UPPSALA
UNIVERSITET

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology 1990

Expression and synthetic activation of [FeFe]-hydrogenases in cyanobacteria

ADAM WEGELIUS

ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2020

ISSN 1651-6214
ISBN 978-91-513-1073-2
urn:nbn:se:uu:diva-425911

Dissertation presented at Uppsala University to be publicly examined in Polhemsalen, Ångströmlaboratoriet, Lägerhyddsvägen 1, Uppsala, Wednesday, 27 January 2021 at 13:15 for the degree of Doctor of Philosophy. The examination will be conducted in English. Faculty examiner: Professor Matthew Posewitz (Department of Chemistry, Colorado School of Mines).

Abstract
Wegelius, A. 2020. Expression and synthetic activation of [FeFe]-hydrogenases in cyanobacteria. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology* 1990. 51 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-1073-2.

Photosynthetic microbes can be utilized for hydrogen production, generating a clean, carbon neutral energy carrier from abundant substrates. Cyanobacteria are photosynthetic prokaryotes with large potential for biotechnological energy applications and several strains are capable of hydrogen production. This production is catalysed by a bi-directional [NiFe]-hydrogenase, or by nitrogenase during nitrogen fixation. However, nature's foremost hydrogen producing enzymes, the [FeFe]-hydrogenases, are not present in these organisms. Many [FeFe]-hydrogenases boast incredible catalytic activities and high bias towards proton reduction. Introduction of a suitable [FeFe]-hydrogenase in a cyanobacterial host could greatly improve the hydrogen production capacity. Unfortunately, generation and characterisation of cyanobacterial strains carrying active [FeFe]-hydrogenases is stalled by the intricate maturation process associated with these enzymes.

In this thesis, I investigate heterologous expression and artificial maturation of [FeFe]-hydrogenases in cyanobacteria. Genetic tools to reliably express [FeFe]-hydrogenases were developed and tested in the unicellular cyanobacterium *Synechocystis* PCC 6083, and in heterocysts of the filamentous cyanobacterium *Nostoc punctiforme* ATCC 29133. Following heterologous expression, functional, semisynthetic [FeFe]-hydrognases operating *in vivo* in cyanobacterial cells were generated by synthetic activation. The procedure proved successful in both the unicellular and filamentous strain, and for [FeFe]-hydrogenases from different groups and subclasses. The semisynthetic enzymes proved capable of hydrogen production under different environmental conditions and links to the metabolism of the host cell. Hydrogen production capacity proved long-lived and was retained for several days. In *Nostoc punctiforme*, synthetic activation was confirmed to generate active [FeFe]-hydrogenase in both vegetative cells and heterocyst.

The results presented in this thesis demonstrate a novel way to explore *in vivo* hydrogen production from heterologous [FeFe]-hydrogenases in cyanobacteria. In the search for suitable candidates for H_2 production systems, synthetic activation may be used to investigate a wide range of [FeFe]-hydrogenases, strains and cultivation conditions, circumventing the need of elaborate maturation machinery optimisation.

Adam Wegelius, Department of Chemistry - Ångström, Molecular Biomimetics, Box 523, Uppsala University, SE-75120 Uppsala, Sweden.

ISSN 1651-6214
ISBN 978-91-513-1073-2
urn:nbn:se:uu:diva-425911 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-425911)

List of Papers

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

I Lindblad, P., Fuente, D., Borbe, F., Cicchi, B., Conejero, J., Couto, N., Čelešnik, H., Diano, M., Dolinar, M., Esposito, S., Evans, C., Ferreira, E., Keller, J., Khanna, N., Kind, G., Landels, A., Lemus, L., Noirel, J., Ocklenburg, S., Oliveira, P., Pacheco, C., Parker, J., Pereira, J., Pham, T., Pinto, F., Rexroth, S., Rögner, M., Schmitz, H., Benavides, A., Siurana, M., Tamagnini, P., Touloupakis, E., Torzillo, G., Urchueguía, J., **Wegelius**, **A**., Wiegand, K., Wright, P., Wutschel, M., Wünschiers, R. (2019) CyanoFactory, a European consortium to develop technologies needed to advance cyanobacteria as chassis for production of chemicals and fuels. *Algal Research* 41: 101510.

II **Wegelius**, **A**.,* Khanna, N.,* Esmieu, C., Davide Barone, G., Pinto, F., Tamagnini, P., Berggren, G., Lindblad, P. (2018) Generation of a functional, semisynthetic [FeFe]-hydrogenase in a photosynthetic microorganism. *Energy and Environmental Sciences* 11: 3163-67.

III **Wegelius**, **A**., Land. H., Berggren, G., Lindblad, P. (2021) Novel semisynthetic [FeFe]-hydrogenase with stable expression and H_2 production capacity in a photosynthetic microbe. *Manuscript*.

IV **Wegelius**, **A**.,* Li, X.,* Turco, F., Stensjö, K. (2018) Design and characterization of a synthetic minimal promoter for heterocyst-specific expression in filamentous cyanobacteria. *PloS One* 13(9): e0203898.

V **Wegelius**, **A**., Khanna, N., Zamader, A., Berggren, G., Lindblad, P. (2021) [FeFe]-hydrogenase synthetically activated in the filamentous cyanobacterium *Nostoc punctiforme* ATCC 29133. *Manuscript*.

VI Miao, R., **Wegelius**, **A**., Durall, C., Liang, F., Khanna, N., Lindblad, P. (2017) Engineering cyanobacteria for biofuel production. In: Hallenbeck, P. (Ed.), Modern Topics in the Phototrophic Prokaryotes, Environmental and Applied Aspects. Chapter 11: 351-393. Springer International Publishing, Switzerland. ISBN: 978-3-319-46259-2.

*Joint first authors

Contents

Abbreviations

ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BCD	Bicictronic design
bp	Base pair
CCM	Carbon concentration mechanism
CO_2	Carbon dioxide
DBMIB	2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone
DCC	*N,N´*-dicyclohexylmethanediimine
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DNA	Deoxyribonucleic acid
GFP	Green fluorescent protein
H_2	Molecular hydrogen
IPCC	Intergovernmental Panel on Climate Change
IPTG	Isopropylthio-β-galactoside
MCS	Multiple cloning site
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
O_2	Molecular oxygen
PAGE	Polyacrylamide gel electrophoresis
PCC	Pasture Culture Collection
PSI	Photosystem I
PSII	Photosystem II
RBS	Ribosomal binding site
RNA	Ribonucleic acid
SD	Shine-Dalgarno
SDS	Sodium dodecyl sulphate
TSS	Transcription start site
UTR	Untranslated region
YFP	Yellow fluorescent protein

Introduction

Motivation

Human activities have caused an approximately 1.0° C global increase in average temperature compared to pre-industrial levels, and this warming has been estimated to reach 1.5° C around year 2040 [1]. Unfortunately, greenhouse gas emissions are not decreasing. In fact, they are still increasing every year [2], and the rate of global temperature rise might be underestimated [3]. The recent report from Intergovernmental Panel on Climate Change (IPCC), 'Special Report on the Ocean and Cryosphere in a Changing Climate' [4], describes that many of the physical impacts of the warming escalates in a non-linear fashion in relation to the temperature increase. Ice sheet and glaciers around the world are losing mass, permafrost temperatures are increasing, oceans are warming up and sea levels are rising. Even a small further increase in global temperature can have catastrophic consequences through cascade effects [5]. The difference between a 1.5° C and a 2° C global warming is predicted to be immense [4, 5]. Global society needs to take drastic measures to keep the ongoing climate change to a minimum.

Carbon dioxide emission is a main driver of climate change and we urgently need to escape our dependence on fossil carbon sources for energy production. Solar irradiation offers a practically inexhaustible source of energy, and a wide and unassuming search for sustainable and efficient ways to harvest this energy needs to be undertaken. This thesis, where I have investigated hydrogen production from cyanobacteria, is my small contribution in this search.

Cyanobacteria as fuel producers

Cyanobacteria are photosynthetic Gram-negative bacteria found in various environments all over the globe. Previously (and sometimes still inaccurately) called 'blue-green algae', cyanobacteria are famous for their role in the Great Oxygenation Event 2.5 billion years ago [6] and for the endosymbiotic relationship with eukaryotic organisms [7] that resulted in today's chloroplasts and life on land as we know it. Cyanobacteria are hugely important as primary producers and, with some strains capable of diazotrophic growth, vital in the

biogeochemical nitrogen cycle. The phylum Cyanobacteria is vast and diverse, with morphologies ranging from unicellular to filamentous. Among the filamentous cyanobacteria, we find strains capable of differentiating all, or a subset of, their cells into specialized, morphologically different compartments to tackle various changes in their environment [8].

Cyanobacteria have several unique characteristics that makes them interesting as fuel producers. Their microbial, photosynthetic nature enables photoautotrophic growth with very minimal substrate requirements. Electrons from the photocatalytic water splitting by PSII are channelled in the photosynthetic electron transport chain to PSI, facilitating the creation of a proton gradient over the thylakoid membrane. In a second light driven reaction, electrons are used to produce the high energy electron carrier ferredoxin, and subsequently NADPH. The energy stored in the gradient over the thylakoid membrane drives the creation of the cellular energy carrier ATP by ATP-synthase. The NADPH and ATP are used for CO_2-fixation and synthesis of all biomolecules that the cell is made of.

Compared to plants, that also perform oxygenic photosynthesis, cyanobacteria are more attractive as fuel producers for many reasons. Their ability to grow on no-arable land, and thus not compete with food production, is often brought up [9, 10]. Cyanobacteria could even be cultivated in dessert regions and/or in seawater. They also have higher solar energy capturing efficiencies [10] and boasts an impressive carbon concentration mechanism (CCM) that enhances the photosynthetic efficiency by reducing photorespiration [11–13]. Compared to higher organisms like plants, cyanobacteria are also, generally speaking, more readily approachable with genetic engineering and recombinant technologies.

The work presented in this thesis employs two cyanobacterial strains, *Nostoc punctiforme* ATCC 29133 (*N. punctiforme*) and *Synechocystis* PCC 6803 (*Synechocystis*). *N. punctiforme* is a filamentous strain with a large, almost 10 Mbp genome [14], isolated from coralloid roots of the cycad *Macrozamia* native to Australia [15]. Upon combined nitrogen starvation, it differentiates a subset of the vegetative cells along the filaments into specialized microaerobic compartments, called heterocysts. These cells host the oxygen sensitive nitrogenase that catalyzes the nitrogen fixation reaction. After differentiation, the filaments are capable of photoautotrophic, diazotrophic growth, where vegetative cells perform oxygenic photosynthesis and provide carbon substrate for nitrogen fixation in the heterocyst [16]. *N. punctiforme* is also capable of heterotrophic growth and occurs symbiotically with fungi and terrestrial plants [14].

Synechocystis is a well-studied unicellular non-diazotrophic cyanobacterium, isolated from freshwater. It is one of the most popular photosynthetic model organisms worldwide. The *Synechocystis* strain used in this work is

glucose tolerant and capable of photoautotrophic, mixotrophic and heterotrophic growth. It's 3,6 Mbp genome was the first among photosynthetic autotrophs to ever be fully sequenced [17].

Metabolic engineering and genetic tools

Metabolic engineering is often defined (for example by Stephanopoulus 2012, Kerkhoven *et al.* 2015 and Teo *et al*. 2017 [18–20]) as *"the directed modulation of metabolic pathways using methods of recombinant technology for the purpose of overproducing fuels and chemical and pharmaceutical products"*. This definition is assigned to James E. Bailey's article in Science 1991 [21]. However, these words do not appear in Bailey's publication, and the origin of this definition is unknown. Regardless, it summarizes well the field of metabolic engineering, where the cellular metabolism is rewired, using advanced technical approaches, to produce novel compounds or increase the production of naturally occurring ones [22]. Metabolic engineering has been used to great effect on countless occasions [22, 23], especially in heterotrophic model organism like *Escherichia coli* (*E. coli*) and yeast.

One of the pillars that metabolic engineering rests upon is synthetic biology. This science field specifically deals with designing and combining well characterized genetic elements using engineering principles to accelerate construction of new biological systems. Ideally, these genetic elements can be used in different applications and behave in a predictable manner. In the context of metabolic engineering, synthetic biology can be described as the development and employment of reliable genetic tools for controlled expression of native or heterologous genes. To advance fuel production from cyanobacteria, and cyanobacterial biotechnology in general, we need better genetic tools for strain development and expression regulation. Compared to the situation in heterotrophic model organisms, the toolbox for genetic engineering of cyanobacteria is still limited [24].

Genetic tools are DNA sequences that mediate biological functions within the host. Examples are vectors, promoters, ribosomal binding sites (RBS), transcription factor binding sites, terminators and so on. Below, I briefly discuss a few genetic tools of special interest for this work and for cyanobacterial genetic engineering in general.

Promoters and transcription initiation

Promoters are binding sites for RNA polymerase in the genetic material which determines the position and frequency of transcription initiation. The recognition of specific promoters and regulation of transcription initiation are meditated by sigma factors, which form complexes with RNA polymerase. In bacteria, the sigma factor recognizes and binds to the -10 and -35 regions of the promotor and initiates transcription at the transcription start site (TSS), defined as position +1 [25]. In cyanobacteria (as well as in other bacteria) one

RNA polymerase is responsible for all transcription in the cell [26], and sigma factor switching can be used as a global response to environmental changes and stress conditions [27].

The choice of promoter is extremely important for the successful production of a selected compound in a biological system. Much effort has been made to discover and create promoter regions for use in cyanobacteria [28]. So far, a large part of the promoters utilized have been native sequences, originating from the same or a closely related strain. Many compounds interesting for fuel or chemical industry has been produced from cyanobacteria by employing such promoters [29]. Promoter regions related to the photosystems or the phycobilisome, such as the phycocyanin promoter P*cpc*, are popular due to their high activity, and has been used with great success, in e.g. *Synechocystis* [24, 30–32]. The light regulated nature of these promoters, as well as the very high activity, can of problematic for some applications. To satisfy the need of more moderate expression levels, Markley *et al*. (2015) developed a library of constitutive promoters with varying activity, based on the P*cpc* [33].

When the complexity of genetic engineering advances, or when products or intermediates are toxic, inducible promoters are strongly desirable. Even in this arena, usage of native cyanobacterial promoters has been widespread. Micronutrient and metal induced promoters, like the nickel induced P*rnsB* [34], offers wide dynamic range and inducibility [24, 35]. Apart from these, inducible promoter systems from other organisms have been adapted and deployed in cyanobacteria [24, 33, 36–39]. Such systems are advantageous as they are free of any native sequences and not laden with native regulation. Orthogonality is often brought up as one of the cornerstones of synthetic biology, but sometimes practicality forces our hands, and we need to rely on the well-repressed native metal induced promoters. In the common model strain *Synechocystis*, where the induction of lac-operated promoters with IPTG is not well-working, there is often little choice, especially considering the light sensitivity of anhydrotetracycline used to induce TetR-regulated promoters, described in [37] and reviewed in [24] and [35].

Translation initiation and ribosomal binding site

The ribosome is a complex molecular machine that, *via* the 16S ribosomal RNA, recognizes and binds to the Shine-Dalgarno (SD) sequence of the ribosomal binding site (RBS) in the mRNA. Following the recognition of the RBS, the ribosome translates the coding sequence, codon by codon, into a peptide chain. This is another key process to consider when designing genetic constructs. Most commonly, a high translation initiation is desirable in cyanobacteria, since intricate regulation and fine tuning of expression most often is engineered on transcriptional level. Looking at heterologous expression in cyanobacteria, native RBSs are commonly used, and are often deployed in expression constructs together with the associated native promoter. But also more systematic approaches, with library creation and evaluation, have been

explored [33, 40]. In *Synechocystis*, the engineered, synthetic RBS* (RBS-'star') [41] with exactly complementary SD sequence to the *Synechocystis* anti-SD, is a well-used alternative.

Plasmids as vectors

Plasmids are circular, self-maintained DNA molecules, that can be used as vectors to transfer recombinant genetic material to host cells. Vectors can be designed to be self-replicating in the target organism, or to integrate DNA into the existing genetic material. The work presented in this thesis employs only the former of the two. The RFS1010 [42] derived broad host range shuttle vector pPMQAK1 [43] is used for expression in *Synechocystis*, and variants of plasmids containing the pDC1 cyanobacterial origin of replication [44–46] are used for expression in *N. punctiforme*.

Interactions between parts

It has been noted in the field, that the usefulness of reporter-protein based characterization of genetic tools, exemplified by some of the works referred to earlier in this chapter, is questionable [40, 47, 48]. The performance of a certain promoter-RBS combination often differs greatly depending on what gene product is being expressed. This is true for cyanobacteria as well as for *E. coli* and can be an obstacle for advanced synthetic biology and metabolic engineering. Due to the longer generation time, longer segregation time and a more complicated strain generation process in cyanobacteria compared to *E. coli*, combinatorial "trial and error" approaches are not practical if we want to advance the research field. Reliability of genetic parts is another cornerstone in synthetic biology, and here, we have a long way to go.

A large part of the context dependent variation observed is suggested to be the result of secondary structures in the mRNA, obscuring the RBS and the SD in unpredictable ways [47, 49]. It is easy to imagine secondary structures, even close to the 5' end, differing dramatically between mRNAs with different coding sequence, even if the 5' UTRs remain identical. In *E. coli*, much of this unpredictability can be circumvented be the employment of standardized insulator sequences and adaptors between the 5' UTR and the CDS. Bicistronic design (BCD) and RiboJ are two examples [49, 50]. Both these devices have been investigated in *Synechocystis* [51], providing vital information for further research. Apart from that, context dependent expression variation in cyanobacteria remains largely unaddressed.

Fuel production

Many of the molecules created by cyanobacteria in the light driven autotrophic biosynthesis are directly useful as fuel compounds or fuel precursors. Certain strains have high native production of fatty acids that can be used for biodiesel

production, others produce molecular hydrogen (H_2). Unfortunately, this native production is generally too low to satisfy the yield requirements in large scale production. The lion's share of the cellular energy and reduction power generated by the photosynthetic light reaction is used for cell proliferation and survival, not to produce large amounts of high energy compounds suitable as fuels.

The metabolic network in a cell consists of a myriad of reactions catalyzed by thousands of enzymes, all expressed in a tightly regulated manner. By careful engineering of the metabolic pathways in the cyanobacterial cell, energy and reductant power can be redirected to allow substantial fuel production directly from CO_2, light, and water [52–55]. Several fuel producing strains have been created using genetic engineering of cyanobacteria [52, 53]. Some of the most promising, recent projects are utilizing advanced metabolic engineering and involves production of ethanol [56], isopropanol [57], 1-butanol [31], isobutanol [58], and hydrogen [59]. Still, to make large scale production feasible, higher yields and better energy balances are generally required.

H_2 metabolism and production in cyanobacteria

Several characteristics make H_2 promising as energy carrier in future energy systems. The energy dense gas can be used as feedstock for production of synthetic fuels, or directly converted to electrical energy in a fuel cell [60]. Electricity production from H_2 is clean and releases water as only exhaust product. In contrast to electricity, that requires large and expensive batteries for energy storage, H_2 can be transported and/or long time stored in, for example, pressurized tanks. In the fossil fuel dependent transport sector, introduction of H_2-powered vehicles could drastically reduce the harmful emissions [61] and several automobile manufacturers offer fuel cell electrical vehicles today. These vehicles display many of the beneficial characteristics of the battery propelled electrical cars, but have comparable range to traditional combustion engine cars. Aside from the promising prospects in transportation, large quantities of H_2 is used in refinery processes and as feedstock in chemical industry. Today, H_2 is produced nearly exclusively from fossil sources, primary natural gas.

Solar driven water splitting is a promising approach to produce clean, carbon neutral H_2. To be sustainable in the long run, production needs to be independent of rare earth metals, easy to implement in large scale, and not produce unrecyclable waste materials. Biological H_2 production from photosynthetic microorganisms is one of few technologies that has the potential to truly fulfill these requirements.

Native production

Many cyanobacterial strains have the inherent capability to produce H_2. This is a rare feature among photoautotrophs, and offers unique possibilities of direct production of a fuel, using only sunlight and water as substrates. Three enzymes, all oxygen intolerant metalloproteins, are directly involved in H_2 metabolism in cyanobacteria: nitrogenase, uptake hydrogenase (Hup), and bidirectional hydrogenase (Hox).

Nitrogenase is the enzyme enabling nitrogen fixation in diazotrophic cyanobacteria. It catalyzes the break of the covalent N_2 bond and the subsequent formation of ammonia, an ATP dependent process in which H_2 is produced as a biproduct. The uptake hydrogenase, Hup, is closely associated with this process, and recovers electrons wasted on H_2 production during nitrogen fixation. It is a [NiFe]-hydrogenase that catalyzes the oxidation of H_2, and this enzyme is exclusively found in nitrogen fixing strains.

Like the uptake hydrogenase, the cyanobacterial Hox is a [NiFe]-hydrogenase. It can catalyze both the oxidation of H_2 and the reduction of protons, and thus be involved in H_2 production or uptake. The enzyme consists of a catalytic and a diaphorase complex, and the catalyzation is not dependent on ATP. The physiological function of the enzyme has long been under debate [62], but it is essential for dark aerobic growth [63] and in mixotrophic, nitrate-deprived conditions [64]. The exact electron donor to the enzyme has also been heatedly debated, see for example [64], [65] and [66]. Regardless, the bidirectional Hox is known to produce H_2 to balance the redox state of the cell during dark fermentation, and to remove excess electrons from the photosynthetic electron transport chain during transition to light [67].

Despite the their native H_2 production capacity, cyanobacteria are still far from being implemented in large scale production systems. The oxygen sensitivity of the H_2 producing enzymes, and the low energy conversion efficiencies due to competitions for electrons with other pathways, are major obstacles [68].

Engineering for increased H_2 production

There are several ways to enhance the H_2 production from cyanobacteria. Deleting the *hup* genes, encoding the uptake hydrogenase, from the genome, and thus removing the H_2 uptake capacity, has been shown to increase the net H_2 evolution in numerous filamentous strains [69–74]. Mutating the nitrogenase, targeting either the active site [75, 76] or the presumed gas channel [77], has also been shown to increase the production. Nonetheless, H_2 production from nitrogenase is still restricted by the high energy demand of the enzyme, and this remains an uncertainty for future applications.

The bidirectional Hox hydrogenase in *Synechocystis* has been engineered to tolerate higher O_2 levels, and the changes also resulted in better reaction

bias for production [78]. Recently, Appel *et al*. (2020) reported the development of a *Synechocystis* strain where the catalytic complex of the Hox hydrogenase was fused to the PsaD subunits of PSI [59]. The fusion facilitated *in vivo* light driven H_2 production using electrons directly from PSI, and showed no uptake activity due to the lack of the diaphorase subunits. Previously, such fusions had only been reported *in vitro* [79, 80]. Although it is presented as a proof of concept study, follow up investigations and upscaling reports will hopefully follow soon.

[FeFe]-hydrogenases

Hydrogenases are divided into classes after the metal composition of the cofactor in the active site. The hydrogenases occurring in cyanobacteria belong, as discussed above, to the [NiFe] class. However, another class, the [FeFe]-hydrogenases, have long been consider superior for usage in biological and biohybrid H_2 production systems. This is attributed to very impressive catalytic activity, several thousand H_2 s^{-1} [81, 82], and a high reaction bias towards production. [FeFe]-hydrogenase research is a very active field, with new enzymes still being discovered and detailed aspects of the H_2 turnover still being discussed [83]. All [FeFe]-hydrogenases harbors a biologically unique cofactor called the H-cluster. This cluster consists of a [4Fe-4S] cluster connected to a diiron subcluster via a cysteine bridge. The complete biosynthesis of this cofactor requires three specific maturases, HydE, HydF and HydG [84, 85], that always accompanies the [FeFe]-hydrogenase (HydA) in the natural hosts. The maturation factors assemble the diiron subcluster and insert it to the active site of the unmatured HydA. Heterologous expression of HydA without the maturases, results in an inactive *apo*-enzyme without catalytic activity [86].

Employment of a suitable [FeFe]-hydrogenase in cyanobacteria could enable efficient H_2 production, not hampered by the energy demand of nitrogenase or the H_2 consumption activity of the [NiFe]-hydrogenases. This strategy has long been considered a possible way to move cyanobacterial H_2 production forward, but the maturation machinery co-expression requirement makes it challenging to pull off.

In 2011, Ducat *et al*. reported light dependent H_2 production from a *Clostridium acetobutylicum* [FeFe]-hydrogenase operating *in vivo* in *Synechococcus elongatus* PCC 7942 [87]. Unfortunately, no follow up studies or upscaling tests of this strain have been reported so far. Seven years later, in 2018, Avilan *et al*. showed light driven H_2 production from a similar [FeFe]-hydrogenase operating in heterocysts of the filamentous cyanobacterium *Nostoc* PCC 7120 [88]. In the same study, the heterocyst metabolism was engineered to better protect the heterologous enzyme from O_2 during oxygenic photosynthesis. This is an encouraging example of metabolic engineering towards sustainable H_2 production in cyanobacteria, and hopefully we will see more investigations of this strain or similar ones in the near future.

Aim

The aim of this thesis can be divided into three parts:

I Develop tools to reliably express [FeFe]-hydrogenases in different cyanobacterial strains,

II Generate functional [FeFe]-hydrogenase operating *in vivo* in cyanobacteria, focusing on alternative maturation methods, and

III Explore hydrogen production from semisynthetic [FeFe]-hydrogenases in unicellular and filamentous cyanobacteria under different environmental conditions

Results and Discussion

Expression of [FeFe]-hydrogenase in cyanobacteria (paper I and V)

The promoter P*trc*$_{core}$ (P*trc*O1-core in [89]) is known to mediate constitutive expression (in the absence of Lac-repressor) in *Synechocystis* and other strains [41, 89]. RBS* (RBS-'star'), with exactly complementary SD sequence to the *Synechocystis* anti-SD, is specifically engineered to be suitable RBS for protein expression in *Synechocystis* [41]. The specific combination of P*trc*$_{core}$ and RBS* has been evaluated in our lab, and is known to give reliable expression of a GFP reporter. However, when we used the same transcription and translation initiation unit (P*trc*$_{core}$-RBS*) to expresses other genes in *Synechocystis*, substantial variations in performance became apparent. When P*trc*$_{core}$-RBS* was used to express a codon optimized *hydA1*-gene encoding the [FeFe]-hydrogenase HydA1 from *Chlamydomonas reinhardtii* (*Cr* HydA1) in *Synechocystis*, confirmed positive transformants showed no sign of protein expression.

RBS34 (BBa_B0034, Registry of Standard Biological Parts [90]) is another RBS known to facilitate translation of mRNA in *Synechocystis* and has successfully been used in combination with P*trc* [41]. When P*trc*$_{core}$-RBS34 was used to drive the expression of an *hydEFG* operon, encoding the three maturation factors HydE, HydF and HydG from *Clostridium acetobutylicum*, only minimal levels of HydG was expressed in confirmed positive transformants. The fact that the expressions strategies described above failed, even though the regulatory parts used have been proven to function for expression of other genes in the same host, was surprising and exemplifies the problems that can be caused by context dependence of genetic tools.

Bicistronic design

Looking for a way to achieve satisfying levels of expression, the 5' UTR regions in both constructs discussed above were replaced with the bicistronic design insulator sequence BCD2 [49]. The BCD is designed to temporally melt secondary structures around the junction between the 5' UTR and the CDS, by the translation of a standardized leader peptide, containing a second RBS imbedded in the coding sequence followed by a hybrid start/stop codon TAATG [49]. Comparison between the expression strategies is shown in Figure 1a.

Western immunoblot analysis of SDS-PAGE separated protein extracts, revealed expression of HydA1 only in strains harboring the BCD construct (Figure 1b). Similar immunoblot analysis, targeting the flag tagged (DYKDDDDK) HydG protein from the *hydEFG* operon, revealed barely detectable expression from the original construct, but strongly evident expression from the BCD counterpart (Figure 1b).

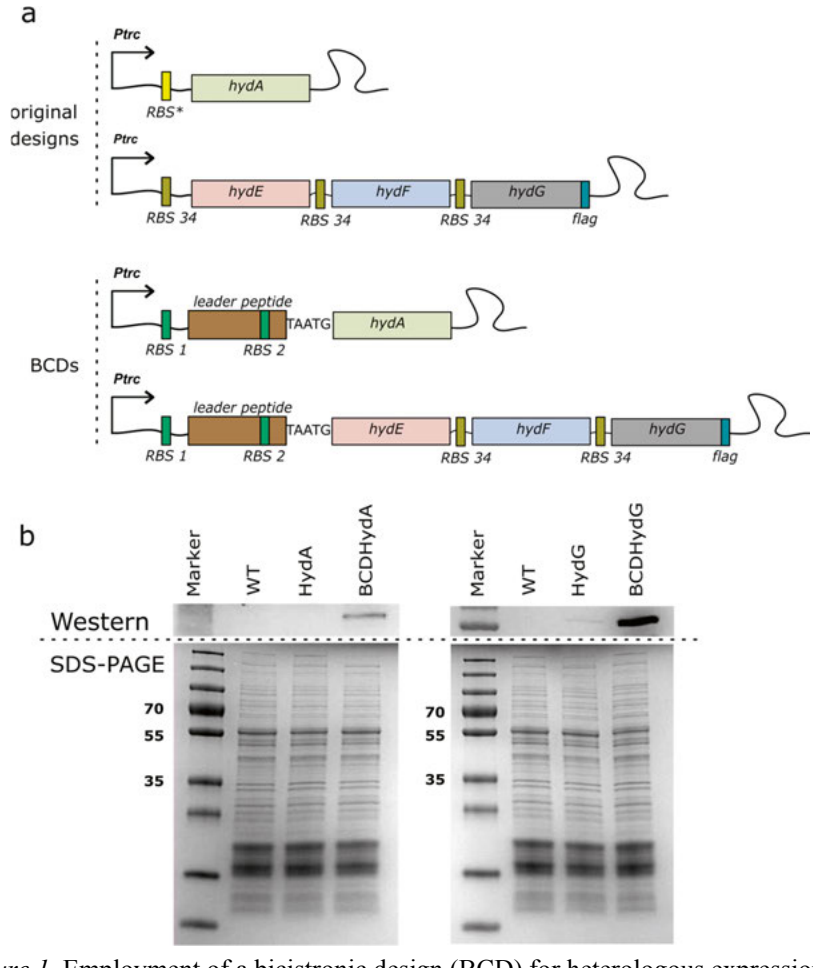

Figure 1. Employment of a bicistronic design (BCD) for heterologous expression of HydA1 and HydEFG in *Synechocystis*. (A) Representation of *hydA* and *hydEFG* expression construct designs, before (original) and after (BCDs) modification with BCD. Elements are not depicted in scale. (B) Western immunoblot analysis of crude protein extracts from *Synechocystis* wild type and engineered strains using HydA antibodies (HydA) and flag tag antibodies ($HydG_{flag}$). SDS-PAGE gels are included for loading reference control and numbers indicate reference molecular weights in kDa. Molecular weight of HydA1 and $HydG_{flag}$ is 46.4 and 54.6 kDa, respectively. Figure adapted from paper I.

Semiquantitative RT-PCR confirmed the existence of mRNA from all four constructs (Figure 2), and no difference in mRNA levels were detected. It is apparent that the expression levels from P*trc*$_{core}$ and RBS*/RBS34, that is known to facilitate clear expression of GFP in *Synechocystis*, change dramatically when employed in a different genetic context. In the case above, the combination failed to express detectable levels of HydA and satisfying levels of HydG, even though mRNA was confirmed from both constructs. When BCD was used, translation was enabled and protein was accumulated. This indicates secondary structure covering the RBS in the mRNA, and demonstrates the effectiveness of BCD for expression of heterologous genes in *Synechocystis*. Based on these conclusions, bicistronic designs were employed for all expressions of [FeFe]-hydrogenases in papers II, III and V.

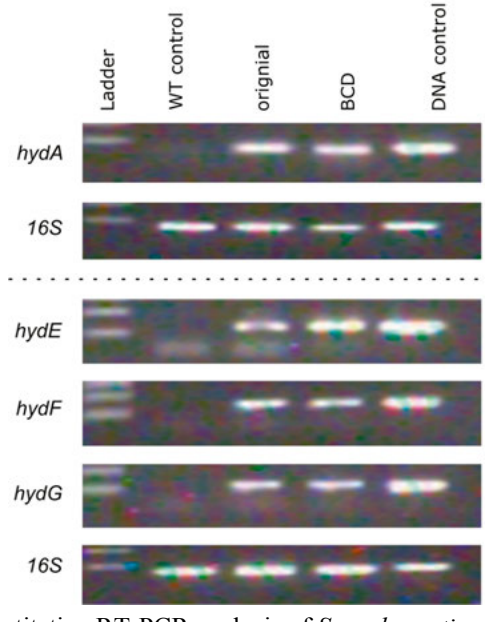

Figure 2. Semi-quantitative RT-PCR analysis of *Synechocystis* wild type (WT control) and engineered strains expressing HydA1 (above) and HydE, F and G (below) without (original) or with (BCD) use of BCD. DNA control is included as size reference. 16s amplification is included as loading reference. Figure adapted from paper I.

Interestingly, changing the RBS34 before *hydE* to a BCD in the maturation factor operon, facilitated efficient translation of the flag tagged *hydG*, starting more than 3500 bp downstream in the mRNA. I conclude that the distance between the stop codons and the following RBSs in the operon (11 bp) is small enough for the ribosome, translating the upstream CDS, to melt any secondary structures that might hinder recruitment to the downstream RBS.

Both P*trc*$_{core}$, RBS* and RBS34 are functional genetic tools also in *E. coli*. To our surprise, both HydA1 and HydG were well expressed in the heterotopic bacterium, also from the original constructs without BCD. It appears that *Synechocystis*, by some mechanism, is a more delicate expression system when it comes to translation initiation. Whether this is true in general, or only for the [FeFe]-hydrogenase related genes in this work, needs further investigation.

Heterocyst specific expression of [FeFe]-hydrogenase

When designing a genetic construct for expression of *Cr* HydA1 in heterocysts of *N. punctiforme* (paper V), the expression problems described above were repeated, and this time with a native promoter fragment. A 316 bp P*hupSL* fragment from *N. punctiforme*, reported to facilitate heterocyst specific expression of GFP [91], was employed, together with RBS*, upstream a *hydA1-gfp* fusion CDS. RBS* is known to be a functional RBS in *N. punctiforme* and was successfully used in paper IV. Here however, when the construct was assembled and electroporated into *N. punctiforme*, heterocysts of confirmed positive transformants displayed no GFP expression. Inspired by the successful employment of BCD in *Synechocystis*, we applied the same strategy for this construct and changed the RBS* of the original construct to BCD2 [49]. The new strain exhibited significant GFP expression, and confocal microscopy confirmed a preserved heterocyst enhanced expression pattern (Figure 3).

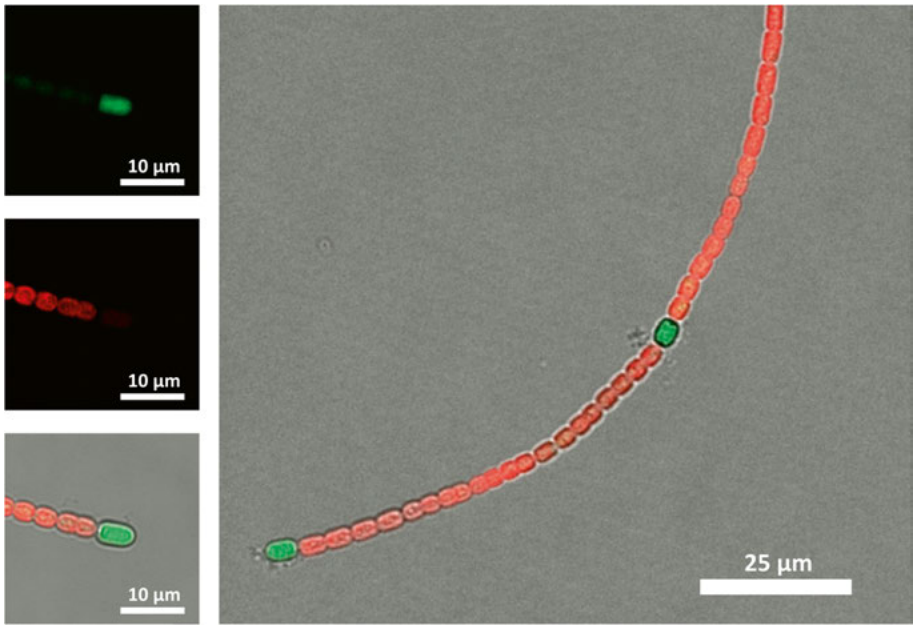

Figure 3. Confocal fluorescence images of *Nostoc punctiforme* filaments expressing the *apo*-[FeFe]-hydrogenase HydA1 from *Chlamydomonas reinhardtii*, fused to a green fluorescent protein (GFP). Green channel depicts florescence from GFP (500-520 nm), red channel depicts autofluorescence (600-700nm). Overlaid images display both fluorescence channels overlaid with the bright-field channel. The large cells with increased GFP fluorescence and reduced autofluorescence are heterocysts. The white bars (10 and 25 µm) indicate scale.

Evidently bicistronic translation initiation design can offer a solution when more traditional construct designs fail to express heterologous proteins in cyanobacteria. This is true even in the more special case of cell specific expression.

Semisynthetic [FeFe]-hydrogenases in *Synechocystis* (papers II and III)

Heterologous expression of an [FeFe]-hydrogenase in cyanobacteria, without the co-expression of the three maturases HydE, F and G, results in an inactive *apo*-enzyme. This is an inconvenience when developing [FeFe]-hydrogenase based H_2 producing strains. Reliable and controlled expression of all four genes in the same cell is required to allow *in vivo* H_2 production. Only two examples of this being executed in cyanobacteria exists in literature (discussed in Introduction) [87, 88]. In both cases the group A, subclass M3, *Clostridium acetobutylicum* [FeFe]-hydrogenase was utilized. Investigations of new or

modified [FeFe]-hydrogenases, in cyanobacteria or other microbes, could potentially be simplified by bypassing the biological maturation system.

Generation of semisynthetic hydrogenase

A few years back, it was discovered that a purified *apo*-HydA can be matured by direct introduction of a synthetic analog of the diiron subcluster $[Fe_2(adt)(CO)_4(CN)_2]^{2-}$ (from now on $[2Fe]^{adt}$, adt = $^-SCH_2NHCH_2S^-$) (Figure 4) [92, 93], a process referred to as artificial maturation or synthetic activation. This technique was later expanded to enable *in vivo* synthetic activation of heterologous expressed, unmatured *Cr* HydA1 in *E. coli* [94].

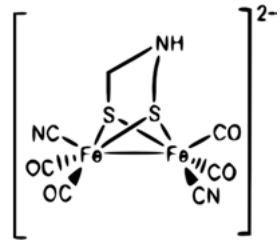

Figure 4. Schematic representation of the inorganic diioron subcluster mimic $[Fe_2(adt)(CO)_4(CN)_2]^{2-}$ ($[2Fe]^{adt}$ adt = $^-SCH_2NHCH_2S^-$), used to artificially maturate *apo*-HydA.

Paper II describes the first artificial maturation of an [FeFe]-hydrogenase in cyanobacteria. A *Chlamydomonas reinhardtii* HydA1 (*Cr* HydA1), expressed and synthetically activated in a hydrogenase deficient (*ΔhoxYH*) [95] strain of the unicellular cyanobacterium *Synechocystis* (*Synechocystis Δhox*), proved capable of H_2 production under fermentative conditions (Figure 5). Fermentative H_2 production from WT *Synechocystis* was improved by expressing and activating *Cr* hydA1, side-by-side to the native Hox hydrogenase (Figure 5). However, the *Cr* hydA1-expressing strain without the bidirectional [NiFe]-hydrogenase produced the same amount of H_2, or more, as the strain with two active hydrogenases. The artificially maturated *Cr* HydA1 is, to my knowledge, the first semisynthetic enzyme to ever operate *in vivo* in cyanobacteria. Further, it is the first example of a subclass M1 [FeFe]-hydrogenase active *in vivo* in cyanobacteria.

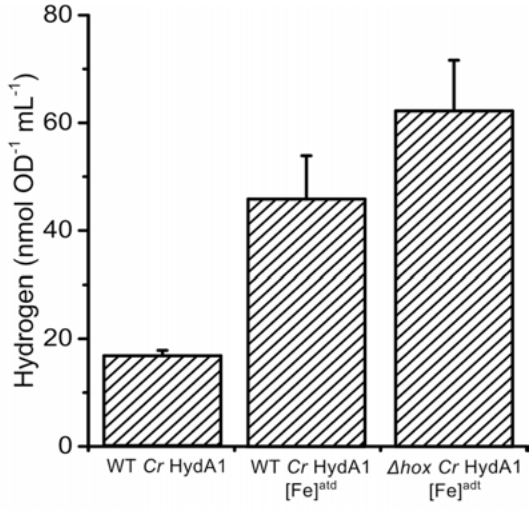

Figure 5. Accumulated H_2 from *Synechocystis* wild type (WT) and *Synechocystis Δhox* expressing *Cr* HydA1. Columns represent nmol H_2 accumulated per mL culture after 48 h glucose supplemented, dark incubation with ($[2Fe]^{adt}$) or without synthetic activation, normalized to optical cell density. Data points represent means of 2-4 independent experiments. Error bars represent s.d. Figure adapted from paper II.

Cr HydA1 belongs to the group A prototypical [FeFe]-hydrogenase, subclass M1. This is the structurally simplest subclass known and has only one protein domain. This domain is called the H-domain and is present in all [FeFe]-hydrogenases [83]. In paper III, we expressed and synthetically activated two newly discovered [FeFe]-hydrogenases [96], *Sm* HydA from *Solobacterium moorei* and *Tam* HydS from *Thermoanaerobacter mathranii*, in *Synechocystis Δhox*. Like *Cr* HydA1 and the *Clostridium acetobutylicum* HydA, *Sm* HydA belongs to the group A [FeFe]-hydrogenase, but is of a different subclass called M2. Enzymes from the M2 subclass encompass an N-terminal domain with two [4Fe-4S]-cluster binding sites, in addition to the H-domain. *Sm* HydA was, upon discovery, partially characterized in *E. coli* where it showed indications of a specific activity surpassing that of *Cr* HydA1 [96]. *Tam* HydS, belongs to group D and subclass M2e. Enzymes of this subclass has the same domains as the M2 subclass, but features an additional uncharacterized C-terminal domain. It is a putative sensory hydrogenase and exhibits only low activity compared to *Sm* hydA and *Cr* HydA1when expressed in *E. coli* [96, 97].

In vivo H_2 production activity was detected from both *Sm* HydA and *Tam* HydA after activation with $[2Fe]^{adt}$ in *Synechocystis Δhox*. *Tam* HydS showed only very limited activity, and only in light with DCMU inhibition. The *Sm* HydA showed significant activity under a number of environmental conditions. Before, no [FeFe]-hydrogenases of the M2 or M2e subclasses had been employed in a cyanobacterium. It is evident that artificial maturation in *Syn-*

echocystis is a reliable and convenient way to investigate novel [FeFe]-hydrogenases in a photosynthetic context. I believe that this will be of great use for further developments in the field.

Effect of light and nitrate

When different incubation conditions were investigated, it was quickly discovered that darkness was far more favorable for H_2 production, than light with DCMU inhibition. This was true for both *Sm* HydA and *Cr* HydA1 in *Synechocystis Δhox*. This was a surprising observation, since the light condition (80-100 µmol photons m^{-2} s^{-1}) would allow for anoxygenic photoreduction of ferredoxin. This was anticipated to be favorable for H_2 production, especially for *Cr* HydA1 considering that photosynthetically reduced ferredoxin is an electron donor to the enzyme in the native host [98, 99]. However, especially in nitrate-deprived conditions without addition of glucose, H_2 production in darkness vastly outperformed the production in light.

The highest H_2 production, both in light with DCMU inhibition and in darkness, was measured without nitrate in the cultivation medium ($BG11_0$). This is due to elimination of the reductant-demanding nitrate assimilation, freeing up reduction capacity for H_2 production [100]. Similar behavior has been observed for Hox catalyzed H_2 production in WT *Synechocystis* [101]. The fact that H_2 production from the synthetically activated hydrogenases increases and decreases in response to environmental changes is a clear indication that the semisynthetic enzyme links to the metabolism of the cells. It is an artificially activated, semisynthetic enzyme that operates *in vivo* and accepts metabolically produced reduction power for H_2 production.

In paper III, the *in vivo* activities of *Cr* HydA1 and *Sm* HydA were compared under nitrogen deprived conditions. The *Cr* HydA1 used in paper II differs slightly from the one used in paper III. The former one is a differently truncated and untagged version, expressed from a differently codon optimized gene. The *Cr* HydA1 in paper III (from now on *Cr* $HydA1_{strep}$) is carrying a step tag II (WSHPQEF) for fair activity comparison to the similarly tagged *Sm* HydA. However, no differences in *in vivo* activity could be detected between *Cr* HydA1 and *Cr* $HydA1_{strep}$ when the enzymes were activated in *Synechocystis Δhox*. When comparing H_2 accumulation data after 48 h dark incubation with $[2Fe]^{adt}$ in nitrate free media ($BG11_0$) without glucose, no significant differences are noted between the two (Figure 6). H_2 accumulation from *Synechocystis Δhox* expressing *Sm* HydA (paper III) was also very similar under the corresponding condition (Figure 6).

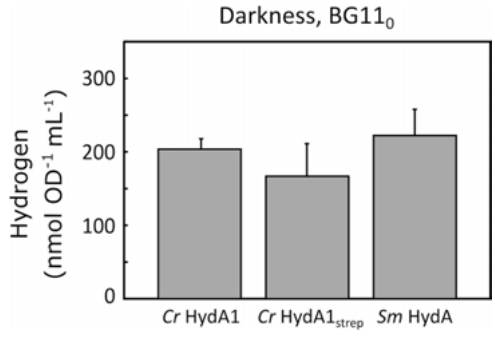

Figure 6. Comparison of H_2 production from *Synechocystis Δhox* cells individually harboring one of the three, synthetically activated, group A *apo*-HydA used in this work. Columns represent nmol H_2 accumulated per mL culture after 48 h dark incubation in BG11$_0$ (nitrate free cultivation media), normalized to optical cell density. Data points represent means of 2-4 individual experiments and error bars represent s.d.

In darkness, as seen in Figure 6, strains expressing *Cr* HydA1$_{strep}$ and *Sm* HydA had accumulated similar amounts of H_2 48 h after synthetic activation. Looking at time resolved H_2 accumulation, the strains production pattern was observed to be very similar in all tested nitrogen deprived dark conditions (Figure 7).

Interestingly, the highest H_2 production was recorded from cultures in BG11$_0$ without glucose supplementation. In these culture vials, H_2 levels in the headspace were above 2% at the 72 h measurement. These samples have not, before or during the experiment, been exposed to external organic carbon sources. Consequentially, this production, albeit evidently fermentative, is thus facilitated completely by reduction power from photosynthetically accumulated substrates, an indirect photobiological H_2 production. We propose that these conditions force complete shutdown of most anabolic cell processes, forcing the cells to rely fully on catabolism of internal substrates resulting in a very reduced cellular environment, suitable for H_2 production.

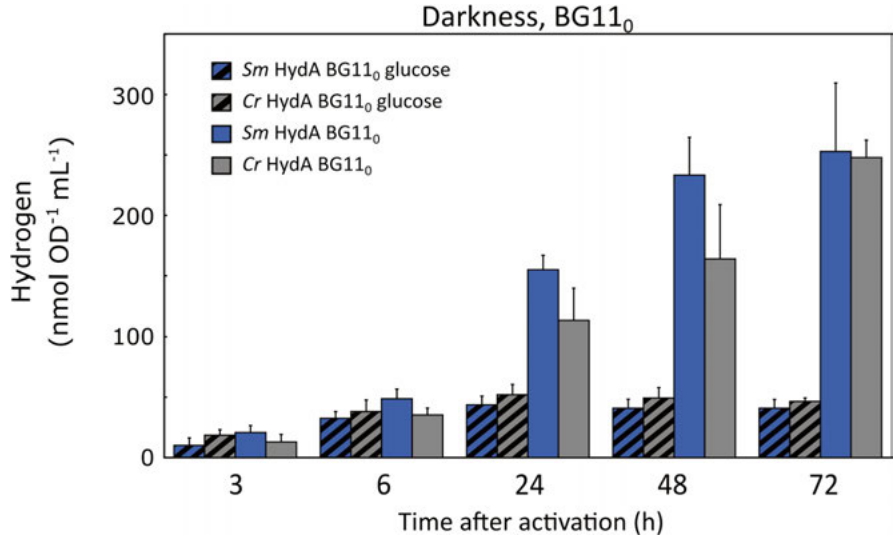

Figure 7. H_2 production comparison between synthetically activated *Sm* HydA, (blue bars) and *Cr* $HydA_{flag}$ (grey bars) in cells of *Synechocystis Δhox*. Bars represent accumulated H_2 in $BG11_0$ in darkness with (black-striped bars) or without (clean bars) glucose. Figures display nmols of accumulated H_2 per mL culture, normalized to optical density. Data points represent means of 2-4 individual experiments and error bars depicts s.d. Figure adapted from paper III.

In glucose free $BG11_0$ under light (80 µmol photons m^{-2} s^{-1}) with DCMU inhibition, the strains expressing *Cr* $HydA1_{strep}$ and *Sm* HydA produced only limited amount H_2, below 3 nmol H_2 OD^{-1} mL^{-1} (data not shown). Contrary to the situation in darkness (Figure 7), glucose supplementation increased this production. Time resolved production for glucose supplemented strains under light in $BG11_0$ can be seen in Figure 8. *Synechocystis Δhox* harboring an activated *Cr* $HydA1_{strep}$ did not show any signs of H_2 production after 24 h. Cells harboring the *Sm* HydA1 hydrogenase however, shows significant increase in accumulated H_2 between 24 and 72 h (Figure 8). This reveals a clear difference in production performance between the two group A hydrogenases. This is, so far, the only example of sustained H_2 production in light from a synthetically activated [FeFe]-hydrogenase in *Synechocystis*. Possibly, the different domain setup of the M2 subclass hydrogenase facilitates more favorable electron donation to the hydrogenase under these conditions, however this need further investigation.

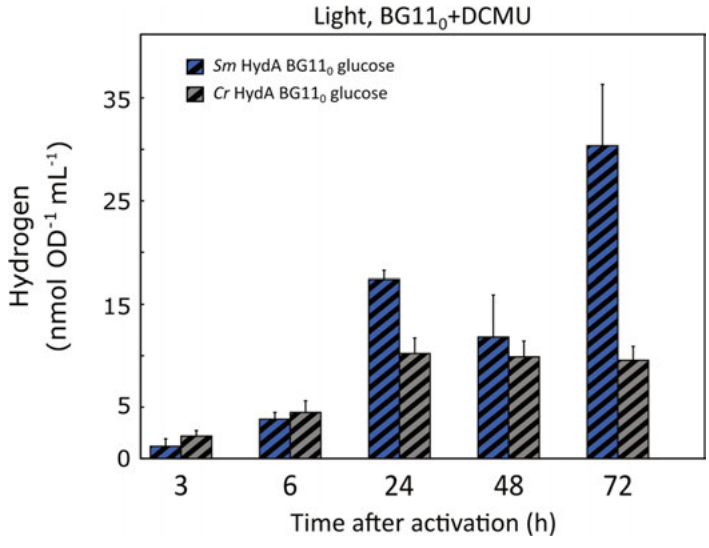

Figure 8. H_2 production comparison between synthetically activated *Sm* HydA, (blue bars) and *Cr* $HydA_{flag}$ (grey bars) in living cells of *Synechocystis Δhox*. Bars represent accumulated H_2 in $BG11_0$ with DCMU under light (80 µmol photons m^{-2} s^{-1}) with glucose supplementation. Figures display nmols of accumulated H_2 per mL culture, normalized to optical density. Data points represent means of 2-4 individual experiments and error bars depicts s.d. Figure adapted from paper III.

It is interesting to note that the best condition for H_2 production in darkness, $BG11_0$ without addition of glucose, facilitates almost no production in light with DCMU. Control experiment in darkness has shown that the PSII inhibitor does not have any direct negative effect on H_2 production from the activated [FeFe]-hydrogenases. In light with DCMU inhibition, circular electron flow around PSI is operating, allowing light driven ATP production. The low production of H_2 in light with DCMU, compared to darkness, indicates competition for electrons between the hydrogenase and cyclic electron flow. Also, high levels of ATP could inhibit glycolytic pathways [102], possibly limiting the production of fermentatively reduced ferredoxin or NADH/NADPH. To address the limited production in light, and possibly elucidate the electron flow towards the activated hydrogenase, H_2 production from *Synechocystis Δhox* harboring an activated *Cr* HydA1 was investigated under the influence of specific inhibitors (Figure 9a). Experiments were carried out under light (100 µmol photons m^{-2} s^{-1}) in $BG11_0$ with and without glucose.

Inhibition of cyclic electron flow by DBMIB, in addition to the DCMU inhibition, increased the H_2 production. Under these conditions, both linear and cyclic photosynthetic electron transport is inhibited. The increase was most dramatic in $BG11_0$ without glucose, where 34 times higher H_2 accumulation was recorded after 48 h, compared to only DCMU inhibition (Figure 9b). ATPase inhibition by DCC resulted in an approximately 5-fold increase

both in presence and absence of glucose (Figure 9 b and c). Under these conditions, cyclic electron flow is operating, but light driven ATP production is limited. The increase in H_2 production is possibly a result of released ATP inhibition of glycolytic reactions, increased NADPH/$NADP^+$ ratio [100], or a combination of both.

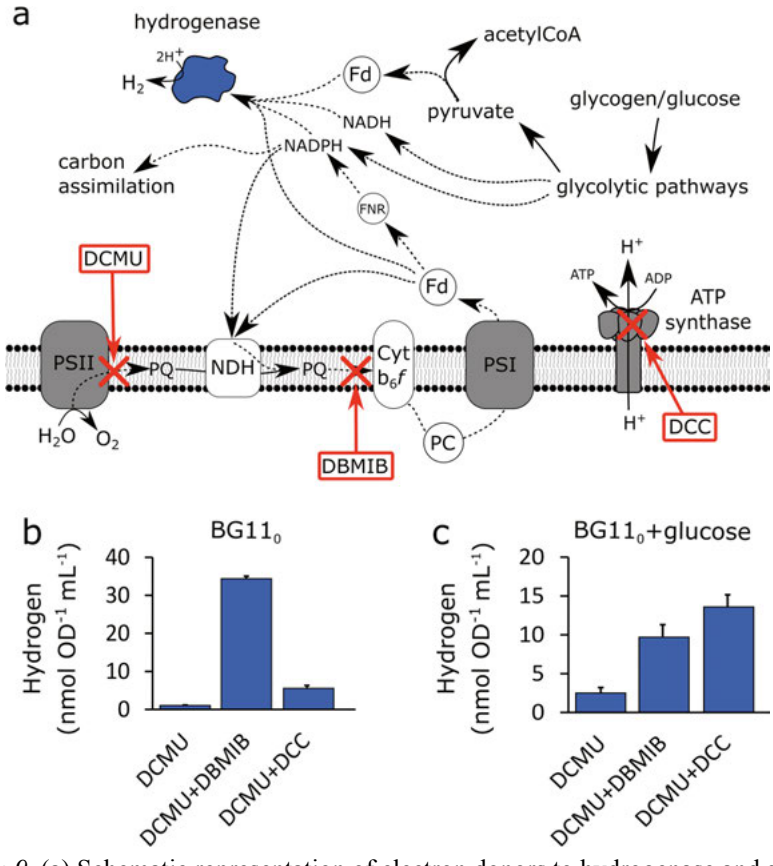

Figure 9. (a) Schematic representation of electron donors to hydrogenase and selected parts of cell metabolism in unicellular cyanobacterium *Synechocystis*. Inhibitors used in this study, DCMU, DBMIB and DCC, are indicated in red. (b and c) Accumulated H_2 from *Synechocystis Δhox* harboring an activated HydA after 48 h under light (100 µmol photons m^{-2} s^{-1}) with different inhibitor combinations in (b) $BG11_0$ and (c) $BG11_0$ with glucose. Data points represent means of 2–4 independent experiments. Error bars represent s.d. Abbreviations: Cyt b6f: Cytochrome b6f; Fd: Ferredoxin; FNR: Ferredoxin-NADP reductase; NDH: NADPH dehydrogenase I; PC: Plastocyanin; PSI and PSII: Photosystem I and II. Figure adapted from paper II.

The high production in darkness, together with the clear positive effect of DBMIB inhibition, indicate a fermentative, PQ independent source of electrons as the main driver of the H_2 production. Considering the similarities in production pattern between *Cr* HydA1 and *Sm* HydA (Figures 6, 7 and 8), I

propose the same for the M2 subclass enzyme. However, strains carrying activated *Sm* HydA has not been investigated in presence of DBMIB or DCC, and the difference between this enzyme and *Cr* HydA1 in glucose supplemented, nitrate deprived light condition (Figure 8) needs to be further investigated.

Long lived production capacity

The inhibition studies and the H_2 production patterns in light and dark, indicates a reductant supply limitation as the main factor for the limited production in light with DCMU inhibition. However, it does not completely rule out other explanations. To investigate the lifetime of the synthetically activated hydrogenase in light, and possibly further elucidate the reasons for the limited production, *Synechocystis Δhox* harboring activated *Sm* HydA was exposed to a period of darkness after a 72 h incubation in light. After synthetic activation with $[2Fe]^{adt}$ in non-glucose supplemented $BG11_0$ with DCMU, cell culture vials were incubated under light (80 μmol photons m^{-2} s^{-1}) for 72 h. During this initial incubation, H_2 production was monitored and is shown in Figure 10a. After the initial incubation, cultures were either sparged with argon and moved to darkness (Figure 10b), sparged with argon and put back in light (Figure 10c) or moved to darkness without sparging with argon (Figure 10c).

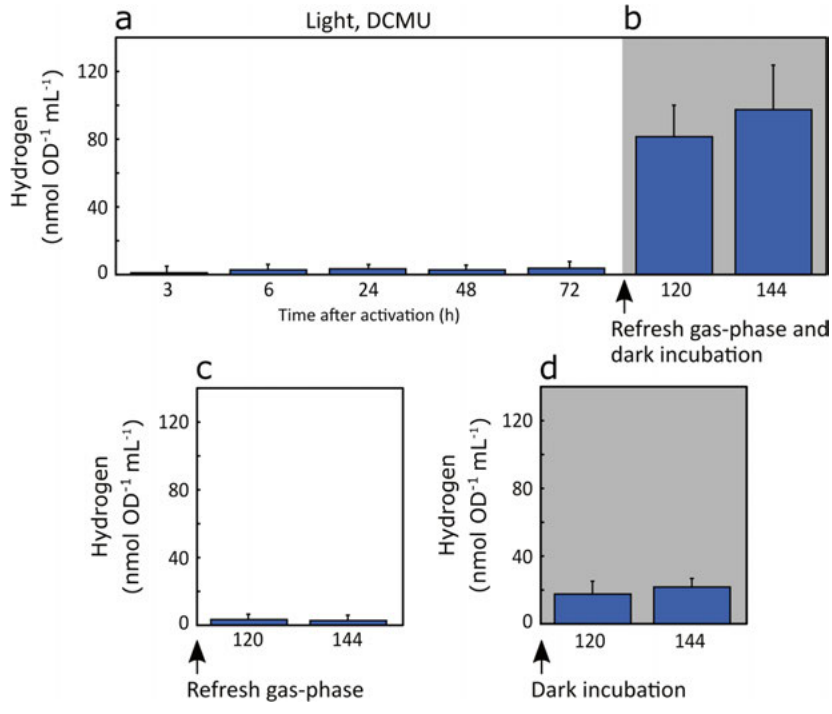

*Figure 10.*Accumulated H_2 by *Synechocystis Δhox* harbouring a synthetically activated *Sm* HydA under nitrogen starved conditions. H_2 accumulation was monitored during (a) initial incubation in light with DCMU inhibition in an argon atmosphere followed by (b) sparging with argon gas and transition to darkness, (c) sparging with argon gas and continued incubation in light and (d) transition to dark incubation without any sparging. Blue bars display nmols of accumulated H_2 gas per mL culture, normalized to optical density. Data points represent means of 2-4 individual experiments and error bars depicts s.d. Figure reproduced from paper III.

During the initial light incubation, net H_2 production was, as observed before, low and stopped after 24 h. However, cultures showed a dramatic increase in H_2 production when moved to dark incubation after sparging with argon (Figure 10b). When sparged and put back under light, H_2 production recovered but accumulation stopped at the same levels as during the initial incubation (Figure 10c). When transferred to dark incubation without sparging, production increased but accumulated H_2 did not reach the levels of samples sparged before transfer to darkness (Figure 10d).

This result proves the presence of functional hydrogenase in the cells beyond 72 h after artificial maturation, even in light. The limited production in light can therefore not be attributed to high degree of damage to the activated enzyme or incorporated $[2Fe]^{adt}$ during these conditions. It also confirms the effectiveness of the DCMU inhibition, since oxygen is expected to permanently damage a matured [FeFe]-hydrogenase [103, 104]. The positive effect

of sparging is interesting and indicates accumulation of inhibiting, but not permanently damaging, gaseous compounds during light incubation.

Genetic tools for H_2 production in *N. punctiforme* (paper IV)

The heterocysts of filamentous cyanobacteria provide a micro-aerobic environment for the oxygen sensitive nitrogenase to operate in. These specialized cells are of particular interest for biological H_2 production for mainly two reasons. First, nitrogenase is an obligate H_2 producer during nitrogen fixation, as described above. Second, the heterocyst environment could be hijacked for expression and protection of a heterologous hydrogenase or other oxygen sensitive enzymes [88, 105].

The P*trc* promoter can be used to express proteins in all cells of the filaments, including heterocysts [43]. However, coordinating the expression of a heterologous protein, for example a hydrogenase, with the heterocyst differentiation offers a neater solution that does not burden vegetative cells with unnecessary protein expression in an unsuitable environment.

Up to this point, all heterocyst specific expressions have been carried out using native promoter sequences found upstream heterocyst specific genes [88, 106–109]. These, often poorly characterized and several hundred nucleotides long, native sequences can be heavily regulated and give rise to unforeseen behavior. Also, long sequences are, naturally, less convenient in cloning. In paper IV, a minimal synthetic promoter for heterocyst specific expression in *N. punctiforme* was developed together with a pair of self-replicating cloning vectors.

Vector for promoter studies

Genetic tools developed specifically for use in for *N. punctiforme* are rare to say the least. Fortunately, tools developed for other more commonly used cyanobacteria often work reasonably well. The broad host range, self-replicating shuttle vector pPMQAK1 [43] and its descendant plasmids pEEK and pEEC [110], are example of this. They are RSF1010 [111] based plasmids, specifically engineered for convenient cloning and application in cyanobacteria. Unfortunately, The RSF1010 derived plasmids are not suitable for electroporation which is the most reliable and time efficient method of transformation in *N. punctiforme*. pDC1 [44] derived plasmids, like pSCR and pSUN [45] are easily electroporated into *N. punctiforme*, but lacks many of the convenient features of pPMQAK1, pEEK and pEEC.

For the promoter study in paper IV, I created an improved shuttle vector, convenient to use in construct assembly and easy to electroporate into *N. punctiforme*, based on pSCR119 [45]. The plasmid, $pSAW_{ccdB}$ (Figure 11), contains the kanamycin resistance gene *ntp* and origin of replication for propagation in both *E. coli* (ColE1) and *N. punctiforme* (pDC1). The multiple cloning site (MCS) consists of a *ccdB* toxin encoding, killer gene [112], flanked by restriction sites. In restriction-digestion, SacI, XbaI and SalI cut upstream the *ccdb*, while XhoI, PstI and KpnI cut downstream. The MCS is in turn flanked by terminators to eliminate unwanted transcriptions in or out of the cloning site. The two innermost restriction sites, SalI and XhoI, have compatible overhangs after digestion and facilitates multiple, step-by-step restriction-ligation insertions, in a BioBrick [90] like manner .

The similar $pSAW_{yfp}$ (Figure 11) was created to enable convenient and standardized promoter studies in *N. punctiforme*. In this plasmid, an *eyfp* reporter gene is cloned downstream the *ccdB* gene. In cloning, *ccdB* is cut out and replaced with a promoter-RBS fragment, using one of the upstream restriction sites and XhoI. The reformed XhoI site will act as a 6 bp spacer between the RBS and the start codon of *eyfp*. The vector can also be useful for expression of YFP-fusion proteins, where the reformed XhoI-site will be translated into Leu-Glu.

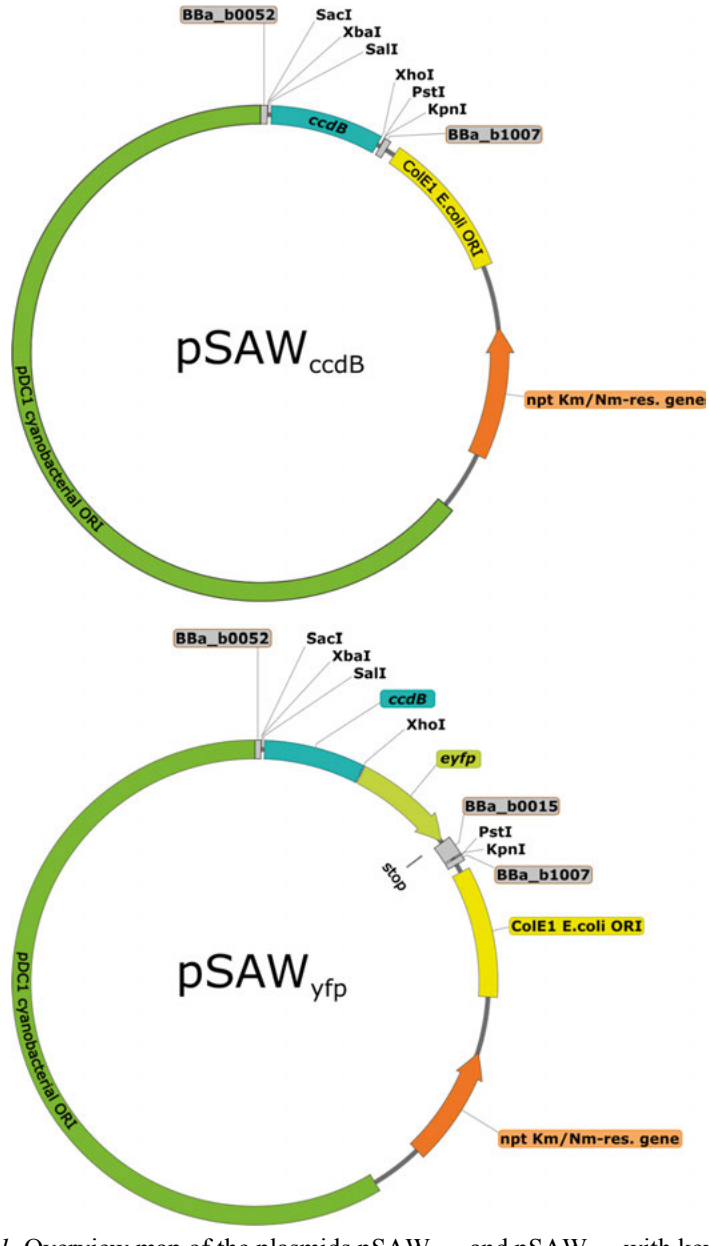

Figure 11. Overview map of the plasmids $pSAW_{ccdb}$ and $pSAW_{yfp}$, with key elements and recommended restrictions sites indicated. Figure adapted from paper IV.

A detailed description of the plasmid creation is available in paper IV, Materials and Methods. Information about all parts of the plasmids are available in paper IV, Supplementary Information.

Minimal, synthetic heterocyst-specific promoter

The DIF$^+$ class promoters are native to filamentous cyanobacteria and controls the expression of various heterocyst specific or heterocyst enhanced gene products [108, 113]. The DIF$^+$ class promoters all display a conserved sequence pattern at the -35 region with consensus sequence 5'-TCCGGA-3'. This sequence is called the DIF1 motif [113]. In paper IV, we hypothesized that the heterocyst-specific expression pattern of the DIF$^+$ promoters should be a consequence only of conserved sequence patterns in the promoter regions, and wanted to use this to develop the first synthetic heterocyst-specific promoter. 58 unique DIF$^+$ class promoters, identified by Mitschke *et al.* in 2011 [108], were aligned and visualized as a weighted sequence logo (WebLogo 3.0) [114].

Except the already discovered DIF1 motif around position -35, a stretch of 13 conserved nucleotides was identified between position -13 and the TSS. This was interpreted as a conserved extended -10 region. Apart from this, no conserved regions were found. That fact that no conserved regions were present upstream the DIF1 motif, indicated that a very compact promotor could be enough to facilitate heterocyst-specific expression. A minimal promoter design was developed based on the conserved regions in the weighted sequence logo, including a randomized sequence between the DIF1 motif and the -10 region, and a short, randomized spacer upstream the DIF1 motif. The promoter was named P$_{synDIF}$, and the design is visualized in Figure 12.

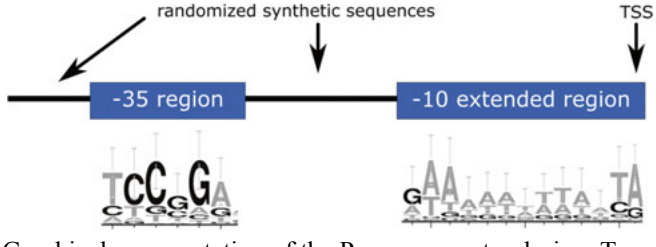

Figure 12. Graphical representation of the P$_{synDIF}$ promoter design. Transcription start site (TSS), extended -10 region, -35 region and randomized spacer sequences are indicated. Consensus sequences from the alignment of 58 DIF$^+$ promoters are depicted as WebLogos underneath their respective part.

To investigate the promoter, it was cloned, together with RBS* [41], into the self-replicating fluorescence reporter vector pSAW$_{yfp}$ described above. To identify the isolated functions of the DIF1 motif and the conserved extended -10 region in P$_{synDIF}$, two minimal control promoters were developed in parallel. In these control promoters, the nucleotides making up either the extended -10 region, or the -35 region, were exchanged into the corresponding nucleotides from P*trc*. The two control promoters, in this text called P-*10c* and P-*35c* respectively, were similarly deployed in pSAW$_{yfp}$. The three resulting reporter constructs can be seen in Figure 13.

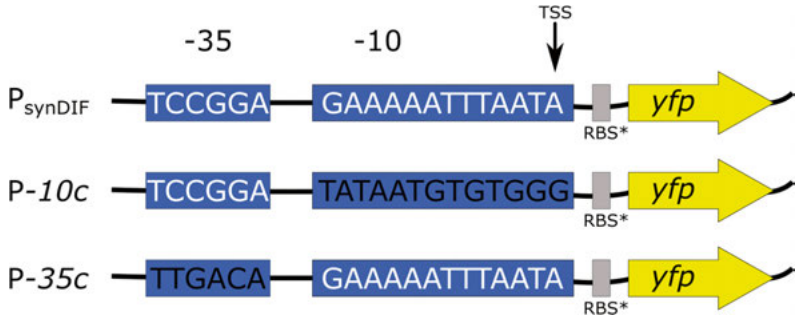

Figure 13. Schematic representations of the synthetic promoter-reporter constructs used to determine the roll of the DIF1 motif and the conserved extended -10 region. Black colored letters indicate nucleotides changed from the P_{synDIF} to the corresponding nucleotides from P*trc*.

After sequence confirmation, the reporter plasmids were electroporated into *N. punctiforme.* Strains carrying the plasmids were, after 12 h of combined nitrogen starvation, investigated using fluorescence confocal microscopy (Figure 14). Filaments carrying the P*synDIF* reporter construct displayed clear YFP fluorescence, distinctly restricted to cells identified as heterocyst according to their reduced autofluorescence (Figure 14). All cells of the filaments carrying the reporter construct with P-*35c* showed clear YPF expression, and niter higher or lower expression could be distinguished in heterocyst. As seen in Figure 14, exchanging the DIF1 motif to -35 region from P*trc* while keeping the extended -10 from P_{synDIF}, rendered the promoter inactive in both vegetative cells and heterocysts.

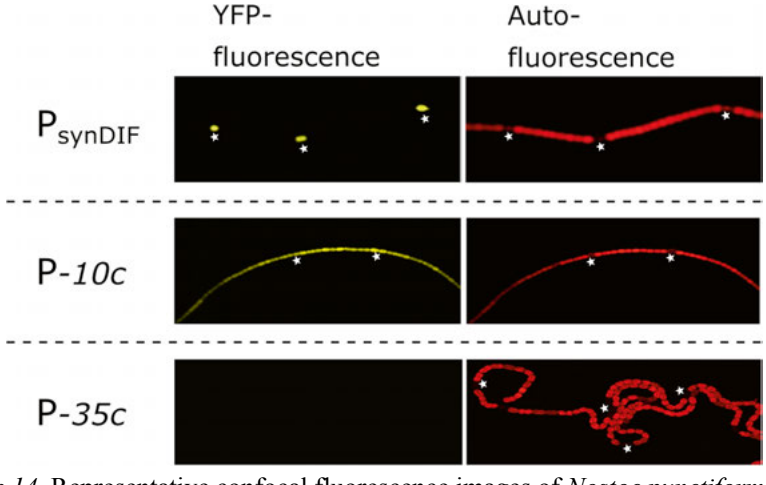

Figure 14. Representative confocal fluorescence images of *Nostoc punctiforme* filaments with fluorescence from the three promoter-YFP constructs. YFP fluorescence is shown in yellow (530-540nm), and autofluorescence in red (600-700nm). Heterocysts, identified by the reduced autofluorescence, are indicated by stars. Figure adapted from paper IV.

The results from the fluorescence investigation shows, without doubt, that the DIF1 motif at -35 must be combined with a suitable -10 region to mediate heterocyst-specific expression. DIF1 motif alone is not, as speculated previously, responsible for the heterocyst-enhanced transcription.

To investigate the activity during different stages of heterocyst differentiation, single filaments were monitored during adaption to diazotrophic growth. Ammonium grown filaments were transferred to $BG11_0$ agar plates, and images of individual filaments were taken at 6, 12, 24 and 48 h. Figure 14 depicts a representative filament during the differentiation process. Heterocysts displayed district YFP fluorescence at 48 h after start of nitrogen starvation. Considering that the lifetime of the fluorescent protein use in this study (EYFP) have been confirmed to have an *in vivo* half-life of about 13 h in a cyanobacterial host [115], we can conclude that P_{synDIF} is active in mature heterocysts.

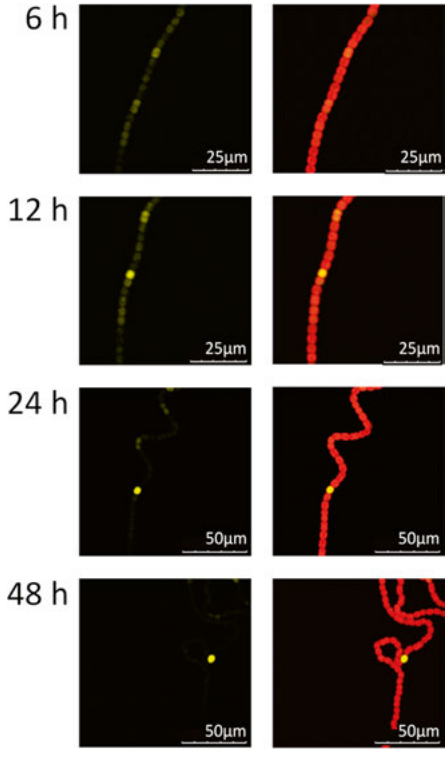

Figure 15. Confocal fluorescence images of a representative *Nostoc punctiforme* filament expressing YFP from the P_{synDIF} reporter construct, at 6, 12, 24 and 48 h after nitrogen deprivation immobilized on agar plate. Autofluorescence (600-700nm) is shown in red, YFP fluorescence (530-540nm) is shown in yellow. Rightmost column shows overlaid images of autofluorescence (600-700nm) and YFP fluorescence (530-540nm). Figure adapted from paper IV.

The P_{synDIF} is a fully synthetic promoter for heterocyst-specific expression. It is active from relatively early during the heterocyst differentiation process,

and stays active in mature heterocysts. With its 48 nucleotides, including the upstream spacer, it is a minimal promoter that can easily be added in any cloning strategy without extra steps, for example in the overhang of an amplification primer.

Synthetic [FeFe]-hydrogenase in *N. punctiforme* (paper V)

Papers II and III describe *in vivo* H_2 production from two different group A [FeFe]-hydrogenases artificially matured in *Synechocystis*. In both cases, the highest H_2 production was observed from nitrogen-starved cells, incubated in darkness without glucose. In paper V, the [FeFe]-hydrogenase *Cr* HydA1, C-terminally fused to GFP, was expressed and activated in *N. punctiforme*.

To direct the expression to heterocysts, the *hydA1* gene was preceded in the construct by the 316 bp long, native promoter fragment P_{hupS} [91] from the genome of *N. punctiforme*. As described above, a BCD device had be deployed to express the heterologous hydrogenase-GFP fusion (Figure 3). Investigation with fluorescent confocal microscopy revealed the *Cr* HydA1-GFP chimeric protein to be highly expressed in the cytoplasm of heterocyst, but with lower levels also in vegetative cells. *N. punctiforme* has no bidirectional hydrogenase, like some other filamentous and unicellular strains, but the heterocysts harbor an uptake hydrogenase besides the nitrogenase. Upon successful synthetic activation of the *Cr* HydA1, the diazotrophic filaments would harbor three different enzymes directly involved in the H_2 metabolism, a slightly more complex situation compared to paper II and III.

Similarly to the procedure described for *Synechocystis*, synthetic activation was carried out in diazotrophic filaments of *N. punctiforme*. Before addition of $[2Fe]^{adt}$, cultures were kept 1 h in anaerobic darkness to reduce nitrogenase activity. After the synthetic activation, cultures were incubated in either darkness or under continuous illumination (80 µmol photons m^{-2} s^{-1}). The cultures under light where incubated with or without DCMU in the culture media. H_2 accumulation in the culture vials were measured 6 h after activation, and then every day for 5 continuous days (Figure 16). In parallel to the samples treated with $[2Fe]^{adt}$, control samples without addition were identically prepared to monitor H_2 evolution from nitrogenase. In light without DCMU inhibition limited H_2 production from nitrogenase was detected (Figure 16). In darkness and in light with DCMU inhibition, no net H_2 production was observed without addition of $[2Fe]^{adt}$.

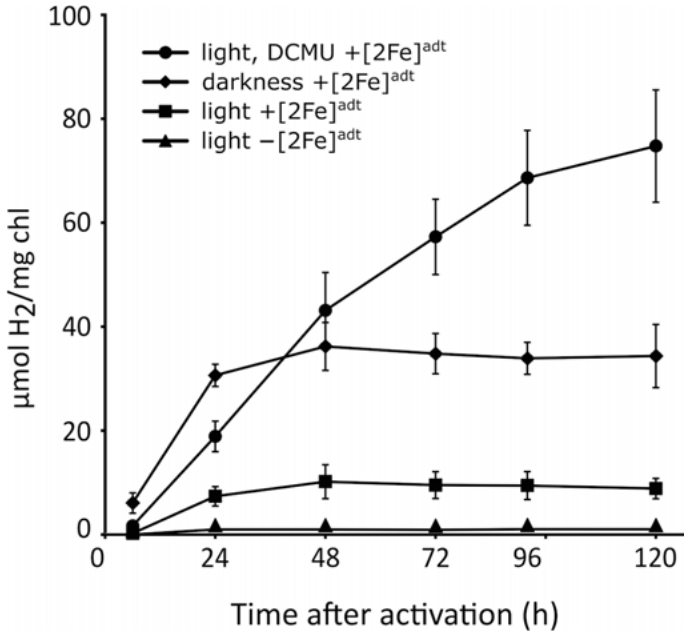

Figure 16. Time resolved H_2 accumulation from diazotrophic *N. punctiforme* cultures expressing the *Cr* HydA1-GFP fusion protein, in light with DCMU inhibition and with addition of $[2Fe]^{adt}$ (circles), darkness with addition of $[2Fe]^{adt}$ (diamonds), light without DCMU inhibition with addition on $[2Fe]^{adt}$ (cubes) and light without DCMU without $[2Fe]^{adt}$ (triangles). Figure displays µmol accumulated H_2, normalized to chlorophyll *a* content. Data points represent means of 2 individual experiments and error bars represent s.d. Figure adapted from paper V.

As seen in Figure 16, sustained H_2 production was measured from activated samples in light with DCMU inhibition, whereas the production in darkness, albeit initially faster, is comparably short-lived. This result is intriguing considering that the opposite pattern was observed when the same [FeFe]-hydrogenase was activated in *Synechocystis* (Figure 7 and 8). Clearly the prerequisites for efficient H_2 production from [FeFe]-hydrogenases, differs completely between the two different cyanobacteria. The sustained production in light with DCMU observed here proves an impressive, *in vivo* life-time of the artificially induced H_2 production capacity. This is the first report of light driven H_2 production from an artificially maturated [FeFe]-hydrogenase.

Given the lack of PSII activity in these conditions, it is possible that some part of the accumulated H_2 in light with DCMU inhibition, originates from activated *Cr* HydA1 in vegetative cells. The H_2 production in light without DCMU inhibition however, we attribute to *Cr* HydA1 in heterocyst (Figure 14). Any activated [FeFe]-hydrogenase in vegetative cells would be quickly inactivated by oxygen evolution under these conditions [103, 104], and only very low nitrogenase activity was observed. It can therefore be concluded that the production arises from activated *Cr* HydA1 in heterocyst, protected by the microaerobic environment and the spatial separation from water splitting by PSII.

Conclusions and Future Directions

This thesis demonstrates how [FeFe]-hydrogenases can be reliably expressed and synthetically activated *in vivo* in cyanobacteria. I have demonstrated successful synthetic activation and subsequent *in vivo* H_2 production in unicellular *Synechocystis* PCC 6803, as well as in both vegetative cells and heterocysts of filamentous *Nostoc punctiforme* ATCC 29133. In total, enzymes from three different subclasses of [FeFe]-hydrogenases have been activated and investigated under different environmental conditions and metabolic regimes. The semisynthetic [FeFe]-hydrogenases are evidently linked to the cellular metabolism and are capable of significant and sustained H_2 production. Artificially maturated enzymes stay functional *in vivo* beyond 72 h after activation by $[2Fe]^{adt}$, an impressive life-time for a hydrogenase-based technology with artificial components.

The highest and most long-lived H_2 production from semisynthetic [FeFe]-hydrogenase in *Synechocystis* was recorded in darkness, during nitrogen deprivation without glucose. Thus, the production appears to be highest in conditions where neither light, nitrate or external carbon substrates are provided. Heterologously expressed, synthetically activated [FeFe]-hydrogenase in *N. punctiforme* was capable of sustained, light driven proton reduction when PSII was inhibited. A photoH_2 production based on anoxygenic photosynthesis. These results emphasize the importance of carefully investigating different hosts when engineering cyanobacterial H_2 production strains. Likewise, different subclasses of [FeFe]-hydrogenases have shown different response to cultivation conditions in our experiments. Our results indicate that different subclasses might be suitable for H_2 production in cyanobacteria under vastly different environmental circumstances. They are, possibly, accepting electrons for proton reduction from different parts of the cell metabolism, opening up for interesting co-expressions of different [FeFe]-hydrogenases in the same cell to maximize reduction power absorption.

Overall, the results presented here demonstrates a new way to investigate [FeFe]-hydrogenases *in vivo* in a photosynthetic context. Using synthetic activation, a wide range of hydrogenases, engineered strains and cultivation conditions can readily be investigated in search of suitable candidates for future H_2 production systems.

Svensk Sammanfattning

Klimatförändringarna utgör ett stort hot mot världen och dess befolkning. Temperaturen på jorden idag är ungefär 1° C högre än vid industriella revolutionen, och det är helt fastslaget att mänskliga utsläpp av fossilt kol i form av koldioxid är den största orsaken till detta. Skrämmande nog fortsätter våra koldioxidutsläpp att öka. Varje år släpper vi ut mer koldioxid än föregående år. Inte lika mycket, utan mer. Trots en rad åtaganden och löften från många av världens länder lyckas vi alltså inte ens stoppa ökningen av koldioxidutsläpp, än mindre minska utsläppen.

Isarna runt polerna har börjat smälta. Temperaturen i permafrosten på norra halvklotet ökar. Isläggningar försenas och snötäckens utbredning minskar. Havstemperaturen höjs och vattennivån stiger. Förändringen sker snabbast närmast polerna. Den senaste rapporten från FN:s klimatpanel (IPCC) belyser hur många av klimatförändringarnas negativa effekter ökar icke-linjärt med en ökande temperatur, och hur bara en liten ytterligare höjning riskerar att få extremt stort utslag genom kaskadeffekter. Skillnaden mellan en ökning på 1,5°C, som vi väntas nå innan år 2040, och en ökning på 2°C bedöms vara enorm. Tyvärr förefaller en ökning med endast 2°C osannolik med tanke på de fortsatt ökande utsläppen.

För att bryta utvecklingen och förhindra en global katastrof måste vi snabbt ställa om till förnyelsebar energiproduktion. Solen förser jorden med praktiskt taget obegränsade mängder energi varje dag och vi måste förutsättningslöst undersöka effektiva och hållbara sätt att fånga och lagra denna energi. Omställningen har redan börjat och ”solcellsrevolutionen” har redan förändrat energisituationen på många platser i världen. För att på lång sikt inte bli beroende av sällsynta jordartsmetaller, och för att kunna tillfredsställa världens ökande behov av inte bara elektricitet, utan även bränsle, krävs andra lösningar. Biobränslen kan vara en väg framåt, men landbaserad odling av energigrödor har visat sig problematisk då den konkurrerar med både matproduktion och ekologiskt och klimatmässigt viktig naturskog. Dessutom sker stora utsläpp av markbunden koldioxid när energigrödorna skördas.

Biobränslen producerade av fotosyntetiserande mikrober, vars odling inte behöver belasta odlingsbar mark, kan vara en väg framåt. Cyanobakterier är prokaryota fotosyntetiserande mikrober som, med små tillsatser av näringssalter, kan växa på luft, solljus och vatten. Vissa stammar producerar naturligt biobränslen, men i för små mängder för att kunna användas i storskalig pro-

duktion. Genom genetisk modifiering kan man styra om metaboliska reaktionsvägar och energiflöden i cellen, och på så vis öka produktionen av bränslen. Vätgas (H_2) är en av de bränslemolekyler som produceras naturligt av cyanobakterier. H_2 bildas antingen av enzymet nitrogenas som bioprodukt under kvävefixeringen, eller av hydrogenaser. Dessa enzym är syrekänsliga och kräver anaeroba förhållanden för att katalysera bildandet av H_2. Hydrogenaser är särskilt intressanta ur H_2-produktionssynpunkt då de, olikt nitrogenas, inte är beroende av ATP för katalysen.

H_2 spås bli en viktig energibärare i framtida hållbara energisystem. Genom att låta den energitäta gasen passera genom en bränslecell produceras el med enbart vatten som biprodukt. Till skillnad från rena elektriska system, där stora dyra batterier krävs för energilagring, kan H_2 lagras i t.ex. enkla kolfiberförstärkta tankar. Utvinningen idag sker nästan uteslutande från naturgas eller andra fossila råvaror, med stora koldioxidutsläpp som följd.

De hydrogenaser som finns naturligt i cyanobakterier är [NiFe]-hydrogenaser, där den katalytiskt aktiva kärnan innehåller Ni- och Fe-atomer. Dessa enzym katalyserar oxidation av H_2 eller reduktion av protoner, och är således inblandade i både upptag och framställning av H_2. De är väl integrerade i den fotosyntetiska metabolismen där de bidrar till cellens redoxbalans och återvinner reduktionspotential "bortslösad" på H_2-produktion under kvävefixeringen.

I andra organismgrupper hittar vi andra typer av hydrogenaser. De mest lovande ur produktionssynpunkt är de syrekänsliga [FeFe]-hydrogenaserna, med ett dijärnkomplex i den aktiva kärnan. Enzym ur denna grupp har visat sig vara otroligt aktiva H_2-producerare och är högintressanta inom energiforskning. Introduktion av ett [FeFe]-hydrogenas har länge setts som en lovande strategi för att öka H_2-produktionen från cyanobakterier. Med genetiska tekniker kan vi relativt lätt uttrycka ett ofullständigt [FeFe]-hydrogenas i en cyanobakteriecell, men för att det aktiva sätet ska byggas upp och ett aktivt enzym ska bildas krävs att tre chaperoner uttrycks i samma cell. Detta komplexa molekylära maskineri utgör ett hinder för att effektivt kunna testa olika [FeFe]-hydrogenaser i olika stammar av cyanobakterier.

Forskningen i denna avhandling behandlar i huvudsak en ny metod att få fram aktivt [FeFe]-hydrogenas i cyanobakterier. Metoden, som kallas syntetisk aktivering, går ut på att de delar av det aktiva sätet som saknas när ett [FeFe]-hydrogenas uttrycks utan chaperoner, tillsätts till levande celler och aktiverar enzymet. Vi har lyckats visa att denna metod fungerar i cyanobakterier, och att det delvis artificiella enzymet kopplar till den fotosyntetiska metabolismen.

Först visades detta med det välstuderade [FeFe]-hydrogenaset HydA1 från eukaryota grönalgen *Clamydomonas reinhardtii*, som aktiverades i den encelliga cyanobakterien *Synechocystis*. Den fermentativa H_2-produktionen från celler med aktiverat [FeFe]-hydrogenas var tydligt högre än från icke-modifierade celler med enbart [NiFe]-hydrogenas. Det syntetiskt aktiverade enzymet producerade H_2 främst i mörker, och den högsta produktionen uppmättes från

kvävesvälta cellodlingar. Under dessa förhållanden var produktionen långlivad och fortgick under flera dagar. Detta är det första semisyntetiska enzym som rapporterats i cyanobakterier, och livslängden är förvånande lång med tanke på de icke-biologiska komponenterna som inte kan återskapas i cellen.

Försök i ljus kräver inhibering av fotosystem II för att syrgas (O_2) inte ska inaktivera det syrekänsliga [FeFe]-hydrogenaset. Under ljus med PSII inhibition var produktionen mycket lägre än i mörker och dessutom kortvarig. Inhibitorstudier i ljus, där även den cykliska fotosyntetiska elektrontransporten runt fotosystem I (PSI) blockerades, avslöjade en negativ inverkan på H_2-utvecklingen från det cykliska elektronflödet.

I en uppföljande studie uttrycktes och aktiverades två nyupptäckta [FeFe]-hydrogenaser, *Sm* HydA från *Solobacterium moorei* och *Tam* HydS från *Thermoanaerobacter mathranii* i *Synechocystis*. *Sm* HydA visade sig acceptera elektroner för H_2-production under samma förhållanden som *Cr* HydA1 och producera ungefär lika mycket, men med högre produktion under ljus från kvävesvälta celler med glukos i odlingsmediet. *Synechocystis* med aktiverat *Tam* HydA producerade mindre H_2, men produktionen skedde bara i ljus utan kvävesvält. Resultaten visar hur olika [FeFe]-hydrogenaser kopplar samman med cellens metabolism på olika sätt och understryker vikten av att utforska fler och varierande [FeFe]-hydrogenaser i cyanobakterier.

Vi har också bevisat att syntetisk aktivering fungerar i den filamentösa cyanobakterien *Nostoc punctiforme*. Under kvävesvält differentierar en del av cellerna längs filamentet till heterocyster, mikroanaeroba celler med tjock cellvägg som möjliggör kvävefixering katalyserad av nitrogenas. *Cr* HydA1 aktiverades i filamentens heterocyster, och den mikroanaeroba miljön skyddade [FeFe]-hydrogenaset från O_2 under pågående fotosyntes.

Sammanfattningsvis beskriver denna avhandling en ny metod för att generera aktiva, fungerande [FeFe]-hydrogenaser i cyanobakterier. Metoden skapar semisyntetiska enzym som kopplar till cellens metabolism och producerar H_2 under olika förhållanden. Denna metod kan användas för att effektivt utforska nya [FeFe]-hydrogenaser i olika cyanobakteriestammar, utan att behöva uttrycka och optimera det komplexa maskineri som utför aktiveringen i de ursprungliga värdorganismerna. Förhoppningsvis kan resultaten presenterade i denna avhandling bidra till utvecklandet av effektivare, hållbara system för biologisk H_2-produktion.

Acknowledgments

I offer my first thanks to **Peter Lindblad**, my primary supervisor and mentor during these years. Your help has been invaluable. During my time in your group, you have tirelessly been supporting me and my colleagues in science and other matters.

I want to thank **Karin Stensjö** for her supervision, support, and inspiration. Everyone is facing their own problems and difficulties, but you tossed yours aside and did what you had to do when it mattered the most. Your unyielding positive energy spreads throughout the group and makes our days better.

I thank **Namita Khanna** for her patient guidance and warm friendship. You don't remember this, but when I arrived in the lab and you took me under your wings, I didn't have a clue about anything. If someone ever thinks that I have done anything in science that is worthy of praise, they should know that I learned everything I know from you.

When I came to the Cyano-group, I met a collection of people so talented, selfless and caring that I couldn't believe it was true. Most of you left the lab years ago, but I remember you, and always will.

Thank you **Claudia Durall de la Fuente** for making me feel like I belonged in the group from the start. Ever since you graduated and left, I can't shake the felling of something important missing. Thank you **Feiyan Liang** for your friendship, your insightful advice, and all your support in the lab. I miss your stupid jokes and fierce temperament. Thank you **Rui Miao** for friendship and support. You are an incredible researcher, but more importantly, a bastion of kindness and compassion. The world needs more people like you. Thank you **Elias Englund** for always freeing up time to help, even while you were busy with your own ambitious projects. Your openness and selfless confidence make people around better persons. Thank you **Bagmi Pattanaik** for the uncountable times you have helped me out. I asked you thousands of questions, and you had the answer to all of them. I have never met a more knowledgeable person. Thank you **Christoph Howe** for your advice and guidance. You are a good friend and I have greatly enjoyed out talks and correspondence. Thank you **Xin Li**, it was a pleasure to work with you. Your kindness and generosity are something truly out of the ordinary.

I would like to thank my current comrades in the Cyano-group for being awesome people and always making me feel happy. A special thanks for feeding me the uncountable times I neglected to bring lunch.

Thank you **Kateryna Kukil** for being such a great and accepting colleague. You are a talented, hard-working scientist, and I have greatly enjoyed our friendship. Thank you **João Rodrigues** for all always being encouraging and not being afraid of letting your enthusiasm show. Thank you **Hao Xie** for your honesty and patience. Talking with you is always a pleasure. Thank you **Matina Roussou** for your sympathetic and progressive disposition. It has been uplifting to have a person like you around. Thank you **Xufeng Liu**, you are an amazing scientist and a good person. Thank you **Haojie Jin** for being such a positive and warm colleague. Thank you **Amit Kugler** for bringing new energy to the group.

I want to thank **Pia Lindberg** for her help with everything from *Nostoc* cultivation to writing study plan. Your incredible ability to immediately understand scientific studies and point out problems and gaps in the argumentation is an asset for the whole group. Thank you **Gustav Berggren** for all your advice regarding writing and for being a supportive and positive collaboration partner. Thank you **Henrik Land** for pleasant collaboration and helpful advice. Thank you **Holly Redman**, **Afridi Zamader** and **Charléne Esmieu** for synthesising subcluster mimic. Thank you **Marco Lorenzi** for your kind help with the glovebox and related matters. Thank you **Giovanni Davide Barone** for collaboration, snus and friendship. **Robin Hoheneder** and **Federico Turco**, thank you for being wonderful colleagues and friends. **Casper de Lichtenberg**, thank for your loud friendship. **Dennis Dienst**, thanks for being an awesome person and an example for everyone. Thank you **Daniel Camsund** for always being enthusiastic, it's always a pleasure talking to you. Thank you **Sven Johansson** for keeping the lab running.

Stort tack till **mamma**, **pappa**, **Julia**, **Hannes**, **farmor**, **mormor** och **morfar** för stöd och kaotisk underhållning under åren. Tack **Victor** och **Kajsa** för systematisk uppmuntran och regelbundna skrattanfall. Stort tack till **Michelle**, det har varit kul.

References

1. IPCC. *Global Warming of 1.5°C*. 2018.
2. Friedlingstein P, Jones MW, O'Sullivan M, Andrew RM, Hauck J, Peters GP, Peters W, Pongratz J, Sitch S, Le Quéré C, et al. Global carbon budget 2019. *Earth Syst Sci Data* 2019; 11: 1783–1838.
3. Ramanathan V, Victor DG. Global warming will happen faster than we think. *Nature* 2018; 564: 30–32.
4. IPCC. *IPCC Special Report on the Ocean and Cryosphere in a Changing Climate*. 2019.
5. Lenton TM, Rockström J, Gaffney O, Rahmstorf S, Richardson K, Steffen W, Schellnhuber HJ. Climate tipping points — too risky to bet against. *Nature* 2019; 575: 592–595.
6. Lyons TW, Reinhard CT, Planavsky NJ. The rise of oxygen in Earth's early ocean and atmosphere. *Nature* 2014; 506: 307–315.
7. Keeling PJ. The endosymbiotic origin, diversification and fate of plastids. *Philos Trans R Soc B Biol Sci* 2010; 365: 729–748.
8. Flores E, Herrero A. Compartmentalized function through cell differentiation in filamentous cyanobacteria. *Nat Rev Microbiol* 2010; 8: 39–50.
9. Case AE, Atsumi S. Cyanobacterial chemical production. *J Biotechnol* 2016; 231: 106–114.
10. Dismukes GC, Carrieri D, Bennette N, Ananyev GM, Posewitz MC. Aquatic phototrophs: efficient alternatives to land-based crops for biofuels. *Curr Opin Biotechnol* 2008; 19: 235–240.
11. Bauwe H, Hagemann M, Kern R, Timm S. Photorespiration has a dual origin and manifold links to central metabolism. *Curr Opin Plant Biol* 2012; 15: 269–275.
12. Durall C, Lindblad P. Mechanisms of carbon fixation and engineering for increased carbon fixation in cyanobacteria. *Algal Res* 2015; 11: 263–270.
13. Badger MR, Price GD. CO2 concentrating mechanisms in cyanobacteria: Molecular components, their diversity and evolution. *J Exp Bot* 2003; 54: 609–622.
14. Meeks JC, Elhai J, Thiel T, Potts M, Larimer F, Lamerdin J, Predki P, Atlas R. An overview of the Genome of *Nostoc punctiforme*, a multicellular, symbiotic Cyanobacterium. *Photosynthesis Research* 2001; 70: 85–106.
15. Rippka R, Deruelles J, Waterbury JB. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J Gen Microbiol* 1979; 111: 1–61.
16. Magnuson A. Heterocyst Thylakoid Bioenergetics. *Life* 2019; 9: 13.

17. Kaneko T, Sato S, Kotani H, Tanaka A, Asamizu E, Nakamura Y, Miyajima N, Hirosawa M, Sugiura M, Sasamoto S, et al. Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions (supplement). *DNA Res* 1996; 3: 185–209.
18. Stephanopoulos G. Synthetic biology and metabolic engineering. *ACS Synth Biol* 2012; 1: 514–525.
19. Kerkhoven EJ, Lahtvee PJ, Nielsen J. Applications of computational modeling in metabolic engineering of yeast. *FEMS Yeast Res* 2015; 15: 1–13.
20. Teo WS, Heng YC, Chen B, Lee HL, Chua N, Chang MW. Synthetic Biology for Biofuels in Saccharomyces cerevisiae. In: Lee SY (ed) *Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Production of Fuels and Chemicals*. Cham: Springer International Publishing. 2016. DOI: 10.1007/978-3-319-31421-1.
21. Bailey JE. Toward a Science of Metabolic Engineering. *Science* 1991; 252: 1668–1675.
22. Nielsen J, Keasling JD. Engineering Cellular Metabolism. *Cell* 2016; 164: 1185–1197.
23. Jullesson D, David F, Pfleger B, Nielsen J. Impact of synthetic biology and metabolic engineering on industrial production of fine chemicals. *Biotechnol Adv* 2015; 33: 1395–1402.
24. Ramey CJ, Barón-Sola Á, Aucoin HR, Boyle NR. Genome Engineering in Cyanobacteria: Where We Are and Where We Need to Go. *ACS Synth Biol* 2015; 4: 1186–1196.
25. Saecker RM, Record MT, Dehaseth PL. Mechanism of bacterial transcription initiation: RNA polymerase - Promoter binding, isomerization to initiation-competent open complexes, and initiation of RNA synthesis. *J Mol Biol* 2011; 412: 754–771.
26. Iyer LM, Koonin E V., Aravind L. Evolution of bacterial RNA polymerase: Implications for large-scale bacterial phylogeny, domain accretion, and horizontal gene transfer. *Gene* 2004; 335: 73–88.
27. Imamura S, Asayama M. Sigma Factors for Cyanobacterial Transcription. *Gene Regul Syst Bio* 2009; 3: 65–87.
28. Stensjö K, Vavitsas K, Tyystjärvi T. Harnessing transcription for bioproduction in cyanobacteria. *Physiol Plant* 2018; 162: 148–155.
29. Zhou J, Zhu T, Cai Z, Li Y. From cyanochemicals to cyanofactories: a review and perspective. *Microb Cell Fact* 2016; 15: 2.
30. Zhou J, Zhang H, Meng H, Zhu Y, Bao G, Zhang Y, Li Y, Ma Y. Discovery of a super-strong promoter enables efficient production of heterologous proteins in cyanobacteria. *Sci Rep* 2014; 4: 4500.
31. Liu X, Miao R, Lindberg P, Lindblad P. Modular engineering for efficient photosynthetic biosynthesis of 1-butanol from CO2 in cyanobacteria. *Energy Environ Sci* 2019; 12: 2765–2777.
32. Valsami E-A, Psychogyiou ME, Pateraki A, Chrysoulaki E, Melis A, Ghanotakis DF. Fusion constructs enhance heterologous β-phellandrene production in *Synechocystis* sp. PCC 6803. *J Appl Phycol* 2020. DOI: 10.1007/s10811-020-02186-1.

33. Markley AL, Begemann MB, Clarke RE, Gordon GC, Pfleger BF. Synthetic biology toolbox for controlling gene expression in the cyanobacterium *Synechococcus sp.* strain PCC 7002. *ACS Synth Biol* 2015; 4: 595–603.
34. Peca L, Kós PB, Vass I. Characterization of the activity of heavy metal-responsive promoters in the cyanobacterium *Syne*chocystis PCC 6803. *Acta Biol Hung* 2007; 58: 11–22.
35. Berla BM, Saha R, Immethun CM, Maranas CD, Moon TS, Pakrasi HB. Synthetic biology of cyanobacteria: Unique challenges and opportunities. *Front Microbiol* 2013; 4: 1–14.
36. Jin H, Lindblad P, Bhaya D. Building an Inducible T7 RNA Polymerase/T7 Promoter Circuit in *Synechocystis* sp. PCC6803. *ACS Synth Biol* 2019; 8: 655–660.
37. Huang H-H, Lindblad P. Wide-dynamic-range promoters engineered for cyanobacteria. *J Biol Eng* 2013; 7: 10.
38. Kelly CL, Taylor GM, Hitchcock A, Torres-Méndez A, Heap JT. A Rhamnose-Inducible System for Precise and Temporal Control of Gene Expression in Cyanobacteria. *ACS Synth Biol* 2018; 7: 1056–1066.
39. Behle A, Saake P, Germann AT, Dienst D, Axmann IM. Comparative Dose-Response Analysis of Inducible Promoters in Cyanobacteria. *ACS Synth Biol* 2020; 9: 843–855.
40. Oliver JWK, Machado IMP, Yoneda H, Atsumi S. Combinatorial optimization of cyanobacterial 2,3-butanediol production. *Metab Eng* 2014; 22: 76–82.
41. Heidorn T, Camsund D, Huang HH, Lindberg P, Oliveira P, Stensjö K, Lindblad P. Synthetic biology in cyanobacteria: Engineering and analyzing novel functions. *Methods Enzymol* 2011; 497: 539–579.
42. Taton A, Unglaub F, Wright NE, Zeng WY, Paz-Yepez J, Brahamsha B, Palenik B, Peterson TC, Haerizadeh F, Golden SS, et al. Broad-host-range vector system for synthetic biology and biotechnology in cyanobacteria. *Nucleic Acids Res* 2014; 42.
43. Huang HH, Camsund D, Lindblad P, Heidorn T. Design and characterization of molecular tools for a Synthetic Biology approach towards developing cyanobacterial biotechnology. *Nucleic Acids Res* 2010; 38: 2577–2593.
44. Lambert G, Carr N. A restriction map of plasmid pDC1 from the filamentous cyanobacterium *Nostoc* sp. MAC PCC 8009. *Plasmid* 1983; 10: 196–198.
45. Argueta C, Yuksek K, Summers M. Construction and use of GFP reporter vectors for analysis of cell-type-specific gene expression in *Nostoc punctiforme*. *J Microbiol Methods* 2004; 59: 181–188.
46. Summers ML, Wallis JG, Campbell EL, Meeks JC, Summers ML, Wallis JG, Campbell EL, Meeks JC. Genetic evidence of a major role for glucose-6-phosphate dehydrogenase in nitrogen fixation and dark growth of the cyanobacterium *Nostoc* sp . strain ATCC 29133 . Genetic Evidence of a Major Role for Glucose-6-Phosphate Dehydrogenase in Nitrogen Fixation a. *J Bacteriol* 1995; 177: 6184–6194.
47. Mutalik VK, Guimaraes JC, Cambray G, Mai QA, Christoffersen MJ, Martin L, Yu A, Lam C, Rodriguez C, Bennett G, et al. Quantitative estimation of activity and quality for collections of functional genetic elements. *NatMethods* 2013; 10: 347–353.

48. Thiel K, Mulaku E, Dandapani H, Nagy C, Aro E-MM, Kallio P. Translation efficiency of heterologous proteins is significantly affected by the genetic context of RBS sequences in engineered cyanobacterium *Synechocystis* sp. PCC 6803. *Microb Cell Fact* 2018; 17: 34.
49. Mutalik VK, Guimaraes JC, Cambray G, Mai Q-AA, Christoffersen MJ, Martin L, Yu A, Lam C, Rodriguez C, Bennett G, et al. Precise and reliable gene expression via standard transcription and translation initiation elements. *Nat Methods* 2013; 10: 354–60.
50. Lou C, Stanton B, Chen YJ, Munsky B, Voigt CA. Ribozyme-based insulator parts buffer synthetic circuits from genetic context. *Nat Biotechnol* 2012; 30: 1137–1142.
51. Englund E, Shabestary K, Hudson EP, Lindberg P. Systematic overexpression study to find target enzymes enhancing production of terpenes in *Synechocystis* PCC 6803, using isoprene as a model compound. *Metab Eng* 2018; 49: 164–177.
52. Angermayr SA, Gorchs Rovira A, Hellingwerf KJ. Metabolic engineering of cyanobacteria for the synthesis of commodity products. *Trends Biotechnol* 2015; 33: 352–361.
53. Oliver NJ, Rabinovitch-Deere CA, Carroll AL, Nozzi NE, Case AE, Atsumi S. Cyanobacterial metabolic engineering for biofuel and chemical production. *Curr Opin Chem Biol* 2016; 35: 43–50.
54. Machado IMP, Atsumi S. Cyanobacterial biofuel production. *J Biotechnol* 2012; 162: 50–56.
55. Miao R, Xie H, Liu X, Lindberg P, Lindblad P. Current processes and future challenges of photoautotrophic production of acetyl-CoA-derived solar fuels and chemicals in cyanobacteria. *Curr Opin Chem Biol* 2020; 59: 69–76.
56. Luan G, Qi Y, Wang M, Li Z, Duan Y, Tan X, Lu X. Combinatory strategy for characterizing and understanding the ethanol synthesis pathway in cyanobacteria cell factories. *Biotechnol Biofuels* 2015; 8: 1–12.
57. Hirokawa Y, Suzuki I, Hanai T. Optimization of isopropanol production by engineered cyanobacteria with a synthetic metabolic pathway. *J Biosci Bioeng* 2015; 119: 585–590.
58. Li X, Shen CR, Liao JC. Isobutanol production as an alternative metabolic sink to rescue the growth deficiency of the glycogen mutant of *Synechococcus* elongatus PCC 7942. *Photosynth Res* 2014; 120: 301–310.
59. Appel J, Hueren V, Boehm M, Gutekunst K. Cyanobacterial in vivo solar hydrogen production using a photosystem I–hydrogenase (PsaD-HoxYH) fusion complex. *Nat Energy* 2020. DOI: 10.1038/s41560-020-0609-6.
60. Rosen MA, Koohi-Fayegh S. The prospects for hydrogen as an energy carrier: an overview of hydrogen energy and hydrogen energy systems. *Energy, Ecol Environ* 2016; 1: 10–29.
61. Staffell I, Scamman D, Velazquez Abad A, Balcombe P, Dodds PE, Ekins P, Shah N, Ward KR. The role of hydrogen and fuel cells in the global energy system. *Energy Environ Sci* 2019; 12: 463–491.
62. Carrieri D, Wawrousek K, Eckert C, Yu J, Maness PC. The role of the bidirectional hydrogenase in cyanobacteria. *Bioresour Technol* 2011; 102: 8368–8377.

63. De Rosa E, Checchetto V, Franchin C, Bergantino E, Berto P, Szabò I, Giacometti GM, Arrigoni G, Costantini P. [NiFe]-hydrogenase is essential for cyanobacterium *Synechocystis* sp. PCC 6803 aerobic growth in the dark. *Sci Rep* 2015; 5: 12424.
64. Gutekunst K, Chen X, Schreiber K, Kaspar U, Makam S, Appel J. The bidirectional NiFe-hydrogenase in *Synechocystis* sp. PCC 6803 Is reduced by flavodoxin and ferredoxin and is essential under mixotrophic, nitrate-limiting conditions. *J Biol Chem* 2014; 289: 1930–1937.
65. Aubert-Jousset E, Cano M, Guedeney GG, Richaud P, Cournac L. Role of HoxE subunit in *Synechocystis* PCC6803 hydrogenase. *FEBS J* 2011; 278: 4035–4043.
66. Dutta I, Vermaas WFJ. The electron transfer pathway upon H2 oxidation by the NiFe bidirectional hydrogenase of *Synechocystis* sp. PCC 6803 in the light shares components with the photosynthetic electron transfer chain in thylakoid membranes. *Int J Hydrogen Energy* 2016; 41: 11949–11959.
67. Gutekunst K, Schulz R. The Physiology of the Bidirectional NiFe-hydrogenase in Cyanobacteria and the Role of Hydrogen Throughout the Evolution of Life. In: *Comprehensive Series in Photochemical and Photobiological Sciences*. 2018, pp. 107–138.
68. Sadvakasova AK, Kossalbayev BD, Zayadan BK, Bolatkhan K, Alwasel S, Najafpour MM, Tomo T, Allakhverdiev SI. Bioprocesses of hydrogen production by cyanobacteria cells and possible ways to increase their productivity. *Renew Sustain Energy Rev* 2020; 133: 110054.
69. Happe T, Schütz K, Böhme H. Transcriptional and mutational analysis of the uptake hydrogenase of the filamentous cyanobacterium *Anabaena variabilis* ATCC 29413. *J Bacteriol* 2000; 182: 1624–1631.
70. Lindberg P, Schütz K, Happe T, Lindblad P. A hydrogen-producing, hydrogenase-free mutant strain of *Nostoc punctiforme* ATCC 29133. *Int J Hydrogen Energy* 2002; 27: 1291–1296.
71. Masukawa H, Mochimaru M, Sakurai H. Hydrogenases and photobiological hydrogen production utilizing nitrogenase system in cyanobacteria. *Int J Hydrogen Energy* 2002; 27: 1471–1474.
72. Yoshino F, Ikeda H, Masukawa H, Sakurai H. High photobiological hydrogen production activity of a *Nostoc* sp. PCC 7422 uptake hydrogenase-deficient mutant with high nitrogenase activity. *Mar Biotechnol* 2007; 9: 101–112.
73. Khetkorn W, Lindblad P, Incharoensakdi A. Inactivation of uptake hydrogenase leads to enhanced and sustained hydrogen production with high nitrogenase activity under high light exposure in the cyanobacterium *Anabaena siamensis* TISTR 8012. *J Biol Eng* 2012; 6: 19.
74. Lindberg P, Devine E, Stensjö K, Lindblad P. Hupw protease specifically required for processing of the catalytic subunit of the uptake hydrogenase in the cyanobacterium *Nostoc* sp. Strain PCC 7120. *Appl Environ Microbiol* 2012; 78: 273–276.
75. Masukawa H, Inoue K, Sakurai H, Wolk CP, Hausinger RP. Site-directed mutagenesis of the *Anabaena* sp. strain PCC 7120 nitrogenase active site to increase photobiological hydrogen production. *Appl Environ Microbiol* 2010; 76: 6741–6750.

76. Masukawa H, Sakurai H, Hausinger RP, Inoue K. Sustained photobiological hydrogen production in the presence of N2 by nitrogenase mutants of the heterocyst-forming cyanobacterium *Anabaena*. *Int J Hydrogen Energy* 2014; 39: 19444–19451.
77. Weyman PD, Pratte B, Thiel T. Hydrogen production in nitrogenase mutants in *Anabaena variabilis*. *FEMS Microbiol Lett* 2010; 304: 55–61.
78. Cano M, Volbeda A, Guedeney G, Aubert-Jousset E, Richaud P, Peltier G, Cournac L. Improved oxygen tolerance of the *Synechocystis* sp. PCC 6803 bidirectional hydrogenase by site-directed mutagenesis of putative residues of the gas diffusion channel. *Int J Hydrogen Energy* 2014; 39: 16872–16884.
79. Ihara M, Nakamoto H, Kamachi T, Okura I, Maeda M. Photoinduced hydrogen production by direct electron transfer from photosystem I cross-linked with cytochrome c3 to [NiFe]-hydrogenase. *Photochem Photobiol* 2006; 82: 1677–1685.
80. Ihara M, Nishihara H, Yoon K-S, Lenz O, Friedrich B, Nakamoto H, Kojima K, Honma D, Kamachi T, Okura I. Light-driven hydrogen production by a hybrid complex of a [NiFe]-hydrogenase and the cyanobacterial photosystem I. *Photochem Photobiol* 2006; 82: 676–682.
81. Cammack R. Hydrogenase sophistication. *Nature* 1999; 397: 214–215.
82. Lubitz W, Ogata H, Rüdiger O, Reijerse E. Hydrogenases. *Chem Rev* 2014; 114: 4081–4148.
83. Land H, Senger M, Berggren G, Stripp ST. Current State of [FeFe]-Hydrogenase Research: Biodiversity and Spectroscopic Investigations. *ACS Catal* 2020; 10: 7069–7086.
84. Posewitz MC, King PW, Smolinski SL, Zhang L, Seibert M, Ghirardi ML. Discovery of two novel radical S-adenosylmethionine proteins required for the assembly of an active [Fe] hydrogenase. *J Biol Chem* 2004; 279: 25711–25720.
85. Peters JW, Schut GJ, Boyd ES, Mulder DW, Shepard EM, Broderick JB, King PW, Adams MWW. [FeFe]- and [NiFe]-hydrogenase diversity, mechanism, and maturation. *Biochim Biophys Acta - Mol Cell Res* 2015; 1853: 1350–1369.
86. King PW, Posewitz MC, Ghirardi ML, Seibert M. Functional studies of [FeFe] hydrogenase maturation in an *Escherichia coli* biosynthetic system. *J Bacteriol* 2006; 188: 2163–2172.
87. Ducat DC, Sachdeva G, Silver PA. Rewiring hydrogenase-dependent redox circuits in cyanobacteria. *Proc Natl Acad Sci U S A* 2011; 108: 3941–3946.
88. Avilan L, Roumezi B, Risoul V, Bernard CS, Kpebe A, Belhadjhassine M, Rousset M, Brugna M, Latifi A. Phototrophic hydrogen production from a clostridial [FeFe] hydrogenase expressed in the heterocysts of the cyanobacterium *Nostoc* PCC 7120. *Appl Microbiol Biotechnol* 2018; 102: 5775–5783.
89. Camsund D, Heidorn T, Lindblad P. Design and analysis of LacI-repressed promoters and DNA-looping in a cyanobacterium. *J Biol Eng* 2014; 8: 4.
90. Knight T. Idempotent Vector Design for Standard Assembly of Biobricks. *MIT Libr* 2003; 1–11.
91. Holmqvist M, Stensjö K, Oliveira P, Lindberg P, Lindblad P. Characterization of the hupSL promoter activity in *Nostoc punctiforme* ATCC 29133. *BMC Microbiol* 2009. DOI: 10.1186/1471-2180-9-54.

92. Berggren G, Adamska A, Lambertz C, Simmons TR, Esselborn J, Atta M, Gambarelli S, Mouesca JM, Reijerse E, Lubitz W, et al. Biomimetic assembly and activation of [FeFe]-hydrogenases. *Nature* 2013; 499: 66–69.
93. Esselborn J, Muraki N, Klein K, Engelbrecht V, Metzler-Nolte N, Apfel U-P, Hofmann E, Kurisu G, Happe T. A structural view of synthetic cofactor integration into [FeFe]-hydrogenases. *Chem Sci* 2016; 7: 959–968.
94. Khanna N, Esmieu C, Mészáros LS, Lindblad P, Berggren G. In vivo activation of an [FeFe] hydrogenase using synthetic cofactors. *Energy Environ Sci* 2017; 10: 1563–1567.
95. Pinto F, van Elburg KA, Pacheco CC, Lopo M, Noirel J, Montagud A, Urchueguía JF, Wright PC, Tamagnini P. Construction of a chassis for hydrogen production: Physiological and molecular characterization of a *Synechocystis* sp. PCC 6803 mutant lacking a functional bidirectional hydrogenase. *Microbiology* 2012; 158: 448–464.
96. Land H, Ceccaldi P, Mészáros LS, Lorenzi M, Redman HJ, Senger M, Stripp ST, Berggren G. Discovery of novel [FeFe]-hydrogenases for biocatalytic H2-production. *Chem Sci* 2019; 10: 9941–9948.
97. Land H, Sekretareva A, Huang P, Redman HJ, Németh B, Polidori N, Mészáros LS, Senger M, Stripp ST, Berggren G. Characterization of a putative sensory [FeFe]-hydrogenase provides new insight into the role of the active site architecture. *Chem Sci* 2020. DOI: 10.1039/d0sc03319g.
98. Melis A, Happe T. Trails of green alga hydrogen research - From Hans Gaffron to new frontiers. *Photosynthesis Research*. 2004; 80.
99. Ghirardi ML, Posewitz MC, Maness P-C, Dubini A, Yu J, Seibert M. Hydrogenases and Hydrogen Photoproduction in Oxygenic Photosynthetic Organisms. *Annu Rev Plant Biol* 2007; 58: 71–91.
100 Hauf W, Schlebusch M, Hüge J, Kopka J, Hagemann M, Forchhammer K. Metabolic Changes in *Synechocystis* PCC6803 upon Nitrogen-Starvation: Excess NADPH Sustains Polyhydroxybutyrate Accumulation. *Metabolites* 2013; 3: 101–118.
101. Gutthann F, Egert M, Marques A, Appel J. Inhibition of respiration and nitrate assimilation enhances photohydrogen evolution under low oxygen concentrations in *Synechocystis* sp. PCC 6803. *Biochim Biophys Acta - Bioenerg* 2007; 1767: 161–169.
102. Knowles VL, Smith CSCR, Smith CSCR, Plaxton WC. Structural and Regulatory Properties of Pyruvate Kinase from the Cyanobacterium *Synechococcus* PCC 6301. *J Biol Chem* 2001; 276: 20966–20972.
103. Kubas A, Orain C, De Sancho D, Saujet L, Sensi M, Gauquelin C, Meynial-Salles I, Soucaille P, Bottin H, Baffert C, et al. Mechanism of O2 diffusion and reduction in FeFe hydrogenases. *Nat Chem* 2017; 9: 88–95.
104. Stripp ST, Goldet G, Brandmayr C, Sanganas O, Vincent KA, Haumann M, Armstrong FA, Happe T. How oxygen attacks [FeFe] hydrogenases from photosynthetic organisms. *Proc Natl Acad Sci U S A* 2009; 106: 17331–17336.
105. Ehira S, Takeuchi T, Higo A. Spatial separation of photosynthesis and ethanol production by cell type-specific metabolic engineering of filamentous cyanobacteria. *Appl Microbiol Biotechnol* 2018; 102: 1523–1531.

106. Mariscal V, Herrero A, Flores E. Continuous periplasm in a filamentous, heterocyst-forming cyanobacterium. *Mol Microbiol* 2007; 65: 1139–1145.
107. Li X, Sandh G, Nenninger A, Muro-Pastor AM, Stensjö K. Differential transcriptional regulation of orthologous *dps* genes from two closely related heterocyst-forming cyanobacteria. *FEMS Microbiol Lett* 2015; 362: 1–8.
108. Mitschke J, Vioque A, Haas F, Hess WR, Muro-Pastor AM. Dynamics of transcriptional start site selection during nitrogen stress-induced cell differentiation in *Anabaena* sp. PCC7120. *PNAS* 2011; 108: 20130–20135.
109. Videau P, Ni S, Rivers OS, Ushijima B, Feldmann EA, Cozy LM, Kennedy MA, Callahan SM. Expanding the direct HetR regulon in *Anabaena* sp. strain PCC 7120. *J Bacteriol* 2014; 196: 1113–1121.
110. Miao R, Liu X, Englund E, Lindberg P, Lindblad P. Isobutanol production in *Synechocystis* PCC 6803 using heterologous and endogenous alcohol dehydrogenases. *Metab Eng Commun* 2017; 5: 45–53.
111. Scholz P, Haring V, Wittmann-Liebold B, Ashman K, Bagdasarian M, Scherzinger E. Complete nucleotide sequence and gene organization of the broad-host-range plasmid RSF1010. *Gene* 1989; 75: 271–288.
112. Bernard, P. Gabant, P. Bahassi, E.M. Couturier M, Mustapha E. Positive-selection vectors using the F plasmid *ccdB* killer gene. *Gene* 1994; 148: 71–74.
113. Muro-Pastor AM, Brenes-Álvarez M, Vioque A. A combinatorial strategy of alternative promoter use during differentiation of a heterocystous cyanobacterium. *Environ Microbiol Rep* 2017; 9: 449–458.
114. Crooks GE, Hon G, Chandonia J-M, Brenner SE. WebLogo: a sequence logo generator. *Genome Res* 2004; 14: 1188–90.
115. Chabot JR, Pedraza JM, Luitel P, Van Oudenaarden A, Oudenaarden A Van. Stochastic gene expression out-of-steady-state in the cyanobacterial circadian clock. *Nature* 2007; 450: 1249–1252.

Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology 1990

Editor: The Dean of the Faculty of Science and Technology

A doctoral dissertation from the Faculty of Science and Technology, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology. (Prior to January, 2005, the series was published under the title "Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology".)

Distribution: publications.uu.se
urn:nbn:se:uu:diva-425911

ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2020