

## **Dissertação**

# **Development of molecular tools for the study of the maturation of cyanobacterial hydrogenases**

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**Porto**

**2011**



**Faculdade de Ciências da Universidade do Porto**

**Mestrado em Biologia**

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Dissertação submetida à Faculdade de  
Ciências UP como requisito parcial para  
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Dissertação defendida em ..... / .... / .....

**O Presidente do Júri**

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# Table of Contents

List of Abbreviations .....	vii
Resumo .....	1
Abstract .....	3
1. Introduction .....	5
2. Material and Methods .....	13
2.1. Organisms and growth conditions.....	13
2.2. DNA isolation .....	13
2.3. Primer Design and Polymerase Chain Reactions (PCRs) .....	13
2.4. Creation of A-tailed ends and cloning .....	17
2.5. Transformation of <i>Escherichia coli</i> .....	18
2.6. Amplification and isolation of plasmid DNA .....	18
2.7. Screening for positive clones by digestion of DNA and sequencing .....	18
2.8. Insertion of the selection cassettes into pNhupWSF and pNhoxW(SF/LF).19	
2.9. Protein extraction .....	20
2.10. Protein gel electrophoresis and Western blot .....	20
3. Results and Discussion .....	23
4. Conclusions .....	31
5. Future Perspectives .....	33
6. References .....	35



## List of Abbreviations

<b>ATP</b>	Adenosine Triphosphate
<b>ADP</b>	Adenosine Diphosphate
<b>bp</b>	Base Pair
<b>BSA</b>	Bovine Serum Albumin
<b>dATP</b>	Deoxyadenosine Triphosphate
<b>DNA</b>	Deoxyribonucleic Acid
<b>dNTP</b>	Deoxynucleotide Triphosphate
<b>DTT</b>	Dithiothreitol
<b>EDTA</b>	Ethylenediaminetetraacetic Acid
<b>GTP</b>	Guanosine Triphosphate
<b>HEPES</b>	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
<b>IgG</b>	Immunoglobulin G
<b>IPTG</b>	isopropyl- $\beta$ -D-1--thiogalactopyranoside
<b>LB</b>	Luria-Bertani
<b>NAD<sup>+</sup>/NADH</b>	Nicotinamide Adenine Dinucleotide
<b>NADP<sup>+</sup>/NADPH</b>	Nicotinamine Adenine Dinucleotide Phosphate
<b>NCBI</b>	National Center for Biotechnology Information
<b>PAGE</b>	Polyacrylamide Gel Electrophoresis
<b>PBS</b>	Phosphate Buffered Saline
<b>PCC</b>	Pasteur Culture Collection
<b>PCR</b>	Polymerase Chain Reaction
<b>PSII</b>	Photosystem II
<b>kDa</b>	Kilodaltons

<b>rpm</b>	Revolutions Per Minute
<b>SDS</b>	Sodium Dodecyl Sulphate
<b>TAE</b>	Tris-acetate-EDTA
<b>UV</b>	Ultraviolet
<b>X-Gal</b>	5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside

## Resumo

As cianobactérias podem conter até duas hidrogenases de Ni e Fe (i) uma hidrogenase de assimilação (codificada pelos genes *hupSL*), responsável pelo consumo do H<sub>2</sub> produzido pela nitrogenase e (ii) uma hidrogenase bidireccional (codificada pelos genes *hoxEFUYH*) que pode consumir ou produzir H<sub>2</sub>. A biossíntese/maturação destas hidrogenases é um processo complexo para o qual é necessária a acção de pelo menos sete proteínas auxiliares para a obtenção de uma enzima funcional. Muito provavelmente, o último passo na maturação das subunidades maiores de ambas as hidrogenases consiste na clivagem de um péptido C-terminal, a qual possibilita a junção subsequente das subunidades maior e menor. Esta clivagem é, provavelmente, efectuada pelas endopeptidases específicas HupW e HoxW, que actuarão respectivamente nas subunidades maiores da hidrogenase de assimilação e da hidrogenase bidireccional. Até ao momento, contudo, não é claro se estas endopeptidases são de facto específicas para cada hidrogenase, uma vez que os únicos estudos foram realizados em *Synechocystis* sp. PCC 6803, uma estirpe que possui apenas a enzima bidireccional. Para clarificar a especificidade desta interacção têm de ser realizados estudos em estirpes contendo ambas as hidrogenases, como por exemplo *Nostoc* sp. PCC 7120. A deleção dos genes *hupW* e/ou *hoxW* nesta estirpe serão elucidativos acerca dos efeitos de cada endopeptidase na maturação de cada hidrogenase.

Neste trabalho foram construídos os vectores integrativos que serão utilizados para gerar mutantes  $\Delta hupW$  e/ou  $\Delta hoxW$  de *Nostoc* sp. PCC 7120. Além disso, no sentido de estabelecer ferramentas para a futura caracterização dos mutantes, foi avaliada a possibilidade de estudar o último passo da maturação das subunidades maiores (clivagem do péptido C-terminal) através de Western blot usando anticorpos específicos para a forma não-processada e processada das hidrogenases. Os resultados revelaram que os anticorpos produzidos contra a forma não processada e processada da hidrogenase bidireccional de *Synechocystis* sp. PCC 6803 não são ideais para *Nostoc* sp. PCC 7120 mas a estratégia é viável e pode ser aplicada no trabalho subsequente.



## Abstract

Cyanobacteria may harbour up to two different [NiFe]-hydrogenases (i) an uptake hydrogenase (encoded by *hupSL*), responsible for the consumption of the H<sub>2</sub> produced by the nitrogenase complex and (ii) a bidirectional hydrogenase (encoded by *hoxEFUYH*) that may consume or produce H<sub>2</sub>. The biosynthesis/maturation of these hydrogenases is a highly complex process in which at least seven accessory proteins are required for the assembly of a functional enzyme. The last step in the maturation of the large subunits of both hydrogenases is, most likely, the cleavage of a C-terminal peptide, which allows the subsequent assembly of the large and small subunits. This cleavage is putatively achieved by specific proteases HupW and HoxW, for the large subunits of the uptake and bidirectional hydrogenase, respectively. However, until now it is not clear if these endopeptidases are indeed specific for each cyanobacterial hydrogenase, since the only studies addressing this subject were performed in *Synechocystis* sp. PCC 6803, a strain harbouring only the bidirectional enzyme. To clarify the specificity of this interaction, studies on strains harbouring both the uptake and bidirectional hydrogenase, such as *Nostoc* sp. strain PCC 7120, have to be performed. The deletion of the *hupW* and/or *hoxW* genes in this strain will clarify the role of each putative endopeptidase in the maturation of each hydrogenase.

In this work the integrative vectors that will be used to generate  $\Delta hupW$  and/or  $\Delta hoxW$  mutants of *Nostoc* sp. strain PCC 7120 were constructed. Moreover, in order to provide tools for the future characterization of the mutants, the feasibility to study the last step in the maturation of the large subunits (C-terminal cleavage) by Western blot using specific antibodies for the unprocessed and processed form was evaluated. The data obtained show that the antibodies raised against the unprocessed and processed form of *Synechocystis* sp. 6803 bidirectional hydrogenase are not ideal for *Nostoc* sp. strain PCC 7120, but the strategy revealed to be viable and can be applied in the subsequent work.

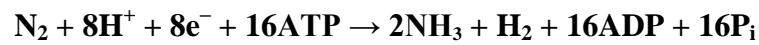


# 1. Introduction

Cyanobacteria constitute the group of oxygenic photosynthetic prokaryotes with a remarkable diversity, geographical distribution and metabolism. This group has a very ancient origin, dated based on analyses of fossil traces in at least 3.5 billion years ago (Schopf, 2000) and it is widely believed that they played a major role in the creation of the Earth's aerobic atmosphere (Schopf, 2000). This long evolutionary history could explain the adaptation to a large diversity of habitats including aquatic (fresh and saltwater), terrestrial and extreme environments (Whitton and Potts, 2000). Besides their wide geographic distribution, cyanobacteria also exhibit a great morphological diversity including unicellular, colonial and multicellular filamentous forms. Interestingly, the multicellular morphotype, present only in few prokaryotic phyla, appeared early during cyanobacterial evolution and the modern forms, including the majority of the unicellular strains, evolved from multicellular lineages (Schirromeister *et al.*, 2011). This early multicellularity could have been beneficial for the adaptation to new niches and, thus, lead to diversification (Schirromeister *et al.*, 2011). The filamentous cyanobacteria may also exhibit a certain degree of cellular differentiation; vegetative cells may develop into specialized cells such as akinetes or heterocysts (Whitton and Potts, 2000). The akinetes are resting cells that are produced under starving conditions (nutrient and/or light) and remain viable for long periods of time under adverse conditions. The heterocysts are cells specialized in the fixation of atmospheric nitrogen ( $N_2$ ) and arise when the availability of the combined form of this element is reduced. The heterocysts possess a set of modifications, namely the absence of activity of PSII, increased respiratory rate and the presence of a thick envelop surrounding the cell (Fay, 1992; Wolk *et al.*, 1994) in order to create a microaerobic environment since the nitrogenase, the enzymatic complex responsible for nitrogen fixation, is very oxygen labile. Some non-heterocystous strains can also fix  $N_2$ . However, in those strains this process occurs only under specific conditions, for e.g. temporal separation with photosynthesis taking place during the photoperiod (Berman-Frank *et al.*, 2003).

The nitrogenase complex (encoded by the *nif* genes) consists of two protein components namely the dinitrogenase, that contains the active site, and the dinitrogenase reductase, that mediates the transfer of electrons from the donor (a ferredoxin or a flavodoxin) to the dinitrogenase (Fay, 1992; Tamagnini *et al.*, 2002; Bothe *et al.*, 2010). This complex catalyzes

the reduction of nitrogen (N<sub>2</sub>) to ammonia (NH<sub>3</sub>) with consumption of ATP and production of hydrogen (H<sub>2</sub>) according to the following overall equation:

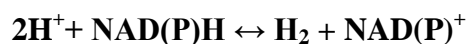


Although H<sub>2</sub> is produced as a by-product during nitrogen fixation, in general it does not evolve from the cells since it is rapidly consumed by the uptake hydrogenase (encoded by the *hupSL* genes) that is present in almost all the N<sub>2</sub>-fixing cyanobacteria, with the exception of three *Synechococcus* strains (Ludwig *et al.*, 2006; Steunou *et al.*, 2008). The uptake hydrogenase catalyses the simple oxidation of hydrogen as shown in the following equation:



The strong correlation between the activity of the nitrogenase and the activity of the uptake hydrogenase (Wolk *et al.*, 1994; Troshina *et al.*, 1996; Schütz *et al.*, 2004) suggests that the main physiological function of the latter is precisely the recycling of the H<sub>2</sub> produced by the former. This recycling was suggested to be beneficial for the nitrogenase itself since it supplies it with reduction equivalents (electrons) as well as the electrons produced can be driven to the respiratory chain leading to the production of ATP and reduction of oxygen, both conditions required for nitrogenase activity (Bothe *et al.*, 1977).

The N<sub>2</sub>-fixing cyanobacteria can also harbour a second hydrogenase, named bidirectional hydrogenase (encoded by the *hoxEFUYH* genes), which is ubiquitous among the non-N<sub>2</sub>-fixing strains, with the exception of the strain *Gloeobacter violaceus* PCC 7421 (Tamagnini *et al.*, 2007). The bidirectional hydrogenase is a pentameric enzyme, constituted by two parts, namely the hydrogenase (HoxYH) and the diaphorase (HoxEFU), and catalyses the following overall reaction:



Whereas the hydrogenase part is responsible for the oxidation of H<sub>2</sub>/reduction of H<sup>+</sup>, the diaphorase part is the enzymatic redox partner for the hydrogenase, donating electrons from the oxidation of NAD(P)H to hydrogenase/ accepting electrons from the hydrogenase for the reduction of NAD(P)<sup>+</sup> (Schmitz *et al.*, 2002; Carrieri *et al.*, 2011) Although the bidirectional hydrogenase can work effectively in both directions, producing or consuming H<sub>2</sub>, recent data

pointed that it works with a bias to its production (McIntosh *et al.*, 2011). In contrast with the uptake hydrogenase, the physiological function of this hydrogenase is not totally clear. Roles in different cellular process have been proposed, such as in photosynthesis, by acting as electron valve (Appel *et al.*, 2000) and in fermentation, by mediating the release of excess reducing power (Troshina *et al.*, 2002). These roles in different redox stages of the cell as well as the likelihood of this enzyme to work in both directions suggest a role as redox regulator (Carrieri *et al.*, 2011).

The cyanobacterial hydrogenases are metalloproteins containing nickel and iron in their active centre and, both the uptake and the hydrogenase part of the bidirectional, are heterodimeric enzymes, constituted by a large subunit (HupL-uptake hydrogenase / HoxH-bidirectional hydrogenase) and a small subunit (HupS-uptake hydrogenase / HoxY-bidirectional hydrogenase). Whereas the large subunits of these enzymes contain the bimetallic active site, whose coordination is very complex, involving carbon monoxide (CO) and cyanide (CN<sup>-</sup>), two biologically uncommon ligands (Böck *et al.*, 2006; Forzi and Sawers, 2007; Higuchi *et al.*, 1997; Volbeda *et al.*, 1996), the small subunits contain Fe-S clusters, involved in the transfer of electrons between the donor/acceptor of electrons and the active site (Vignais and Colbeau, 2004).

Although catalyzing a simple chemical reaction, the biosynthesis/maturation of the [NiFe]-hydrogenases is a highly complex process requiring several proteins for the processing of both small and large subunits. The majority of the knowledge regarding the maturation of the [NiFe]-hydrogenases comes from studies focusing on the maturation of the large subunit. This process has been extensively studied for the hydrogenase 3 of *Escherichia coli* (Casalot and Rousset, 2001; Vignais and Colbeau, 2004; Forzi and Sawers, 2007) and revealed to follow a complex pathway (Fig. 1), in which at least seven proteins are involved, namely the Hyp proteins (HypA, HypB, HypC, HypD, HypE and HypF) and a specific endopeptidase.

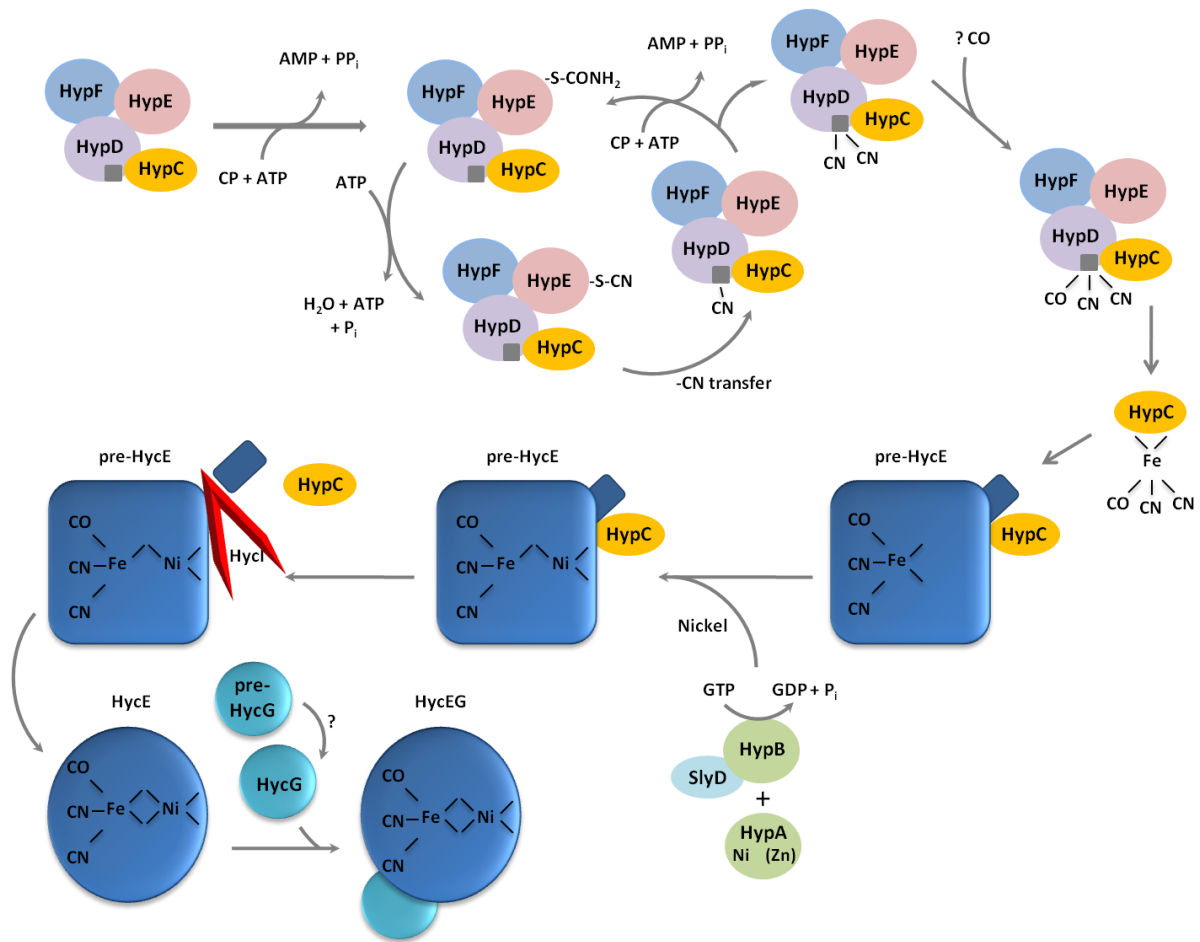


Fig. 1. Postulated model for the biosynthesis/maturation of the hydrogenase 3 of *Escherichia coli* (HycEG). The question marks indicate that it is unclear the metabolic origin of the CO ligand as well as the mechanisms involved in the maturation of the small subunit (HycG). The gray square in the HypD represents a Fe-S cluster. Abbreviations: CP, carbamoyl phosphate; PPi, pyrophosphate; Pi, inorganic phosphate; CO, carbonyl ligand; CN, cyano ligand. Adapted from: Magalon and Böck (2000b); Blokesch and Böck (2002); Vignais and Colbeau (2004) and Forzi and Sawers (2007).

The Hyp proteins affect hydrogenases pleiotropically and play a crucial role in the incorporation of the metal ions, Ni and Fe, and the ligands, CO and CN<sup>-</sup>, into their active centre. From the two metal ions, the Fe is thought to be the first to be inserted into the enzyme (Maier and Böck, 1996). This metal has one CO and two CN<sup>-</sup> ligands and it is not clear which is inserted first. However, it is most likely to be the CN<sup>-</sup> (Pickett *et al.*, 2004; Forzi and Sawers, 2007). Two Hyp proteins are required for the synthesis of the CN<sup>-</sup> ligands,

namely the HypF and the HypE. The HypF in the presence of ATP catalyzes the transfer of the carbamoyl group from carbamoyl phosphate (CP) to the thiol group of the C-terminal cysteine of HypE, with the consequent formation of AMP and pyrophosphate (Paschos *et al.*, 2002). In a reaction dependant on ATP, the HypE dehydrates its new formed carbamoyl group, leading to the formation of a CN<sup>-</sup> group (Fig.1) (Reissmann *et al.*, 2003). Although the origin of the CO can theoretically be the same as the origin of CN<sup>-</sup>, i.e. the carbamoyl phosphate (Paschos *et al.*, 2001), these two ligands seem to have different metabolic origins (Roseboom *et al.*, 2005). However, the source of CO is not clear (Forzi and Sawers, 2007). The CO and CN<sup>-</sup> ligands are subsequently accepted by a complex formed by HypD and HypC (Fig. 1), where the liganding of Fe is likely to occur (Blokesch and Böck, 2002, Blokesch *et al.*, 2004a). The HypD might provide the electrons required for the attachment of the Fe to the three ligands, since it contains a FeS cluster (Blokesch and Böck, 2002; Blokesch *et al.*, 2004a). The HypC is a chaperone-type protein and interacts with the precursor of the large subunit of the hydrogenase 3 of *E. coli* (pre-HycE), delivering to it the liganded Fe (Magalon and Böck, 2000a; Blokesch and Böck, 2002). The interaction between HypC and pre-HycE leads to the release of HypD (Blokesch and Böck, 2006), but HypC maintains attached to the pre-HycE, keeping it in an open conformation, required for the subsequent insertion of nickel (Fig. 1) (Drapal and Böck, 1998). The insertion of this second metal requires the concerted action of HypA and HypB (Olson *et al.*, 2001). The HypA is a zinc metalloprotein that binds nickel (Atanassova and Zamble, 2005) and the HypB is a metal binding (zinc and nickel), GTP binding and GTP hydrolysis protein (Maier *et al.*, 1995; Leach *et al.*, 2005; Gasper *et al.*, 2006). It has been suggested that HypA functions as nickel chaperone and HypB mediates the transfer of this metal to the hydrogenase active site (Blokesch *et al.*, 2004a; Gasper *et al.*, 2006). This process may be done through a HypB GTP-induced dimer that, after the insertion of nickel, releases the complex from hydrogenase via GTP hydrolysis activity (Gasper *et al.*, 2006). Recently, it was shown that SlyD, a peptidyl-prolyl cis/trans-isomerase with a metal-binding domain, forms a complex with HypB (Zhang *et al.*, 2005). Although the deletion of the *slyD* gene does not abolish hydrogenase activity, it leads to its clear reduction due to impaired metal centre assembly and, therefore, SlyD improves the kinetics of Ni insertion (Zhang *et al.*, 2005).

The insertion of nickel allows the maturation of the large subunit to proceed to the last step, which consists on the cleavage of a C-terminal peptide by a specific endopeptidase that uses this metal as a part of its recognition motif (Theodoratou *et al.*, 2000a). However, this cleavage can only occur after the dissociation of HypC from the precursor of the large subunit

(Magalon and Böck, 2000a; b). Together with nickel, this endopeptidase recognises the highly conserved consensus sequence DPCxxCxx(H/R) and cleaves the precursor of the large subunit at the histidine or arginine residue releasing a short peptide that varies both in length and in sequence between different hydrogenases and organisms (Wünschiers *et al.*, 2003). However, substitutions to chemically different amino acids close to the recognition sequence, as well as the exchange of the C-terminal between isoenzymes leads to impaired cleavage in *E. coli* (Theodoratou *et al.*, 2000b). Therefore, the recognition of the hydrogenase large subunit by its specific endopeptidase depends not only on the cleavage site consensus sequence but also on the overall three-dimensional structure of the hydrogenase and endopeptidase (Theodoratou *et al.*, 2000b). In fact, studies in *E. coli* revealed a high specificity of this interaction since its hydrogenases 1, 2 and 3 are only processed by the endopeptidase HyaD, HybD and HycI, respectively (Menon *et al.*, 1991; 1994; Rossmann *et al.*, 1995). This cleavage triggers a change in conformation in which the free thiol of the most C-terminally located cysteine residue closes the bridge between the two metal ions, completing the formation of the active centre (Magalon and Böck, 2000a; Theodoratou *et al.*, 2005). Subsequent to this conformational switch, the mature large subunit can be assembled with the mature small subunit yielding a functional enzyme (Magalon and Böck, 2000b).

The knowledge about the maturation of the small subunit is very scarce (Casalot and Rousset, 2001; Böck *et al.*, 2006). As referred above, the main feature of this subunit is the presence of Fe-S clusters. Although three pathways for the assembly of Fe-S clusters into proteins were identified in bacteria, namely the Isc (iron-sulfur cluster) system, the Suf (sulfur formation) system, and the Nif (nitrogen fixation) system (Ayala-Castro *et al.*, 2008; Frazzon and Dean, 2003; Johnson *et al.*, 2005), the specific mechanism by which these clusters are assembled in the small subunits of the hydrogenases is still unknown (Böck *et al.*, 2006). However, it would be interesting to study a possible involvement of the Isc system, since, besides the nitrogenase specific system (Nif), it has been related with the assembly of the Fe-S clusters of the nitrogenase (Frazzon and Dean, 2003).

Despite being relatively well established for *E. coli* hydrogenases, the biosynthesis/maturation of the cyanobacterial hydrogenases is a process scarcely known. However, homologues of the *E. coli* *hyp* genes (*hypFCDEAB*) have been identified in cyanobacterial genomes (Hansel *et al.*, 2001; Tamagnini *et al.*, 2007; Agervald *et al.*, 2008) and so they might fulfill similar functions as it has been reported for other organisms (Casalot and Rousset, 2001). In fact, in *Synechocystis* sp. PCC 6803, a cyanobacterium harbouring only the bidirectional hydrogenase, the products of these genes are required for hydrogenase activity

(Hoffmann *et al.*, 2006). The presence of a single copy of most of the *hyp* genes in the genome of cyanobacteria possessing both the uptake and bidirectional hydrogenases suggests that the Hyp proteins might be responsible for the biosynthesis/maturation of both enzymes (Tamagnini *et al.*, 2007). In fact, these proteins show a notable broad activity since the *E. coli* native Hyp proteins, with the exception of HypA, can partially assure the functions of the Hyp proteins of *Synechocystis* sp. PCC 6803 in the maturation of the bidirectional hydrogenase of this cyanobacterium (Wells *et al.*, 2011).

The genes encoding the putative hydrogenases-specific endopeptidases were also identified in the available cyanobacteria genome sequences, and were named *hupW* and *hoxW*, for the endopeptidase processing the uptake and bidirectional hydrogenase, respectively (Wünschiers *et al.*, 2003). This genomic analysis revealed that *hupW* and *hoxW* are only found in cyanobacteria harbouring both the uptake and bidirectional hydrogenase (Wünschiers *et al.*, 2003). Moreover, in cyanobacteria harbouring only the uptake hydrogenase only *hupW* is present and in cyanobacteria harbouring only the bidirectional hydrogenase only *hoxW* is present (Wünschiers *et al.*, 2003). These data corroborate the hypothesis that HupW and HoxW are specific for the uptake and bidirectional hydrogenases, respectively. However, the unique studies reported so far addressing the role of these genes were done or in *Synechocystis* sp. PCC 6803 (Hoffmann *et al.*, 2006) or in *E. coli* hydrogenases-null mutant expressing the hydrogenase and respective maturation system of this cyanobacterium (Wells *et al.*, 2011) and revealed that *hoxW* is essential for the hydrogenase activity. Recently, antibodies specific for the unprocessed and processed form of *Synechocystis* sp. 6803 bidirectional hydrogenase were developed and can be used as molecular tool to study the C-terminal cleavage of the large subunit (Yu *et al.*, 2010). Since *Synechocystis* sp. PCC 6803 possesses only the bidirectional hydrogenase, these studies do not allow to take conclusions about the specificity of this endopeptidase. To prove the actual involvement and specificity of the HupW and HoxW endopeptidases on the maturation of the cyanobacterial hydrogenases, studies on strains containing both the uptake and the bidirectional hydrogenases are required.

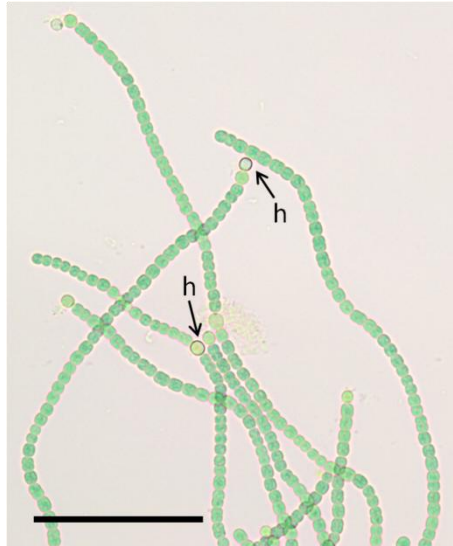


Fig. 2. Light micrograph showing a general view of *Nostoc* sp. strain PCC 7120 grown in BG11<sub>0</sub> medium. h – heterocyst. Bar = 50  $\mu$ m.

For the study of the maturation/regulation of cyanobacterial hydrogenases the filamentous heterocystous *Nostoc* sp. PCC 7120 (Fig. 2) is going to be used as model organism. Besides harbouring both hydrogenases, the genome of this strain is fully sequenced and there are molecular tools available for its genetic manipulation.

The major aim of this work was to develop molecular tools to study the maturation of cyanobacterial hydrogenases. To prove the specificity of the putative endopeptidases,  $\Delta hupW$  and/or  $\Delta hoxW$  mutants of *Nostoc* sp. strain PCC 7120 will be generated. Therefore, the integrative vectors that will be used to generate these mutants were constructed. Moreover, to evaluate the feasibility to study the last step of the maturation of the large subunits (C-terminal cleavage) using specific antibodies, the ones raised against the unprocessed and processed form of *Synechocystis* sp. 6803 bidirectional hydrogenase were tested.

## **2. Material and Methods**

### **2.1. Organisms and growth conditions**

For comparative purposes cultures of *Nostoc* sp. strain PCC 7120 and *Synechocystis* sp. 6803 were established for one week in both BG11 and BG11<sub>0</sub> medium (Rippka *et al.*, 1979) under 16 hours light ( $\sim 10 \mu\text{E}\cdot\text{m}^{-1}\cdot\text{s}^{-2}$ ) / 8 hours dark regimen at 25 °C on an orbital shaker at 80 rpm.

### **2.2. DNA isolation**

Cyanobacterial genomic DNA extraction was performed according to the previously described phenol-chloroform method (Tamagnini *et al.*, 1997). Cells were resuspended in 10 mM EDTA, 50 mM Tris-HCl, pH 8.0, and disrupted by 5 times 30 seconds vigorous vortexing, alternated with 1 minute cooling steps on ice, with 0.6 g of acid-washed glass beads (425-600  $\mu\text{m}$  diameter) in the presence of 0.25% (w/v) SDS, and 500  $\mu\text{L}$  of phenol:chloroform (1:1; v/v). The aqueous phase was collected after separation by centrifugation at  $16.000 \times g$  for 10 min, at 6 °C, and re-extracted by adding an equal volume of chloroform following by 5 seconds vortexing and centrifugation at  $16.000 \times g$  for 3 minutes, at 6 °C. The upper phase was recovered and the DNA was precipitated with 1/10 volumes of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ice cold 100% ethanol, during ca. 1-2h at -20 °C. After precipitation, DNA was collected by centrifugation at  $16.000 \times g$  for 15 minutes, at 6 °C, washed with ice cold 70% ethanol, dried and dissolved in sterile water.

### **2.3. Primer Design and Polymerase Chain Reactions (PCRs)**

Based on the genome sequence of *Nostoc* sp. strain PCC 7120, available on NCBI database, primers for the amplification of *hupW* and *hoxW* flanking regions were designed

(Table 1), in order to obtain flanking regions of either approximately 500 or 1000 base pairs. In the primers located closer to the target gene was inserted a 5' complementary sequence containing *Xma*I recognition site (Fig. 3A)

**Table 1.** Oligonucleotide primers used in this work.

Name	Sequence (5' → 3')	Reference
N.hupW5F-S	CGACTATGCTCATCGCAAAC	This work
N.hupW5R	<u>CCCGGGC</u> ATTCTAGAGCAGGTAGAACAGGTATTACTC	This work
N.hupW3F	TCTAGAATG <u>CCCGGG</u> ATGGGGATTGGGGATTAG	This work
N.hupW3R-S	TCAATGTAGCGGTATTGGTG	This work
N.hoxW5F-L	TCATGGTGCTACAATCCTAC	This work
N.hoxW5F-S	ATCGCCCAATTTTCGTAACC	This work
N.hoxW5R	<u>CCCGGGC</u> ATTCTAGAGGCTCTTGTGCAAATTTTCAG	This work
N.hoxW3F	TCTAGAATG <u>CCCGGG</u> TTTGAAGTTGGAGCAGTAG	This work
N.hoxW3R-S	AATCGCCGCAATTGAAGG	This work
N.hoxW3R-L	CGCATCACCAATTGATTCC	This work
Km.KmScFwd	CTGAC <u>CCCGGG</u> TGAATGTCAGCTACTGG	Pinto, F. J. V. (personal communication)
KmRev	CAA <u>ACCCGGG</u> CGATTACTTTTCGACCTC	Pinto, F. J. V. (personal communication)
KmScRev	ACAGAC <u>CCCGGG</u> CAAGCGGATGGCTGATG	Pinto, F. J. V. (personal communication)
Primer Pair	Application	Expected Product Size (bp)
N.hupW5F-S N.hupW5R	Amplification of the <i>hupW</i> 5' short flanking region	556
N.hupW3F N.hupW3R-S	Amplification of the <i>hupW</i> 3' short flanking region	571
N.hupW5F-S N.hupW3R-S	Amplification of the fused <i>hupW</i> short flanking regions in the overlap PCR	1112
N.hoxW5F-S N.hoxW5R	Amplification of the <i>hoxW</i> 5' shorter flanking region	589
N.hoxW3F N.hoxW3R-S	Amplification of the <i>hoxW</i> 3' shorter flanking region	577
N.hoxW5F-S N.hoxW3R-S	Amplification of the fused <i>hoxW</i> shorter flanking regions in the overlap PCR	1151
N.hoxW5F-L N.hoxW5R	Amplification of the <i>hoxW</i> 5' longer flanking region	1025
N.hoxW3F N.hoxW3R-L	Amplification of the <i>hoxW</i> 3' longer flanking region	1045
N.hoxW5F-L N.hoxW3R-L	Amplification of the fused <i>hoxW</i> longer flanking regions in the overlap PCR	2055
Km.KmScFwd KmRev	Amplification of the Km cassette	1381
Km.KmScFwd KmScRev	Amplification of the KmSc cassette	3317

Underlined sequence – *Xma*I recognition site.

The *hoxW* and *hupW* flanking regions were amplified by PCR in reactions of 50  $\mu$ L constituted by 10 ng of *Nostoc* sp. PCC 7120 genomic DNA, 0.2  $\mu$ M of each primer (see table 1), 250  $\mu$ M of dNTPs, 1X *Pfu* DNA Polymerase Buffer with  $MgSO_4$  (Promega) and 0.03 units/ $\mu$ L of *Pfu* DNA polymerase (Promega). The PCR profile used was: initial denaturation of 2 minutes at 95  $^{\circ}C$ ; 35 cycles of 95  $^{\circ}C$  for 30 seconds, 60  $^{\circ}C$  for 45 seconds, 72  $^{\circ}C$  for 1 minute and 30 seconds (shorter flanking regions) or 2 minutes and 30 seconds (longer flanking regions), followed by a final extension at 72  $^{\circ}C$  for 7 minutes. The products obtained were separated by horizontal gel electrophoresis in 1% (w/v) agarose gels using 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA), according to standard protocols (Sambrook and Russell, 2001). The fluorescent dye ethidium bromide was added to the gels and the stained DNA was directly visualized under UV light. The GeneRuler<sup>TM</sup> DNA Ladder Mix, 100-10000 bp (Fermentas) was used as molecular marker. The DNA from the specific bands was purified from the agarose gels using the illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare), according to the manufacturer's instructions.

The flanking regions of each target gene were joined together by overlap PCR (Wurch *et al.*, 1998), taking advantage of the complementary sequences inserted, that act as primers (Fig. 3B). These PCRs were done in reactions of 50  $\mu$ L constituted by 50-80 ng of each flanking region, 0.2  $\mu$ M of each primer (see table 1), 250  $\mu$ M of dNTPs, 0.2  $\mu$ g/ $\mu$ L of BSA, 1X *Pfu* DNA Polymerase Buffer with  $MgSO_4$  (Promega) and 0.03 units/ $\mu$ L of *Pfu* DNA polymerase (Promega). The PCR profile used was: initial denaturation of 2 minutes at 95  $^{\circ}C$ ; 35 cycles of 95  $^{\circ}C$  for 30 seconds, 60  $^{\circ}C$  for 30 seconds, 72  $^{\circ}C$  for 2 minutes and 30 seconds (overlap with the shorter flanking regions) or 4 minutes and 20 seconds (overlap with the longer flanking regions), followed by a final extension at 72  $^{\circ}C$  for 7 minutes. The products obtained were separated by horizontal gel electrophoresis and the DNA from the specific bands was purified from the agarose gels, using the same procedure as the used for the separation and purification of the *hupW* and *hoxW* flanking regions.

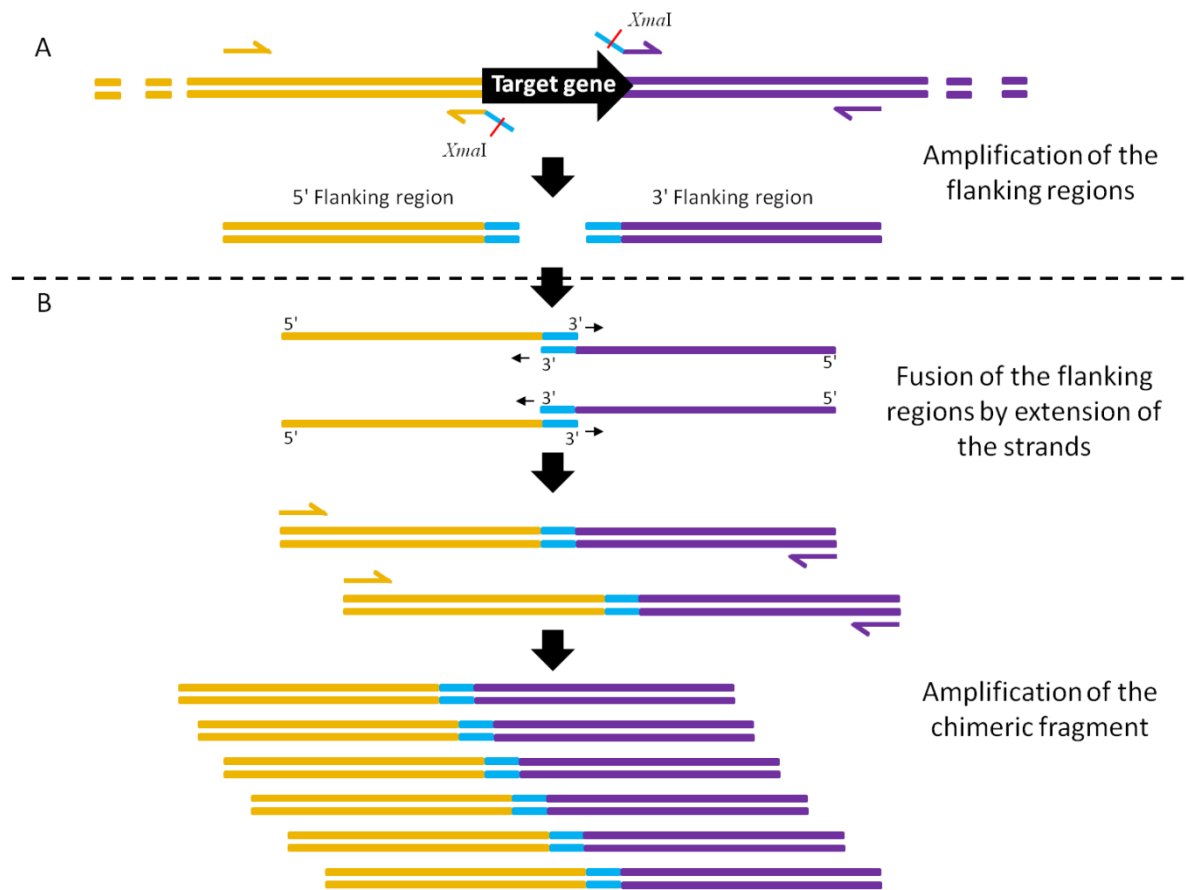


Fig. 3. Strategy used in this work for the creation of a chimeric fragment without the target gene. The first PCR step amplifies the two flanking regions of the target gene (A). The primers represented in gold and purple are 100% identical to the template (5' and 3' flanking regions, respectively) and the primers represented in gold-blue and purple-blue contain a complementary 5' sequence with a *XmaI* recognition site (represented in blue). The second step is an overlap PCR reaction (B), in which the complementary sequences inserted on the flanking regions act as primers and lead to the formation of a unique fragment by extension of the strands. This fragment is amplified with the primers identical to the template, previously used to amplify each flanking region. To simplify, the *XmaI* recognition site is only represented in the primers but it is present in all blue regions of the fragments obtained.

Selection cassettes, containing the *nptII* gene (encoding a neomycin phosphotransferase that confers resistance to neomycin and kanamycin) [Km cassette] and the *nptII* gene plus the *sacB* gene (encoding a levansucrase that confers sucrose sensitivity) [KmSc cassette] were amplified from the plasmid pK18mobsacB (NBRP (NIG, Japan):

*E. coli*), using primer pairs containing a *Xma*I recognition site at the 5' ends, for cloning purposes. These amplifications were done in reactions of 50  $\mu$ L constituted by 10 ng of pK18mobsacB, 0.2  $\mu$ M of each primer (see table 1), 250  $\mu$ M of dNTPs, 1.5 mM of MgCl<sub>2</sub>, 1X Colorless GoTaq® Flexi Buffer (Promega) and 0.025 units/  $\mu$ L of GoTaq® DNA Polymerase (Promega). The PCR profile used was: initial denaturation of 2 minutes at 95 °C; 35 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 3 minutes and 30 seconds, followed by a final extension at 72 °C for 7 minutes. The products obtained were separated by horizontal gel electrophoresis and the DNA from the specific bands was purified from the agarose gels, using the same procedure as the used for the separation and purification of the *hupW* and *hoxW* flanking regions

All PCRs described above were performed in the thermal cycler MyCycler™ (Bio-Rad Laboratories, Inc.).

## 2.4. Creation of A-tailed ends and cloning

The blunt ended DNA fragments generated in the overlap PCR reactions by *Pfu* DNA polymerase were modified with *Taq* DNA polymerase in order to introduce an A-tail at the 3' ends. These modifications were done in 50  $\mu$ L reactions constituted by 1-2  $\mu$ g of DNA, 200  $\mu$ M of dATP, 1.5 mM of MgCl<sub>2</sub>, 1X Colorless GoTaq® Flexi Buffer (Promega) and 0.025 units/  $\mu$ L of GoTaq® DNA Polymerase (Promega) and incubated at 72 °C for 20 minutes. The DNA products were purified from the enzymatic reactions using the illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare), according to the manufacturer's instructions.

The overlap A-tailed fragments and the selection cassettes were ligated into pGEM®-T Easy Vector (Promega) yielding **pNhupWSF** (*hupW* short flanking regions), **pNhoxWSF** (*hoxW* shorter flanking regions), **pNhoxWLF** (*hoxW* longer flanking regions), pKm and pKmSc. These ligation reactions were performed with T4 DNA ligase (Promega) according to the manufacturer's instructions and, subsequently, the enzyme was heat-inactivated.

## **2.5. Transformation of *Escherichia coli***

Competent cells of *Escherichia coli* DH5 $\alpha$  were transformed by the heat shock method. Aliquots of 100  $\mu$ L of competent cells were mixed with the DNA from the ligation reactions and incubated on ice for 30 minutes. After this incubation, the mixture competent cells-DNA was incubated at 42  $^{\circ}$ C in a water bath for 90 seconds and chilled again on ice for 2 minutes. The recovery of the cells was made by adding 890  $\mu$ L of LB broth liquid medium followed by incubation at 37  $^{\circ}$ C with orbital shaking of about 180 rpm for 1 hour. Finally, 100  $\mu$ L of cells were plated on Petri dishes containing LB-Agar medium supplemented either with 100  $\mu$ g/mL of ampicillin, 50  $\mu$ g/mL of X-Gal and 100  $\mu$ M of IPTG (cells transformed with pNhupWSF, pNhoxWSF and pNhoxWLF) or 100  $\mu$ g/mL of ampicillin and 50  $\mu$ g/mL of kanamycin (cells transformed with pKm and pKmSc) and incubated overnight at 37  $^{\circ}$ C.

## **2.6. Amplification and isolation of plasmid DNA**

Positive colonies resulting from the transformation procedure were used to inoculate LB broth liquid medium supplemented either with 100  $\mu$ g/mL of ampicillin (pNhupWSF, pNhoxWSF and pNhoxWLF transformants) or 100  $\mu$ g/mL of ampicillin and 50  $\mu$ g/mL of kanamycin (pKm and pKmSc transformants) and were incubated overnight at 37  $^{\circ}$ C with orbital shaking. The amplified plasmid DNA was isolated from the liquid cultures using the GenElute<sup>TM</sup> Plasmid Miniprep Kit (Sigma-Aldrich), according to the manufacturer's instructions.

## **2.7. Screening for positive clones by digestion of DNA and sequencing**

In order to screen for positive clones the plasmid DNA isolated from the liquid cultures was digested with *Eco*RI (Fermentas), according to the manufacturer's instructions and, subsequently, the enzyme was heat-inactivated. The products of the digestions were resolved by agarose gel electrophoresis as described in section 2.3.

The fidelity of the sequence of the positive clones was checked by sequencing. The sequencing reactions were performed with the M13 primers at STAB Vida (Lisboa, Portugal) and the plasmid DNA samples were sent according to the provider's instructions.

## **2.8. Insertion of the selection cassettes into pNhupWSF and pNhoxW(SF/LF)**

The selection cassettes conferring resistance to kanamycin and resistance to kanamycin plus sensitivity to sucrose were isolated from the pKm and from the pKmSc, respectively. Whereas the kanamycin resistance cassette was isolated by simple digestion of pKm with *XmaI* (Fermentas) the kanamycin resistance plus sucrose sensitivity cassette was isolated by digestion of pKmSc with *XmaI* (Fermentas) followed by DNA purification from the enzymatic reaction using illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare), and a second digestion with *ScaI* (Fermentas). Simultaneously to the isolation of the selection cassettes, the vectors pNhupWSF, pNhoxWSF and pNhoxWLF, whose sequencing results revealed the absence of errors in their sequences, were digested with *XmaI* (Fermentas). The products of the digestions were separated by agarose gel electrophoresis and the fragments corresponding to the selection cassettes and to the linearized vectors were recovered from the gel using illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare).

Both selection cassettes were ligated into both linearized vectors producing **pNhupWSFKm**, **pNhupWSFKmSc**, **pNhoxW(SF/LF)Km** and **pNhoxW(SF/LF)KmSc**. These ligation reactions were performed with T4 DNA ligase (Promega) according to the manufacturer's instructions and, subsequently, the enzyme was heat-inactivated.

Competent cells of *Escherichia coli* DH5 $\alpha$  were transformed with the products of the ligation reactions as described in section 2.5., and the cells were plated in LB-Agar medium supplemented with 100  $\mu$ g/mL of ampicilin and 50  $\mu$ g/mL of kanamycin.

Positive colonies resulting from the transformation procedure were used to inoculate LB broth liquid medium supplemented with 100  $\mu$ g/mL of ampicilin and 50  $\mu$ g/mL of kanamycin and were incubated overnight at 37 °C with orbital shaking. The amplified plasmid DNA was isolated from the liquid cultures as described in section 2.6.

The plasmid DNA isolated from the liquid cultures was digested with *NcoI* (Fermentas) in order to screen for positive clones as well as to determine the orientation of the selection cassettes. The products of the digestion reactions were resolved by agarose gel electrophoresis as described in section 2.3.

All the digestions and DNA purifications were done according to the manufacturer's instructions.

## **2.9. Protein extraction**

Cells of *Nostoc* sp. PCC 7120 and *Synechocystis* sp. PCC 6803 cultured in BG11 or BG11<sub>0</sub> were harvested at two different time points (middle of the light period [L8] and middle of the dark period [D4]). The protein extracts were prepared in lysis buffer [10 mM HEPES, 0.5% Triton X-100, 10mM EDTA, 2 mM DTT, pH 8.0, supplemented with protease inhibitor cocktail (Roche Diagnostics GmbH)] by sonication on ice (2x15 s, output 3, 50% duty cycle, in Branson Sonifier 250), and centrifuged at maximum speed for 10 minutes at 4 °C. The determination of the protein content was performed using BCA<sup>TM</sup> Protein Assay (Pierce), using bovine serum albumin (BSA) as standard.

## **2.10. Protein gel electrophoresis and Western blot**

The protein samples from *Nostoc* sp. PCC 7120 and *Synechocystis* sp. PCC 6803 (20 µg) were prepared (by adding 5 µL of 3x sample buffer [0.1875M Tris-HCl, pH 6.8, 6% (w/v) SDS, 30% (v/v) glycerol, 15% β-mercaptoethanol, 0.006% (w/v) bromophenol blue] plus distilled water to a final volume of 30 µL and incubated at 95 °C for 5 minutes) and then separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 3% (w/v) stacking gel and 12.5% (w/v) resolving gel. After separation, proteins were either stained with Coomassie Blue or transferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences), in a semi-dry system during 1 h at 0.8 mA/cm<sup>2</sup>, using 30 mM glycine, 48 mM Tris, 0.0375% (w/v) SDS, 20% (v/v) methanol as transfer buffer. The success of the transfer was checked by incubation of the membrane in Ponceau solution [0.2% (w/v) Ponceau S, 3% (v/v) trichloroacetic acid, 3% (v/v) sulfosalicylic acid] for a few seconds and

then washed with distilled water. After the transfer, the membrane was blocked for 2 h with 5% (w/v) milk powder in TPBS (PBS supplemented with 0.05% (v/v) Tween 20), with orbital shaking. The membrane was incubated overnight with polyclonal rabbit-anti-HoxH C-terminal antiserum at 1:1000 dilution in TPBS with 5% (w/v) milk powder. After two washes of 15 min with TPBS, the membrane was incubated for 1 h with goat-anti-rabbit IgG linked to horseradish peroxidase (Amersham Biosciences) at a 1:5000 dilution in TPBS with 5% (w/v) milk powder. The membrane was washed twice with TPBS for 15 min and twice with PBS for 15 min and the immunodetection was performed using ECL Western blotting detection reagents (Amersham Biosciences). The stripping of the membrane was done by sequential washes in four solutions of 5% (v/v) acetic acid plus 0.1% (v/v) Ponceau solution for 5 min in each, followed by a 5 min wash in TPBS, and then blocked for 2 h in 5% (w/v) milk powder in TPBS, with orbital shaking. Subsequently, the membrane was incubated overnight with polyclonal rabbit-anti-HoxH antiserum at a 1:2500 dilution in TPBS with 5% (w/v) milk powder. After washing twice with TPBS for 15 min, the membrane was incubated for 1 h with a 1:5000 dilution in TPBS with 5% (w/v) milk powder of goat-anti-rabbit IgG linked to horseradish peroxidase (Amersham Biosciences). After two washes with TPBS for 15 min followed by two washes with PBS for 15 min, the immunodetection was performed using ECL Western blotting detection reagents (Amersham Biosciences).



### 3. Results and Discussion

The last step in the maturation of the large subunit of [NiFe]-hydrogenases is a cleavage of the C-terminal peptide by a hydrogenase-specific endopeptidase. Although little is known about the biosynthesis/maturation of the cyanobacterial hydrogenases, the genes encoding the putative hydrogenase-specific endopeptidases (*hupW* and *hoxW*) were identified. However, until now the studies cannot clarify if, similarly to other organisms, these endopeptidases are indeed specific for each cyanobacterial hydrogenase. In *Synechocystis* sp. PCC 6803 the inactivation of the *hoxW* leads to the loss of the bidirectional hydrogenase activity (Hoffmann *et al.*, 2006). However, since this cyanobacterium harbours only the bidirectional enzyme, the disruption of these genes on strains containing both the uptake and the bidirectional hydrogenases, such as *Nostoc* sp. PCC 7120, are essential to evaluate the actual involvement and specificity of the HupW and HoxW endopeptidases on the maturation of the cyanobacterial hydrogenases.

In order to construct vectors to generate  $\Delta hupW$  and  $\Delta hoxW$  mutants of *Nostoc* sp. PCC 7120, the DNA of this strain was isolated and the flanking regions of each gene were amplified by PCR, in which the primers located closer to the target gene had 5' complementary sequences containing *XmaI* recognition site. As shown in fig. 4, these reactions were characterized by a high specificity, since just one amplicon was obtained with high yield, with exception for the amplification of the *hupW* 5' short flanking region. However, even in the latter case the yield achieved was enough to proceed to the subsequent steps.

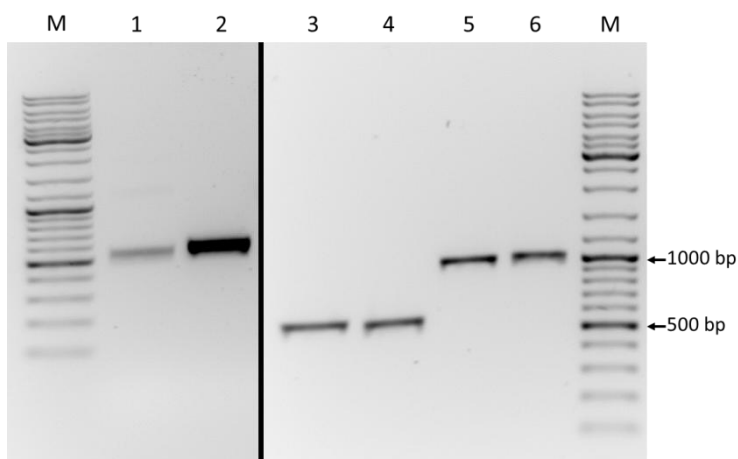


Fig. 4. PCR amplification of the *hupW* and *hoxW* flanking regions of *Nostoc* sp. PCC 7120. Lane 1: *hupW* 5' short flanking region; Lane 2: *hupW* 3' short flanking region; Lane 3: *hoxW* 5' shorter flanking region; Lane 4: *hoxW* 3' shorter flanking region; Lane 5: *hoxW* 5' longer flanking region; *hoxW* 3' longer flanking region. M - GeneRuler™ DNA Ladder Mix, 100-10000 bp (Fermentas).

In a second reaction the flanking regions of each gene were joined together by overlap PCR, producing a chimeric fragment with the same structure as the original of the genome, with the difference that instead of the target gene it is present the sequence containing the *Xma*I recognition site. This procedure has been used to successfully produce chimeric fragments required for several applications (Nagy *et al.*, 2006; Simionatto *et al.*, 2009; Zhang *et al.*, 2010), including the creation of integrative vectors to transform cyanobacteria (Nagarajan *et al.*, 2011) and, it is characterized to be an efficient and rapid method (Zhang *et al.*, 2010). In contrast to the amplification of the *hupW* and *hoxW* flanking regions, the fusion of the respective two flanks of each gene led to the formation of unspecific products with low efficiency (Fig. 5). This was probably due to the reduced length of the overlapping region used (only 15 bp), which might have paired unspecifically. Although overlapping sequences with similar lengths as the used in this work, and even less, have been used successfully in other works (Simionatto *et al.*, 2009; Zhang *et al.*, 2010), longer sequences improve the specificity and, thereby increase the yield of the specific product (Nagarajan *et al.*, 2011).

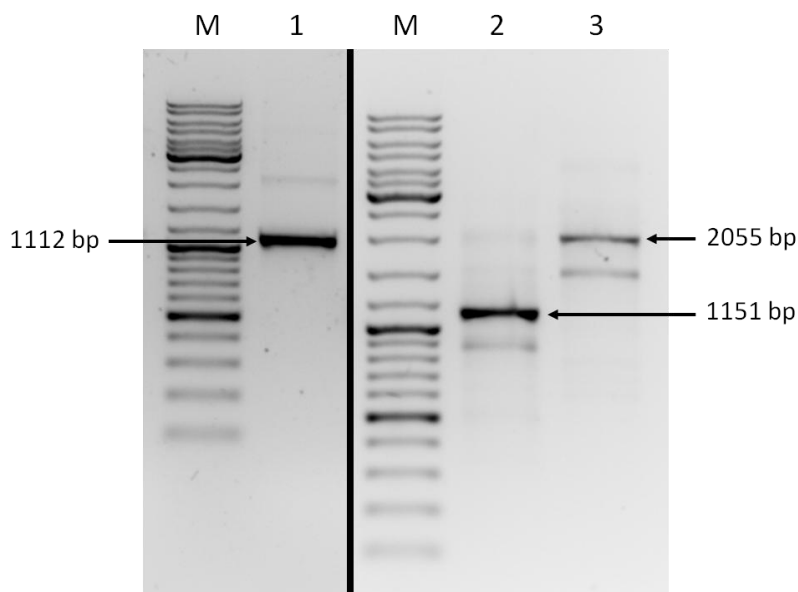


Fig. 5. Fusion of the respective *hupW* and *hoxW* flanking regions by overlap PCR. Lane 1: *hupW* short flanking regions (1112 bp); Lane 2: *hoxW* shorter flanking regions (1151 bp); Lane 3: *hoxW* longer flanking regions (2055 bp). M - GeneRuler™ DNA Ladder Mix, 100-10000 bp (Fermentas). The specific products are indicated by arrows.

The specific blunt ended DNA fragments generated in the overlap PCR reactions by *Pfu* DNA polymerase were purified and, modified with *Taq* DNA polymerase via terminal transferase activity, in order to introduce an A-tail at the 3' ends. The A-tailed fragments were then cloned into pGEM®-T Easy Vector (Promega) yielding the **pNhupWSF**, **pNhoxWSF** and **pNhoxWLF** (Fig. 6). The pGEM®-T Easy has been used in several works as a vector for carrying constructs to disrupt genes in *Nostoc* sp. PCC 7120 (Picossi *et al.*, 2005; Olmedo-Verd *et al.*, 2006; Flores *et al.*, 2007). Although in the method used all the polymerase chain

reactions were performed with *Pfu* DNA polymerase (Promega), an enzyme characterized by a low error rate and so, high fidelity (Cha and Thilly, 1993; Cline *et al.*, 1996), the sequencing results of the *hupW* construct revealed point mutations. One could think that the published genome sequence might be wrong. However, this construct was repeated for several times and every clone sequenced revealed mutations in different sites, so that hypothesis was excluded. Due to the insertion of mutations during the PCR the *hupW* construct was not done with the longer flanking regions and, for the amplification of the shorter flanking regions the primers were designed to amplify as little from coding sequence as possible, in order to avoid mutations or, at least improve the chances of being non significant. Despite this approach, the *hupW* construct was done in a subsequent round without mutations in both coding and non-coding regions.

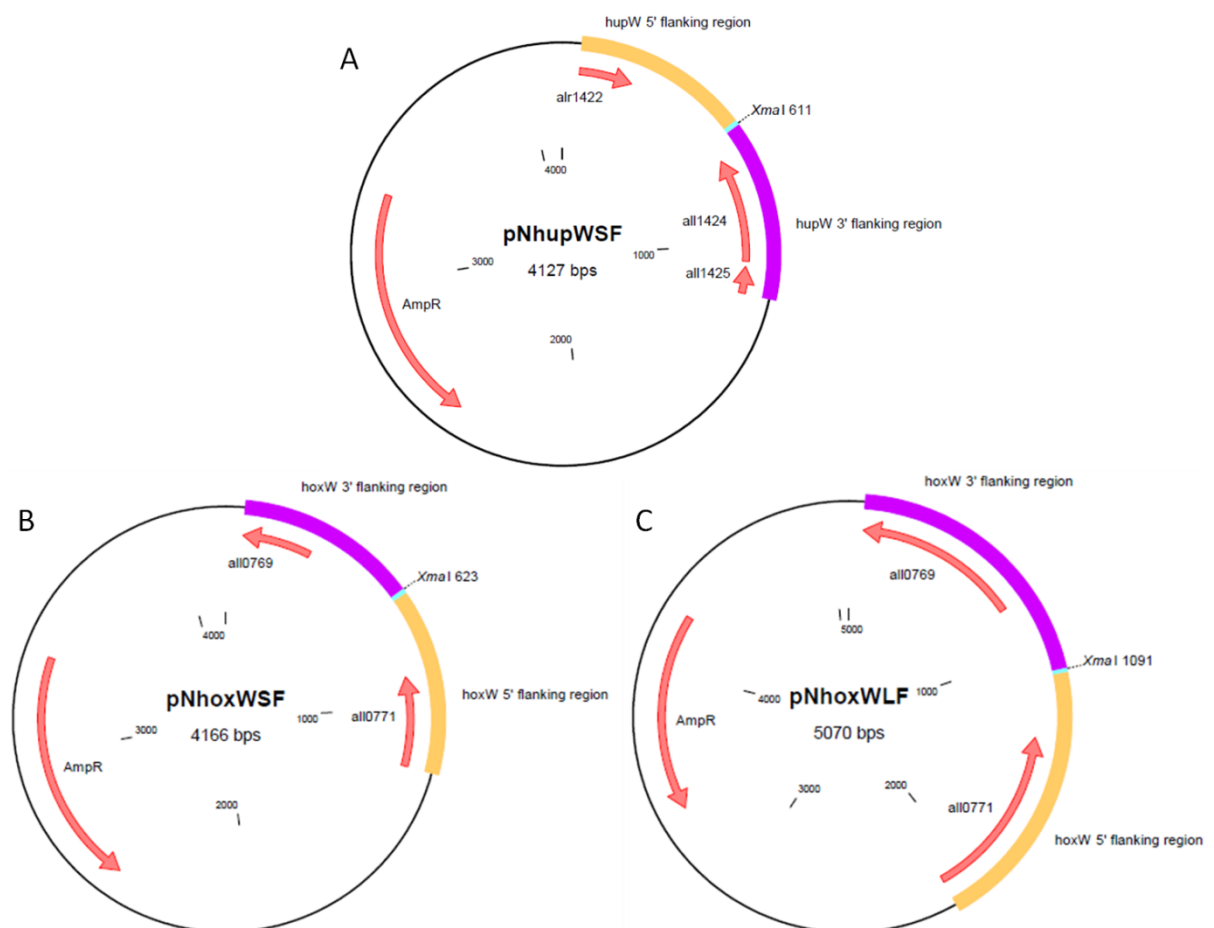


Fig. 6. Maps of the vectors containing the overlap-generated chimeric fragment constituted by the *hupW* short flanking regions – pNhupWSF (A); the *hoxW* shorter flanking regions – pNhoxWSF (B) and the *hoxW* longer flanking regions – pNhoxWLF (C).

Selection cassettes conferring resistance to kanamycin (containing the *nptII* gene) and resistance to kanamycin plus sucrose sensitivity (containing the *nptII* and the *sacB* genes), both compatible and widely used in cyanobacteria as selectable markers (Cai and Wolk, 1990; Vermaas, 1996; Hoffmann *et al.*, 2006; Paz-Yepes *et al.*, 2009), were amplified from the plasmid pK18mobsacB (NBRP (NIG, Japan): *E.coli*), using primer pairs containing a *XmaI* recognition site and cloned into pGEM®-T Easy, yielding pKm and pKmSc, respectively (data not shown). These cassettes were then recovered from pKm and pKmSc and cloned into the *XmaI* recognition site of the pNhupWSF, pNhoxWSF and pNhoxWLF, producing the **pNhupWSFKm**, **pNhupWSFKmSc**, **pNhoxW(SF/LF)Km** and **pNhoxW(SF/LF)KmSc**. As examples, in the fig. 7 is shown the maps of the pNhoxWLFKm and pNhoxWLFKmSc, all the other vectors constructed follow the same pattern.

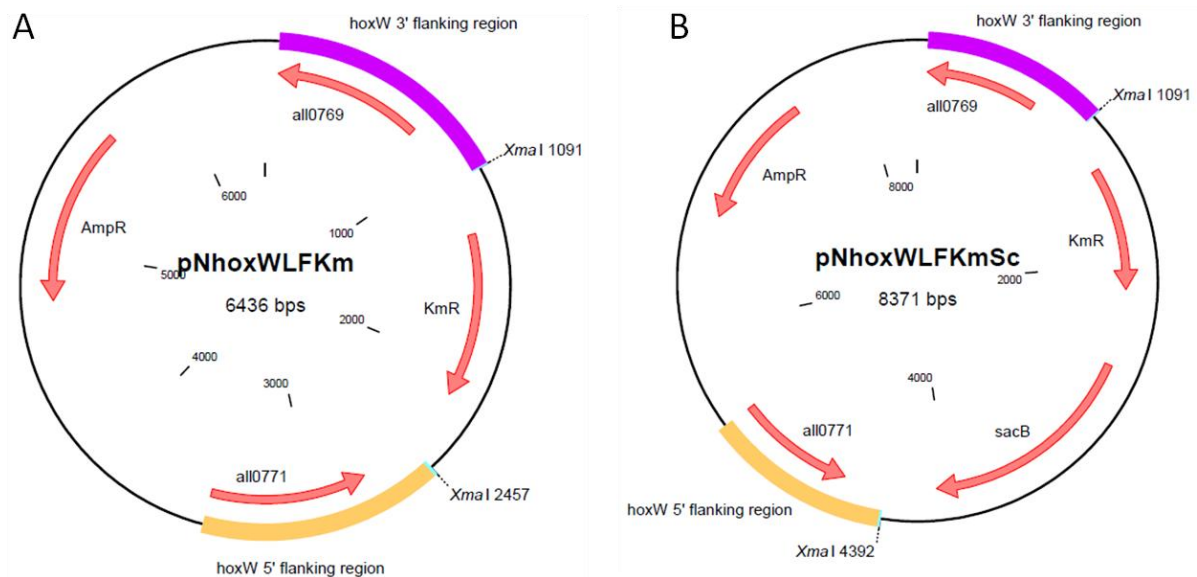


Fig. 7. Maps of the integrative vectors constructed with the longer flanking regions for the generation of  $\Delta$ *hoxW* mutants of *Nostoc* sp. strain PCC 7120. These vectors contain a cassette conferring resistance to kanamycin – pNhoxWLFKm (A) or resistance to kanamycin plus sensitivity to sucrose – pNhoxWLFKmSc (B) flanked by the flanking regions of the *hoxW* gene.

The vectors constructed contain a selection cassette flanked by the flanking regions of the respective target gene for which they are designed to knockout. Therefore, after being introduced into *Nostoc* sp. PCC 7120 the flanking regions are able to recombine with the native homologous sequence and lead to the replacement of the target gene by the selection cassette through double homologous recombination (Fig. 8). In this work the vectors were

constructed with flanking regions of different lengths (either approximately 500 or 1000 base pairs) and this will allow to determine the transformation efficiency for different lengths of homology, providing useful information for future transformations of *Nostoc* sp. PCC 7120. Since these vectors contain no cyanobacterial origin of replication, the colonies that after transformation grow on the selective medium would have integrated the construction in the genome.

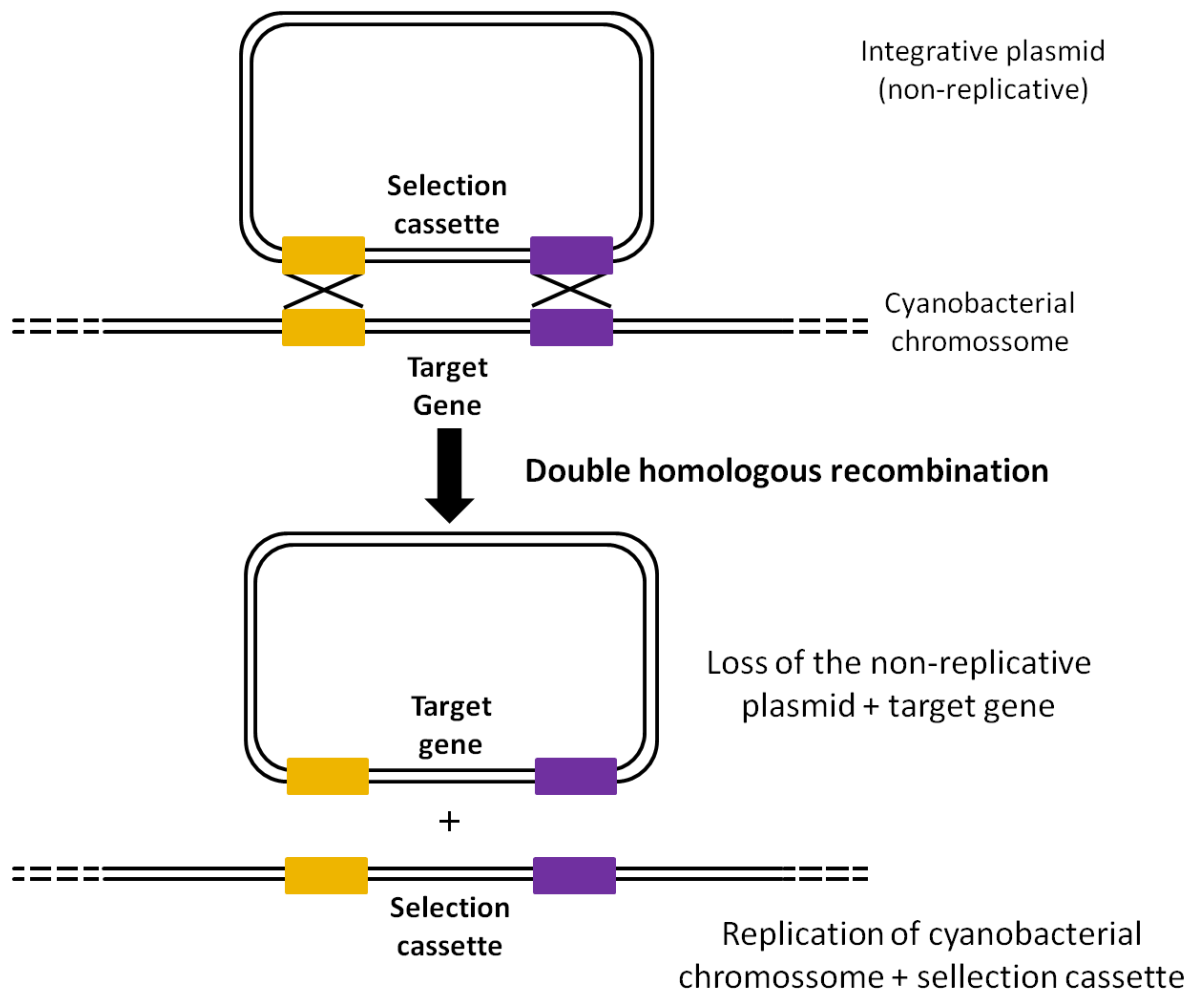


Fig. 8. Schematic representation of gene disruption through double homologous recombination in cyanobacteria. A non-replicative plasmid carries a selection cassette flanked by the flanking regions of the target gene (gold and purple rectangles). These regions recombine with the homologous sequences in the cyanobacterial chromosome leading to DNA exchange between the two molecules. The cyanobacterial chromosome loses the target gene and gains the selective cassette. The plasmid, with the target gene, is lost since it does not replicate in the cyanobacterium. Adapted from Vermaas (2004).

The most straightforward approach to construct vectors to produce deletion mutants through double homologous recombination is to amplify the target gene or a portion of it and to insert the selectable marker on the middle, at restriction sites that are already present natively. In fact, the majority of cyanobacteria deletion mutants are produced through this method (Masukawa *et al.*, 2002; Flores *et al.*, 2007; Paz-Yepes *et al.*, 2009). However, using this procedure the target gene is not removed but disrupted. In this work the vectors were designed to actually remove the target gene. Moreover, the use of the double marker cassette, providing resistance to kanamycin and sucrose sensitivity allows the generation of mutants without selectable markers in a two-steps procedure. In the first step the target gene is removed; the cells are transformed with the vectors containing the selection cassettes flanked by the flanking regions of the target gene (see the example given at fig. 7B) and the transformants are selected on medium containing kanamycin. In the second step the positive clones from the first step are transformed with the respective vector without the selection cassette (to follow the example given see fig. 6C) and the transformants that grow on medium containing sucrose would have lost the selection cassette. After the removal of the cassette it is possible to undergo to other cycle of transformations with other construct containing the same selectable markers, in order to create double mutants. Through this method it is virtually achievable the creation of strains containing an unlimited number of gene disruptions/replacements. This would not be possible if the plasmids were constructed by the manner described above since the selection cassette, once integrated, could not be removed from the genome and subsequent mutations in the same strain would be limited by the availability of other compatible selectable markers. Alternatively to the *sacB*, the *rps12* gene (conferring sensitivity to streptomycin) can also be used, in an identical manner, to produce cyanobacteria mutants free from markers (Matsuoka *et al.*, 2001).

One difficulty in the generation of cyanobacteria mutants arises from the fact that some strains are polyploid; i.e. possesses multiple copies of the chromosome (Binder and Chisholm, 1990; Schneider *et al.*, 2007; Griese *et al.*, 2011) and so, to obtain a desired strain, all the wild-type copies have to be replaced by the mutated ones. This segregation is achieved by culturing the transformants in successive increased concentrations of the selective agent. Therefore, the generation of cyanobacteria mutants is a slow process, especially when marker-free mutants are intended.

In order to study the effects of the deletions that will be produced by the plasmids constructed in this work, antibodies specific for the unprocessed and processed (C-terminal cleaved) form of the large subunit would be an important tool. In fact, a similar strategy has

been successfully applied in *E. coli* for the study of the C-terminal cleavage (Magalon and Böck, 2000a, Magalon and Böck, 2000b; Theodoratou *et al.*, 2000b). In this work the antibodies raised against the unprocessed and processed form of *Synechocystis* sp. 6803 bidirectional hydrogenase (kindly provided by Pin-Ching Maness, Biosciences Center, National Renewable Energy Laboratory, Colorado 80401 USA) were tested for *Nostoc* sp. strain PCC 7120, in order to evaluate the possibility to use them for the future characterization of the mutants by Western blot. For this purpose both strains were cultured for one week in BG11 (with nitrate) and BG110 (without nitrate) under 16 hours light ( $\sim 10 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) / 8 hours dark regimen at 25 °C with orbital shaking (80 rpm) and the samples were collected at two different time points (middle of the light period [L8] and middle of the dark period [D4]). As expected the antibodies anti-HoxH C-terminal and anti-HoxH are highly specific for the unprocessed and processed form of the hydrogenase large subunit of *Synechocystis* sp. 6803, respectively. However, the anti-HoxH can also detect the unprocessed form (Fig. 9A). Whereas the unprocessed form was more abundant in cells cultured in the presence of nitrate, the processed form was more abundant in cells cultured in dark and in the absence of nitrate (Fig. 9A), which is consistent with the data from hydrogenase activity measurements (Baebprasert *et al.*, 2010). Although these antibodies can effectively detect the unprocessed and processed form of the bidirectional hydrogenase large subunit of *Nostoc* sp. strain PCC 7120, there were clearly visible unspecific bands of about 25 kDa (Fig. 9B). Interestingly, the 25 kDa bands are half the size of the large subunits and follow the same pattern of expression; the meaning of this finding is at the moment unknown.

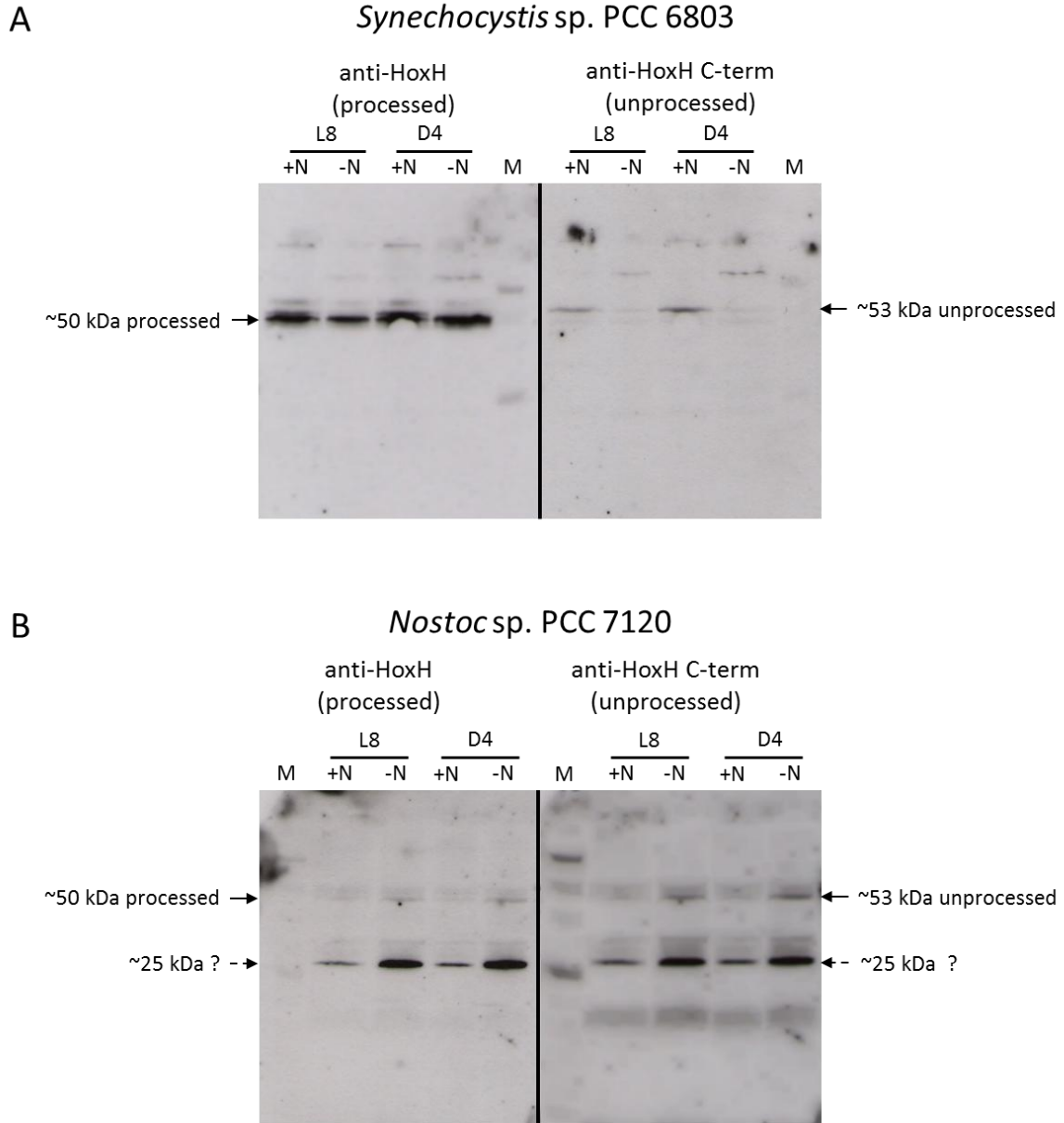


Fig. 9. SDS-PAGE/immunoblots of *Synechocystis* sp. PCC 6803 (A) and *Nostoc* sp. PCC 7120 (B) protein extracts. The cells were collected at distinct time points (L8 - 8h into the light period, D4 - 4h into the dark period; cycles of 16h light/8h dark) from BG11 (with nitrate; +N) or BG11<sub>0</sub> medium (without nitrate; -N). The membrane was incubated with polyclonal rabbit antiserum raised against the processed and unprocessed form of the large subunit of the bidirectional hydrogenase (HoxH) of *Synechocystis* sp. PCC 6803. M - marker: Precision Plus Protein Dual Color Standards, Biorad.

## 4. Conclusions

In this work a set of integrative vectors were constructed for the generation of  $\Delta hupW$  and/or  $\Delta hoxW$  mutants of *Nostoc* sp. strain PCC 7120. The deletion of these genes in *Nostoc* sp. strain PCC 7120, a strain harbouring both the uptake and bidirectional hydrogenase will allow to clarify the specificity of the endopeptidases HupW and HoxW for each hydrogenase.

Moreover, in order to provide tools for the future characterization of the mutants, the feasibility to study the last step in the maturation of the large subunits (C-terminal cleavage) by Western blot using specific antibodies was evaluated. The data obtained show that the antibodies raised against the unprocessed and processed form of *Synechocystis* sp. 6803 bidirectional hydrogenase are not ideal for *Nostoc* sp. strain PCC 7120. However, this strategy revealed to be viable and, therefore, antibodies specific for both the unprocessed and processed form of the uptake and bidirectional hydrogenase of the latter strain were designed and ordered.



## 5. Future Perspectives

In order to proceed with the study of the specificity of the endopeptidase-hydrogenase interaction, *Nostoc* sp. strain PCC 7120 will be transformed with the vectors constructed in this work, via triparental mating (Elhai and Wolk, 1988). In the mutants the arrest of the maturation of the hydrogenases large subunits is expected and will be confirmed by Western blot analyses using the antibodies developed specifically for this cyanobacterium. Additionally, the activity of each hydrogenase may also be evaluated by gas chromatography.

It should be taken in consideration that the studies addressing the *hyp* genes were also done in *Synechocystis* sp. 6803; therefore, it would be interesting to delete these genes in *Nostoc* sp. strain PCC 7120 in order to evaluate their involvement in the biosynthesis/maturation of both hydrogenases, particularly in the uptake enzyme.



## 6. References

**Agervald A, Stensjö K, Holmqvist M, Lindblad P** (2008) Transcription of the extended *hyp*-operon in *Nostoc* sp. strain PCC 7120. *BMC Microbiology*. **8**: 69.

**Atanassova A, Zamble DB** (2005) *Escherichia coli* HypA is a zinc metalloprotein with a weak affinity for nickel. *Journal of Bacteriology*. **187**: 4689-4697.

**Ayala-Castro C, Saini A, Outten FW** (2008) Fe-S cluster assembly pathways in bacteria. *Microbiology and Molecular Biology Reviews*. **72**: 110-125.

**Appel J, Phunpruch S, Steinmüller K** (2000) The bidirectional hydrogenase of *Synechocystis* sp. PCC 6803 works as an electron valve during photosynthesis. *Arch. Microbiol.* **173**: 333-338.

**Baebprasert W, Lindblad P, Incharoensakdi A** (2010) Response of H<sub>2</sub> production and Hox-hydrogenase activity to external factors in the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803. *International Journal of Hydrogen Energy*. **35**: 6611-6616.

**Berman-Frank I, Lundgren P, Falkowski P** (2003) Nitrogen fixation and photosynthetic oxygen evolution in cyanobacteria. *Research in Microbiology*. **154**: 157-164.

**Binder BJ, Chisholm SW** (1990) Relationship between DNA cycle and growth rate in *Synechococcus* sp. strain PCC 6301. *Journal of Bacteriology*. **172**: 2313-2319.

**Blokesch M, Albracht SPJ, Matzanke BF, Drapal NM, Jacobi A, Böck A** (2004a) The complex between hydrogenase-maturation proteins HypC and HypD is an intermediate in the supply of cyanide to the active site iron of [NiFe]-hydrogenases. *J. Mol. Biol.* **344**: 155-167.

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

**Blokesch M, Böck A** (2006) Properties of the [NiFe]-hydrogenase maturation protein HypD. *FEBS Letters*. **580**: 4065-4068.

**Blokesch M, Rohrmoser M, Rode S, Böck A** (2004b) HypF, a zinc-containing protein involved in NiFe hydrogenase maturation. *Journal of Bacteriology*. **186**: 2603- 2611.

**Böck A, King PW, Blokesch M, Posewitz MC** (2006) Maturation of hydrogenases. *Advances in Microbial Physiology*. **51**: 1-71.

**Bothe H, Schmitz O, Yates MG, Newton WE** (2010) Nitrogen fixation and hydrogen metabolism in cyanobacteria. *Microbiol. Mol. Biol. Rev.* **74**: 529-551.

**Bothe H, Tennigkeit J, Eisbrenner G** (1977) The utilization of molecular hydrogen by the blue-green alga *Anabaena cylindrica*. *Archives of Microbiology*. **114**: 43-49.

**Cai Y, Wolk P** (1990) Use of a conditionally lethal gene in *Anabaena* sp. strain PCC 7120 to select for double recombinants and to entrap insertion sequences. *Journal of Bacteriology*. **172**: 3138-3145.

**Carrieri D, Wawrousek K, Eckert C, Yu J, Maness P-C** (2011) The role of the bidirectional hydrogenase in cyanobacteria. *Bioresource Technology*. *In press*.

**Casalot L, Rousset M** (2001) Maturation of the [NiFe] hydrogenases. *Trends in Microbiology*. **9**: 228-237.

**Cha RS, Thilly WG** (1993) Specificity, efficiency, and fidelity of PCR. *PCR Methods Appl.* **3**: 18-29.

**Cline J, Braman JC, Hogrefe HH** (1996) PCR fidelity of *Pfu* DNA polymerase and other thermostable DNA polymerases. *Nucleic Acids Research*. **24**: 3546-3551.

**Drapal N, Böck A** (1998) Interaction of the hydrogenase accessory protein HypC with HycE, the large subunit of *Escherichia coli* hydrogenase 3 during enzyme maturation. *Biochemistry*. **37**: 2941-2948.

**Elhai J, Wolk CP** (1988) Conjugal transfer of DNA to cyanobacteria. *Methods Enzymol.* **167**: 747-754.

**Fay P** (1992) Oxygen Relations of Nitrogen Fixation in Cyanobacteria. *Microbiological Reviews.* **56**: 340-373.

**Flores E, Pernil R, Muro-Pastor AM, Mariscal V, Maldener I, Lechno-Yossef S, Fan Q, Wolk CP, Herrero A** (2007) Septum-localized protein required for filament integrity and diazotrophy in the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120. *Journal of Bacteriology.* **189**: 3884-3890.

**Forzi L, Sawers RG** (2007) Maturation of [NiFe]-hydrogenases in *Escherichia coli*. *Biometals.* **20**: 565-578.

**Frazzon J, Dean DR** (2003) Formation of iron-sulfur clusters in bacteria: an emerging field in bioinorganic chemistry. *Curr. Opin. Chem. Biol.* **7**: 166-173.

**Gasper R, Scrima A, Wittinghofer A** (2006) Structural insights into HypB, a GTP-binding protein that regulates metal binding. *The Journal of Biological Chemistry.* **281**: 27492-27502.

**Griese M, Lange C, Soppa J** (2011) Ploidy in cyanobacteria. *FEMS Microbiology Letters.* **323**: 124-131.

**Hansel A, Axelsson R, Lindberg P, Troshina OY, Wünschiers R, Lindblad P** (2001) Cloning and characterisation of a *hyp* gene cluster in the filamentous cyanobacterium *Nostoc* sp. strain PCC 73102. *FEMS Microbiol. Lett.* **201**: 59-64.

**Higuchi Y, Yagi T, Yasuoka N** (1997) Unusual ligand structure in Ni-Fe active center and an additional Mg site in hydrogenase revealed by high resolution X-ray structure analysis. *Structure.* **5**: 1671-1680.

**Hoffmann D, Gutekunst K, Klissenbauer M, Schulz-Friedrich R, Appel J** (2006) Mutagenesis of hydrogenase accessory genes of *Synechocystis* sp. PCC 6803 - Additional

homologues of *hypA* and *hypB* are not active in hydrogenase maturation. FEBS Journal. **273**: 4516-4527.

**Johnson DC, Dean DR, Smith AD, Johnson MK** (2005) Structure, function, and formation of biological iron-sulfur clusters. Annu. Rev. Biochem. **74**: 247-281.

**Leach MR, Sandal S, Sun H, Zamble DB** (2005) Metal binding activity of the *Escherichia coli* hydrogenase maturation factor HypB. Biochemistry. **44**: 12229-12238.

**Ludwig M, Schulz-Friedrich R, Appel J** (2006) Occurrence of hydrogenases in cyanobacteria and anoxygenic photosynthetic bacteria: Implications for the phylogenetic origin of cyanobacterial and algal hydrogenases. J. Mol. Evol. **63**: 758-768.

**Magalon A, Böck A** (2000a) Analysis of the HypC-HycE complex, a key intermediate in the assembly of the metal center of the *Escherichia coli* hydrogenase 3. The Journal of Biological Chemistry. **275**: 21114-21120.

**Magalon A, Böck A** (2000b) Dissection of the maturation of the [NiFe] hydrogenase 3 from *Escherichia coli* taking place after nickel incorporation. FEBS Letters. **473**: 254-258.

**Maier T, Böck A** (1996) Generation of active [NiFe] hydrogenase *in vitro* from nickel-free precursor form. Biochemistry. **35**: 10089-10093.

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

**Matsuoka M, Takahama K, Ogawa T** (2001) Gene replacement in cyanobacteria mediated by a dominant streptomycin-sensitive *rps12* gene that allows selection of mutants free from drug resistance markers. Microbiology. **147**: 2077-2087.

**Maier T, Lottspeich F, Böck A** (1995) GTP hydrolysis by HypB is essential for nickel insertion into hydrogenases of *Escherichia coli*. Eur. J. Biochem. **230**: 133-138.

**McIntosh CL, Germer F, Schulz R, Appel J, Jones AK** (2011) The [NiFe]-hydrogenase of the cyanobacterium *Synechocystis* sp. PCC 6803 works bidirectionally with a bias to H<sub>2</sub> production. Journal of the American Chemical Society. *In press*.

**Menon NK, Chatelus CY, Dervartanian M, Wendt JC, Shanmugam KT, Peck Jr HD, Przybyla AE** (1994) Cloning, sequencing, and mutational analysis of the *hyb* operon encoding *Escherichia coli* hydrogenase 2. Journal of Bacteriology. **176**: 4416-4423.

**Menon NK, Robbins J, Wendt JC, Shanmugam KT, Przybyla AE** (1991) Mutational analysis and characterization of *Escherichia coli* *hya* operon, which encodes [NiFe] hydrogenase 1. Journal of Bacteriology. **173**: 4851-4861.

**Nagarajan A, Winter R, Eaton-Rye J, Burnap R** (2011) A synthetic DNA and fusion PCR approach to the ectopic expression of high levels of the D1 protein of photosystem II in *Synechocystis* sp. PCC 6803. Journal of Photochemistry and Photobiology B: Biology. **104**: 212-219.

**Nagy ZB, Varga-Orvos Z, Szakál B, Tamás L, Puskás LG** (2006) Assembling and cloning genes for fusion proteins using reverse transcription one-step overlap extension PCR method. Analytical Biochemistry. **351**: 311-313.

**Olmedo-Verd E, Muro-Pastor AM, Flores E, Herrero A** (2006) Localized induction of the *ntcA* regulatory gene in developing heterocysts of *Anabaena* sp. strain 7120. Journal of Bacteriology. **188**: 6694-6699.

**Olson JW, Mehta NS, Maier RJ** (2001) Requirement of nickel metabolism proteins HypA and HypB for full activity of both hydrogenase and urease in *Helicobacter pylori*. Molecular Microbiology. **39**: 176-182.

**Paschos A, Bauer A, Zimmermann A, Zehelein E, Böck A** (2002) HypF, a carbamoyl phosphate-converting enzyme involved in [NiFe] hydrogenase maturation. The Journal of Biological Chemistry. **277**: 49945-49951.

**Paschos A, Glass RS, Böck A** (2001) Carbamoylphosphate requirement for synthesis of the active center of [NiFe]-hydrogenases. *FEBS Letters*. **488**: 9-12.

**Paz-Yepes J, Flores E, Herrero A** (2009) Expression and mutational analysis of the *glnB* genomic region in the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120. *Journal of Bacteriology*. **191**: 2353-2361.

**Pickett CJ, Vincent KA, Ibrahim SK, Gormal CA, Smith BE, Fairhurst SA, Best SP** (2004) Synergic binding of carbon monoxide and cyanide to the FeMo cofactor of nitrogenase: relic chemistry of an ancient enzyme? *Chem. Eur. J.* **10**: 4770-4776.

**Picossi S, Montesinos ML, Pernil R, Lichtlé C, Herrero A, Flores E** (2005) ABC-type neutral amino acid permease N-I is required for optimal diazotrophic growth and is repressed in the heterocysts of *Anabaena* sp. strain PCC 7120. *Molecular Microbiology*. **57**: 1582-1592.

**Reissmann S, Hochleitner E, Wang H, Paschos A, Lottspeich F, Glass RS, Böck A** (2003) Taming of a poison: biosynthesis of the NiFe-hydrogenase cyanide ligands. *Science*. **299**: 1067-1070.

**Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY** (1979) Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol.* **111**:1-61.

**Roseboom W, Blokesch M, Böck A, Albracht SPJ** (2005) The biosynthetic routes for carbon monoxide and cyanide in the Ni-Fe active site of hydrogenases are different. *FEBS Letters*. **579**: 469-472.

**Rossmann R, Maier T, Lottspeich F, Böck A** (1995) Characterisation of a protease from *Escherichia coli* involved in hydrogenase maturation. *Eur. J. Biochem.* **227**: 545-550.

**Sambrook J., Russell D.W.** 2001. *Molecular Cloning: A Laboratory Manual*, 3<sup>rd</sup> ed. Cold Spring Harbor Laboratory Press, New York, USA.

**Schirrmeister BE, Antonelli A, Bagheri HC** (2011) The origin of multicellularity in cyanobacteria. *BMC Evolutionary Biology*. **11**: 45.

**Schmitz O, Boison G, Salzmann H, Bothe H, Schütz K, Wang S-H, Happe T** (2002) HoxE – a subunit specific for the pentameric bidirectional hydrogenase complex (HoxEFUYH) of cyanobacteria. *Biochimica et Biophysica Acta*. **1554**: 66-74.

**Schneider D, Fuhrmann E, Scholz I, Hess WR, Graumann PL** (2007) Fluorescence staining of live cyanobacterial cells suggest non-stringent chromosome segregation and absence of a connection between cytoplasmic and thylakoid membranes. *BMC Cell Biology*. **8**: 39.

**Schopf JW** (2000) The fossil record: tracing the roots of the cyanobacterial lineage, p. 13-35. *In* The Ecology of Cyanobacteria. Edited by Whitton BA and Potts M. Kluwer Academic Publishers, The Netherlands.

**Schütz K, Happe T, Troshina O, Lindblad P, Leitão E, Oliveira P, Tamagnini P** (2004) Cyanobacterial H<sub>2</sub> production – a comparative analysis. *Planta*. **218**: 350-359.

**Simionatto S, Marchioro SB, Galli V, Luerce TD, Hartwig DD, Moreira ÂN, Dellagostin** (2009) Efficient site-directed mutagenesis using an overlap extension-PCR method for expressing *Mycoplasma hyopneumoniae* genes in *Escherichia coli*. *Journal of Microbiological Methods*. **79**: 101-105.

**Steunou AS, Jensen SI, Brecht E, Becraft ED, Bateson MM, Kilian O, Bhaya D, Ward DM, Peters JW, Grossman AR & Kühl M** (2008) Regulation of nif gene expression and the energetics of N<sub>2</sub> fixation over the diel cycle in a hot spring microbial mat. *ISME J*. **2**: 364-378.

**Tamagnini P, Axelsson R, Lindberg P, Oxelfelt F, Wünschiers R, Lindblad P** (2002) Hydrogenases and Hydrogen Metabolism of Cyanobacteria. *Microbiology and Molecular Biology Reviews*. **66**: 1-20.

**Tamagnini P, Leitão E, Oliveira P, Ferreira D, Pinto F, Harris DJ, Heidorn T, Lindblad P** (2007) Cyanobacterial hydrogenases: diversity, regulation and applications. *FEMS Microbiol. Rev.* **31**: 692-720.

**Tamagnini P, Troshina O, Oxelfelt F, Salema R, Lindblad P** (1997) Hydrogenases in *Nostoc* sp. Strain PCC 73102, a Strain Lacking a Bidirectional Enzyme. *Applied and Environmental Microbiology.* **63**: 1801-1807.

**Theodoratou E, Huber R, Böck A** (2005) [NiFe]-hydrogenase maturation endopeptidase: structure and function. *Biochem. Soc. Trans.* **33**: 108-111.

**Theodoratou E, Paschos A, Magalon A, Fritsche E, Huber R, Böck A** (2000a) Nickel serves as a substrate recognition motif for the endopeptidase involved in hydrogenase maturation. *Eur. J. Biochem.* **267**: 1995-1999.

**Theodoratou E, Paschos A, Mintz-Weber S, Böck A** (2000b) Analysis of the cleavage site specificity of the endopeptidase involved in the maturation of the large subunit of hydrogenase 3 from *Escherichia coli*. *Arch Microbiol.* **173**: 110-116.

**Troshina O, Serebryakova L, Sheremetieva M, Lindblad P** (2002) Production of H<sub>2</sub> by the unicellular cyanobacterium *Gloeocapsa alpicola* CALU 743 during fermentation. *International Journal of Hydrogen Energy.* **27**: 1283-1289.

**Troshina OY, Serebryakova LT, Lindblad P** (1996) Induction of H<sub>2</sub>-uptake and nitrogenase activities in the cyanobacterium *Anabaena variabilis* ATCC 29413: effects of hydrogen and organic substrate. *Current Microbiology.* **33**: 11-15.

**Vermaas W** (1996) Molecular genetics of the cyanobacterium *Synechocystis* sp. PCC 6803: principles and possible biotechnology applications. *Journal of Applied Phycology.* **8**: 263-273.

**Vermaas WFJ** (2004) Targeted genetic modification of cyanobacteria: new biotechnological applications, p. 457-470. *In Handbook of Microalgal Culture: Biotechnology and Applied Phycology.* Edited by Amos Richmond. Blackwell Science.

**Vignais PM, Colbeau A** (2004) Molecular biology of microbial hydrogenases. *Curr. Issues Mol. Biol.* **6**: 159-188.

**Volbeda A, Garcin E, Piras, C, Lacey AL, Fernandez VM, Hatchikian EC, Frey M, Fontecilla-Camps JC** (1996) Structure of the [NiFe] hydrogenase active site: evidence for biologically uncommon Fe ligands. *Journal of American Chemical Society.* **118**: 12989-12996.

**Wells MA, Mercer J, Mott RA, Pereira-Medrano AG, Burja AM, Radianingtyas H, Wright PC** (2011) Engineering a non-native hydrogen production pathway into *Escherichia coli* via a cyanobacterial [NiFe] hydrogenase. *Metab. Eng. In press.*

**Whitton BA, Potts M** (2000) Introduction to the Cyanobacteria, p. 1-11. *In The Ecology of Cyanobacteria.* Edited by Whitton BA and Potts M. Kluwer Academic Publishers, The Netherlands.

**Wolk CP, Ernst A, Elhai J** (1994) Heterocyst metabolism and development, p. 769-823. *In The Molecular Biology of Cyanobacteria.* Edited by Bryant DA. Kluwer Academic Publishers, The Netherlands.

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

**Wurch T, Lestienne F, Pauwels PJ** (1998) A modified overlap extension PCR method to create chimeric genes in the absence of restriction enzymes. *Biotechnology Techniques.* **12**: 653-657.

**Yu J, Eckert C, Wawrousek K, Pennington G, Maness P-C** (2010) The construction of a recombinant system for solar hydrogen production in *Synechocystis* sp. PCC 6803. The 9<sup>th</sup> International Hydrogenase Conference. Book of Abstracts. Page 7.

**Zhang A-L, Zhang L, Zhang L-Z, Chen H, Lan X-Y, Zhang C-L, Zhang C-F** (2010) An efficient and rapid method for gene cloning from eukaryotic genomic DNA using overlap-

PCR: with an example of cattle *Ghrelin* gene. Biochemical and Biophysical Research Communications. **391**: 1490-1493.

**Zhang JW, Butland G, Greenblatt JF, Emili A, Zamble DB** (2005) A role for SlyD in the *Escherichia coli* hydrogenase biosynthetic pathway. The Journal of Biological Chemistry. **280**: 4360-4366.

