Maturation and Regulation of Cyanobacterial Hydrogenases

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Abstract

Accelerated global warming plus an increasing need for energy is an equation not easily solved, thus new forms of sustainable energy production are urgently requested. In this context hydrogen production based on a cyanobacterial system offers an environmentally friendly alternative for energy capture and conversion. Cyanobacteria can produce hydrogen gas from sun light and water through the combination of photosystems and hydrogenases, and are suitable to cultivate in large scale.

In the present thesis the maturation process of [NiFe]-hydrogenases is investigated with special focus on transcription of the accessory genes encoding proteins needed for assembly of the large and possibly also for the small hydrogenase subunit. The cyanobacteria used are two N2-fixing, filamentous, heterocystous strains; Nostoc sp. strain PCC 7120 and Nostoc punctiforme PCC 73102.

For a biotechnological exploration of hydrogen production tools for regulatory purposes are important. The transcription factor CalA (cyanobacterial AbrB like) (Alr0946 in the genome) in Nostoc sp. strain PCC 7120 was found to be involved in hydrogen metabolism by regulating the transcription of the maturation protein HypC. Further the bidirectional hydrogenase activity was down-regulated in the presence of elevated levels of CalA, a result important to take into account when optimizing cyanobacteria for hydrogen production.

CalA regulates at least 25 proteins in Nostoc sp. strain PCC 7120 and one of the down-regulated proteins was superoxide dismutase, FeSOD. The characterization of FeSOD shows that it has a specific and important function in the oxidative stress tolerance of Nostoc sp. stain PCC 7120. Since CalA is involved in regulation of both the hydrogen metabolism as well as stress responses these findings indicate that Alr0946 is an important transcription factor in Nostoc sp. strain PCC 7120 active on a global level in the cell.

This thesis adds more knowledge concerning maturation and regulation of cyanobacterial hydrogenases which might be useful for future large scale hydrogen.

Keywords: Biohydrogen, Cyanobacteria, Nostoc sp. PCC 7120, Nostoc punctiforme PCC 73102, Hydrogenase, Maturation, Transcriptional regulation, CyAbrB, CalA, FeSOD

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


III. Holmqvist, M., Agervald, Å., K. Stensjö, and P. Lindblad. Transcript level analysis of five ORFs putatively involved in the maturation of the uptake hydrogenase small subunit in two N2-fixing cyanobacteria, Nostoc sp. strain PCC 7120 and Nostoc punctiforme ATCC 29133. Manuscript.


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<td>5´Rapid Amplifications of cDNA ends</td>
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<td>abrB</td>
<td>Antibiotic resistance gene B</td>
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<td>ATP</td>
<td>Adenosine-5'-triphosphate</td>
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<td>BLAST</td>
<td>Basic local alignment search tool</td>
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<td>bp</td>
<td>Base pair</td>
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<td>Cyanobacterial AbrB like gene A</td>
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<td>EMSA</td>
<td>Electorostatic mobility shift assay</td>
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<td>IMAC</td>
<td>Ion metal affinity chromatography</td>
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<td>NHL</td>
<td>NCL-1, HT2A and Lin-41</td>
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<td>mRNA</td>
<td>Messenger RNA</td>
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<td>Mass spectrometry</td>
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<td>NADH</td>
<td>Nikotinamid adenine dinucleotide</td>
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<td>ORF</td>
<td>Open reading frame</td>
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<td>PCC</td>
<td>Pasteur culture collection</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>ppm</td>
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<td>PSI / PSII</td>
<td>Photosystem I / Photosystem II</td>
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<td>PTM</td>
<td>Post-translational modification</td>
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<td>RT-PCR</td>
<td>Reverse transcriptase PCR</td>
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<td>RTq-PCR</td>
<td>Reverse transcriptase quantitative PCR</td>
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<td>SOD</td>
<td>Superoxide dismutase</td>
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<td>TPR</td>
<td>Tetra/trico peptide repeat</td>
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<td>TSP</td>
<td>Transcriptional start point</td>
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Introduction

Immediate changes are required

The population on Earth increases every day and United Nations predict that there will be approximately 9 billion people year 2050, which can be compared with 6.8 billion people 2010 [United Nations, 1999]. During the 21st century the population in the western industrialized countries will not change significantly, but the increase will come in countries where the industrial development is progressing fast, especially in China and India. Many people will benefit from this development and poverty can be decreased. As a consequence the global energy consumption is estimated to at least double until year 2050, a fact which points at two central energy challenges. Firstly, the supply of reliable and affordable energy sources has to be secured and secondly, a rapid transformation to an efficient, low-carbon environmentally friendly system has to take place [International Energy Agency, 2008].

It is no news that consumption of fossil fuels potentially produces significant global issues with irreversible damage to the global climate. CO₂ emissions have been accumulating in the atmosphere on a timescale of many centuries. The CO₂ equilibrates between the atmosphere and the near-surface layer of the oceans in approximately 10-30 years, which gives the answer to why only 50% of the CO₂ emissions have remained in the atmosphere. However, the near-surface layer of the oceans are now saturated and it takes somewhere between 400 and several thousand years to get a relevant mixing between the surface and the deep oceans [Lewis and Nocera, 2006]. During the Earth’s history the changes in temperature and gas compositions have been many and the CO₂ level peaks fluctuate in a rather periodic pattern with approximately 1000 years intervals. The atmospheric CO₂ concentrations, which are considered natural, have varied between 210 and 300 ppm for the past 420 000 years. Although the CO₂ fluctuations are highly debated, the value for 2006, 380 ppm, is above the highest peak in history and the trend is still increasing [Lewis and Nocera, 2006]. There are many climate models made to predict what will happen on the Earth when the atmospheric CO₂ concentration goes up. Some of them predict minor changes, while others predict serious scenarios with for example rising sea levels, accelerated loss of permafrost with release of more greenhouse gases (CO₂ and CH₄) as a consequence and changes in the hydrological cycle [Lewis and Nocera,
The member countries of the European Union are working towards the goal of 450 ppm CO\textsubscript{2} in the atmosphere 2050, which would end up to an estimated increase in mean temperature with +2 °C [International Energy Agency, 2008; European Commission, 2008]. Other calculations based on stabilizing atmospheric CO\textsubscript{2} in the 550-650 ppm range predict an increase in temperature with +3 °C, but if nothing is done values reaching over 750 ppm with +6 °C higher temperatures as a consequence, are not far fetched [International Energy Agency, 2008; Lewis and Nocera, 2006]. What can be said for certain is that the level of CO\textsubscript{2} will continue to rise and that this will influence life on Earth considerably. Notably is that these changes probably will happen before the reserves of fossil fuels are finished.

Calculations made on the fossil fuel reserves on Earth, based on 1998 consumption rates, predict that there are oil reserves enough for 40-80 years, natural gas for 60-160 years, and supply of coal for 1000-2000 years. However, the calculations made in 1998 did not cover the increasing need for energy in Asia, resulting in a further increase in global energy consumption [Lewis and Nocera, 2006]. These predictions of the fossil fuel reserves may sound comforting, but with the emerging knowledge concerning the effects of increasing CO\textsubscript{2} levels and a predicted twofold increase in energy consumption alternatives to fossil has to be developed. Furthermore, hundreds of millions of businesses, households and motorists have to change their ways of living to reduce their individual energy consumptions. Governments have to take their responsibilities and create innovative regulatory frameworks as well as invest in infrastructure to integrate climate-policy goals with a steady supply of reliable and affordable energy. All countries have to start up long-termed cooperation plans to implement the changes and ensure broad participation in these. Everyone has to understand that all have to participate to make difference. The global carbon market has to be further developed and more investments into energy research and development are needed [International Energy Agency, 2008].

Molecular hydrogen (H\textsubscript{2}) is an energy carrier possible to convert via fuel cells to power without CO\textsubscript{2} emission and with high efficiency. H\textsubscript{2} can be produced from many different energy sources for example biomass, nuclear energy or solar and wind energy. In addition H\textsubscript{2} provides national security since most countries can produce this energy carrier independent of other countries. The European Union has developed a roadmap and action plan, HyWays, where impacts on environment, society and economy of a large scale introduction of hydrogen are analyzed. The Roadmap covers forty years, 2010 to 2050, and contains for example technology development of hydrogen production, vehicle development and building of infrastructure suited for hydrogen gas [European Commission, 2008]. In Sweden the short
term goal is that 10% of the energy used in the transport sector 2010 should come from renewable energy [Energimyndigheten, 2008].

In this thesis the production of hydrogen gas will be highlighted. The approach is to produce hydrogen on a sustainable basis by using solar energy, water and a system based on cyanobacteria. These microorganisms are able to convert the solar energy into hydrogen gas via the enzyme hydrogenase and/or nitrogenase, while at the same time reducing the amount of CO₂ in the atmosphere. The only by-products are oxygen gas and biomass, of which the latter can be dried, processed into pellets and burned thereby providing more energy or be used as fertilizer. This thesis focus especially on maturation and regulation processes of hydrogenases in the model organism *Nostoc* sp. strain PCC 7120.

**Solar bio-hydrogen, H₂ from sun and water**

In the search for alternative and sustainable energy carriers an obvious energy source is the sun. Solar energy has enormous potential since it is clean, abundant and cheap. Hydrogen gas is often considered the optimal energy carrier of the future since it provides energy with water vapor as the only combustion emission. Cyanobacteria and green algae are the only organisms capable of performing oxygenic photosynthesis and producing molecular hydrogen from sun and water via the enzymes hydrogenase and nitrogenases (Fig. 1) [Happe and Naber, 1993; Houchins, 1984; Tamagnini et al., 2007; Tsygankov, 2007].

![Figure 1: Schematic picture of the energy transfer from solar energy to hydrogen via the photosystems (PSI and PSII) and the bidirectional hydrogenase in cyanobacteria.](image-url)
Important parameters for hydrogen production are; i) efficiency i.e. high turnover number, ii) robustness of the system i.e. a long-term stability, and iii) a low over-potential. Before hydrogen production in large scale based on a cyanobacterial approach can be a reality, there are some modifications requested. The native hydrogenases in cyanobacteria are not very active and to raise the productivity introduction of foreign hydrogenases into cyanobacterial systems are of interest. Reaction rates for certain hydrogenases can be very high with turnover rates in the range of $10^3$-$10^4$ per second at 30ºC [Pershad et al., 1999]. Furthermore, hydrogenases can be as effective as platinum (Pt) for reduction of protons when applied to graphite electrodes [Leger et al., 2002], an interesting fact since platinum is both highly priced and existing in low amounts. Hydrogenases on the other hand contain abundant and inexpensive metals as iron (Fe) and nickel (Ni). These metals are positioned in the hydrogenase active site, which requires a complex maturation process to become functional. To optimize this process research, for example presented in this thesis, is focusing on which proteins and regulatory networks are necessary for the organism. A drawback is that most hydrogenases, with the exception of three hydrogenases in *Ralstonia eutropha*, are sensitive to oxygen which inactivates the enzyme [Buhrke et al., 2005]. However, [NiFe]-hydrogenases can be reactivated again when oxygen is removed, which is not possible for the more efficient [FeFe]-hydrogenase. The exact mechanism for the inactivation is not known, but it is proposed that oxygen use the same gas channel as hydrogen to the active site, thereby blocking the pathway. Much effort is put into modification of the amino acids in the channel to prohibit the oxygen molecule to enter. The chemical reaction performed by hydrogenases, to split or produce H$_2$, is reversible and the direction depends on the redox potential of existing substrates. The presence of low-potential electron donors are essential to drive the reaction towards hydrogen formation from water [Vignais and Billoud, 2007]. Electrons are the limiting factor in hydrogen production and mapping electron pathways are necessary to be able to re-rout the electrons and focus the energy to hydrogen production instead of production of biomass. Furthermore, continuous removal of produced H$_2$ is necessary since the hydrogenase work close to its chemical equilibrium and gets inhibited above a certain partial pressure of H$_2$ [Angermayer et al., 2009].

Parallel to the development of hydrogen production effort must be put on fuel cells, by raising the efficiency and lower the device cost, as well as addressing the development and cost of the new infrastructure needed, for example storage and transport.
Cyanobacteria

“Cyanobacteria” is partly a Greek word, kyanós, which means blue while the second part classifies the organisms into the bacterial kingdom. Initially cyanobacteria were classified as algae and they are often still referred to as blue-green algae. Ancestors to today’s cyanobacteria developed early in evolution and fossil traces have been found dating back ~3.8 billion years [Knoll, 2008]. Somewhere around 2.8-2.4 billion years ago cyanobacteria evolved photosystem II, the start point for development of today’s atmospheric oxygen, which dramatically changed the life conditions on Earth provoking an explosion of biodiversity [Knoll, 2008]. Through this evolutionary step cyanobacteria did not need any external electron acceptors and were able to perform oxygenic photosynthesis using water as the electron donor. To adapt the anaerobic electron-transport chains to an aerobic habitat a cytochrome oxidase was evolved [Imlay, 2006]. The fortunate combination of being able to reduce both nitrogen and carbon under aerobic conditions are most likely one of the most important answers to the ecological success of cyanobacteria. Even though cyanobacteria are not part of the eukaryotic kingdom they have contributed significantly, since it is believed that an ancient cyanobacterium has been engulfed by a plant cell and is ancestor to the chloroplast [Miyagishima, 2005; Mulkidjanian et al., 2006].

Cyanobacteria have an extraordinary capacity to adapt to almost any type of conditions on Earth and are therefore found in environments ranging from fresh water to oceans, from terrestrial to arctic environments and from bare rock to soil [Witton and Potts, 2000]. A few cyanobacteria are endosymbionts in for example lichens and plants. In such associations nitrogen is fixed by the cyanobacteria and is exchanged with the plant for carbohydrates. Additionally the plant offers a safe haven for the cyanobacteria [Meeks et al., 2002].

Among cyanobacteria there are a wide range of morphs including unicellular, colonial and filamentous forms. Some filamentous strains show the ability to differentiate into four different cell types: vegetative cells, akinetes, hormogonia and heterocysts. Most cells are vegetative cells, photosynthetic cells formed under favorable growing conditions. Hormogonia, involved in symbiosis, are short and mobile filaments formed in response to different environmental stresses. When the environmental conditions become extremely harsh akinetes, a spore, can be formed. In this cell type high level of valuable substances such as nitrogen and glycogen are stored and the akinete is very resistant to both cold and draught. When the environment gets more favorable the akinete can develop into a vegetative cell [Meeks et al., 2002]. The fourth cell type, the heterocyst, occur in a semi-regular pattern in some filamentous strains at a frequency of 5-10% of
the total cells, when there is no combined nitrogen source in the surrounding environment. The irreversible development of a vegetative cell into a heterocyst takes approximately 24 hours [Yoon and Golden, 1998; Huang et al., 2004]. In the heterocysts the oxygen level is kept very low and the micro-oxygenic environment is created mainly in three ways: there is no, or possibly low amounts of photosystem II which means that oxygen is present only at a very low level, heterocyst has a thick envelope consisting of an inner layer composed of glycolipids and an outer layer composed of polysaccharides protecting the cell from oxygen penetration and finally the respiration is high with oxidases quickly consuming present oxygen [Bergman et al., 1997; Meeks et al., 2002; Meeks and Elhai, 2002; Tamagnini et al., 2007; Tsygankov, 2007; Cardona et al., 2009]. Heterocysts harbor the oxygen sensitive nitrogenase, the enzyme capable of fixing the atmospheric nitrogen, and hydrogenases.

Cyanobacteria are classified as Gram-negative, although many species have cell envelopes with features specific to a Gram-positive envelope, in terms of thickness of the peptidoglycan layer, lipid components etc. Cyanobacteria are covered with a gelatinous sheath followed by an outer membrane, a peptidoclycan layer and a cytoplasmic or plasma membrane [Liberton and Pakrasi, 2008].

The model organisms

_Nostoc_ sp. strain PCC 7120

_Nostoc_ sp. strain PCC 7120, also called _Anabaena_ sp. strain PCC 7120, (here-after referred to as _Nostoc_ PCC 7120) is a photoautotrophic, filamentous and heterocyst-forming cyanobacterium capable of N₂-fixation (Fig. 2). The genome is fully sequenced, approximately 7.21 Mb, and divided on one chromosome and six plasmids [Sazuka et al., 1999; Kaneko et al. 2001]. Techniques for genetic manipulation including an efficient conjugation system are available, which have made this strain suitable for studies of genetics and physiology of cellular differentiation, pattern formation and nitrogen fixation. It is possible to grow _Nostoc_ PCC 7120 in bioreactors suitable for large scale production of hydrogen. _Nostoc_ PCC 7120 may contain both an uptake and a bidirectional hydrogenase, and one set of _hyp_ genes.

_Nostoc punctiforme_ ATCC 29133

_Nostoc punctiforme_ ATCC 2913, also called _Nostoc_ sp. PCC 73102 or _Nostoc punctiforme_ (here-after referred to as _N. punctiforme_), is a photoautotrophic, filamentous and heterocyst-forming cyanobacteria capable of nitro-
N. punctiforme can induce the formation of hormogonia and akinetes when needed. N. punctiforme was originally isolated from the roots of a cycad of the Macrozamia species, where it lives in a symbiotic association with the plant. In the dark roots the cyanobacteria fix atmospheric nitrogen and exchange this compound for carbohydrates produced by the plant during photosynthesis [Meeks et al., 2002]. The N. punctiforme genome is sequenced, 9.06 Mb divided on one chromosome and five plasmids, which is significantly larger than any other sequenced cyanobacteria so far pointing at an underlying complexity not yet understood [Meeks et al., 2001; Anderson et al., 2006]. N. punctiforme contains an uptake hydrogenase and one set of hyp-genes.

Figure 2: The filamentous, heterocystous, N₂-fixing cyanobacteria Nostoc sp. strain PCC 7120. The majority of the cells in the filaments are vegetative cells photosynthetically active (dark green cells), while 5-10% are heterocysts, harboring the oxygen sensitive nitrogenase responsible for N₂-fixation (bigger light green cells).

Hydrogen metabolism in cyanobacteria

The enzymes directly involved in hydrogen metabolism in cyanobacteria are hydrogenases and nitrogenases. Depending on cyanobacterial strain, a single cell can harbor a bidirectional hydrogenase, an uptake hydrogenase or both (Fig. 3).

Nitrogenase

Organisms harboring a nitrogenase are very fortunate since they can survive even if the concentrations of combined nitrogen get low or non-existing.
Nitrogenase fixes nitrogen gas into ammonia (NH\textsubscript{3}), which is later converted into proteins and nucleic acids [Meeks et al., 2002]. There are different types of nitrogenases based on the metal composition in the active site. The molybdenum (Mo) containing nitrogenase fixes the atmospheric nitrogen according to following formula: \( \text{N}_2 + 8e^- + 8 \text{ATP} \rightarrow 2\text{NH}_3 + \text{H}_2 + 8 \text{ADP} + 8 \text{Pi} \) [Tamagnini et al., 2007]. Since eight ATP are consumed per nitrogen molecule fixed this process is very costly for the cell. As by-product of nitrogen fixation molecular hydrogen is formed and this energy rich molecule is instantly recycled by the uptake hydrogenase. So far Synechococcus sp. BG 043511 is the only exception found, which is able of N\textsubscript{2}-fixation but is lacking an uptake hydrogenase [Ludwig et al., 2006]. Nitrogenases as well as hydrogenases are sensitive to O\textsubscript{2}, which inactive the proteins. In order to protect these enzymes cyanobacteria have developed different strategies based on either spatial or temporal separation, to part O\textsubscript{2} evolving photosynthesis from N\textsubscript{2}-fixation. In filamentous heterocystous strains the enzymes are located in heterocyst and in unicellular or filamentous strains lacking heterocysts the enzymes function under anaerobic conditions only e.g. during the night when there is no oxygen produced by PSII [Bergman et al. 1997; Tsygankov, 2007].

**Hydrogenases**

Hydrogenases are enzymes which catalyze the reaction where elemental hydrogen is oxidized into protons and electrons \( \text{H}_2 \leftrightarrow 2\text{H}^+ + 2e^- \), a process not directly dependent on ATP. The reaction can also go in the opposite direction, to reduce protons into a hydrogen molecule. The redox-potential of the individual electron acceptors or donors able to interact with the enzyme determines which way the reaction goes [Vignais and Colbeau, 2004; Böck et al., 2006; Vignais and Billoud, 2007]. Hydrogenases are involved in three main tasks: participate in energy conservation, provide a sink for electrons, and function as regulatory or H\textsubscript{2}-sensing hydrogenases. The H\textsubscript{2}-sensing hydrogenases control hydrogenase gene expression in response to the stimuli of elemental hydrogen, but are not present in cyanobacteria [Böck et al., 2006; Tamagnini et al., 2007].

On the basis of the metal found in the active site of the enzyme hydrogenases are classified into three different groups: [FeFe]-hydrogenases, [NiFe]-hydrogenases and [Fe]-hydrogenases [Volbeda et al., 1995; Peters, 1999; Lyon et al., 2004; Vignais and Billoud 2007]. All cyanobacterial hydrogenases belong to the group [NiFe]-hydrogenases [Vignais et al., 2001; Shestakov and Mikheeva, 2006; Tamagnini et al., 2007]. The active site has a very complex structure with one Ni and one Fe atom, to which the biochemically unusual ligands of CN and CO are bound. The Ni atom has a vacant site which is believed to be the binding position for the substrate hy-
Hydrogen gas and a hydrophobic gas channel leading directly from the Ni atom to the surface of the protein has been modeled in experiments performed under high levels of xenon (Xe) gas and with molecular dynamic calculations. This channel is assumed to be blocked by oxygen or CO which inactivate the protein [Lubitz et al., 2008]. To produce a fully active and mature active site at least seven proteins are required (see maturation of hydrogenases below). The active site is buried deeply in the hydrogenase large subunit. The small subunit, HupS, contains three iron-sulphur [FeS]-clusters which are involved in electron transport to and from the active site [Vignais and Billoud, 2007].

Since no cyanobacterial hydrogenase has been crystallized, most conclusions regarding the structure of the active site are drawn from the X-ray structure of the [NiFe]-hydrogenases from *Desulfovibrio gigas* [Volbeda et al., 1995]. Results from other organisms largely support these structures and it is likely that the general features are the same also for cyanobacteria.

![Diagram of enzymes involved in hydrogen metabolism](image)

*Figure 3*: Schematic picture of the enzymes involved in hydrogen metabolism in *Nostoc* PCC 7120. In diazotrophic growth atmospheric nitrogen (N₂) is fixed into ammonia (NH₄⁺). As a by-product molecular hydrogen (H₂) is formed, which is consumed by the uptake hydrogenase. The bidirectional hydrogenase can either split or form H₂ depending on the redox potential.
Uptake hydrogenase

The cyanobacterial uptake hydrogenase consists of at least two subunits, encoded by the structural genes hupSL (hydrogen uptake) [Vignais et al., 2001]. The hupSL are transcribed together as an operon and are in most cases located in close vicinity to the maturation genes of the active site, the hyp-genes, see below. The active site is positioned in HupL and HupS harbors [FeS]-clusters, transporting electrons in and out from the active site [Volbeda et al., 1995; Vignais et al., 2001]. In other organisms containing an uptake hydrogenase, the enzyme consists of three subunits, the third subunit being a b-type cytochrome involved in the electron transport to the terminal acceptor oxygen. It also connects the functional parts of the hydrogenase to the plasma membrane. The cyanobacterial uptake hydrogenase is membrane associated and the presence of a third subunit is therefore likely, though not yet identified [Tamagnini et al., 2007].

The uptake hydrogenase is localized in heterocysts and the hupSL-operon is only transcribed under N₂-fixing conditions [Hansel et al., 2001; Tamagnini et al., 2007]. The uptake hydrogenase recycles the energy rich molecular hydrogen produced by the nitrogenase and as a consequence also supplies reducing power in the form of electrons to other cell functions, but it may also function as a protector of the micro-oxygenic level in the heterocyst, since oxygen is reduced in the respiration via the “Knallgas” reaction [Tamagnini et al., 2007].

Bidirectional hydrogenase

The bidirectional hydrogenase is a pentameric enzyme, consisting of a hydrogenase and a diaphorase part, encoded by the hoxEFUYH-genes (hydrogen oxidation). The active site positioned in the large subunit, HoxH, and the electron transporting [FeS]-clusters are located in the small subunit, HoxY [Schmitz et al., 2002]. hoxEFU are the structural genes for a diaphorase unit, which in a bidirectional way accepts or deliver electrons to produce or reduce NAD(P)H used as reducing power. In some cyanobacterial strains all hox-genes are clustered in one operon but with multiple transcription start points, like in Synechocystis PCC 6803, or are divided on two operons, like in Nostoc PCC 7120 [Sjöholm et al., 2007].

Different from the uptake hydrogenase, the bidirectional hydrogenase is found in vegetative cells as well as in heterocysts and its presence is not connected to N₂-fixation [Hallenbeck and Beneman, 1978; Houchins and Burris, 1981]. The bidirectional hydrogenase can either split or form H₂ depending on the redox-potential [Tamagnini et al. 2007]. Its biological function is not fully understood, but there are three main possible functions considered: it may function as a valve for low-potential electrons generated dur-
ing the light reaction of photosynthesis, be responsible for H₂ oxidation in
the periplasm and electron delivery to the respiratory chain [Schmitz et al.,
1995], or remove the excess reductants under anaerobic conditions [Trostina
et al., 2002; Tamagnini et al., 2007]. It is debated whether the bidirecctional
hydrogenase is soluble or loosely associated to either the thylakoid and/or the
cytoplasmic membrane, since different investigations have come up with
contradictory results [Hallenbeck and Beneman, 1978; Houchins and Burris,
1981; Kentemich et al., 1989; Serebryakova et al., 1994; Appel et al., 2000].

Maturation of [NiFe]-hydrogenases

Structural and maturation genes

Production of a functional hydrogenase requires genes encoding proteins
building the skeleton of the enzyme (structural genes), as well as maturation
genomes encoding chaperons and co-factors necessary for assembly of the ac-
tive site and correct folding. The hyp-genes (hyp for hydrogenase pleiotrop-
phic) are a set of genes encoding proteins responsible for some of these func-
tions. For the synthesis and the insertion of the metallocentre the gene prod-
ucts of hypFCDEAB are needed as well as ATP, GTP, carbamoyl phosphate
and a specific protease, hupW/hoxW, needed to cleave off the last amino
acids of the C-terminal of the large subunit. [Böck et al., 2006; Vignais and
Billoud, 2007].

The hyp-genes are conserved and can either be clustered, e.g. Nostoc PCC
7120 and N. punctiforme or spread in the genome as in Synechocystis sp.
PCC 6803 [Kaneko et al., 2001]. Independent of type and number of hydro-
genases there is only one set of hyp-genes indicating a co-regulation of the
hyp-genes on the assembly of both types of hydrogenases. How this is done
is not known, but the hyp-genes are most likely regulated differently depend-
ing on the organism, the environment and the type of hydrogenase.

The specific functions of the Hyp-proteins

When it comes to the maturation process of hydrogenases most investiga-
tions have been made in Escherichia coli (E. coli). However, the high ho-
mosity of cyanobacterial and E. coli hyp-genes make it reasonable to rest on
analogy assumptions for the function in cyanobacteria. Indeed, these
hypothesis were confirmed in a study made in Synechocystis sp. PCC 6803
where insertion and deletion mutants of hyp-genes showed no activity
[Hoffman et al., 2006].
Figure 4: Schematic picture of the maturation process of active site in [NiFe]-hydrogenases. HypEF are involved in the CN-ligands synthesis and transfer to the iron atom which is escorted by HypCD to the large subunit apo-protein (1). HypC interacts with the apo-protein of the large subunit working as a chaperon to stabilize the protein. The nickel atom is inserted into the active site by HypAB (2) and correctly inserted nickel is a checkpoint for the protease to cleave of the C-terminal end (3) which enables a conformal change of the large subunit making it possible for the small subunit to attach (4).

Synthesis and maturation of complex metalloenzymes can be divided into three basic processes: formation of an apoprotein, uptake of metals and assembly of the active site [Böck et al., 2006]. Hydrogenases have also a fourth step, where part of the C-terminal end of the large subunit is removed by a hydrogenase specific protease enabling an interaction between the large and small subunits (Fig. 4 step 3-4) [Magalon and Böck, 2000].

Iron atom insertion and synthesis of its CN- and CO-ligands
To the Fe-atom in the active site three biochemically unusual diatomic ligands are coordinated. Since these ligands are toxic to its “host” an interesting question is how the organism manage to synthesize, transport and incorporate them into the active centre. A possible candidate for CN-synthesis is HypF which has a sequence motif also found in O-carbamoyl transferases. HypF has been shown to hydrolyse carbamoylphosphate and
cleave ATP into AMP in the presence of purified HypE and ATP. HypE then activates the oxygen of the carboxamide and after phosphorylation/ dephosphorylation the group is converted to a thiocyanate. The mechanisms for how the cyano (CN) ligand is transferred is not well known, but thiocyanates are known to be good donors of cyanide groups to iron and therefore it is likely that HypE is responsible for this transfer. Since there are two cyanide ligands bound to the iron this cycle might be repeated once more before the carbonmonoxide is bound to the iron. It is unknown which mechanisms are involved in the synthesis of the carbonyl ligand and its metabolic origin. However it is realistic to assume that the carbonyl is incorporated after the CN-ligands since the CN-ligands synergistically enhances CO binding to the complex [Pickett et al., 2004]. It is in theory possible that the carbamoyl phosphat is the metabolic origin of the CO group, but studies of the [NiFe]-hydrogenase from *Allochromatium vinosum* indicate that CN- and CO-ligands are synthesized via different paths. The generation of the carbonyl ligand will require energy, and this pathway will probably be the same for all bacteria, suggesting either further biochemical functions of one or several of the hyp-genes or other proteins. Since only very small amounts of CO is needed, it could be generated and collected from more than one source [Roseboom et al., 2005; Forzi and Sawers, 2007]. The carbamoylphosphate in *E. coli* is the gene product of *carAB*, and mutations in the *carAB*-operon resulted in a non-functioning hydrogenase [Paschos et al., 2001]. In *Nostoc* PCC 7120 the homologues to the *carAB*-genes are alr1155 and alr3809 and they are found in different locations in the genome [Kaneko et al., 2001].

The HypC-HypD complex has been shown to interact with HypF and HypE. The HypC protein has an N-terminal cysteiny1 residue vital for the interaction with the large subunit and HypD. A hypothesis is that this cysteiny1 residue acts as an acceptor of the modified iron. HypD on the other hand harbors a [4Fe-4S]-cluster and seven essential cysteiny1 residues which are very important for the function of the protein. It is suggested that HypD directly transfers the CN-ligand from HypE to a Fe atom in the N-terminal cysteiny1 residue of HypC. Where this iron comes from is not yet clear. Alternatively HypD can act as a scaffold for the cyanide transfer by using one of the irons in the in the [4Fe-4S]-cluster [Vignais and Colbeau, 2004, Böck et al., 2006; Forzi and Sawers, 2007]. HypC acts as a chaperone stabilizing the large subunit of the hydorgenase and it is suggested that HypC might deliver the Fe(CN)$_2$(CO) into the active site locted in the large subunit of the hydrogenase.

**Nickel insertion into the active site and protease cleavage**

Nickel is delivered into the active site by HypA which is a nickel-binding zink metalloenzyme. Mutational studies has shown that nickel is inserted
after the introduction of the modified iron cluster into the active site [Maier
and Böck, 1996]. HypB, a metal- and GTP-binding and hydrolysis protein, is
also required for successful insertion of the nickel atom. Interestingly HypA
and HypB probably function to improve the fidelity or kinetics of the
insertion, since mutants lacking these genes still get functional hydrogenases
if the medium contains high concentrations of nickel [Waugh and Boxer,
1986; Hube et al., 2002]. Another proposed function for HypB is the switch
model where HypB upon GTP hydrolysis makes sure that the HypA-HypB
complex releases the active site in the large subunit [Gasper et al., 2006]. In
E. coli the SlyD, a peptidyl-propyl cis/trans isomerase, further improves the
kinetics of the nickel insertion. The mechanism of the interaction with HypB
is not well known, but SlyD might either aid correct folding and the
conformal change of the large subunit after nickel insertion or act in the
assembly of the complex involved in nickel insertion. No homologues of
slyD has been found in cyanobacteria [Sazuka et al., 1999; Zhang et al.,
2005; Forzi and Sawers, 2007].

The nickel insertion event functions as a checkpoint and it is only after
this is done the cleavage of the C-terminal peptide of the large subunit
precursor takes place [Theodoratou et al., 2000]. This is achieved by a
hydrogenase specific protease, HupW or HoxW, where HupW acts on the
uptake and HoxW on the bidirectional hydrogenase respectively. The
cleavage enables a conformal change of the large subunit resulting in a
hidden location for the active site within the protein. It might also signal to
the small subunit that the large subunit is ready for interaction and assembly
[Magalon and Böck, 2000; Theodoratou et al., 2000; Böck et al., 2006;
Vignais and Billoud, 2007; Devine et al., 2008].

Maturation of the small subunit

While the maturation process of the hydrogenase large subunit is extensively
studied, the process for assembly and maturation of the small subunit is still
unclear. The small subunit of most [NiFe]-hydrogenases contains three [FeS]-
clusters, two [4Fe4S]-clusters and one [3Fe4S]-cluster which transports the
electrons to and from the active site to an external electron acceptor [Lubitz
et al., 2008]. From studies made on the maturation process of nitrogenases,
which also harbor [FeS]-clusters, two proteins, NifS and NifU, were found
to be essential. NifS forms from the L-cysteine substrate the sulfur used for
the [FeS]-cluster assembly and NifU provides the molecular scaffold of the
[FeS] cluster. In cyanobacteria the NfU protein is essential as a scaffold
protins for assembly of the [FeS]-clusters [Vignais and Billund, 2007].
Hence the maturation of the small subunit most likely requires gene products
harboring domains similar to NfU.
Regulatory RNA and CRISPR

Regulatory RNAs in bacteria is gaining much attention lately. These RNAs have been shown to be involved in all steps of the central dogma of molecular biology from DNA maintenance or silencing to modulating transcription, mRNA stability and translation. There are many different mechanistic functions behind regulation including interactions with DNA, change in RNA conformation, base pairing with other RNAs and protein binding [Waters and Storz, 2009]. One group of regulatory RNA recently discovered are CRISPR (Clustered, Regulatory Interspaced Short Palindromic Repeats) which have been identified as a system providing required resistance against phages and prevention of plasmid conjugation most likely by targeting the homologous foreign DNA through an unknown mechanism (Fig. 5) [Sorek et al., 2008; Marraffini and Sontheimer, 2008].

CRISPR array

CRISPR systems are found in bacteria and archea and consist of arrays of short conserved direct repeats interspersed by non-repetitive sequences called spacers. The sequences in the CRISPR array vary between different microbial species. Other known components of the CRISPR system are the CAS-genes (CRISPR-associated complex for antiviral defence) and a leader sequence [Sorek et al., 2008; Young, 2008]. The short conserved direct repeats of the CRISPR array vary in length between 24-47 bp and repeat themselves from 2 to 249 times [Grissa et al., 2007]. The repeats are not truly palindromic but usually show some dyad symmetry and can often form 5-7 bp hairpin-like secondary structure [Sorek et al., 2008; Young, 2008; Waters and Storz, 2009]. Many repeats have a conserved 3’end of GAAA(C/G) a motif possibly involved as binding site for the CAS proteins [Kunin et al., 2007].

Spacers

The spacers vary in length, 26-72 bp, and the sequences are usually unique in the genome. Some spacer sequences match sequences found in phage genomes and might therefore be derived from previous phage infections. These spacer sequences appear to originate from both coding and non-coding parts of the phage genome [Sorek et al., 2008]. The spacers seem to be constantly changing, sometimes within months, but the reasons and exact mechanisms for the exchange are not yet known [Young, 2008].
Leader

5’ of most CRISPR is an AT-rich sequence of approximately 550 bp, directly adjoining the first repeat. This sequence is called leader and is usually not conserved between species. When a new spacer is incorporated in the array it is almost always incorporated between the leader and the previous repeat. This finding suggests a putative role for the leader as a recognition sequence for new spacers. Another possible function is that the leader functions as a promoter of the array [Tang et al., 2002; Tang et al., 2005; Sorek et al., 2008].

CAS-genes

The CRISPR systems have been characterized and divided into 12 subgroups and each subgroup has 2-6 subgroup specific CAS-genes in close vicinity. There are also examples where the CAS-genes, which never have been found in strains lacking repeats, are found in distant parts of the genome with respect to the CRISPR array [Waters and Storz, 2009]. Both the number of CAS-genes and the respective sequences vary between different microbial species, but CAS1 seems to be essential and is present in all known CRISPR systems with one exception [Sorek et al., 2008]. There is also another protein family called Repeat Associated Mysterious Protein (RAMP), only identified in genomes with CRISPR. The functions of these proteins are not known [Haft et al., 2005]. The entire CRISPR array is transcribed as one full-length RNA strand, which is processed into shorter fragments corresponding to single spacer units called crRNA (CRISPR-RNA) by CasE [Brouns et al., 2008]. The exact molecular functions of the CAS-proteins are not known but common features are that they contain DNA/RNA binding domains, exo- or endonuclease domains, and helicase motives, RNA and DNA binding domains and domains involved in transcript regulation [Waters and Storz, 2009].
**Figure 5:** Schematic picture of the putative CRISPR defence mechanism. A) Infecting phage DNA or RNA or DNA insertion through conjugation leads in most cases to cell death. However, sometimes the bacteria manage to degrade the foreign DNA and insert the pieces into its genomic DNA in a CRISPR. The bacterial DNA is transcribed into mRNA and the CRISPR array is processed by the CAS proteins into crRNA (CRISPR-RNA). When the bacterium gets infected again the DNA is recognised by the crRNA and is subsequently degraded, resulting in survival of the bacterium.

**Stress response in cyanobacteria**

Cyanobacteria are in their natural habitat continuously exposed to changes in the environment and have thus developed an enormous capacity to adapt to various stress conditions. Examples of environmental stresses are changes in light intensities, temperature, salt concentrations or nutrient limitations. Individual cells or/and organisms are equipped with sensors and signal transducers which might be general or specific to individual environmental changes, which are triggered when a certain threshold level is exceeded. One type of responses to stress is to activate or deactivate certain genes resulting
in changes in the enzyme composition in order to acclimate to the new environment [Los et al., 2008].

DNA microarray analysis is a useful tool to analyze the global response in transcript level towards stress. Expression of oxidative stress-induced genes in *Synechocystis* PCC 6803 have been investigated by several groups demonstrating that at least 160 genes were significantly enhanced with at least doubled expression levels within 15 min after being transferred from 20 to 300 μmol photons m$^{-2}$ s$^{-1}$ [Hihara et al., 2001; Huang et al., 2002; Hsiao et al., 2004]. Oxidative stress mainly caused by reactive oxygen species (ROS) such as the superoxide anion (O$_2^-$), singlet oxygen (¹O₂), hydroxyl radical (OH), hydrogen peroxide (H$_2$O$_2$) cause damage on DNA, proteins and lipids [Imlay, 2003; Zhao et al., 2007]. To survive, all aerobic organisms have developed various defence mechanisms against ROS, ranging from enzymatic reactions with peroxidases, catalases and superoxide dismutases (SODs) to non-enzymatic, using carotenoids or vitamins. Generally oxidative stress represses gene expression coupled to pigment synthesis and photosynthesis [Sing et al., 2005].
Aim of my PhD thesis

The aim of my work is to extend and deepen the understanding of the hydrogen metabolism in cyanobacteria with the future goal to use this knowledge to produce hydrogen as an energy carrier on a commercial scale. Likely enzymes for such processes are hydrogenases, even though nitrogenases also can produce molecular hydrogen. Even though the nitrogenase is more active than hydrogenases, the nitrogenase alternative is most likely far too costly since at least eight ATP-molecules are needed per hydrogen molecule. The amount of hydrogen produced by hydrogenases in cyanobacteria today is far from the levels needed for commercial production. To improve the hydrogen levels it is likely that a modified cyanobacterial strain will be used in the future. Examples of possible modifications are insertion of multiple copies of the structural genes for hydrogenases or insertion of foreign hydrogenases with higher efficiency. As a consequence several practical questions arise needed to be answered before the vision can be realized. For example, if multiple copies of the structural genes are inserted do they also require multiple copies of the genes encoding the proteins involved in the different maturation processes, or if its is enough to know how to regulate these genes with for example transcription factors. If so which are these transcription factors? Further, if foreign hydrogenases are inserted which maturation proteins are then needed? In either case more fundamental knowledge has to be gathered concerning the maturation processes and the regulation of the hydrogen metabolism. So far, the assembly and maturation processes of the hydrogenase active site in the large subunit of the enzyme are explored, but little is known concerning corresponding processes for the small subunit.

In my work I have been using two different strains of cyanobacteria as model organisms; *Nostoc* PCC 7120 and *N. punctiforme*. These two stains were chosen since they are closely related, filamentous, heterocystous and N$_2$-fixing, but differ in the sense that *Nostoc* PCC 7120 contains both the uptake as well as the bidirectional hydrogenase, while *N. punctiforme* only contains the uptake hydrogenase. Both strains harbor only one set of *hyp*-genes, encoding the proteins needed for the assembly of the hydrogenase active site. These resemblances and differences make these model organisms suitable for comparison of the expression of the maturation genes as well as their regulation.
Results

Photosynthetic electron transfer (Paper I)

In order to analyze the electron flow in the thylakoid membranes in vegetative cells from *N. punctiforme* a protocol for isolation of the oxygen evolving thylakoid membranes was developed based on pneumatic pressure-drop lysis. With this method it is possible to isolate active, oxygen evolving thylakoid membranes since neither mechanical shearing nor friction are created resulting in heat release and membrane damage. The method does also allow a homogenous rupture of the whole cell and is independent of sample volume and concentration. The thylakoid membranes were analyzed with respect to biochemically and biophysically aspects of photosynthetic electron transfer. The oxygen evolving capacities of the isolated thylakoid membranes were measured to check if they still were intact and active before further characterizations were performed. Intact cells were compared with isolated thylakoid membranes and the rates from the mean oxygen evolution were about a third in the isolated thylakoids. To examine the photosynthetic complexes of the isolated thylakoid membrane fraction as compared to intact cells, fluorescence emission spectra were obtained at 77K. The spectra were quite similar with PSII found at 680 and 690 nm and PSI around 730 nm. The peaks from the phycobilisomes, 541 and 657 nm, were as expected lost in the thylakoid membrane fraction. An X-band EPR spectra was recorded at room temperature to analyze the PSI/PSII ratio which was calculated to 3.9/1.

Furthermore a proteomic study was also initiated for separation and identification of protein complexes in *N. punctiforme* thylakoid membrane using 2D gel electrophoresis based on Blue Native electrophoresis in the first dimension followed by SDS-PAGE in the second. In the thylakoid membrane fraction proteins from all major thylakoid membrane proteins complexes were found, i.e. ATP synthase, cytochrome b$_6$f complex, NDH-1, PSI and PSII.
Maturation process of hydrogenases (Paper II and III)

The hyp-operon and its five upstream ORFs

The maturation of hydrogenases into active enzymes is a complex process and a correctly assembled active site requires the involvement of at least seven proteins, encoded by hypABCDEF and a hydrogenase specific protease, encoded by either hupW or hoxW. The N2-fixing cyanobacterium Nostoc PCC 7120 may, depending on growth condition, contain both an uptake and a bidirectional hydrogenase, but always only one set of hyp-genes. During non N2-fixing condition in Nostoc PCC 7120 only vegetative cells are present and thus only the bidirectional hydrogenase. Upon a shift to N2-fixing conditions heterocyst differentiation is induced and genes involved in uptake hydrogenase maturation are expressed, making it possible to compare the expression levels of genes putatively involved in maturation of the uptake hydrogenase [Hansel et al., 2001]. In order to identify genes important for hydrogenase maturation and to investigate their regulations the expression of hyp-genes were investigated in Nostoc PCC 7120.

Figure 6: Schematic picture of the extended hyp-operon, covering in total 14 kb. The hyp-genes involved in maturation of the hydrogenase active site are depicted in black. The five upstream ORFs of the hyp-operon, putatively also involved in hydrogenase maturation are depicted in light grey. The structural gene for the small subunit hupS is depicted in white. R1-R6 indicates where the conserved short intergenic sequences are positioned.

Semi-quantitative PCRs demonstrated that the six hyp-genes together with one ORF, asr0697, may be transcribed as a single operon (Fig. 6). In addition, five upstream ORFs located in between hupSL, encoding the structural proteins for the uptake hydrogenase, and the hyp-operon together with two ORFs downstream from the hyp-genes, asr0701-alr0702, were shown to be part of the same transcriptional unit, covering 14 kb. Transcriptional start points (TSPs) were identified with 5`RACE 280 bp and 445 bp upstream from hypF and hypC respectively, demonstrating the existence of several transcripts. A third TSP was identified 45 bp upstream of asr0689, the first
of five ORFs in the extended hyp-operon. Putative regulatory sequences were identified in the respective promoter areas: for \textit{asr0689} an NtcA binding site and an extended -10 box, for \textit{hypF} an extended -10 box only and for \textit{hypC} a -35 and a -10 box.

The upstream hyp-gene cluster, \textit{asr0689-alr0693}, is conserved in filamentous cyanobacteria and interestingly absent in non N\textsubscript{2}-fixing cyanobacteria. Three of the proteins, Alr0691-Alr0693, encoded by the upstream hyp-genes, harbor domains which may be important for maturation of the small hydrogenase subunit; a NifU-like domain (Alr0692), TPR-repeats (Tetratrico Peptide Repeats) (Alr0691) and NHL repeats (abbreviation of NCL-1, HT2A and Lin-41) (Alr0693). To investigate if the upstream ORFs were specifically involved in the maturation process of the small subunit of the uptake hydrogenase, \textit{Nostoc} PCC 7120 and \textit{N. punctiforme} were grown in a non N\textsubscript{2}-fixing environment before being transferred to N\textsubscript{2}-fixing condition. Total RNA was prepared at day 0 (just before nitrogen depletion), after 24 hours, 48 hours and 72 hours in order to follow the gene expression during heterocyst differentiation as well as in mature heterocysts. Heterocyst preparations were performed after 48 hours for \textit{Nostoc} PCC 7120. The cell morphology was studied microscopically during the experiment and light micrographs were taken at 0, 24, 48 and 72 hours. Semi-quantitative PCRs with specific primers for all five ORFs in the upstream hyp-gene cluster together with \textit{hypF, hypC, hupS} and 16\textsubscript{S} or 23\textsubscript{S} for \textit{Nostoc} PCC 7120 and \textit{N. punctiforme} were performed. Strong up-regulations were observed after 24 hours for \textit{N. punctiforme} and after 48 hours \textit{Nostoc} PCC 7120 for all five ORFs in the upstream hyp-gene cluster as well as for \textit{hypF, hypC} and \textit{hupS}.

\textbf{Short conserved sequences and CRISPR}

Short conserved sequences were found in six intergenic regions of the 14 kb extended hyp-operon, appearing between 11 and 79 times in the genome. Some of the sequences are able to form putative secondary structures and all are positioned the intergenic regions between transcriptional and translational start points. None of the conserved sequences had at the time of publication known functions. One of the conserved sequences, R5, positioned in the intergenic region of \textit{asr0701} and \textit{alr0702} was later identified as CRISPR (Clustered Regulatory Interspaced Short Palindromic Repeats) sequence NC_003272_3 (Fig 6). This CRISPR belongs to the shortest type of array consisting of two repeats with a single spacer in between (Fig. 7).
Intergenic region (asr0701- alr0702)

caaattcttgctgtgattggttttcagcatttttgaggtgtactctttgacaaagcagtgcctcaaaagc
taccttagtcacaaagttccagcaaccgcacctttgaaacccaaatatataagctttccagcttgagcc
attgcaattaactaaatccctatcagggatttgaacatccccaggttattcagccattgctattgcatc
attgcaattaactaaatccctatcagggatttgaacgttaagaaacaatagtttgtttactcgcaaca
ttgatccattaatacaacgcgaatctttagcccttagcccttttatcatgtagttgagcgtgcgcaaac
cgagaaatatggcaactttcac

Figure 7: The CRISPR in the intergenic region of asr0701 and alr0702 is the shortest type of CRISPR known with one spacer region flanked by CRISPR repeat. Part of spacer sequence is identical to DNA from a retrovirus.

No known CAS genes are located in close vicinity and the spacer sequence is only found once in the Nostoc PCC 7120 genome. Part of the spacer sequence, 44%, was found to share 100% nucleotide identity with a Porcine edogenous type C retrovirus (Accession number AJ279057).

The last 22 nt counting from the 3’ end of the identified CRISPR (NC_003272_3) is conserved and is 100% identical to other CRISPR sequences in Nostoc PCC 7120 with id NC_003272_8, NC_003272_16, NC_003272_20 and a new putative CRISPR sequence, identified in this work, called new (Fig. 9A). The secondary structure of a single CRISPR repeat is in all cases forming a putative hairpin like structure and the structures can be divided into two groups based on their shapes (Fig. 9B-C). In silico analysis show that homologues of four out of six CAS-genes identified in other bacteria are found in the Nostoc PCC 7120 genome and they are divided in three clusters located in different parts of the genome (Table 1).

The other conserved sequences found in the intergenic regions of the extended hyp-operon are today still mysterious with no answers concerning their existence or functions. Still it is possible to speculate and these sequences might be related to trans-encoded base pairing sRNA (small RNA) targeting mRNA for cleavage or transcriptional regulation [Waters and Storz, 2009]. They might also have something to do with transposition event, either existing as a result of such insertions or quite the opposite to defend the organism against unwanted insertion of DNA possibly by a base pairing mechanism leading to degradation [Wagner and Flärdh, 2002].
Table 1: A summary of the CRISPR associated proteins (CAS) and Repeat Associated Mysterious Protein (RAMP) proteins in *Nostoc* PCC 7120.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein family</th>
<th>CRISPR in close vicinity</th>
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<tbody>
<tr>
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<td></td>
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<tr>
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<td>CAS 1</td>
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Regulation by the transcription factor CalA (Paper IV and V)

*Characterization of transcription factor CalA*

DNA-binding proteins can regulate transcription by interaction with the promoter region. In a protein-DNA affinity assay five proteins were found to interact with the upstream region of *hypC*, encoding one of the maturation genes for the hydrogenase active site and were identified by MS-MS as streptavidin, an α- and a β- subunit of phycocyanin, a biotin carrier protein and Alr0946. The gene product of *alr0946* is annotated as a soluble conserved hypothetical protein with a molecular weight of 16 kDa and a theoretical pI of 5.5 [Kaneko et al., 2001]. In proteomic studies with iTRAQ-Based Quantitative Analysis the homologue in *Nostoc* PCC 7120 as well as in *N. punctiforme* were identified, demonstrating the existence of a bona fide protein, interestingly present in quite high abundance, which is unusual for transcription factors [Stensjö et al., 2007; Ow et al., 2008; Ow et al., 2009]. Alr0946 harbors a conserved region similar to a protein family of transcriptional regulators called AbrB (*Antibiotic Resistance*) and homologues are found in several organisms from *Bacillus* to Cyanobacteria. The protein has been given a name reflecting its function, *calA* (*cyanobacterial AbrB like from clade A*), in the genome *alr0946* in *Nostoc* PCC 7120. Further, *calA*
and its homologues in other cyanobacteria are highly uniform in their genomic locations, where they are followed by a gene encoding a protease, in *Nostoc* PCC 7120 encoded by *alr0947*. Northern Blot and semi-quantitative PCR analysis of *calA* and *alr0947* show that they are co-transcribed as one operon. Two transcriptional start points, TSP, including two putative extended -10 boxes were identified with 5´RACE 599 nt and 42 nt upstream of the translational start point of *calA*. The protein encoded by *alr0947* has a molecular weight of 30.4 kDa and has a theoretical pI of 7.6. Its function is not known, but it has eight membrane spanning regions and a conserved motive called Abi, (Abortive infection protein) [IPR003675]. Members of this family are probably proteases from the CAAX amino terminal protease family [PF02517], which is a large and diverse superfamily of putative membrane-bound proteins. The protease is as the AbrB-like protein conserved in filamentous cyanobacteria.

**Transcriptional regulation by CalA and its DNA-binding mechanisms**

EMSA and quantitative real time PCR (RTqPCR) verified that CalA is binding specifically to the upstream region of *hypC* and also to its own upstream region, in both cases acting as a repressor of expression. No consensus binding site has been identified for CalA or other proteins of the same family of transcription factors [Bobay et al., 2004; Bobay et al., 2006]. The hypothesized interaction mechanism is thought to be due to the topology of the regulated DNA sequence. There are no crystal structures of cyanobacterial AbrB but several from *Bacillus subtilis*. A 3D-model based on the DNA binding part of the crystal structure from *Bacillus subtilis* combined with alignments of corresponding part of CalA was created and several similarities and dissimilarities between the two homologues were identified. The 3D model suggests that the cyanobacterial AbrB-like protein most likely is present as a dimer in *Nostoc* PCC 7120 and not as a tetramer as in *Bacillus subtilis*. Furthermore, the loop-hinge regions typical for the AbrB superfamily, which enables a flexible binding to DNA, seems to be conserved and analysis of the surface electrostatic potential of the putative DNA-binding domain in CalA indicates that the surface is mainly positively charged.

**Over-production of CalA in Nostoc PCC 7120**

A CalA over-producing strain of *Nostoc* PCC 7120 harboring the vector *p nir* with *calA* (OV) was created and characterized. Additionally an OV was created including a 6xHis-tag in the N-terminal end and a strain harboring only *p nir* (EV) to be used as a control were created. The over-producing strain, OV, has a different phenotype when compared to the control strain, EV, and
wild type strain, WT, exhibiting a lower growth rate and higher sensitivity to elevated light intensities. These differences are seen 2, 4 and 7 days after induction and get more pronounced when the cells are exposed to elevated light intensities, resulting in a higher oxidative stress. The morphology of the cells changes in OV resulting in rounder and sometimes disc shaped cells and individual as well as entire filaments get bleached. The bleaching phenotype is caused by phycobilisome degradation. When comparing total-protein extracts from 0, +2, +4 and +7 days after induction with NO₃⁻ by separation on 1D SDS-PAGE eight proteins, all part of the phycobilisome complex, were decreasing in OV while increasing in EV. Of the identified proteins were two actual light harvesting phycobiliproteins, Alr0021 and Alr0022, while the others were linker proteins involved in assembly, functional organisation and modulation of the phycobiliproteins absorption characteristics; Alr0020, Alr0525, Alr0530, Alr0534, Alr0535 and Alr0537 [Bald et al., 1996; Liu et al., 2005].

Using another proteomic approach with 2D gel electrophoresis, IEF followed by SDS-PAGE, approximately 200 protein spots were visible of which 10 were significantly up-regulated and 15 down-regulated in OV compared to EV four days after induction. Six proteins were chosen for identification and were found to be: iron superoxide dismutase (FeSOD) encode by alr2938, hypothetical protein CalA (alr0946 in the genome), neomycin/kanamycin resistance protein, phycocyanin α subunit, phycoerythrocyanin α chain and phycocyanin β chain. FeSOD is soluble and one of the most abundant proteins except for the phycobiliproteins and was down-regulated by approximately 40%. Nostoc PCC 7120 genome contains also a second membrane bound SOD, MnSOD, but this protein was interestingly not found to be regulated by CalA.

Since the transcription of the hypC, encoding one of the accessory proteins needed for a mature hydrogenase, is negatively affected by elevated levels of CalA the effect of this down-regulation on the bidirectional hydrogenase activity was investigated. Interestingly, CalA reduces H₂ evolution significantly in OV as compared to EV with the relative activity decreasing from 1.2 just before induction to 0.53 and 0.68 +2 and +4 days after induction.

In order to study protein-protein interactions and putative modifications of CalA His-tagged CalA was prepared from OV. When purified with IMAC using a low salt concentration buffer protocol to maintain protein-protein interactions to a higher extent and separated with 1D SDS-PAGE or with 2D gel electrophoresis (IEF followed by SDS-PAGE) three proteins were co-purified, identified as neomycin and kanamycin resistance protein, hypothetical protein All2080 and nucleoside diphosphate kinase. Furthermore at
least five different spots with the same molecular size but with separate pIs were observed in the 2D gel electrophoresis study, all identified as CalA (Fig. 8).

*Figure 8: SDS-PAGE of over-expressed and purified CalA. A) In the 1D SDS-PAGE analysis four bands were observed not present in the control marked in the picture by arrowheads. B) In the 2D SDS-PAGE with the same protein sample as used in the 1D SDS-PAGE eight protein spots A-H were identified not present in the control. Five of the spots were identified as CalA (a-e), All2080 (f), nucleoside diphosphate kinase (g), and neomycin and kanamycin resistance protein (h).*
Discussion

The hydrogenase maturation process

Maturation of hydrogenases is a biochemically complex process requiring assistance of components specially designed for assembly of the active site located in the large subunit HupL as well as some maturation process for [FeS]-cluster assembly. The proteins encoded by the hyp-genes, shown to be involved in assembly and maturation of the active site, are in filamentous N₂-fixing cyanobacteria as Nostoc PCC 7120 and N. punctiforme clustered and located in close vicinity to the structural genes of the uptake hydrogenase. This is in contrast to the situation in Synechocysits PCC 6803, a unicellular cyanobacterium, where the hyp-genes are spread in the genome. Further, the presence of only one set of hyp-genes in N. punctiforme as well as Nostoc PCC 7120 suggests that they are responsible for maturation of both the uptake as well as the bidirectional hydrogenase. For maturation of a certain hydrogenase the hyp-genes are most likely are regulated by transcription factors for example CyAbrB, NtcA and LexA, which in turn are stimulated by various environmental signals e.g. presence of combined nitrogen source) [Agervald et al., 2008; Agervald et al., 2009b; Oliveira and Lindblad, 2005; Oliveira and Lindblad, 2008; Sjöholm et al., 2007; Ferreira et al., 2007]. In Nostoc PCC 7120 the six hyp-genes, hypFCDEAB have been shown to be co-transcribed with two downstream ORFs, asr0701-alr0702, five upstream ORFs, asr0689-alr0693, and an additional ORF asr0697 between hypC and hypD, an operon in total covering 14 kb. This is in line with investigations of the corresponding hyp-gene clusters in N. punctiforme and Lyngbya majuscula [Hansel et al., 2001; Leitão et al., 2006]. At least three transcriptional start points are identified within the operon of Nostoc PCC 7120 pointing at the possibility for specific regulation of the different components of the maturation machinery. One TSP is positioned upstream the first ORF, asr0689, the second upstream hypF and the third upstream hypC. HypF and HypC are both active in the assembly of the active site, but their respective functions are different. Since HypF is involved in the synthesis of the CN-ligands of the iron atom and HypC is a chaperone, stabilizing the large subunit during assembly of the active site existence of individual regulation of the respective genes is therefore not surprising. The TSP upstream asr0689 is probably regulating some, if not all of the five ORFs, asr0689-alr0693. In contrast to N. punctiforme no TSP was found upstream alr0693.
and the homologues of all five ORFs in *N. punctiforme* are present in a slightly different order as compared to *Nostoc* PCC 7120, indicating that the regulation might be different in the two strains. The homologues to the five ORFs upstream the *hyp*-genes are conserved in filamentous N₂-fixing cyanobacteria, always located between the structural genes for the uptake hydrogenase and the *hyp*-genes. With the exception of *alr0692*, which is encoding a protein annotated as NifU-like/NfU involved in [FeS]-cluster assembly in cyanobacteria, all ORFs are encoding proteins without any known functions [Sazuka et al., 1999; Vignais and Billoud, 2007]. Interestingly *Ralstonia eutropha* and *Rhizobium leguminosarum* (*R. leguminosarum*), both containing an [NiFe] uptake hydrogenase, show similarities in the genomic arrangement with seven and four ORFs respectively, positioned between the structural genes and the *hyp*-operon [Burgdorf et al., 2005; Manyani et al., 2005]. Mutational analysis in *R. leguminosarum* show that some of the ORFs are involved in maturation of the hydrogenase small subunit and affinity tag experiments of some of the proteins encoded by the ORFs in *Ralstonia eutropha* demonstrate that they are interacting with proteins involved in the maturation of both the large and the small subunit [Manyani et al., 2005; Schubert et al., 2007]. Neither *Ralstonia eutropha* nor *R. leguminosarum* are closely related to cyanobacteria and therefore no homologues are identified. However, conserved domains found in *R. leguminosarum* are also present in filamentous cyanobacteria. *Alr0692* and homologues contain a NifU-like/NfU related domain involved in [FeS]-cluster assembly and *Alr0691* and *Alr0693* with homologues contain TPR and NHL repeats respectively present in chaperons and in proteins active in stabilization of multi-protein complexes [Das et al., 1998; Blatch et al., 1999; Wagner, 2000; D’Andrea et al., 2003].

To further investigate the functions of the proteins encoded by the five upstream ORFs *Nostoc* PCC 7120 and *N. punctiforme* were chosen. Since it is possible to induce the signal for induction of heterocysts and consequently also uptake hydrogenase synthesis, by shifting the nitrogen source from the preferred NH₄⁺ to N₂-fixation, the expression patterns for the five upstream ORFs and the *hyp*-genes would give answer to possible specificity towards the uptake hydrogenase. Indeed the semi-quantitative PCR showed a strong up-regulation of the five upstream ORFs in a similar fashion for the *hyp*-genes in *N. punctiforme* already after 24 hours and after 48 hours in *Nostoc* PCC 7120. These results show that the five upstream ORFs are regulated in a similar way as the *hyp*-genes in both strains, further strengthening the hypothesized role in maturation of the uptake hydrogenase. Of the five ORFs conserved in N₂-fixing heterocystous cyanobacteria only the homologue including the NifU-like/NfU related domain is present in *Synechocystis* PCC 6803. This is not surprising since *Synechocystis* PCC 6803 contains a bidirectional hydrogenase with a small subunit harboring [FeS]-clusters which
requires an NiU protein for that assembly. Since no homologues of the other four ORFs are present this indicates that they are not needed for maturation of the bidirectional hydrogenase in *Synechocystis* PCC 6803.

Since the presence of uptake hydrogenases are strictly connected to the presence of nitrogenases it is also possible that the five ORFs conserved in N$_2$-fixing cyanobacteria are involved in mechanisms connected to the nitrogenase.

**CRISPR and other conserved sequences in the extended hyp-operon**

The conserved sequence (aaatccctatcagggattgaaac) found in the intergenic of *asr0701* and *alr0702*, the two most downstream genes in the hyp-operon, turned out to be a CRISPR [Grissa et al., 2007; Agervald et al., 2008]. The main questions arising from this discovery are why there is a CRISPR system in the hyp-operon; does it have a function, and if so is that putative function coupled to hydrogen metabolism or is it connected to something completely different. Bioinformatics show that the sequence is 100% identical to CRISPR sequence NC_003272_3 (attaactaaatccctatcagggattgaaac) and consists of two CRISPR repeats with one spacer sequence in the middle [Grissa et al., 2007]. The spacers are thought to be the result of previous attacks of foreign DNA from infecting plasmids or phages [Sorek et al., 2008] and indeed, 44% of the spacer of the NC_003272_3 CRISPR system was found to share 100% nucleotide identity with a retrovirus. The exact mechanisms for recognition and degradation of foreign DNA is not known, but they must be quite effective relying on only one spacer copy. Another possibility is that many spacers derive from the same phage DNA and together can be efficient enough to stop the infection.

Approximately half of the CRISPR sequence is conserved and can be found in five out of 19 of the other CRISPR arrays existing in *Nostoc* PCC 7120. This implies either a so far unknown important mechanistic function or that it has spread in the genome successfully by for example transposome activities. Only part of the 5′ ends of these five CRISPR systems is conserved which make the putative hairpin structures look different. Based on these differences the five CRISPR arrays can be divided into two subgroups depending on the structure of their respective hairpins. The reasons for these differences are not known but they could play a role in the production of crRNA. Possible reasons can for example be that group A and B are processed by different sets of the CAS-genes or that group A and B are active under different conditions.
There are three clusters of CAS proteins (CRISPR associated proteins) in the Nostoc PCC 7120 genome. The first cluster of CAS-genes is complete with all the different CAS-genes found in Nostoc PCC 7120. The second CAS-gene cluster is the largest one with CAS proteins belonging to another CAS-protein family called RAMP, while the third cluster is consisting only
of CAS1 and CAS2. The existing homologues and different types of CAS-families indicate complex regulation mechanisms not understood.

If it is true that CRISPR systems also work as protection against infecting DNA in cyanobacteria it may offer a possible way to make cyanobacteria immune against infections. This is of great interest for cultivation in future large production plants where cyanobacteria will be used for production of for example different energy carriers as for example biodiesel, ethanol or molecular hydrogen. It is likely that many of the production plants will be positioned in the ocean since this offers a possibility to cost reduction compared to the more expensive land area. Another reason is that seawater is more abundant than fresh water and thus also less expensive. All cost reductions are of significant importance since the produced energy carriers must be able to compete in price with the existing ones. However the dense cultures used in production units are more sensitive to infections than normal populations and a drawback with seawater is that it contains high amounts of phages, 5-10 phages/bacterial cell [Wommak and Colwell, 2000; Sorek et al., 2008]. Here the CRISPR systems can be of interest, since if continuous screenings the environment are done, in this case the seawater, the presence of infectious phages can be detected. Spacers with DNA from the phage can be inserted into the cyanobacterial genome and the modified "immune" cyanobacteria can be used in the production plant. An alternative is also that the cyanobacteria itself during the screening phase picks up new parts of the phage genome and modifies itself and part of the screening culture can be introduced in the production plant offering resistance. Important knowledge is today lacking concerning for example on which criteria the bacterium chooses the spacer sequences, how the spacers are inserted, why the spacers are inserted at the different locations, which factors triggers that process and how long the spacer sequences are kept in the CRISPR array. Another question is how effective the different CRISPR systems are as compare to each other. Is it so that a system with a long CRISPR array is more active since it has incorporated more spacers, or do the different CRISPR systems have different functions or are specialised on different this? There are many questions remaining to be answered concerning this type of recently discovered regulatory RNA. As soon as more knowledge is gathered about the CRISPR systems and their machinery they might become a very useful genetic tool, both on a lab scale and in large commercial production plants. Phage infections in commercial cultivations are both costly and difficult to defend against. Therefore it is very welcome to be able to act and make the bacteria immune against some of the infections and hopefully CRISPR systems will provide that service.
The CyAbrB transcription factor in *Nostoc* PCC 7120

In investigations of transcriptional regulation, promoter regions from several cyanobacterial strains performed in our laboratory reviled the presence of a small protein, annotated as hypothetical with a conserved domain belonging to a family of transcription factors called AbrB [Sazuka et al., 1999; Oliveira and Lindblad, 2008; Agervald et al., 2009b; Holmqvist et al., unpublished]. The protein has been given the name *calA* (cyanobacterial AbrB like) (*alr0946* in the *Nostoc* PCC 7120 genome). Since the promoter regions used were all from genes involved in different parts of the hydrogen metabolism, the findings of the DNA binding protein indicated a possible common regulatory function connected to hydrogen metabolism.

**Comparison of AbrB and CyAbrB**

The AbrB (antibiotic resistance) protein is extensively studied in the genus of *Bacillus* (e.g. *Bacillus subtilis*), bacteria of the Bacilli class very distantly related to cyanobacteria, where it initially was found to be involved in transition states and initiation of spore formation. Further investigations demonstrated that this transcription factor is also active on a global level, directly or indirectly is regulating more than 60 genes involved in metabolic pathways concerning nutrient limitations [Phillips and Strauch, 2002; Bobay et al., 2004; Strauch et al., 2005]. Active regulation is a key function for survival for bacteria, which often face rapid changes in the surrounding environment. Hence they are dependent upon their capacity to quickly evaluate metabolic and environmental information to be able to adapt to the new situation [Bobay et al., 2004; Strauch et al., 2005]. The AbrB protein is described as “promiscuous” since no DNA binding consensus region has been identified, but while the DNA binding properties of AbrB are broad, the protein certainly does not bind randomly [Bobay et al., 2004]. Instead specific binding is hypothesised to be decided by the 3D structure of DNA target. Additionally, the DNA binding affinities are different for individual promoters, implying other factors involved in the discrimination [Bobay et al., 2004].

The *Bacillus subtilis* AbrB protein is a homotetramer, consisting of four identical 10.5 kDa subunits. The DNA binding domain is located in the N-terminal end, while the C-terminal primarily hypothesised to be the multimerization domain [Bobay et al., 2005]. In the cyanobacterial homologues of AbrB the DNA-binding domain is located in the C-terminal with a higher predicted molecular weight, e.g. 16.06 kDa in *Nostoc* PCC 7120. With respect to these differences the number of subunits and the putative nature of the DNA binding mechanism of CalA was investigated in *Nostoc* PCC 7120 resulting in a 3D model of the DNA-binding domain. The model is based on
alignments of the DNA-binding part in CalA and *Bacillus subtilis* combined with the the crystal structure of *Bacillus subtilis* (1Z0R). The 3D model suggests that the protein exists as a dimer, which is in line with investigations made on the *Synechocystis* PCC 6803 homologue and with general structural analysis of prokaryotic transcription factors demonstrating that most are present as homodimers [Huffman and Brennan, 2002; Ishii and Hihara, 2008; Agervald et al., 2009a]. The putative DNA binding domain is predicted to be positively charged which is of special interest since strong DNA binding to negative DNA helix is usually considered beneficial. This characteristic differs compared to AbrB from *Bacillus subtilis*, which has both negatively charged and positively charged areas. Further the 3D model predicts that the flexible loop-hinge regions typical for the AbrB transcription factor family is conserved, allowing the protein to bind specifically to unrelated DNA sequences. Since the AbrB homologues in cyanobacteria seem to be different from the *Bacillus* species, the cyanobacterial homologues often are referred to as CyAbrB.

In cyanobacteria the interest for homologues of AbrB and their functions is quite recent and not so much is known. However, there are some investigations done where CyAbrB homologues are shown to regulate genes encoding key proteins in several physiological and metabolic processes e.g. hydrogen metabolism, oxidative stress, nitrogen and carbon fixation, and production of the toxin cylindrospermopsin [Oliveira and Lindblad, 2008; Ishii and Hihara, 2008; Shalev-Malul et al., 2008; Lieman-Hurwitz et al., 2009; Agervald et al., 2009a; Agervald et al., 2009b]. *In silico* analysis of the cyanobacterial genomes currently available revealed the presence of at least two genes encoding CyAbrB proteins [Ishii and Hihara, 2008]. Several attempts to create fully segregated knock out mutants of the CyAbrB homologue of Sll0359 and CalA have all been unsuccessful, indicating that the protein might be essential to the organism for survival [Oliveira and Lindblad, 2008; Ishii and Hihara, 2008; Lieman-Hurwitz et al., 2009; Agervald et al., 2009b]. However, a fully segregated mutant of the *Synechocystis* PCC 6803 homologue sll0822 was possible to obtain, and interestingly upon deletion of sll0822 increasing expression levels of slr0359 were detected. The increasing transcription levels of slr0359 can be interpreted as a way for the cyanobacteria to compensate for the loss of sll0822, indicating that the two homologues at least partly are involved in the same regulatory pathways [Ishii and Hihara, 2008].

**CalA is a key regulator in the hydrogen metabolism**

CalA was in *Nostoc* PCC 7120 found to interact with the promoter region of hypC, one of the accessory genes needed for a mature hydrogenase [Agervald et al., 2009b]. Specific binding of CalA to the hypC promoter as well as
to its own promoter region was confirmed by EMSA, the latter also in accordance with investigations in *Synechocystis* PCC 6803 [Oliveira and Lindblad, 2008]. Northern Blot and semi-quantitative RT-PCR results show that *calA* is co-transcribed with the downstream gene, *alr0947*, encoding a putative protease of the Abi superfamily. Homologues of CalA and Alr0947 are both conserved proteins within cyanobacteria with similarities ranging from 60 to 99% and 41 to 92% respectively on amino acid level [Agervald et al., 2009b]. Interestingly also the genomic location of the operon is conserved, indicating a possible co-function not yet understood [Oliveira and Lindblad, 2008]. RTqPCR experiments using RNA from the CalA over-producing strain, OV, and control strains showed a down-regulation of *hypC* and *alr0947* in OV as compared with the control strains demonstrating that CalA in both cases is working as a repressor and the existence of a feedback regulation on the *calA-alr0947*-operon. Feedback regulation pathways for transcription factors are common to ensure suitable levels of the regulating protein [Wagner, 2000]. In the specific case of CalA the feedback regulation mechanism are not known, but since CalA is interacting with its own promoter some autoregulation seems to be present [Agervald et al., 2009b]. This is in accordance with studies made in *Bacillus subtilis*, where *abrB* has been shown to be autoregulated during exponential growth keeping the protein concentration within a certain range. In response to stress and subsequent entry of the transition state a regulatory cascade is activated. During this process the intracellular concentration of another regulatory protein, Spo0A, is increased, which when phosphorylated is a repressor of *abrB* transcription [Phillips and Strauch, 2002]. The levels of AbrB are decreased below its threshold for regulatory effectiveness thereby also stopping its autoregulation [Phillips and Strauch, 2002].

The identification of CalA demonstrated the active presence of this Cy-AbrB protein in *Nostoc* PCC 7120 and an over-producing strain was constructed to further investigate its functions. Since the transcript levels of the maturation gene *hypC*, was shown to be repressed by CalA it was investigated if the decrease consequently would affect the bidirectional hydrogenase activity. Indeed, the activity of the bidirectional hydrogenase was +2 and +4 days after induction with NO₃⁻ shown to decrease to 53% and 68% respectively as compared to the control, a *Nostoc* PCC 7120 strain harboring the *pnir* without *calA* (EV), demonstrating a connection either directly or indirectly to the elevated levels of CalA. If the regulation is indirect it may be as a consequence of decreased levels of the maturation protein. In our study in *Nostoc* PCC 7120 the expression level of *hoxE*, encoding one of the diaphorase subunits of the bidirectional hydrogenase, increases strongly when NO₃⁻ is added. The increase was also observed in the control strains and is therefore likely a result of the change of nitrogen source and corresponding nitrogen metabolisms. A possible regulation by CalA on *hoxE* will
probably be masked by the strong increase in expression created by the NH4+/NO3- shift. The expression pattern of hoxY, encoding the small subunit of the hydrogenase, is decreasing in OV following the same trend as hypC, leading to the conclusion that CalA is acting as a repressor on both hypC and hoxY. This is not in line with investigations made in Synechocystis PCC 6803 where the the hox-operon was shown to be activated by the CyAbrB homologue, Sll0359 [Oliveira and Lindblad, 2008]. However, there are several important differences between the model organism in this study, Nostoc PCC 7120, and Synechocystis PCC 6803. Firstly, Nostoc PCC 7120 is a N2-fixing strain while Synechocystis PCC 6803 is not, secondly are the hox-genes in Nostoc PCC 7120 divided on two clusters with several individual transcriptional start points which may be regulated individually, whereas Synechocystis PCC 6803 has only one hox-cluster [Oliveira and Lindblad, 2005; Sjöholm et al., 2007], and thirdly additional transcription factors may be involved, for example CyLexA which has been found binding to one of the regulatory upstream regions of the hox-operon in Synechocystis PCC 6803 and Nostoc PCC 7120 [Oliveira and Lindblad, 2005; Gutekunst et al., 2005; Sjöholm et al., 2007].

CalA is involved in regulation of oxygen stress

To broaden the search for processes regulated by CalA a 2D gel electrophoresis proteomic study was performed. Total-protein extracts from OV and EV were separated by isoelectric focusing followed by SDS-PAGE and the protein patterns from day 0 and day +4 after inductions with NO3- were compared. At day +4 at least 25 proteins showed significant changes in abundance in OV as compared to the control, EV, of which 10 were up-regulated and 15 were down-regulated [Agervald et al., 2009a]. One of the proteins identified was FeSOD, iron superoxide dismutase, encoded by alr2938, which was down-regulated with 40% at day +4 in OV as compared to EV. Superoxide dismutases are proteins involved in scavenging reactive oxygen species, ROS, more specifically O2•-, arising at the reducing side of PSI. Nostoc PCC 7120 contains two types of SODs, one soluble FeSOD, encoded by alr2938, present in high amounts and a membrane bound SOD, MnSOD, encoded by all0070, present in 5-8 times lower amount than FeSOD [Sazuka et al., 1999; Regelsberger et al.,2004 ]. Both SODs are present and active in vegetative cells and in heterocysts [Regelsberger et al, 2004; Zhao et al., 2007]. The presence of FeSOD in Nostoc PCC 7120 is known, but the transcriptional regulation of the gene has not been studied. Semi-quantitative RT-PCR demonstrated that alr2938 may be co-transcribed with an upstream ORF, asr2937, encoding a small protein of unknown function [Agervald et al., 2009a]. Transcriptional start points including putative -10 boxes were identified upstream both asr2937 and alr2938. In addition a putative NtcA
binding site was identified centered -28 bp upstream the TSP of *alr2938*. The presence of multiple TSPs within the operon points at the possibility for individual regulation, but without mutational analysis it is not possible to conclude about the functions of the operon. This since Asr2937 does not have any useful characteristics such as conserved domains or membrane spanning regions, which may give any suggestions for a putative function.

Since FeSOD is active in oxygen stress the effects of induced light intensities were studied. The CalA over-expression strain showed a bleaching phenotype with lower growth rate and truncated filaments already two days after induction of over-expression (Fig. 10). During stress conditions, caused either by nutrient limitations or by excess of light, degradation of phycobilisomes is a known process to prevent damage of ROS on photosynthetic reaction centres [Collier and Grossman, 1992; Grossman et al., 2001]. Phycobiliproteins are very abundant and can make up to 60% in extracts of soluble proteins [Williams et al., 1980]. The severe bleaching of the filaments is at least partly explained by the extensive degradation of the phycobilisome complexes seen when the amount and activity of FeSOD is down-regulated in OV by CalA. However, additional impacts of CalA can not be excluded. The reason for the fragmentation is unknown, but one could speculate that cell defence mechanisms similar to plant programmed cell death (PCD) might be causing the fragmentation, where the filamentous cyanobacteria removes severely damaged cells by simply breaking the filament [Berman-Frank et al., 2004]. The morphology of individual filaments were also changed resulting in rounder, sometimes disc shaped structures. The cause for this behaviour is not known, but cell elongation processes or growth rate might be affected by the elevated levels of CalA. The bleaching phenotype was even more pronounced when illumination was increased almost four times (35 to 125 μmol m⁻² s⁻¹), which is in accordance with an increased need of FeSOD during a stronger oxidative stress (Fig. 10). The results indicate that CalA is involved in regulation of stress responses and that FeSOD has a specific and important function in the oxidative stress tolerance of the multi-cellular cyanobacterium *Nostoc* sp. PCC 7120.
Figure 10: Phenotype study of the CalA over-expressing strain in Nostoc PCC 7120 (OV) and the control strain EV. The OV cultures get a bleaching phenotype with fragmented cells already after two days induction of CalA. The phenotype differences get more pronounce over time and increased light, demonstrating the fatal effects of elevated levels of CalA.

To distinguish whether the differences in abundance of FeSOD in the CalA over-expression study was due to direct regulation by promoter interaction of CalA EMSAs were performed with the FeSOD promoter and purified CalA. Specific shifts were observed demonstrating a direct interaction. Semi-quantitative PCR analysis with gene specific primers for *alr2938*, *all0070*, *calA* and 23S were performed with RNA from the CalA over-expression strain from cells harvested at 0, +4 and +7 days after induction. Interestingly the down-regulation of FeSOD observed at protein level was also seen on transcriptional level in OV in contrast to EV where the transcript level was up-regulated over time while the protein level was constant. The expression levels of *all0070* encoding MnSOD were increasing in both OV and EV over time. Activity measurements of FeSOD and MnSOD supported the results observed on transcriptional and protein levels, indicating that MnSOD it is not regulated by CalA and further cannot compensate for the decreased levels of FeSOD, despite that they are present within the same cellular compartment. This is of particular interest since prokaryotes lacking specific cell organelles, still can have proteins with specific functions in their respective cell localization. These results supports the theory about SOD specificity in cyanobacteria [Li et al., 2002; Regelsberger et al, 2004; Zhao et al., 2007] where the soluble FeSOD might be the main $O_2^-$ protector and
MnSOD due to lower expression as well as being immobilized in the membranes has other specific functions.

*Putative modifications of CalA and interacting proteins*

Cyanobacteria are organisms which promptly have to adapt to changing environments. As a consequence they constantly have to balance their metabolic and energetic processes resulting in activation and deactivation of gene expression. Transcriptional regulation is often achieved by binding of transcription factors to consensus regions in the promoter region [Wagner, 2000]. Since the same gene may be regulated by several transcription factors questions arise how the organism is distinguishing which transcription factor that may bind. Since the DNA sequence forming the 3D structure to which CyAbrB is interacting may include consensus binding sequences of other transcription factors a possible way to alter the binding properties of CyAbrB may be post-translational modifications (PTM). Another possibility for regulation may be by protein-protein interaction with other regulating proteins. To investigate if CalA is modified or interacts with other proteins Histagged CalA was over-expressed in *Nostoc* PCC 7120 and purified. The proteins in the eluted fraction were separated with 1D SDS-PAGE and 2D gel electrophoresis (IEF followed by SDS-PAGE) and compared to a control. Interestingly three bands not present in the control were observed and the three proteins were identified as hypothetical protein All2080, nucleoside diphosphate kinase and neomycin/kanamycin resistance protein. The neomycin/kanamycin resistance protein is an artefact from the over-expression since the selection for $pnir$ is based on resistance to neomycin. The co-purification of nucleoside diphosphate kinase is puzzling, since this protein normally is catalyzing the exchange of phosphate groups between different nucleoside diphosphates [Parks and Agarwal, 1973]. Interestingly All2080 is the CyAbrB homologue in *Nostoc* PCC 7120 and the results indicate that the two proteins interact, which is in accordance with results from similar experiments in *Synechocystis* PCC 6803 [Ishii and Hihara, 2008]. All2080 and CalA may form a heterodimer active in regulation, but it is jet premature to speculate in why and in what extent.

Since PTMs leads to a change in pIs of the proteins change pI identifications of such are possible with 2D gel electrophoresis (IEF followed by SDS-PAGE). The same protein extract as used for 1D SDS-PAGE was separated in 2 DE and at least five different spots with separate pI were all identified as CalA, indicating the presence of PTMs. The putative PTMs need to be identified with LC-MS.
Electron flow in thylakoid membranes isolated from vegetative cells of *N. punctiforme*

Thylakoid membranes are the site of active photosynthesis and all known cyanobacteria, with the one exception of *Glyobacter violaceus PCC 7421*, contain thylakoid membranes [Nakamura et al., 2003]. Since the lack of electrons is one of the bottlenecks for increased H₂ production knowledge concerning the bioenergetics is needed. Further, by mapping the electron flow in vegetative cells and heterocysts in filamentous cyanobacteria this information can be used to re-route electrons from biomass production to hydrogen production pathways. To achieve this, detailed characterizations of thylakoid membranes are important.

Proteomic investigations have been initiated of *N. punctiforme* [Anderson et al., 2006; Ow et al., 2009] and *Nostoc PCC 7120* [Stensjö et al., 2007; Ow et al., 2008], but total protein-extraction methods and shot gun proteomics used are not optimal for examinations of the composition of membrane-bound enzyme complexes, for example respiratory enzymes and photosynthetic reaction centers.

The extraction method and protein separation developed for this investigation was successful for identification of specific proteins involved in electron transfer over the thylakoid membrane and further to categorize them to the different important membrane enzyme complexes in *N. punctiforme*. 
Summary and future outlooks

Hydrogen production based on a cyanobacterial system is working and promising, but needs further optimization before large scale production is of commercial interest. A proposed way to increase hydrogen production is to insert extra copies of the structural genes for the hydrogenase to get a higher production. Since these proteins need assistance of multiple proteins to mature into functional enzymes, knowledge about the number and regulation of maturation genes are highly requested to prohibit a bottle neck at the maturation step. The maturation process of the small hydrogenase subunit is not well studied and thus the five upstream ORFs of the hyp-operon are promising candidates to shred light into this field. Investigations of the individual expression levels as well as mutational analysis would provide useful information.

To be able to increase hydrogen production fine tuning of transcription is highly important, which may be achieved by for example transcription factors and small RNAs (sRNA). Based on the results presented in this thesis transcription factors of the CyAbrB family are shown to be key players involved in hydrogen metabolism and stress response. However, there are probably many other transcription factors involved in the regulations and the cross talk between these proteins as well as their individual regulations are not known. Thus competition studies performed on promoters with known interaction of several transcription factors would give hints to how some of these regulatory networks function. It would also be highly interesting to study the action performed by transcription factors and their cross talk in vegetative cells as compared to heterocysts. This information could directly be applied to metabolically engineer hydrogen metabolism to reach the future goal of large scale hydrogen production. Further, understanding of the regulation mechanisms on the transcription factor itself would provide a way to regulate the transcription factors and thus the transcription of genes of interest. Investigations of this kind can for example be done by performing DNA-affinity assays with the promoter region of cyAbrB with protein extracts form different growth conditions. Putative differences in interacting proteins might give a clue concerning the regulation and a suggestion for further analysis. Protein binding to DNA regulated by post-translational modifications is lately gaining increasing interest and thus the putative modifications of CyAbrB provide a model system for this type of investigations.
The presence of small RNAs (sRNA) and their regulatory functions in transcription regulation has lately turned into a hot topic. Since cyanobacteria often face changes in the surrounding environment they need to have a well working system for stress response. Quick adaption is necessary and a RNA based regulatory system is thus useful since it is generally faster than a protein based mechanism with transcription factors. One example of these sRNA are CRIPRS, and the identification of CRISPR system in the hyp-operon introduced the knowledge and awareness that the system also is present in the cyanobacteria used in our research. Even though CRISPR systems, putatively used by the organism as protection against infections, is poorly understood right now, it might in the future develop into a very useful tool for basic research as well as on applied level in large scale cyanobacterial power plants. The CRISPR research field is novel and results are getting a lot of attention even though much is unknown. Biotechnology will most likely develop new applications based on these mechanisms in the future.

vändas för storskalig produktion och det är här forskningen beskriven i denne avhandlig tar vid.

Målet med mitt arbete är att vidga och fördjupa kunskapen och förståelsen för hur vägtgasmetabolismen i cyanobakterier fungerar. Jag har i detta syfte använt två olika stammar: *Nostoc* sp. strain PCC 7120 and *Nostoc punctiforme*. Dessa två stammar valdes då de är nära besläktade, filamentösa, heterocystbildande och kvävefixerande. En viktig skillnad är att *Nostoc* PCC 7120 har två typer av hydrogenaser, medan *Nostoc punctiforme* bara har en. Vidare har båda stammarna bara en uppsättning hyp-gener som kodar för proteiner nödvändiga för sammansättningen av hydrogenasens aktiva säte. Dessa likheter och skillnader gör dessa två stammar till lämpliga kandidater för att jämföra uttrycken av gener inblandade i mognadsprocessen liksom för deras respektive reglering.

Sammanfattat visar resultaten från denna avhandlig att:

- *hyp*-generna skrivs ut som ett operon i *Nostoc* PCC 7120 och att de kan skrivas ut som ett operon tillsammans med ytterligare sju förmodade gener varav fem är belägna uppströms och två är belägna nedströms *hyp*-generna. Tre transkripsstartpunkter identifieras inom detta stora operon, kallat ”extended *hyp*-operon”, som sträcker sig över 14 kb, vilket visar på möjligheter till individuell reglering av innefattade gener.
- Fem av de uppströms belägna generna uppregleras när heterocystdifferentiering induceras i *Nostoc* PCC 7120 och i *Nostoc punctiforme* vilket indikerar inblandning i mognadsprocessen för upptagshydrogenaset.
- Korta konserverade sekvenser identifierades på sex olika ställen mellan gener i ”extended *hyp*-operon”. En av dessa sekvenser visade sig vara en CRISPR sekvens, ett nyligen upptäckt system för bakterier att skydda sig mot infekterande virus och bakterier via konjugering.
- En transkriptionsfaktor tillhörande familjen CyAbrB identifierades i *Nostoc* PCC 7120 då den band till promoter regionen av *hypC*. När överuttryckt visade den sig i en proteomikstudie signifikant reglera åtminstone 25 proteiner.
- Järnsuperoxiddismutas, FeSOD, identifierades som ett av dessa proteiner vilket nedreglerades med 40 % jämfört med kontrollen efter fyra dagars överuttryck av CalA. Nedreglering av FeSOD visade sig få fatala konsekvenser för cyanobakterien med fragmenterade och bleka filament speciellt tydligt vid ökad ljusexponering samt tid, vilket visar på den essentiella betydelsen av FeSOD vid oxidativ stress.
- Aktiviteten hos det bidirektionella hydrogenaset nedregleras också jämfört med kontrollen med 68 % respektive 53 % +2 och +4 dagar.
efter inducering av vid överuttryck av Arl0946. Detta kan bero på direkta och/eller indirekta konsekvenser av förhöjda nivåer av CalA.

- En metod för preparation av thylakoidmembran arbetades fram för *Nostoc punctiforme*. Thylakoidmembranen kunde avge syrgas vilket demonstrerar att de är intakta vilket är av stor betydelse vid studier av elektronflödena över membranen.
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United Nations. 1999. The World at Six Billion. Table 1


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