Metabolic versatility of [NiFe]-hydrogenases in *Thiocapsa roseopersicina*

PhD Thesis

Judit Maróti

Supervisors:
Prof. Kornél L. Kovács
Dr. Rákhely Gábor

Institute of Biophysics, Biological Research Centre,
Hungarian Academy of Science
Department of Biotechnology, University of Szeged

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Abbreviations

BLAST: Basic Local Alignment Search Tool
BV: benzyl-viologen
CTAB: N-cetyl-N,N,N-trimethyl-ammonium bromide
DNA: deoxyribonucleic acid
DNSA: dinitrosalicylic acid
DTT: dithiothreitol
EDTA: ethylenediaminetetraacetic acid disodium dihydrate
EtBr: ethidium bromide
FMN: flavin mononucleotide
FNR: Fumarate Nitrate Reductase
H-D exchange: Hydrogen-Deuterium exchange
MBH: membran bound hydrogenase
mRNA: messenger ribonucleic acid
MTT: membran targeting and translocation
MV: methyl-viologen
NADH: nicotinamide adenine dinucleotide (reduced form)
NADPH: nicotinamide adenine dinucleotide phosphate (reduced form)
OD: optical density
ORF: open reading frame
PCR: polymerase chain reaction
qPCR: qantitative polymerase chain reaction
RH: regulatory hydrogenase
RNA: ribonucleic acid
RT-PCR: reverse transcription-coupled polymerase chain reaction
SDS: sodium dodecyl sulfate
SH: soluble hydrogenase
TAT: twin-arginine translocation
TCD: thermal conductivity detector

Introduction

In the global context of oil depletion, sustainability of energy supply is a major current issue. The world's energy demand is expected to grow rapidly in the next decades, and the reserves of fossil fuels are not sufficient to supply this growing need. Novel safe energy carriers have to be introduced. The most plausible energy source is the Sun, efficient conversion of sunlight has to be solved in the foreseeable future. It is widely accepted that an efficient way for harnessing sunlight could significantly help in solving the world's energy problems for the long run. Many microbes are able to carry out reactions leading to the accumulation of high-energy content compounds, which could be used as alternative energy vectors. The largest commonly available resource for making bioenergy is the cellulosic biomass derived from plants. This energy source is ultimately fixed light energy, sunlight being converted to chemical energy by plants, and accumulated in several organs. Similarly, the presently used fossil fuels represent converted sunlight as well. The main differences are the duration of accumulation (millions of years in the case of fossil fuels) and the rate of accumulation and utilization of these energy carriers. One of the possibilities to overcome the efficacy and price barriers of sunlight harvesting is the usage of microorganisms. Biomass can be converted into bioethanol, biogas or biohydrogen by thermochemical or by biological processes on which a growing interest has developed during the last decades. Even more direct ways exist for biological sunlight conversion, hydrogen production by direct and indirect photolysis is possible using photosynthetic microbes. Direct light energy capture process wires electrons - coming from light energy - to special enzymes, which produce biohydrogen. Indeed, some microorganisms possess features which would confer the potential for their exploitation in mass biohydrogen production. These features have to be adequately controlled, fine-tuned and in certain cases modified for the desired purposes. The related features and functions are embodied by multiple enzymes and metabolic pathways in the microorganisms, such as the photosynthetic system, respiration complexes, hydrogenase and nitrogenase enzymes.

1. Scientific background

1.1. Hydrogen metabolism of the cells

Microbial hydrogen production and consumption implicate hydrogenase enzymes as biocatalysts for the reversible oxidation of molecular hydrogen. Most of the organisms - capable of metabolizing hydrogen - are prokaryotes belonging to the Bacteria and Archea domains of life. However, hydrogenases had also been identified in the subcellular organelles of eukaryotes (Vignais et al., 2001, Horner et al., 2002). Hydrogenases catalyse either proton reduction or hydrogen oxidation *in vivo* in many different metabolic contexts. H_2 is consumed as a source of energy and reductant during chemolithoautotrophic growth and is produced as a fermentation product during anaerobic, heterotrophic growth. Many anoxygenic phototrophs can utilize H_2 as an electron donor for photoautotrophic growth. Various functions are often associated with different cellular localisations of hydrogenase enzymes. H_2 evolution is often cytoplasmic, whereas H_2 uptake is usually periplasmic and membrane-associated (Vignais et al., 2001).

Nitrogen fixation is one of the main processes of biogenic H_2 production. It is unique for the reason that it does not involve a specialized H_2-forming enzyme, hydrogenase, but based on the function of nitrogenase complex. Many oxygenic and anoxygenic phototrophs fix dinitrogen and produce H_2 concomitantly. By recycling the hydrogen produced by the nitrogenase, uptake hydrogenases save energy which otherwise would be lost by the cell.

1.2. Hydrogenases

It is now generally accepted that the presently known hydrogenases belong to three groups of independent phylogenetic origin. These groups are defined on the basis of their metal content: [NiFe]-hydrogenases, [FeFe]-hydrogenases and [Fe]-hydrogenases (formerly referred to as `metal-free hydrogenases`); each characterised by a distinctive

functional core that is conserved within each class (Vignais et al., 2001, 2007). The core consists of the subunits minimally required for structure and function.

1.2.1. [Fe]-hydrogenases

Certain methanogens harbour [Fe]-hydrogenases, also called Hmd, since these homodimeric enzymes function as H_2 forming methenyltetrahydromethanopterin dehydrogenases. Unlike the classical iron hydrogenases, Hmd enzymes harbour cofactor-bound, redox inactive iron but no iron sulphur clusters (Lyon et al., 2004). Associated with a specific cofactor, they have catalytic properties different from [NiFe]- and [FeFe]-hydrogenases; they do not catalyze the reversible reaction: $2H^+ + 2e^- \Leftrightarrow H_2$.

1.2.2. [FeFe]-hydrogenases

[FeFe]-hydrogenases are found in anaerobic, primarily H_2-evolving organisms, including fermentative bacteria, sulphate reducers and known as the only type of hydrogenase found in eukaryotes. They are characterised by extreme high oxygen sensitivity, high turnover rate and low affinity for the substrate hydrogen (Adams et al., 1990, Vignais et al., 2001, Horner et al., 2002). [FeFe]-hydrogenases are quite heterogeneous in structure and domain organisation. Mono-, di-, tri- and tetrameric enzymes have also been described (Vignais et al., 2001). The metallo-catalytic cluster of [FeFe]-hydrogenases, the so-called H-cluster, consists of a [4Fe-4S] cube linked through a protein cysteine residue to a 2Fe subcluster (Nicolet et al., 1999). The iron atoms of the [4Fe-4S] center are coordinated to the protein structure by three additional conserved cysteine residues, which are found in motifs L1-L3. Non-protein ligands, CN^- and CO, are attached to both iron atoms. Additional domains, which differ remarkably in size and cofactor content, are often present in this class of hydrogenases.

1.2.3. [NiFe]-hydrogenases

1.2.3.1. Structure

The biggest and best studied class of hydrogenases are [NiFe]-hydrogenases. The basic module of a catalytically active [NiFe]-hydrogenase consists of an αβ heterodimer with an approximately 60 kDa large subunit (α-subunit) hosting the binuclear NiFe active site and a small subunit (β-subunit) of ca. 30 kDa hosting the Fe-S clusters. The three dimensional structures of [NiFe]-hydrogenases from *Desulfovibrio gigas* (Volbeda et al., 1995, Volbeda et al., 1996) and *Desulfovibrio vulgaris* Miyazaki F (Higuchi et al., 1997) *Desulfovibrio fructosovorans* (Montet et al., 1997) *Desulfovibrio desulfuricans* (Matias et al., 2001) and the [NiFe(Se)] *Desulfomicrobium baculatum* (formerly *Desulfovibrio baculatus*; Garcin et al., 1999) have been studied. The crystal structure of *D. gigas* (Figure 1.) showed that the two subunits interact very extensively through a large contact surface and form a globular heterodimer. The [NiFe] active site is coordinated by an N- and a C-terminal pair of cysteines. Two of the thiolate groups (provided by Cys68 and Cys533) form a bridge between the two metals (Volbeda et al., 1995). The Ni ligating cysteine is occupied by a selenocysteine in the [NiFe(Se)] hydrogenase of *D. baculatum* (Garcin et al., 1999). Infrared spectroscopic studies revealed the presence of three non protein ligands, 1 CO and 2 CN^- bound to the Fe atom (Volbeda et al., 1996; Happe et al., 1997). In *D. vulgaris* hydrogenase, one of the three nonprotein ligands was modelled as SO (Higuchi et al., 1997). Two extra CN moieties: one bound to the Fe and one bound to the Ni atom, exist at the active site of the soluble hydrogenase (SH) of *Ralstonia eutropha* (Burgdorf et al., 2005/A). This special architecture may account for the O_2 and CO insensitivity of this particular hydrogenase.

The small subunit contains up to three linearly arranged cubic Fe-S clusters, which conduct electrons between the H_2-activating center and the (physiological) electron acceptor/donor of hydrogenase. The N-terminal part of the small electron-transfer subunit contains the binding site for the proximal [4Fe-4S]. The proximal [4Fe-4S] cluster could directly exchange electrons with the catalytic site, they are both buried deep in the protein (Volbeda et al., 1995). The type of interaction with a specific redox partner can often be deduced from the structural features of the hydrogenase small subunit.

A few [NiFe]-hydrogenases, including the NAD-reducing multimeric hydrogenases and the *E. coli* type-3 hydrogenases, contain a minimal version of a functional hydrogenase module consisting of a [NiFe] active site in the large subunit and one proximal [4Fe-4S] cluster in the small subunit. It was demonstrated with *R. eutropha* mutants that this subform meets the minimal requirements for being catalytically active (Massanz et al., 1998).

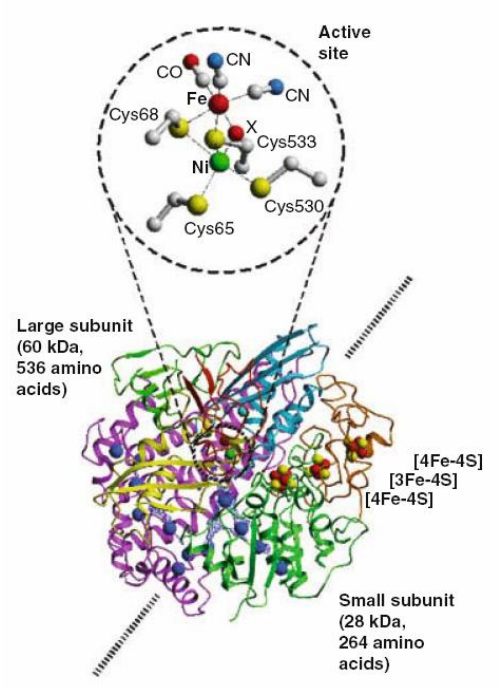

Figure 1. Three-dimensional structure of the *Desulfovibrio gigas* [NiFe] hydrogenase. The large subunit harbours the active site. The small subunit coordinates three iron-sulphur clusters (red: iron; yellow: sulphur). Cysteine residues which participate in coordinating the active-site metals are labelled. The structure of the active site is shown as a blow-up.

1.2.3.2. Classification

Sequence comparison of the large subunits of [NiFe]-hydrogenases revealed two highly conserved regions surrounding the two pairs of cysteine ligands of the [NiFe] center, near the N- and C-termini of the sequence. The pattern, inferred from multiple sequence alignments of two specific motifs, L1 and L2, provided the basis of the recent classification of [NiFe]-hydrogenases (Vignais et al., 2001).

Group 1. Uptake [NiFe]-hydrogenases

Membrane bound uptake hydrogenases link the oxidation of hydrogen to the reduction of anaerobic electron acceptors, such as NO_3^-, SO_4^{2-}, fumarate or CO_2 (anaerobic respiration) or to O_2 (aerobic respiration), with the recovery of energy in the form of protonmotive force (Vignais et al.,2004). Membrane-bound, periplasmically oriented [NiFe] hydrogenases was found in many proteobacteria and characterized by a highly conserved (approx. 50 amino acid) segment at the C-terminus of the small subunit (Figure 2.). This hydrophobic region is essential for binding the hydrogenase to the membrane and coupling the electron flow to the quinone pool of the respiratory chain. A membrane-integrated cytochrome *b* has been isolated as a complex with the heterodimeric [NiFe]-hydrogenase from *Wolinella succinogenes* (Dross et al., 1992; Dross et al., 1993). This cytochrome, designated as HydC, binds two heme groups. Analysis of site-directed mutants revealed that substitution of three histidine residues (His25, His67 and His186) in HydC, which are predicted to be heme B ligands, abolished quinone reactivity of the *W. succinogenes* hydrogenase, while benzylviologen reduction was retained. A similar phenotype was observed by mutating two conserved histidine residues in the small hydrogenase subunit HydA, one is located in the membrane-integrated C-terminal helix of HydA (His 305) and the other is supposed to be involved in the ligation of the distal [4Fe-4S] cluster. The data convincingly show that these components are necessary for electron transport from H_2 to either fumarate or polysulfide, and for quinone reactivity (Gross et al, 1998). Moreover, using cytochrome *b* deficient mutants of *R. eutropha*, it was demonstrated that the *b*-type cytochrome is bifunctional. In addition to its electron-transfer function, it anchors the hydrogenase to the membrane (Bernhard et al., 1997). To the contrary, disruption of HupC in *Pseudomonas hydrogenovora* did not alter the location of the HupSL dimer (Ohtsuki et al., 1997). The homologous b-type cytochrome in *Rhodobacter capsulatus* was shown to be required for the stabilization of HupSL (Cauvin et al., 1991).

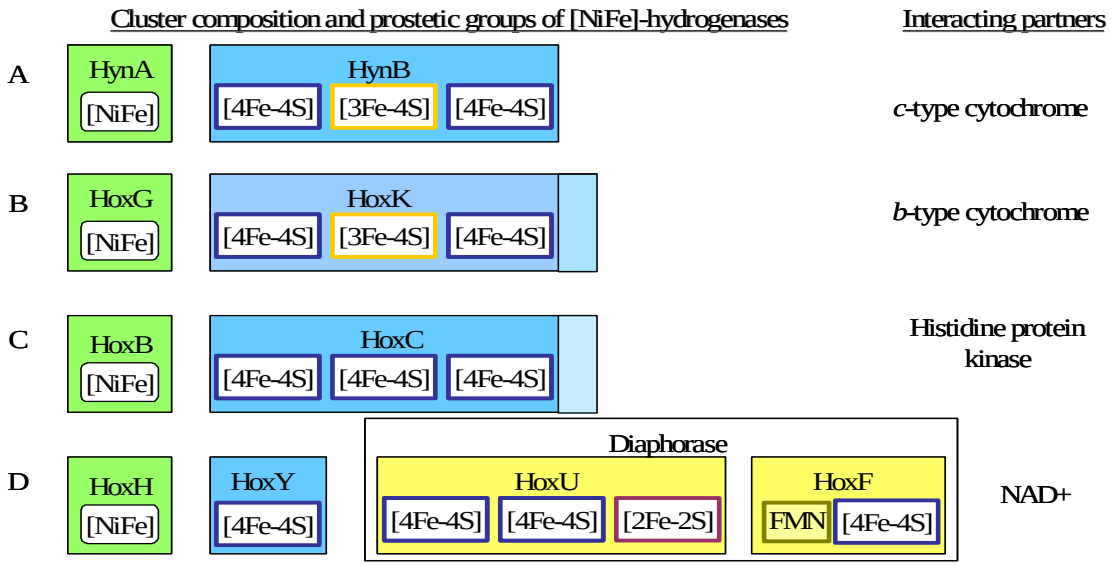

Figure 2. Domain organization of selected [NiFe] hydrogenases. (A) *D. gigas* [NiFe] hydrogenase. (B) *R. eutropha* membrane-bound hydrogenase (MBH). Hatched area symbolizes the characteristic C-terminal region of the small subunit. (C) *R. eutropha* regulatory hydrogenase (RH). Hatched area symbolizes the characteristic C-terminal region of the small subunit. (D) *R. eutropha* soluble hydrogenase (SH). The diaphorase moiety is boxed.

Another type of uptake hydrogenases is represented by the periplasmic hydrogenase of *Desulfovibrio* species (Figure 2.). It is able to interact with low-potential *c*-type cytochromes and a transmembrane redox protein complex encoded by the *hmc* operon (Rossi et al., 1993). It also participates in the creation of proton gradient across the membrane for energy conservation. The Hmc complex was proposed to transfer electrons from the periplasmic hydrogen oxidation to the cytoplasmic sulphate reduction (Rossi et al., 1993). Two subunits of the *E. coli* hydrogenase-2 encoded by the *hybOABCDEFG* operon (Sargent et al., 1998/B) slightly resemble the subunits of the Hmc complex.

Characteristic feature of the uptake hydrogenases is the presence of a special signal peptide (30-50 amino acid residues) at the N-terminus of their small subunit. The signal peptide contains a conserved RRxFxK motif recognised by a specific protein translocation pathway designated as membrane targeting and translocation (Mtt) or twin-

arginine translocation (Tat) (Rodrigue et al., 1999, Sargent et al., 1998/A, Wu et al., 2000) pathway.

Group 2. Cytoplasmic H_2 sensors and cyanobacterial uptake [NiFe]-hydrogenases

One of the main features distinguishing these hydrogenases from the members of group 1 is the missing signal peptide at the N-terminus of the small subunit, since these enzymes are not exported but reside in the cytoplasm.

The H_2-sensing regulatory [NiFe] hydrogenases, HupUV/HoxBC, had been identified in *Bradyrhizobium japonicum* (Black et al., 1994), *Rhodobacter capsulatus* (Elsen et al., 1996), *Ralstonia eutropha* (Lenz et al., 1997) and *Rhodopseudomonas palustris* (Rey et al., 2006). They function as hydrogen sensors in the regulatory cascade which controls the biosynthesis of some uptake hydrogenases in response to H_2.

Regulatory hydrogenases also contain a C-terminal region of approx. 50 amino acids in the small subunit (Figure 2.). Although, the sequence of this peptide is highly conserved within this group of proteins, it is completely distinct from the C-terminal small subunit region of membrane-bound [NiFe] hydrogenases (Group 1.). This observation points to a specific role of the C-terminal extension in partner recognition and hence signal transduction. In fact, the formation of a tight complex between the H_2-sensing hydrogenase of *R. eutropha* and its cognate signal-transmitting histidine protein kinase has been demonstrated *in vitro* using purified components (Bernhard et al., 2001). Unlike the standard energy-converting [NiFe] hydrogenases, which are isolated as simple heterodimers ($\alpha\beta$), the H_2-sensing hydrogenase of *R. eutropha* forms a tetramer consisting of two dimeric species ($\alpha_2\beta_2$) (Bernhard et al., 2001).

In contrast to the majority of hydrogenases, regulatory hydrogenases are insensitive towards oxygen (Vignais et al., 1997, Buhrke et al., 2004), similarly to the soluble hydrogenase (SH) of *R. eutropha* (Van der Linden et al., 2004, Burgdorf et al., 2005/A). Oxygen tolerant hydrogenases are in the focus of biotechnological interest, since oxygen insensitivity is one of the main requirements for hydrogenase application in *in vitro* systems. Unfortunately, the regulatory hydrogenases have extreme low activity.

Although cyanobacterial uptake hydrogenases (HupSL) fulfil the same function as other uptake hydrogenases of group 1, they appear to be more closely related to the H_2 sensing

hydrogenases of group 2. In the absence of the N-terminal signal peptide of the small subunit, these enzymes are localised on the cytoplasmic side of either the cytoplasmic or thylakoid membrane and induced under nitrogen fixing conditions (Tamagnini 2002).

Group 3. Bidirectional, heteromultimeric cytoplasmic [NiFe]-hydrogenases

Heteromultimeric [NiFe] hydrogenases generally reside in the cytoplasm or are bound to the inner surface of the cytoplasmic membrane. A typical feature of these enzymes is a tight association of the hydrogenase module with a second redox-active moiety that binds coenzymes such as F_{420} (8-hydroxy-5-deazaflavin), NAD^+ or $NADP^+$ which are reversibly reduced by H_2. Group 3 comprehends F_{420}-reducing hydrogenases of methanogenic archaea and F_{420}-nonreducing hydrogenases of *Methanothermobacter* and *Methanococcus* strains (Vignais et al., 2001). A group of tetrameric NADP-reactive [NiFe]-hydrogenases has been found in the hyperthermophilic archaeal species, such as *Pyrococcus furiosus* (Ma et al., 1993, Ma et al., 2000) and *Thermococcus litoralis* (Rákhely et al., 1999). The HydA and HydD subunits of this type of enzyme constitute the hydrogenase module, whereas the HydB and HydG subunits form the flavin-containing NADP-reactive moiety of the protein. It is remarkable that this group of [NiFe]-hydrogenases possess both H_2-oxidizing and S^0-reducing activities *in vitro*. During fermentation, the enzyme accepts both polysulfides and protons as electron acceptors and is therefore designated as sulfhydrogenase (Ma et al., 1993). Cytoplasmic, NAD^+-reducing hydrogenases were identified in *R. eutropha* (Schneider et al., 1976), *Rhodococcus opacus* (Grzeszik et al., 1997) and in the methanotroph *Methylococcus capsulatus* (Hanczár et al., 2002). These enzymes consist of four distinct subunits. The hydrogenase module contains a truncated form of the small electron-transferring subunit with only one Fe-S cluster as prosthetic group (Figure 2). This moiety is associated with a heterodimeric iron flavoprotein, the so-called diaphorase module. The diaphorase consists of a large (approx. 65-kDa) polypeptide, HoxF, and a small (approx. 25-kDa) subunit, HoxU. The diaphorase moiety accommodates three to four iron-sulphur clusters and one flavin mononucleotide (Figure 2.). In *R. eutropha* the latter was shown to contribute to the stabilization of the enzyme (Massanz 1998). It should also be noted, that soluble hydrogenase (SH) of *R. eutropha* was originally described as tetrameric

enzyme, consisting of HoxFUYH, and could be activated only by NADH. The hexameric form of the enzyme has recently been isolated and shown to be activated also by NADPH (Burgdorf et al., 2005/B). The two additional HoxI subunits may provide a binding site for NADPH. Likewise, the regulatory hydrogenases (RH) of *R. eutropha* and *R. capsulatus* and the soluble hydrogenase of *R. eutropha* were also described as O_2 insensitive enzyme (Burgdorf et al., 2005/A).

Homologous enzymes were discovered in cyanobacteria (Appel et al., 2000, Tamagnini et al., 2002) and in *Allochromatium vinosum* (Long et al., 2007). In these cases the above mentioned tetrameric structure is supplemented with a HoxE subunit. Sequence alignments revealed a close relationship between the HoxEFU subunits and three peripheral subunits of bacterial and mitochondrial NADH ubiquinone oxidoreductases (Friedrich et al., 2000).

Group 4. H_2-evolving, energy-conserving, membrane-associated [NiFe]-hydrogenases

The H_2-evolving [NiFe]-hydrogenases expressed under strictly anoxic conditions are constituents of multicomponent membrane-bound enzyme complexes which share the ability to couple the oxidation of carbonyl groups derived from formate, acetate or carbon monoxide to the reduction of protons yielding H_2. The most extensively studied example is the formate-hydrogenlyase complex in *E. coli* comprising eight *hyc* gene products. The hydrogenase module of the so-called hydrogenase-3 consists of the active-site-containing subunit: HycE and the small subunit: HycG, which harbours only the proximal [4Fe-4S] cluster. Both the formate dehydrogenase moiety of the complex as well as the two hydrogenase subunits are attached to the inner side of the cytoplasmic membrane via intrinsic membrane proteins, which serve as membrane anchors and electron mediators between the two redox proteins (Böhm et al., 1990; Sauter et al., 1992). So far, it has not been possible to isolate the entire hydrogenase-3 complex. The CO-induced [NiFe]-hydrogenase of *Rhodospirillum rubrum* is a constituent of the CO-oxidizing enzyme system which permits the organism to grow on carbon monoxide as the sole energy source in the dark . Like *E. coli* hydrogenase-3, the CooLH hydrogenase complex of *R. rubrum* is rather labile, and hence biochemical data are limited. Multisubunit [NiFe]-hydrogenase complexes of the *E. coli* type-3 are also present in archaea such as

Methanothermobacter species (Tersteegen and Hedderich, 1999), in *Methanosarcina barkeri* strain Fusaro (Künkel et al., 1998), *Thermococcus litoralis* (Takács et al., 2008) and in *Carboxidothermus hydrogenoformans* (Soboh et al., 2002).

1.2.3.3. [NiFe]-hydrogenases of *Ralstonia eutropha* H16

The β-proteobacterium *R. eurtopha* strain H16 is a hydrogen oxidizing bacterium harbouring three distinct [NiFe]-hydrogenases that each serves unique physiological roles: a membrane-bound hydrogenase (MBH), a cytoplasmic soluble NAD^+ reducing hydrogenase (SH) and a regulatory hydrogenase (RH). Special emphasis on these hydrogenases in this chapter is sound since they are all characteristic and well studied representatives of the above mentioned [NiFe]-hydrogenase groups and considered as homologous of *T. roseopersicina* hydrogenase enzymes. The domain organisation of *R. eutropha* hydrogenases are shown on Figure 2. The strain utilizes hydrogen for its chemolitoautotrophic growth via the hydrogenases. Both MBH and SH are able to oxidize H_2 and transfer electrons to the quinone pool (MBH) and NAD^+ (SH) (Bernhard et al., 1997. Massanz et al., 1998, Burgdorf et al., 2005/B). According to the physiological requirements, they have higher hydrogen uptake than evolution activities.

1.2.3.4. Regulation of [NiFe]-hydrogenases

The control of hydrogenase synthesis represents a means to quick and efficient respond to changes in the environment an in particular to new energy demands. Transcriptional control involves usually one or several two-component regulatory systems which may act either positively or neagatively.

Hydrogen-specific regulatory cascade controls the transcription of uptake hydrogenase genes (*hupSL* in *R. capsulatus* and *B. japonicum* and *hoxKG* in *R. eutropha*) (Elsen et al., 1996, Black et al., 1994, Lenz et al., 1997). It comprises a hydrogen sensor (Regulatory Hydrogenase, RH) encoded by the *hupUV/hoxBC* genes in connection with a two-component regulatory system – consists of a protein histidine kinase HupT/HoxJ and a response regulator HupR/HoxA.

Oxygen can affect hydrogenase gene expression through various mechanisms. The ArcA/ArcB (aerobic respiratory control) two-component system controls the transcription of hydrogenase-1 and -2 in the absence of oxygen in *E. coli* (Iuchi and Lin, 1993). Functionally similar two-component systems, RegA(PrrA)/RegB(PrrB), are involved in the redox regulation of hydrogenases in *R. capsulatus* (Elsen et al., 2000). The fumarate and nitrate reduction regulator (FNR) was shown to be involved in the expression regulation of the *hyp* genes in *E. coli*. In *B. japonicum* the *hupSL* hydrogenase is regulated by microoxic conditions through the FixLJ and $FixK_2$ proteins (Durmowicz and Maier, 1998).

The hydrogenase operon of *R. leguminosarum* is upregulated under nitrogen fixing conditions by the NifA protein (Brito et al., 1997). Nitrate, acting via the NarX/NarL and NarQ/NarP systems, represses the synthesis of the *hya* (hydrogenase-1) and *hyb* (hydrogenase-2) hydrogenases of *E coli* (Richard et al., 1999). The availability of metals, found in the active centre of hydrogenases can also have a regulatory affect (Axelsson and Lindblad 2002). The circadian clock controlled expression of *hoxEF* and *hoxUYH* operons of *Synecococcus* sp. PCC 7942 was also described (Schmitz et al., 2001).

1.2.3.5. Biosynthesis of [NiFe]-hydrogenases

The subunits, coded by the structural genes, have to undergo different modification processes mediated by accessory proteins. Most of our knowledge on this biosynthesis process is based on the studies of the maturation of the hydrogenase-3 (HycGE) of *E. coli* (Maier et al., 1996; Magalon et al.,2001; Magalon and Böck., 2000/B) and of the [NiFe]-hydrogenases of *R. eutropha* (Jones et al., 2004; Winter et al., 2005; Schubert et al., 2007). Although, the principles and general sequence of molecular events are similar in each [NiFe]-hydrogenase, there are distinct features in the maturation process among various organisms. In the *E. coli* hydrogenase-3 (HycGE) model, the liganded Fe atom is transferred to the immature large subunit (pre-HycE) with the contribution of HypC, HypD, HypE and HypF proteins. During these processes, there is a complex between the HypC and the precursor form of the large subunit (Magalon and Böck, 2000/A), which terminates only after the Ni incorporation into pre-HycE mediated by the HypB-HypA-

SlyD complex. As the last step of the maturation of the large subunit, the C-terminal extension is cleaved off, and the large subunit gets the final conformation containing the perfectly assembled active centre. After the independent maturation processes of the small and large subunits, the subunit oligomerization is the last step of the entire hydrogenase biosynthesis process. Finally, hydrogenases have to find their intracellular location, the soluble enzymes accumulate in the cytoplasm, while the periplasmic enzymes have to be transported through the cytoplasmic membrane.

1.3. Thiocapsa roseopersicina BBS

1.3.1. General features

Our model organism, *Thiocapsa roseopersicina* BBS is a purple sulphur photosynthetic γ-proteobacterium belonging to the Chromatiaceae family (The Prokaryotes 3rd Springer 2006). It was isolated from cold see water of the North Sea. The 1.2-3 μm diameter sphere cells are nonmotile. The optimal growth temperature is 24-28 ^{o}C, and growth is inhibited over 30 ^{o}C (Bogorov 1974). The wild-type strain needs 4-5 days for the growth in liquid culture, and about 10 days for forming visible colonies on plates. *T. roseopersicina* is a phototrophic bacterium can not cleave water and photosynthesis takes place under anoxygenic conditions. Since anoxygenic photosynthetic bacteria absorb light at longer wavelengths than cyanobacteria, yielding less energy for photosynthesis, they need electron donors like reduced sulphur, organic compounds or molecular hydrogen (Sasikala et al., 1993). They do not produce oxygen; the photosynthetic final product is usually elemental sulphur or sulphate. Besides photosynthetic growth, aerobic chemolithoautotrophic metabolism allows *T. roseopersicina* to grow in the dark (Kondratieva et al., 1976). The nitrogenase enzyme complex makes the organism capable of nitrogen fixation, which process is accompanied by H_2 production (Bogorov 1974). Hydrogen metabolism of the cells is based on its [NiFe]-hydrogenases and nitrogenase(s).

1.3.2. Hydrogenases of *T. roseopersicina* BBS

Thiocapsa roseopersicina BBS is a representative of the group of microorganisms in which multiplicity of hydrogenases had been identified. These all belong to the group of [NiFe]-hydrogenases, but show different characteristics in localisation, *in vivo* function and subunit composition of the *in vivo* active enzyme. Besides the hydrogenase small and large subunits, additional hydrogenase related proteins have been identified (Kovács et al., 2002, 2005). These participate in posttranslational maturation of the hydrogenase polypeptides (Fodor et al.,2001; Maróti et al., 2003; Maróti et al., 2010), transcriptional regulation (Kovács et al., 2005/A; Kovács et al., 2005/B) or function as electron-transfer subunits (Rákhely et al., 2004; Palágyi-Mészáros et al., 2009).

Two membrane-bound hydrogenases have been identified in *T. roseopersicina* which belong to the group of uptake [NiFe]-hydrogenases (Group 1.) (Vignais et al., 2001). The deduced amino acid sequences of the two small (*hupS* and *hynS* (formerly *hydS*)) and large subunit (*hupL* and *hynL* (formerly *hydL*)) sequences share 46 and 58% identity respectively. The *hup* and *hyn* genes differ in the arrangement of accessory genes (Colbeau et al., 1994, Rákhely et al., 1998,). Both HynS and HupS, the small subunits, possess tat-signal sequence, they are likely transported through the membrane by the Tat secretion system (Sargent et al., 1999) and anchored to the membrane on the periplasmic side. They show distinct stability and biochemical properties (Kovács & Bagyinka 1990).

1.3.2.1. Hyn hydrogenase

Hyn hydrogenase (formerly Hyd) is a bidirectional enzyme. Outstanding stability under extreme conditions brings this enzyme into the focus of biotechnological interest. It is resistant to oxygen inactivation and remains active after removal from the membrane (Kovács et al., 1988; Kovács & Bagyinka 1990). HynSL is more active at 80 °C than at the growth temperature of the host organism (25-28 °C). The *hynS* and *hynL* genes, encoding for the hydrogenase small and large subunits, are separated by a 1,979 bp intergenic region (IS) (Figure 3.) (Rákhely et al., 1998). In this section, two *orf*s, *isp1* and *isp2*, have been recognised which show significant homology to ORF5 and ORF6 of

D. vulgaris hmc operon (Rákhely et al., 1998). Isp1 is considered as a transmembrane redox protein, while Isp2 is a cytoplasmic enzyme resembling the heterodisulphide reductases (Rákhely 1998). Recent study has demonstrated that these genes code for functional proteins, Isp1 and Isp2, which are both required for the *in vivo* functional HynSL hydrogenase, but neither for its *in vitro* activity or expression (Palágyi-Mészáros et al., 2009). The Isp proteins are likely to play an electron transfer role to/from the HynSL hydrogenase and may connect this enzyme to sulphur metabolism. Since Isp1 is adverted as a membrane-integrated protein it was presumed to anchor the HynSL dimer to the membrane. Unexpectedly, experimental results failed to confirm this assumption (Palágyi-Mészáros et al., 2009). The *hynS-isp1-isp2-hynL* genes are transcribed together (Rákhely et al., 1998) and regulated by oxygen through the global redox regulator FnrT (Kovács et al., 2005/A).

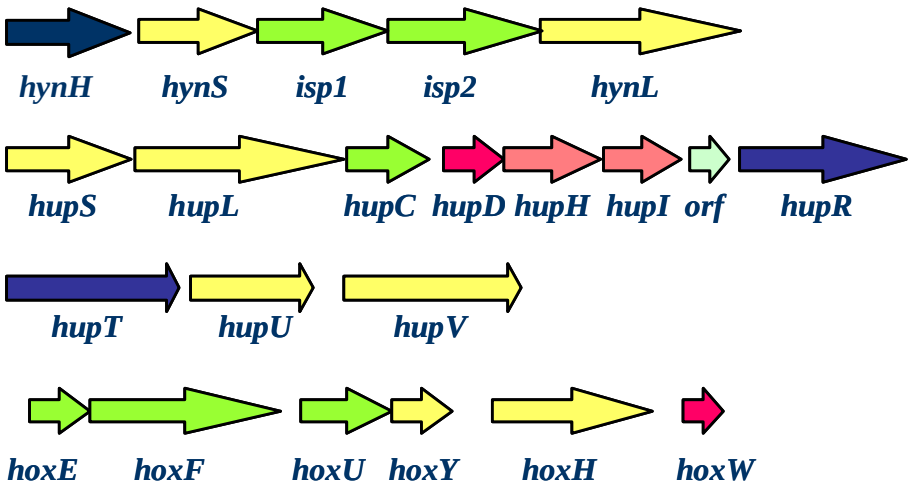

Figure 3. Genomic context of hydrogenase genes in *T. roseopersicina*

1.3.2.2. Hup hydrogenase (HupSL)

Hup represents the other membrane-bound hydrogenase in *T. roseopersicina* (Colbeau et al., 1994). It belongs to the class of [NiFe]-hydrogenases which act physiologically as hydrogen uptake (Hup) hydrogenases recycling hydrogen present in the environment or produced by the nitrogenase enzyme complex as a means of energy recovery (Vignais et al., 2001). Hup hydrogenase is essential for hydrogen dependent growth of *T. roseopersicina* (Palágyi-Mészáros et al., 2009). The hydrogenase small and large subunits are encoded in the *hupSLCDHIR* operon by the *hupS* and *hupL* genes (Figure

3.). The gene product of the *hupC* gene shows homology to *b-type* cytochromes which are considered as membrane anchors and electron transfer subunits of Hup hydrogenases. Nevertheless, the exact role of HupC in *T. roseopersicina* was not examined so far. The regulation of this enzyme was shown to be independent of molecular hydrogen due to the absence of functional H_2 sensor and kinase components of the regulatory cascade (Kovács et al., 2005/B). By oxidizing hydrogen and generating low potential electrons, hydrogenases participate in cellular redox metabolism. Global regulatory systems (FNR (Fumarate Nitrate Reduction Regulator), ArcAB, RegAB), which respond to alterations in oxygen content and redox conditions of the environment, play an important role of hydrogenase regulation of several bacteria (Kovács et al., 2005/C). The other membrane-bound hydrogenase, Hyn, is regulated by the FNR homologue: FnrT. Preliminary data suggest that redox regulation also takes place in the case of Hup hydrogenase, however, mining in the practically finished genome sequence did not result data indicating the presence of the described regulators. Identification of the components involved in the redox regulation of the Hup hydrogenase in *T. roseopersicina* is in progress.

1.3.2.3. Regulatory hydrogenase (HupUV)

The genes showing similarity to sequences coding for the hydrogen-sensing, regulatory hydrogenases (HupUV/ HoxBC, RH (Group 2.)) of other bacteria (Vignais et al., 1997, Buhrke et al., 2004) were identified in *T. roseopersicina* (*hupUV*) (Figure 3.) (Kovács et al., 2005/B). The genes encoding the additional necessary elements (*hupR* and *hupT)* for hydrogen sensing were also found in the genome. However, no hydrogen-dependent regulation of any known [NiFe]-hydrogenases was observed in *T. roseopersicina*. The lack of expression of the *hupTUV* genes would explain the hydrogen independent phenotype. RT-PCR experiments were carried out to test the existence of the *hupTUV* mRNA. No mRNA corresponding to the *hupTUV* genes could be identified, although HupUV was active in the H-D exchange assay when expressed from plasmid (Kovács et al., 2005/C) Since the *hupUV* genes are silent, the wild type *T. roseopersicina* strain lacks the regulatory hydrogenase (HupUV), thus `Hup hydrogenase` in this thesis stands for the HupSL enzyme hereafter.

1.3.2.4. Hox hydrogenase

The third known hydrogenase in *T. roseopersicina* is localised in the soluble fraction. This [NiFe]-hydrogenase belongs to the group of the cytoplasmic NAD^+ reducing hydrogenases (Group 3.) (Rákhely et al., 2004). The enzyme consists of 5 subunits (HoxEFUYH), the hydrogenase part, having the well-known two subunits organization (HoxYH), is associated with the diaphorase subunits (HoxFU), and there is an additional protein (HoxE) connected to the diaphorase part. The Hox hydrogenase functions as a truly bidirectional hydrogenase; it evolves hydrogen under nitrogenase-repressed conditions, and it recycles hydrogen produced by the nitrogenase in cells fixing nitrogen. In-frame deletion of the *hoxE* gene eliminated hydrogen evolution derived from the Hox enzyme *in vivo*, but did not alter hydrogenase activity *in vitro*. This suggests that HoxE has a hydrogenase-related role; it likely participates in the electron transfer processes (Rákhely et al., 2004; Rákhely et al., 2007). Through the use of a tagged HoxE protein, the whole Hox hydrogenase pentamer could be purified as an intact complex (Palágyi-Mészáros et al., 2009). This was the first example to show the presence of a cyanobacterial-type (heteropentameric), NAD-reducing hydrogenase in phototrophic bacteria other than cyanobacteria. A similar enzyme has recently been discovered in *Allochromatium vinosum* (Long et al., 2007).

1.3.3. Hydrogenase maturation in *T. roseopersicina*

The basic principles of [NiFe]-hydrogenase maturation are expected to be valid for the hydrogenases of *T. roseopersicina*, as these ancient enzymes are well conserved. However, variations in the specificity of accessory proteins may be expected, when the cells express more than one hydrogenase associated with distinct metabolic functions, as in the case of *T. roseopersicina* (Maróti et al., 2010). A few accessory genes have been identified in the proximity of the hydrogenase structural genes (*hupDHI*, *hoxW*, *hynH*) (Colbeau et al., 1994, Rákhely et al., 2004), others were found using transposon mutagenesis (*hypC*$_1$, *hypC*$_2$, *hypD*, *hypE*, *hypF*, *hynD* and *hupK*) (Fodor et al., 2001, Maróti et al., 2003), and recently, the mining in the genome sequence of *T.*

roseopersicina revealed the presence of the *hypA* and *hypB* genes in a distant genomic region (unpublished data). Mutational analysis showed that the HypD, HypE and HypF proteins are essential for the maturation of all hydrogenases in *T. roseopersicina*, HupK is selective for the two membrane-bound enzymes (Hyn and Hup), while both $HypC_1$ and $HypC_2$ chaperones proved to be pleiotrophic (Maróti et al., 2003, Maróti et al., 2010). In the latter case, further protein-protein interaction studies were performed. Both HypC variants interacted with the HynL and HoxH subunits, while only $HypC_2$ interacted with HypD and HupK (Maróti et al., 2010). It was demonstrated that the three endopeptidase enzymes (HynD, HupD and HoxW) are truly specific accessory proteins in *T. roseopersicina* essential for the last step of the assembly of large hydrogenase subunits HynL, HupL and HoxH (Figure 4.) (Maróti et al., 2010).

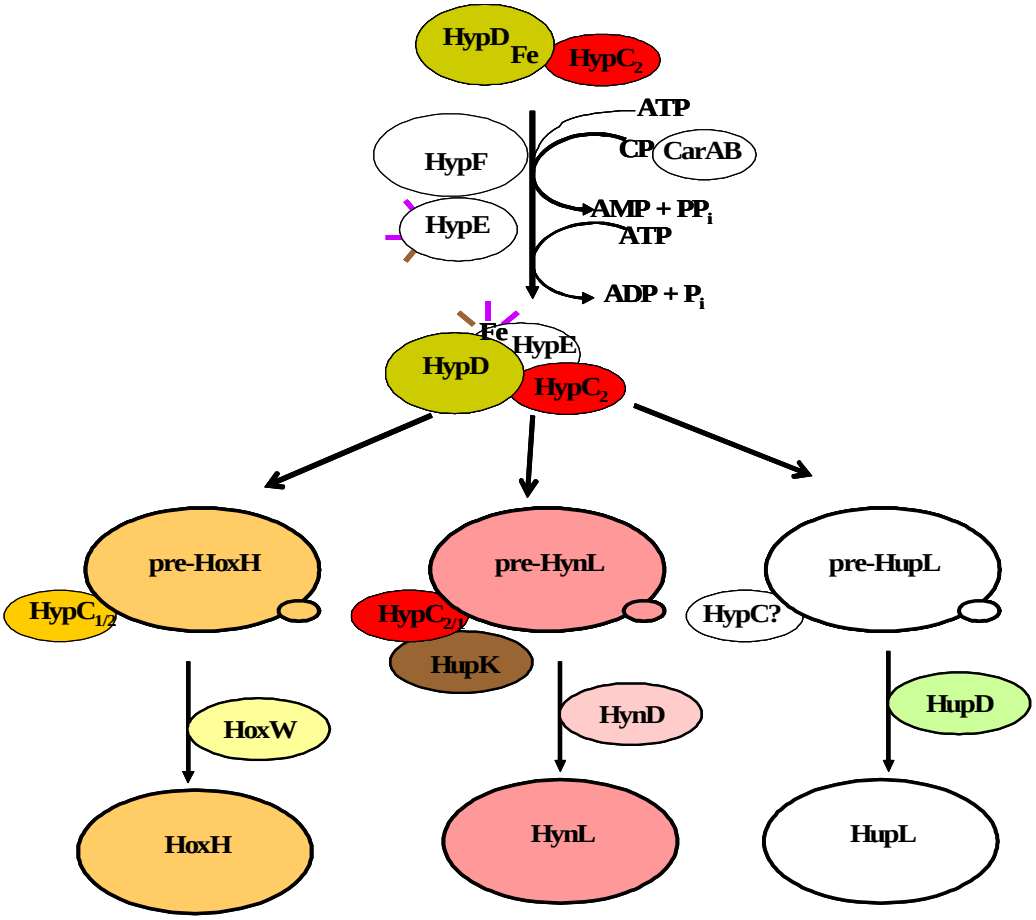

Figure 4. Schematic representation of the roles of HypC variants and specific endopeptidases in *T. roseopersicina*.

2. Aims of the thesis work

T. roseopersicina was known to harbour two membrane-associated (HynSL and HupSL) and one soluble (HoxEFUYH) [NiFe]-hydrogenases. Whole genome sequencing of *T. roseopersicina* revealed the coding sequence of an additional soluble hydrogenase enzyme. Therefore, this strain has one of the most complex hydrogen metabolisms, hydrogenase network. Our aim is to disclose the molecular background of the biosynthesis of the individual hydrogenases, their expression regulation, their metabolic linkages for understanding their physiological role and metabolic interactions.

Within this global project, the aims of this work were the followings:

- Examination of the role of the HupC protein in order to obtain better insight into the *in vivo* roles of HupSL hydrogenase
- Demonstration of the *in vivo* electron transfer function of the HupC domain of the functional HupSLC complex
- Establishment of the presence of the newly discovered soluble hydrogenase (Hox2) at RNA and protein level as well
- Characterisation of the Hox2 hydrogenase by studying domain organisation, examining *in vivo* and *in vitro* activity properties
- Determination of the factors regulating the expression and activity of the Hox2 complex
- Comparison and analysis of the possible *in vivo* roles of the Hox1 and Hox2 bidirectional hydrogenases
- Description of the role of Hox2 enzyme in the hydrogenase network in *T. roseopersicina*

3. Materials and Methods

Bacterial strains and plasmids

Strains and plasmids are listed in Table 1. *T. roseopersicina* strains were maintained in Pfennig's mineral medium: 20g NaCl, 1g KH_2PO_4, 1g $MgCl_2$, 1g KCl, 1g NH_4Cl, 2g $NaHCO_3$, 4g $Na_2S_2O_3$, 20µl B_{12} (1000 µg/ml), 1ml Trace elements, 1ml 20 mM Fe-EDTA (Pfenning et al., 1991).

Trace elements: 2975mg Na_2-EDTA, 300mg H_3BO_4, 200mg $CaCl_2.6\ H_2O$, 100mg $ZnSO_4.7H_2O$, 30mg $MnCl_2.4H_2O$, 30mg $Na_2MoO_4.2H_2O$, 20mg $NiCl_2.6H_2O$, 10mg $CuCl_2.2H_2O$ / 1L water.

Pfennig's medium was modified by changing the sodium-thiosulphate ($Na_2S_2O_3$, anhydrous form) concentration (PC4, PC2 and PC1, 4 g L^{-1}, 2 g L^{-1} and 1 g L^{-1} sodium-thiosulphate, respectively) and by supplementing the medium with various organic substrates glucose (PC4G and PC2G), sodium-pyruvate, sodium-acetate, sucrose, sodium-formate, sodium-succinate, sodium-lactate and sodium-fumarate (all added in 5 g L^{-1} final concentration). Cells were grown anaerobically in liquid cultures illuminated with continuous light using incandescent light bulbs of 60W, or in the dark at 27-30ºC. Plates were supplemented with sodium-acetate (2 g L^{-1}) and solidified with Phytagel (7 g L^{-1}). Plates were incubated in anaerobic jars by means of the AnaeroCult (Merck) for two weeks.

E. coli strains were maintained on LB-agar plates (Sambrook et al., 1989). Antibiotics were used in the following concentrations (µg mL^{-1}): for *E. coli*: ampicillin (100), kanamycin (25); for *T. roseopersicina*: gentamicin (5), kanamycin (25), streptomycin (5), erythromycin (50).

Strain / plasmid	Relevant genotype or phenotype	Reference or source
Thiocapsa roseopersicina		
BBS	wild type	Bogorov et al., 1974
M539	BBS, *hypF*::miniTn5, Km^r	Fodor et al., 2001
GB112131	*hynSL*::Sm^r,*hupSL*::Gm^r,*hoxH*::Em^r ,(Em^r) oriented as *hox* operon	Rákhely et al., 2004
GB2131	*hupSL*::Gm^r,*hoxH*::Em^r	Rákhely et al., 2004
GB1121	*hynSL*::Sm^r,*hupSL*::Gm^r	Rákhely et al., 2004
GB1131	*hynSL*::Sm^r ,*hoxH*::Em^r	Palágyi-Mészáros et al., 2009
HCMG4	*hynSL*::Sm^r , *hoxH*::Em^r Δ*hupC*	Palágyi-Mészáros et al., 2009
HCMG4/pMHE6C	*hynSL*::Sm^r , *hoxH*::Em^r , Δ*hupC* harbouring pMHE6C	Palágyi-Mészáros et al., 2009
GB11213141	*hynSL*::Sm^r ,*hupSL*::Gm^r, *hoxH*::Em^r ,Δ*hoxH2*	This work
GB112141	*hynSL*::Sm^r,*hupSL*::Gm^r, *hoxH*::Em^r, Δ*hoxH2*	This work
GB113141	*hynSL*::Sm^r, *hoxH*::Em^r, Δ*hoxH2*	This work
GB213141	*hupSL*::Gm^r, *hoxH*::Em^r, Δ*hoxH2*	This work

Strain / plasmid	Relevant genotype or phenotype	Reference or source
GB11213141/ pMHE6hoxH2c	*hynSL*::Sm^r, *hupSL*::Gm^r, *hoxH*::Em^r, Δ*hoxH2* harbouring pMHE6hoxH2c	This work
E. coli		
S17-1(λpir)	294 *(recA pro res mod)* Tp^r, Sm^r (pRP4-2-Tc::Mu-Km::Tn7), λ*pir*	Herrero et al., 1990
XL1-Blue MRF'	Δ(*mcrA*)183, Δ(*mcrCB-hsdSMR-mrr*)173, *endA1, supE44, thi-1, recA1, gyrA96, relA1 lac* [F' *proAB lacI*^{q}ZΔM15 Tn10 (Tc^r)]^c	Stratagene
Vectors		
pBluescript SK (+)	Cloning vector, Amp^r	Stratagene
pBhox2	6038bp *Eco*RV-*Hind*III fragment cloned into pBtSK+	This work
pK18*mobsacB*	*sacB*, RP4 *oriT*, ColE1 *ori*, Km^r	Schafer et al., 1994
pHCD2	In frame up- and downstream regions of *hupC* in pK18*mobsacB*, Km^r	Palágyi-Mészáros et al., 2009
phoxH2_2	up- and downstream regions of *hoxH2* in pK18*mobsacB*, Km^r	This work
pMHE6crtKm	Broad host range expression vector containing the promoter region of *crtD*, Km^r	Fodor et al., 2004
pMHE6C	*hupC* gene cloned into pMHE6crtKm, Km^r	Palágyi-Mészáros et al., 2009
pMHE6hoxH2c	*hox2H* gene cloned into pMHE6crtKm, Km^r	This work

Conjugation

T. roseopersicina was grown in Pfennig's mineral medium at 27-30 °C for 3-4 days until reach late logarithmic, early stationary phase of growth (OD_{650} = 1.8). *E. coli* was grown to mid logarithmic phase (OD_{600}=0.7) in LB medium at 37 °C. The cells were collected by centrifugation, washed three times with 5 mL of Salt solution (20g NaCl, 1g KH_2PO_4, 1g $MgCl_2$, 1g KCl L^{-1}) and resuspended in 200 μL Salt solution. The mixed *T. roseopersicina* and *E. coli* cells were incubated overnight on PNA plates (Pfennig's mineral medium, supplemented with 0.2% acetate and 0.2% Nutrient Broth (BBL) solidified with 1.5% agar) in light room, aerobically at 27-30 ^{o}C. Selection was done on Pfennig's mineral medium supplemented with 0.2% acetate and the appropriate antibiotics, solidified with 0.7% Phyta gel. Plates were incubated in anaerobic jars (Oxoid) in light room, at 27-30 °C for two weeks.

Isolation of genomic DNA from *T. roseopersicina*

Cells were suspended in TE buffer (Tris/HCl 10 mM, EDTA 1 mM, pH= 7.5), treated with proteinase K in the presence of SDS. NaCl and CTAB were added to the samples, and after incubation at 65 ^{o}C for 20 minutes phenol-chloroform extraction was performed. The mixture was precipitated with isopropanol, washed with 70 % ethanol and the dried pellets were suspended in water (Ausubel et al., 1996).

Isolation of plasmid DNA from *E. coli*

QIAGEN Plamid kit (Qiagen), GenElute (Sigma), PerfectPrep (Eppendorf) plasmid purification kits or the alkaline method were used for plasmid purification.

DNA manipulation

Digestion of DNA with restriction endonucleases, blunting of the DNA ends with Klenow polymerase or T4 DNA polymerase, phosphate treatment with alkaline phosphatase and polynucleotide kinase and ligation of DNA ends with T4 DNA ligase

were performed according to the manufacturer's instructions (Fermentas, Stratagene, Amersham Biosciences).

Agarose gel electrophoresis

Agarose gel electrophoresis was done in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH = 8.0) using 1-2% agarose gel containing 0.5 μg/mL EtBr as described in Current Protocols in Molecular Biology (Ausubel et al., 1996).

Isolation of DNA fragments

Isolation of DNA fragments was performed with the DNA Extraction Kit (Fermentas) as described in manufacturers instructions.

Polimerase chain reaction (PCR)

PCR-s were carried out in Eppendorf 5415R and Hybaid PCRExpress thermocyclers. DyNAzyme (Finnzymes), Phusion (Finnzymes) or Pfu (Fermentas) DNA polymerases were used. The final concentrations were: primers 1 μM each, dNTPs 200 μM each, buffer, enzyme and Mg^{2+} according to the manufacturer's instructions.

Preparation of competent cells and transformation

Preparation and transformation of *Escherichia coli* competent cells were carried out according to the SEM method (Inoue et al., 1996).

Isolation of the *hox2* gene cluster in *T. roseopersicina*

A BLAST search was performed in our local *T. roseopersicina* genome data bank using the *hox1H* gene as query sequence. A genomic locus was identified which harboured putative genes coding for proteins similar to the Hox2FUYH subunits of the soluble NAD^+-reducing hydrogenases. No other hydrogenase related gene could be identified in

this locus. *hox2W* was found similarly in a distant genomic locus. The sequences were submitted to the Genbank; accession numbers are the following: GU560006 (*hox2FUYH* locus), GU560007 (*hox2W* locus).

A cosmid library (created from *T. roseopersicina* genomic DNA as part of the ongoing genome project (unpublished data)) was screened by colony PCR using the primers OHOX2HFW (5`-TCCACGAGGAGATCAAGTCC-3`) and OHOX2HREV (5`-CAGTGCAGCAACTCGATCAT-3`) located in the *hox2H* gene. A cosmid giving positive signal (p751) was selected and a 6038 bp *EcoR*V-*Hind*III digested fragment, which harbours the *hox2FUYH* genes was cloned into pBtSK+ (pBhox2).

Mutant strains and complementation

Deletion of the *hox2H* gene

The deletion construct was derived from the pK18*mobsacB* vector (Schafer 1994). The upstream region of the *hox2H* gene was amplified with the ohox225 (5`-GTCTCCAGATTCTTAGTCATG-3`) and ohox226 (5`-CATCCTGCAGCTGGTCGATC-3`) primers and the 742 bp PCR product was cloned into the *Sma*I-digested pK18*mobsacB* (phoxH2_1). The downstream region was amplified with the hox2forw*Sal* (5`-ATCGTATCGTCGACAGTCCATCCGCCGCGTTGCG-3`) and the hox2rev*Hind* (5`-CAACGTCAAAGCTTTCGGCACCGTCGTCCATAAC-3`) primers and digested with *Sal*I-*Hind*III enzymes. The 766 bp fragment was cloned into the *Sal*I-*Hind*III digested phoxH2_1 yielding phoxH2_2. The phoxH2_2 plasmid was transformed into *E. coli* S17-1(λpir) strain then conjugated into *T. roseopersicina* GB1121, GB1131, GB2131 and GB112131 mutant strains. Single and double recombinants were selected based on kanamycin resistance and the *sacB* positive selection system (Schafer 1994) yielding the following mutant strains: GB112141, GB113141, GB213141 and GB11213141 (Table 1).

***Δhox2H* complementation**

The *hox2H* gene was amplified with the ohoxH2c1 (5`-GAACGAGCCATATGACTAAGAATCTGGAGACC-3`) and the ohoxH2c2 (5`-

CGTATATCAAGCTTGGCGTCGATCGAACCGTC-3`) primers containing *Nde*I and *Hind*III restriction sites. The PCR product was digested with *Nde*I-*Hind*III enzymes and the 1484 bp fragment was cloned into the corresponding sites of pMHE6crtKm vector (Fodor et al., 2004) resulting in pMHE6hox2c. The construct was transformed to *E. coli* S17-1(λpir) and conjugated into *T. roseopersicina* GB11213141 strain (Table 1.)

In-frame deletion of the *hupC* gene

For deletion of the *hupC* gene, the pHCD1 and pHCD2 in-frame deletion constructions were created as follows. The upstream region of *hupC* was amplified with the ohup20 (5'-CGAGCAGGCCAAGTATTC-3') and ohup19 (5'-TGTTGGTCAGGCGGATCT-3') primers and the 836 bp PCR product was cloned into the *Sma*I-digested pK18*mobsacB* (pHCD1). The downstream region was amplified with the ohup21 (5'-GGCGGATGTTCAAGGACG-3') and ohup22 (5'-TCGACCACGACACTGAAG-3') primers. The 800 bp fragment was cloned into the *Pst*I digested, polished pHCD1 (pHCD2). The pHCD2 plasmid was transformed to *E. coli* S17-1(λpir) and conjugated into *T. roseopersicina* GB1131 strain yielding the in-frame deletion mutant HCMG (Δ*hupC* GB1131) strain (Table1).

***Δhup*C complementation**

The *hupC* gene was amplified with the ohupc1 (5'-CATATGTCGCGAGCTGCGTCGCG-3') and ohupc2 (5'-AAGCTTTGGCCGATCGTCCTTGAACAT-3') primers containing *Nde*I and *Hind*III restriction sites. The 777bp PCR product was cloned into the *Eco*RV digested pBtSK+ (pBtC). The 777bp *Nde*I-*Hin*dIII digested fragment was cloned into the corresponding sites of pMHE6crtKm (Fodor et al., 2004) resulting pMHE6C. It was transformed into *E. coli* S17-1(λpir) strain, than conjugated into the HCMG strain.

RNA isolation and DNaseI treatment

15 ml of *T. roseopersicina* liquid cell cultures were pelleted and resuspended in 300 µL of SET buffer (50mM EDTA pH = 8.0, 50mM Tris/HCl pH = 8.0 and 20% sucrose) and 300 µL SDS buffer (20%SDS, 1% $(NH_4)_2SO_4$ pH = 4.8) was added. After the addition of 500 µL saturated NaCl the sample was centrifuged at 20,000 x g for 10 min. The supernatant was transferred into a new tube, mixed with 2-propanol (70% of the total volume of the supernatant) and centrifuged at 20,000 x g for 20 min. The pellet was washed twice with 1 mL of 70% ethanol. The dried pellet was suspended in 20 µL diethylpyrocarbonate-treated water. DNase treatments were performed by DNase I (Fermentas) according to the manufacturer's instructions.

Since the expression level of the *hox2* genes are extremely low, and RNA isolated by the above mentioned procedure was not suitable for successful RT-PCR and quantitative PCR, RNA was also isolated and treated with DNase I using the RiboPure Bacteria kit (Ambion) by following the manufacturer's protocol.

Reverse transcription (RT)

Reverse transcription was performed by TaqMan Reverse Transcription Reagents (ABi and Roche) and Omnisript® Reverse transcriptase Kit (Qiagen) according to the manufacturers instructions. Either random hexamers or specific primers were used for the cDNA synthesis.

RT-PCR

For the *hox2* operon, the reverse transcription was initiated at primer OHOXHQRT (5`-GTTGTTGTTGGTGGTGGACA-3`) located at the C-terminal of the *hox2H* gene, while amplification was performed with the following primers: OHOX2FFW (5`-GGAATACGACCTGAGCGAGATG-3`) and OHOX2FREV (5`-GGAATTTGTCGAGCGTGTTGA-3`) located in the *hox2F* gene.

Quantitative PCR

For the expression analysis of *hox2* genes in GB112131 strain, the reverse transcription was initiated either at OHOXHQRT or by using random primers. Expression levels of

hox2 genes (*hox2F* with primers OHOX2FFW and OHOX2FREV, *hox2U* with primers OHOX2UFW and OHOX2UREV, *hox2Y* with primers OHOX2YFW and OHOX2YREV, *hoxH2* with primers OHOX2HFW and OHOX2HREV), *hox* genes *(hoxF* with primers OHOX1FFW (5`-GGTGTATGGGCCTATGTTCG-3`) and OHOX1FREV (5`-TGATTGGTCGGACAACGTAA-3`), *hoxU* with primers OHOX1UFW (5`-GATGCAGATCCAGACCAACA-3`) and OHOX1UREV (5`-GGTAGCTCGCCTGACGATAG-3`), *hoxY* with primers OHOX1YFW (5`-GGCTGTCACATGTCCTTCCT-3`) and OHOX1YREV (5`-ACCAGGATCTTGCAGTGCTT-3`), *hoxH* with primers OHOX1HFW (5`-CCGTCGAGGACTTCAGCTAC-3`) and OHOX1HREV (5`-GACCAGTCGGGCATAGTGAT-3`) and 16SrDNA genes with primers RRN01 (5`-GCAACGCGAAGAACCTTACC-3`) and RRN02 (5`-CCAAGGCATCTCTGCCAAGT-3`) of *T. roseopersicina* were analyzed in various samples. Quantitative PCR experiments were performed using the ABI Step One Plus PCR machine.

For the expression analysis of the *hup* operon reverse transcription was initiated from the huprto2 (5`-CGCTTGAGCCGATTCTGAACAT-3`) primer specific for the *hupL* gene. Quantitative PCR was performed using the ohupSRT1 (5`-GGACAAGGGCAGCTTCTATCA-3`) and ohupSRT2 (5`-CGCATTGGCCTCGATACC-3`) primers located in the *hupS* gene. The experiments were performed in the 7500 real-time PCR instrument (Applied Biosystems). Calibration curves were generated using sixfold dilutions of pKK48 plasmid DNA (containing the sequence of the *hupS* gene) in the 100 to 0.001 $ng \cdot \mu L^{-1}$ concentration range.

Activity measurements

The gas chromatograph used for the activity measurements was Agilent 6890 with TCD detector using nitrogen as carrier gas. The gas chromatograph was equipped with a 30 m long 0.53 mm inner diameter Plot Molecularsieve 5/A column, oven temperature was 60ºC.

***In vivo* hydrogen evolution activity measurement**

T. roseopersicina cultures (60 mL) were grown in various Pfennig medium (PC4, PC2, PC4G, PC2G) under nitrogen atmosphere in sealed 100 mL Hypo-Vial glasses. Anaerobiosis was established by flushing the gas phase with N_2 for 10 minutes. H_2 production was followed by gas chromatograph on each day of growth starting at the 5^{th} day and ending at the 15^{th} day.

In vivo hydrogen uptake activity measurements

T. roseopersicina (60 mL) were cultured in 100 mL sealed hypovials using two distinct approaches.

- H_2 is injected into the bottles

For the measurement of the *in vivo* H_2 uptake activity of the HupSL hydrogenase the strains were grown in Pfennig medium (60 mL) containing different amount of thiosulphate (PC4, PC2, and PC1). The gas phase was flushed with N_2 for 10 minutes and 5 ml of pure H_2 was injected into the bottles. The cultures were grown under illumination and the H_2 content of the gas phase was measured at the 6^{th} day by gas chromatograph.

- H_2 is produced by the nitrogenase enzyme complex

In case of the same activity measurement of the Hox2 hydrogenase, strains were cultured in Pfennig medium (60 mL) containing 2 g L^{-1} thiosulphate and supplemented with 5 g L^{-1} glucose but lacking ammonium-chloride (NC2G) under nitrogen atmosphere. H_2 production was followed by gas chromatograph on each day of growth starting at the 5^{th} day ending at the 15^{th} day. Hydrogen uptake was calculated from the total hydrogen amount produced by the nitrogenase complex in the absence and presence of the hydrogenase.

Preparation of membrane and soluble protein fractions of *T. roseopersicina*

T. roseopersicina culture (60 mL) was harvested by centrifugation at 7,000 x g. The cells were suspended in 3 mL of 20 mM K-phosphate buffer (pH = 7.0), and sonicated 8 times for 10 seconds on ice using 15 W power with a mechanical amplitude of 50 μm. The broken cells were centrifuged at 10,000 x *g* for 15 min. The debris (remaining whole cells and sulphur crystals) was discarded and the supernatant was centrifuged at 100,000

x g for 1.5 hours. The pellet was washed twice with 20 mM K-phosphate buffer (pH = 7.0) and used as membrane fraction. The supernatant was regarded as the soluble fraction.

In vitro methyl-viologen dependent hydrogen evolution activity measurement

T. roseopersicina cultures (60 mL, grown in PC2G medium in sealed 100 mL Hypo-Vials flushed with N_2) were harvested and the soluble fraction was prepared as described above. Soluble fraction (200 μL) was used for the measurement, 1.76 mL 20 mM K-phosphate buffer (pH = 7.0) and 40 μL 40 mM methyl-viologen (MV) was added. The mixture was flushed with nitrogen for 10 min, the reaction was initiated by injecting 100 μL anaerobic 50 mg mL^{-1} sodium-dithionite. Samples were shaken gently at room temperature for 1 h, hydrogen content of the gas phase was determined by gas chromatograph.

In vitro NADH/NADPH dependent hydrogen evolution activity measurement

T. roseopersicina cultures (60 mL) were harvested (grown in PC2G medium in sealed 100 mL Hypo-Vials flushed with N_2), soluble fraction was prepared as described above. To 500 μL soluble fraction, 1.46 mL 20 mM K-phosphate buffer (pH = 7.0) containing 4 mM DTT and 20 μL 200 mM FMN was added. The mixture was flushed with nitrogen for 10 min, and incubated at 37^{o}C for 1 h. The reaction was initiated by injecting 20 μL 120 mM anaerobic NADH or NADPH. Samples were shaken gently at 37^{o}C for 6 h, hydrogen content of the headspace was tested by injecting 500 μL samples into the gas chromatograph in every 60 minutes.

In vitro hydrogen uptake activity measurement

Hox2 hydrogenase: *T. roseopersicina* cultures (60 mL, grown in PC2G medium in sealed 100 mL Hypo-Vials flushed with N_2) were harvested and soluble fraction was prepared as described above. To the soluble fraction (200 μL) used in the measurement, 1.76 mL 20 mM K-phosphate buffer (pH = 7.0) and 40 μL 40 mM benzyl-viologen (BV)

was added. The mixture was flushed with nitrogen for 5 min followed by flushing with 100% hydrogen for another 5 min. Samples were incubated at 60 ^{o}C in spectrophotometer, rate measurement of hydrogenase activity was performed by following the absorbance at 600 nm in time.

HupSL hydrogenase: *T. roseopersicina* cultures (60 ml, grown in Pfennig medium supplemented with different amount of thiosulphate in sealed 100 mL Hypo-Vials flushed with N_2) were harvested, soluble and membrane fractions were created as described above. The membrane fraction, as a pellet, was suspended in 1.96 mL of 20 mM K-phosphate buffer (pH = 7.0) and 40 μL 40 mM benzyl-viologen (BV) was added. In case of the soluble fraction, the reaction mixture was prepared and the latter experimental procedure was performed.

Determination of glucose content

Changes in glucose concentration were followed by two approaches. Beside DNSA method (Miller et al., 1959), we applied a simple tool developed for blood sugar tests (GlucoVal). Device was calibrated for Pfennig medium containing glucose (dilution series for glucose), measurements proved to be accurate for Pfennig medium containing 0 - 5 g L^{-1} glucose.

Determination of thiosulphate content

Samples of 1 mL volume were taken from 60 mL *T. roseopersicina* cultures on each day of growth. Cell densities were spectrophotometrically measured at 600 nm, then 1 mL samples were pelleted by centrifugation at 10,000 x g for 5 min. Supernatant was used for thiosulphate determination of the medium. Thiosulphate was identified clearly by UV absorption at 230 nm using quartz cuvettes. Calibration curve for thiosulphate was linear for PCG medium supplemented with 0 to 1.5 g L^{-1} thiosulphate. Absorbance values were normalized with cell densities.

Determination of bacteriochlorophyll content

Methanol extraction procedure was used for the determination of the bacteriochlorophyll content, as described previously (Stahl et al., 1984). The absorption of the samples at 772 nm was measured; the extinction coefficient was 8.41 $g^{-1} \cdot L \cdot cm^{-1}$. The *in vivo* and *in vitro* activities of the HupSL hydrogenase were normalised to the bacteriochlorophyll content of the samples.

4. Results

4.1. Characterization of the HupC protein in T. roseopersicina

4.1.1. Optimisation of the growth conditions suitable for the activity measurements of the Hup hydrogenase

T. roseopersicina harbours at least three different hydrogenases. For the characterisation of the individual enzymes, different mutant strains have been constructed. The *ΔhynSL*, *ΔhoxH* strain (GB1131) is suitable for the measurement of the *in vivo* H_2 uptake activity of the Hup hydrogenase without the contribution of the two other hydrogenases. Under standard growth conditions (photoautotroph, cultivated in Pfennig`s mineral medium supplemented with 4 $g \cdot L^{-1}$ sodium thiosulphate (PC4)) the activity of the Hup hydrogenase could hardly be detected. The following facts were taken under consideration to solve this issue. Thiosulphate serves as reducing power for the photosynthetic carbon fixation via the central quinone pool (Dahl et al., 1994). Our previous investigations indicated that the *in vivo* activities of the Hox1, Hyn and Hup hydrogenases were dependent on the nature and quantity of the electron sources used in the growth medium (Rákhely et al., 2007, Laurinavichene et al., 2007). Multiplicity of hydrogenases influence the redox status of the wild type *T. roseopersicina* cells by H_2 evolution (e^- consumption) or H_2 uptake (e^- donation). The relatively high (4 $g \cdot L^{-1}$) thiosulphate content of the medium and the absence of the other three hydrogenases, especially Hox1, which is considered as the main H_2 evolving hydrogenase, may cause electron accumulation in the quinone pool. Reduced quinone pool was presumed as the reason for decreased Hup hydrogenase activity, since it functions as a H_2 uptake, electron donating enzyme. In order to confirm this assumption, Hup hydrogenase activity was measured in cells grown in the presence of various amounts of thiosulphate (PC4, PC2 and PC1 containing 4, 2 and 1 $g \cdot L^{-1}$ $Na_2S_2O_3$) (Table 2).

The results of both *in vivo* and *in vitro* activity measurements demonstrated that lower thiosulphate concentration in the medium resulted in higher Hup hydrogenase activity. However, regressive cell growth was also observed in the presence of 1 $g \cdot L^{-1}$ thiosulphate in the medium.

Medium	*In vivo* H_2 uptake (%)	*In vitro* H_2 uptake (%)
PC4	0.0	0.0
PC2	45.0 ± 2.6	83.6 ± 34.2
PC1	100 ± 5.6	100 ± 11.1

Table 2. Relative *in vivo* and *in vitro* H_2 uptake activity of the GB1131 (*ΔhynSL*, *ΔhoxH*) strain grown at various $Na_2S_2O_3$ concentrations (PC4, PC2 and PC1 containing 4, 2 and 1 $g \cdot L^{-1}$ $Na_2S_2O_3$). The results are given in percentages of the values of sample grown in PC1 medium.

The effect of the thiosulphate concentration on the expression level of the *hupSL* genes has also been monitored. The data of quantitative RT-PCR (Table 4.) revealed that the *hupSL* mRNA level was higer by diminishing the thiosulphate content from 4 to 2 $g \cdot L^{-1}$. The results of the activity measurements and the expression analysis correlate well and confirm the hypothesis outlined above. When Hup is the only active hydrogenase in the cell, both the activity and the expression levels of the enzyme strongly depend on the thiosulphate content of the growth medium. Further experiments on Hup activity were performed with samples cultivated in PC2 medium.

4.1.2. Mutational analysis of the HupC protein

HupC homologues are reviewed as third subunits of uptake [NiFe]-hydrogenases, which are not merely redox carriers but also anchor for the binding of the uptake hydrogenases to the membrane. The role of the HupC protein in *T. roseopersicina* was studied by in-frame deletion mutagenesis. The *hupC* gene was deleted in the *ΔhynSL*, *ΔhoxH* (GB1131) mutant strain (HCMG4) and the mutant was used in several different activity measurements. H_2 uptake activity measurements on whole bacterial cells demonstrated

that the activity of Hup hydrogenase in the *ΔhupC* (HCMG4) strain decreased substantially *in vivo*, while it was doubled *in vitro* compared to the strain harbouring the *hupC* gene (GB1131) (Table 3.). For verification of the relationship between the HupC protein and the observed changes in the activity, complementation was performed by introducing an expression cassette containing the *hupC* gene driven by the *crtD* promoter. Table 4 shows, that the plasmid-borne HupC (pMHE6C/HCMG4) partially restored the Hup hydrogenase activity *in vivo*. GB112131 strain lacking Hyn, Hox1 and Hup hydrogenases was used as negative control under the described growth conditions (PC2), in line with M539 strain in which the HypF protein is not able to fulfil its function during the maturation process of [NiFe]-hydrogenases.

Strain	***In vivo* H_2 uptake activity**	***In vitro* H_2 uptake activity**
GB1131(*ΔhynSL*, *ΔhoxH*)	100 ± 2.6	100.0 ± 14.5
HCMG4(*ΔhynSL*, *ΔhoxH*,*ΔhupC*)	40.4 ± 5.5	198.9 ± 5.5
pMHE6C/HCMG4	68.3 ±10.3	231.2 ± 33.5
GB112131(*ΔhynSL*, *ΔhoxH*,*ΔhupSL*)	0 ± 0	0 ± 0
M539(*ΔhypF*)	0 ± 0	0 ± 0

Table 3. Relative *in vivo* and *in vitro* hydrogen uptake activities of the Hup hydrogenase in the presence and absence of the HupC protein. The hydrogenase activity values are normalised to the bacteriochlorophyll content. The results are given as percentage of the level for GB1131.

Inspection of the *hup* expression level in the presence and absence of HupC revealed elevated *hupS* mRNA level of the *ΔhupC* strain (Table 4.). This corrobates the above-mentioned possible control of the Hup expression. Without HupC, the HupSL can not donate electrons to the quinone pool, and its low level of reduction may signal the regulatory system to upregulate the Hup expression.

Strain	**PC4**	**PC2**
GB1131(*ΔhynSL*, *ΔhoxH*)	100.0 ± 0.0	1650.0 ± 44.5
HCMG4(*ΔhynSL*, *ΔhoxH*, *ΔhupC*)	300.0 ± 20.0	2700.0 ± 102.8

Table 4. Relative mRNA levels of the *hup* operon in the presence and absence of the *hupC* gene at various $Na_2S_2O_3$ concentrations (PC4 and PC2 containing 4 and 2 $g \cdot L^{-1}$ $Na_2S_2O_3$). The results are given in percentages of the mRNA level of GB1131 (*ΔhynSL*, *ΔhoxH*) strain. The values are normalised to the total RNA content.

Membrane and soluble fractions were separated - using the same (GB1131, HCMG4 and pMHE6C/HCMG4) strains and growth conditions - and used for *in vitro* H_2 uptake activity measurements (Table 5.). Although Hup activity in the *ΔhupC* strain (HCMG4) decreased dramatically in the membrane fraction, concomitant increase of the activity in the soluble fraction could not be detected. In the HupC complementing strain (pMHE6C/HCMG4) the HupSL activity was significantly higher in the soluble than in the membrane fraction.

Strain	***In vitro* relative uptake activity**	
	Membrane fraction	**Soluble fraction**
GB1131 (*ΔhynS-isp1-isp2-hynL, ΔhoxH)*	100.0 ± 12.9	36.0 ± 8.5
HCMG4 (*ΔhupC, ΔhynS-isp1-isp2-hynL, ΔhoxH)*	1.8 ± 0.24	11.3 ± 1.8
pMHE6C HCMG4 (*ΔhupC, ΔhynS-isp1-isp2-hynL, ΔhoxH,* pMHE6C*)*	47.3 ± 0.9	115.0 ± 7.1

Table 5. Relative *in vitro* H_2 uptake activity of the membrane and soluble fractions of different mutant strains.

4.2. Identification and characterisation of the Hox2 hydrogenase in T. roseopersicina

4.2.1. Discovery of the *hox2* genes in the genome of *T. roseopersicina*

Small, but reproducible hydrogenase activity of the triple hydrogenase mutant strain (GB112131 lacking Hyn, Hup and Hox hydrogenases) occurred under specific photomixotrophic conditions (PC2G). Since this activity was not manifested in the *ΔhypF* strain, it was attributed to an additional functional [NiFe]-hydrogenase in *T. roseopersicina*.

Mining in the available data of *T. roseopersicina* genome sequence revealed 4 *orf*s which encode putative proteins showing significant homology to the subunits of bidirectional heteromultimeric cytoplasmic [NiFe]-hydrogenases (Vignais et al., 2001, Vignais et al., 2007). Since, a member of this group has already been identified and characterised in this organism as Hox hydrogenase (Rakhely et al., 2004), it was renamed as Hox1, and the newly found genes were labelled as *hox2*. The *hox2FUYH* genes encode putative hydrogenase small and large subunits (*hox2YH*) and two diaphorase subunits (*hox2FU*). Although several members of the group of bidirectional heteromultimeric cytoplasmic [NiFe]-hydrogenases harbour additional subunits, like the HoxE of the Hox1 (Hox1EFUYH) hydrogenase, mining in the genome sequence failed to find coding sequence for additional Hox2 hydrogenase subunits. However, an *orf* showing homology to endopeptidase genes was identified in a distant genomic region. Endopeptidases are responsible for posttranslational cleavage of the C-terminal extension of the large hydrogenase subunit as the final step of the maturation process. Since three other endopeptidase genes were discovered earlier and their gene products were shown to be strictly specific for the certain hydrogenases (Hyn, Hup and Hox1) (Maróti et al., 2010),

the newly identified *orf* was supposed to be involved in the maturation process of the deduced Hox2H subunit, and was termed as *hox2W*.

In silico analysis of the *hox2FUYH* genes revealed that the deduced Hox2 enzyme show highest homology to the NAD^+ reducing hydrogenase of *Methylococcus capsulatus* (Bath) (Hanczár et al., 2002) (Table 6). The corresponding subunits of the two Hox-type hydrogenases of *T. roseopersicina* were compared, the relative identity values are shown in Table 6.

	Thiocapsa roseopersicina BBS Hox1					*Thiocapsa roseopersicina* BBS Hox2			
	E	**F**	**U**	**Y**	**H**	**2F**	**2U**	**2Y**	**2H**
Ralstonia eutropha H16	-	35	33	45	40	44	41	48	47
Methylococcus capsulatus Bath	-	38	33	49	46	56	63	65	66
Synechococcus PCC 7002	53	60	57	46	51	28	38	43	42
Nostoc PCC 7422	55	61	58	49	54	28	33	41	43
Thiocapsa roseopersicina BBS Hox2	-	**28**	**31**	**49**	**46**	100	100	100	100

Table 6. Relative sequence identity values between Hox subunits of various organisms (in %).

4.2.2. Mutational analysis of the Hox2 hydrogenase

Deletion of *hox2H* gene was performed in order to examine whether Hox2 is responsible for the hydrogenase activity observed in GB112131 under photomixotrophic growth conditions (PC2G). Mutation of the *hox2H* gene in GB112131 (*ΔhynSL*, *ΔhupSL*, *ΔhoxH*) strain completely eliminated hydrogen evolution, the GB11213141 (*ΔhynSL*, *ΔhupSL*, *ΔhoxH*, *Δhox2H*) strain is devoid of any hydrogenase activity. Complementation with the plasmid-borne Hox2H using pMHE6crtKm broad host range plasmid (pMHEhoxH2c /GB11213141) restored the hydrogen production, which finally corroborated that Hox2 was the source of the residual hydrogen production in GB112131 (Figure 5.).

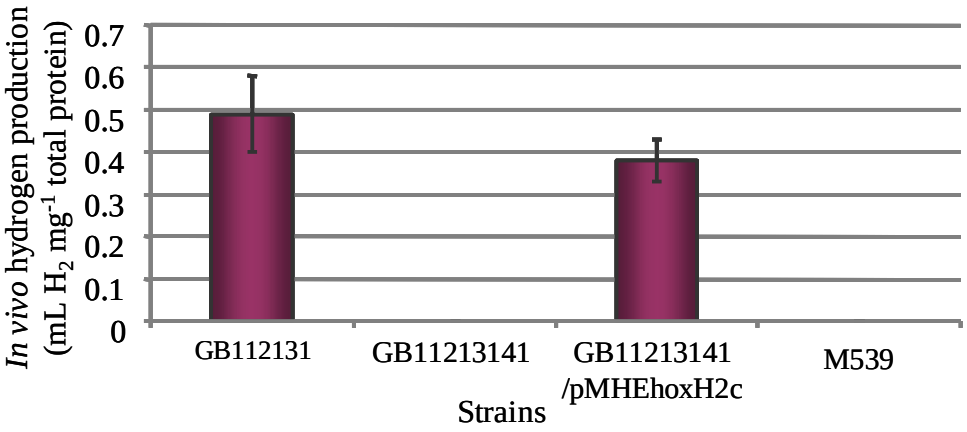

Figure 5. Hox2 is responsible for residual hydrogen production observed in GB112131. Strains were cultivated in PC2G medium. The *ΔhypF* strain (M539), lacking all functional NiFe hydrogenases, was used as negative control.

4.2.3. Characterisation of the *in vivo* Hox2 hydrogenase activity

Under the generally used growth conditions (photoautotroph, cultivated in Pfennig`s mineral medium supplemented with 4 $g \cdot L^{-1}$ $Na_2S_2O_3$ (PC4)), the triple hydrogenase mutant strain (GB112131) showed no hydrogenase activity. As it was indicated earlier, thiosulphate strongly influences the hydrogen metabolism of the cells (Rákhely et al., 2004). However, alterations in the thiosulphate content of the medium did not result in observable Hox2 activity. Thus, the presence of further electron donating component(s) was predicted. After testing numerous compounds, such as glucose, pyruvate, acetate, sucrose, formate, succinate, lactate and fumarate (data not shown), glucose proved to be the inducer of the Hox2 mediated *in vivo* hydrogen production. Additionally, 5 $g \cdot L^{-1}$ sodium pyruvate also made Hox2 to be metabolically active, however, the amount of hydrogen was approximately 45% of the hydrogen produced in the presence of glucose. Since pyruvate is an intermediate of glycolysis, it is not considered as an alternative metabolic linkage.

Hydrogen evolution of the GB112131 strain was induced in the presence of glucose but only with the concomitant reduction of the generally used (4 $g \cdot L^{-1}$) thiosulphate concentration in the medium. Glucose and thiosulphate concentrations were both optimised for GB112131 mediated hydrogen production.

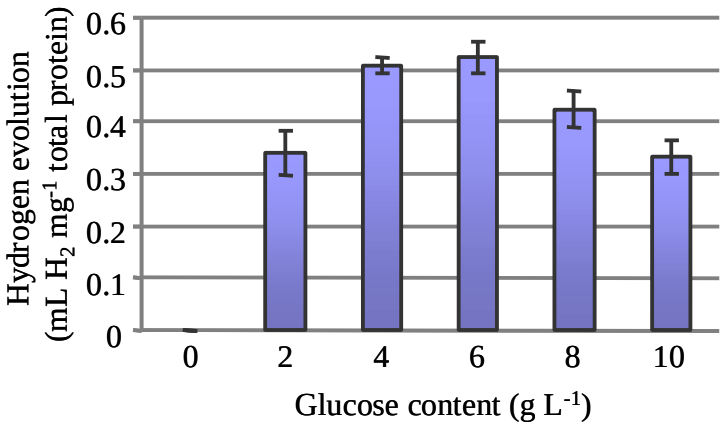

Figure 6. Determination of the optimal glucose content for Hox2-based hydrogen evolution. GB112131 strain was cultivated in PC2 supplemented with 0-10 g L^{-1} glucose. *In vivo* hydrogen production was measured on each day of growth. Hydrogen accumulated between the 7th and 10th day of growth is shown.

Optimal glucose concentration was determined in the range of 4 to 8 g·L^{-1} glucose (Figure 6.). Consequently, 5 g·L^{-1} glucose was used in all further experiments.

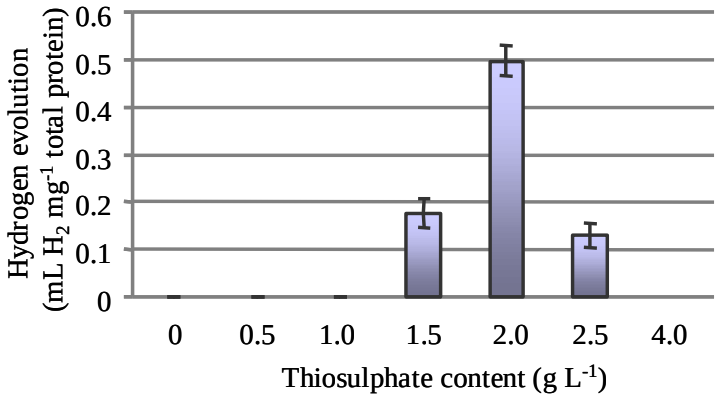

Figure 7. Determination of the optimal thiosulphate content for Hox2-based hydrogen evolution. GB112131 strain was cultivated in PCG supplemented with 0-4 g L^{-1} thiosulphate. *In vivo* hydrogen production was measured on each day of growth. Hydrogen accumulated between the 7th and 10th day of growth is shown.

Optimal thiosulphate concentration in the presence of glucose was defined as 2 g·L^{-1} (Figure 7.). Cell growth was quite weak below 1 g·L^{-1} thiosulphate even in the presence of glucose. Thus, Pfennig medium containing 2 g·L^{-1} sodium thiosulphate supplemented with 5 g·L^{-1} glucose (PC2G medium) represented the optimal growth condition for hydrogen production of the GB112131 strain.

The amount of hydrogen produced by the Hox2 hydrogenase *in vivo* is very low, approximately 0.45-0.55 µL H_2·mg^{-1} total protein, accumulated in 3-4 days. Furthermore, hydrogen production starts at the 7th day of growth, which is the early period of the

stationary phase of photomixotrophic growth (concomitant presence of carbonate and organic substrate in the medium) and lasts until the 10th day.

Changes in thiosulphate and glucose concentrations of the PC2G medium was followed during the life cycle of the culture (GB112131). Complete thiosulphate consumption was observed by the 6th day of growth (Figure 8.). No hydrogen production could be observed when cultures were supplemented with an additional 2 $g \cdot L^{-1}$ thiosulphate at the 5th day of growth. At the same time, initial glucose concentration (5 $g \cdot L^{-1}$) did not change until the 5th day of growth (Figure 9.). Glucose consumption took place from the 6th to 9th day almost reaching the 2 $g \cdot L^{-1}$ final concentration. The measurement of the glucose content lasted until the 13th day of growth, but no further concentration change was observed after the 9th day. Minimal glucose consumption was observed when the GB112131 strain was grown in PC4G medium (4 $g \cdot L^{-1}$ thiosulphate and 5 $g \cdot L^{-1}$ glucose) (Figure 9.). The timing of glucose addition, which took place either at the time of culture inoculation or at the 5th day of growth, did not influence the observed phenomena.

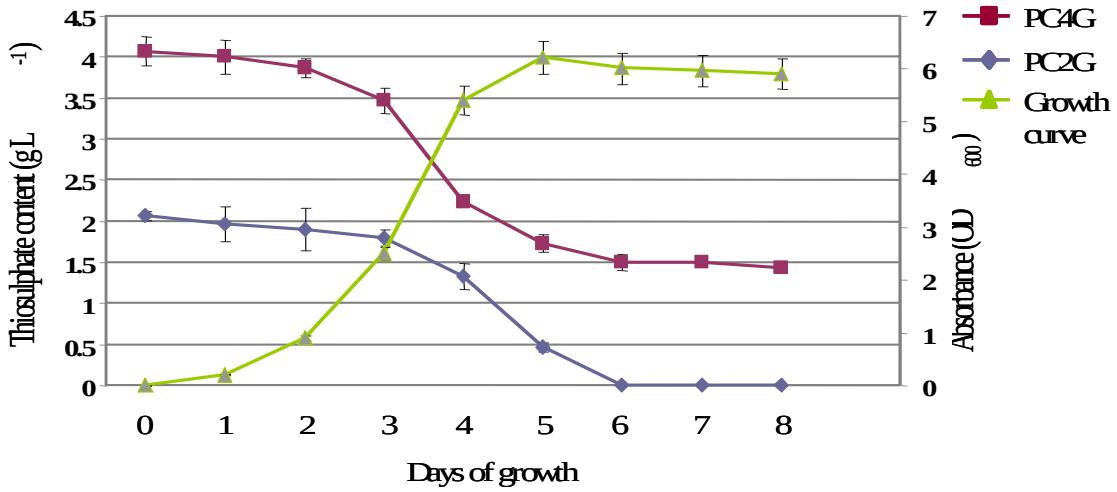

Figure 8. Thiosulphate consumption of GB112131 strain as a function of time. Samples were taken on each day of growth, thiosulphate content was determined as described in Materials and Methods.

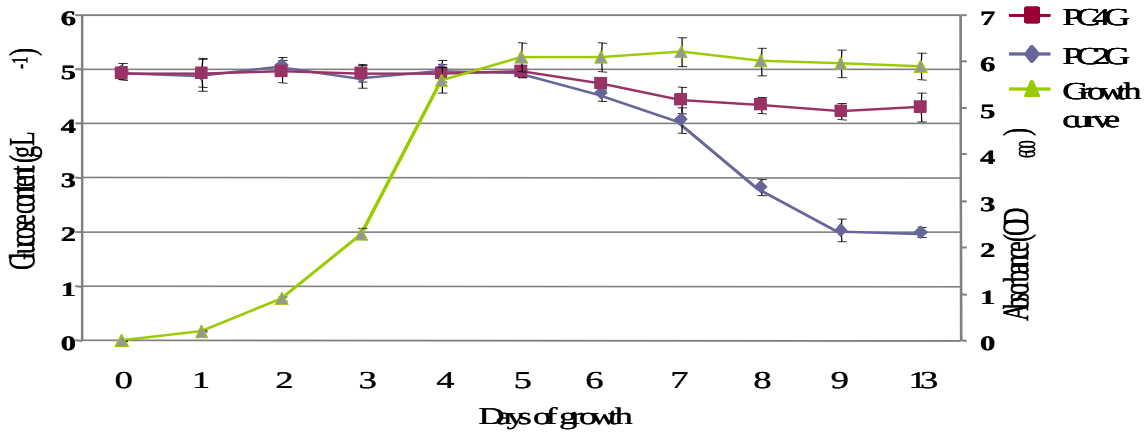

Figure 9. Glucose utilization by GB112131 strain as a function of time. Samples were taken on each day of growth, glucose content was determined as described in Materials and Methods.

Light dependence of *in vivo* hydrogen production by Hox2 was also tested: the samples were illuminated until the beginning of stationary growth phase (5th day), then half of the samples were wrapped in aluminium foil and were cultivated further in dark. GB112131 evolves hydrogen under continuous illumination, no hydrogen production was observed in dark.

Hox2 was also shown to be a real bidirectional enzyme, *in vivo* hydrogen uptake mediated by Hox2 could be measured under nitrogen-fixing conditions (NC2G medium: PC2G medium lacking ammonium-chloride) (Figure 10.)

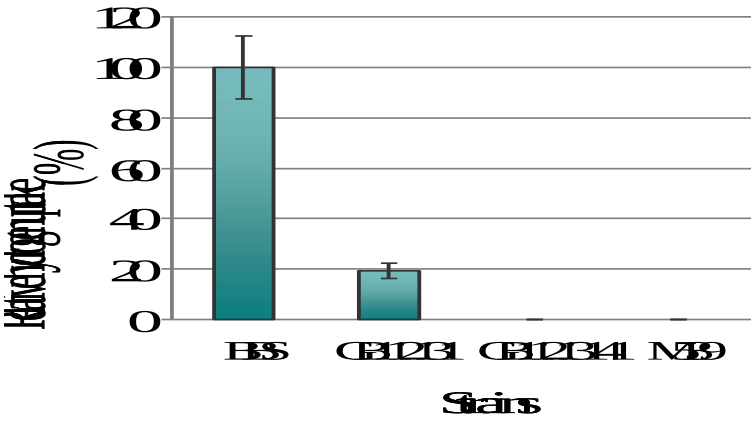

Figure 10. *In vivo* hydrogen uptake mediated by Hox2. Strains were cultivated in NC2G (nitrogen-fixing medium) medium, uptake hydrogenase activity of Hox2 was calculated as described in Materials and Methods. BBS: wild type *T. roseopersicina*; GB112131: *ΔhynSL*, *ΔhupSL*, *Δhox1H*; GB11213141: *ΔhynSL*, *ΔhupSL*, *Δhox1H*, *Δhox2H*; M539: *ΔhypF*

4.2.4. Comparison of the *in vivo* hydrogenase activities of Hox1 and Hox2

Hox1 activity was also examined as function of thiosulphate and glucose concentration (Figure 11.). GB112141 strain (*ΔhynSL*, *ΔhupSL*, *Δhox2H* (Hox1$^+$)) cultivated in various media was used in these experiments. It was previously demonstrated that Hox1 is able to evolve hydrogen in the generally used Pfennig medium (PC4) indicating that thiosulphate can serve as electron donor in this case (Rákhely et al., 2004). Under illumination, lower thiosulphate concentration (PC2) resulted in decreased hydrogen production. Escalated hydrogen production of Hox1 in the glucose containing media were independent of the thiosulphate concentration (PCG2, PCG4). Moreover, Hox1 was able to use glucose – grown in PC2G – in a slightly earlier growth phase than Hox2. Elevated hydrogen production was observed in the late logarithmic growth phase, around the 6th day of culturing and the additive effect caused by glucose lasted until the 8th day. No significant difference could be observed in the kinetics of thiosulphate utilization by GB112141 (Hox1$^+$) compared to GB112131 (Hox2$^+$) (data not shown). It is notable, that Hox1 showed twenty-fold *in vivo* hydrogen evolution activity relative to Hox2 cultivated in PC2G medium.

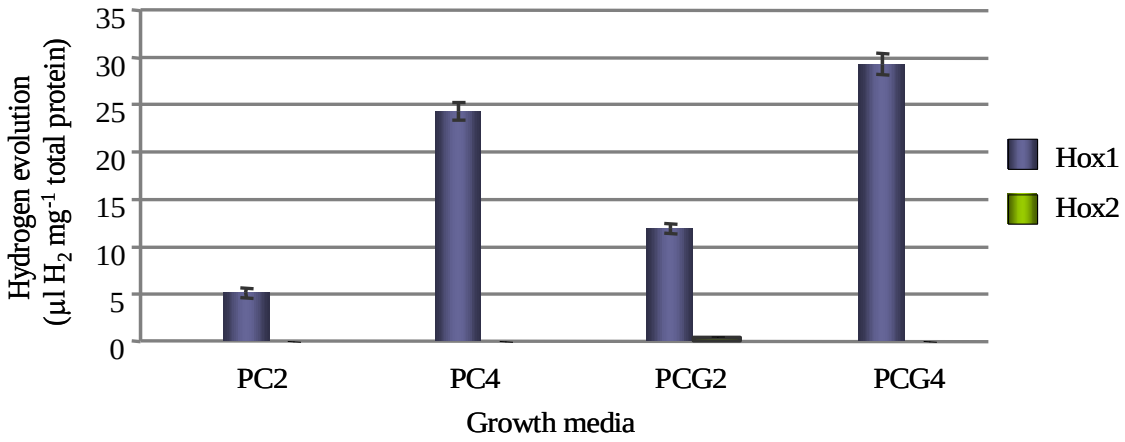

Figure 11. Comparison of *in vivo* hydrogen evolution of Hox1 (GB112141) and Hox2 (GB112131) under various growth conditions. Accumulated hydrogen production was measured at the end of the 8th day of growth.

4.2.5. *In vitro* activity of the Hox2 hydrogenase

In vitro activity of the Hox2 hydrogenase was also examined. GB112131 strain was grown under conditions favouring *in vivo* hydrogen production (PC2G medium). Cells were harvested at the 5th and 10th days of growth and used for the separation of soluble and membrane fractions. Hox2 hydrogenase activity always located in the soluble fraction as it was predicted by the *in silico* analysis of the *hox2* genes. Not only the location but the enzyme activity levels were also independent of the growth phase. Hydrogen evolution capability of the Hox2 hydrogenase in the presence of artificial electron donors is very weak (0.07 μL H_2 h^{-1} mg^{-1} total protein) (Figure 12.), furthermore, hydrogen uptake could not even be detected. *In vitro* NAD^+/$NADP^+$ reducing and NADH/NADPH oxidizing activities of the Hox2 complex were also studied, only NADH-dependent hydrogen evolution could be detected (Table 7.). No hydrogen-uptake coupled NAD^+/$NADP^+$ reduction could be measured.

Strain	NADH dependent H_2 evolution	NADPH dependent H_2 evolution
GB112131	0.053 ± 0.011	0
GB11213141	0	0

Table 7. *In vitro* NADH/NADPH dependent hydrogen evolution measurement of Hox2. Strains are listed in Table 1. Results are given in μL H_2 h^{-1} mg^{-1} total protein.

4.2.6. Comparison of the *in vitro* activities of Hox1 and Hox2

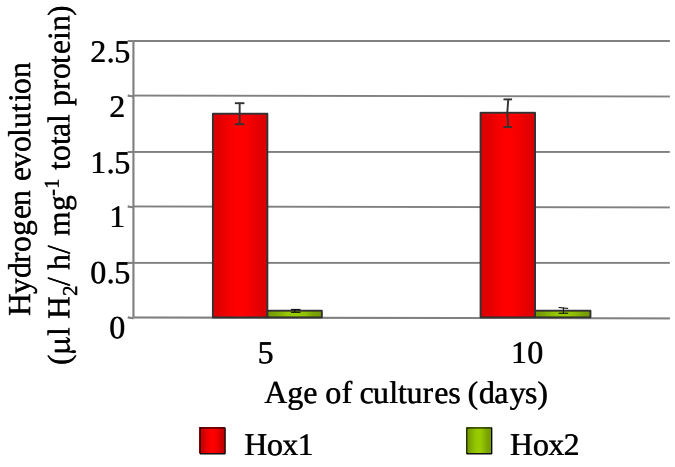

Figure 12. Comparison of *in vitro* methyl-viologen dependent hydrogen evolution rates of Hox1 (GB112141) and Hox2 (GB112131). Soluble fractions prepared on the 5th and 10th days of growth were used.

The *in vitro* evolution activity of Hox1 (1.85 μL H_2 h^{-1} mg^{-1} total protein isolated from cells grown under identical conditions) is orders of magnitude higher than that of Hox2 (Figure 12.).

4.3. Expression analysis of the hox2 genes

Hydrogen evolution and consumption by the Hox2 hydrogenase takes place under specific growth conditions. The concentration of thiosulphate, as the main electron and energy source of the cells, and the attendance of glucose as an additional electron donating component, seems to be the key factors for Hox2 activity. Quantitative PCR analysis was performed to follow the expression levels of the *hox2* genes under various growth conditions, altering the presence and amount of these two components (PC2, PC4, PC2G, PC4G). Expression of the *hox2H* gene, encoding for the Hox2 hydrogenase large subunit, was observed under all conditions and found to be approximately 5-6 orders of magnitude lower than that of 16S rDNA. Significantly higher *hox2H* expression level could be detected in the GB112131 strain when grown under growth condition favouring *in vivo* hydrogen evolution (PC2G) compared to those values from GB112131 grown in PC4, PC2, PC4G (Figure 13.). Interestingly, neither elevated thiosulphate concentration in the presence of glucose (PC4G) nor glucose deficiency (PC2 and PC4) abolished the expression level of the *hox2H* gene completely. However, GB112131 strain lacked *in vivo* hydrogenase activity under these circumstances.

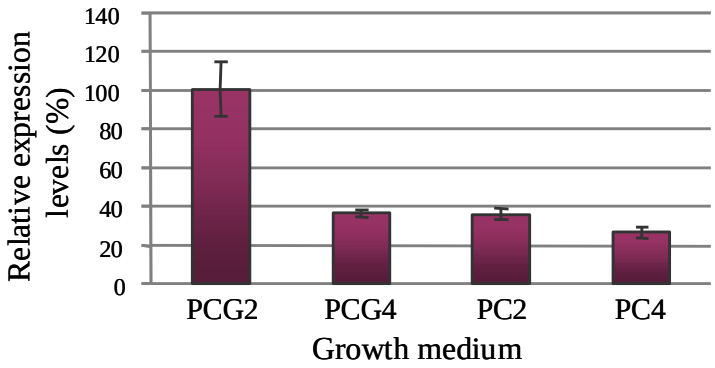

Figure 13. Real-time quantitative PCR analysis of *hox2H* gene expression. GB112131 strain was grown in various media (PCG2, PCG4, PC2, PC4), cells were harvested at the 5th and 10th days of growth (not shown here).

In line with the experimental procedure of the *in vitro* activity measurements cells were harvested at the 5th and 10th day of growth, but no difference could be detected in the expression pattern depending on the actual growth phase (not shown).

Preparation of cDNA for quantitative RT-PCR experiments was performed in two different ways. Reverse transcription was initiated from *hox2H* specific oligonucleotide or by using random hexamers. In the latter case, the expression level of *hox2F* was always considerably higher than that of *hox2H* (Figure 14.). In the presence of glucose (PC2G, PC4G), this difference was 40-45 fold, while in the absence of glucose (PC2, PC4) only 7-8 fold. The same experiments for the two other *hox2* genes resulted in very similar expression level of the *hox2F* and *hox2U* genes. The mRNA level of *hox2Y* and *hox2H* was also alike, pointing towards a dissimilar expression of the diaphorase and hydrogenase dimers.

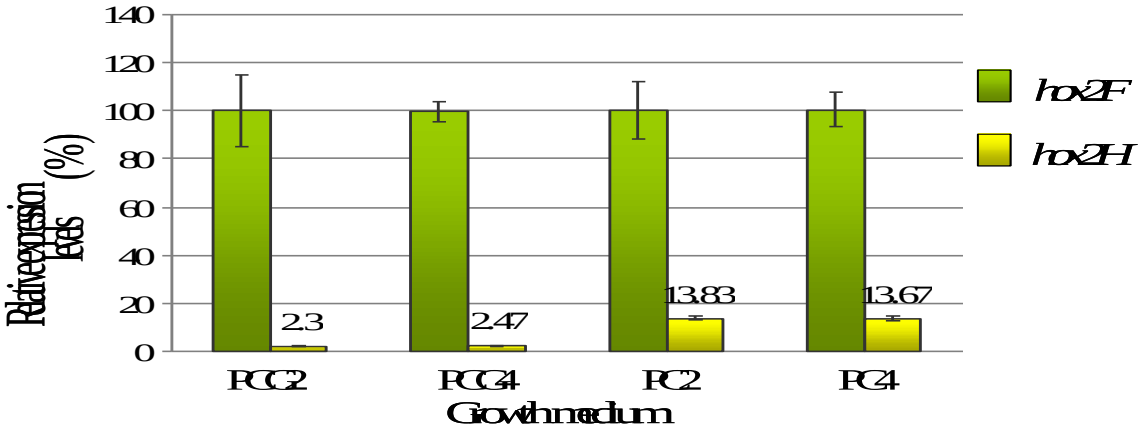

Figure 14. Ratios of *hox2F*/*hox2H* gene expression levels. GB112131 strain was grown in different media (PCG2, PCG4, PC2, PC4). Random hexamers were used for reverse transcription.

This difference did not occur when *hox2H* specific oligonucleotide was used for the initiation of the cDNA synthesis. This cDNA was used as template of a RT-PCR with primers located at the *hox2F* gene (Figure 15.).

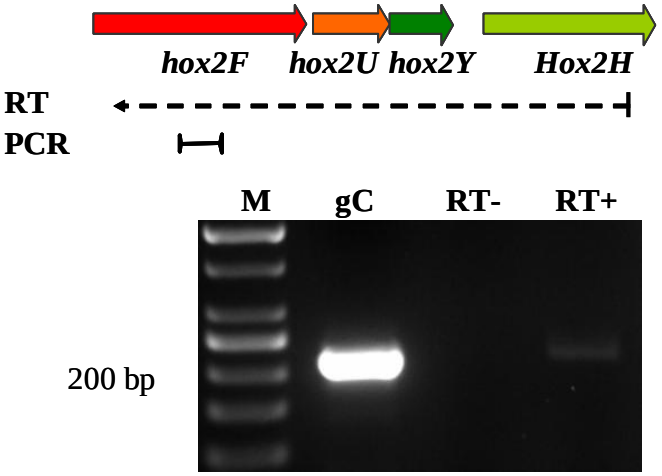

Figure 15. Reverse transcription PCR analysis of the *hox2* operon. Reverse transcription was initiated at the *hox2H* gene, the PCR was performed with primers located at the *hox2F* gene. M: marker; gC: genomic control; RT-: Reverse transcriptase is not present in the reaction mixture; RT+: Reverse transcriptase is present in the reaction mixture.

Since *in vivo* activity of the Hox2 hydrogenase solely occurred under illumination, light dependence of the *hox2* expression level was also measured, but no difference could be detected in cells grown under light and dark conditions (data not shown).

4.4. Comparison of the expression levels of Hox1 and Hox2 hydrogenases

Expression levels of Hox1 and Hox2 hydrogenases were compared, likewise *in vivo* and *in vitro* activities. Contrary to the activity measurements, where different mutant strains were used to follow hydrogen evolution/uptake of the individual hydrogenases, the wild type and the triple mutant GB112131 *T. roseopersicina* strains were used for comparative expressional studies. Cells were grown under the growth conditions used for activity measurements (PC2, PC4, PC2G and PC4G). The upregulation of *hox2H* gene in cells grown in PC2G medium was weaker by 40-45% in wild type strain (BBS) than it was in GB112131 ($Hox2^{+}$). In addition, in PC2G medium, relative to PC4, PC2 and PC4G, expression of *hox1H* gene was upregulated in BBS to similar extent as the *hox2H* gene.
The expression levels of the *hoxFU*, and *hoxYH* genes of the two hydrogenases were also compared (Figure 16.). The diaphorase encoding *hox2FU* and *hox1FU* genes were expressed at very similar level. In case of the genes coding for hydrogenase subunits, much higher expression levels marked the *hox1YH* genes compared to *hox2YH*, which is coherent with the hydrogen production capabilities of the two enzymes.

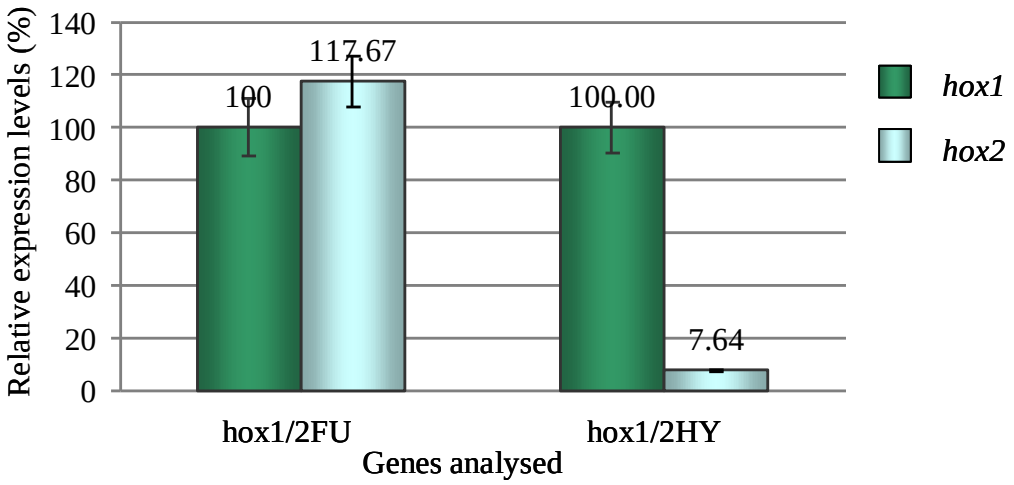

Figure 16. Ratios of *hox1*/*hox2* gene expression levels. BBS strain was grown in PCG2 medium.

5. Discussion

Numerous microbes have been described which synthesize several hydrogenases having similar sequences and structures. Peculiarities of their synthesis, functions and interactions in the cells are still not fully understood. *T. roseopersicina* BBS harbours four functional [NiFe]-hydrogenases and the silent genes of the so-called regulatory hydrogenase. These enzymes were studied in many different aspects. At the genetic level hydrogenase structural and accessory genes were identified, wild variety of mutant strains has been created and used for the exploration of the regulation, maturation, localisation and function of each hydrogenase in this organism.

Hup hydrogenase has been characterised mainly at the gene level. The membrane fraction of the Hyn deficient mutant strain (GB11) was previously used for the demonstration of *in vitro* Hup hydrogenase activity (Kovacs et al., 2005). The role of HupC protein as a membrane anchor and distinct electron transfer subunit of the HupSL dimer has been studied in GB1131 ((*ΔhynSL*, *Δhox1H*) strain. Experimental results are summarized and discussed in this chapter.

Genome sequencing of *T. roseopersicina* disclosed further genes related to hydrogen metabolism leading to the enrichment of its hydrogenase set. Identification and characterisation of Hox2 hydrogenase and comparison of the two Hox-type hydrogenases are also interpreted below.

5.1. Characterization of the HupC protein in T. roseopersicina

HupC protein is encoded in the *hupSLCDHIR* operon, downstream from the *hupSL* genes, encoding for hydrogenase small and large subunits. It shows homology to *b*-type cytochromes which are reviewed as anchor electron transmitters between the hydrogenase dimer and the quinone pool of the respiratory chain (Vignais et al., 2004).

Reduced *in vivo* activity caused by the elimination of the HupC protein in *T. roseopersicina* confirms its postulated electron transfer role, the electron flow from the hydrogenase is blocked in the absence of HupC.

As a premise of the Hup hydrogenase related experiments, optimisation of the growth conditions was performed. Hydrogen metabolism of the cells strongly depends on the nature and quantity of available electron sources (Rákhely et al., 2007, Laurinavichene et al., 2007) and the cooperative function of present hydrogenase enzymes. The relatively high thiosulphate content of the generally used Pfennig medium (4 g L^{-1}), as primary electron source, in the absence of Hyn and Hox1 enzymes and the related hydrogen production (electron consumption), proved to be an inhibitor of the Hup hydrogenase at both the expression, *in vivo* and *in vitro* activity level. These factors might cause the overreduction of the quinone pool of the cells, which makes Hup hydrogenase superfluous, as an electron donating enzyme. Reduction of the thiosulphate content of the medium accommodated Hup expression and activity in the GB1131 strain.

Similarly to the effect of the reduced thiosulphate content of the medium, deletion of the *hupC* gene also upregulated the *hupSL* expression level. It is assumed that the missing HupC is not able to transmit electrons from the HupSL subunits which leads to a more oxidised quinone pool. Increased electron requirement is reflected in a higher expression level of the electron donating Hup hydrogenase. Elevated *hupSL* expression level of the HupC mutant strain is in good agreement with the high *in vitro* Hup hydrogenase activity of the same strain measured in intact cells.

Elimination of *hupC* resulted in the detachment of HupSL from the membrane, however, concomitant increase of the activity could not be detected in the soluble fraction. The lower total activity in the HupC minus cell fraction might be explained by the lower stability of the HupSL enzyme in the absence of HupC in the disrupted and fractionated cells relative to the wild type cells. The stability of HupSL could be restored by the plasmid-borne HupC, however, the majority of the activity remained in the soluble fraction.

Thus HupC is considered as the third subunit of the *in vivo* active Hup complex in *T. roseopersicina*, catalysing the hydrogen dependent reduction of quinones. It is also required for the stability and membrane association of the enzyme.

5.2. Identification and characterisation of the Hox2 hydrogenase in T. roseopersicina

Discovery of the *hox2* genes had been made possible by the genome sequencing project of *T. roseopersicina*. Hox2 represents the last hydrogenase gene set in this organism. The *hox2FUYH* genes encode diaphorase (FU) and hydrogenase (YH) dimers, which are the primary subunits of bidirectional heteromultimeric cytoplasmic [NiFe]-hydrogenases. Reverse transcription coupled PCR experiments revealed transcript harbouring all four genes. *hox2W* was also identified in the genome and is likely to represent the coding region of a Hox2 specific endopeptidase.

Characterisation of Hox2 activity was performed under various growth conditions in order to reveal the *in vivo* role of this particular hydrogenase. Hox2 was not able to evolve/consume hydrogen when cells were grown in original Pfennig medium. This is the reason why GB112131 strain was an appropriate negative control of several previous experiments. However, low but detectable expression level of the *hox2* operon was observed under these circumstances. Thiosulphate is considered as the primary electron source for photochemolithoautotrophic growth, but unambiguously not able to donate electrons for the Hox2 hydrogenase. The initial 2 $g \cdot L^{-1}$ thiosulphate in the medium was established as minimally required concentration for normal cell growth. Pfennig medium containing 2 $g \cdot L^{-1}$ thiosulphate proved to be the good platform to test additional electron rich compounds without the risk for encountering the inhibiting, masking effects of excessive thiosulphate. Glucose was determined as the inducer of Hox2 at both the expression and *in vivo* activity level, but only in the presence of 2 $g \cdot L^{-1}$ thiosulphate. Elevated thiosulphate concentration (4 g·L-1), even in the presence of glucose, extinguished Hox2 mediated hydrogen production and reduced the expression level of the *hox* operon, demonstrating the masking phenomenon caused by the excessive amount of thiosulphate. Therefore, the simultaneous presence of low thiosulphate concentration and glucose is required and sufficient for the *in vivo* activation of Hox2 hydrogenase.

In vivo and *in vitro* activity of the Hox2 hydrogenase and expression pattern of the *hox2* genes were followed during the life cycle of the cells in line with the glucose and thiosulphate concentration of the medium (Figure 17.).

In accordance with the *in vitro* activity, transcripts were also detected at the early growth phase (5th day), indicating the presence of matured, operable Hox2 hydrogenase complex. Delayed *in vivo* hydrogen producing activity (7th day) appeared shortly after the complete consumption of thiosulphate and the simultaneously activated glucose metabolism (6th day).

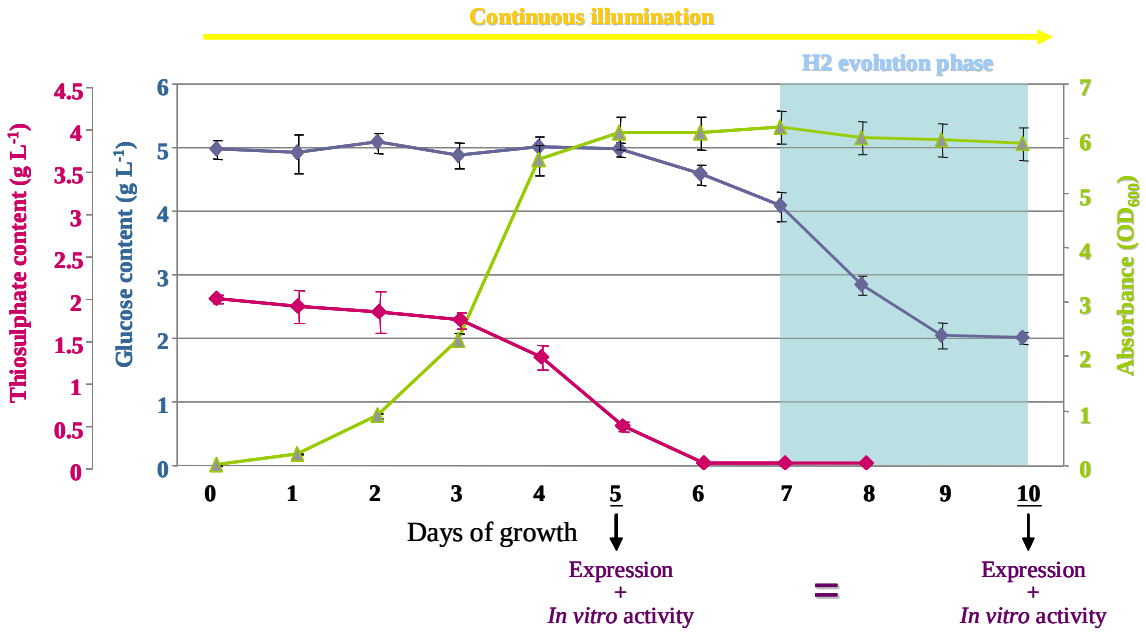

Figure 17. Summary of the experimental results disclosing the *in vivo* function of Hox2 hydrogenase. Cells were cultivated in Pfennig medium containing 2 g·L^{-1} thiosulphate supplemented with 5 g·L^{-1} glucose.

These results indicates the assuption that thiosulphate is the major electron source utilized by the cells until the late the logarithmic growth stage. Glucose metabolism takes over at the stationary phase and donates electrons to the previously accumulated Hox2 hydrogenase.

Light also seems to play a role in this process, since continuous illumination was additionally required for hydrogen evolution catalysed by Hox2, without any effect of light on the transcript level of *hox2* genes.

Comparison of the expression levels of the *hox2* genes in the wild type and triple hydrogenase mutant strains indicated an interplay between the hydrogenases present in the cell. Lower expression level of *hox2* genes was observed in BBS (wild type) as compared to that in GB112131 (*ΔhynSL*, *ΔhupSL*, *Δhox1H*) which might be due to the

presence and concerted action of three additional functional hydrogenases, with special emphasis on the Hox1.

Expression analysis of the ancient *hox2* genes revealed dissimilar expression levels of the diaphorase and hydrogenase encoding genes for the benefit of diaphorase subunits. The significantly higher expression of *hox2FU* relative to *hox2YH* implies the presence of two transcriptional units or distinct stability of the mRNA parts harbouring *hox2FU* and *hox2YH* genes. Similar phenomenon was described in the case of *R. eutropha* Hox hydrogenase (Oelmüller et al., 1990). An unstable *hox2YH* transcript might explain the weak hydrogen producing capability of Hox2 hydrogenase.

Hox2 was shown to be a real bidirectional enzyme located in the cytoplasm. Similarly to the Hox enzyme of *M. capsulatus* Bath, it was shown to utilize only NADH but not NADPH.

It is notable that *hox2FUYH* genes represent the 5^{th} gene set in the genome of *T. roseopersicina* encoding a [NiFe]-hydrogenase. Since the *hupUV* genes are silent in the wild type strain, Hox2 represents the 4^{th} functional [NiFe]-hydrogenase in this organism.

5.3. Comparison of the Hox1 and Hox2 hydrogenases

Discovery of Hox2 did not only raise the number of functional [NiFe]-hydrogenases in *T. roseopersicina* but the concomitant presence of two bidirectional heteromultimeric cytoplasmic [NiFe]-hydrogenases of distinct composition in the same organism is also noteworthy. Sequence analysis of Hox1 and Hox2 hydrogenases revealed unambiguous differences (identity values are shown in Table 6.). Hox1 belongs to the group of cyanobacterial-type Hox hydrogenases (Rákhely et al., 2004) (Table 6.) known as heteropentameric enzymes (HoxEFUYH). HoxE, as the fifth subunit, was suggested to represent a third redox gate of Hox1 in *T. roseopersicina*, since it is essential for the *in vivo*, but irrelevant for the *in vitro* activity of this enzyme (Rákhely et al.,2004). The soluble hydrogenase (SH) of *R. eutropha* shows unique organisation in terms of subunit stoichiometry, two HoxI proteins possibly provide the binding domain for NADPH, thereby creating $HoxFUYHI_2$ (Burgdorf et al., 2004). Hox2 was identified as heterotetrameric hydrogenase in *T. roseopersicina*, composed of a diaphorase (Hox2FU)

and a hydrogenase (Hox2YH) dimer. It shows highest homology to the tetrameric soluble hydrogenase of *M. capsulatus* Bath (Hanczár et al., 2002) and resembles the corresponding subunits of the soluble hydrogenase (SH) of *R.eutropha* H16 (Schneider et al., 1976) (Table 6.).

Diversity of the two Hox-type hydrogenases in *T. roseopersicina* can be observed not only in their structural organisation. The expression level of *hox1YH* was remarkably higher than that of *hox2YH*, in accordance with their hydrogen producing capability. Interestingly, this difference in the expression level of the diaphorase encoding genes was not observed. Both Hox1 and Hox2 were proved to be real bidirectional enzymes, able to produce or consume hydrogen depending on the actual redox state of the cell. They were shown to be able to utilize NADH but not NADPH.

Hox1 enzyme is strongly linked to the sulphur metabolism (Rákhely et al., 2007), while Hox2 is apparently not able to use electrons derived from thiosulphate for hydrogen generation directly. Glucose metabolism proved to be the inducer of *in vivo* Hox2 activity, although this induction is not specific for Hox2; the *hox1* genes were also shown to be upregulated in PC2G medium and Hox1 was also shown to be able to use glucose as electron source for hydrogen production. Therefore, the two Hox enzymes might link the glucose metabolism to distinct bioenergetic pathways: Hox2 simply connects it to the NAD+/NADH housekeeping, while Hox1 might have a more complex role which is to be elucidated in future studies.

In summary Hox2 is the second bidirectional Hox-type hydrogenase in this bacterium, making *T. roseopersicina* the first organism harbouring more than one Hox-type [NiFe]-hydrogenases. Moreover this is the first known organism possessing one four-subunit and one five-subunit NAD^+-reducing/NADH-oxidizing [NiFe]-hydrogenases, both functional.

6. Summary

Multiple [NiFe]-hydrogenases participate in the hydrogen metabolism of the purple sulphur photosynthetic bacterium *Thiocapsa roseopersicina*. These enzymes are connected to various bioenergetic/redox processes of the cells. Functional investigation of the electron transfer subunit (HupC) of the membrane-associated Hup hydrogenase and detailed characterisation of the recently identified Hox2 hydrogenase were in the focus of my PhD work.

The following summarizing statements are concluded based on my results:

I. I created *ΔhupC* in frame deletion mutant strain in *T. roseopersicina* in order to examine the possible role of this protein. HupC was shown to be part of the *in vivo* functional Hup hydrogenase complex, as an electron transfer subunit. It is also required for the stability and membrane association of the Hup enzyme.

II. Lack of the HupC protein resulted in the elevated expression level of the *hupSL* genes. I postulated that in the absence of HupC, the electron transfer, from the Hup hydrogenase to the quinone pool of the cell is blocked, leading to a more oxidised quinone pool which upregulates the expression of the electron-donating Hup hydrogenase.

III. I demonstrated that the amount of the available external electron source (thiosulphate) in the growth medium has a regulatory effect on Hup expression, which is likely realised through the quinone pool.

IV. Further hydrogenase structural and accessory genes were discovered in the genome of *T. roseopersicina* encoding for a bidirectional heteromultimeric cytoplasmic [NiFe]-hydrogenase (Hox2FUYH Hox2) and a supposedly Hox2 specific endopeptidase (Hox2W).

V. I optimized the growth conditions for the *in vivo* Hox2 hydrogenase activity. The simultaneous presence of low thiosulphate concentration and glucose is required and sufficient for the *in vivo* metabolic activation of

the Hox2 hydrogenase. Hox2 mediated hydrogen production appears at early the stationary phase of photomixotrophic growth.

VI. The role of thiosulphate and glucose was investigated by following their concentrations during the life cycle of the cells in line with Hox2 activity and expression pattern. The results indicate that thiosulphate is the major electron source utilized by the cells at the logarithmic growth stage. Glucose metabolism takes over at the stationary phase and donates electrons to the previously accumulated Hox2 hydrogenase.

VII. I proved that Hox2 is a real bidirectional hydrogenase - able to catalyse *in vivo* hydrogen production and consumption. It is located in the cytoplasm and able to catalyse NADH- but not NADPH-dependent hydrogen evolution *in vitro*.

VIII. Expression analysis revealed dissimilar expression of the genes encoding for the hydrogenase (*hox2YH*) and diaphorase (*hox2FU*) dimers of the Hox2 hydrogenase which implies the presence of two transcriptional units and/or distinct stability of the mRNA harbouring the *hox2FU* and *hox2YH* genes. The considerably low level of *hox2YH* transcript may explain the low hydrogen producing capability of Hox2 hydrogenase. In addition, the transcript levels of the *hox2* genes are different in the wild-type and triple (Δ*hyn*,Δ*hup*,Δ*hox1H*) hydrogenase mutant strains indicating an interplay between the hydrogenases present in the cell.

IX. I investigated the structural differences between the two Hox-type hydrogenases representing a heteropentameric and heterotetrameric forms of this group of [NiFe]-hydrogenases. I pointed out the novelty of the simultaneous presence of two functional Hox-type hydrogenases of various subunit composition in an organism.

X. I also observed the functional distinction of these enzymes. Both the hydrogen producing activity of the Hox1 hydrogenase and the expression level of the *hox1YH* genes are considerably higher than that of Hox2/*hox2YH*. Nevertheless, the expression level of the diaphorase encoding genes did nod differ from eachother.

XI. I have shown, that Hox2 gets metabolically active after the start of glycolysis, while Hox1 is able to use both glucose and thiosulphate as electron source for hydrogen production.

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8. Publications

8.1. Publications covering the content of the thesis

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8.2. Other publications

Maróti, G., Rákhely, G., Maróti, J., Dorogházi, E., Klement, E., Medzihradszky, F. K. and Kovács K. L. (2010) Specificity and selectivity of HypC chaperonins and endopeptidases in the molecular assembly machinery of [NiFe] hydrogenases of *Thiocapsa roseopersicina*. *Int. J. Hydrogen Energy* **35**: 3358-3370.

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10. Summary in Hungarian

T. roseopersicina hidrogén anyagcseréjében számos [NiFe]-hidrogenáz játszik szerepet. Ezek az enzimek a sejt különböző bioenergetikai/redox folyamataival állnak kapcsolatban. Munkám során a membránkötött Hup hidrogenáz elektron transzport alegységét (HupC) illetve a szolubilis Hox2 hidrogenázt vizsgáltam. Kísérleteim eredményeit az alábbi állításokban foglalom össze:

1. A HupC fehérje szerepének vizsgálatához *ΔhupC* delációs mutáns törzset készítettem. Bizonyítottam, hogy a HupC elektrontovábbító alegységként része az *in vivo* funkcionális Hup hidrogenáz komplexnek. Emellett szükséges a Hup hidrogenáz stabilitásához és membránhoz való kötődéséhez.

2. A HupC fehérje hiánya hatással van a *hupSL* gének expressziós szintjére. Hiányában feltételezhetően elzáródik az elektronok útja a Hup hidrogenáztól a kinonraktár irányába, az oxidáltabb állapotú kinonraktár pedig pozitívan regulálhatja a Hup hidrogenázt kódoló géneket.

3. Bizonyítottam, hogy a sejtek számára rendelkezésére álló külső elektron forrás (tioszulfát) mennyisége a tápoldatban szintén befolyásolja a *hupSL* gének expresszióját. Ez a regulátor hatás valószínűleg a kinonraktáron keresztül realizálódik.

4. A *T. roseopersicina* genomjában azonosítottam heterotetramer citoplazmatikus [NiFe]-hidrogenáz génjeit (*hox2FUYH*) és egy feltehetően Hox2 specifikus endopeptidázt kódoló gént (*hox2W*).

5. Optimalizáltam a növesztési körülményeket a Hox2 hidrogenáz *in vivo* aktivitásának mérhetőségéhez. Csökkentett tioszulfát koncentráció (2 g L^{-1}) és glükóz egyidejű jelenléte szükséges és elégséges feltételek a Hox2

hidrogenáz mérhető *in vivo* aktivitásának megjelenéséhez. A Hox2 által katalizált hidrogéntermelés fotomixotróf növesztési körülmények között a stacioner növekedési fázisban jelenik meg.

6. Vizsgáltam a tioszulfát és a glükóz Hox2 hidrogenáz aktivitásában játszott szerepét. Kimutattam, hogy a tioszulfát a sejtek által használt elsődleges elektronforrás az exponenciális növekedési fázisban. A stacioner fázisban a glükóz veszi át ezt a szerepet és a glikolízisből származó elektronok használja - a már korábban szintetizálódott - Hox2 hidrogenáz a hidrogén termeléshez.

7. Bizonyítottam, hogy a Hox2 egy valóban két irányban működő hidrogenáz – ami képes az *in vivo* hidrogéntermelést és felvételt is katalizálni. Az enzim a citoplazmában lokalizálódik és képes *in vitro* NADH- (de nem NADPH-) függő hidrogén termelésre.

8. Az expressziós vizsgálatok eredményei a hidrogenáz (*hox2YH*) és diaforáz (*hox2FU*) alegységeket kódoló gének eltérő expressziós szintjét tárták fel, ami két transzkripciós egységre vagy a *hox2FU* és *hox2YH* géneket hordozó mRNS eltérő stabilitására utal. A *hox2YH* transzkript jelentősen alacsonyabb szintje magyarázhatja a Hox2 hidrogenáz alacsony hidrogéntermelő képességét. *A hox2* gének eltérő transzkript szintje a vad típusú és a Δ*hyn*,Δ*hup*,Δ*hox1H* hidrogenáz mutáns törzsekben a jelenlévő hidrogenázok közötti összehangolt működésre utal.

9. Megvizsgáltam a szerkezeti különbségeket a Hox1 és Hox2 hidrogenázok között, melyek a Hox-típusú hidrogenázok csoportjának egy négy és egy öt alegységes tagját képviselik *T. roseopersicina*-ban. Két működőképes, struktúrális felépítésében különböző Hox-típusú hidrogenáz egyidejű jelenléte egy organizmusban mindezidáig egyedülálló.

10. Bemutattam, hogy a Hox1 hidrogenáz hidrogéntermelő aktivitása nagymértékben meghaladja a Hox2 enzimét és a *hox1YH* gének expressziós szintje is jelentősen magasabb mint a *hox2YH* géneké. Ez a különbség a diaforáz alegységeket kódoló gének esetén nem észlelhető.

11. Bizonyítottam, hogy a Hox2 hidrogenáz csak a glikolízis kezdete után válik metabolikusan aktívvá, míg a Hox1 egyaránt képes glükózt és tioszulfátot elektrondonorként felhasználni hidrogéntermelés során.