Comprehensive Summaries of Uppsala Dissertations
from the Faculty of Science and Technology 888

Cyanobacterial Hydrogen Metabolism – Transcriptional Regulation of the Hydrogenases in Filamentous Strains

BY

RIKARD AXELSSON

ACTA UNIVERSITATIS UPSALIENSIS
UPPSALA 2003

Dissertation presented at Uppsala University to be publicly examined in Lecture hall, room 3041, Department of Physiological Botany, Uppsala, Friday, October 10, 2003 at 10:00 for the degree of Doctor of Philosophy. The examination will be conducted in English.

Abstract

Axelsson, R. 2003. Cyanobacterial Hydrogen Metabolism – Transcriptional Regulation of the Hydrogenases in Filamentous Strains. Acta Universitatis Upsaliensis. *Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology* 888. 49 pp. Uppsala. ISBN 91-554-5743-6

Cyanobacteria are a heterogeneous group of phototrophic microorganisms. Many cyanobacteria have the capacity to fix atmospheric nitrogen. During the process of nitrogen fixation, molecular hydrogen is produced. Three enzymes are directly involved in hydrogen metabolism in cyanobacteria. A nitrogenase, evolving hydrogen during nitrogen-fixation, an uptake hydrogenase, recycling the hydrogen produced by nitrogenase, and a bidirectional hydrogenase that has the capacity to both take up and produce hydrogen. The main objective in this thesis was to examine the transcriptional regulation of both the uptake and the bidirectional hydrogenase in filamentous cyanobacteria.

The transcriptional regulation of the uptake hydrogenase was demonstrated to be influenced by external conditions in *Nostoc muscorum* and *Nostoc punctiforme*. Nickel, molecular hydrogen, and anaerobic conditions all induced the relative amount of uptake hydrogenase transcript. In addition, a transcript could be detected in nitrogen-fixing, but not in non-nitrogen fixing conditions.

The transcriptional regulation of the bidirectional hydrogenase in *N. muscorum* and *Anabaena* PCC 7120 was also examined. The relative amount of transcript from the bidirectional hydrogenase in both strains was demonstrated to increase during anaerobic conditions. Moreover, experiments using *N. muscorum* demonstrated that addition of nickel also increase the amount of transcript. However, no change in the relative amount of transcript from the bidirectional hydrogenase could be observed by additional hydrogen or during a shift from non-nitrogen fixing to nitrogen fixing conditions.

The genes responsible for maturation of the hydrogenase were identified, cloned and sequenced in *N. punctiforme*. The transcription of the genes was examined and all genes were located on a single transcript. Like the uptake hydrogenase, a transcript could be detected under nitrogen-fixing but not under non-nitrogen fixing conditions.

Initial studies, using microarrays, were used to analyse and compare the transcription of a large set of *Anabaena* PCC 7120 genes under non-nitrogen and nitrogen-fixing conditions. Both up- and down-regulated genes could be identified.

This thesis advances the knowledge about the transcriptional regulation of the hydrogenases in filamentous cyanobacteria and can be used as a platform for further experiments aiming at a modified hydrogen metabolism.

Rikard Axelsson, Department of Evolutionary Biology, Physiological Botany, Villav. 6, Uppsala University, SE-75236 Uppsala, Sweden

ISSN 1104-232X
ISBN 91-554-5743-6
urn:nbn:se:uu:diva-3590 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-3590)

Printed in Sweden by Eklundshofs Grafiska, Uppsala 2003

This thesis is based on the following six papers, which will be referred to in the text by their respective Roman numerals.

I Axelsson R, Oxelfelt F, and Lindblad P. 1999. Transcriptional regulation of *Nostoc* uptake hydrogenase. FEMS Microbiology Letters 170:77-81.

II Axelsson R, and Lindblad P. 2002. Transcriptional regulation of *Nostoc* hydrogenases: Effects of oxygen, hydrogen, and nickel. Applied and Environmental Microbiology 61:444-447.

III Hansel A, Axelsson R, Lindberg P, Troshina O, Wünschiers R, and Lindblad P. 2001. Cloning and characterization of a *hyp* gene cluster in the filamentous cyanobacterium *Nostoc* sp. strain PCC 73102. FEMS Microbiology Letters 201:59-64.

IV Axelsson R, and Lindblad P. 2003. The genes encoding the bidirectional hydrogenase in *Anabaena* sp. strain PCC 7120 are transcribed as two polycistronic operons. Manuscript.

V Axelsson R, Lindblad P, and Wünschiers R. 2003. Expression analysis of genes in *Anabaena* sp. strain PCC 7120 during nitrogen-fixing and non-nitrogen fixing conditions. Manuscript.

VI Tamagnini P, Axelsson R, Lindberg P, Oxelfelt F, Wünschiers R, and Lindblad P. 2002. Hydrogenases and hydrogen metabolism of cyanobacteria. Microbiology and Molecular Biology Reviews 66:1-20.

CONTENTS

INTRODUCTION

Hydrogenases and hydrogen metabolism

Hydrogenases are enzymes that catalyse the following reactions:

$$2e^- + 2H^+ \leftrightarrow H_2$$

Generally, hydrogenases can catalyse both directions of the reaction *in vitro*. *In vivo*, however, the enzymes usually catalyse either uptake or evolution of molecular hydrogen (H_2). Based on the metal composition of the active site, hydrogenases are divided into three distinct classes: nickel-iron [NiFe]-hydrogenases, iron [Fe]-hydrogenases, and metal free hydrogenases. Each class is characterised by a distinct functional core (Vignais et al 2001).

[NiFe]-hydrogenases may be divided into four subclasses, first suggested by Wu and Mandrand (1993), and recently modified by Vignais et al (2001). A majority of the known hydrogenases are [NiFe]-hydrogenases, distributed among archea and bacteria (Vignais et al 2001), and maybe one example in a green alga (Zinn et al 1994, Wünschiers et al 2001). The number of subunits in [NiFe]-hydrogenases varies from two to at least six (Vignais et al 2001). The first published crystal structure of a [NiFe]-hydrogenase was that of the heterodimeric hydrogenase of *Desulfovibrio gigas* (Volbeda et al 1995).

[Fe]-hydrogenases are restricted to bacteria and eukaryotes. In contrast to [NiFe]-hydrogenases, which consist of at least two subunits, many [Fe]-hydrogenases are monomeric proteins. Dimeric, trimeric and tetrameric enzymes have also been described (Malki et al 1995, Verhagen et al 1999, Nicolet et al 1999, Vignais et al 2001). [Fe]-hydrogenases are more difficult to divide into distinct groups on the basis of sequence, structure or function (Vignais 2001). The first resolved crystal structure of an [Fe]-hydrogenase was that of *Clostridium pasteurianum* (Peters et al 1998).

Metal free hydrogenases have been described in a few methanogens. The knowledge about these hydrogenases is limited, and no crystal structure is available. The enzyme is a monomer encoded by a monocistronic gene (Thauer et al 1996). An organic cofactor seems to be bound to the enzyme and influence the catalytic capacity (Buurman et al 2000).

Hydrogenases have different functions in different organisms. In fermentative

bacteria, e.g. bacteria of the clostridial type, the function is to dispose the excess of reducing equivalents (Adams 1990). Other microorganisms use H_2 as an electron source, both aerobically and anaerobically (Bowien and Schlegel 1981, Thauer et al 1996). In N_2-fixing bacteria, e.g. rhizobia and cyanobacteria, a specific uptake hydrogenase is used to recycle the H_2 produced by nitrogenase during nitrogen fixation (Baginsky et al 2002, Wünschiers and Lindblad 2003). [Fe]-hydrogenases have also been found in organelles of eukaryotic organisms such as hydrogenosomes of protozoa and chloroplast of green algae and are active under anaerobic conditions (Horner et al 2000, Happe et al 2002).

Regulation of hydrogenases

The regulation of hydrogenases has been studied in many different microorganisms under different environmental conditions. Molecular hydrogen (H_2), has been shown to be important for the transcription of hydrogenases. In nitrogen-fixing *Bradyrhizobium japonicum* the expression of the hydrogenase genes requires the presence of at least 0.1% hydrogen for optimal expression (Kim et al 1991). In *Rhodobacter capsulatus*, the transcription of the hydrogenase genes is induced by the hydrogen produced by the nitrogenase during nitrogen fixation or by exogenously added hydrogen (Colbeau and Vignais 1992). The transcription of both the soluble (SH) and the membrane bound hydrogenases (MBH) in *Ralstonia eutropha* are also regulated by hydrogen. In all these three bacteria, multicomponent regulatory systems that mediate the sensing of hydrogen have been characterized. The regulatory system in *Ralstonia eutropha* consists of three protein components encoded by four genes (Lenz and Friedrich 1998); (1) a regulatory hydrogenase, RH, encoded by *hoxBC*, (2) a sensor kinase, encoded by *hoxJ*, (3) and a response regulator encoded by *hoxA* (Fig. 1). Similar multi-component systems have also been described in *Bradyrhizobium japonicum* (van Soom et al. 1993, 1997 and 1999, Black and Maier 1994) and *Rhodobacter capsulatus* (Elsen et al 1996, 1997, Dischert et al 1999). HoxB and HoxC are similar to the small and large subunit of [NiFe]-hydrogenases. HoxC contains motifs found in [NiFe]-hydrogenases for coordination of nickel and iron (Kleihues et al 2001). HoxJ shows homology to histidine kinases of bacterial two component regulatory systems and HoxA is a member of the NtrC family of response

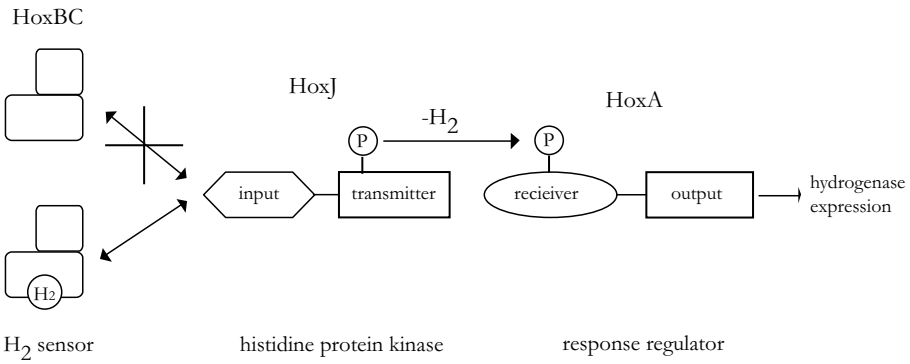

Fig. 1. Multicomponent regulatory system sensing molecular hydrogen in *Ralstonia eutropha*. Upon binding of H_2, HoxBC interacts with HoxJ and no phosporylation of HoxA occurs. The non-phosporylated HoxA induces transcription of the hydrogenases genes (adapted from Lenz and Friedrich 1998).

regulators (Eberz & Friedrich 1991) and has been shown to bind to promotor regions upstream of the membrane bound as well as the soluble hydrogenase operons in *Ralstonia eutropha* (Kleihues et al 2001). Molecular hydrogen is sensed by the RH-hydrogenase (HoxBC). Deletions of *hoxB* and *hoxC* prevent the other hydrogenases to be expressed. HoxBC interacts with HoxJ. Deletions of *hoxJ* results in a high level of hydrogenase expression suggesting that the autophosphorylation acts negatively on hydrogenase expression (Lenz and Friedrich 1998).

Nickel has also been demonstrated to influence the activity and transcription of [NiFe] hydrogenases. If nickel is absent in the active centre, the hydrogenase will not be catalytically active, and the hydrogenase genes are minimally transcribed as described in *Bradyrhizobium japonicum* and *Escherishia coli* (Kim et al 1991, Wu and Mandrand-Berthelot 1986). Nickel was also demonstrated to be essential for an active hydrogenase in *Frankia* (Sellstedt and Smith 1990).

Two major types of Ni^{2+} import systems have been described. The Nik system of *Escherichia coli* is a member of the ABC transporter family and provides Ni^{2+} for e.g. the biosynthesis of hydrogenases (Navarro et al 1993). The regulation of the *nik*-operon shows that it is induced under anaerobic conditions and is strictly dependent of the global regulatory protein Fnr, which is known to control several anaerobic respiratory and fermentative metabolic activities. In

addition, inactivation of the nickel transport results in a loss of hydrogenase activity in *E. coli* (Wu et al 1989). A second type of nickel transporter identified is HoxN, that is a transition metal permease found in *Ralstonia eutropha* (Eberz et al 1989, Eitinger and Friedrich 1991).

Oxygen (O_2) has also been reported to influence both activity and transcription of hydrogenases. In *Bradyrhizobium japonicum*, the transcription of the hydrogenase genes was optimal at 0.2 to 3% O_2. At lower or higher concentrations, the genes were minimally expressed (Kim et al 1991). In addition, the transcription of hydrogenase 1 and hydrogenase 2 in *E. coli* has been demonstrated to be induced under anaerobic conditions (Richard et al 1999).

Cyanobacteria

Cyanobacteria are microorganisms that can be found in very different environments such as fresh-, and seawater, in the soil, deserts, polar regions, hot water springs, and saline environments (Ward and Castenholz 2000, Vincent 2000, Wynn-Williams 2000). They also have the capacity to form symbiotic relations with other organisms (Adams 2000).

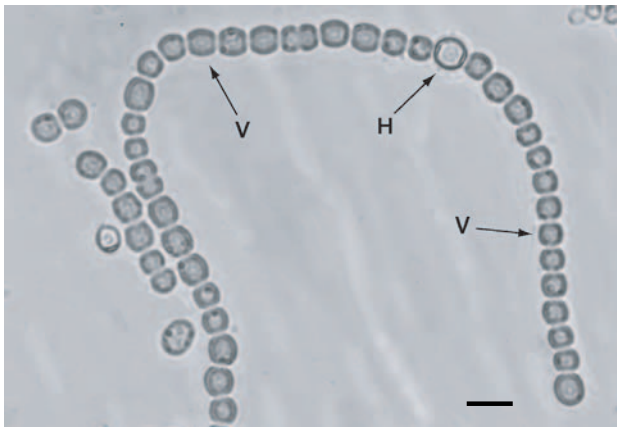

Fig. 2. Light microphotograph of the cyanobacterium *Anabaena* strain PCC 7120. V = vegetative cells, H = heterocyst. Bar = 10 μm.

The morphological variation of cyanobacteria is considerable. Both unicellular and filamentous forms are known, and variation within these morphological

types occurs (Rippka et al 1979) (Fig. 2). Cyanobacteria are able to perform chlorophyll *a* based, oxygenic photosynthesis using a photosynthetic apparatus similar to that of chloroplasts of algae and higher plants. Some cyanobacteria are obligate photoautotrophs, while others can grow heterotrophically in the dark (Rippka et al 1979). Many cyanobacteria, both filamentous and unicellular strains, have the capacity to fix atmospheric nitrogen, a capability only found in prokaryotic organisms. Some filamentous strains can also develop a specialized cell, the heterocyst, where nitrogen fixation takes place. The heterocyst is slightly larger and rounded compared to the vegetative cell, and evenly distributed along the filament (Wolk 1996).

Enzymes directly involved in cyanobacterial hydrogen metabolism

Three enzymes have been described to be directly involved in hydrogen metabolism in cyanobacteria; (1) nitrogenase(s), catalysing the production of molecular hydrogen concomitantly with the reduction of nitrogen to ammonia, (2) an uptake hydrogenase, catalysing the consumption of hydrogen produced by the nitrogenase, (3) a bidirectional hydrogenase, that has the capacity to take up and produce hydrogen (Fig. 3). The three enzymes can be distributed in different ways. However, multiple copies of single hydrogenases have not been found so far in any cyanobacterium (Wünschiers and Lindblad 2003). Several nitrogen-fixing strains have all three enzymes, e.g. the two filamentous, heterocystous strains *Anabaena* PCC 7120 (Houchins and Burris 1981b, Kaneko et al 2001) and *Anabaena variabilis* ATCC 29413 (Happe et al 2000, Schmitz et al 1995). Others have only an uptake hydrogenase and a nitrogenase, e.g. the filamentous, heterocystous strain *Nostoc punctiforme* (also *Nostoc* PCC 73102, *Nostoc* ATCC 29133) (Tamagnini et al 1997, Meeks et al 2001) while only a bidirectional hydrogenase is present in e.g. in the unicellular strain *Synechocystis* PCC 6803 (Kaneko et al 1996, Appel and Schulz 1998).

An uptake hydrogenase is consistently present when a cyanobacterium has the capacity to fix atmospheric nitrogen. An exception might be the unicellular *Synechococcus* PCC 6301 (also *Anacystis nidulans*). It has been reported that it may possess an uptake hydrogenase despite it is a non-nitrogen fixing strain (Boison

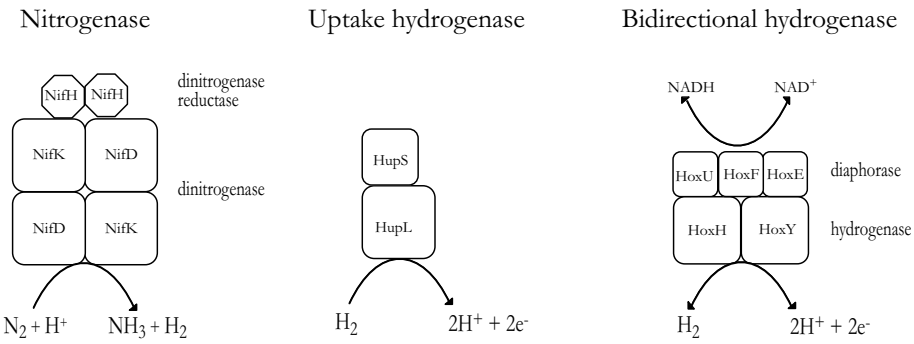

Fig. 3. The three enzymes directly involved in cyanobacterial hydrogen (H_2) metabolism. The nitrogenase consists of two proteins: dinitrogenase reductase and dinitrogenase (Berman-Frank et al 2003). Nitrogenase fixes atmospheric nitrogen and at the same time molecular hydrogen is produced, recycled by an uptake hydrogenase. The third enzyme is a bidirectional hydrogenase, consisting of a diaphorase and a hydrogenase component, with the capacity to both take up and produce hydrogen (Wünschiers and Lindblad 2003).

et al 1996). The distribution of the bidirectional hydrogenase is not as clear as in the case of the uptake hydrogenase and it is missing in several strains (Tamagnini et al 1997, Tamagnini et al 2000).

All hydrogenases identified in cyanobacteria belong to [NiFe]-hydrogenases. However, no cyanobacterial hydrogenase has yet been purified and crystallised.

Nitrogen fixation and nitrogenase

Nitrogenase is an enzyme that is directly involved in hydrogen metabolism as a consequence of its production of molecular hydrogen during nitrogen fixation.

The overall reaction of nitrogen fixation can be written as follows:

$$N_2 + 8H^+ + 16\ ATP \rightarrow 2NH_3 + H_2 + 16\ ADP + 16P_i$$

The above reaction is catalysed by nitrogenase, which consists of two separate protein components: dinitrogenase and dinitrogenase reductase. The dinitrogenase is a heterotetramer consisting of two subunits of NifK and two

subunits of NifD (Fig. 3). The dinitrogenase reductase is a homodimer of NifH and plays a role in transferring electrons from a ferredoxin, or a flavodoxin, to the dinitrogenase, where the actual reduction of N_2 occurs (Howard and Rees 1996). The reaction requires ATP and low-potential electrons. ATP may be generated by either cyclic photophosporylation or oxidative phosporylation and the low potential electrons can come from NADPH that may be generated from the degeneration of carbohydrates produced during photosynthesis (Haselkorn and Buikema 1992). In heterocystous cyanobacteria, carbohydrates are imported from vegetative cells and products of nitrogen fixation are exported to the vegetative cells (Böhme 1998). Nitrogen fixation is oxygen sensitive since nitrogenase becomes inactivated by O_2 (Pienkos et al 1983). Since oxygen is produced by photosystem II during photosynthesis, these two processes must be separated either temporally or spatially by cyanobacteria. Heterocystous cyanobacteria separate the oxygen evolution and nitrogen fixation spatially by performing photosynthesis in the vegetative cells and nitrogen fixation in the heterocysts. To provide an environment with low oxygen the heterocyst lacks photosystem II activity, is surrounded by a thickened cell wall to reduce the diffusion of oxygen, and has a higher respiration rate (Böhme 1998).

Nitrogenase is subject to strict regulatory controls. Nitrogen fixation is not only inhibited by O_2, but also by ammonium and nitrate (Halbleib and Ludden 2000). A key protein in the control of nitrogen metabolism in cyanobacteria is NtcA, a transcriptional regulator that belongs to the CAP family (Herrero et al 2001). NtcA is present in both unicellular and filamentous, heterocystous cyanobacteria (Frias et al 1993, Herrero et al 2001), and has been demonstrated to be essential for heterocyst development (Wei et al 1994). NtcA binds to a palindromic target motif GTA (N_8) TAC upstream of the target gene. Studies of the motif in *Synechococcus* PCC 7942 demonstrated that some variation in the sequence was possible while still maintaining the binding capacity. However, GT(N_{10})AC was found to be essential for binding NtcA (Vázquez-Bermúdez et al 2002). Examples of known genes regulated by NtcA are *glnA* (glutamine synthetase), *nir* (nitrate assimilation), *ntcA* (autoregulatory), and genes important for heterocyst development, e.g. *hetC* and *devBCA* (Herrero et al 2001). In addition, NtcA has also been demonstrated to bind upstream of *xisA* which is a site-specific recombinase responsible for the excision of an 11.5 kb DNA element located within *nifD* in *Anabaena* PCC 7120 (Ramasubramanian et al 1994).

Cyanobacterial uptake hydrogenase, function and activity

All known cyanobacterial uptake hydrogenases consist of two subunits, encoded by *hupS* and *hupL*, respectively (Fig. 4). The small subunit, HupS, contains the iron-sulphur [Fe-S] cluster necessary for electron transfer to the active site, which is located in the large subunit, HupL. HupS also contains cysteins that

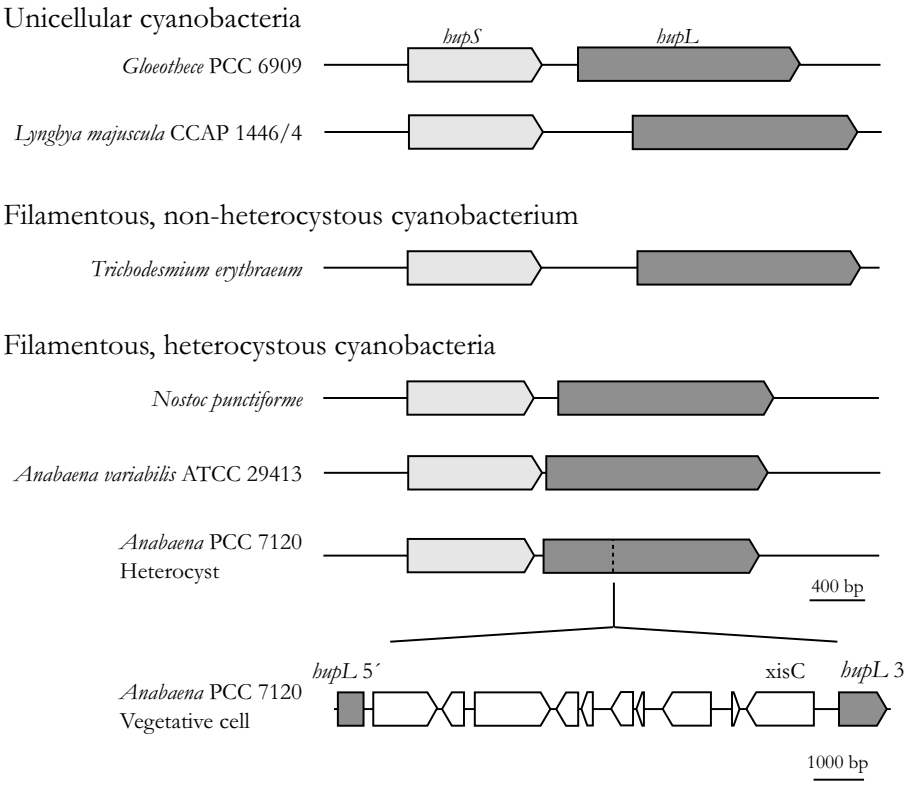

Fig. 4. Physical map of the genes encoding the uptake hydrogenase (*hupSL*) in unicellular, filamentous non-heterocystous, and filamentous heterocystous cyanobacteria. All genes are contigous except *hupL* in *Anabaena* PCC 7120. In vegetative cells, a 10.5 kb DNA fragment is located within *hupL*. However, this DNA fragment is excised by the site specific recombinase which is XisC located within the fragment. *Gloeothece* PCC 6909 (GenBank AY260103), *Lyngbya majuscula* CCAP 1446/4 (GenBank AF368526), *Trichodesmium erythraeum* (http://spider.jgi-psf.org/JGI_microbial/html/trichodesmium/trichod_homepage.html), *Nostoc punctiforme* (Oxelfelt et al 1998), *Anabaena variabilis* ATCC 29413 (Happe et al 2001), and *Anabaena* PCC 7120 (Carrasco et al 1995).

are involved in the coordination of the [Fe-S] clusters. The small subunit in cyanobacteria differs from other small subunits from other microorganisms since the signal peptide in the N-terminus part of HupS is missing (Oxelfelt et al 1998). HupL contains two putative nickel-binding sites (R x CG x C) necessary for the coordination of the nickel in the active site (Carrasco et al 1995, Oxelfelt et al 1998, Happe et al 2000, Wünschiers and Lindblad 2003).

The physiological function of the uptake hydrogenase in cyanobacteria is to catalyse the consumption of hydrogen produced by nitrogenase (Happe at al 2000, Lindberg et al 2002, Houchins 1984, Oxelfelt et al 1995, Troshina et al 1996). A strong correlation between the activities of uptake hydrogenase and nitrogenase has been demonstrated in filamentous cyanobacteria. It is believed that the electrons derived from the hydrogen oxidation catalysed by the uptake hydrogenase recombine, through the respiratory chain, with oxygen in the oxyhydrogen reaction to form water. The advantage would be that energy from the H_2 produced by nitrogenase can be recaptured and oxygen will be consumed and protect nitrogenase from oxygen. The recycling of hydrogen would also supply reducing equivalents to nitrogenase and other cell functions (Wünschiers and Lindblad 2003). However, the uptake hydrogenase is not essential for diazotrophic growth (Happe et al 2000, Lindberg et al 2002, Masukawa et al 2002). Inactivation of the uptake hydrogenase in *Anabaena variabilis* resulted in a lower rate of nitrogen fixation and slightly reduced growth rate compared to the wild type (Happe et al 2000).

In heterocystous cyanobacteria the uptake hydrogenase is located in the heterocyst with no activity in the vegetative cells (Peterson and Wolk 1978, Houchins and Burris 1981a, Houchins 1984, Carrasco et al 1995). The enzyme has been suggested to be membrane bound, located in the thylakoid (Eisbrenner et al 1978) or in the cytoplasmic membrane (Houchins and Burris 1981b). Since all identified cyanobacterial HupS are missing the N-terminal signal peptide important for membrane translocation, it has been suggested that the uptake hydrogenase is located on the cytoplasmic side of the thylakoid or cytoplasm membrane (Appel and Schultz 1998).

The activity of the uptake hydrogenase has been demonstrated to be influenced by different external factors such as nickel, molecular hydrogen, carbon and nitrogen. Addition of extra nickel resulted in an increased hydrogen uptake activity in *Nostoc punctiforme*, *Anabaena cylindrica*, *Oscillatoria subbrevis*, and *Anabaena* strains CA and 1F (Xiankong et al 1984, Daday et al 1985, Kumar and

Polasa 1991, Oxelfelt et al 1995). However, a nickel concentration above 10 μM does not stimulate the hydrogen uptake (Kumar and Polasa 1991, Oxelfelt et al 1995). A direct dependence of nickel was demonstrated for the induction of the uptake hydrogenase in *Anabaena* strains C1 and 1F (Xiankong et al 1984) and *Anabaena cylindrica* (Daday et al 1985).

Molecular hydrogen has also been demonstrated to induce higher uptake activities in cyanobacteria. Studies on *Anabaena* PCC 7120 (Houchins and Burris 1981a), *Nostoc punctiforme* (Oxelfelt et al 1995), *Anabaena cylindrica* and *Nostoc muscorum* (Eisbrenner et al 1978) demonstrated an increase of the hydrogen uptake activity when a fraction of the air was replaced by molecular hydrogen. However, in *Anabaena variabilis* only a slight stimulatory effect on the hydrogen uptake activity was observed when exogenous hydrogen was added. It was suggested that the hydrogen produced from the nitrogenase is sufficient for hydrogenase induction (Troshina et al 1996).

A stimulation of the hydrogen uptake activity, together with nitrogenase activity, could be observed in *Nostoc punctiforme* when organic carbon was added to the medium (Oxelfelt et al 1995). However, no effect could be observed in *Anabaena variabilis* after addition of carbon (Troshina et al 1996). Addition of ammonium decreased the activity of both the uptake hydrogenase and the nitrogenase in *Nostoc punctiforme* (Oxelfelt et al 1995). A similar observation was made in *Anabaena variabilis* (Troshina et al 1996).

Transcription of the genes encoding the uptake hydrogenase

In the two cyanobacteria examined, *hupS* and *hupL* are located on a single transcript containing no additional ORFs (Happe et al 2000, Lindberg et al 2000). The size of the transcript was determined by Northern blotting in *Anabaena variabilis* ATCC 29413 to be approximately 2.7 kb (Happe et al 2000). In *Nostoc punctiforme*, *hupSL* was shown to be a transcript unit and a putative transcriptional terminator could be identified downstream of *hupL*. The intergenic region contains 7 bp repeats putatively forming a hairpin structure. The function of a hairpin formation in cyanobacterial *hupSL* is unknown but may be involved in transcript stability or translational coupling between the structural genes (Lindberg et al 2000). Studies on the localisation of the *hupSL*-transcript in *Anabaena* PCC 7120 demonstrated the presence in the heterocysts only (Carrasco et al 1995). No *hupSL*-transcript could be detected in vegetative cells of *Anabaena*

variabilis using either Northern blot or RT-PCR (Happe et al 2000). However, another study was able to detect a low level of *hupL*-transcript in vegetative cells from a nitrogen-fixing culture of *Anabaena variabilis*. The authors suggested that this could be a result of a basal activity of the *hupSL* promotor not necessarily resulting in translation (Boison et al 2000). A low H_2 uptake in ammonia grown cells of *Anabaena variabilis*, an activity thought to be due to the bidirectional hydrogenase has been reported (Troshina et al 1996). In addition, *Anabaena variabilis* contains an alternative nitrogenase expressed in vegetative cells under anaerobic conditions (Thiel et al 1995, 2001). In the study where a *hupL*-transcript was detected in vegetative cells (Boison et al 2000), no investigations of e.g. the presence of an alternative nitrogenase or uptake hydrogenase were performed. Moreover, there is no data on the regulation of the transcription of *hupSL* in the vegetative cells of *Anabaena variabilis* during anaerobic conditions.

Transfer of non-nitrogen fixing vegetative cells to nitrogen-fixing conditions induces a *hupL* transcript in *Anabaena* PCC 7120 and *Anabaena variabilis* (Carrasco et al 1995, Happe et al 2000, Boison et al 2000). Prior to expression of *hupSL* in *Anabaena* PCC 7120, a 10.5 kb DNA fragment is excised from within *hupL*. In *Anabaena* PCC 7120, two additional gene rearrangements occur (Golden et al 1985). Each excision requires a site-specific recombinase. XisC is responsible for the excision of the 10.5 kb in *hupL* and its gene is located on the excised fragment. Studies of the upstream region of another site-specific recombinase, *xisA*, reveaed a binding site of the global nitrogen regulator NtcA. However, no obvious NtcA binding site could be detected upstream of *xisC* (Carrasco et al 1995). In contrast to *Anabaena* PCC 7120, no programmed rearrangement occurs in *Anabaena variabilis* ATCC 29413 or *Nostoc punctiforme* (Happe et al 2000 and Oxelfelt et al 1998). As in *Anabaena* PCC 7120, a *hupL* transcript was not detected in non-nitrogen fixing cells but was induced during nitrogen fixing conditions (Happe et al 2000).

The transcription start site of *hupSL* has been determined in *Anabaena variabilis* ATCC 29413 and *Nostoc punctiforme* using primer extension and 5´RACE, respectively (Happe et al 2000, Lindberg et al 2000). The transcription start of *hupSL* in *Anabaena variabilis* ATCC 29413 was located 103 bp upstream of the translation start site. One half of a putative Fnr binding site was found 144 bp upstream of the translational start site (Happe et 2000). Fnr is a transcription factor playing a major role in altering the gene expression between aerobic and anaerobic conditions to facilitate changes in energy metabolism (Kiley and

Beinert 1999). The transcription start in *Nostoc punctiforme* was located 259 bp upstream of the translation start. The promotor region has putative binding sites for NtcA and integration host factor (IHF). NtcA is the global nitrogen regulator in cyanobacteria and IHF is a DNA-binding protein consisting of two subunits that upon binding creates a sharp (more than 160°) bend. This allows proteins that bind further upstream to interact with the promotor region. In addition, IHF can also act directly as a repressor or activator (Wagner 2000).

Cyanobacterial bidirectional hydrogenase, function and activity

Initially, the bidirectional hydrogenase was suggested to be an enzyme with four subunits, consisting of a diaphorase part, HoxFU, and a hydrogenase part, HoxYH. These subunits are homologous to the heterotetrameric NAD^+-reducing hydrogenase of *Ralstonia eutropha* (Schmitz et al 1995). Recently, it was suggested that a fifth subunit, HoxE, belongs to the diaphorase part of the bidirectional hydrogenase. Thus, the cyanobacterial bidirectional hydrogenase is encoded by *hoxEFUYH* (Schmitz et al 2002).

The physiological function of the bidirectional hydrogenase in cyanobacteria is not completely clear. The bidirectional hydrogenase has been suggested to act as an electron valve during photosynthesis in *Synechocystis* PCC 6803. Inactivation of the bidirectional hydrogenase resulted in a higher fluorescence of photosystem II compared to the wild type (Appel et al 2000). The enzyme has also been proposed to play a role in fermentation by functioning as a mediator in the release of excess reducing power during anaerobic conditions (Stal and Moezelaar 1997, Troshina et al 2002). In addition, it has been suggested that the bidirectional hydrogenase is part of the respiratory complex I (Appel and Schulz 1996, Schmitz and Bothe 1996). In cyanobacteria, only 11 subunits out of 14 conserved subunits of a prokaryotic complex I have been identified. Some of the subunits of the bidirectional hydrogenase show sequence similarities with the missing subunits of the respiratory complex I (Schmitz et al 1995). However, the bidirectional hydrogenase has been demonstrated to be absent from several cyanobacterial strains (Tamagnini et al 1997, Tamagnini et al 2000) and studies of the respiration of *Nostoc punctiforme*, a strain naturally lacking the bidirectional hydrogenase (Tamagnini et al 1997), demonstrated rates of respiration

comparable to cyanobacteria having a bidirectional hydrogenase (Boison et al 1999). In addition, mutants of *hoxU* in *Synechocystis* PCC 6301 (Boison et al 1998) and *hoxF* in *Synechocystis* PCC 6803 showed non-impaired respiratory O_2 uptake whilst being affected in H_2 evolution (Howitt and Vermaas 1997). In general, it seems that the bidirectional hydrogenase does not play an essential role in those strains where it is present. Inactivation of *hoxH* in *Synechocystis* PCC 6803 and *Anabaena* PCC 7120 resulted in a small decrease in growth rate compared to the wild type (Appel et al 2000, Masukawa et al 2002).

The bidirectional hydrogenase is present in both vegetative cells and in heterocysts (Hallenbeck and Beneman 1978, Houchins and Burris 1981a). In *Anabaena* PCC 7120 the bidirectional hydrogenase appeared in the soluble fraction after cell disruption and it was suggested that the enzyme is soluble (Houchins and Burris 1981b, Hallenbeck and Beneman 1978). However, investigations in other cyanobacteria suggest an association with cell membranes. In *Anabaena variabilis* and *Synechocystis* PCC 6803, an association with the thylakoid membrane was demonstrated (Serebryakova et al 1994, Appel et al 2000). However, based on immunological data, an association with the cytoplasmic membrane in *Synechocystis* PCC 6301 has been suggested (Kentemich et al 1989).

The activity of the bidirectional hydrogenase has been examined in both unicellular and filamentous cyanobacteria. *In vivo*, NADH supports H_2 evolution in *Synechocystis* PCC 6301. NADPH also supports H_2 evolution but with less than 50% of the activity obtained using NADH. For H_2 uptake, NAD^+ is the preferred electron acceptor (Schmitz and Bothe 1996).

The activity of the bidirectional hydrogenase has in several studies been demonstrated to be induced by anaerobic conditions (Schmitz and Bothe 1996, Serebryakova et al 1994, Houchins and Burris 1981a). The bidirectional hydrogenase in *Anabaena* PCC 7120 is active in both vegetative cells and in heterocysts in aerobically grown filaments, with a several-fold higher activity in heterocysts. Transferred to anaerobic conditions, the activity of the bidirectional hydrogenase increased with about two orders of magnitude with approximately the same activities in both cell types (Houchins and Burris 1981a). Similar results have been observed in *Anabaena variabilis* (Serebryakova et al 1994). In contrast to the filamentous cyanobacteria, the activity of the bidirectional hydrogenase in the unicellular *Gloeocapsa alpicola* is not directly dependent on oxygen. Higher activity is observed under nitrogen starvation and low light, and it was suggested that the bidirectional hydrogenase could act as an alternative electron donor to

photosystem I after inactivation of photosystem II due to nitrogen starvation. Under dark anoxic conditions the unicellular cyanobacterium *Gloeocapsa alpicola* produces H_2 catalysed by the bidirectional hydrogenase (Troshina et al 2002). In addition, the unicellular strain *Chroococcidiopsis thermalis* contains a bidirectional hydrogenase with some catalytic properties more similar to an uptake hydrogenase. It is not inducible under anaerobic conditions or under nitrate starving conditions (Serebryakova et al 2000).

In contrast to the uptake hydrogenase, the bidirectional hydrogenase in *Anabaena* PCC 7120 did not respond to added H_2 in aerobically grown cells (Houchins and Burris 1981a).

Transcription of the genes encoding the bidirectional hydrogenase

The genes encoding the bidirectional hydrogenase, *hoxEFUYH*, in cyanobacteria are organised in a similar way in many strains. In some strains the genes are not adjacent and must thus be located on at least two operons. It is also possible to identify ORFs that are located between the *hox*-genes (Fig. 5).

The information about the transcription and regulation of the *hox*-genes is limited in cyanobacteria. Transcript(s) of the bidirectional hydrogenase is present in both vegetative cells and heterocysts under nitrogen-fixing conditions and in vegetative cells during non-nitrogen fixing conditions in *Anabaena variabilis* ATCC 29413. In addition, *hoxFUYH* were shown to be transcribed as a transcript unit together with two ORFs with unknown function. These experiments were performed using RT-PCR and do not exclude additional promotors within the operon (Boison et al 2000). The *hox*-genes in the unicellular *Synechocystis* PCC 6301 are located on two different transcripts. *hoxEF* form one transcript and at least 16 kb downstream of *hoxF*, *hoxUYH* is forming a second transcript together with *hoxW*, *hypA*, and *hypB* (Boison et al 2000). In *Synechococcus* PCC 7942, *hoxEF* and *hoxUYHW* are located on two different transcripts. Using real time PCR and reporter gene constructs, it was suggested that a second promotor might be present between *hoxH* and *hoxW*. It was also demonstrated that the *hox*-genes had a circadian clock expression (Schmitz et al 2001).

Very few regulatory studies have been performed on the transcriptional regulation of the *hox*-genes in cyanobacteria. Studies of the transcription of *hoxY* and *hoxH Gloeocapsa alpicola* CALU 743 during nitrogen-limiting growth conditions demonstrated an increase in the enzyme activity but no regulation on

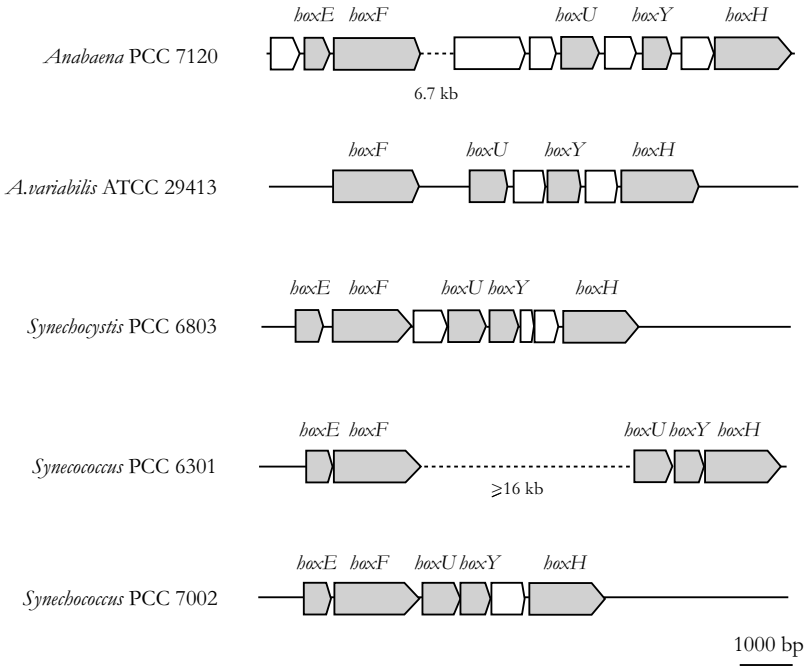

Fig. 5. Physical map of the genes encoding the bidirectional hydrogenase (*hox EFUYH*) in cyanobacteria. *Anabaena* PCC 7120 (Kaneko et al 2001), *Anabaena variabilis* ATCC 29413 (Schmitz et al 1995), *Synechocystis* PCC 6803 (Appel and Schulz 1996), *Synecococcus* PCC 6301 (also *Anacystis nidulans*) (Boison et al 1996), and *Synechococcus* PCC 7002 (GenBank AF381045).

the transcriptional level (Sheremetieva et al 2002). In contrast, transfer to a low level of oxygen in *Anabaena variabilis* induced both the enzyme activity as well as the relative amount of *hoxH* (Sheremetieva et al 2002).

Maturation of hydrogenases

The maturation of hydrogenases is a complex process in which several proteins, encoded by e.g. the *hyp*-genes, are involved. The respective genes involved in the maturation of [NiFe]-hydrogenases have been identified,

sequenced, and characterized but the corresponding genes for the maturation of [Fe]-hydrogenases remain to be identified. Very little is known in cyanobacteria, most of the knowledge about hydrogenase maturation is from other microorganisms such as *Escherichia coli* (Lutz et al 1991), *Ralstonia eutropha* (Wolf et al 1998), *Bradyrhizobium japonicum* (Olson and Maier 1997), and *Rhizobium leguminosarum* (Rey et al 1993). Homologues of the *hyp*-genes have been identified in cyanobacteria but their role in maturation of the hydrogenases remains to be demonstrated. The Hyp-proteins are involved in the insertion of Ni, Fe, and the ligands, CO and CN, into the active site of the large subunit. Other genes involved in the maturation of the hydrogenases, not belonging to the *hyp*-genes, encode endopeptidases, which are responsible for a specific cleavage of a C-terminal part of the large subunit of the hydrogenase.

One of the earliest steps in the maturation is the formation of the complex between HypC and the large subunit of the hydrogenase. HypC is assumed to act as a chaperone, maintaining the large subunit in a conformation accessible for metal insertion. A *hypC* mutant results in a metal free hydrogenase. The next step is the insertion of the ligands CN and CO into the large subunit. This is performed by HypF and of HypE (Reissman et al 2003). HypF has been shown by mutational studies to be absolutely required for hydrogenase maturation. It has been demonstrated that the CN and CO ligands are derived from carbamoylphosphate. Proteins catalysing O-carbamoylations contain a sequence motif (VxHHxAH) that is also found in HypF. In addition, HypF contains two zinc finger motifs. It is possible that HypF interacts with the large subunit and that the acyl-phosphatase and the carbomolphosphate domains synthesise and insert the ligands in the active site. Using a two-hybrid method, it was shown that HypF and HypE interact in *Helicobacter pylori* (Rain et al 2001). Inactivation of HypE results in a non-mature hydrogenase, so HypE is also essential for hydrogenase maturation. HypB is suggested to be the main contributor of insertion of nickel. Deletions of *hypB* leads to nickel free hydrogenase precursors and an inactive hydrogenase in *E. coli* (Maier et al 1993). In *R. leguminosarum* (Rey et al 1994), *B. japonicum* (Olson et al 1997), HypB has also been shown to have a function in nickel storage, as a result of histidine rich domains in the amino terminus. The function of HypA is to cooperate with HypB during nickel insertion (Hube et al 2002). In *H. pylori* it was demonstrated that HypA and HypB form a heterodimer (Mehta et al 2003). The role of HypD is unclear and remains to be identified. However, the protein has been demonstrated to form a complex with HupC in

E. coli (Blokesch and Böck 2002).

The last identified step in the maturation of the large subunit is the proteolytic cleavage of the C-terminus. Recently, two ORFs putatively encoding hydrogenase specific endopeptidases, HoxW and HupW, were identified in *Anabaena* PCC 7120. It was suggested that they are responsible for the specific proteolytic cleavage of the C-terminal part of the bidirectional hydrogenase and uptake hydrogenase, respectively. Putative endopeptidases were also found in the unicellular *Synechocystis* PCC 6803, containing only the bidirectional hydrogenase, and the filamentous, heterocystous *Nostoc punctiforme*, containing the uptake hydrogenase only. Only one, though specific, putative endopeptidase was found in each of these two strains supporting the hypothesis that each hydrogenase has a specific endopeptidase (Wünschiers et al 2003).

AIM OF THE THESIS

The aim of this thesis was to investigate the transcription of genes encoding hydrogenases in filamentous, heterocystous cyanobacteria. At the time this thesis was initiated, there was only one publication concerning the transcription and regulation of a cyanobacterial hydrogenase (Carrasco et al 1995). In addition, the sequence information about cyanobacterial hydrogenases was limited. Only one sequence from an uptake (*hupSL*) (Carrasco et al 1995) and one from a bidirectional hydrogenase (*hoxFUYH*) was available (Schmitz et al 1995). The transcription had been investigated in other microorganisms and together with the knowledge from physiological studies of cyanobacterial hydrogenases this was used as a starting point for transcriptional studies in cyanobacteria. The initial work was performed using *Nostoc punctiforme* and *Nostoc muscorum*, two strains available in the laboratory since several years. It was known that the bidirectional hydrogenase was absent in *Nostoc punctiforme* (Tamagnini et al 1997), which made it a good candidate for examining the uptake hydrogenase. In contrast, *Nostoc muscorum* contains both the uptake and the bidirectional hydrogenase and could be used for studies of the latter enzyme. During the last couple of years, several cyanobacterial genomes have been sequenced, and when the genome of *Anabaena* PCC 7120 became available (Kaneko et al 2001), this strain became the model organism for further studies of the transcription of cyanobacterial *hox*-genes.

There is a biotechnological aspect in working with cyanobacteria, their hydrogen metabolism and its transcriptional regulation. Today, there are only a few cyanobacterial strains that have been genetically modified and characterized with the aim of producing and evolving molecular hydrogen. Hydrogen is an ideal future energy carrier that could be used for energy purposes. Although not directly being the topic of my thesis, detailed knowledge of the transcriptional regulation of the cyanobacterial hydrogenases may be useful when exploring the possibilities of cyanobacterial biohydrogen as well as genetically modified cyanobacteria evolving significant amount of hydrogen.

METHODOLOGY

Several methods, e.g. Northern blot, reverse transcription-PCR (RT-PCR), microarray techniques, are available to examine the transcription of a gene, a set of genes, or even expression of a complete genome. All methods have advantages as well as disadvantages. The transcriptional studies in this thesis have been performed using RT-PCR (papers I-IV) and a microarray technique (paper V). A general conclusion about the presence of transcripts from the uptake and bidirectional hydrogenase as well as the *hyp*-genes, is that they are present in low copy number and/or are short-lived. These features restrict the use of Northern blot since sensitivity is lower compared to e.g. RT-PCR. Despite several attempts with Northern blot it was not possible to convincingly visualise a transcript, a result also found in other laboratories (Carrasco et al 1995, Boison et al 2000, Happe et al 2000).

RT-PCR is a sensitive method able to detect very low amounts of a specific transcript. Theoretically, a single transcript can be detected since there is an amplification step in the PCR. RNA is reverse transcribed into cDNA using a reverse transcriptase. The cDNA is used as a template in the subsequent PCR. The final PCR-product may be analysed using an ethidium bromide stained agarose gel. Due to the sensitivity of RT-PCR, it is absolutely necessary to remove all contaminating DNA from the RNA, since the DNA also is a template in a PCR.

One important conclusion from my RT-PCR, 5´RACE and microarray experiments is the absolute need of RNA of high quality. The experience from preparing RNA from different cyanobacteria is that many strains require specific adjustments in the RNA preparation procedure to give optimal RNA quality and amount. There are several possible explanations for these individual differences among the different strains. One could be that some cyanobacteria have a thick polysaccaride layer that surrounds the cell that makes it difficult to prepare high quality RNA. These individual differences among the cyanobacterial strains used in this thesis lead e.g. to the use of two different protocols when preparing RNA from *Nostoc muscorum* and *Nostoc punctiforme*, respectively (papers I-III).

The microarray technique was used to investigate the transcription of selected genes during nitrogen-fixing and non-nitrogen fixing conditions (paper

V). The principle of microarrays is simple but requires more complicated facilities and software in order to perform the analysis. The microarray technique used in these experiments removed all tRNA and rRNA, and the remaining mRNA was amplified according to a modified Eberwine protocol (Eberwine et al 1992), before being hybridised to the probes. The RNA was labelled with streptavidin-phycoerythrin and detected using a CCD camera. Considering the number of genes that may be analysed, a relatively small amount (10 μg) of total RNA was used in the microarray experiments.

Determination of the transcription start site was performed using a 5´RACE method (Rapid Amplification of cDNA ends). In summary, the target RNA is reverse-transcribed into cDNA. An enzyme (terminal deoxynucleotidyl transferase, TdT) adds a stretch of nucleotides (dCTPs) in the 5´end. Subsequent amplification with PCR, cloning of the PCR-fragment and sequencing allow easy identification of the transcription start site.

In vivo hydrogen uptake activity was measured using an H_2-electrode (for details see Oxelfelt et al 1995).

RESULTS AND DISCUSSION

Transcription of *hupSL* in *N. punctiforme* and *N. muscorum* (papers I-III)

Several physiological experiments demonstrated a correlation of the nitrogenase and uptake hydrogenase activities suggesting that a function of the uptake hydrogenase to recycle the H_2 produced by the nitrogenase (Happe at al 2000, Lindberg et al 2002, Houchins 1984, Oxelfelt et al 1995, Troshina et al 1996). As described above, *hupL* in *Anabaena* PCC 7120 is interrupted by a 10.5 kb DNA fragment. Complete sequencing of *hupSL* from *N. punctiforme* and partial sequencing of *hupL* from *N. muscorum*, demonstrated a contiguous *hupL* in all cells (Oxelfelt et al 1998, paper I). In addition, no *xisC* homologous sequences, a gene encoding the site-specific recombinase responsible for the excision of the DNA within *hupL* in *Anabaena* PCC 7120, could be detected using low stringency Southern hybridisation in *N. muscorum* and *N. punctiforme* (paper I). These results show that no rearrangement of *hupL* occurs before *hupL* is expressed in these two strains. In order to examine the regulation of the genes encoding the uptake hydrogenase (*hupSL*), RNA was prepared from non-nitrogen fixing and nitrogen-fixing cultures. RT-PCR experiments demonstrated the presence of *hupSL*-transcript in both *Nostoc muscorum* and *Nostoc punctiforme* during nitrogen-fixing conditions only (papers I, III). In *N. muscorum*, a *hupL*-transcript was detected approximately 24 h after a transfer to nitrogen-fixing conditions (paper I), in agreement with earlier results using *Anabaena* PCC 7120 (Carrasco et al 1995). Adding ammonium to a nitrogen-fixing culture of *N. muscorum* resulted in the disappearance of the *hupL*-transcript after 4 to 5 days (unpublished data).

Four environmental conditions (H_2, Ni, O_2, and N) were examined for their respective influence on the transcription of *hupSL*. Molecular hydrogen (H_2) was found to positively regulate regulates the transcription of the uptake hydrogenase genes in *N. muscorum* and *N. punctiforme* (paper II). Nine percent of the air was replaced by H_2. In both cyanobacteria the relative amount of *hupL*-transcript increased as well as the *in vivo* hydrogen uptake activity. Previous studies demonstrated that addition of H_2 resulted in an increase of the *in vivo* hydrogen uptake activity in nitrogen-fixing cultures of *Anabaena* PCC 7120,

Anabaena cylindrica, *N. punctiforme*, and *N. muscorum* (Oxelfelt et al 1995, Houchins and Burris 1981a, Eisbrenner et al 1978).

Nickel is part of the active centre in the large subunit and has been demonstrated to be essential for hydrogenase activity. Several physiological studies in other organisms demonstrated an increase in the hydrogen uptake activity when external nickel was added and subsequent loss of the activity when nickel was removed. To investigate if nickel regulates the transcription of the uptake hydrogenase genes in cyanobacteria, 0.5 μM of $NiSO_4$ was added to the growth medium (paper II). An increase of the *in vivo* hydrogen uptake activity as well as the relative amount of *hupL*-transcript could be observed in both *N. punctiforme* and *N. muscorum*. The Ni-stimulated *in vivo* hydrogen uptake activity in *N. punctiforme* is in agreement with a previous study (Oxelfelt et al 1995), but also found in *Anabaena cylindrica*, *Oscillatoria subbrevis*, and *Anabaena* strains CA and 1F (Xiankong et al 1984, Daday et al 1985, Kumar and Polasa 1991). This demonstrates that at least part of the nickel regulation of the uptake hydrogenases is on the transcriptional level.

Anaerobic and microaerobic conditions have been demonstrated to influence the expression of *hupSL* in other microorganisms such as *Escherichia coli*, and *Bradyrhizobium japonicum* (Richard et al 1999, Kim et al 1991). The effect of reduced level of O_2 on the transcription of *hupSL* was examined in *Nostoc muscorum* and *Nostoc punctiforme* by replacing the air with argon and CO_2. After the transfer to a low oxygen concentration the *in vivo* hydrogen uptake activity, as well as the relative amount *hupL*-transcript, increased in both *N. muscorum* and *N. punctiforme*. Thus, oxygen regulates the uptake hydrogenase on the transcriptional level.

As described above, and in detail in papers I-III, four environmental conditions, H_2, Ni, O_2, and N, have been identified resulting in increased level of *hupL*-transcription. The transcription of the uptake hydrogenase genes after the addition of hydrogen has been shown to be induced in e.g. *Ralstonia eutropha*, *Bradyrhizobium japonicum*, and *Rhodobacter capsulatus* and regulated by a hydrogen sensor system (Lenz and Friedrich 1998, van Soom et al. 1993, 1997 and 1999, Black and Maier 1994, Elsen et al 1996, 1997, Dischert et al 1999). An induction of a cyanobacterial *hupSL*-transcript, due to addition of H_2, suggests the presence of a cyanobacterial hydrogen sensor. However, no hydrogen sensor has so far been identified in cyanobacteria. A search for the corresponding sensor genes, comparing both protein and nucleotide sequences from *R. eutropha* and

A

```
Alcaligenes      GVAHELNNPIS_QLLQVLMNLIQNGYDA_DNGSGIPPQNLSRIFDPFFTTKPVGKGTGLGLSIS
Ralstonia        GVAHELNNPIS_HVQQVMMNLIQNAYDA_DNGPGIAAEHLARVFDPFFSTKPVGKGTGLSLSIS
Rhodobacter      GVAHELNNPIS_HIQQVVMNLVQNALDA_DTGPGVAEDVAPTIFDPFFTTKDVGKGTGLGLSIS
alr3225          GVAHEINNPVN_QLNQVFMNLISNAIDA_DNGVGIPPEILSKLFDPFFTTKSVGKGTGLGLSIS
alr2739          GVAHEINNPVS_QLNQVFMNIINNAVDA_DNGPGINQLVMQKLFDPFFTTKPVGQGTGLGLSIS
alr2481          GVAHEINNPVN_QLNQVFMNILANAIDA_DNGMGMSEKTQQQIFNPFFTTKPVGKGTGMGMSIS

Consensus           HExxxPL     QxxxNxxxNA     DxGxG          FxPF         GxGLGL
```

B

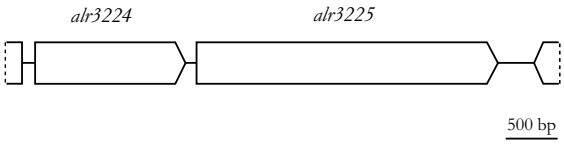

Fig. 6. Putative histidine protein kinases in *Anabaena* PCC 7120. (A) C-terminal transmitter domain from known histidine protein kinases in described hydrogen sensor systems in *Alcaligenes hydrogenophilus*, *Ralstonia eutropha*, and *Rhodobacter capsulatus*, and putative candidates in *Anabaena* PCC 7120 (adapted from Lenz and Friedrich 1998). (B) The putative histidine protein kinase *alr3225* is located adjacent to *alr3224*, a putative *xisC*.

R. capsulatus, in the completely sequenced genome of *Anabaena* PCC 7120, and sequences of *Nostoc punctiforme* failed to discover any obvious candidates that may fulfil these functions. However, the ORFs *alr3225*, *alr2739*, and *alr2481* in *Anabaena* PCC 7120 share the conserved motifs of HoxJ/HupT of the response regulator in *Ralstonia eutropha* and *Rhodobacter capsulatus* (Fig. 6A). The translated ORFs contain a large N-terminal part not present in HoxJ/HupT. This additional part could hypothetically act as a hydrogen sensor. However, if working as a hydrogen sensor, it must act in a novel way since it does not contain the conserved motifs for the coordination of nickel described in *Ralstonia eutropha* hydrogen sensor (Kleihues et al 2001). Using RT-PCR it could be demonstrated that the ORF *alr3225* is located on the same operon as *alr3224* (unpublished data) (Fig 6B) , which is annotated in CyanoBase as a putative *xisC* homologue. XisC is the recombinase responsible for the excision of the extra DNA within *hupL* in *Anabaena* PCC 7120. However, inactivation of *xisC* by the insertion an antibiotic cassette resulted in a HupL minus strain (Lindblad et al 2002). If *alr3224* is a second copy of *xisC*, it could not complement the function of the

inactivated recombinase, at least under the conditions that were tested.

If there is a cyanobacterial hydrogen sensor, it may regulate transcription of the uptake hydrogenase in response to the hydrogen produced by nitrogenase. This may occur during a shift from non-nitrogen fixing to nitrogen-fixing conditions. During non-nitrogen fixing conditions, no hydrogen is produced by nitrogenase and no hydrogenase activity or *hup*-transcript, can be detected. During a transfer to nitrogen-fixing conditions, nitrogenase is induced and when active produces hydrogen that could be sensed by a hydrogen sensor system, which activate the transcription of the uptake hydrogenase genes (Fig 7B). This has been observed in *Rhodobacter capsulatus*, where the hydrogen produced by the nitrogenase, or added exogenously, induced the transcription of the hydrogenase genes (Colbeau and Vignais 1992).

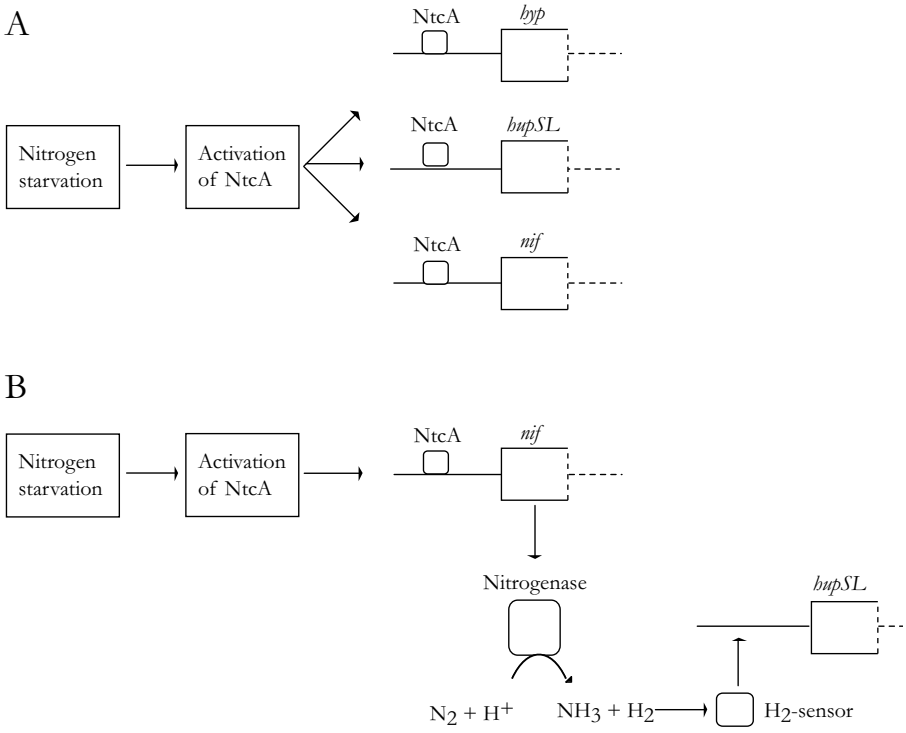

Fig. 7. Two models for the activation of the transcription of the genes encoding the uptake hydrogenase in cyanobacteria during a transfer to nitrogen-fixing conditions (nitrogen starvation). (A) Direct activation of the uptake hydrogenase (*hupSL*), nitrogenase (*nif*), and hydrogen maturation (*hyp*) genes by NtcA (the global nitrogen regulator). (B) Activation of the uptake hydrogenase genes through a hydrogen sensor.

Another possibility during nitrogen-shifts is a transcriptional regulation of *hupSL* by NtcA (Fig. 7A). A putative NtcA binding site was identified upstream of *hupSL* in *Nostoc punctiforme* (Lindberg et al 2000). This would ensure a coordination of heterocyst development, nitrogen fixation, and hydrogen uptake. This could be similar to the situation suggested in *Rhizobium leguminosarum* (Brito et al 1997). By using heterologous *nifA* from *Klebsiella pneumoniae* and *Rhizobium meliloti*, a NifA dependent transcription of the uptake hydrogenase genes could be observed. NifA is a key regulatory protein in the activation of *nif*-genes in e.g. rhizobial species (Fischer 1994). Regulation of the *nif* and *hup*-genes by NifA would coordinate the expression under nitrogen-fixing conditions. However, a coordinate regulation of the transcription of the *nif* and *hup*-genes by NtcA may not be a general feature in cyanobacteria. Sequence analysis of the region upstream of *hupS* in *Anabaena variabilis* was unable to detect any NtcA binding sites (Happe et al 2000).

Components and mechanisms involved in the regulation by nickel are not well understood in cyanobacteria. In other organisms, nickel import seems essential for full hydrogenase activity. As described above, inactivation of the Nik system in *Escherichia coli* results in a non-active hydrogenase (Wu et al 1989). However, no cyanobacterial nickel import systems have been described and investigations, using nickel transport systems from *E. coli* and *R. eutropha* as models, of the genomes of *Nostoc punctiforme* and *Anabaena* PCC 7120 do not reveal any clear candidates. Nevertheless, genes involved in nickel tolerance have been identified and characterized in *Synechocystis* PCC 6803. The genes, *nrsABCD*, were shown to be induced when nickel was added to the growth medium resulting in a tolerance to high nickel concentrations. Thus, *nrsABCD* encodes an efflux system in *Synechocystis* PCC 6803 (Garcia-Domingues et al 2000). These genes have been demonstrated to be under control of a two-component regulatory system encoded by *nrsR* and *nrsS* (Lópes-Maury 2002). NrsR is the response regulator and was demonstrated to bind upstream of *nrsABCD*. NrsS is the putative nickel sensor (Garcia-Domingues et al 2000). Corresponding genes are putatively present in *Anabaena* PCC 7120 (Keneko et al 2001). If NikRS also influence the transcription of the hydrogenase genes is unknown. In addition, to have an efflux system seems to be contradicting when comparing with *Escherichia coli* and *Ralstonia eutropha*. These strains have a nickel import system and e.g. the hydrogenase activity is dependent on a functional nickel import (Wu et al 1989). However, in the natural environments of cyanobacteria there might be sufficient

amount of nickel and no specific nickel import is needed. In addition, nickel has been demonstrated to be toxic when present in high concentration (see for example Oxelfelt et al 1995). Nickel may be co-transported into the cells by an unspecific transport system and when reaching a high intracellular concentration, a specific nickel efflux transporter is induced. It has been shown that under artificial conditions (high nickel concentrations), nickel can be taken up by magnesium transport systems (Smith and Maguire 1998). In natural conditions the magnesium concentration is at least three orders of magnitude higher than nickel and the nickel taken up by magnesium transport systems contribute very little to nickel uptake under physiological conditions (Eitinger et al 2000).

The transcription of the uptake hydrogenase was demonstrated to be induced when air was replaced by argon and CO_2. How changed levels of oxygen can be mediated and affect the transcription of the uptake and the bidirectional hydrogenase (see below) is unknown in cyanobacteria. In *Escherichia coli*, several genes have been demonstrated to be regulated by altered oxygen concentrations. The regulation has been shown to be under the control of the transcriptional regulator Fnr (Gunsalus and Park 1994). Intrestingly, a putative Fnr binding site was identified upstream of *hupS* in *Anabaena variabilis* (Happe et al 2000), but not in *Nostoc punctiforme* (Lindberg at al 2000). However, if a corresponding cyanobacterial Fnr protein binds to the promotor region of the cyanobacterial hydrogenases remains to be demonstrated. An oxygen dependent transcription of the uptake hydrogenase, mediated by NifA in *Rhizobium leguminosarum* has been demonstrated (Brito et al 1997). In addition, experiments using *Bradyrhizobium japonicum* showed that the optimal oxygen concentration for maximum transcription of the hydrogenase genes was 0.2-3%. At both lower and higher concentrations the expression is significantly lower (Kim et al 1991).

Transcription of *hox* in *N. muscorum* and *Anabaena* PCC 7120 (papers I, II, IV)

There are a few obvious differences in the organisation of the genes encoding the uptake hydrogenase (*hupSL*) compared to the bidirectional hydrogenase (*hoxEFUYH*) in cyanobacteria. In strains with an uptake hydrogenase, the two structural genes are located next to each other and located on the same transcript.

With the sequences available from a number of different cyanobacteria it is clear that different cyanobacterial strains have different gene arrangements of the bidirectional hydrogenase and thus must have different regulation of transcription of the structural genes. The transcription of the genes encoding the bidirectional hydrogenase in *Anabaena* PCC 7120 was investigated (paper IV). From the genomic sequence, two clusters of *hox*-genes can be identified. One cluster with *hoxE* and *hoxF* and one cluster with *hoxU*, *hoxY*, and *hoxH*. Between *hoxF* and *hoxU*, there is approximately 8.8 kb of DNA containing several ORFs in both directions. The transcription units were determined by RT-PCR and two different transcripts could be identified. One transcript was formed by *alr0752*, *hoxF* and *hoxE*, and one by *hoxU*, *alr0753*, *hoxY*, *alr0765*, and *hoxH*. The *hox*-genes in the unicellular *Synechococcus* PCC 6301 and *Synechococcus* PCC 7942 were also demonstrated to be located on two different transcripts (Boison et al 2000, Schmitz et al 2001). However, the *hox*-genes in *Anabaena variabilis* are transcribed as one transcript (Boison et al 2000).

A transcription start site was determined upstream of *hoxU* in *Anabaena* PCC 7120, using 5´RACE (paper IV). It was located 55 bp from the translation start site. A putative Fnr-binding site was identified 209 bp upstream of the transcription start site.

The functions of the three ORFs (*alr0750*, *alr0763*, and *alr0765*) that are transcribed together with the *hox*-genes in *Anabaena* PCC 7120 are unknown. No obvious connection to the bidirectional hydrogenase or hydrogen metabolism can be identified. Interestingly, a comparison with the filamentous heterocystous *Nostoc punctiforme*, a strain lacking the bidirectional hydrogenase and the unicellular non-nitrogen fixing *Synechocystis* PCC 6803, containing the bidirectional hydrogenase, reveal that both *alr0750* and *alr0765* are present in the *Nostoc punctiforme* genome. In addition, it is possible to find the homologues to all three genes in *Synechocystis* PCC 6803 genome. However, the similarities are significantly lower than the corresponding comparison to the *Nostoc punctiforme* genome. Some information about a possible function can be obtained by analysing the sequence. A putative UspA motif can be identified in *alr0750*. UspA has been described as a general stress protein and induced under several stress conditions (Nyström and Neidhart 1992). No putative motifs can be identified in *alr0763*. In *alr0765*, a CP12 motif can be found. CP12 contains three conserved cysteines and a histidine suggesting a possible a zinc-binding domain.

The transcription of *hoxH* was examined in both *Nostoc muscorum* and

Anabaena PCC 7120 during different environmental conditions. In contrast to *hupL*, no significant change in the relative amount of *hoxH* transcript was observed during transfer from non-nitrogen fixing to nitrogen-fixing conditions, which may indicate that the bidirectional hydrogenase does not play a role during nitrogen fixation. A *hoxH*-transcript is present in vegetative cells (paper I), in contrast to *hupSL* that is present in heterocysts only (Carrasco et al 1995). In *Anabaena variabilis* it was demonstrated that the *hox*-transcript also is present in the heterocyst (Boison et al 2000). However, it is difficult to study the transcription of genes in the heterocysts only, using RT-PCR. It requires heterocyst preparations, which is absolutely free from vegetative cells.

Previous studies demonstrated that addition of hydrogen did not have any influence on the activity of the bidirectional hydrogenase in *Anabaena* PCC 7120 (Houchins and Burris 1981a). When replacing 9% of the air with molecular hydrogen in *Nostoc muscorum*, no change in the relative amount *hoxH*-transcript could be detected, in agreement with the activity measurements in *Anabaena* PCC 7120 (Houchins and Burris 1981a). Adding hydrogen to a culture may have some similarities to a transfer from non-nitrogen fixing to nitrogen-fixing conditions, no molecular hydrogen is produced during non-nitrogen fixing but during nitrogen-fixing conditions.

The bidirectional hydrogenase is an enzyme also containing nickel in the active centre. When 0.5 μM of nickel was added to the growth medium of a culture of *Nostoc muscorum*, the relative amount of *hoxH* transcript was increased, a result also observed for e.g. *Bradyrhizobium japonicum* (Kim et al 1991).

Several studies have been performed where cyanobacteria were transferred to anaerobic conditions resulting in an induction of the bidirectional hydrogenase activity. In agreement, nitrogen-fixing cells of *Nostoc muscorum* and *Anabaena* PCC 7120 transferred to an anaerobic condition, demonstrated a significantly increased level of *hoxH*-transcript

The transcriptional studies of the cyanobacterial *hox*-genes demonstrate differences compared to the *hupSL*-genes and thus suggests another regulation of the bidirectional hydrogenase in the cyanobacterial cell. One obvious difference is the presence of transcripts from the bidirectional hydrogenase in vegetative cells. From transcriptional data, it seems that the bidirectional hydrogenase is not involved in nitrogen fixation. No transcriptional regulation occurs during either a transfer from non-nitrogen fixing to nitrogen-fixing conditions or after addition of external hydrogen. Like the *hupSL*-genes, the transcription of the

	Nostoc muscorum		*Nostoc punctiforme*	*Anabaena* PCC 7120
	hupL	*hoxH*	*hupL*	*hoxH*
Condition				
+Ni	⇧	⇧	⇧	—
$+H_2$	⇧	⇨	⇧	—
$-O_2$	⇧	⇧	⇧	⇧
-N	⇧	⇨	⇧	—

Summary of results. Transcriptional regulation of *hupL* and *hoxH* in *Nostoc muscorum, Nostoc punctiforme,* and *Anabaena* PCC 7120 when exposed to different environmental conditions (papers I-IV).

+Ni = addition of 0.5 μM of $NiSO_4$
$+H_2$ = addition of 9% hydrogen
$-O_2$ = air replaced with argon and CO_2
-N = transfer from non- nitrogen to nitrogen-fixing conditions
⇧ = induction of the relative amount of transcript
⇨ = no change of the relative amountof transcript
— = not determined

genes encoding the bidirectional hydrogenase are regulated by nickel. This might be an important feature of nickel containing enzymes. This observation has been made in e.g. the nickel containing urease in *Helicobacter pylori* that was shown to be regulated on the transcriptional level by nickel (van Vliet et al 2001a, 2001b). Since the enzyme will be inactive if nickel is absent, it could be an investment for the cyanobacterium to restrict the transcription and translation.

The induction of the *hox*-genes during a transfer from aerobic to anaerobic conditions could be the result of a suggested physiological function, fermentation, of the bidirectional hydrogenase. Cyanobacteria encounter occasions where the environment becomes anaerobic, e.g. in microbial mats, and must therefore be able to deposit excess reducing power in another way than to use oxygen as the terminal electron acceptor. One way of doing this could be to produce hydrogen

through the presence of an active hydrogenase. Fermentative pathways that include hydrogen production have been suggested in several cyanobacteria (Stal and Moezalaar 1997, Troshina et al 2002). The enzymes involved in fermentation are constitutively expressed so that the cell can quickly respond to an anaerobic environment (Stal and Moezalaar 1997). In agreement, all conditions examined until today have demonstrated a *hox*-transcript.

Identification, cloning, and transcription of *hyp*-genes in *N. punctiforme* (paper III)

hyp-genes, putatively involved in the maturation of the uptake hydrogenase, were identified, cloned and sequenced in *Nostoc punctiforme*. *hypF*, *hypC*, *hypD*, *hypA*, and *hypB* were found to be located in a cluster with all genes orientated in the same direction (Fig. 8), approximately 3.8 kb from *hupSL*, however the

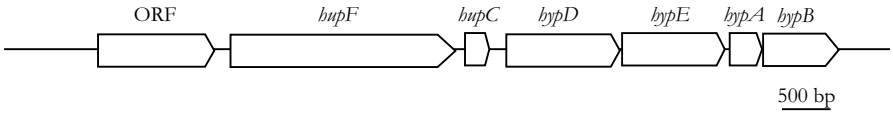

Fig. 8. Physical map of *hypFCDEAB* in *Nostoc punctiforme*. All *hyp*-genes, including the upstream ORF, are located on a single transcript (paper III).

opposite direction. Using RT-PCR with PCRs covering the intergenic regions of two adjacent genes, it was demonstrated that all genes are present on, at least, one transcript. In addition, upstream of *hypF* an ORF with unknown function was found to be part of the transcript containing all *hyp*-genes. All attempts to find a transcription start upstream of *hypF* and *hypC* were unsuccessful. However, a transcription start point was found 17 bp upstream of the putative first putative translation start of the upstream ORF. When examining the promotor region upstream of ORF-*hypFCDAB*-transcript, –10 and – 35 elements were identified as well as a putative NtcA binding site. A putative NtcA binding site was also identified upstream of *hupSL* in *Nostoc punctiforme* (Lindberg et al 2000).

Transcriptional studies were performed using non-nitrogen fixing and nitrogen-fixing conditions and a *hyp*-transcript was only detectable in nitrogen-

fixing cells. Similarly, a *hupL*-transcript is also present during nitrogen-fixing conditions (papers II, III). These results are in agreement with transcriptional studies in *Anabaena* PCC 7120, where RT-PCR experiments demonstrated the presence of a *hypB*-transcript in the heterocyst only (Gubili and Bothakur 1998).

As in other organisms, e.g. *Ralstonia eutropha*, *Rhizobium leguminosarum*, *Escherichia coli*, the *hyp*-genes in *Nostoc punctiforme* are located on one transcript (see Casalot and Rousset 2001). In *Nostoc punctiforme* the transcription may be under the control of NtcA, putatively coordinated with *hupSL*.

The *hyp*-genes, and predicted proteins, identified in *Nostoc punctiforme* contain conserved motifs that have been shown in other microorganisms to be essential for the maturation process. Even though likely, if the genes have an identical role in cyanobacteria remains to be demonstrated.

Large scale analysis of the gene expression in non-N_2 fixing and N_2-fixing conditions (paper V)

Based on the *Anabaena* PCC 7120 genome sequence, a large scale transcriptional study of selected genes, using microarrays was initiated. Total RNA was isolated from nitrogen-fixing and non-nitrogen fixing cultures of *Anabaena* PCC 7120, and used in a novel microarray technique where the respective probe is automatically synthesized and the hybridization is performed in a microchannel. In addition, both tRNA and rRNA are removed from the RNA preparation and the remaining mRNA is amplified using a modified Eberwine protocol (Eberwine et al 1992), before being hybridized.

We analyzed the expression of 1276 genes (approximately 20% of the complete genome) from all functional classes. Of the selected genes, 205 showed a significant difference in the two conditions. In nitrogen-fixing conditions 101 genes, mainly involved in energy metabolism, photosynthesis, respiration and nitrogen-fixation, were found to be stronger expressed. Even though the selected novel technique requires further improvements and optimization, it was possible to confirm, with a few exceptions, some of the results obtained in previous findings demonstrating that the technique used may be used to detect differences in gene expression under different environmental conditions.

FUTURE PERSPECTIVES

Most of the work in this thesis has focused on the transcriptional regulation of hydrogenases in filamentous, heterocystous cyanobacteria. The transcription has been examined during changes of important environmental conditions to understand the transcriptional regulation of the cyanobacterial uptake and bidirectional hydrogenase. All four environmental conditions examined (H_2, Ni, O_2, and N), positively induced the transcription of *hupSL* (papers I-III), while the transcription of the bidirectional hydrogenase was induced by Ni and O_2 (papers I, III, IV). These results raise interesting questions that may open up new directions within this area of research in cyanobacteria.

The four environmental conditions investigated are probably sensed and regulate the transcription of the hydrogenase genes in different ways. Future efforts could be to identify and characterize the genes that are involved in these processes. In a near future, investigations of a putative hydrogen sensor, nickel sensor and transport, the role of NtcA, and what components are involved in the transcriptional regulation of the hydrogenases at a low level of O_2, could be initiated. In addition, detailed studies of the promotor regions of the hydrogenases using fusions with a reporter gene could increase the understanding of the transcriptional regulation.

The roles of the ORFs that are located on the same transcript as the *hox*-genes in *Anabaena* PCC 7120 and *Anabaena variabilis* could be investigated and determine whether these genes are important for the bidirectional hydrogenase or not. Possible differences in the transcriptional regulation of hydrogenases in other cyanobacterial strains should also be investigated. There are some indications that the regulation might be different in different strains. For example, a NtcA binding site can be found upstream of *hupS* in *Nostoc punctiforme* but not in *Anabaena variabilis*.

The increasing number of completed cyanobacterial genomes will facilitate the search for novel genes and allow comparisons between the strains. With the microarray technique, it will be possible to set up experiments where the transcription of all expressed genes can be detected under different conditions. This might result in the discovery of novel genes involved in the regulation of the hydrogenases.

ACKNOWLEDGEMENTS

I wish to express my gratitude to:

My supervisor, Professor Peter Lindblad for your support, encouragement, and contagious ”positivism”.

The Cyanogroup, both present and former members.
Pia, José, Fernando, Per, Olga, Karin, Paulo, Marie, Ellinor, Åsa, Kjell, Caisa, Mehtap Alfred, Fredrik. You have all been great friends and created a nice atmosphere at Fysbot. Special thanks to Pia and José for all help with computer programs.
Röbbe, for all help with the manuscript and for yours and Catherine’s hospitality during my stay at your place in Cologne.

All the guests that have visited the Cyanogroup during the years.

Professor Peter Engström, for accepting me as a student.

Birgitta, Anita and Björn for all practical help at the department.

”The Plant people”.

All the people that have been involved in teaching.

All those who have helped me proof-reading the thesis.

Till sist ett stort tack till familj och vänner.
Mamma och pappa, som hela tiden stöttat och hjälpt till trots att ni bor så långt bort.
Riitta-Liisa och Janne, för all hjälp som jag och Anna fått under mitt skrivande.
Kalle, för omslagsbilden.
Fredrik och Ulrika med familjer.
Anna-panna, hur skulle det ha gått utan dig?
”Ville”, som har fått mig att sätta kommande händelser i rätt proportioner.

REFERENCES

Adams DG. 2000. Symbiotic interactions. In: The Ecology of Cyanobacteria (Whitton BA and Potts M, eds) pp 523-561. Kluver Academic Publishers, Dordrecht.

Adams MWW. 1990. The structure and mechanism of iron-hydrogenases. Biochim Biophys Acta 1020:115-145.

Appel J, and Schulz R. 1996. Sequence analysis of an operon of a NAD(P)-reducing nickel hydrogenase from the cyanobacterium *Synechocystis* sp. PCC 6803 gives additional evidence for direct coupling of the enzyme to NAD(P)H-dehydrogenase (complex I). Biochim Biophys Acta 1298:141-147.

Appel J, and Schulz R. 1998. Hydrogen metabolism with oxygenic photosynthesis: hydrogenases as important regulatory devices for a proper redox poising? J Photochem Photobiol B:Biol 47:1-11.

Appel J, Phupruch S, Steinmüller K, and Schulz R. 2000. The bidirectional hydrogenase of *Synechocystis* sp. works as an electron valve during photosynthesis. Arch Microbiol 173:333-338.

Baginsky C, Brito B, Imperial J, Palacios JM, and Ruiz-Argüeso T. 2002. Diversity and evolution of hydrogenase systems in rhizobia. Appl Environ Microbiol 68:4915-4924.

Berman-Frank I, Lundgren P, and Falkowski P. 2003. Nitrogen fixation and photosynthetic oxygen evolution in cyanobacteria. Res Microbiol 154:157-164.

Black LK, Fu C, and Maier RJ. 1994. Sequences and characterization of *hupU* and *hupV* genes of *Bradyrhizobium japonicum* encoding a possible nickel-sensing complex involved in hydrogenase expression. J Bacteriol 176:7102-7216.

Blokesch M, and Böck A. 2002. Maturation of [NiFe]-hydrogenases in *Escherichia coli*: the HypC cycle. J Mol Biol 324:287-296.

Boison G, Schmitz O, Mikheeva L, Shestakov S, and Bothe H. 1996. Cloning, molecular analysis and insertional mutagenesis of the bidirectional hydrogenase genes from the cyanobacterium *Anacystis nidulans*. FEBS Letters 394:153-158.

Boison G, Schmitz O, Schmitz B, and Bothe H. 1998. Unusual gene arrangement of the bidirectional hydrogenase and functional analysis of its diaphorase subunit HoxU in respiration of the unicellular cyanobacterium *Anacystis nidulans*. Curr Microbiol 36:253-258.

Boison G, Bothe H, Hansel A, and Lindblad P. 1999. Evidence against a common use of the diaphorase subunits by the bidirectional hydrogenase and by the respiratory complex I in cyanobacteria. FEMS Microbiol Lett 174:159-165.

Boison G, Bothe H, and Schmitz O. 2000. Transcriptional analysis of hydrogenase genes in the cyanobacteria *Anacystis nidulans* and *Anabaena variabilis* monitored by RT-PCR. Curr Microbiol 40:315-321.

Bowien B, and Schlegel HG. 1981. Physiology and biochemistry of aerobic hydrogen-oxidizing bacteria. Annu Rev Microbiol 35:405-52.

Brito B, Martinez M, Fernandez D, Rey L, Cabrera E, Palacios JM, Imperial J, and Ruiz-Argüeso T. 1997. Hydrogenase genes from *Rhizobium leguminosarum* bv. viciae are controlled by the nitrogen fixation regulatory protein NifA. Proc Natl Acad Sci 94:6019-6024.

Buurman, Shima S, and Thauer RK. 2000. The metal-free hydrogenase from methanogenic archea: evidence for a bound cofactor. FEBS Lett 485:200-204.

Böhme H. 1998. Regulation of nitrogen fixation in heterocyst-forming cyanobacteria. Trends Plant Sci. 9:346-351.

Carrasco CD, Buettner JA, and Golden JW. 1995. Programed DNA rearrangement of a cyanobacterial *hupL* gene in heterocysts. Proc Natl Acad Sci 92:791-795.

Casalot L, and Rousset M. 2001. Maturation of the [NiFe] hydrogenases. Trends Microbiol 5:228-236.

Colbeau A, and Vignais PM. 1992. Use of *hupS*::*lacZ* gene fusion to study regulation of hydrogenase expression in *Rhodobacter capsulatus*: stimulation by H_2. J Bacteriol 174:4258-4264.

Daday A, Mackerras AH, and Smith GD. 1985. The effect of nickel on hydrogen metabolism and nitrogen fixation in the cyanobacterium *Anabaena cylindrica*. Journ of Gen Microbiol 131:231-238.

Dischert W, Vignais PM, and Colbeau A. 1999. The synthesis of *Rhodobacter capsulatus* HupSL hydrogenase is regulated by the two-component HupT/HupR system. Mol Microbiol 34:995-1006.

Eberwine J, Yeh H, Miyashiro K, Cao Y, Nair S, Finnell R, Zettel M, and Coleman P. 1992. Analysis of gene expression in single live neurons. Proc Natl Acad Sci 89:3010-3014.

Eberz G, Eitinger T, and Friedrich B. 1989. Genetic determinants of a nickel-specific transport system are part of the plasmid-encoded hydrogenase gene cluster in *Alcaligenes eutrophus*. J Bacteriol 171:1340-1345.

Eberz G, and Friedrich B. 1991. Three trans-acting regulatory functions control hydrogenase synthesis in Alcaligenes eutrophus. J Bacteriol 173:1845-1854.

Eisbrenner G, Distler E, Floener L, and Bothe H. 1978. The occurance of the hydrogenase in some blue-green algae. Arch Microbiol 118:177-184.

Eitinger T, and Friedrich B. 1991. Cloning, nucleotide sequence, and heterologous expression of a high-affinity nickel transport gene from *Alcaligenes eutrophus*. J Biological Chemistry 266:3222-3227.

Eitinger T, and Mandrand-Berthelot M-A. 2000. Nickel transport in microorganisms. Arch Microbiol 173:1-9.

Elsen S, Colbeau A, Chabert J, and Vignais PM. 1996. The *hupTUV* is involved in negative control of hydrogenase synthesis in *Rhodobacter capsulatus*. J Bacteriol 171:5174-5181.

Elsen S, Colbeau A, and Vignais PM. 1997. Purification and in vitro phosphorylation of HupT, a regulatory protein controlling hydrogenase gene expression in *Rhodobacter capsulatus*. J Bacteriol 179:968-971.

Fischer HM. 1994. Genetic regulation of nitrogen fixation in rhizobia. Microbiol Rev 58:352-386.

Frias JE, Merida A, Herrero A, Martin-Nieto J, and Flores E. 1993. General distribution of the nitrogen control gene *ntcA* in cyanobacteria. J Bacteriol 175:5710-5713.

García-Domíngues M, Lopez-Maury L, Florencio FJ, and Reyes JC. 2000. A gene cluster involved in metal homeostasis in the cyanobacterium *Synechococcus* sp. strain PCC 6803. J Bacteriol 182:1507-1514.

Golden JW, Robinson SJ, and Haselkorn R. 1985. Rearrangement of nitrogen fixation genes during heterocyst differentiation in the cyanobacterium *Anabaena*. Nature. 1985 314:419-423.

Gubili J, and Borthakur D. (1998) Organization of the *hupDEAB* genes within the hydrogenase gene cluster of *Anabaena* sp. strain PCC7120. J of Appl Phyc 10:163-167.

Gunsalus RP, and Park SJ. 1994. Aerobic-anaerobic gene regulation in *Escherichia coli*: control by the ArcAB and Fnr regulons. Res Microbiol 145:437-50.

Halbleib CM, and Ludden PW. 2000. Regulation of biological nitrogen fixation. J Nutr 130:1081-1084.

Hallenbeck PC, and Beneman JR. 1978. Characterization and partial purification of the reverible hydrogenase of *Anabaena cylindrica*. FEBS Lett 94:261-264.

Happe T, Schütz K, and Böhme H. 2000. Transcriptional and mutational analysis of the uptake hydrogenase of the filamentous cyanobacterium *Anabaena variabilis* ATCC 29413. J Bacteriol 182:1624-1631.

Happe T, Hemschemeier A, Winkler M, and Kaminski A. 2002. Hydrogenases in green algae: do they save the algae's life and solve our energy problems? Trends Plant Sci 7:246-50.

Haselkorn R, and Buikema WJ. 1992. Nitrogen fixation in cyanobacteria. In: Biological nitrogen fixation. (Stacey G, Burris RH, and Evans HJ, eds) pp 166-190. Chapman and Hall, London.

Herrero A, Muro-Pastor AM, and Flores E. 2001. Nitrogen control in cyanobacteria. J Bacteriol 183:411-425.

Horner DS, Foster PG, and Embley TM. 2000. Iron hydrogenases and the evolution of anaerobic eukaryotes. Mol Biol Evol 17:1695-1709.

Houchins JP, and Burris RH. 1981a. Occurrence and localization of two distinct hydrogenases in the heterocystous cyanobacterium *Anabaena* sp. strain 7120. J Bacteriol 146:209-204.

Houchins JP, and Burris RH. 1981b. Comparative characterization of two distinct hydrogenases from *Anabaena* sp. strain 7120. J Bacteriol 146:215-221.

Houchins JP 1984. The physiology and biochemistry of hydrogen metabolism in cyanobacteria. Biochim Biophys Acta 768:227-255.

Howard HB, and Rees DC. 1996. Structural basis of biological nitrogen fixation. Chem Rev 96: 2965-2982.

Howitt CA, and Vermaas WFJ. 1997. Analysis of respiratory mutants of *Synechocystis* 6803. In: Book of abstracts of the IX International Symposium on Photosynthetic Prokaryotes (Peschek G A, Löffelhardt W, and Schmetterer G A, Eds, p 36, Vienna, Austria.

Hube M, Blokesch M, and Böck A. 2002. Network of hydrogenase expression in *Eschericha coli*: Role of accessory proteins HypA and HybF. J Bacteriol 14: 3879-3885.

Kaneko T, Sato S, Kotani H, Tanaka A, Asamizu E, Nakamura Y, Miyajima N, Hirosawa M, Sugiura M, Sasamoto S, Kimura T, Hosouchi T, Matsuno A, Muraki A, Nakazaki N, Naruo K, Okumura S, Shimpo S, Takeuchi C, Wada T, Watanabe A, Yamada M, Yasuda M, and Tabata S. 1996. Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. DNA Res 3:185-209.

Kaneko T, Nakamura Y, Wolk CP, Kuritz T, Sasamoto S, Watanabe A, Iriguchi M, Ishikawa A, Kawashima K, Kimura T, Kishida Y, Kohara M, Matsumoto M, Matsuno A, Muraki A, Nakazaki N, Shimpo S, Sugimoto M, Takazawa M, Yamada M, Yasuda M, and Tabata S. 2001. Complete genomic sequence of the filamentous nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120. DNA Res 8:205-213.

Kentemich T, Bahnweg M, Mayer F, and Bothe H. 1989. Localization of the reversible hydrogenase in cyanobacteria. Z Naturforch 44c:384-391.

Kiley PJ, and Beinert H. 1999. Oxygen sensing by the global regulator, FNR: the role of the iron-sulfur cluster. FEMS Microbiol Rev 22:341-352.

Kim HC, Yu C, and Maier RJ. 1991. Common cis-acting region responsible for transcriptional regulation of *Bradyrhizobium japonicum* hydrogenase by nickel oxygen, and hydrogen. J Bacteriol 173:3993-3999.

Kleihues L, Lenz O, Bernard M, Buhrke T, and Friedrich B. 2001 The H_2 sensor of *Ralstonia eutropha* is a member of the subclass of regulatory [NiFe] hydrogenases. J Bacteriol 182: 2716-2724.

Kumar S, and Polasa H. 1991. Influence of nickel and copper on photobiological hydrogen production and uptake in *Oscillatoria subbrevis* strain 111. Proc Indian Natn Sci Acad 281-285.

Lenz O, and Friedrich B. 1998. A novel multicomponent regulatory system mediates H_2 sensing in *Alcaligenes eutrophus*. Proc Natl Acad Sci 95:12474-12479.

Lindberg P, Hansel A, and Lindblad P. 2000. *hupS* and *hupL* constitute a transcription unit in the cyanobacterium *Nostoc* sp. PCC 73102. Arch Microbiol 174:129-133.

Lindberg P, Schütz K, Happe T, and Lindblad P. 2002. A hydrogen-producing, hydrogenase-free mutant strain of *Nostoc punctiforme* ATCC 29133. Int J Hydrogen Energ 27:1291-1296.

Lindblad P, Christensson K, Lindberg P, Fedorov A, Pinto F, Tsygankov A. 2002. Photoproduction of H_2 by wildtype *Anabaena* PCC 7120 and a hydrogen uptake deficient mutant: from laboratory experiments to outdoor culture. Int J Hydrogen Energ 27:1271-1281.

Lópes-Maury L, García-Domíngues M, Florencio FJ, and Reyes JC. 2002. A two-component signal transduction system involved in nickel sensing in the cyanobacterium *Synechocystis* sp. PCC 6803. Mol Microbiol 43:247-256.

Lutz S, Jacobi A, Schlensog V, Böhm R, Sawers G, and Böck A. 1991 Molecular characterization of an operon (*hyp*) necessary for the activity of the three hydrogenase isoenzymes in *Escherichia coli*. Mol Microbiol 5:123-35.

Maier T, Jacobi A, Sauter M, and Böck A. 1993. The product of the *hypB* gene, which is required for nickel incorporation into hydrogenases, is a novel guanine nucleotide-binding protein. J Bacteriol 175:630-635.

Malki S, Saimmaime I, De Luca G, Rousset M Dermoun Z, and Belaich J-P. 1995. Characterization of an operon encoding an NAPD-reducing hydrogenase in *Desulfovibrio fructosovorans*. J Bacteriol 177:2628-2636.

Masukawa H, Mochimaru M, and Sakurai H. 2002. Disruption of the uptake hydrogenase gene, but not of the bidirectional hydrogenase gene, leads to enhanced photobiological hydrogen production by the nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120. Appl Microbiol Biotechnol 58:618-624.

Meeks JC, Elhai J, Thiel T, Potts M, Larimer F, Lamerdin J, Predki P, and Atlas R. 2001. An overview of the genome of *Nostoc punctiforme*, a multicellular, symbiotic cyanobacterium. Photosynth Res 70:85-106.

Mehta N, Olson JW, and Maier RJ. 2003. Characterization of *Helicobacter pylori* nickel metabolism accessory proteins needed for maturation of both urease and hydrogenase. J Bacteriol 185:726-734.

Navarro C, Wu L-F, and Mandrand-Berthelot M-A. 1993. The *nik*-operon of *Escherichia coli* encodes a periplasmic binding-protein-dependent transport system for nickel. Mol Microbiol 9:1181-1191.

Nicolet Y, Piras C, Legrand P, Hatchikian CE, and Fontecilla-Camps JC. 1999. *Desulfovibrio desulfuricans* iron hydrogenase: the structure shows unusual coordination to an active site Fe binuclear center. Structure 7:13-23.

Nyström T, and Neidhart F C. 1992. Cloning, mapping and nucleotide sequencing of a gene encoding a universal stress protein in *Escherichia coli*. Mol Microbiol 6:3187-3198.

Olson JW, and Maier RJ. 1997. The sequences of *hypF*, *hypC* and *hypD* complete the *hyp* gene cluster required for hydrogenase activity in *Bradyrhizobium japonicum*. Gene 199:93-99.

Oxelfelt F, Tamagnini P, Salema R, and Lindblad P. 1995. Hydrogen uptake in *Nostoc* strain PCC 73102: Effects of nickel, hydrogen, carbon and nitrogen. Plant Physiol Biochem 33: 617-623.

Oxelfelt F, Tamagnini P, and Lindblad P. 1998. Hydrogen uptake in *Nostoc* sp. strain PCC 73102. Cloning and characterization of a *hupSL* homologue. Arch Microbiol 169:267-274.

Peters JW, Lanzilotta WN, Lemon BJ, and Seefeldt LC. 1998. X-ray crystal structure of the Fe-only hydrogenase (Cpl) from *Clostridium pasteurianum* to 1.8 angstrom resolution. Science 282:1853-1858.

Peterson RB, and Wolk PC. 1978. Localization of an uptake hydrogenase in *Anabaena*. Plant Physiol 61:688-691.

Pienkos PT, Bodmer S, and Tabita FR. 1983. Oxygen inactivation and recovery of nitrogenase activity in cyanobacteria. J Bacteriol 153:182-90.

Rain JC, Selig L, De Reuse H, Battaglia V, Reverdy C, Simon S, Lenzen G, Petel F, Wojcik J, Schachter V, Chemama Y, Labigne A, and Legrain P. 2001. The protein-protein interaction map of *Helicobacter pylori*. Nature. 409:211-215.

Ramasubramanian TS, Wei TF, and Golden JW. 1994. Two *Anabaena* sp. strain PCC 7120 DNA-binding factors interact with vegetative cell- and heterocyst-specific genes. J Bacteriol 176:1214-1223.

Reissmann S, Hochleitner E, Wang H, Paschos A, Lottspeich F, Glass RS, and Böck A. 2003. Taming of a Poison: Biosynthesis of the NiFe-Hydrogenase Cyanide Ligands. Science 299:1067-1070.

Rey L, Murillo J, Hernando Y, Hidalgo E, Cabrera E, Imperial J, and Ruiz-Argüeso T. 1993. Molecular analysis of a microaerobically induced operon required for hydrogenase synthesis in *Rhizobium leguminosarum* biovar viciae. Mol Microbiol 8:471-481.

Rey L, Imperial J, Palacios JM, and Ruiz-Argüeso T. 1994. Purification of *Rhizobium leguminosarum* HypB, a nickel-binding protein required for hydrogenase synthesis. J Bacteriol 176:6066-6073.

Richard D J, Sawers G, Sargent F, McWalter L, and Boxer DH. 1999. Transcriptional regulation in response to oxygen and nitrate of the operons encoding the [NiFe] hydrogenases 1 and 2 of *Escherichia coli*. Microbiol 145: 2903-2912.

Rippka R, Deruelles J, Waterbury JB, Herdman M, and Stanier RY. 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria.

J Gen Microbiol 111:1-61.

Schmitz O, Boison G, Hilscher R, Hundeshagen B, Zimmer W, Lottspeich F, and Bothe H. 1995. Molecular biological analysis of a bidirectional hydrogenase from cyanobacteria. Eur J Biochem 233:266-276.

Schmitz O, and Bothe H. 1996. NAD(P)+-dependent hydrogenase activity in extracts from the cyanobacterium *Anacystis nidulans*. FEMS Microbiol Lett 135:97-101.

Schmitz O, Boison G, and Bothe H. 2001. Quantitative analysis of expression of two circadian clock-controlled gene clusters coding for the bidirectional hydrogenase in the cyanobacterium *Synechococcus* sp. PCC 7942. Mol Microbiol 41:1409-1417.

Schmitz O, Boison G, Salzman H, Bothe H, Schütz K, and Happe T. 2002. HoxE-a subunit specific for the petameric bidirection hydrogenase coplex (HoxEFUYH) of cyanobacteria. Biochim Biophys Acta 1554:66-74.

Sellstedt A, and Smith GD. 1990. Nickel is essential for active hydrogenase in free-living *Frankia* isolated from *Casuarina*. FEMS Microbiol Lett 70:137-140.

Serebryakova LT, Zorin NA, and Lindblad P. 1994. Reversible hydrogenase in *Anabaena variabilis* ATCC 29413: presence and localization in non-N_2 fixing cells. Arch Microbiol 161:140-144.

Serebryakova LT, Sheremetieva M, and Lindblad P. 2000. H_2-uptake and evolution in the unicellular cyanobacterium *Chroococcidiopsis thermalis* CALU 758. Plant Physiol Biochem 38:525-530.

Sheremetieva M, Troshina O, Serebryakova LT, and Lindblad P. 2002. Identification of *hox* genes and analysis of their transcription in the unicellular cyanobacterium *Gloeocapsa alpicola* CALU 743 growing under nitrate-limiting conditions. FEMS Microbiol Lett 214:229-233.

Smith RL, Maguire ME. 1998 Microbial magnesium transport: unusual transporters searching for identity. Mol Microbiol 28:217-26.

Stal L J and Moezelaar R. 1997. Fermentation in cyanobacteria. FEMS Microbiol Rev 21: 179-211.

Tamagnini P, Troshina O, Oxelfelt F, Salema R, and Lindblad P. 1997. Hydrogenases in *Nostoc* sp. PCC 73102, a strain lacking a bidirectional hydrogenase. Appl Environ Microbiol 63:1801-1807.

Tamagnini P, Costa J-L, Almeida L, Oliveria M-J, Salema R, and Lindblad P. 2000. Diversity of cyanobacterial hydrogenases, a molecular approach. Curr Microbiol 40:356-360.

Thauer RK, Klein AR, and Hartmann GC. 1996. Reactions with molecular hydrogen in microorganisms: Evidence for a purely organic hydrogenation catalyst. Chem Rev 96:3031-3042.

Thiel T, Lyons EM, Erker JC, and Ernst A. 1995. A second nitrogenase in vegetative cells of a heterocyst-forming cyanobacterium. Proc Natl Acad Sci 92:9358-9362.

Thiel T, and Pratte B. 2001. Effect on heterocyst diffrentiation of nitrogen fixation in vegetative cells of the cyanobacterium *Anabaena variabilis* ATCC 29413. J Bacteriol 183:280-286.

Troshina OY, Serebryakova LT, and Lindblad P. 1996. Induction of H_2-uptake and nitrogenase activities in the cyanobacterium *Anabaena variabilis* ATCC 29413: effects of hydrogen and organic substrate. Curr Microbiol 33:11-15.

Troshina O, Serebryakova L, Sheremetieva M, and Lindblad P. 2002. Production of H_2 by the unicellular cyanobacterium *Gloeocapsa alpicola* CALU 743 during fermentation. Int J Hydrogen Energ 27:1283-1289.

van Soom C, de Wilde P, and Vanderleyden J. 1997. HoxA is a transcriptional regulator for expression of the hup structural genes in free-living *Bradyrhizobium japonicum*. Mol Microbiol 23:967-977.

van Soom C, Lerouge I, Vanderleyden J, Ruiz-Argüeso T, Palacios JM. 1999. Identification and characterization of *hupT*, a gene involved in negative regulation of hydrogen oxidation in Bradyrhizobium japonicum. J Bacteriol 181:5085-5089.

van Soom C, Verreth C, Sampaio MJ, Vanderleyden J. 1993. Identification of a potential transcriptional regulator of hydrogenase activity in free-living *Bradyrhizobium japonicum* strains. Mol Gen Genet. 239:235-40.

van Vliet AH, Poppelaars SW, Davies BJ, Stoof J, Bereswill S, Kist M, Penn CW, Kuipers EJ, Kusters JG. 2001a. NikR mediates nickel-responsive transcriptional induction of urease expression in *Helicobacter pylori*. Infect Immun 69:4891-4897.

van Vliet AH, Kuipers EJ, Waidner B, Davies BJ, de Vries N, Penn CW, Vandenbroucke-Grauls CM, Kist M, Bereswill S, and Kusters JG. 2001b. Nickel-responsive induction of urease expression in *Helicobacter pylori* is mediated at the transcriptional level. Infect Immun 69:4891-4897.

Vázquez-Bermúdez MF, Flores E, Herrero A. 2002. Analysis of binding sites for the nitrogen-control transcription factor NtcA in the promoters of *Synechococcus* nitrogen-regulated genes. Biochim Biophys Acta 1578:95-98.

Verhagen FJM, O´Rourke T, and Adams MWW. 1999. The hyperthermophilic bacterium, *Thermotoga maritima*, contains unusually complex iron-hydrogenase: amino acid sequence analyses versus biochemical characterization. Biochim Biophys Acta 1412:212-229.

Vignais P, Billoud B, and Meyer J. 2001. Classification and phylogeny of hydrogenases. FEMS Microbiol Rev 25:455-501.

Vincent WF. 2000. Cyanobacterial dominance in the polar regions. In: The Ecology of Cyanobacteria (Whitton BA and Potts M, eds) pp 321-340. Kluver Academic Publishers, Dordrecht.

Volbeda A, Charon MH, Piras C, Hatchikian EC, Frey M, and Fontecilla-Camps JC. 1995. Crystal structure of the nickel-iron hydrogenase from *Desulfovibrio gigas*. Nature 373:580-587.

Wagner R. 2000. The master-bender integration host factor (IHF) and its family of DNA-binding proteins. In: Transcription regulation in prokaryotes pp 227-232. Oxford University Press.

Ward DM, and Castenholz RW. 2000. Cyanobacteria in geothermal habitats. In: The Ecology of Cyanobacteria (Whitton BA and Potts M, eds) pp 37-59. Kluver Academic Publishers, Dordrecht.

Wei TF, Ramasubramanian TS, and Golden JW. 1994. *Anabaena* sp. strain PCC 7120 *ntcA* gene required for growth on nitrate and heterocyst development. J Bacteriol 176:4473-4482.

Wolf I, Buhrke T, Dernedde J, Pohlmann A, and Friedrich B. 1998. Duplication of *hyp* genes involved in maturation of [NiFe] hydrogenases in *Alcaligenes eutrophus* H16. Arch Microbiol 170:451-459.

Wolk CP. 1996. Heterocyst formation. Annu Rev Genet 30:59-78.

Wu LF, and Mandrand-Berthelot MA. 1986. Genetic and physiological characterization of new Escherichia coli mutants impaired in hydrogenase activity. Biochimie 68:167-179.

Wu LF, Mandrand-Berthelot MA, Waugh R, Edmonds CJ, Holt SE, and Boxer DH. 1989. Nickel deficiency gives rise to the defective hydrogenase phenotype of *hydC* and *fnr* mutants in *Escherichia coli*. Mol Microbiol 3:1709-1718.

Wu LF, and Mandrand MA. 1993. Microbial hydrogenases: primary structure, classification, signatures and phylogeny. FEMS Microbiol Rev 10:243-269.

Wünschiers R, Senger H, and Schulz R. 2001. Electron pathways involved in H_2-metabolism in the green alga *Scenedesmus obliquus*. Biochim Biophys Acta 1503:271-278.

Wünschiers R, Batur M, and Lindblad P. 2003. Presence and expression of hydrogenase specific C-terminal endopeptidases in cyanobacteria. BMC Microbiol 3:8

Wünschiers R and Lindblad P. 2003. Light-dependent hydrogen uptake and generation by cyanobacteria. Handbook of Photochemistry and Photobiology, in press

Wynn-Williams DD. 2000. Cyanobacteria in deserts – Life at the limit? In: The Ecology of Cyanobacteria (Whitton BA and Potts M, eds) pp 341-366. Kluver Academic Publishers, Dordrecht.

Xiankong Z, Tabita, and van Baalen C. 1984. Nickel control of hydrogen production and uptake in *Anabaena* spp. strains CA and 1F. J Gen. Microbiol 130:1815-1818.

Zinn T, Schnackenberg J, Haak D, Roemer S, Schulz R, and Senger H. 1994. Evidence for nickel in the soluble hydrogenase from the unicellular green algae *Scenedesmus obliquus*. Z Naturforsch 49c:33-38.

Acta Universitatis Upsaliensis

Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology

Editor: The Dean of the Faculty of Science and Technology

A doctoral dissertation from the Faculty of Science and Technology, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series *Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology*. (Prior to October, 1993, the series was published under the title "Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science".)

Distribution:
Uppsala University Library
Box 510, SE-751 20 Uppsala, Sweden
www.uu.se, acta@ub.uu.se

ISSN 1104-232X
ISBN 91-554-5743-6