Occurrence of Hydrogenases in Cyanobacteria and Anoxygenic Photosynthetic Bacteria: Implications for the Phylogenetic Origin of Cyanobacterial and Algal Hydrogenases

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Abstract. Hydrogenases are important enzymes in the energy metabolism of microorganisms. Therefore, they are widespread in prokaryotes. We analyzed the occurrence of hydrogenases in cyanobacteria and deduced a FeFe-hydrogenase in three different heliobacterial strains. This allowed the first phylogenetic analysis of the hydrogenases of all five major groups of photosynthetic bacteria (heliobacteria, green nonsulfur bacteria, green sulfur bacteria, photosynthetic proteobacteria, and cyanobacteria). In the case of both hydrogenases found in cyanobacteria (uptake and bidirectional), the green nonsulfur bacterium *Chloroflexus aurantiacus* was found to be the closest ancestor. Apart from a close relation between the archaebacterial and the green sulfur bacterial sulfhydrogenase, we could not find any evidence for horizontal gene transfer. Therefore, it would be most parsimonious if a *Chloroflexus*-like bacterium was the ancestor of *Chloroflexus aurantiacus* and cyanobacteria. After having transmitted both hydrogenase genes vertically to the different cyanobacterial species, either no, one, or both enzymes were lost, thus producing the current distribution. Our data and the available data from the literature on the occurrence of cyanobacterial hydrogenases show that the cyanobacterial uptake hydrogenase is strictly linked to the occurrence of the nitrogenase. Nevertheless, we did identify a nitrogen-fixing *Synechococcus* strain without an uptake hydrogenase. Since we could not find genes of a FeFe-hydrogenase in any of the tested cyanobacteria, although strains performing anoxygenic photosynthesis were also included in the analysis, a cyanobacterial origin of the contemporary FeFe-hydrogenase of algal plastids seems unlikely.

Key words: Heliobacteria — *Chloroflexus* — Oxygenic photosynthesis — Photosynthetic proteobacteria

Introduction

In the world of microorganisms, hydrogen is an important intermediate. It can be either a rich source of energy used to deliver electrons to a kind of respiratory chain or a means of disposing of a surplus of reducing equivalents during fermentation. This second process—like respiration—allows the production of additional ATP. Therefore, a large number of enzymes handling hydrogen (hydrogenases) can be found in the genomes of bacteria and unicellular eukaryotes sequenced to date. They are classified according to their metal content as NiFe-hydrogenases, FeFe-hydrogenases (Vignais et al. 2001), or FeS-cluster-free hydrogenases (Shima et al. 2004). The latter seem to be restricted to methanogenic archaebacteria, whereas the other two classes are widespread among all major bacterial divisions. Both types need accessory proteins for posttranslational processing to yield an active enzyme. NiFe-hydrogenases depend on the action of six

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proteins encoded by the hyp genes (*hy*drogenases-*p*leiotropic genes) and a protease. The different steps of NiFe-hydrogenase maturation are best described in *Escherichia coli* (for review see Blokesch et al. 2002). In contrast FeFe-hydrogenases seem to need only two or three additional proteins encoded by *hydE*, *hydF* (or *hydEF*), and *hydG* to get active (Posewitz et al. 2004).

Cyanobacteria are known to contain two different NiFe-hydrogenases, called uptake and bidirectional enzymes, according to their preferred reaction under physiological conditions (Houchins 1984, Appel and Schulz 1998, Tamagnini et al. 2002). The occurrence of the uptake hydrogenase (*hup*) is coupled to nitrogen fixation and is needed for the recycling of H_2, inevitably produced by the nitrogenase during its catalytic cycle. This was confirmed in an investigation of the distribution of hydrogenases in nitrogen-fixing filamentous strains (Tamagnini et al. 2000). The bidirectional enzyme encoded by the *hox* genes fulfills a role as an electron valve to avoid the accumulation of electrons in the photosynthetic electron transport chain during transition states (Appel and Schulz 1996; Appel et al. 2000; Cournac et al. 2002, 2004), and it is also part of the fermentative metabolism in cyanobacteria (van der Oost and Cox 1987).

Investigation of the total genomes available (http://www.kazusa.or.jp/cyano/cyano.html, http://genome.jgi-psf.org/) and the above-mentioned study on filamentous strains suggests that approximately half of the cyanobacterial strains have the *hox* genes, whereas the other half does not. Its distribution seems to be erratic.

The plastids of green algae which have been derived from cyanobacteria by endosymbiosis (Delwiche and Palmer 1997) have long been known to produce hydrogen in the light (Gaffron and Rubin 1942). These organelles contain a peculiar FeFe-hydrogenase confined to the domain with the active site without any additional FeS clusters (Florin et al. 2001). One major question is still unanswered: How was this type of enzyme acquired by algae (Horner et al. 2002)?

To gain further insight into the distribution of hydrogenases in cyanobacteria, we deduced their occurrence in cyanobacterial strains, covering a wide range of cyanobacteria, by Southern hybridization and PCR. We also investigated different heliobacterial strains for the presence of different hydrogenase genes and their enzyme activities, and we tested the expression of the hydrogenase genes found in the draft genome sequence of *Chloroflexus aurantiacus* (http://genome.jgi-psf.org/). The operons of the uptake and bidirectional hydrogenases are not clustered in any of the genomes of photosynthetic bacteria sequenced. Thus, simultaneous acquisition by lateral gene transfer (LGT) seems improbable. Investigation of their phylogenetic affiliation might be an indication of the evolutionary fate of photosynthetic bacteria.

Materials and Methods

Cultivation and Growth Experiments

The cyanobacterial strains *Synechocystis* sp. PCC 6803, *Nostoc punctiforme* PCC 73102, *Chamaesiphon* SAG 5.82, *Synechococcus* sp. Miami BG 043511, *Chroococcus turgidus*, *Tolypothrix distorta*, *Nostoc* sp., *Hapalosiphon hibernicus*, and *Cylindrospermum* sp. were grown in BG-11 medium at 28°C and 50 µE m^{-2} s^{-1} as described (Appel et al. 2000). *Gloeobacter violaceus* PCC 7421 was grown in the same medium but at 5 µE m^{-2} s^{-1} and 20°C. For the induction of nitrogen fixation, cells were inoculated in BG-11$_0$ (Rippka et al. 1979). *Aphanothece halophytica* and *Oscillatoria limnetica* were grown in Turks Islands Salt medium (Oren and Padan 1978) at 28°C and 50 µE m^{-2} s^{-1}. *Acaryochloris marina* was grown in artificial seawater (Jones et al. 1963) at 28°C and 5 µE m^{-2} s^{-1}. If no activity of the bidirectional hydrogenase was detectable under normal growth conditions, cells were induced by bubbling with nitrogen either in the dark or in the light for 24 h.

A. halophytica and *O. limnetica* were received from E. Padan (Hebrew University of Jerusalem, Jerusalem, Israel), *A. marina* was a gift from S. Miyachi (Marine Biotechnology Institute, Tokyo, Japan), *Synechococcus* sp. Miami BG 043511 was obtained from A. Mitsui (University of Miami, Miami, FL, USA), and *Synechocystis* sp. PCC 6803 from L. McIntosh (Michigan State University, East Lansing, MI, USA). All other cyanobacterial strains were obtained from the Sammlung of Algen (Göttingen, Germany).

Heliobacterial strains (*Heliobacillus mobilis* DSM 6151 and *Heliobacterium gestii* DSM 11169) were grown in medium 370 (http://www.dsmz.de; DSMZ, Braunschweig) at 35°C and 10 µE m^{-2} s^{-1}. *Heliobacterium undosum* DSM 13378 was grown in the same medium at 28°C and 10 µE m^{-2} s^{-1}. *Chloroflexus aurantiacus* J-10FL was grown in medium 87 (http://www.dsmz.de; DSMZ, Braunschweig) at 46°C and 10 µE m^{-2} s^{-1}.

Cloning Procedures and Sequencing

The degenerated primers (FEN1a, FEN1b, and FEC) used for the amplification of the genes of a FeFe-hydrogenase are listed in Table 1. DNA cloning and PCR amplification were performed by standard procedures (Sambrook and Russell 2001). For sequencing, the Cycle Reader Auto DNA Sequencing kit (MBI Fermentas, St. Leon-Rot, Germany) was used and the reactions were read in a Li-Cor DNA Sequencer Long Readir 4200 (MWG, Ebersberg, Germany).

Isolation of DNA and Southern Blot Hybridization

For isolation of genomic DNA cells were resuspended in 100 µl TE buffer (10 mM Tris, 1 mM Na$_2$-EDTA, pH 8.0). The suspension was supplemented with an equal volume of glass beads (0.5 mm diameter), 2 µl of a 10% sodium dodecyl sulphate (SDS) solution, and 100 µl of phenol-chloroform-isoamylalcohol (25:24:1). The mixture was vortexed three times for 10 sec and then centrifuged at 10,000g for 10 min. The supernatant was extracted once with phenol-chloroform-isoamylalcohol and twice with chloroform-isoamylalcohol (24:1) and centrifuged at 10,000g for 2 min, respectively. The DNA was precipitated with 0.1 vol sodium acetate (3 M, pH 6.5) and 2.5× volume of 100% ethanol for 2 h at –20°C. After centrifugation (15,700g at –9°C for 15 min), the pellet was washed with 70% ethanol. Then the pellet was dried in a vacuum centrifuge and resuspended in 20 µl TE buffer overnight at 4°C. Southern hybridization was carried out with the Dig system (Roche Diagnostics GmbH, Mannheim, Germany) under the most stringent conditions as described by the manufacturer. The primers

Table 1. Primers for the amplification of Dig-labeled probes for Southern hybridization or RT-PCR and the degenerated primer used for the amplification of FeFe-hydrogenase specific sequences

Name	Sequence	Position in genome or ORF
HoxH1	TTCTGCCTTAGCTCGACAAT	1672407–1672426
HoxH2	GTGCAAATCCCTCCAGCCGG	1673230–1673249
HupL1	AACGCCTGGACACACGCTGA	90–110
HupL2	AGCAGTCCGAAAACGCGCCA	1463–1483
cfl_hupLtag1	AGAGGCTGTGCGGCTTATTCGAGAGGGTCA	136–157
cfl_hupLtag2	AGAGGCTGTGCGGCTTATTCG	
cfl_hupLgp	CCTCAATGTTTCGCGGCTTCG	470–489
cfl_hoxHtag1	AGAGGCTGTGCGAAACCGAGCATCAGATCA	51–78
cfl_hoxHtag2	AGAGGCTGTGCGAAACCGAG	
cfl_hoxHgp	TCCGGTGACACGCATCGAG	381–400
FEN1a	AGYTGYTGYCCYGSYTGG	
FEN1b	TCYTGYTGYCCYGSYTGG	
FEC	CCACCGGGACAGGCCAT	

HoxH1 and HoxH2 were used to amplify a Dig-labeled probe against the gene of the large subunit of the bidirectional hydrogenase *hoxH*, HupL1 and HupL2 were used for a probe against the gene of the large subunit of the uptake hydrogenase *hupL*, and FEN1b and FEC were used to amplify a probe against the gene of the FeFe-hydrogenase of *Heliobacillus mobilis* (Table 1).

RNA Isolation and RT-PCR

Total RNA was isolated as described by Gutekunst et al. (2005). cDNA was synthesized with a tagged primer (Table 1) that annealed with 15–19 nucleotides specific to the corresponding gene and carried a tag of 13 or 14 nucleotides at its 5′-end as a target for subsequent PCR according to the method of Cobley et al. (2002). Five hundred nanograms of RNA was incubated with 5 pmol of primer and 2 mM dNTPs at 65°C for 5 min. The reaction was chilled on ice. Subsequently, 5× buffer (Invitrogen, Karlsruhe, Germany) and 40 U RNase inhibitor were added. After incubating the mixture at 42°C for 2 min, 200 U Superscript II (Invitrogen) was added. In the control reaction, the reverse transcriptase was replaced by water. Reverse transcription (RT) was performed at 42°C for 50 min. The reaction was terminated by incubation at 70°C for 15 min. Then the reaction was immediately chilled on ice and treated with 3 U RNaseH (MBI Fermentas, St. Leon-Rot, Germany). An aliquot of 2 μl of the RT reaction was used to amplify the cDNA with a gene-specific and an adapter-specific primer (see Table 1).

Hydrogen Measurements

Hydrogenase activity was measured as previously described (Appel et al. 2000), but with a Clark-type electrode from Hansatech (DW 1 Liquid Clark electrode; Hansatech Institute, Norfolk, UK). The electrode was connected to a lab-made control box with a voltage of –600 mV. The activity of the uptake hydrogenase was measured in the same setup during illumination with 800 μE m^{-2} s^{-1}.

Phylogenetic Analysis

Sequence alignments were made with ClustalW (Thompson et al. 1994) or T-Coffee (Notredame et al. 2000). Irrespective of slight differences in the alignments between these two methods, the same phylogenetic trees were obtained.

After manual optimization and removal of gaps from the alignments, parsimony, maximum likelihood, and distances were calculated with the 3.63 release of the PHYLIP package (Felsenstein 2005), using the Jones-Taylor-Thornton matrix and the algorithm of Fitch and Margoliash (1967). Maximum parsimony and distances were calculated for 1000 bootstraps and maximum likelihood for 200. Alignments are available as supplementary information.

Results

Hydrogenases in Cyanobacterial Strains

All the cyanobacterial strains were grown under nitrogen-replete conditions. The nitrogen-fixing strains were also grown in BG-11$_0$ to induce nitrogen fixation and the uptake hydrogenase. Some heterocystous strains are known to perform a genetic rearrangement in *hupL* under these conditions (Carrasco et al. 1995). The activity of the bidirectional hydrogenase was determined in the presence of methylviologen and dithionite in cells grown under normal conditions or after bubbling with nitrogen for 24 h. The activity of the uptake hydrogenase was measured as hydrogen uptake in the light. DNA was isolated from the same cells and subjected to Southern hybridization and PCR. The results are given in Table 2.

The unicellular strains tested (*Chamaesiphon* SAG 5.82, *Synechococcus* sp. BG 043511, *Aphanothece halophytica*, *Chroococcus turgidus*, and *Acaryochloris marina*) all contained the genes for the bidirectional hydrogenase. In all of these strains except *Chamaesiphon*, we could also detect the activity of the enzyme. In the latter, even bubbling with nitrogen for 24 h in the dark or in the light did not elicit any activity.

All of the heterocystous nitrogen-fixing strains (*Tolypothrix distorta, Nostoc* sp., *Hapalosiphon hibernicus*, and *Cylindrospermum* sp.) were found to have

Table 2. Distribution of uptake and bidirectional hydrogenases in the cyanobacterial strains tested by Southern hybridization and activity measurements

| | Uptake hydrogenase | | Bidirectional hydrogenase | | FeFe-hydrogenase |
	Southern hybridization	Activity	Southern hybridization	Activity	Southern hybridization
Control strains					
Synechocystis sp. PCC 6803	–	–	+	+	–
Nostoc punctiforme PCC 73102	+	+	–	–	–
Gloeobacter violaceus PCC 7421	–	–	–	–	–
Tested strains					
Chamaesiphon SAG 5.82	–	–	+	–	–
Synechococcus sp. BG 043511	–	–	+	+	–
Aphanothece halophytica	–	–	+	+	–
Chroococcus turgidus	–	–	+	+	–
Oscillatoria limnetica	–	–	+	+	–
Tolypothrix distorta	+	+	–	–	–
Nostoc sp.	+	+	+	+	–
Hapalosiphon hibernicus	+	+	–	–	–
Cylindrospermum sp.	+	+	–	–	–
Prochlorothrix hollandica	–	n.d.	+	n.d.	–
Acaryochloris marina	–	–	+	+	–

Note. The occurrence of the FeFe-hydrogenase was tested by Southern hybridization and PCR using degenerated primers. Completely sequenced cyanobacterial strains were used as control strains. n.d., not determined.

the genes for the uptake hydrogenase and to express its activity under nitrogen depletion. This behavior resembles the control strain *Nostoc punctiforme*, which was shown to have only the uptake hydrogenase (Tamagnini et al. 1997).

Surprisingly, the nitrogen-fixing unicellular strain *Synechococcus* sp. BG 043511 was found to have only the bidirectional enzyme and not the uptake hydrogenase.

FeFe-hydrogenases are known to be mainly restricted to anaerobic habitats. Therefore, cyanobacterial strains (*A. halophytica* and *Oscillatoria limnetica*) which are known to thrive under anoxygenic conditions were also included in the analysis. These strains were shown to be able to perform anoxygenic photosynthesis and to produce hydrogen under anoxic conditions (Belkin and Padan 1978; Padan 1979). But neither Southern hybridizations nor the PCR experiments with a set of different degenerated primers derived from highly conserved sequences could deduce the presence of a respective gene in any strain tested, although the same set of primers prooved to be effective when used with DNA isolated from algae and heliobacteria (see below). Thus, it seems unlikely that these cyanobacteria do have a FeFe-hydrogenase.

Heliobacterial Hydrogenases

Heliobacteria are the only gram-positive bacteria known to perform photosynthesis. They harbor a primitive homodimeric photosystem of the FeS type, which is similar to photosystem I of cyanobacteria (Liebl et al. 1993). Since some scenarios discuss heliobacteria as putative predecessors of cyanobacteria (most recently reviewed in Olsen and Blankenship 2004), we investigated the occurrence of hydrogenases in three heliobacterial strains (*Heliobacillus mobilis*, *Heliobacterium gestii*, and *Heliobacterium undosum*).

PCR products were amplified with primers specific for FeFe-hydrogenases. The product for *Hc. mobilis* was sequenced and turned out to be part of a FeFe-hydrogenase gene. The whole gene cluster including a FeFe-hydrogenase gene and a fusion of *nuoE* (*hoxE*) and *nuoF* (*hoxF*) was sequenced at Integrated Genomics (Chicago, IL, USA). The sequence was deposited in GenBank (AM162647). A schematic representation of the gene cluster of *Hc. mobilis* is shown in Fig. 1 in comparison to similar clusters in other bacteria.

Southern hybridization of the other two heliobacteria using a Dig-labeled probe against the gene of *Hc. mobilis* revealed the presence of strong signals in both strains (Fig. 2), confirming the occurrence of the same hydrogenase.

In the same strains, hydrogenase activity was measured in the presence of methylviologen/dithionite and different concentrations of carbon monoxide. Fifty percent inhibition was determined at 0.9 µM for *Hc. mobilis*, 0.5 µM for *H. undosum*, and 2.5 µM for *H. gestii*. This is close to the values for hydrogenases I and II of *Clostridium pasteurianum*, 0.4 and 2.8 µM, respectively, and are well below the 35 µM needed for NiFe-hydrogenases (Adams 1990). Therefore, all these heliobacterial strains do not express an active NiFe-hydrogenase under the tested conditions.

A phylogenetic analysis of the H-cluster domains of the FeFe-hydrogenases of a number of bacteria,
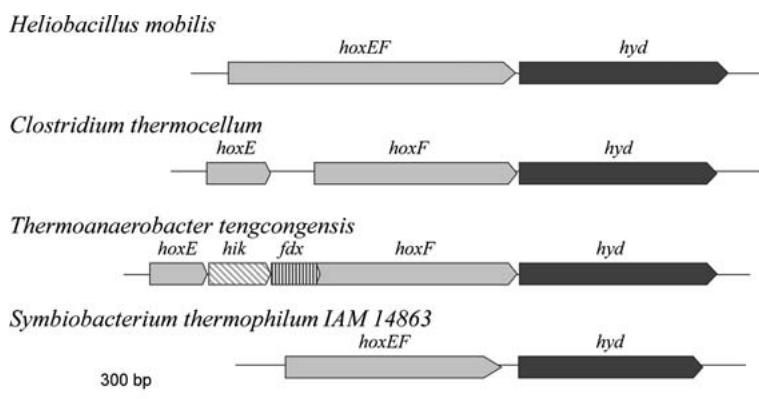
Fig. 1. Schematic drawing of FeFe-hydrogenase gene cluster found in *Heliobacillus mobilis* in comparison to other gram-positive bacteria that include homologues to *hoxE* (*nuoE*) and hoxF (*nuoF*) or fusions of both genes.
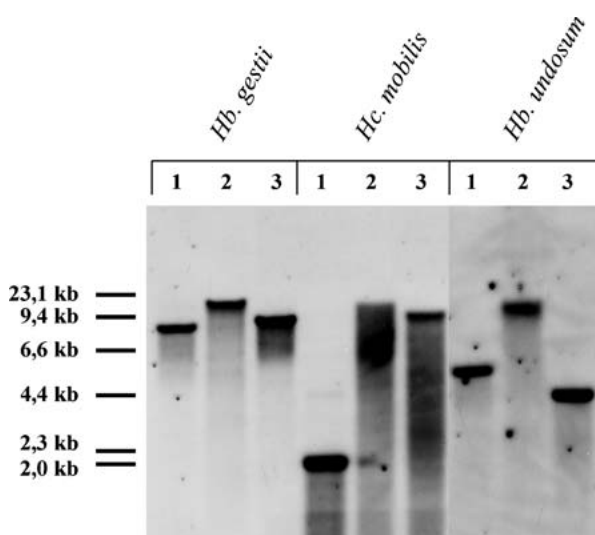

Fig. 2. Southern hybridization of genomic DNA of *H. gestii*, *Hc. mobilis*, and *H. undosum* with a probe specific for the *hydA* gene of *Hc. mobilis*. One hundred nanograms of DNA was digested with either *Bam*HI (1), *Hin*dIII (2), or *Eco*RI (3).

the algal sequences, all available eukaryotes, the diatom *Thalassiosira pseudonana* (Armbrust et al. 2004), and *Heliobacillus mobilis* did not reveal a close ancestry of the algae and *Hc. mobilis* (data not shown). Overall this tree was poorly resolved and eukaryotic sequences were intermixed with bacterial sequences excluding any direct conclusions to the origin of eukaryotic hydrogenases as already noted by Horner et al. (2000). Only in the case of green algae, all sequences clustered with a high bootstrap support, which is already evident from their structure, which is restricted to the active-site FeS cluster, the so-called H cluster (Horner et al. 2002).

Hydrogenases in Chloroflexus aurantiacus

The main representative of the photosynthetic green nonsulfur bacteria is *Chloroflexus aurantiacus*. It contains a Q-type photosystem similar to photosystem II of cyanobacteria. Investigation of the draft genome of *Chloroflexus aurantiacus* (http://genome.jgi-psf.org/)

revealed the presence of *hup* and *hox* genes. No gene for a FeFe-hydrogenase could be found by similarity searches. PCR experiments and Southern hybridizations did not detect a FeFe-hydrogenase-specific sequence either.

Since *C. aurantiacus* is not known to fix nitrogen, we performed RT-PCR experiments to deduce if both hydrogenase genes are transcriptionally expressed. As depicted in Fig. 3, transcripts for *hupL* as well as *hoxH* were detected. The uptake hydrogenase is actively expressed since it was reported that *Chloroflexus* is capable of growing photolithoautotrophically on hydrogen (Pierson and Olson 1987). We found an average hydrogenase activity of 60 nmol H_2/min/ OD_{750} in the presence of methylviologen and dithionite in all cultures tested, which corresponds to the expression of the bidirectional enzyme.

Phylogenetic Origin of Cyanobacterial Hydrogenases

Phylogenetic analysis of hydrogenases shows that the *hup* and *hox* hydrogenases form two well-defined clusters (Vignais et al. 2001). Therefore, we investigated the phylogeny of these hydrogenases separately. All three methods used (distance, parsimony and maximum likelihood) produced the same overall tree irrespective of the type of hydrogenase. In the case of the uptake hydrogenases (Fig. 4), the green sulfur bacteria and the photosynthetic proteobacteria form two clusters on a well-defined branch separated from cyanobacteria and *Chloroflexus* that is supported by a high bootstrap value. *Chloroflexus* forms the deepest branch in the cyanobacterial cluster. The phylogenetic resolution of the cyanobacteria is poor. Only the heterocystous cyanobacteria cluster with a high bootstrap value.

Because gram-positive bacteria have been suggested to represent a more ancient development and to be the ancestors of gram-negative bacteria (Gupta 1998), the uptake hydrogenases of two *Frankia* strains and *Streptomyces avermitilis* were included in the analysis and were consistently placed close to the root of the cluster of hydrogenases.
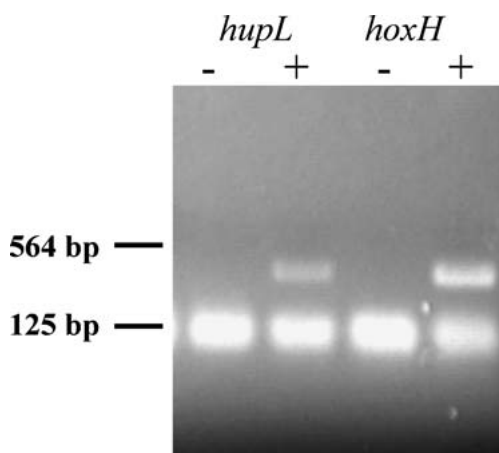
Fig. 3. Gel electrophoresis of RT-PCR samples with primers specific for *hupL* and *hoxH* of *Chloroflexus aurantiacus*. A negative control without reverse transcriptase (–) and a sample with reverse transcriptase (+) were applied. The sizes of the molecular weight markers are given on the left.

The root was defined by subunit D (NdhH) of the bacterial complex I. The latter subunits were used as an outgroup since they are known to be of distant similarity to the large hydrogenase subunits and thought to be the result of divergent evolution (Albracht and Hedderich 2000; Dupuis et al. 2001).

The tree calculated for the bidirectional hydrogenase is shown in Fig. 5. In this case, the HyhL subunits of archaebacteria and green sulfur bacteria were included in the analysis because they are also able to reduce $NAD(P)^+$ and form a tetrameric complex which is similar to the soluble hydrogenases of the Knallgas bacteria or the pentameric bidirectional hydrogenases of cyanobacteria.

Thiocapsa roseopersicina is the only photosynthetic proteobacterium known to contain a bidirectional hydrogenase. To analyze its phylogenetic affiliation more properly, a selection of other sequences from nonphotosynthetic proteobacteria was included in the analysis. The overall topology of the tree did not change, even when all currently available proteobacterial HoxH sequences were included.

Again, the sequences of the subunit D of the complex I were used as an outgroup.

All the HyhL sequences form a well-defined cluster where the archaebacterial sequences are clearly separated from the green sulfur bacteria. Concerning the HoxH sequences, the Knallgas bacteria form a separate cluster from all other bacteria, which is also supported by three different insertions in these bacteria (see Supplementary Information). In this tree *Chloroflexus* is again the most deeply branching in the cluster of cyanobacterial sequences, whereas the only photosynthetic proteobacterium, *T. roseopersicina* (Kovacs et al. 2005), is in a different cluster with other proteobacteria. The δ-proteobacteria *Geobacter metallireducens* and *G. sulfurreducens* seem to branch off the cyanobacterial and *Chloroflexus* cluster. However, the lower bootstrap values indicate that this affiliation is of lower statistical significance.

The heterocystous strains *A. variabilis* and *Nostoc* sp. PCC 7120 are clearly separated from the other cyanobacteria by an insertion of nine amino acids between residue P285 and residue D286 of the *Synechocystis* sp. PCC 6803 sequence (see Supplementary Information).

Discussion

Occurrence of Hydrogenases in Cyanobacteria

Our results confirm and expand previous findings on the distribution of cyanobacterial hydrogenases. The bidirectional enzyme was detectable in non-nitrogen-fixing and nitrogen-fixing strains (Table 2). We could not detect an uptake hydrogenase in *Synechococcus* sp. BG 043511, although it is a nitrogen-fixing unicellular cyanobacterium. This finding is in accordance with observations of recurrent periods of hydrogen production in the dark elicited by the nitrogenase in this *Synechococcus* strain (Suda et al. 1992; Mitsui and Suda 1995). It therefore represents a natural uptake hydrogenase-deficient diazotrophic cyanobacterium. Since this type of hydrogenase is expressed to recycle the hydrogen produced by nitrogenase and to omit the concomitant energy loss, it should be a selective disadvantage under nitrogen-fixing conditions. Although there are a lot of diazotrophic cyanobacteria expressing an uptake hydrogenase, our findings suggest that the selection pressure for the presence of this enzyme is not valid under all environmental conditions. This is supported by competition studies of wild-type cells and uptake hydrogenase-free mutants of *Anabaena* sp. PCC 7120 that found no clear advantage for the wild-type cells (Lindblad et al. 2002).

All non-nitrogen-fixing strains and *Synechococcus* sp. BG 043511 showed a strong hybridization with a probe against *hoxH*, and all of them except *Chamaesiphon* also expressed an active bidirectional enzyme. Compared to the complete genomes of unicellular strains available and to a study on the distribution of hydrogenases in filamentous cyanobacteria (Tamagnini et al. 2000), this is a high proportion. From the available sequence information it is obvious that especially marine cyanobacteria isolated from the open ocean (*Prochlorococcus* MED4, *Prochlorococcus* MIT9313, *Prochlorococcus* SS120, *Synechococcus* WH8102, *Synechococcus* sp. CC9311, *Crocosphaera watsonii*, and *Trichodesmium erythraeum* IMS101) have no need for this enzyme. This is a good indication that the bidirectional hydrogenase is especially needed when the cells have to face microaerobic or anaerobic conditions, which are highly unlikely in the open ocean.
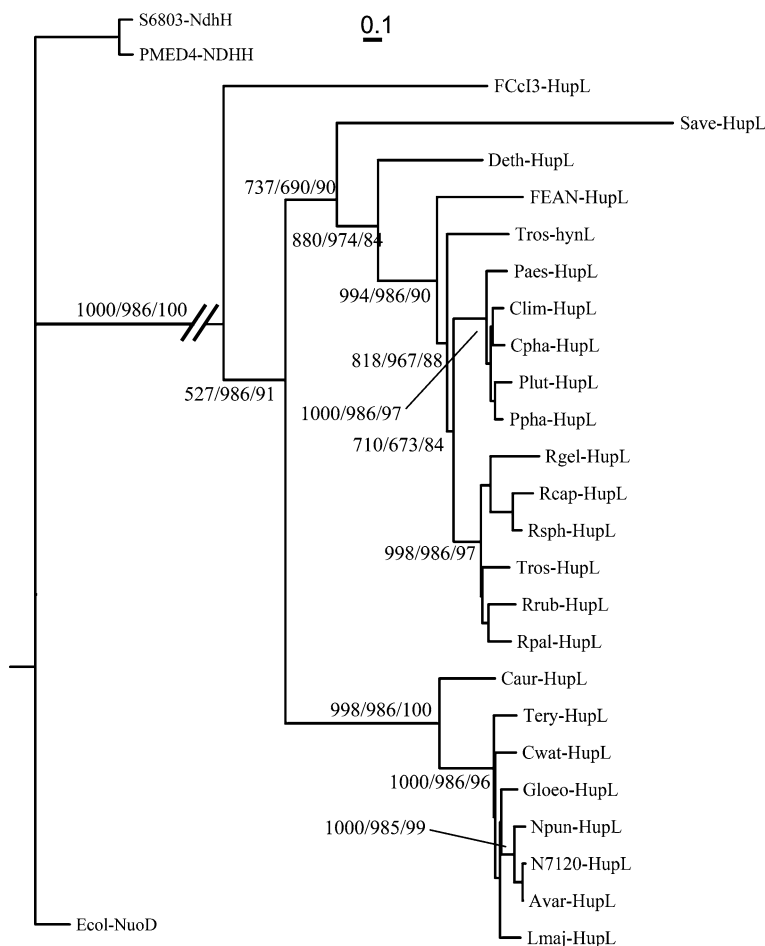
Fig. 4. Phylogenetic tree of the uptake hydrogenases (HupL) of the five major groups of photosynthetic bacteria. At the most important nodes the bootstrap values are given for the distance method, maximum parsimony, and maximum likelihood. The abbreviations and the accession numbers of the sequences used are as follows: Avar, *Anabaena variabilis* (HupL, ZP_00159099; HoxH, CAA55878); Amax, *Arthrospira maxima* (AAQ63961); Apla, *Arthrospira platensis* (the different strains FACHB341 [AAQ63964], FACHBOUQDS6 [AAQ63959], FACHB439 [AAQ63960], and *platensis* [AAQ91344] correspond to HoxH1, HoxH2, HoxH3, and HoxH4); Cagg, *Chlorochromatium aggregatum* (ABB27521); Clim, *Chlorobium limicola* DSM 245 (HupL, ZP_00511757; HyhL, ZP_00511993); Caur, *Chloroflexus aurantiacus* J-10-fl (HupL, ZP_00767078; HoxH, ZP_00766172); Cnec, *Cupriavidus necator* (H16) (AAP85844); Cpha, *Chlorobium phaeobacteroides* DSM 266 (HupL, ZP_00527994; HyhL, ZP_00528294); Ctep, *Chlorobium tepidum* (NP_662771); Cwat, *Crocosphaera watsonii* (ZP_00174297); Deth, *Dehalococcoides ethenogenes* (AAW40581); Dpsy, *Desulfotalea psychrophila* (CAG36941); Ecol, *Escherichia coli* (NuoD, CAA48363); FCcI3, *Frankia* sp. CcI3 (YP_483571); FEAN, *Frankia* sp. EAN1pec (ZP_00571318); Galp, *Gloeocapsa alpicola* (AAO85440); Gloeo, *Gloeothece* sp. PCC 6909 (AAP04005); Gmet, *Geobacter metallireducens* (ZP_00299938); Gsul, *Geobacter sulfurreducens* (NP_953763); Lmaj, *Lyngbya majuscula* CCAP 1446/4 (HupL, AAO66476; HoxH, AAT07678); N7120, *Nostoc* sp. PCC 7120 (HupL, all0687; HoxH, alr0766); Npun, *Nostoc punctiforme* (ZP_00112356); Paes, *Prosthecochloris aestuarii* DSM 271 (HupL, ZP_00591696; HyhL, ZP_00590995); Pfur, *Pyrococcus furiosus* (HyhL1, NP_578623; HyhL2, NP_579061); Plut, *Pelodictyon luteum* DSM 273 (YP_375349); PMED4, *Prochlorococcus* MED4 (NdhH, PMM0172); Phol, *Prochlorothrix hollandica* (AAB53705); Ppha, *Pelodictyon phaeoclathratiforme* BU-1 (HupL, ZP_00589592; HyhL, ZP_00588240); Rcap, *Rhodobacter capsulatus* (CAA31870); Rgel, *Rubrivivax gelatinosus* (ZP_00243708); Rmet, *Ralstonia metallidurans* (CH34) (ZP_00271447); Ropa, *Rhodococcus opacus* (AAB57892); Rpal, *Rhodopseudomonas palustris* CGA009 (NP_946314); Rsph, *Rhodobacter sphaeroides* 2.4.1 (YP_353568); Rrub, *Rhodospirillum rubrum* (YP_426250); S6301, *Synechococcus* sp. PCC 6301 (CAA66383); S6803, *Synechocystis* sp. PCC 6803 (NdhH, slr0261; HoxH, sll1226); S7002, *Synechococcus* sp. PCC 7002 (AAN03569); S7942, *Synechococcus* sp. PCC 7942 (ZP_00165515); Save, *Streptomyces avermitilis* (NP_828543); Spla, *Spirulina platensis* (AAQ63963); Ssub, *Spirulina subsulsa* (AAQ63962); Susi, *Solibacter usitatus* (ZP_00525369); Tery, *Trichodesmium erythraeum* IMS 101 (ZP_00328546); Tros, *Thiocapsa roseopersicina* (HupL, AAA27410; HynL, AAC38282; HoxH, NP_579061).

Possible Ancestry of Algal Hydrogenases

A number of cyanobacterial strains are known to be able to perform anoxygenic photosynthesis (Padan 1979). Probably an even higher number of strains is able to switch to different kinds of fermentative metabolism including hydrogen production via the pyruvate:ferredoxin oxidoreductase and hydrogenase (Stal and Moezelaar 1997). This is indicative of the presence of FeFe-hydrogenases, which are known to use ferredoxin as electron donors (Adams 1990). Despite a major effort to detect the respective genes by Southern hybridization and PCR, we did not find any in the cyanobacteria investigated. Furthermore,
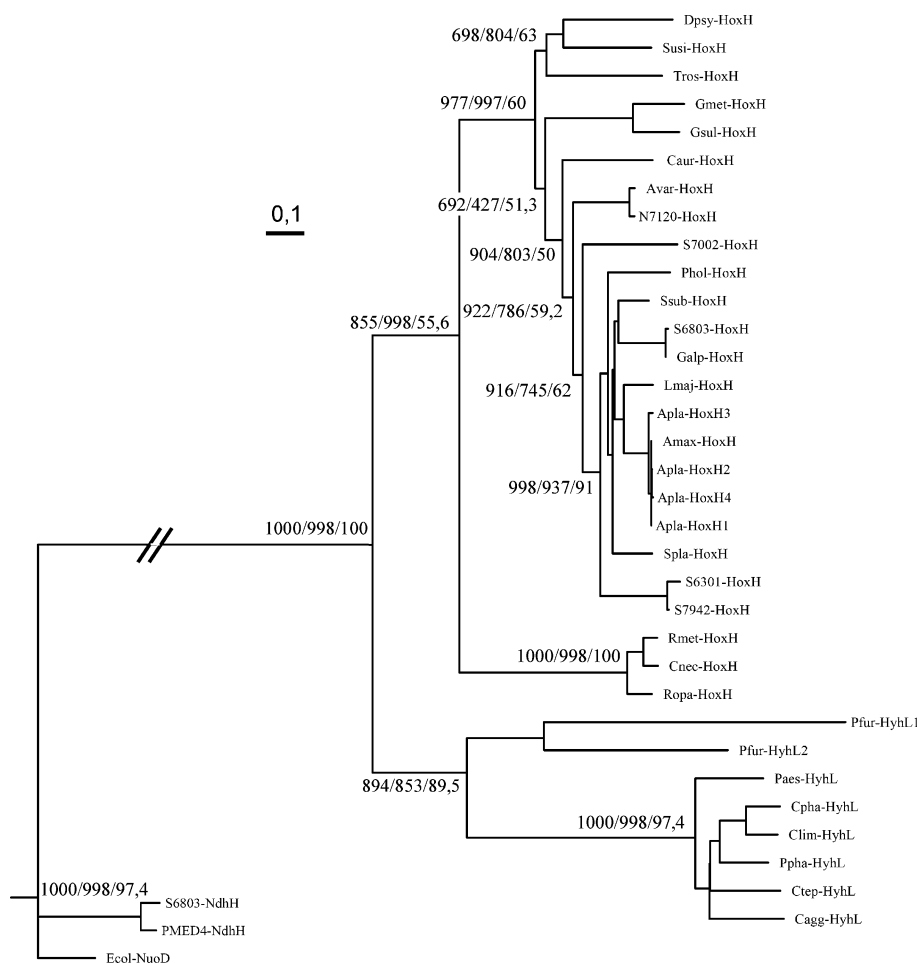
Fig. 5. Phylogenetic tree of the cytoplasmatic multimeric reversible hydrogenases (HoxH, HyhL) classified as group 3, according to Vignais et al. (2001), of the five major groups of photosynthetic bacteria. This enzyme is called bidirectional hydrogenase in cyanobacteria. At the most important nodes the bootstrap values are given for the distance method, maximum parsimony, and maximum likelihood. The abbreviations and the accession numbers of the sequences used are given in the legend to Fig. 4.

none of the completely sequenced genomes have FeFe-hydrogenase genes.

Nevertheless, using the same methods we did detect this type of hydrogenase in three heliobacterial strains (Fig. 2). Heliobacteria are the only group of gram-positive bacteria known to perform photosynthesis. A number of investigations suggest that cyanobacteria are related to gram-positives. This is especially found for transcriptional regulation (Figge et al. 2000). We could show that transcription of the operon of the bidirectional hydrogenase of *Synechocystis* sp. PCC 6803 is activated by LexA (Gutekunst et al. 2005). Since Mazon et al. (2004) showed that the LexA binding sites of gram-positive bacteria and cyanobacteria are similar and a distant ancestry of the uptake hydrogenase to gram-positive bacteria is suggested by our phylogenetic analysis (Fig. 4), a special relationship with respect to the hydrogenase genes seems to be valid. However, our phylogenetic analysis of FeFe-hydrogenases could not deduce a heliobacterial ancestry, neither for the FeFe-hydrogenases of green algae nor for the diatom *Thalassiosira pseudonana*. This renders the possibility highly unlikely that a heliobacterial enzyme passed on to a putative cyanobacterium via vertical inheritance or LGT could have reached an algal cell via endosymbiosis.

Our results do not exclude that there is a cyanobacterium with an FeFe-hydrogenase somewhere out there. Nevertheless, they suggest that the FeFe-hydrogenase of algae has not been acquired together with the cyanobacterial endosymbiont, although nowadays the hydrogenase is localized to the plastid.

Hydrogenases are of a pivotal role in the hydrogen and syntrophy hypothesis of eukaryogenesis and the development of mitochondrial endosymbiosis (Martin and Müller 1998; Moreira and Lopez-Garcia 1998; Anderson and Kurland 1999). In both scenarios hydrogen was the glue for a symbiosis between a hydrogen-consuming archeon and a hydrogen-producing proteobacterium that finally became a mitochondriated eukaryote. Therefore, the endosymbiotic uptake of the mitochondrial predecessor also might have inherited a FeFe-hydrogenase to the algal cell. But since the phylogenetic resolution of the FeFe-hydrogenases is low, the ancestry of the plastidial enzyme has to await further investigation and might be even of a third, still unknown descent.

Origin of Cyanobacterial Hydrogenases

The large number of available prokaryotic genomes increasingly showed the importance of LGT. It is

now discussed as a major driving force for the invention and evolution of prokaryotic genomes and is thought also to include main physiological traits such as photosynthesis, respiration, nitrogen fixation, and sulfur metabolism (Ochman et al. 2000; Boucher et al. 2003). Hydrogenases are no exception, as is obvious by the transfer of large operons of archaebacterial type hydrogenases to bacteria (Calteau et al. 2005). The close relation between the archaebacterial and the green sulfur bacterial sulfhydrogenase (HyhL) found in this study also suggests that there has been interspecies gene transfer (Fig. 5).

Phylogenetic analysis of whole genomes of photosynthetic bacteria of all five classes (cyanobacteria, heliobacteria, green nonsulfur bacteria, green sulfur bacteria, and purple bacteria) did not show a clear relationship of the different groups. Thus, photosynthetic ancestry is hidden by a large amount of LGT (Raymond et al. 2002, 2003).

The phylogenetic trees deduced in this work (Figs. 4 and 5) reveal that *Chloroflexus* is the closest ancestor of cyanobacteria since it was found to be the most deeply branching in the cyanobacterial clusters in case of both hydrogenases. In all cyanobacterial genomes sequenced to date and in the genome of *Chloroflexus*, the two hydrogenase operons are widely separated on the chromosome, rendering simultaneous gene transfer unlikely. Investigations on the occurrence of signature sequences, so-called indels, restricted to housekeeping genes which are thought to be not or only rarely amenable to horizontal transfer, suggest a certain branching order of the bacterial groups. According to these analyses, the photosynthetic bacteria appeared in the order heliobacteria, green nonsulfur bacteria (*Chloroflexus*), cyanobacteria, green sulfur bacteria, and proteobacteria (purple bacteria) (Gupta et al. 1999; Gupta 2003).

Taking together the latter analysis and the results presented in this work, *Chloroflexus* has to be placed at the root of cyanobacteria. It would be most parsimonious to suggest that the current distribution of the two hydrogenases in the cyanobacterial strains reflects differential loss of the genes of their last common ancestor. They were either kept in the genome or lost in the different strains according to their ecological needs or constraints. Especially the marine cyanobacteria do not to require the bidirectional hydrogenase since they rarely encounter the anaerobic conditions which activate this enzyme (Appel et al. 2000; Cournac et al. 2002, 2004). In case of the *Prochlorococcus* strains and *Synechococcus* WH8102, the low nitrogen supply in the oligotrophic oceanic regions inhabited by these cyanobacteria exerts considerable pressure to reduce their genomes since DNA synthesis consumes a large amount of nitrogen. Therefore, they lost many genes not absolutely necessary for growth (Partensky et al. 1999), which is also true for the hydrogenases since they can be deleted from the genome without affecting the growth of the mutant (Appel et al. 2000; Lindblad et al. 2002).

The bidirectional hydrogenase was shown to work in transition from anaerobiosis in the dark to aerobic conditions in the light (Appel et al. 2000; Cournac et al. 2002, 2004). It possibly plays a role to avoid an overload of low potential electrons in the photosynthetic electron transport. It might be an interesting question for the future whether this enzyme could have been a prerequisite for the invention of an electron transport including two different photosystems that might not have worked at the same pace or without tight control by the dark reaction when oxygenic photosynthesis was in its infancy.

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