-sequence hairpin structures are also indicated (for details, see panel B), as well as positions and directions of putative ORFs, labeled 1 to 3, downstream of *hupL*. Note the presence of *xisC* and the subsequent rearrangement within *hupL* in *Anabaena* strain PCC 7120. (B) *hupSL* intergenic sequence hairpin structures. The stop codon of *hupS* and the start codon of *hupL* are boxed, and calculated $\Delta G$ values for each hairpin structure are shown below the respective structures (112).
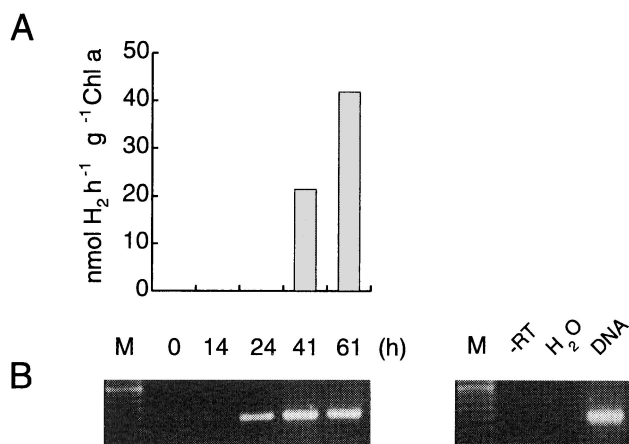
FIG. 4. Induction of an in vivo light-dependent hydrogen uptake in *Nostoc muscorum* cells during a shift from non-nitrogen-fixing to nitrogen-fixing conditions. Ammonia-grown cells were transferred to nitrogen-fixing conditions (time = 0) and analyzed with a Clark-type electrode for the appearance of an in vivo light-dependent hydrogen uptake (A) and by RT-PCR for the presence of a *hupL* transcript (B) after 14, 24, 41, and 61 h (17). M, marker (100-bp DNA ladder). Controls (right panel) include only a PCR (-RT; all RNA samples, only $t = 0$ h shown, negative control), replacement of RNA with water ($H_2O$, negative control), and use of genomic DNA instead of RNA (DNA, positive control).

ingly, when exposed to anaerobic conditions *A. variabilis* synthesizes a second Mo nitrogenase cellularly localized in the vegetative cells (199). The concomitant induction of the already identified uptake hydrogenase (79) or a possible alternative uptake hydrogenase deserves further studies. It was previously shown that ammonia-grown cells of *A. variabilis* have low but consistent in vivo hydrogen uptake, an activity that was attributed to the bidirectional enzyme (203). Moreover, some uptake hydrogenase activity could be detected in vegetative cells of *Anabaena* spp. grown microaerobically or anaerobically (88, 157).

The *hupSL* transcript in *A. variabilis* ATCC 29413 is 2.7 kb, and the transcription start site is located 103 bp upstream of the start codon, whereas in *Nostoc* strain PCC 73102 it is situated 259 bp upstream. Both start sites are preceded by putative −10 sequences, and putative promoter elements have been identified (Fig. 3A) (79, 112). In the *A. variabilis* sequence it is possible to identify half of a consensus FNR-binding sequence, and in *Nostoc* strain PCC 73102 there are possible integration host factor (IHF) and NtcA-binding sites (Fig. 3A) (79, 112). An FNR (fumarate nitrate reductase regulator)-binding site is involved in regulation of the *Escherichia coli hyp* operon (119), and the protein induces several operons during anaerobic growth (186). IHF is known to be involved in transcriptional activation of *nif* genes in purple bacteria (84), and the global cyanobacterial nitrogen regulator NtcA is necessary for heterocyst differentiation and expression of some of the genes involved in nitrogen fixation in *Anabaena* strain PCC 7120 (62, 82, 215).

The cyanobacterial *hupSL* genes differ from those of other microorganisms in being separated by longer intergenic regions (79, 150). These regions consist largely of 7-bp repeated sequences belonging to different families of short tandemly

repeated repetitive sequences commonly found in heterocystous cyanobacteria (112, 131). Even though the specific short tandemly repeated repetitive sequences are not conserved, sequence analyses revealed that the possibility of a hairpin formation is a common feature, indicating a conserved two-dimensional structure rather than a specific sequence of the repeat itself (Fig. 3B) (112). The specific function(s) and origin of the repeats are not known (93, 131). One possible function of hairpins could be to increase the stability of the transcript. Another suggestion is that the hairpin structure may confer a translational coupling between the two structural genes (112).

## Cyanobacterial Bidirectional Hydrogenases

The soluble or loosely membrane-associated cyanobacterial bidirectional hydrogenase is a common enzyme in both nitrogen-fixing and non-nitrogen-fixing cyanobacteria (75, 96, 97, 117, 177). However, recent data clearly demonstrated that, at least in nitrogen-fixing strains, it is not an universal enzyme and that strains missing the bidirectional enzyme have nothing obvious in common (192, 194). The Ni-containing bidirectional hydrogenase, partially purified and characterized from *A. variabilis* ATCC 29413 (178) differs from the uptake hydrogenase in its physical and catalytic properties (178, 202). In general, cyanobacterial bidirectional hydrogenases are characterized by their sensitivity to oxygen, thermotolerance, and high affinity to hydrogen (49, 87, 89, 163, 178). Methyl viologen, chemically reduced by dithionite, supports hydrogen evolution by all examined bidirectional hydrogenases and is usually used as an electron donor in the assay of the enzyme. Exposure to hydrogen during growth does not elicit additional activity of the bidirectional hydrogenase. Combined nitrogen in the growth medium also has little effect on the level of activity of the enzyme, suggesting that its function is independent of nitrogen fixation. Supporting this theory, Howarth and Codd (90) demonstrated that the biosynthesis of nitrogenase was not a prerequisite for the bidirectional hydrogenase biosynthesis and hydrogen evolution in several unicellular strains. The in vivo activity of the bidirectional enzyme in heterocystous strains increases considerably under anaerobic or microaerobic conditions (177), whereas in the unicellular non-nitrogen-fixing *Gloeocapsa alpicola* and *Chroococcidiopsis thermalis* the partial pressure of oxygen does not seem to have any significant influence (179, 180).

The physiological role of the bidirectional hydrogenase has been a matter of speculation and is still unclear. One possibility is that it functions as a mediator of the release of excess of reducing power in anaerobic environments. Kentemich et al. (97) and Schmitz et al. (173) reported that the low $K_m$ for hydrogen indicates that the enzyme generally operates in the direction of hydrogen uptake. Since the proton gradient in cyanobacteria is directed outward and the bidirectional hydrogenase has high affinity for hydrogen, they suggested that there may be a function in the oxidation of hydrogen at the periplasmic side and that the enzyme may allocate electrons to the respiratory chain (97, 173). Furthermore, it has been proposed that the enzyme functions as a valve for low-potential electrons generated during the light reaction of the photosynthesis, thus preventing the slowing of the electron transport chain under stress conditions (11). An incomplete version of respiratory
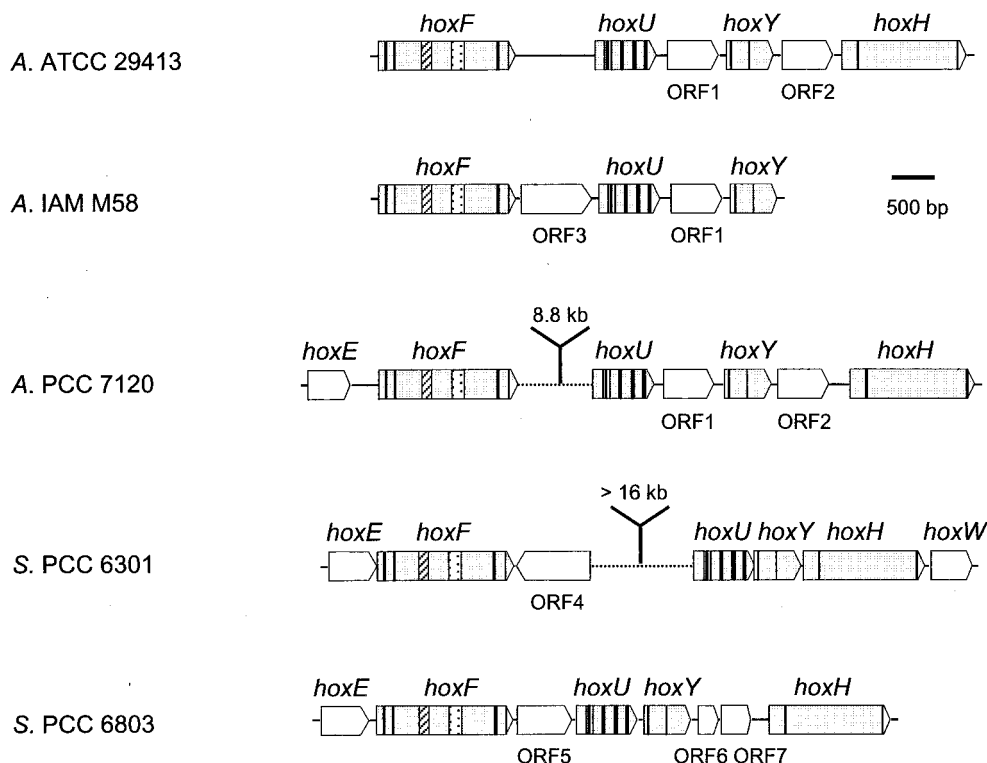
FIG. 5. Physical map of the genes encoding the bidirectional hydrogenase (*hox*) and additional ORFs (labeled 1 to 7, with identical numbers indicating homologous ORFs) in the cyanobacteria *A. variabilis* (two strains; *A.* ATCC 29413 [173] and *A.* IAM M58 [Gen. Bank accession no. AB057405]), *Anabaena* strain PCC 7120 (*A.* PCC 7120) (http://www.kazusa.or.jpn/cyano/anabaena), *Synechococcus* PCC 6301 (*S.* PCC 6301) (29, 30), and *Synechocystis* PCC 6803 (*S.* PCC 6803) (12, 95, 144). Vertical lines correspond to the positions of triplets encoding conserved cysteine residues compared to other microorganisms (for more details, see Table 4). ▨ and ▦ represent the NAD- and flavin mononucleotide-binding regions, respectively.

complex I (11 subunits out of 14 strictly conserved in other prokaryotes like *E. coli*) was identified in cyanobacteria, and the bidirectional hydrogenase was suggested to fulfill the functions of the missing subunits (11, 12, 30, 174). However, other data do not support this theory (27, 64, 91, 189), including the fact that the bidirectional enzyme is not a universal cyanobacterial enzyme (192, 194). Since the bidirectional hydrogenase is absent in a significant set of strains, it seems unlikely that it plays a common, central role in cyanobacterial respiratory complex I, unless different cyanobacteria have adopted different strategies. Some of the conserved sequence motifs of the bidirectional hydrogenase are similar in the two corresponding complex I subunits, but apart from this there are only low sequence similarities (64). These authors proposed that the cyanobacterial and chloroplastidial complex, which both have 11 subunits in common with respiratory complex I, might work as a NADPH:plastoquinone oxidoreductase, possibly involved in the cyclic photosynthetic electron transport, and that the sequence similarities observed between the NADH dehydrogenase part of complex I and the bidirectional hydrogenase are due to a common ancestor. It should be pointed out that, at present, the existence of a respiratory 14-subunit complex I in cyanobacteria cannot be ruled out. *Nostoc* strain PCC 73102, a strain lacking the bidirectional hydrogenase (27, 194), respires at rates comparable to those of other cyanobacteria (27), and bidirectional hydrogenase-minus mutants of *Synechocystis*

strain PCC 6803 exhibited growth and respiration rates comparable to those of the wild type (91). It is obvious that more data are necessary to clarify the function of the bidirectional hydrogenase in cyanobacteria.

**hox genes and the deduced proteins.** In 1995, Schmitz et al. (173) sequenced a set of structural genes (*hox* genes) encoding a bidirectional hydrogenase in *A. variabilis* ATCC 29413. These authors suggested that the bidirectional enzyme is a heterotetrameric enzyme consisting of a hydrogenase part (encoded by *hoxYH*) and a diaphorase part (encoded by *hoxFU*) (Fig. 1 and 5). In subsequent works, *hox* genes have been sequenced and characterized in the unicellular *Synechocystis* strain PCC 6803, *Synechococcus* strain PCC 6301 (12, 28–30, 95, 144, 174), and (partially) *C. thermalis* CALU 758 (178), as well as in the filamentous strains *Anabaena* strain PCC 7120 (www.kazusa.or .jp./cyano/anabaena) and (partially) *A. variabilis* IAM M58 (GenBank accession no. AB057405). The physical organizations of the structural genes encoding the bidirectional enzyme are similar (Fig. 5). In *A. variabilis, Anabaena* strain PCC 7120, *Synechococcus* strain PCC 6301, and *Synechocystis* strain PCC 6803, one or several additional ORFs have been identified between some of the structural genes. In *A. variabilis* IAM M58, an additional ORF is localized between *hoxF* and *hoxU* (Fig. 5). The intergenic distances between *hoxF* and *hoxU* in both *Synechococcus* strain PCC 6301 and *Anabaena* strain PCC 7120 are longer (at least 16 and approximately 9 kb, respec-

tively) than in other strains (Fig. 5). An ORF positioned upstream of *hoxF* and termed *hoxE* has been identified and sequenced in *Synechococcus* strain PCC 6301 and *Synechocystis* strain PCC 6803, and is also present in *Anabaena* strain PCC 7120 (http://www.kazusa.or.jp./cyano/anabaena). It has been suggested to encode a third diaphorase subunit (28). Moreover, in *Synechococcus* strain PCC 6301, an ORF termed *hoxW* is localized downstream of *hoxH*, putatively encoding a protease (Fig. 5) (30).

As with the uptake hydrogenase, molecular studies helped to clarify the picture of the subunit composition and molecular mass of the bidirectional hydrogenase. In agreement with the molecular data, previous work referred to large subunits of about 50 to 56 kDa in *A. variabilis, Synechococcus* strain PCC 6301, *M. aeruginosa*, and *Spirulina plantensis* (15, 96, 118) and to small subunits of about 17 kDa in *A. variabilis* and *Synechococcus* strain PCC 6301 (96). In *A. variabilis*, *hoxH* and *hoxY* encode predicted polypeptides of 54.8 and 22.5 kDa, respectively (173). Concerning the holoenzyme, earlier chromatographic studies reported an apparent molecular mass of 230 kDa for the hydrogenase of *A. cylindrica* (73). However, as pointed out by the authors, the enzyme was probably bound in a complex with other proteins. Considerably lower molecular masses (165 and 113 kDa) were reported for the bidirectional hydrogenase of *Anabaena* strain PCC 7120, with two-thirds of the activity being found in the 165-kDa peak (89). Native polyacrylamide gel electrophoresis followed by in vitro activity staining demonstrated the presence of a functional enzyme of about 118 kDa in induced cells of *A. variabilis, Anabaena* strain PCC 7120, and *N. muscorum* (177, 194). It is believed that in *A. variabilis* the four predicted polypeptides (Fig. 1) are assembled to form a tetrameric enzymatic complex, which is consistent with the molecular masses mentioned above.

The cellular and subcellular localization of the bidirectional hydrogenase is a topic that needs further investigation. Earlier work indicated that the enzyme is present in both the vegetative cells and heterocysts and that through gentle cell disruption procedures it was easily solubilized and thus located in the cytoplasm (73, 88, 89). However, other works suggest an association of the bidirectional hydrogenase with cell membranes. The enzyme of the unicellular *Synechococcus* strain PCC 6301 seems to be loosely associated with the cytoplasmic membrane (96, 97), whereas associations with the thylakoid membranes have been demonstrated in *A. variabilis* and *Synechocystis* strain PCC 6803 (11, 177).

Nucleotide sequence comparisons have shown that there is a reasonably high degree of homology between the *hox* genes of cyanobacteria and genes encoding the $NAD^+$-reducing hydrogenase from the chemolithotrophic hydrogen-metabolizing bacterium *R. eutropha* as well as those encoding methyl viologen-reducing hydrogenases from species of the archaeal genera *Methanobacterium*, *Methanococcus*, and *Methanothermus* (173). Sequence comparisons between the bidirectional hydrogenase genes or deduced proteins of *A. variabilis* ATCC 29413, *A. variabilis* IAM M58, *Anabaena* strain PCC 7120, *Synechococcus* strain PCC 6301, and *Synechocystis* strain PCC 6803 reveal that they are highly homologous, with *A. variabilis* ATCC 29413 and *Anabaena* strain PCC 7120 being at least 95% identical when the deduced amino acid sequences of proteins are compared (Table 3). The two *A. variabilis* strains

are less similar (78 to 88% identical), and all other comparisons are in the range of 58 to 71% identical amino acids of the deduced proteins, with the exception of 80 to 88% identical amino acids when comparing *Anabaena* strain PCC 7120 and *A. variabilis* IAM M58 (Table 3).

Some of the characteristics of the deduced proteins are summarized in Fig. 5 and Table 4. HoxH (the large subunit of the hydrogenase dimer) in cyanobacteria harbors six conserved cysteines, four matching the typical cysteine-containing N-terminal and C-terminal conserved motifs involved in the binding of nickel to the active site (174, 212). The unicellular strains contain an additional cysteine positioned 4 amino acids upstream of the C-terminal motif. In the small subunit of the hydrogenase dimer, HoxY, four cysteine residues, believed to be involved in coordinating a putative [4Fe4S] cluster (212), can be identified (Fig. 5; Table 4). The HoxU protein (the small subunit of the diaphorase moiety) contains several conserved cysteine residues putatively involved in the binding of [FeS] clusters. Two of the motifs are located at the N-terminal part of the protein; the first harbors four cysteines probably involved in binding a [2Fe2S] cluster, and the second harbors three cysteines residues putatively binding a [3Fe4S] or [4Fe4S] cluster (12, 174). The final conserved cysteine residue in the latter motif is preceded by an additional cysteine in the filamentous strains. Moreover, the C-terminal part of HoxU possesses two typical [4Fe4S] cluster-binding sites (Fig. 5; Table 4) (12, 174). The large subunit of the diaphorase moiety, HoxF, is generally less highly conserved in its N-terminal part. However, this region harbors two shorter, highly conserved cysteine-containing stretches with a longer nonconserved region in between, which is postulated to be involved in binding a [2Fe2S] cluster (Fig. 5; Table 4) (63). All five cyanobacterial strains contain another conserved cysteine directly upstream of this motif; the filamentous strains also harbor a cysteine immediately downstream. In the middle region of this subunit, typical glycine-rich binding sites for NAD (GxGxxGxxxG) and flavin mononucleotide ($GxGxxxxGx_{10}GxxG$) can be identified (Fig. 5) (173). The five cyanobacterial HoxF proteins contain, in the NAD motif, another glycine following the second conserved one; in addition, in the two unicellular strains a third glycine immediately follows this additional glycine. The C-terminal end of HoxF contains a conserved motif with five cysteines, four of which correspond to the residues postulated to be involved in binding a [4Fe4S] cluster (Table 4) (170, 173). The fifth cysteine is positioned 9 amino acids upstream of the last conserved residue in this motif. In addition, a universal sixth cysteine preceding the conserved C-terminal motif is present in cyanobacterial HoxF.

**Transcription of *hox* genes.** Transcriptional studies (using RT-PCR) indicate that the structural genes form a single transcriptional unit in *A. variabilis* ATCC 29413 (28). In contrast, in the unicellular *Synechococcus* strain PCC 6301 two transcripts were detected, the dicistronic *hoxEF* and the polycistronic *hoxUYHWhypAB* (28). Further promoter activities were identified in the *hox* locus by using β-galactosidase and bacterial luciferase as reporters (28). With the exception of a shift from non-nitrogen-fixing to nitrogen-fixing conditions, which was associated with no significant change in the transcript level of *hoxH* in *N. muscorum* (Fig. 6) (17), the influences of key

environmental conditions on the transcription of the cyanobacterial *hox* genes remain to be examined.

## Genetic Diversity of Cyanobacterial Hydrogenases

The molecular diversity of cyanobacterial hydrogenases can be perceived from the results presented for a selected set of nitrogen-fixing *Anabaena* and *Nostoc* strains (Table 5). Although *hup* gene homologues are present in all strains examined, all kinds of combinations can be detected concerning the presence or absence of sequences homologous to *xisC* and to the bidirectional hydrogenase genes. Interestingly, at present there are no data supporting the existence of either multiple uptake hydrogenases or multiple bidirectional hydrogenases in a single cyanobacterial strain. It should be pointed out that it is not possible to establish any correlation between the presence or absence of the bidirectional hydrogenase and the occurrence of the gene responsible for the rearrangement of *hupL*. Similarly, there is no obvious connection to the different habitats from where the individual strains originally were isolated. The natural molecular variation of hydrogenases in different cyanobacteria is a field both to examine and to explore in an effort to understand the physiological function(s) as well as regulation of the respective enzyme and in an effort to identify and use the "correct genetic background" when constructing and examining a genetically engineered cyanobacterial strain.

## Accessory Genes

The maturation of nickel-containing enzymes, e.g., hydrogenases, ureases, and carbon monoxide dehydrogenases, is a complex process requiring accessory proteins (42, 81, 122, 127, 135, 211, 213). Initial work using *E. coli* revealed five ORFs, designated *hypABCDE*, affecting hydrogenases pleiotropically (119). At present, there is no information on the function of Hyp proteins in cyanobacteria. However, some of the homologous gene products of other bacteria (e.g., *E. coli*, *R. eutropha*, and *B. japonicum*) are well characterized, and in general homologous Hyp proteins fulfill similar functions in different organisms (summarized in Table 6) (42).

**Organization and transcription of cyanobacterial *hyp* genes.** Although hydrogenase genes have been identified and characterized in several cyanobacteria (Tables 1 to 4 and 7), very little is known about the cyanobacterial *hyp* genes and the corresponding Hyp proteins (Fig. 7). Among the first *hyp* genes to be identified were *hypABF* in *Synechococcus* strain PCC 6301 (29) and *hypB* in *Anabaena* strain PCC 7120 (originally published as *hupB* [70]) (Fig. 7). The former are preceded by structural genes encoding the bidirectional hydrogenase of *Synechococcus* strain PCC 6301 (*hoxUYH*) and the putative protease HoxW, which might be involved in the maturation of HoxH (200) (Fig. 5 and 7). *hypF* was only partially sequenced. Transcriptional analysis (RT-PCR) revealed that *hypA* and *hypB* form a polycistronic transcript together with *hoxUYHW*, whereas *hypF* is part of a different transcript (28). *hypB* of *Anabaena* strain PCC 7120 is part of a gene cluster consisting of *hypBAEDF* (originally published as *hupBAEDF* [71]), in which *hypA* and *hypB* overlap by 10 bp (as in *Nostoc* strain PCC 73102 [74; http://jgi.doe.gov/JGI_microbial/html/nostoc

TABLE 3. Sequence comparison of the structural genes *hoxFUYH* and the deduced amino acid sequences, of the cyanobacteria *A. variabilis* ATCC 29413, *A. variabilis* IAM M58, *Anabaena* strain PCC 7120, *Synechococcus* strain PCC 6301, and *Synechocystis* strain PCC 6803.

| Strain | Gene or protein | Sequence comparison[a] with: | | | |
|---|---|---|---|---|---|
| | | *A. variabilis* IAM M58 | *Anabaena* strain PCC 7120 | *Synechococcus* strain PCC 6301 | *Synechocystis* strain PCC 6803 |
| *A. variabilis* ATCC 29413 | *hoxFUYH* | 78/82/76/—[b] | 93/94/91/94 | 63/62/60/63 | 65/64/67/67 |
| | HoxFUYH | 82 (87)/88 (95)/78 (83)/— | 95 (96)/98 (99)/95 (96)/97 (98) | 62 (73)/63 (73)/60 (75)/64 (74) | 65 (76)/62 (75)/63 (77)/68 (77) |
| *A. variabilis* IAM M58 | *hoxFUYH* | | 79/82/76/— | 66/63/64/— | 67/66/65/— |
| | HoxFUYH | | 81 (87)/89 (95)/80 (89)/— | 61 (72)/58 (71)/60 (75)/— | 67 (76)/61 (74)/62 (78)/— |
| *Anabaena* strain PCC 7120 | *hoxFUYH* | | | 63/77/63/63 | 66/65/67/66 |
| | HoxFUYH | | | 60 (71)/61 (72)/60 (74)/65 (74) | 65 (76)/62 (75)/62 (76)/68 (77) |
| *Synechococcus* strain PCC 6301 | *hoxFUYH* | | | | 64/66/63/66 |
| | HoxFUYH | | | | 59 (71)/70 (80)/62 (80)/71 (79) |

[a] Nucleotide identity (%) and amino acid identity (similarity) (%) are given for *hoxF*, *hoxU*, *hoxY*, and *hoxH* (and their proteins) separately.

[b] —, not sequenced in *A. variabilis* IAM M58.

TABLE 4. Positions and putative functions of conserved cysteine residues (Cys) in cyanobacterial bidirectional hydrogenases compared to those in similar hydrogenases in other microorganisms

| Protein | Conserved residues[a] | Function | Reference(s) |
|---|---|---|---|
| HoxF | $Cys_{25}$, $Cys_{30}$, $Cys_{62}$, $Cys_{66}$ | Binding of putative [2Fe2S] cluster | 12, 63, 174 |
| | $Cys_{453-463}$, $Cys_{456-466}$, $Cys_{459-469}$, $Cys_{499-509}$ | Binding of putative [4Fe4S] cluster | 170, 173 |
| HoxU | $Cys_{36}$, $Cys_{47}$, $Cys_{50}$, $Cys_{64}$ | Binding of putative [2Fe2S] cluster | 12, 174 |
| | $Cys_{100}$, $Cys_{103}$, $Cys_{109}$ | Binding of either putative [3Fe4S] or putative [4Fe4S] cluster | 12, 174 |
| | $Cys_{148}$, $Cys_{151}$, $Cys_{154}$, $Cys_{158}$, $Cys_{192}$, $Cys_{195}$, $Cys_{198}$, $Cys_{202}$ | Binding of two putative [4Fe4S] clusters | 12, 174 |
| HoxY | $Cys_{14}$, $Cys_{17}$, $Cys_{86-87}$, $Cys_{150-153}$ | Binding of putative [4Fe4S] cluster | 212 |
| HoxH | $Cys_{62}$, $Cys_{65}$, $Cys_{443-452}$, $Cys_{446-455}$ | Binding of Ni | 159, 174, 212 |

[a] The indicated intervals represent different positions in different strains.

/nostoc_homepage.html]). Analysis of the genome of *Anabaena* strain PCC 7120 (http://www.kazusa.or.jpn/cyano/anabaena) showed that *hupSL* and the *hyp* genes are approximately 3.8 kb apart and directed in opposite directions from each other (Fig. 7). Moreover, an ORF encoding a HypC homologue is located between *hypF* and *hypD*, and another ORF putatively encoding a 4-oxalocrotonate tautomerase homologue (217) is situated between *hypD* and *hypE* (Fig. 7). RT-PCR analysis revealed that *hypB* is expressed in heterocyst-induced but not in non-nitrogen-fixing cultures (71). A *hyp* gene cluster consisting of *hypFCDEAB* and an additional upstream ORF has been cloned from *Nostoc* strain PCC 73102 and sequenced (74). RT-PCR-based gene expression analysis showed that these genes form one transcript. Upstream of the transcriptional start site, putative binding sites for the global nitrogen regulator NtcA have been identified (74). A putative NtcA-binding site has also been identified upstream of the *hupSL* transcriptional start site (112), in agreement with the experimental evidence demonstrating that both the *hyp* and the *hup* operons are transcribed under nitrogen-fixing but not under non-nitrogen-fixing conditions (74). As in *Anabaena* strain PCC 7120, the *hup* operon is located upstream of the *hyp* operon (about 3.9 kb) and oriented in the opposite direction (Fig. 7). In *Synechocystis* strain PCC 6803, the *hyp* genes are scattered over the genome, with the exception of *hypA'* and *hypB'*, which have the same direction and are 61 bp apart (Fig. 7). Two of the six *hyp* genes of *Synechocystis* strain PCC 6803, *hypA* and *hypB*, are duplicated (Fig. 7; Table 7) (95, 144). These are, at present, the only known duplications of cyanobacterial *hyp* genes, although duplication of *hyp* genes is not uncommon among bacteria. In addition, *Synechocystis* strain PCC 6803 contains an identified *hupE* homologue, an accessory gene with similarities to genes encoding both hydrogenase and urease accessory proteins (95, 144). A *hypE* homologue has been cloned and sequenced from *Synechococcus* strain

PCC 7002 (171). The gene is located 213 bp upstream of *ureC* (encoding a putative urease subunit), but in the opposite direction (Fig. 7).

Interestingly, *Anabaena* strain PCC 7120 contains only one set of *hyp* genes, and the presence of both an uptake (39) and a bidirectional hydrogenase (1, 194) in this strain suggests that one copy is enough to fulfill the functions for both hydrogenases. Assuming that this is a general feature for cyanobacteria, the specific regulation of *hyp* genes in response to the differentially regulated hydrogenases in a single cyanobacterial strain deserves future attention.

## CYANOBACTERIAL BIOHYDROGEN

As discussed above, two cyanobacterial enzymes are capable of hydrogen production: the nitrogenase(s) and the bidirectional hydrogenase. Individual strains may harbor both a bidirectional hydrogenase (although this is not a universal cyanobacterial enzyme [Table 5] [192]) and none to several nitrogenases, encoded by different structural genes. There is no report describing the existence of either several uptake hydrogenases or several bidirectional hydrogenases in a single cyanobacterial strain. An efficient photoconversion of water to hydrogen by cyanobacteria is certainly influenced by many other factors, and only an extensive knowledge of this field can lead to improvement of the rates of cyanobacterial hydrogen



FIG. 6. Visualization of a *hoxH* transcript from *N. muscorum* during a shift from non-nitrogen-fixing to nitrogen-fixing conditions (the same experiment as described in the legend to Fig. 4). Transcripts from $t = 0$, 14, 24, 41, and 61 h after the transfer to nitrogen-fixing conditions are shown (17). M, marker (100-bp DNA ladder).

TABLE 5. Molecular diversity of hydrogenases in selected nitrogen-fixing *Anabaena* and *Nostoc* strains[a]

| Group | Strain[b] | hup | xisC | hox | Reference(s) |
|---|---|---|---|---|---|
| A | *Anabaena* strain PCC 7120 | + | + | + | 39, 194 |
| | *Nostoc* strain PCC 6314 | + | + | + | 192 |
| | *Nostoc* strain Mitsui 56111 | + | + | + | 192 |
| B | *A. variabilis* ATCC 29413 | + | − | + | 17, 79, 173, 194 |
| | *N. muscorum* CCAP 1453/12 | + | − | + | 17, 194 |
| | *Nostoc* strain Mitsui 91911 | + | − | + | 192 |
| C | *Nostoc* strain Mitsui 38901 | + | + | − | 192 |
| | *Nostoc* strain CYA 238 | + | + | − | 192 |
| D | *Nostoc* strain PCC 73102 | + | − | − | 26, 150, 194 |
| | *Nostoc* strain CYA 190 | + | − | − | 192 |
| | *Nostoc* strain ACOI 578 | + | − | − | 192 |

[a] Diversity is demonstrated by the presence (+) or absence (−) of sequences homologous to *hup* (uptake hydrogenase), *xisC* (rearrangement in *hupL* in *Anabaena* strain PCC 7120), and *hox* (bidirectional hydrogenase) genes.
[b] Selected strains are arranged in four groups: (A) strains containing *hup*, *xisC*, and *hox*; (B) strains containing *hup* and *hox*; (C) strains containing *hup* and *xisC*; and (D) strains containing only *hup*.

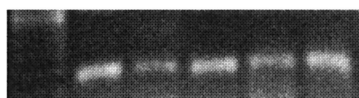TABLE 6. Conserved motifs and putative functions of putative cyanobacterial Hyp proteins

| Protein | Conserved motif(s) | Function(s) | References |
|---|---|---|---|
| HypA | $Cx_{1-2}Cx_{12-13}Cx_{1-2}C$ | Ni incorporation (putative) <br> [FeS] cluster binding or zinc finger motif, regulation | 28, 71, 148, 201 |
| HypB | N-terminal CxxCGC[a] <br> Histidine-rich N terminus[a] <br> $x_2Nx_2SSPGAGKTx_{27}DAx_6Gx_{44-48}Px_5Gx_{28}x_4TKxD$ | Ni incorporation <br> Protein stability <br> Mobilization, carriage and storage of nickel <br> GTPase | 28, 34, 71, 145, 147, 148 |
| HypC | N-terminal MCL(G/A)(L/I)P | Chaperone <br> The cysteine interacts with the precursor of the large hydrogenase subunit and facilitates Ni insertion | 25, 121, 146 |
| HypD | CPVC <br> 5 conserved cysteines | Ni incorporation <br> Potential metal binding <br> Unknown | 71, 146 |
| HypE | G(G/S)GGK(L/A)(M/S)x(Q/D)L$x_{55-62}$x(V/I)NDLA(V/M)(S/A)GaxP(L/R)$x_{74-79}$GDxx(I/L)x(S/N)G(E/D)(L/I)G(N/R) HG$x_{152-157}$(E/D)QLPRIC | Purine derivate binding <br> Potential phosphoribosylformylglycinamidine cycloligase (AIR synthethase; EC 6.3.3.1) | 66, 71, 171 |
| HypF | G(R/T)VOGVGFR$x_{13}$G(D/W)V(C/N)N$x_3$G <br> $Cx_2Cx_{18}Cx_2Cx_{24}Cx_2Cx_{18}Cx_2C$ <br> HHxAH | CO/CN synthesis <br> Potential acylphosphatase <br> Two zinc fingers, transcriptional regulation <br> Histidine motif | 28, 146, 153 |

[a] Not present in HypB' of *Synechocystis* strain PCC 6803.

TABLE 7. Summary of genes related to hydrogenases in cyanobacteria

| Strain | Accession no. (reference) of: | | | | | | |
|---|---|---|---|---|---|---|---|
| | *hupS* | *hupL* | *hoxE* | *hoxF* | *hoxU* | *hoxY* | *hoxH* |
| Unicellular | | | | | | | |
| *Synechococcus* strain PCC 6301 | | | Y13471[a] (30) | Y13471 (30) | X97797 (29, 30) | X97797 (29, 30) | X97797 (29, 30) |
| *Synechococcus* strain PCC 7002 | | | | | | | |
| *Synechocystis* strain PCC 6803 | NP[a] (11, 95, 144) | NP (11, 95, 144) | sll1220,[b] X97610 (12, 95, 144) | sll1221, X97610 (12, 95, 144) | sll1223, X97610 (12, 95, 144.) | sll1224, X97610 (12, 95, 144.) | sll1226, X97610 (12, 95, 144) |
| Filamentous heterocystous | | | | | | | |
| *A. variabilis* ATCC 29413 | Y13216 (79) | Y13216 (79) | | X79285 (173) | X79285 (173) | X79285 (173) | X79285 (173) |
| *A. variabilis* IAM M58 | | | | AB057405 | AB057405 | AB057405 | |
| *Anabaena* strain PCC 7120 | all0688[c], U08013 (39) | all0687N/C, U08013 (39) | alr 0751 | alr0752 | alr0762 | alr0764 | alr0766 |
| *N. punctiforme* PCC 73102 (ATCC 29133) | c651/20, AF030525 (150) | c651/21, AF030525 (150) | NP (27)[d] | NP (27)[d] | NP (27)[d] | NP (194)[d] | NP (194)[d] |

[a] GenBank; http://www.ncbi.nlm.nih.gov/.
[b] CyanoBase; http://www.kazusa.or.jp/cyano/cyano.html.
[c] CyanoBase; http://www.kazusa.or.jp/cyano/anabaena/.
[d] DOE/JGI; http://www.jgi.doe.gov/JGI_microbial/html/nostoc/nostoc_homepage.html.
[e] NP, not present.

production. For example, it is known that immobilized cells produce more hydrogen than do free-living cultures (125) and that non-Mo-containing nitrogenases allocate more electrons to the production of hydrogen (206). The gas phase (214), the age and density of the culture, and the composition, pH, and temperature of the growth medium are also crucial to the final result. Most of the research on cyanobacterial hydrogen production has been carried out with nitrogen-fixing strains (31, 55, 106, 111, 166, 205, 206). In these organisms, the net hydrogen production is the result of hydrogen evolution catalyzed by nitrogenase and of hydrogen consumption catalyzed mainly by an uptake hydrogenase. Consequently, the production and selection of mutants deficient in hydrogen uptake activity is of great interest (76, 79, 115, 136). Moreover, the nitrogenase has a high ATP requirement and this lowers the potential solar energy conversion efficiencies considerably. On the other hand, the bidirectional hydrogenase requires much less metabolic energy but is extremely sensitive to oxygen (14, 15). For reviews on the potential, problems and prospects of hydrogen production by cyanobacteria, see references (19, 20, 72, 75, 113, 117, 120, 125, and 164).

## Rates of Cyanobacterial Hydrogen Production

As discussed above, most of the research on rates of cyanobacterial hydrogen production has been carried out with nitrogen-fixing filamentous strains with typical values ranging from 0.17 to 4.2 nmol of hydrogen produced per $\mu$g of chlorophyll *a* per h (128). Interestingly, nitrogen-fixing cells of *A. variabilis* SPU 003 have the capacity to produce hydrogen mainly in darkness (181). Addition of various sugars stimulated the production of hydrogen, with mannose giving the highest rate, 5.58 nmol of hydrogen produced per mg dry weight per h (181). Since the hydrogen production is the result of hydrogen evolution catalyzed by nitrogenase and a hydrogen consumption catalyzed mainly by an uptake hydrogenase, the obvious improvements are to increase the hydrogen production by using alternative nitrogenases and by inhibiting the activity of the uptake hydrogenase. In a recent study, the hydrogen produc-

tion by autotrophic, vanadium-grown cells (i.e., cells expressing the alternative vanadium-containing nitrogenase) of *A. variabilis* ATCC 29413 (wild type) and of its mutant PK84, impaired in the utilization of molecular hydrogen, was studied in a photobioreactor (204). The highest hydrogen production rates were observed in cultures grown at gradually increased irradiation. The wild-type strain evolved hydrogen only under a argon atmosphere, with the actual rate being as high as the potential rate (61% of oxygenic photosynthesis used for hydrogen production). In contrast, PK84 cells also produced hydrogen during growth under carbon dioxide-enriched air (13% of oxygenic photosynthesis used for hydrogen production, representing 33% of the potential rate) (204). *A. variabilis* PK84 has also been examined under simulated outdoor conditions (31). Use of a 4.35-liter automated helical tubular bioreactor and continuous cultivation for 2.5 months resulted in a maximum hydrogen evolution of 230 ml of hydrogen produced per 12-h light period at a growth density of 3.6 to 4.6 $\mu$g of chlorophyll *a* per ml of cell culture (31). Replacing air with argon doubled the rate of hydrogen evolution. Anoxygenic conditions over the dark periods had a negative effect on hydrogen production. Similarly, under aerobic outdoor conditions operated for 4 months, a maximum rate of 80 ml of hydrogen per reactor (4.35 liters) per hour was obtained from a batch culture on a bright day (57). The maximum efficiency of conversion of light to chemical energy of hydrogen was calculated to be 0.33 and 0.14% on a cloudy and a sunny day, respectively (57).

## Strategies for Improving Cyanobacterial Strains for Photobiological Hydrogen Production

Thorough studies on hydrogen uptake and/or evolution have until now focused on only a few filamentous cyanobacteria, e.g., different *Anabaena* and *Nostoc* strains. However, other cyanobacteria, e.g., *Oscillatoria*, are able to fix nitrogen without forming heterocysts by using the strategy of temporal separation of the oxygen-sensitive nitrogen fixation and the oxygen-evolving photosynthesis. Such strains deserve a thorough ex-

TABLE 7—*Continued*

| | | | | Accession no. (reference) of: | | | | |
|---|---|---|---|---|---|---|---|---|
| hoxW | hypA | hypA' | hypB | hypB' | hypC | hypD | hypE | hypF |
| X97797 (29, 30) | X97797 (29, 30) | | X97797 (29, 30) | | | | | X97797 (29, 30) |
| | | | | | | | AF035751 (171) | |
| | slr1675 (95, 144) | sll1078 (95, 144) | Sll1432 (95, 144) | sll1079 (95, 144) | ssl3580 (95, 144) | slr1498 (95, 144) | sll1462 (95, 144) | sll0322 (95, 144) |
| | c286, AF006594 (71)[d] c651/98, AF325724 (74)[d] | | c286, AF006594 (70, 71)[d] c651/99, AF325724 (74)[d] | | c286[d] c651/95, AF325829 (74)[d] | c286, AF006594 (71)[d] c651/96, AF325724 (74)[d] | c286, AF006594 (71)[d] c651/97, AF325724 (74)[d] | c286[d] c651/94, AF325829 (74)[d] |

amination of their hydrogen metabolism. Considering the versatility of cyanobacteria and their ability to survive under many different environmental conditions, more strains originating from different habitats must be studied with respect to their applicability in biohydrogen production. The genetic backgrounds (e.g., structural and accessory genes including mechanisms of regulation) of the selected strains and their overall capacities to be genetically modified should be determined. Of specific interest might be isolates originating from nitrogen-fixing associations. The situation of the symbiotic cyanobacteria is similar to that of steady-state cultures in bioreactors: the cells almost do not grow, they have a high metabolism in a restricted space, and they export at least one metabolite to the host. In addition, a heterotrophic capacity can be used during conversion of organic wastes into valuable, energy-rich compounds such as molecular hydrogen. Similarly, other cyanobacterial habitats mimicking these conditions, e.g., cyanobacterial mats, are also of great interest.

Several strategies are available for improving existing cyanobacterial strains for the biotechnological production of hy-
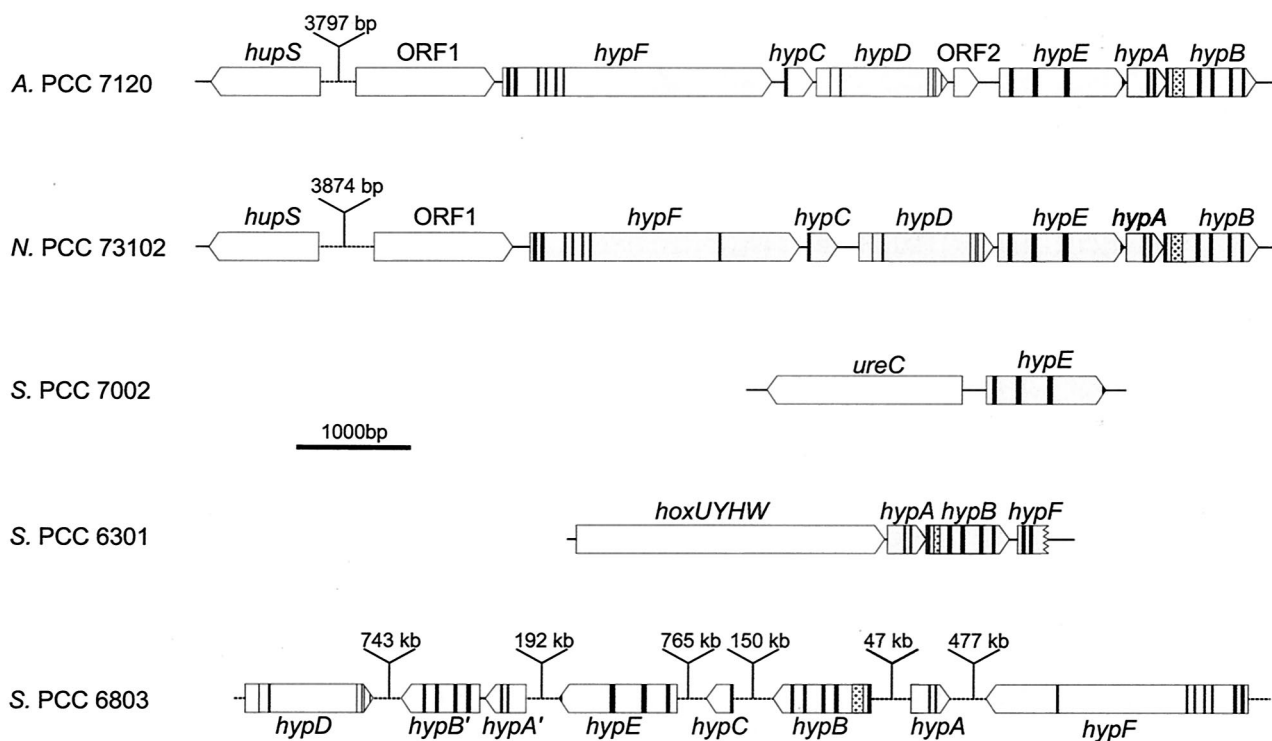


FIG. 7. Physical organization and conserved regions (vertical lines; for further explanations, see Table 6) of hitherto identified cyanobacterial *hyp* genes. The histidine-rich N terminals of *hypB* are indicated (▨). *Anabaena* strain PCC 7120 (*A.* PCC 7120) (70, 71; http://www.kazusa.or.jpn /cyano/anabaena), *Nostoc* PCC 73102 (*N.* PCC 73102) (74; http://www.jgi.doe.gov/JGI_microbial/html/nostoc/nostoc_homepage/html); *Synechococcus* strain PCC 7002 (*S.* PCC 7002) (171), *Synechococcus* strain PCC 6301 (*S.* PCC 6301) (29), and *Synechocystis* strain PCC 6803 (*S.* PCC 6803) (95, 144).

drogen. Inactivation of a gene encoding the uptake hydrogenase leads to a mutant that is not able to recycle the hydrogen evolved by the nitrogenase under nitrogen-fixing conditions. Consequently, hydrogen produced through the action of a nitrogenase will either be oxidized by the bidirectional hydrogenase or, if this enzyme is not present, be evolved from the cells. Recent experiments demonstrated that a nitrogen-fixing culture of an uptake-deficient mutant (a *hup* mutant generated by introducing a cassette of foreign DNA into *hupL*) of *A. variabilis* ATCC 29413 (a strain containing both an uptake hydrogenase and a bidirectional enzyme [Table 5]) produces molecular hydrogen (79). The absence of a bidirectional enzyme in cyanobacteria such as *Nostoc* strain PCC 73102 (Table 5) makes these strains interesting candidates for inactivation experiments. Identification and engineering of an oxygen-stable hydrogen-evolving hydrogenase might result in a photosynthesizing microorganism that evolves significant amounts of hydrogen. Interestingly, replacing *Azotobacter vinelandii* hydrogenase small-subunit cysteines with serines can create insensitivity to inhibition by oxygen and preferentially damage hydrogen oxidation over hydrogen evolution (132). Moreover, overproducing mutants might be obtained by providing genes encoding a selected hydrogenase on an expression vector. Coupling the genes to a strong promoter might lead to an increased amount of the hydrogenase and thus to increased levels of hydrogen produced by the organism. Similarly, overexpression might also be used to increase the nitrogenase activity. A thorough examination of the genes involved in the regulation of hydrogenase expression, e.g., *hyp* genes, might generate knowledge leading to further strategies for improving hydrogen production rates in cyanobacteria.

Genetic engineering has become increasingly important with the establishment of molecular biological tools and techniques for cyanobacteria. A few unicellular strains, including *Synechococcus* strains PCC 6301 and PCC 7942 and *Synechocystis* strain PCC 6803, are naturally transformable. Protocols and vector systems useful for the transfer of DNA into different cyanobacteria are available for nontransformable strains (196). These methods have been used with success in filamentous genera such as *Anabaena* and *Nostoc*, which are interesting candidates for future photobiotechnological applications (75, 76, 79). The introduction of the structural genes encoding a clostridial Fe hydrogenase into the unicellular cyanobacterium *Synechococcus elongatus* PCC 7942 and their subsequent transcription and translation into a functional hydrogen-evolving enzyme in a photosynthetic microorganism (16, 138) is interesting and deserves further studies.

### Future Research and Development Directions and International Cooperation and Networks

The study of biohydrogen is a long-term approach to the development of knowledge and technologies aiming at producing molecular hydrogen from solar energy and water by using a renewable process. The present research and development activities within this field, with its long-term goal, can be promoted, stimulated, and made to advance at a higher rate through collaborations and networks. Besides some national biohydrogen programs and several individual projects, two major international initiatives can be recognized. (i) In the inter-

national program IEA (International Energy Agency; http://www.iea.org) Agreement of the Production and Utilization of Hydrogen, Annex 15 Photobiological Hydrogen Production, the main objectives are to investigate and develop processes and equipment for photobiological production of hydrogen by direct conversion of solar energy (114). The research is organized into four subtasks: light-driven hydrogen production by microalgae, maximization of photosynthetic efficiencies, hydrogen fermentations, and improvement of photobioreactor systems for hydrogen production. (ii) In the European program COST (European Cooperation in the Field of Scientific and Technical Research; http://www.belspo.be/cost/) 8.41 Biological and Biochemical Diversity of Hydrogen Metabolism, the main objective is to pool interrelated European expertise in order to understand the structural and molecular basis of the functions, as well as the factors that influence the activity and stability of hydrogenase enzymes.

### ADDENDUM IN PROOF

The complete genomic sequence of *Anabaena* strain PCC 7120 was recently published by Kaneko et al. (T. Kaneko, Y. Nakamura, C. P. Wolk, T. Kuritz, S. Sasamoto, A. Watanabe, M. Iriguchi, A. Ishikawa, K. Kawashima, T. Kimura, Y. Kishida, M. Kohara, M. Matsumoto, A. Matsuno, A. Muraki, N. Nakazaki, S. Shimpo, M. Sugimoto, M. Takazawa, M. Yamada, M. Yasuda, and S. Tabata, DNA Res. **8:**213, 227–253, 2001).

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