Insights into the assembly of the NiFe hydrogenase enzymes

Ph.D. Thesis

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Abbreviations

ADP= adenosine di-phosphate
AEBSF= 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride
AMP= adenosine monophosphate
Amp= ampicillin
ATP= adenosine triphosphate
bhr= broad-host-range
CFU= colony forming unit
CIAP (CIP) = calf intestinal alkaline phosphatase
CTAB= N-cetyl-N,N,N-trimethyl-ammonium bromide
DIG= digoxigenin
DMSO= dimethylsulfoxid
DNA= deoxyribonucleic acid
dNTP= deoxynucleotide triphosphate
EDTA= ethylenediaminetetraacetic acid disodium dihydrate
Em= erythromycin
F_{420}= F_{420} coenzyme
GC= gas chromatograph
Gm= gentamycin
H_2ase= hydrogenase
IPTG= isopropyl-beta-D-thiogalactopyranoside
Km= kanamycin
MALDI MS= matrix-assisted laser desorption/ionization mass spectrometry
MALDI-TOF MS= matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry
MBH= membrane bound hydrogenase
mRNA= messenger ribonucleic acid
MS= mass spectrometry
NAD^+= nicotinamide adenine dinucleotide
$NADP^+$= nicotinamide adenine dinucleotide phosphate
NBT= nitroblue tetrazolium
OD= optical density
orf= open reading frame
ox. = oxidized
PAGE= polyacrylamide gel electrophoresis
PCR= polymerase chain reaction
P_i= inorganic phosphate
PIPES= piperizine ethane sulfonic acid
PP_i= inorganic diphosphate
RBS= ribosomal binding site
red. = reduced
RT-PCR= reverse transcription linked polymerase chain reaction
SDS= sodium dodecyl sulfate
SDS-PAGE= sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH= soluble hydrogenase
Sm= streptomycin
Tc= tetracycline
TCD= thermal conductivity detector
Tris= tris(hydroxymethyl)aminomethane
X-phosphate= 5-bromo-4-chloro-3-indolyl phosphate

Introduction

Benefits of the biologically produced hydrogen

Energy is indispensable in all fields of life. The demand for the energy is permanently growing, but the reserves of our primary energy-carriers will be depleted within a few decades (Cammack et al., 2001, Sloan 2003). Today we use various fossil fuels, like coal, crude oil and natural gas, which are available due to prehistoric phototrophic organisms. These sources are derivatives of huge amounts of organic material ever lived on the Earth. Eventually they store the energy derived from the Sun and collected during extremely long period of time. The production rate of fossil fuels is much slower than the rate of their utilization today.

The question is arising, what we should do after consuming all of this stored energy, after using most of the fossil fuels available. There are several conceptions to deal with this problem affecting the sustainable development of the whole human society. Before thinking about the possible solutions, some basic principles have to be considered. First, the new energy carriers have to be environmentally friendly; otherwise the extensive pollution of our planet will have disastrous consequences for the living populations and their habitats on Earth. Present technologies for processing the fossil fuels are mostly harmful for the environment; they should be changed by using clean energy sources. The new energy carrier has to be inexpensive and available in large amount, and has to be storable safely for long time (Kovács et al., 2000). One can find a limited number of potentially suitable energy generation technologies, for example the atomic fission used presently in nuclear power plants. However, the most plausible primary source is the energy of the Sun, which is the largest natural energy source that we may be able to harvest in the foreseeable future. Wind turbines converting the energy of wind into electric power, or the hydroelectric power stations producing “white coal”, solar cells, solar batteries exist, but they are quite expensive and ineffective means to exploit the immense energy arriving from the Sun and some of these technologies are applicable only under specific conditions.

The solar energy can also be converted to hydrogen and some photosynthetic organisms have the capability to produce molecular hydrogen (Benemann 1996). Hydrogen fulfils the

requirements mentioned above, it can be burnt by several methods to generate energy and the only by-product is water (Moy 2003). Certain organisms contain ancient enzymes, which can evolve hydrogen from protons and electrons. These enzymes are the hydrogenases, and, besides hydrogen evolution, they can also consume H_2 depending on the actual metabolic processes in the cell (Cammack et al., 2001). Hydrogenases may become useful catalysts for the production of the clean energy carrier, replacing palladium and platinum for conversion of H_2 to protons and electrons. The enzymes can be immobilized on electrodes and used as enzyme electrodes in the biocatalysis (Morozov et al., 2002). Biosensors may be used in various fields, e.g., monitoring environmental pollution, process controls (Bianco 2002). A major limitation of today's enzyme based technologies is the lack of long-term stability of the enzymes used, although various methods of protein immobilization and stabilization are being developed to overcome this problem (Qian et al., 2002). The use of enzymes, which have a high intrinsic stability, may contribute fundamentally to the stability of the bioconversion systems in general. The multiple potential uses of hydrogenase enzymes give them a massive biotechnological value. The rapid development in molecular biology provides many tools to modify, improve, and shuffle these enzymes for our special purposes (Stemmer 1994). To produce environmentally safe, stable, versatile, and reliable biological systems based on hydrogenase enzymes is a real opportunity, and it opens exciting and promising future prospects for basic research and biotechnological applications.

1. Literature background

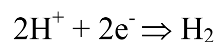
1.1. Hydrogen metabolism in the cell

Hydrogen plays a major role in the energy metabolism of microorganisms. Basically, two enzyme systems participate in hydrogen metabolism, the nitrogenase and the hydrogenase enzymes.

H_2 evolution is an obligate step of the N_2-fixation process, it is carried out by the nitrogenase enzyme itself in order to drive the reaction towards the desired direction of N_2 reduction. Rhizobia oxidize the excess evolved H_2, thereby increasing the metabolic efficiency of anaerobic diazotrophy. Recycling the H_2 evolved by the nitrogenase increases the ATP production and protects the nitrogenase against O_2 under carbon or phosphate limitation (Emerich et al., 1979). The H_2 oxidation process is accomplished by hydrogenase enzyme.

Hydrogenases are key enzymes in hydrogen metabolism, these ancient enzymes also occur in many organisms without N_2-fixing capability. Many of these enzymes do not only consume but can also evolve molecular hydrogen *in vivo*.

Hydrogenases are metalloenzymes catalyzing the oxidation of molecular hydrogen into protons and electrons, and the reverse reaction, the reduction of protons (Vignais et al., 2001, Vignais and Colbeau 2004, Cammack et al., 2001).


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

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

In many cases a single strain possesses several different enzymes involved in hydrogen activation. For example in cyanobacteria up to three enzymes participate in these processes: the nitrogenase, which produces hydrogen during N_2-fixation, the membrane-bound hydrogenase, which reoxidizes the hydrogen, and a bidirectional hydrogenase catalyzing both the oxidation of molecular hydrogen and the reduction of protons (Tamagnini et al., 2002). In filamentous cyanobacteria, net hydrogen production is mainly due to the nitrogenase in the heterocysts and is linked to the reduction of nitrogen to ammonium. However, e.g., *Anabaena* cells oxidize the hydrogen with the uptake hydrogenase via the oxyhydrogen (“Knallgas”) reaction (Amend and Schock 2001).

1.2. Hydrogenase enzymes

1.2.1. Occurrence and physiological role of hydrogenases

Most of the hydrogenases are able to catalyze both H_2 uptake and evolution reactions at least *in vitro*. The *in vivo* roles of the hydrogenases are different depending on the metabolic processes of the host organism, which are related to the hydrogenase function. Usually they prefer to catalyze either the uptake or the evolution of hydrogen *in vivo*. Hydrogenases related to nitrogen fixation catalyze only the oxidation of the hydrogen produced as by-product by the nitrogenase complex.

Hydrogenases, being very ancient enzymes, are harboured by several bacteria and archaea. These enzymes can be localized in the cytoplasm or can also be attached to the cell membrane. They play roles in different metabolic processes in the different organisms. There are hydrogenases in strict anaerobic and facultative anaerobic strains (Vignais et al., 2001). The hydrogen taken up by hydrogenases can be the sole energy source in certain cases (Buhrke et al., 2004), but mostly hydrogen serves as an electron source for the various energy conserving processes like respiration, or photosynthesis. The hydrogenases reduce various cofactors (NAD^+, $NADP^+$, FMN, quinones), which are indispensable sources for the reductive processes in the cell (Vignais et al., 2001). Electron acceptors can be simple oxidized molecules, like sulfates, nitrates, and several organic substrates, e.g., formate and fumarate. Anaerobic, fermentative bacteria usually produce H_2 in order to eliminate the excess electron pool. In the case of enterobacteria H_2 can be produced by the formate-hydrogen lyase enzyme complex using formate as electron donor (Sawers et al., 1985, Andrews et al., 1997).

It is now clear that hydrogenases ([FeFe] hydrogenases) are widely distributed among algae (Horner et al., 2002). Hydrogenases of eukaryotes are usually localized in organelles like mitochondria or chloroplasts. These unique enzymes have thus moved from the margins of eukaryotic biology to become the focus of intense speculation and interest.

The activity of the hydrogenase enzymes can be determined by several methods. The uptake activity measurements are based on redox reactions, artificial redox dyes are used to quantitate spectrophotometrically the redox changes caused by an active hydrogenase enzyme

(Benemann 1996). Methyl-viologen, benzyl-viologen and methylene-blue are the most often used electron acceptors. Hydrogen evolution can be followed by gas chromatography equipped with thermal conductivity detector (TCD) in both *in vitro* and *in vivo* experiments (Vignais et al., 2001). For *in vitro* measurements reduced redox dyes are used as artificial electron donors while *in vivo* the metabolizing cells produce the electrons. Other common method for hydrogenase activity determination is measurement of the hydrogen-deuterium (H-D) exchange (Vignais et al., 2000).

1.2.2. Classification of hydrogenases

Hydrogenase enzymes can be distinguished by several properties. They differ in their physiological role and localization, as it was discussed above, but the basic principle in the classification is the structure, the metal content of the active centre of the enzymes.

There are hydrogenases without any redox active metals in the catalytic centre, some Archaea contain such enzyme named as H_2-forming methylenetetrahydromethanopterin dehydrogenase (Hmd) (Hartmann et al., 1996). These are unusual homodimeric hydrogenases, which harbour a low molecular mass cofactor.

The [FeFe] hydrogenases contain only iron in their active centre (Cammack et al., 2001). There are different [FeFe] hydrogenases, some of them are monomeric, but others are oligomeric. The active site, named as H-cluster, consists of a binuclear [FeFe] center bound to a [4Fe-4S] cluster by a bridging cysteine and attached to the protein by four cysteine ligands (Adams 1990). The H-cluster of the [FeFe] hydrogenase of *Desulfovibrio desulfuricans* is composed of a typical [4Fe-4S] cubane bridged to a binuclear active site Fe center containing putative CO and CN^- ligands and one bridging 1,3-propanedithiol molecule (Nicolet et al., 1999). The monomeric [FeFe] enzymes usually possess additional redox domains containing mainly iron-sulfur clusters. The smallest [FeFe] hydrogenases have been found in green algae. The clostridial type [FeFe] hydrogenases are larger; they contain three additional domains in addition to the H-cluster: at the N terminus a [2Fe-2S] plant ferredoxin-type domain, a unique [4Fe-4S] domain, and a second [4Fe-4S] unit (Peters et al., 1998). The largest catalytic subunit known so far (ca. 130 kDa) is a putative monomeric hydrogenase from the anaerobic eukaryote *Nyctotherus ovalis* (Van Hoek et al., 2000). It possesses all regions known from clostridial-type [FeFe] hydrogenases plus a C-terminal extension, whose parts are homologous to the NuoE and NouF subunits of the NADH-ubiquinon

oxidoreductases. [FeFe] hydrogenases are very versatile with respect to electron donors and acceptors. For example the periplasmic dimeric [FeFe] hydrogenase from *Desulfovibrio vulgaris* reduces cytochrome c_3 (Adams 1990), while the tetrameric cytosolic [FeFe] hydrogenase from *Desulfovibrio fructosovorans* utilizes NADP (Malki et al., 1995).

The [NiFe] hydrogenases compose the largest group (Vignais et al., 2001). The catalytic core of these enzymes is a heterodimeric protein; there are one Ni and one Fe atom in the active centre. A subgroup of the [NiFe] hydrogenases also contains selenium, as selenocysteine coordinated to the nickel atom (Sorgenfrei et al., 1993). The large subunits of the [NiFe] hydrogenases contain two highly conserved regions in the N- and C-terminal regions. Four cysteines can be found in these conserved motifs (at the N-terminal region R-G-[LIVMF]-E-$x_{(15)}$-[QESM]-R-x-**C**-G-[LIVM]-**C**-$x_{(3)}$-H and at the C-terminus [FY]-D-P-**C**-[LIM]-[ASG]-**C**-$x_{(2,3)}$-H/R). The cysteines coordinate the metal atoms in the binuclear centre. These conserved motifs are highly characteristic for the groups of [NiFe] hydrogenases. This classification corresponds to the cellular function and localization of the different [NiFe] hydrogenases. In addition to the basic heterodimeric, additional subunits can be attached to the hydrogenase part, forming an enzyme complex. These subunits usually link the enzyme to the redox processes in the cell (e.g., diaphorase subunits) and/or have electron transferring roles. The [NiFe] hydrogenases have been classified into 4 subgroups (Vignais et al., 2001):

1. Membrane-associated respiratory uptake [NiFe]-hydrogenases
    - Membrane-bound periplasmically oriented [NiFe]-hydrogenases
    - Periplasmic soluble [NiFe]-hydrogenases of sulfate reducers
    - Membrane-bound archaeal uptake [NiFe]-hydrogenases
2. Cytoplasmic heterodimeric [NiFe]-hydrogenases
    - H_2-uptake hydrogenases of cyanobacteria
    - H_2-signaling (sensor) [NiFe]-hydrogenases
3. Cytoplasmic heteromultimeric reversible [NiFe]-hydrogenases
    - F_{420}-reducing [NiFe]-hydrogenases of methanogens
    - Tetrameric bifunctional [NiFe]-hydrogenases of hyperthermophiles
    - Methyl viologen-reducing [NiFe]-hydrogenases
    - Bi-directional NAD-linked [NiFe]-hydrogenases
4. Membrane-associated H_2-evolving respiratory [NiFe]-hydrogenases

1.2.3. [NiFe] hydrogenases

The core of the [NiFe] hydrogenases necessary for the hydrogen evolution/oxidation consists of two subunits, the small and the large subunit (Figure 1.). In the case of the membrane-bound enzymes, the small subunit is attached to the membrane, while the large subunit mostly localized in the periplasm. There are exceptions, for example the *Escherichia coli* hydrogenase 3 is membrane-associated, but the large subunit is in the cytoplasmic side of the membrane (Sauter et al., 1992). Usually the periplasmic hydrogenases work towards the oxidation of molecular hydrogen. Most diazotrophic organisms possess such a hydrogenase enzyme (Brito et al., 1997, Vignais and Colbeau 2004). This arrangement has significance in the energy conservation of the cell (Kovács and Bagyinka, 1990). The [NiFe] hydrogenases localized in the cytoplasm are bidirectional enzymes, and can be linked to the various metabolic processes like aerobic and anaerobic respiration, photosynthesis (Schmitz et al., 1995, Tamagnini et al., 2002). There is a special type of the cytoplasmic [NiFe] hydrogenases in *Rhodobacter capsulatus* and *Ralstonia eutropha*, which can sense the presence of hydrogen in the cell, and can regulate the expression of the other hydrogenases through a signal transduction cascade (Lenz and Friedrich 1998, Elsen et al., 2003).

The [NiFe] hydrogenase large subunit is about 64 kDa; it contains the binuclear metallocenter, which is the active site of the enzyme. The X-ray crystallographic studies of [NiFe]-hydrogenases isolated from *Desulfovibrio gigas* (Volbeda et al., 1995, 1996), *D. vulgaris* Miyazaki (Higuchi et al., 1997), *D. fructosovorans* (Volbeda et al., 2002), *Desulfomicrobium norvegium* (formerly *Desulfomicrobium baculatum*) (Garcin et al., 1999) and *D. desulfuricans* ATCC 27774 (Matias et al., 2001) have revealed the unique binuclear [NiFe] active site of these enzymes. The nickel atom is coordinated by four cysteinate-sulfur atoms, two of which bridge to the iron atom (in the *D. norvegium* enzyme, a selenocysteine instead of a cysteine coordinates the Ni). In the aerobically isolated inactive enzyme, there is an additional μ-oxo or hydroxo (Garcin et al., 1999) or sulfido group (Higuchi et al., 1997; Matias et al., 2001) bridging the Ni and Fe atoms. In addition, as demonstrated by crystallography and spectroscopy, non-protein diatomic ligands bind to the iron atom. The normally poisonous ligands, two CN^- and one CO (Volbeda et al., 1996; Happe et al., 1997; Pierik et al., 1999), or SO, CO and CN^- in *D. vulgaris* (Higuchi et al., 1997) are additional unique features in the exceptional [NiFe] active center. The FTIR and EPR properties of the metallocenter of the cytoplasmic NAD-reducing hydrogenase of *R. eutropha* (SH) suggested the presence of two additional CN^- ligands, so that SH may have a $Ni(CN)Fe(CN)_3(CO)$ at its

active site (Burgdorf et al., 2005). Although the [NiFe] and the [FeFe] hydrogenases have completely different structures and are evolutionary unrelated, they share a common feature, namely the presence of endogenous CO and CN^- ligands bound to the Fe atom in the active site. The presence of these ligands stabilizes iron in a low oxidation and spin state and makes

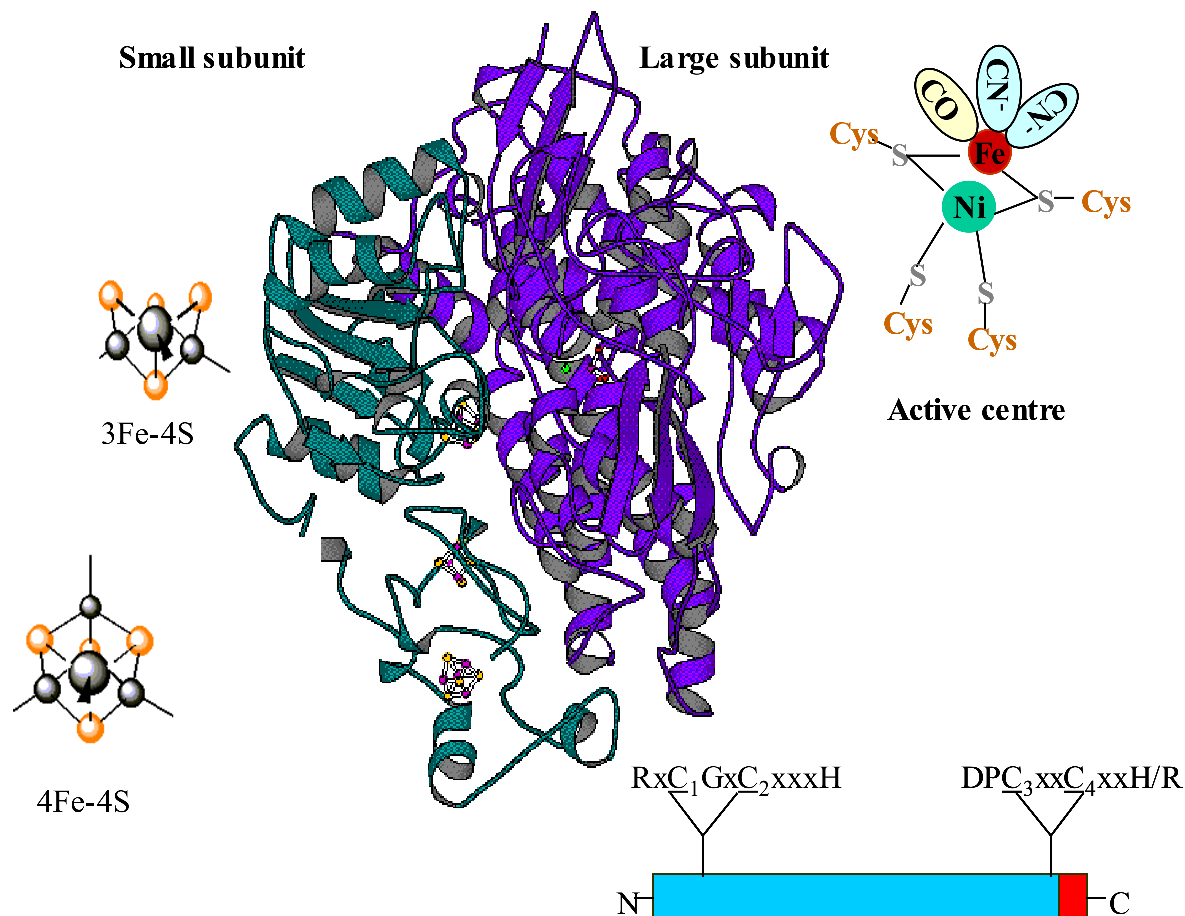


Figure 1. Crystal structure of the [NiFe] hydrogenase of *Desulfovibrio gigas*. The large subunit contains the active centre (magnified), the Fe and Ni atoms are coordinated by four cysteines. The small subunit possesses the [Fe-S] clusters.

it similar to the complexed transition metals (Ru, Pd or Pt) known to be good catalysts for H_2 splitting (Adams and Stiefel, 2000). Another common feature is the presence of a [4Fe-4S] cluster proximal to the binuclear metallocenter, which is then wired to the surface for electron exchange with its redox partners by a conduit of iron-sulfur clusters of the small subunit. The small subunit of 30 kDa holds the conserved [4Fe-4S] cluster located in the proximity of the active site. One or two additional [Fe-S] clusters usually exist on small subunits. These clusters are responsible for transferring electrons to the surface of the enzyme, and are thought to interact with redox partners.

Most of the hydrogenases are very sensitive to oxygen. Well-characterized exceptions are the regulatory hydrogenases (HupUV in *R. capsulatus*, HoxBC in *R. eutropha*) (Vignais et al., 1997, Buhrke et al., 2004) and the SH enzyme (HoxFUYH) of *R. eutropha.* The latter enzyme contains three CN^- ligands in its active centre (Burgdorf et al., 2005). This oxygen resistance is explained with the unique organization of the centre, which probably renders the hydrophobic gas channels inaccessible for the oxygen. The structural information are very useful to identify amino acid residues to be modified to make the enzyme more efficient, stable, and less oxygen sensitive (Szilágyi et al., 2002).

1.3. Biosynthesis of hydrogenases

1.3.1. General properties

Considering the complexity in function and localization, the structural sophistication of hydrogenases and their active sites, it is not surprising that a number of gene products are required for the biosynthesis of these enzymes, in addition to the structural polypeptides. In the case of the [FeFe] hydrogenases only a few such putative genes were identified until now (Posewitz et al., 2004). In contrast, the frequent genomic clustering of the genes involved in [NiFe] hydrogenase synthesis and maturation has facilitated their identification in several cases (Vignais et al., 2001). Of course there are few exceptions, where these additional genes are scattered in the genome (Maróti et al., 2003).

1.3.2. Accessory proteins participating in the [NiFe] hydrogenase maturation process

The formation of an active [NiFe] hydrogenase requires at least 10 different accessory proteins. Some of the auxiliary proteins are needed for the maturation of all [NiFe] hydrogenase enzymes in the cell, these are the pleiotropic accessory proteins (*hyp*-hydrogenase pleiotropic), while others are specific for only one hydrogenase in the host organism. The HypB, HypD, HypE and HypF proteins are always essential in all organisms

harbouring [NiFe] hydrogenase enzymes, whereas the specificity of the HypA, HypC and HypX is questionable.

1.3.2.1. Hyp accessory proteins

HypA is only required for the synthesis of hydrogenase 3 of *E. coli*, and seems to have an inhibitory or repressor role on hydrogenase 2 activity (the uptake hydrogenase encoded by the *hyb* operon) (Hube et al., 2002). The HybF protein, encoded in the *hyb* operon, is highly homologous to HypA (52% identity) and may play a similar role in the synthesis of hydrogenase 2 as HypA does in the synthesis of hydrogenase 3 (Hube et al., 2002). Addition of excess Ni to the medium restores hydrogenase activity of *hypA* mutants both in *E. coli* and *Helicobacter pylori* (Olson et al., 2001). From these experiments it has been concluded that HypA may have a role in Ni metabolism/insertion. HypA, predicted from homologous *hypA* genes, in six different bacterial species contain the conserved $Cx_2Cx_{12}Cx_2C$ cysteine cluster (Mehta et al., 2003). The CxxC motif is a characteristic [Fe-S] cluster coordinating domain, suggesting that HypA contains a [Fe-S] cluster. Amino acid substitution mutants affecting the cysteine cluster will contribute to the elucidation of its function.

All HypB proteins have a conserved GTP-binding motif at the carboxyl-terminus, and GTPase activity has been shown essential for Ni insertion (Maier et al., 1995, Casalot and Rousset 2001). Some organisms, such as *Rhizobium leguminosarum*, *Azotobacter vinelandii* and *Bradyrhizobium japonicum*, have evolved a second - nickel storing - function for HypB (Rey et al., 1994, Garg et al., 1994, Olson et al., 1997). This function is accomplished by histidine-rich domains, which are present at the amino-terminus (24 histidines within a 39 amino acid long stretch). Hence, this protein is involved in nickel binding and accumulation, and utilizes energy (GTP) to mobilize nickel for its subsequent incorporation into hydrogenase. The “Ni binding and accumulation” function in *E. coli* is accomplished by a separate accessory protein named SlyD (Zhang et al., 2005). Hydrogenase activity in *hypB* mutants of *A. vinelandii*, *H. pylori* and *E. coli* could be restored by the addition of excess Ni to the medium. HypB from *R. leguminosarum* and *B. japonicum* were purified by nickel-affinity chromatography, and were demonstrated to bind Ni^{2+} ions: 4 and 9 per monomer, respectively (Rey et al., 1994, Fu et al., 1995).

HypC proteins are intensively studied. These small chaperon-like proteins are needed for functional hydrogenase enzymes. HypC-s have been assumed to act as chaperones,

maintaining the large subunit in a conformation accessible for metal insertion. The N-terminal M-C-[LIV]-[GA]-[LIV]-P-x-[QKR]-[LIV] motif is highly conserved among the HypC-s (Magalon and Böck 2000). The first methionine is removed by methionine aminopeptidase, so the Cys2 becomes the first amino acid in the biologically active protein (Magalon and Böck 2000). This cysteine is essential in the interaction with both HypD and the large subunit (Blokesch and Böck 2002). A detailed analysis of the HypC-preHycE complex in *E. coli* by site-directed mutagenesis has revealed that Cys241 from HycE, which is a nickel ligand, and Cys2 from HypC were involved in the formation of this complex (Drapal and Böck 1998). This interesting result suggests that Cys241 is not available for nickel coordination as long as the complex persists.

HypD contains four conserved cysteines, two of them in a CxxC arrangement. The HypD of *E. coli* is a [Fe-S] protein (Blokesch et al., 2004). It was proven to interact with HypC and later also with HypE during the maturation of hydrogenase-3.

HypE from *E. coli* was shown to be able to hydrolyze ATP to ADP + P_i. HypE first interacts with HypF, and the last amino acid (Cys) of HypE is carbamoylated (Blokesch et al., 2004). The carbamoyl ligand is then dehydrated in an ATP dependent manner by HypE. The emergent CN^- group is transferred to iron through a HypC-HypD-HypE complex (Figure 2.).

HypF is one of the most studied accessory proteins. It has the perfect consensus pattern of acyl-phosphatases (Paschos et al., 2002) and a sequence motif (VXHHXAH) that is present in proteins catalyzing O-carbamoylations. These intriguing features led to the discovery that the CO and CN^- ligands might derive from carbamoylphosphate, as *E. coli carAB* deletion mutants, which are defective in carbamoylphosphate synthesis, produced unprocessed hydrogenase enzymes. However, most recent results suggest that the biosynthetic routes of the CO and CN^- ligands are different. The CN^- groups derive from carbamoylphosphate catalyzed by the HypF-HypE complex in the presence of ATP (Blokesch et al., 2004). The CO derives from acetate as it was shown by ^{13}C labeling infrared studies and ^{14}C labeling experiments with overproduced *E. coli* Hyp proteins (Roseboom et al., 2005). It is not clear, which Hyp protein is involved in the formation of CO. One candidate is the HypD with its [4Fe-4S] cluster (Blokesch et al., 2004). HypF also contains two zinc-finger motifs (Paschos et al., 2002). It is therefore possible that the chaperone domain of HypF interacts with the large subunit precursor and that the acyl-phosphatase and the carbamoylphosphatase domains synthesize and/or insert the CN^- ligands in the active-site cavity. It is to note, that in *R. eutropha* two HypF proteins are present, but only the $HypF_1$ was shown to participate in the [NiFe] active site biosynthesis (Jones et al., 2004).

HypX (HoxX in *R. eutropha*) has been proposed to participate in the generation and transport of the CO and CN^- ligands (Buhrke and Friedrich 1998). Sequence comparison revealed that the amino-terminal half of the protein is highly homologous to tetrahydrofolate (FH_4)-dependent enzymes, which are responsible for carrying C1 molecules. It has therefore been assumed that the formimino (---CHNH) and the formyl (---CHO) groups, which are carried by the N5-formimino-FH_4 and the N5-formyl-FH_4, might be CN^- and CO ligand precursors. However, it is worth noting that *hypX* is not present in all hydrogen-metabolizing bacteria, HypX was only found in *R. leguminosarum* (Rey et al., 1996), and *R. eutropha* (Buhrke and Friedrich 1998). No HypX homolog has been found in other proteobacteria, including *E. coli*. The *hypX* deletion mutants have a reduced but still detectable level of hydrogenase activity (Buhrke and Friedrich 1998). Thus, HypX might not be an essential factor in the maturation process.

1.3.2.2. Specific endopeptidases

Probably the most studied specific accessory proteins are the endoproteases, which play very important role in the anchoring of the metallocenter (Fritsche et al., 1999). Each hydrogenase, except the regulatory hydrogenases, which lack such C-terminal extension, has its own specific endoprotease. The endoproteolytic cleavage is the final step in the processing of the large subunit. After nickel has been transferred to the active site, the endopeptidase removes a C-terminal extension from the large subunit (Rossmann et al., 1994, Magalon et al., 2001) triggering a conformational change, which internalizes the metal center. It was shown in the case of the HycI endopeptidase, processing the *E. coli* hydrogenase 3, that the nickel in the precursor of the large subunit serves as a recognition motif and it has been postulated that the endopeptidase forms a substrate complex with the nickel-containing large subunit by interacting with the metal. Thus, Ni insertion is a prerequisite for the recognition of preHycE (*E. coli* hydrogenase-3) by HycI (*E. coli* hydrogenase-3 specific protease). Interestingly, two third of the C-terminal extension of the preHycE could be truncated without any changes in the maturation process. However, exchanging the C-terminal extension of preHycE to the C-terminal extension of preHybC (hydrogenase-2) abolished processing (Theodoratou et al., 2000).

1.3.2.3. Hup (Hox)-type accessory proteins

There are several additional proteins participating in various steps of the maturation process. These proteins are mostly specific for only one hydrogenase in the host organism. Some of them are present only in a few bacteria, and their role is not always well understood. Mostly in diazotrophic bacteria there is a hydrogen-consuming, membrane-bound, periplasmically oriented [NiFe]-hydrogenase enzyme, named HupSL (HoxKG in *R. eutropha*). The HupSL enzyme requires the above-mentioned essential Hyp proteins and a specific endopeptidase for the maturation. In addition, there are some additional specific proteins participating in the biosynthesis of these enzymes.

The HupD proteins are homologous to other endopeptidases, while the HupF (HoxL in *R. eutropha*) proteins show strong sequence homology to HypC-s. In *R. leguminosarum, R. capsulatus* and *B. japonicum* the HupSL hydrogenase is the only hydrogenase, but there are two homologous small chaperon-type proteins in all of them, the HypC and the HupF (Rey et al., 1993, Colbeau et al., 1993, Fu and Maier 1994).

The presence of the *hupE* gene is limited to a few organisms (*R. leguminosarum, Azotobacter chroococcum and Methylococcus capsulatus* (Du et al., 1992, Csáki et al., 2001). The function of its predicted product is unknown, but it has been proposed that it might act as a nickel transporter in *R. leguminosarum* (Baginsky et al., 2002).

The gene of the HupG (HoxO) putative protein is present in a few organisms (Colbeau et al., 1993, Fu and Maier 1994). Deletion of the *hupG* gene abolished the membrane-bound hydrogenase activity in *R. eutropha* (Bernhard et al., 1996). The *E. coli* homologue HyaE was shown to interact with the Tat-signal peptide-bearing small subunit of hydrogenase-1 (HyaA) (Dubini and Sargent 2003).

Another putative protein is the HupH (HoxQ), the role of this protein is unclear. A mutation of the *orf* resulted in the activity loss of the HoxKG hydrogenase of *R. eutropha* (Bernhard et al., 1996).

HupI (HoxR) showed strong identity to rubredoxin and rubredoxin-like proteins from many other bacteria. The HupI proteins contain two CxxC motifs, which may serve as iron ligands for non-heme iron proteins involved as intermediate electron carriers or in the assembly process for [Fe-S] (or [NiFe]) clusters (Fu and Maier 1994).

Deletion of the gene of the HupJ (HoxT) slightly reduced selectively the membrane bound hydrogenase activity in *R. eutropha* (90% of the wild-type activity remained), but this mutant was able to grow lithoautotrophically in the presence of the soluble hydrogenase (SH,

HoxYH) only. Thus some role in the electron transport is supposed for this putative protein. The *E. coli* homologue HybE interacts with the small subunit of hydrogenase-2 (HybO), which enhances the proposed electron-transporting role of this gene product (Dubini and Sargent 2003).

The examination of the HupK (HoxV) protein led to interesting and somewhat controversial results. Usually, the gene coding for this protein is the last member of the *hup* operon. The deletion of the gene resulted in the complete hydrogenase activity loss in *R. leguminosarum* (Brito et al., 1994), but in *R. eutropha* the activity of the membrane bound hydrogenase (MBH, HoxKG) in the *ΔhoxV* mutant strain was reduced to 30% only (Bernhard et al., 1996). The HupK protein shows sequence similarity to the large subunit of [NiFe] hydrogenases of different organisms (Imperial et al., 1993). The homology is limited to the amino- and carboxyl-termini, and includes zones surrounding the conserved cysteine pairs in the large subunits. The strongest homology is at the C-terminus, but the first cysteinyl residues are exchanged to phenylalanine at both termini. Amino acids involved in the coordination of the diatomic ligands are also present in the putative HupK proteins (Casalot and Rousset 2001). Due to the similarity to the large subunits, it was proposed that HupK proteins have a scaffold function in the assembly of the active centre of hydrogenases.

1.3.3. Biosynthesis of the [NiFe] active centre

The complex assembly of the [NiFe] active centre, embedded in the large subunit, is the subject of a maturation process. The subunits, coded by the structural genes, have to undergo different modification processes mediated by some of the accessory proteins listed above. 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). Of course there are differences in maturation among the various organisms, but the principles and some key proteins are similar in each [NiFe] hydrogenase containing organism. Therefore, a maturation model was set by the extensively studied hydrogenase 3 of *E. coli* (Maier and Böck 1996, Magalon and Böck 2000, Blokesch et al., 2001, Blokesch and Böck 2002). The first step is the synthesis of the large subunit (HycE) as a precursor protein (pre-HycE) together with an extension at the carboxyl-terminus. After insertion of all components of the metallocenter, in the final step of the large subunit maturation an endopeptidase removes the C-terminal extension from the

precursor of the large subunit (Rossmann et al., 1995, Theodoratou et al., 2000, Magalon and Böck 2000).

The metallocenter assembly of hydrogenase 3 requires a number of accessory proteins. The *hyp* gene products, namely HypA, HypB, HypC, HypD, HypE, HypF take part in this process in *E. coli*.

The earliest step identified to date is the formation of a complex between HypC and HypD (containing a [Fe-S] center) (Blokesch and Böck 2002, Blokesch et al., 2004). The N-terminal cysteine of HypC is essential in this interaction. Building of the active site is likely to continue with the insertion of the three diatomic ligands, as the CN^- and CO molecules are stabilized by amino acids that are deeply buried in the active-site cavity. This hypothesis is supported by the observation that the large subunit precursor produced under nickel starvation conditions could be matured *in vitro* by nickel addition (Rossmann et al., 1994), indicating that the Fe-CO-$(CN)_2$ complex was already inserted in this precursor. The liganded Fe atom is transferred to the immature large subunit (pre-HycE) from the HypC-HypD-HypE complex (Figure 2.). During these processes there is another complex between the HypC and the precursor form of the large subunit (Magalon and Böck 2000), 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. In the *E. coli* hydrogenase-3 (HycGE) model, the maturation processes of the small and large subunits are independent, and the subunit oligomerization is the last step of the entire hydrogenase biosynthesis process.

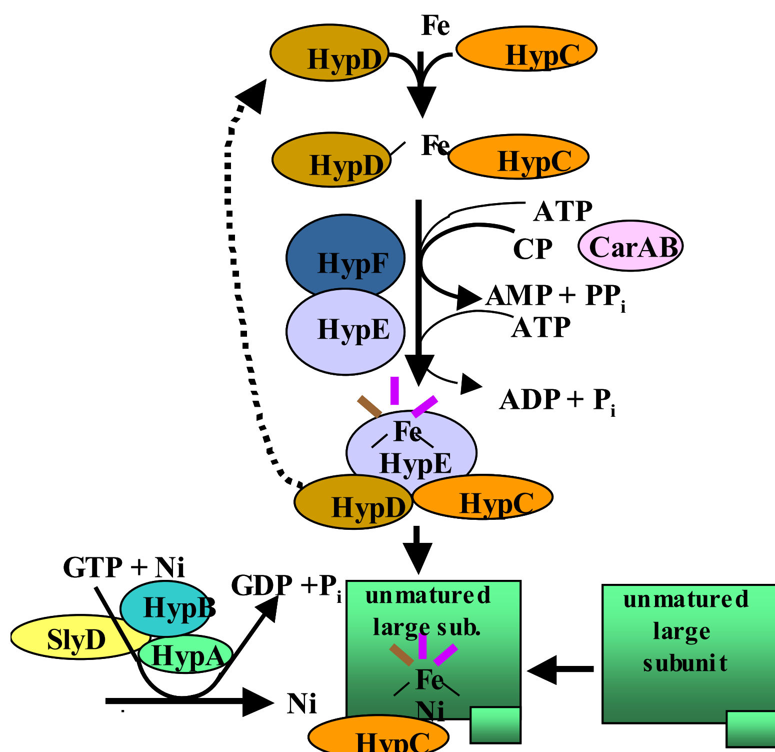


Figure 2. Assembly of the NiFe active centre of the hydrogenase-3 of *E. coli*

1.3.4. Biosynthesis of the small subunit

The main role of the small subunit of the [NiFe] hydrogenases is the electron transfer, which is mediated by [Fe-S] clusters. Little is known about the maturation of the small subunit compared to our knowledge regarding the large subunit. Probably the biosynthetic processes of the two subunits are completely independent from each other (Thiemermann et al., 1996, Massanz et al., 1998, Magalon and Böck 2000). The assembly of the [Fe-S] clusters is the key for answering the open questions of the maturation of the small subunits. Up to 10 proteins may be required, the best-known ones are the NifU-like and NifS-like proteins, which are involved in [Fe-S] and sulfide mobilization (Frazzon et al., 2002). The subcellular localization of these processes in prokaryotes and eukaryotes is currently in the focus of active research.

1.3.5. Transport of the [NiFe] hydrogenases

After assembling the active centre in the large subunit and the redox relay of the small subunit, the independently matured subunits dock to each other, and the hydrogenase enzymes are able to catalyze the hydrogen uptake and evolution from this moment. Hydrogenases have to find their intracellular location, the soluble enzymes stay in the cytoplasm, while the periplasmic enzymes have to be transported through the membrane. There is a special, recently discovered translocation system, the twin-arginin-translocation (Tat) pathway, which is used by the periplasmic hydrogenase enzymes (Rodrigue et al., 1999, Wu et al., 2000, Sargent et al., 2002). Proteins targeted via this pathway have signal sequences bearing a distinctive double arginine-containing amino acid sequence motif (Berks 1996, Stanley et al., 2000). In contrast to the well established Sec apparatus for transmembrane protein traffic in prokaryotes, this twin arginine translocase functions to transport prefolded proteins (Berks et al., 2000). The majority of Tat substrates in bacteria are the peripheral subunits of membrane-associated electron transfer complexes. Some accessory proteins like the HyaE and HybE proteins of *E. coli* (HupG and HupJ in other organisms) were proven to interact with the Tat-signal peptide-bearing small subunit of hydrogenase-1 and 2 (HyaA and HybO) (Dubini and Sargent 2003).

1.4. *Thiocapsa roseopersicina*

1.4.1. General features

Thiocapsa roseopersicina BBS is a Gram-negative, purple, photosynthetic sulfur bacterium belonging to the Chromatiaceae family in the γ-subdivision of proteobacteria (Pfennig and Trüper 1991). The BBS strain was originally isolated from an estuary of the White Sea. The 1-3 μm diameter coccoid cells are nonmotile. The optimal growth temperature is 24-28 ^oC, and growth is inhibited over 30 ^oC (Bogorov 1974). The wild-type strain needs 4-5 days to grow up in liquid culture, and about 10 days for forming visible colonies on plates. *T. roseopersicina* can grow under various environmental conditions; basically it grows phototrophically under anaerobic conditions. In contrast to cyanobacteria (oxygenic phototrophic bacteria), which use water as electron donor and produce oxygen during their photosynthesis, the anoxygenic phototrophic bacteria do not possess photosystem II, they use hydrogen, reduced sulfur compounds or simple organic substrates (e.g., acetate) as electron donors. They do not produce oxygen; the photosynthetic final product is usually elemental sulfur or sulfate. These bacteria live in anoxic environments illuminated by light, in the planktonic and benthic regions of the seas. They possess internal photosynthetic membrane, which is continuous with the cytoplasmic membrane. *T. roseopersicina* is a purple sulfur bacterium; it uses reduced sulfur compounds as electron source. In addition to photosynthetic growth, the strain is capable for grow aerobically, chemolithotrophically, in the dark (Kondratieva et al., 1976). It can fix molecular nitrogen, therefore it can grow diazotrophically as well (Bogorov 1974). Since hydrogen is the byproduct of the nitrogen fixation, this process also affects the gas metabolism of this bacterium.

1.4.2. Hydrogenases in *Thiocapsa roseopersicina*

T. roseopersicina BBS harbours at least three [NiFe] hydrogenases (HupSL, HynSL, HoxEFUYH) (Colbeau et al., 1994, Rákhely et al., 1998, Kovács et al., 2002, Rákhely et al., 2004, K. L. Kovács et al., 2005) and probably a fourth one of the regulatory hydrogenase type

(Fig. 3.). All these enzymes show the characteristics of the [NiFe] hydrogenases, two of them are attached to the periplasmic membrane, while the third and fourth ones are localized in the cytoplasm.

1.4.2.1. HynSL hydrogenase

One of the membrane-associated [NiFe] hydrogenases (HynSL, previously HydSL; Rákhely et al., 1998, Vignais et al., 2001) shows extraordinary stability: it is much more active at 80°C, than around the growth temperature, which is 24-28°C. The enzyme can be purified under air and at room temperature, and can be activated by reduction (Gogotov et al., 1976, Kovács et al., 1988). The expression of the enzyme is regulated by oxygen through the global anaerobic regulator FnrT protein (Á. T. Kovács et al., 2005). The HynSL enzyme functions in the direction of both hydrogen uptake and evolution, depending on the actual

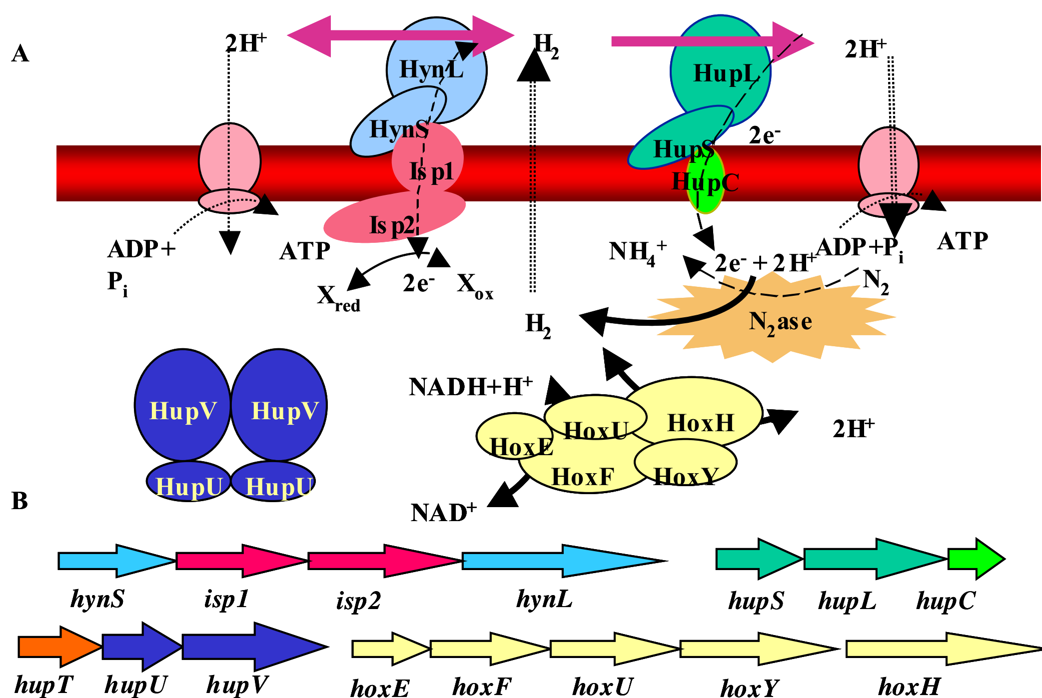


Figure 3. A. NiFe hydrogenases in *T. roseopersicina.* **B**. Organization of the structural genes of the hydrogenases.

metabolic status of the cell. There are two additional *orf-s* (*isp1, isp2*) between the coding regions of the small and large subunits (*hynS, hynL*) (Rákhely et al., 1998). The roles of these extra genes and their products are not clear yet; we have preliminary evidence that they are involved in the electron transfer (L. S. Mészáros et al., personal communication). Sequence homology between the *isp* genes and the ORF5 and ORF6 in the *hmc* operon of *D. vulgaris*

suggests that the corresponding protein products may form parts of a transmembrane electron transfer complex (Rákhely et al., 1998). RT-PCR analysis revealed that a single mRNA species coding for all four *orf*s exists (Á. T. Kovács, personal communication). The translation of Isp1 and Isp2 proteins was proven by ^{35}S labeling of the proteins produced in *E. coli* from the mRNA coding for *hynS-isp1-isp2-hynL* (G. Rákhely, personal communication). Other interesting feature is the absence of accessory proteins in the vicinity of the *hynSL* structural genes, so the organization of the structural genes is similar to that is present in other member of the Chromatiaceae family and in the hyperthermophile *Aquifex aeolicus* (Dahl et al., 1999).

1.4.2.2. HupSL hydrogenase

A *hup* gene cluster coding for a typical hydrogen uptake [NiFe] hydrogenase is also present in *T. roseopersicina* (Colbeau et al., 1994). In the photosynthetic membrane this hydrogenase is also heat stable, although not in the same extent as the HynSL. The optimum *in vitro* working temperature of this enzyme is around 60 ^{o}C, while the temperature optimum of the HynSL is above 80 ^{o}C. The expression of the HupSL hydrogenase is extremely sensitive to oxygen (J. Balogh et al., personal communication), but the enzyme itself can be activated easily under hydrogen after exposing it to air if it is attached to the photosynthetic membrane. A few *hup*-type accessory genes can be found downstream from the *hup* structural genes (*hupCDHIR*). The deduced amino acid sequences of the small (HupS and HynS) and large subunits (HupL and HynL) of the two membrane-bound hydrogenases share 46% and 58% identity.

1.4.2.3. The soluble HoxEFUYH hydrogenase complex

The third known hydrogenase in *T. roseopersicina* is localized in the soluble fraction. This [NiFe] hydrogenase belongs to the group of the cytoplasmic NAD^+ reducing hydrogenases (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 was proven to function as a truly bidirectional hydrogenase; it produced hydrogen under nitrogenase-repressed conditions, and it recycled 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*, although it had no effect on the hydrogenase activity *in vitro* (Rákhely et al., 2004). This suggests that HoxE has a hydrogenase-related role; it likely participates in the electron transfer processes. This is the first example of the presence of a cyanobacterial-type (5 subunits), NAD-reducing hydrogenase in a phototrophic bacterium, which is not a cyanobacterium. Only one accessory gene was identified downstream from the structural genes, the role of the putative specific endopeptidase (HoxW) is discussed in the results section.

1.4.2.4. Regulatory hydrogenase

The genes showing similarity to sequences coding for the hydrogen-sensing, regulatory hydrogenases (HupUV/ HoxBC, RH) of other bacteria (Vignais et al., 1997, Buhrke et al., 2004) were identified in *T. roseopersicina* (*hupUV*). 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 this strain. 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* mRNS. No mRNA corresponding to the *hupTUV* genes could be identified, but HupUV expressed from a plasmid turned out to be active in the H-D exchange assay (Kovács et al., to be published).

1.4.3. Hydrogenase maturation in *T. roseopersicina*

The basic principles of the [NiFe] hydrogenase maturation are expected to be valid in the case of the hydrogenases of *T. roseopersicina*, as the primary structures of the enzymes are well conserved. However, there could be some differences - compared to other systems - in the role and specificity of the accessory proteins responsible for the assembly of the heterobinuclear active centre. This speculation is supported by the facts that at least three different, functioning [NiFe] hydrogenases are present in the cell, and that *T. roseopersicina* can grow under several different conditions.

Transposon-based mutagenesis was adapted for *T. roseopersicina* (Fodor et al., 2001) in order to search for the accessory genes. A mutant library was created and hydrogenase deficient mutants were obtained. One of the hydrogenase deficient mutant strains (M539) was

investigated in detail (Fodor et al., 2001). The Tn5 mini transposon was inserted into the *hypF* gene in this strain, eliminating all hydrogenase activities. The inactivation of the hydrogen uptake activity in the *hypF* deficient mutant resulted in a dramatic increase in the hydrogen evolution capacity of this strain under nitrogen-fixing conditions.

HypF is one of the indispensable, pleiotropic accessory proteins, which creates the CN^- diatomic ligands from carbamoylphosphate. In most cases the *hypF* gene is member of the *hyp* operon, but in *T. roseopersicina* no other accessory genes were found in the vicinity of the *hypF*.

Aims of the thesis work

There is a complicated, multifactor-containing hydrogen metabolism in the purple sulfur bacterium *T. roseopersicina*. At least three hydrogenases work in a coordinated action depending on the metabolism of the cell. Hydrogen metabolism may be connected to other metabolic processes like sulfur metabolism and photosynthesis.

The hydrogenases themselves go through a regulated, multi-step maturation process. At least 30 gene products including structural proteins, accessory proteins and regulatory proteins are involved in the biosynthesis of the [NiFe] hydrogenases in *T. roseopersicina*. The proper concerted action of this protein network provides the possibility for the cells to adapt to the various conditions. My main aim was to understand these biosynthetic processes, to identify the participating proteins, and to establish the roles of these accessory proteins in hydrogenase biosynthesis. The individual steps of this process are probably arranged accurately in time as well, but this is not known in detail to date in any organisms.

The general aim of this study was to understand the biosynthetic processes of hydrogenases, which can open promising future perspectives for the production of a new, biologically generated energy carrier. Microorganisms containing suitable hydrogenase enzymes are amenable to produce clean molecular hydrogen in vast quantities from the ultimate renewable source of Sun. The availability of proper hydrogenases for this task is crucial: stable, inexpensive biological catalysts are needed for all applications. For this purpose the comprehension of the difficult maturation process is a necessary, but not yet sufficient requirement.

The desired functions of the matured hydrogenases are principally determined by the structure of the protein. Structure-function relationship is addressed in laboratory evolution and rational (computational) design studies. On the one hand I aimed to get insight into the molecular basis of protein stability and specificity, and on the other hand to I attempted to use the limited knowledge to optimize the cells of phototrophic bacteria for increased hydrogen production and for specific applications, including biosensors.

The specific goals of this work were as follows:

1. search for the accessory genes in *T. roseopersicina*
2. examination of the specificity of the auxiliary proteins
3. establishment of the roles of the accessory proteins by identifying protein complexes participating in the [NiFe] hydrogenase biosynthesis
4. set up a model for the assembly of hydrogenase enzymes in *T. roseopersicina*
5. understanding the assembly of the [NiFe] hydrogenases in order to create stable enzymes, which can be used *in vitro* or in heterologous hosts for biological hydrogen production

2. Materials and Methods

Bacterial strains and plasmids

Strains and plasmids are listed in Table 1. *T. roseopersicina* strains were grown photoautotrophically in Pfennig's mineral medium, under anaerobic conditions, in liquid cultures with continuous illumination at 27-30 ^{o}C for 4-5 days (Pfennig and Trüper 1991). Plates were solidified with 7 g L^{-1} Phytagel (Sigma) and supplemented with acetate (2 g L^{-1}) when selecting for transconjugants. The plates were incubated in anaerobic jars using the AnaeroCult (Merck) system for two weeks. *E. coli* strains were maintained on LB-agar plates. Antibiotics were used in the following concentrations (µg mL^{-1}): for *E. coli*: ampicillin (100), kanamycin (25), tetracycline (20), for *T. roseopersicina*: kanamycin (25), streptomycin (5), gentamycin (5).

Table 1. Strains and plasmids

Strain / plasmid	Relevant genotype or phenotype	Reference or source
Strains		
Thiocapsa roseopersicina		
BBS	wild type	(Bogorov 1974)
GB11	BBS, Δ(*hynS-isp1-isp2-hynL*):: ΩSm, Sm^r	(Rákhely et al., 2004)
GB21	BBS, Δ(*hupS-hupL*):: ΩGm, Gm^r	(This work)
GB1121	GB11, Δ(*hupS-hupL*):: ΩGm, Sm^r, Gm^r	(Rákhely et al., 2004)
M442	BBS, *hypD*::miniTn5, Km^r	(Maróti et al., 2003)
M1250	BBS, *hypE*::miniTn5, Km^r	(Maróti et al., 2003)
M4711	BBS, *hynD*::miniTn5, Km^r	(Maróti et al., 2003)

M646	BBS, *hynL*::miniTn5, Km^r	(Maróti et al., 2003)
M1343	BBS, *hypD*::miniTn5, Km^r	(Maróti et al., 2003)
M539	BBS, *hypF*::miniTn5, Km^r	(Fodor et al., 2001)
DC1B	BBS, $\Delta hypC_1$	(Maróti et al., 2003)
DC1G	GB11, $\Delta hypC_1$, Sm^r	(Maróti et al., 2003)
DC1H	GB1121, $\Delta hypC_1$, Sm^r, Gm^r	(Maróti et al., 2003)
DC2B	BBS, $\Delta hypC_2$	(Maróti et al., 2003)
DC2G	GB11, $\Delta hypC_2$, Sm^r	(Maróti et al., 2003)
DC2H	GB1121, $\Delta hypC_2$, Sm^r, Gm^r	(Maróti et al., 2003)
DC12B	BBS, $\Delta hypC_1$, $\Delta hypC_2$	(Maróti et al., 2003)
C100	pB6HypC1-Km in DC1B, Km^r	(This work)
C200	pB6HypC2-Km in DC2B, Km^r	(Fodor et al., 2004)
DHKW426	BBS, Δ*hupK*	(Maróti et al., 2003)
DHKG517	GB11, Δ*hupK,* Sm^r	(Maróti et al., 2003)
W400	pB6HupK-Km in DHKW426, Km^r	(Fodor et al., 2004)
DYDB	BBS, Δ*hynD*	(This work)
DYDG12H	GB1121, Δ*hynD,* Sm^r, Gm^r	(This work)
DYDG1	GB11, Δ*hynD,* Sm^r	(This work)
DYDG2	GB21, Δ*hynD,* Gm^r	(This work)
DUDB	BBS, Δ*hupD*	(This work)

DUDG12H	GB1121, Δ*hupD,* Sm^r, Gm^r	(This work)
DUDG1	GB11, Δ*hupD,* Sm^r, Em^r	(This work)
DUDG2	GB21, Δ*hupD,* Gm^r	(This work)
DXDG12H	GB1121, Δ*hoxW,* Sm^r, Gm^r, Em^r	(This work)
DXDG1	GB11, Δ*hoxW,* Sm^r, Em^r	(This work)
DXDG2	GB21, Δ*hoxW,* Gm^r, Em^r	(This work)
YG2YC	pMHEYDCt in DYDG2, Gm^r, Km^r	(This work)
UG1UC	pMHEUDCt in DUDG1, Sm^r, Km^r	(This work)
XG12XC	pMHEXDCt in DXDG12H, Sm^r, Gm^r, Em^r, Km^r	(This work)
FNRTM	*fnrT*::ΩEr, Em^r	(Á. T. Kovács et al., 2005)
Escherichia coli		
DH5α	*endA1, hsdR17, supE44, thi-1, λ- recA1, gyrA96, relA1, ΔlacU169* (ϕ80d*lac*ZΔM15)	Bethesda Research Laboratories
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
S17-1(λ*pir*)	294 (*recA pro res mod*) Tp^r, Sm^r (pRP4-2-Tc::Mu-Km::Tn7), λ*pir*	(Herrero et al., 1990)
BL21(DE3)	*E. coli* B, F¯, *dcm, ompT, hsdS(*r_B^- m_B^-*) gal* λ(DE3)	Novagene
Plasmids		
pBluescript SK (+)	cloning vector, Amp^r	Stratagene
pBBR1MCS-5	bhr cloning vector, Gm^r, backbone of the pMHE series	(Kovach et al., 1995)
pK18*mobsacB*	bhr vector for site directed mutagenesis, Km^r, *sacB*$^+$	(Schafer et al., 1994)

pM42-1	8.1 kb *Bam*HI fragment harboring the transposon from M442 in pBluescript SK, Amp^r, Km^r	(Maróti et al., 2003)
pM42-5	6.3 kb *Not*I-*Bam*HI fragment of the pM42-1 was cloned into the pBluescript SK, Amp^r	(Maróti et al., 2003)
pM42-8	3.5 kb *Pst*I fragment harboring the transposon from M442 in pBluescript SK, Amp^r, Km^r	(Maróti et al., 2003)
pM12-50	4.3 kb *Sal*I fragment harboring the transposon from M1250 in pBluescript SK, Amp^r, Km^r	(Maróti et al., 2003)
pM47-10	7 kb *Sph*I fragment containing the transposon from M4711 in pBluescript SK, Amp^r, Km^r	(Maróti et al., 2003)
pBRHynD	pBBR1MCS-5 carrying the *hynD* gene, Gm^r	(Maróti et al., 2003)
pBRC1	pBBR1MCS-5 carrying the $hypC_1$ gene, Gm^r	(Maróti et al., 2003)
pBRC2	pBBR1MCS-5 carrying the $hypC_2$ gene, Gm^r	(Maróti et al., 2003)
pBRCDE	pBBR1MCS-5 carrying the $hypC_1$, *hypD* and *hypE* genes, Gm^r	(Maróti et al., 2003)
pBRKCDE	pBBR1MCS-5 carrying the *hupK*, $hypC_1$, *hypD* and *hypE* genes, Gm^r	(Maróti et al., 2003)
pDC1	in-frame up and downstream regions of $hypC_1$ in pK18*mobsacB* , Km^r	(Maróti et al., 2003)
pDC2	in-frame up and downstream regions of $hypC_2$ in pK18*mobsacB* , Km^r	(Maróti et al., 2003)
pDHuK	in-frame up and downstream regions of *hupK* in pK18*mobsacB* , Km^r	(Maróti et al., 2003)
pDYD	in-frame up and downstream regions of *hynD* in pK18*mobsacB* , Km^r	(This work)
pDUD	in-frame up and downstream regions of *hupD* in pK18*mobsacB* , Km^r	(This work)
pDXD	Em resistance cassette between the up and downstream regions of *hoxW* in pK18*mobsacB*, Km^r, Em^r	(This work)
pMHE6crtKm	bhr vector, Km^r	(Fodor et al., 2004)

pMHEYDCt	based on pMHE6crtKm; HynD of *T. roseopersicina* with tandem FLAG-tag Strep-tag II fused to the C-terminus can be expressed from T7 or *crtD* promoter regions, Km^r	(This work)
pMHEUDCt	based on pMHE6crtKm; HupD of *T. roseopersicina* with tandem FLAG-tag Strep-tag II fused to the C-terminus can be expressed from T7 or *crtD* promoter regions, Km^r	(This work)
pMHEXDCt	based on pMHE6crtKm; HoxW of *T. roseopersicina* with tandem FLAG-tag Strep-tag II fused to the C-terminus can be expressed from T7 or *crtD* promoter regions, Km^r	(This work)
pB6HupK-Km	based on pMHE6crtKm; HupK of *T. roseopersicina* with tandem FLAG-tag Strep-tag II fused to the C-terminus can be expressed from T7 or *crtD* promoter regions	(Fodor et al., 2004)
pB6HypC1-Km	based on pMHE6crtKm; $HypC_1$ of *T. roseopersicina* with tandem FLAG-tag Strep-tag II fused to the C-terminus can be expressed from T7 or crtD promoter regions	(This work)
pB6HypC2-Km	based on pMHE6crtKm; $HypC_2$ of *T. roseopersicina* with tandem FLAG-tag Strep-tag II fused to the C-terminus can be expressed from T7 or crtD promoter regions	(Fodor et al., 2004)
pHYNDR	*Sal*I-*Hin*dIII digested HYNDP1-HYNDP2 PCR fragment in pFLAC	(This work)

Conjugation

The conjugation method developed for *Allochromatium vinosum* (Pattaragulwanit and Dahl 1995) was modified for *T. roseopersicina* as follows (Fodor et al., 2001). *T. roseopersicina* was grown in Pfennig's mineral medium for 3-4 days to reach late logarithmic, early stationary phase (~10^8-10^9 colony forming unit / mL). *E. coli* was grown to mid logarithmic phase (OD_{600}=0.7) in LB medium. 3 mL of the *E. coli* donor was filtered onto a nitrocellulose membrane, washed three times with 5 mL Pfennig's mineral medium without Na_2S. Then 10 mL of the recipient *T. roseopersicina* was filtered onto the same membrane. Controls contained only donor or recipient, and were handled in the same way. Filters were incubated overnight in light room, aerobically at 27-30 ^oC on PNA plates

(Pfennig's mineral medium without Na_2S, supplemented with 0.2% acetate and 0.2% Nutrient Broth (BBL) solidified with 1.5% agar (Gibco BRL)). Selection was done on Pfennig's mineral medium supplemented with 0.2% acetate and the appropriate antibiotics. Plates were incubated in anaerobic jars for two weeks.

Transposon mutagenesis

The mini transposon delivery plasmid pUT/mini-Tn5Km (Herrero et al., 1990) was mobilised from *E. coli* S17-1(λpir) to *T. roseopersicina* BBS. 100 colonies were randomly selected after each mating and screened for a hydrogenase deficient phenotype (Fodor et al., 2001). In this work, the M442, M1250, M4711, M646 and the M1343 mutants were chosen for detailed molecular analysis.

DNA manipulations

Isolation of genomic DNA. 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. Qiagen / Sigma / Eppendorf plasmid purification kits or the alkaline method were used for plasmid purification.

Digestion of DNA with restriction endonucleases; blunting, phosphatase treatment and ligation of DNA ends; and isolation of DNA fragments. Digestion with restriction endonucleases; blunting with T4 DNA polymerase; treatment of DNA ends with Calf Intestinal Alkaline Phosphatase (CIAP); ligation; and isolation of DNA fragments were preformed according to the manufacturer's instructions (Fermentas, Stratagene, and Amersham-Pharmacia).

Agarose gel electrophoresis.

Agarose gel electrophoresis was done in TAE buffer (50xTAE: in 1 L of water 242 g Tris/HCl, 57.1 mL glacial acetic acid and 100 mL EDTA (0.5 M, pH= 8)) as described in Current Protocols in Molecular Biology (Ausubel et al., 1996).

Preparation of competent cells and transformation.

Competent cells and transformation were prepared according to the SEM method (Inoue et al., 1996).

Polymerase Chain Reaction (PCR)

PCR-s were carried out in a PTC-150 MiniCycler (MJ Research) and in a PCRExpress (Hybaid) thermocycler. The final concentrations were: primers 1 μM each, dNTPs 200 μM each, buffer enzyme and Mg^{2+} according to the manufacturer's instructions.

DNA sequencing and sequence analysis

Sequencing was done on both strands by an automatic Applied Biosystems 373 Stretch DNA sequencer. The searches in the NBRF, SwissProt, combined EMBL-Genbank and Prosite databases were carried out with the various BLAST programs (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple alignments were performed with the CLUSTALW program (DNASIS Max v1.0, Hitachi Genetic System).

Southern blot and hybridization

Southern blotting, hybridization and detection of probes were done according the manufacturers instructions (Roche, Amersham-Pharmacia) or the general practice (Ausubel et al., 1996). After separating DNA by gel electrophoresis, DNA was blotted to HybondN+ membranes (Amersham-Pharmacia). Membranes were washed, dried and baked at 80 ^{o}C under vacuum. After hybridization with the labeled DNA probe and incubation with anti-DIG antibody conjugated with alkaline-phosphatase, detection was done with NBT and X-phosphate.

Isolation of the hydrogenase related genes

Partial genomic libraries were prepared from the various mutants in pBluescript SK+ and ampicillin + kanamycin resistant clones were selected. A list of the positive clones is given in Table 1. The sequenced genes and regions have been deposited in the Genbank, under the accession numbers: AY152822 (M442, M1250) and AY152823 (M4711).

In-frame deletion and insertion mutant constructs

The in frame deletion and insertion vector constructs derived from the pK18*mobsacB* vector (Schafer et al., 1994).

Deletion construct for removal of *hupK*. For deletion of the *hupK* gene, the 932 bp *Eco*RV- *Eco*47III fragment of pM42-5 (downstream region of the *hupK*) was inserted into the *Sma*I site of pK18*mobsacB* (pDHuKA). The polished 878 bp *Bgl*I fragment from pM42-5 (the upstream homologous region) was ligated into the *Hin*dIII digested/blunted pDHuKA resulting in pDHuK.

Deletion constructs for the mutation of $hypC_1$ and $hypC_2$. For removal of $hypC_1$ and $hypC_2$ genes, the pDC1 and pDC2 in-frame deletion constructs were created as follows.

The blunted 1,423 bp *Sac*I fragment (the downstream region of $hypC_1$) was cloned from pM42-5 into the *Sma*I digested pK18*mobsacB* (pDC1A). The upstream region of $hypC_1$ was amplified with the TRHC101 (5' GTTATCCTGAAGCGCGATCA 3') and TRHC102 (5' CTAGACACATGGACAAAAGA 3') primers and the 1,441 bp PCR product was cloned into the *Hin*dIII digested, Klenow filled pDC1A, resulting the pDC1.

The upstream and downstream regions of $hypC_2$ were amplified by PCR using *Pwo* polymerase. The following primers were used:

HYDAZ04 (5' ATCGGGATACCGAGACACAT 3')
HYDAZ05 (5' AATGGGTTGAACGAGAGTCG 3')
TRHC201 (5' TGAGCATGGTCGCAAACACG 3')
TRHC202 (5' GGACGGCTCGAGGTTTGATC 3')

pDC2A was obtained by cloning the HYDAZ04 - HYDAZ05 PCR fragment covering the 951 bp downstream homologous region of $hypC_2$ into the polished *Hin*dIII site of the pK18*mobsacB* vector. The 949 bp upstream homologous region was amplified with the TRHC201 and TRHC202 primers and cloned into the *Sal*I digested, Klenow filled pDC2A (pDC2).

Deletion constructs for mutating the endoprotease-like sequences. For removal of *hynD, hupD* and *hoxW* genes, the pDYD, pDUD in-frame deletion and the pDXD insertion constructs were created as follows.

The upstream and downstream regions of *hynD* were amplified by PCR using *Pwo* polymerase. The following primers were used:

HYDAZ01 (5' TCCGAGAACGATTTCGATCG 3')
TRDC2E (5' ACGGCGCTGGACCTTATGCC 3')
HYDAZ02 (5' CCGCAGTCGAGGCGGCGATT 3')
HYDAZ13 (5' ATATCGAGCACGATCACCTG 3')

The 974 bp upstream homologous region was ligated into the *Pst*I digested, T4 polymerase treated pK18*mobsacB* vector resulting the pHynDA. The pDYD was obtained by ligating the

931 bp downstream homologous region into the *Sph*I digested, T4 polymerase treated pHynDA.

The upstream and downstream regions of *hupD* were amplified by PCR using *Pwo* polymerase. The following primers were used:

TRHUPC01 (5’ ATGTCGCGAGCTGCGTCGCG 3’)

TRHUPD01 (5’ CACGTTGGACGACATCACCC 3’)

TRHUPD02 (5’ TGTCCGCCCGGACCGTCCAA 3’)

TRHUPo3R (5’ CACCTCGCCGACAGAATCTC 3’)

The 930 bp upstream homologous region was ligated into the *Sma*I digested pK18*mobsacB* vector giving the pHupDA. The pDUD was obtained by ligating the 1035 bp downstream homologous region into the *Sal*I digested, Klenow-filled pHupDA.

For constructing the pDXD in first step the 918 bp Klenow-filled *Eco*RI-*Sal*I fragment containing the erythromycin resistance gene was cut from the pRL271 vector. This fragment was ligated into the *Nco*I digested (*Nco*I site is in the middle of the *hoxW* gene); T4 polymerase blunted pTCB4/2 vector containing the whole *hox* operon resulted in the pTCB4/2Em vector. 2190 bp *Bam*HI fragment containing the erythromycin resistance gene and the downstream and upstream homologous regions was cut from the pTCB4/2Em and ligated into the *Bam*HI digested pK18*mobsacB* resulting the pDXD.

The mutant strains

Selection for the first recombination event was based on kanamycin resistance. The selection for the second recombination was based on the *sacB* positive selection system in all cases (Schafer et al., 1994), and on erythromycin resistance in the case of *hoxW* mutants. In *T. roseopersicina* 3 % sucrose was efficient to induce the *sacB* system.

The in frame deletion constructs pDHuK, pDC1, and pDC2 were transformed into *E. coli* S17-1 (λpir) strain, then conjugated into *T. roseopersicina* BBS, GB11 and GB1121 strains. leading to the in frame deletion mutants DHKW426 (Δ*hupK* BBS), DHKG517 (Δ*hupK* GB11), DC1B (Δ$hypC_1$ BBS), DC1G (Δ$hypC_1$ GB11), DC1H (Δ$hypC_1$ GB1121), DC2B (Δ$hypC_2$ BBS), DC2G (Δ$hypC_2$ GB11), DC2H (Δ$hypC_2$ GB1121) and DC12B (Δ$hypC_1$ Δ$hypC_2$ BBS) strains (Table 1.).

The in frame deletion constructs pDYD and pDUD were transformed into *E. coli* S17-1 (λpir) strain, then conjugated into *T. roseopersicina* BBS, GB11, GB21 and GB1121 strains resulting the in frame deletion mutants DYDB (Δ*hynD* BBS), DYDG1 (Δ*hynD* GB11),

DYDG2 (Δ*hynD* GB21) DYDG12H (Δ*hynD* GB1121), DUDB (Δ*hupD* BBS), DUDG1 (Δ*hupD* GB11), DUDG2 (Δ*hupD* GB21) DUDG12H (Δ*hupD* GB1121) strains (Table 1.).

The insertion mutant construct pDXD was transformed into *T. roseopersicina* BBS, GB11, GB21 strains resulting the insertion mutants, DXDG1 (Δ*hoxW* GB11), DXDG2 (Δ*hoxW* GB21) and DXDG12H (Δ*hoxW* GB1121) strains. The in frame deletion mutant clones were verified using PCR, Southern-analysis, and sequencing.

Constructions for complementation

Homologous complementation was performed using pBBR1MCS-5 based vectors (Kovach et al., 1995).

Complementation of the *hynD* transposon mutant strain (M4711). On the pM47-10 template the following primers were used to amplify the 949 bp PCR fragment carrying the *hynD* gene:
HYDAZ04: (5'ATCGGGATACCGAGACACAT 3')
HZDAZ05: (5' AATGGGTTGAACGAGAGTCG 3')

First, this fragment was cloned into the *Hin*cII digested pBluescribe plasmid (pHDS), then it was recloned into pBBR1MCS-5, as a *Sph*I-*Sac*I fragment (pBRHynD).

Complementation of the *hupK* mutant. pBRHupK was constructed by cloning a 2,936 bp *Apa*I-*Cla*I fragment, containing the *hupK* gene with its regulatory region, into the *Apa*I-*Cla*I digested pBBR1MCS-5 vector.

Complementation of the $hypC_1$ and $hypC_2$ mutants. pBRC1 was obtained by inserting the 1,753 bp *Eco*RI-*Pst*I fragment, containing the $hypC_1$ gene, from pM42-5 (pM42-5: the 6.3 kb *Not*I-*Bam*HI fragment of the pM42-1 was cloned into the pBluescript SK+ *Not*I-*Bam*HI sites) into *Eco*RI-*Pst*I digested pBBR1MCS-5. pBRC2 was produced by insertion of the 552 bp *Rsa*I fragment, containing the $hypC_2$ gene from pM47-13, into *Sma*I digested pBBR1MCS-5.

Complementation of the *hynD*, *hupD* and *hoxW* mutant strains. Homologous complementation experiments were performed with constructs amenable also for the affinity protein purification (pMHE6YDCt, pMHE6UDCt, pMHE6XDCt).

For constructing the pMHE6YDCt, in first step the *hynD* was amplified with the following primers:
HYDAZ04: (5' ATCGGGATACCGAGACACAT 3')
HYDAZ17H: (5' AAGCTTAGTGCACCAGCGATCGAG 3')

Using the HYDAZ17H primer the STOP codon of the *hynD* gene was removed and changed for a *Hin*dIII restriction site (underlined). The 614 bp PCR product was ligated into the *Sma*I digested pBluescriptSK+ yielding the pBtYD. 629 bp *Xba*I-*Hin*dIII fragment was cut from the pBtYD and ligated into the *Xba*I-*Hin*dIII digested pMHE6crtKm (Fodor et al., 2004), resulting the pMHE6YDCt.

For constructing the pMHE6UDCt, in first step the *hupD* was amplified with the following primers:

TRHUPD03N: (5' CATATGCGATCCGATCCGGAGATC 3')

TRHUPD04H: (5' AAGCTTTTGACGATCTCCGAGAGG 3')

Using the TRHUPD04H primer the STOP codon of the *hupD* gene was removed and changed for a *Hin*dIII restriction site (underlined). The 672 bp PCR product was ligated into the *Sma*I digested pBluescriptSK+ resulting the pBtUD. 665 bp *Nde*I-*Hin*dIII fragment was cut from the pBtUD and ligated into the *Nde*I-*Hin*dIII digested pMHE6crtKm resulting the pMHE6UDCt.

For constructing the pMHE6XDCt, in first step the *hoxW* was amplified with the following primers:

TCHO10: (5' GATCGCCAGCAGCTTCCACT 3')

TCHO34: (5' GTGAAGCTTGCACGTAGTTGGAAT 3')

The 865 bp PCR product was ligated into the *Sma*I digested pBluescriptSK+ resulting the pBtXD. 877 bp *Xba*I-*Hin*dIII fragment was cut from the pBtXD and ligated into the *Xba*I-*Hin*dIII digested pMHE6crtKm, yielding the pMHE6XDCt.

The constructs were conjugated into the appropriate mutant strains; pMHE6YDCt was conjugated into the DYDG2 strain resulting the YG2YC strain. DUDG1 was complemented by the pMHE6UDCt (UG1UC strain). The DXDG12H *hoxW* mutant GB1121 was complemented with the pMHE6XDCt yielding the XG12XC strain.

Complementation of the M442 transposon mutant strain. pBRCDE homologous complementation vector was constructed in three steps. The 1,753 bp *Eco*RI-*Pst*I fragment from pM42-5 was cloned into the *Eco*RI-*Pst*I digested pBBR1MCS-5, which yielded the pBRC1 construct. The 293 bp *Pst*I-*Bam*HI fragment (part of the *hypD* gene), derived from the pM12-50, and was ligated into the *Pst*I-*Bam*HI digested pBRC1 (pBRCT2). The 1,703 bp *Bam*HI fragment (downstream region of the *hypD* gene and the entire *hypE* gene) from pM42-8 was transferred into the *Bam*HI digested pBRCT2 yielding pBRCDE. pBRKCDE homologous complementation vector was constructed in three steps as well. The 2,936 bp *Apa*I-*Cla*I fragment (harbouring the *hupK* gene) from pM42-5 was inserted into *Apa*I-*Cla*I

digested pBBR1MCS-5 producing pBRHupK. pBRKT2 was obtained by cloning the 1,069 bp *Cla*I-*Bam*HI fragment (containing the *hypC*$_1$ and the 5' region of the *hypD* gene) from pM12-50 into the *Cla*I-*Bam*HI digested pBRHupK. pBRKT2 was digested with *Bam*HI and the 1,703 bp *Bam*HI fragment from pM42-8 was built into this vector (pBRKCDE). The homologous complementation constructs were transformed into *E. coli* S17-1 (λpir) strain, and then conjugated into the appropriate *T. roseopersicina* strains.

Construct for the β-galactosidase assay

The promoter region of the *hynD* gene was amplified from pHDS (Maróti et al., 2003) with primers HYNDP1 (5` GTCCTGGAGCATGAAGAGAT 3`) and HYNDP2 (5` ACGAAGCTTACATTGCCGAGGCCGAGGA 3`) (contains an artificially introduced *Hin*dIII site, underlined). The *Hin*dIII-*Sal*I digested 201 bp product was cloned into the *Sal*I - *Hin*dIII site of pFLAC, yielding pHYNDR.

RNA isolation, reverse transcription (RT) and PCR

RNA was isolated with TRI Reagent (Sigma Aldrich) by following the manufacturer's recommendations. Prior to reverse transcription, the RNA was treated with DNase I as previously described (Fodor et al., 2001). Reverse transcription was performed using MMLV Reverse Transcriptase (Promega).

a, For the analysis of the organisation of *hupK-hypC1* genes, the TRHC102 primer (in *hypC*$_1$, sequence see above) was used for the reverse transcription and PCR. The TRHD04 (5' TTGCGGTTGT TGAGCCGCTG 3') served as the other primer in PCR. Using these primers a 524 bp fragment could be amplified.

b, For the *hox* operon the reverse transcription was initiated at primer TCHO14 (5` CGACATGGCGCTGGCGTCGG 3`) located at the middle of the *hoxW* gene, while the PCR was performed with the following primers: TCHO27 (5` CTGCAGGAGCGCTACGACAT 3`) in the *hoxE* gene and TCHO9 (5` GGATGACTGACAGCTGGCCGCGAGG 3`) in the *hoxF* gene. This generated a 301bp fragment that was run on an agarose gel.

c, For the *hup* operon the reverse transcription was initiated at primer ohup14 (5` AGCAGATCCGGCATTCCA 3`) located at the middle of the *hupI* gene, while the PCR was performed with the following primers: huptro3 (5` TCCGACGTGATCCTCTCCATGA 3`) and ohup3 (5` CACCGCCTTGCAGCTGTCGGC 3`) in the *hupS* gene. This generated a 230bp fragment that was run on an agarose gel.

Quantitative RT-PCR

Reverse transcription was performed using RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas). Quantification of cDNA was performed on ABI PRISM 7000 Sequence Detection System (Applied Biosystem) using SYBR Green PCR Master Mix. The following primers were used: for *hynD*, HYNDRT1 (5` GGCAGTGTGCGCGTCTT 3`) and HYNDRT2 (5` TCATGGACGGTCTTGGCATT 3`), and for *crtD* gene of *T. roseopersicina* (Kovács et al., 2003), caroR (5` CTCCTCGTCGGCGAAGAG 3`) and caroF (5` CGCATCCCTGACCGACTATC 3`). The amount of *hynD* cDNA was normalized to the level of *crtD* cDNA.

β–galactosidase assay

The β-galactosidase activity of the toluene-permeabilized cell extracts was assayed as described earlier for *T. roseopersicina* (Kovács et al., 2003, Miller 1972). Cells were assayed at the late logarithmic growth state. One Miller unit corresponds to 1 µmol of *o*-nitrophenyl-β-galactoside (Sigma-Aldrich) hydrolyzed per minute normalized to the optical density of *T. roseopersicina* at 650nm.

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

300 mL of *T. roseopersicina* culture was harvested in a Sorvall RC5C centrifuge at 7,000 *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. The broken cells were centrifuged at 10,000 *g* for 15 min. The debris (containing whole cells and sulfur crystals) was discarded and the supernatant was centrifuged at 100,000 *g* for 3 hours (Hanczár et al., 2002). The ultracentrifugation pellet was washed with 20 mM K-phosphate buffer (pH = 7.0) and used as membrane fraction. The supernatant was considered as the soluble fraction.

Hydrogen uptake activity assay *in vitro*

H_2 uptake coupled to benzylviologen or methylviologen reduction was assayed spectrophotometrically at 55 °C. The harvested cells, membrane or soluble fractions were suspended in 20 mM K-phosphate buffer (pH = 7.0). 2 mL of this mixture was placed into a cuvette, 18 µL of 20 mM benzylviologen was added, and the cuvettes were sealed with

SubaSeal stoppers. The gas phase was flushed with N_2 for 5-10 min and then with H_2 for 5-10 min.

Hydrogen evolution assay *in vitro*

0.5 mL of sample was suspended in 1.2 mL of 20 mM K-phosphate buffer (pH = 7.0) in Hypo-Vials (10 cm^3 volume, Pierce) and 1 mL of 1 mM methylviologen was added. In order to measure the activity of the Hyn enzyme selectively, cells were heat treated at 80 °C for 30 minutes prior to the assay. The gas phase was flushed with N_2 for 10 min, followed by the anaerobic addition of 0.5 mL of 0.1 g mL^{-1} dithionite. Samples were incubated at 40 °C for 30 min. Hydrogen production was measured by GC.

Affinity purification of accessory proteins

For the purification of the *T. roseopersicina* **HynD, HupK, HypC$_1$, HypC$_2$** proteins fused with tandem FLAG-tag Strep-tag II at the C-terminus 2 g of cell paste (~1 L of culture) from the *T. roseopersicina* YG2YC, DHKW426/pB6HupK-Km (W400), DC2B/pB6HypC2-Km (C200) or DC1B/pB6HypC1-Km (C100) strains (Table 1.) was suspended in ~3 mL TBS (50 mM Tris/HCl, pH= 7.4 and 150 mM NaCl). Lysozyme was added to a 200 µg mL^{-1} final concentration before sonication. Cell debris was removed by centrifugation (10000 x g, 10 min.). Triton X-100 (0.5% final conc.) was optionally added to the supernatant and it was incubated with 100 µL ANTI-FLAG M2 affinity resin (SIGMA) at 4°C for 1 h with gentle shaking. The column was washed 7 times with 1.5 mL TBS (in some cases with 0.5% Triton X-100) and - when further purification was carried out using Strep-Tactin Sepharose avidin was added (final conc.: 100 µg mL^{-1}) at the 6th washing step to block biotinylated proteins. For elution, the slurry was incubated twice in 100 µL TBS with 200 µg mL^{-1} FLAG peptide for 5 and 10 minutes, respectively, and then washed with another 50 µL elution solution. The remaining bound proteins were eluted by adding 200 µl 0.1 M glycine puffer (pH = 3.5) onto the column. The pooled eluate could be purified further by incubating with 50 µL Strep-Tactin Sepharose (IBA) at 4 ^{o}C for 1 h with gentle shaking. The column was washed 4 times with 1 mL of TBS. Bound proteins were eluted 6 times with 50 µL of TBS buffer supplemented with 2.5 mM desthiobiotin.

The whole procedure was carried out parallel with a negative control as well. Aliquots were collected from both the control and the samples at each step and analyzed by SDS-PAGE.

Polyacrylamide gel electrophoresis (PAGE (native and denaturing))

Native and denaturing polyacrylamide gel electrophoresis were performed as described in Current Protocols in Molecular Biology (Ausubel et al., 1996). Samples were applied to the gel in native or SDS loading buffer, in the latter case samples were incubated at 95 ^{o}C for 10 minutes prior to loading. For matrix-assisted laser desorption/ionization (MALDI) MS analysis, protein samples were concentrated by the trichloroacetic acid/deoxycholate precipitation method, washed twice with cold acetone and dried prior to applying them on denaturing gels.

Staining of protein gels

Coomassie and silver staining of proteins was done as described in Current Protocols in Molecular Biology (Ausubel et al., 1996). The modified Coomassie staining method was used for gels prepared for MALDI-MS analysis (Rosenfeld et al., 1992).

Identification of proteins by MALDI-TOF MS

Identification of proteins was done by Ms. Éva Hunyadi-Gulyás, and Ms. Éva Klement in the laboratory of Dr. Katalin F. Medzihradszky (Mass Spectrometry Facility, Biological Research Center, Hungarian Academy of Sciences). Silver stained gel bands were cut. After reduction (dithiotreitol, SIGMA) and alkylation (iodoacetamide, SIGMA) the proteins were in-gel digested with side-chain protected porcine trypsin (Promega). For the protocol see http://donatello.ucsf.edu/ingel.html. The tryptic peptides were extracted from the gel and purified on C18 ZipTip (Millipore). An aliquot of the unfractionated digest was mixed with the saturated aqueous solution of the matrix (2,5-dihydroxy-benzoic-acid, DHB) and applied to the sample target. Mass spectra were recorded on a REFLEX III MALDI-TOF mass spectrometer (BRUKER, Germany) in positive reflectron mode. External calibration was applied using peptide standards. Post source decay (PSD) spectra of selected peptides were acquired in 10-12 steps, lowering the reflectron voltage by 25% at each step. For both the peptide mass fingerprints (PMS) and PSD spectra, a database search was performed in the NCBI protein database using Protein Prospector MS-Fit and MS-Tag, respectively (http://prospector.ucsf.edu/).

3. Results

The examination of the biosynthetic processes of the [NiFe] hydrogenases in *T. roseopersicina* has some prerequisites. First, the participating accessory genes had to be found and isolated. Then the organisation and regulation of each gene had to be established before starting the experiments concerning the functions of the gene products. The last section of the results focuses on the approaches (deletion mutagenesis and protein-protein interaction studies) used to determine the specificities and possible roles of the accessory proteins of choice.

3.1. Identification of hydrogenase accessory genes in *T. roseopersicina*

3.1.1. Accessory genes in the operons of the structural genes

Only a few accessory genes were found in the proximity of the hydrogenase structural genes. The *hupDHI* genes are in the *hup* operon, downstream from the genes of the structural subunits of the Hup hydrogenase (Fig. 8.). The *hupD* gene encodes for an endopeptidase-like protein supposed to play a role in the C-terminal posttranslational modification of the large subunit, HupL. The exact roles of the proteins coded by the *hupHI* genes are unknown.

The *hox* operon consists of six genes, five of them code for the proteins of the hydrogenase and diaphorase structural subunits (*hoxEFUYH*). The last open reading frame, named *hoxW* (Fig. 8.), also shows characteristics of the C-terminal processing endopeptidases.

No accessory proteins could be found in the chromosomal region of the *hynS-isp1-isp2-hynL* genes coding for the stable HynSL hydrogenase (Fig. 3.).

In order to examine the maturation process of the hydrogenase enzymes in *T. roseopersicina* in detail, the accessory genes have to be found in the genome. For this purpose different approaches were used. First transposon-based random mutagenesis was developed and applied, later the genome sequencing of our model organism provided useful data about the localization of some important auxiliary genes and the structure of their gene products.

3.1.2. Transposon-based mutagenesis

Transposon-based mutagenesis was performed in order to create a mutant *T. roseopersicina* library and to find the hydrogenase accessory genes (Fodor et al., 2001). Six of 1,600 mutant colonies showed a hydrogenase deficient phenotype, 4 of them (M442, M1250, M1343, M539) lost all hydrogenase activities and in two cases (M646, M4711) the hydrogenase activities of the cells were dramatically reduced but detectable. The M539 mutant (*hypF*::Km) was characterized earlier (Fodor et al. 2001). The M442 and M4711 strains were selected for detailed analysis.

The *hupK, hypC$_1$, hypD, hypE* genes. An approximately 8.1 kb *Bam*HI genomic fragment from the pleiotropic mutant M442 was isolated, subcloned and sequenced. The *hypC$_1$*, *hypD*, and the *hupK* genes were identified in this clone (Fig. 4., Table 2.).

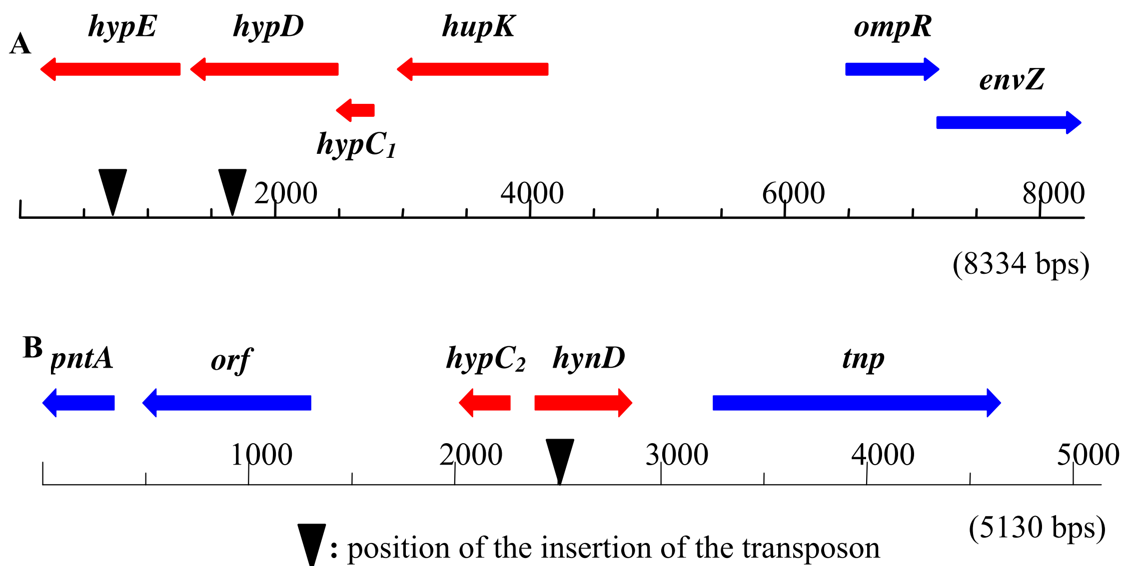


Figure 4. Identified hydrogenase accessory genes in the M442, M1250 (panel A) and M4711 (panel B) transposon mutant strains. PntA is similar to transhydrogenases, Orf is a putative conserved protein, *tnp* seems to encode a transposase. The sequences have been deposited in the Gene Bank, accession numbers: AY152822 (A) and AY152823 (B).

Upstream from the *hupK* gene, no hydrogenase related gene could be identified, but two *orf*-s showed significant homology to the two component regulatory system OmpR-EnvZ (Cai and Inouye 2002). In *T. roseopersicina,* the *hypD* gene starts with a GUG, and the Tn5 transposon was inserted at the 792nd bp of the 1,146 bp long *orf*. As the *Bam*HI fragment from

M442 did not contain the whole *hypD* gene, an overlapping 3.5 kb *Pst*I genomic fragment was cloned and sequenced. The *hypE*-type gene was found downstream from the *hypD* gene (Fig. 4., Table 2.). It is to note, that in a separate hydrogenase-deficient mutant (M1250), the transposon was inserted into the *hypE* gene. No additional accessory genes were found downstream from *hypE*. Together, an 8,334 bp long region was sequenced on both strands.

	Thiocapsa roseopersicina					
	HupK 389 aa	$HypC_1$ 94 aa	HypD 381 aa	HypE 360 aa	$HypC_2$ 81 aa	HynD 156 aa
R. eutropha	30% (HoxV)	55% (HypC)	57%	76%	30% (HypC)	31% (HoxM)
E. coli	-	34% (HypC)	42%	46%	37% (HybG)	29% (HyaD)
R. leguminosarum	27%	50% (HypC)	59%	61%	37% (HypC)	29% (HupD)
A. chroococcum	26%	47% (HypC)	60%	80%	37% (HypC)	30% (HupM)

Table 2. Identity values between the accessory proteins of *T. roseopersicina* and the corresponding proteins from other organisms.

The *hynD* and *hypC₂* genes. A 5,130 bp long chromosomal fragment surrounding the transposon in the M4711 non-pleiotropic mutant was sequenced on both strands. Two [NiFe] hydrogenase related *orf*-s were found. The deduced amino acid sequence of the first *orf* showed similarity to the HypC proteins (Fig. 4., Table 2.), and the characteristic motif at the N-terminus of HypC-s: M-C-(L/I/V)-(G/A)-(L/I/V)-P could also be aligned. The second *orf* (named *hynD*) encoded a putative protein, similar to the hydrogenase specific endoproteases of other microorganisms. Multiple alignment indicated that the putative HynD was similar to the other [NiFe] hydrogenase processing proteases after a GTG codon. The start codon of the *hynD* gene could not be identified, there was a long stretch (148 aa) upstream from this GTG without ATG in frame, but the translated sequence was unrelated to any known proteins. The codon usage of this upstream region is not characteristic of the known codon usage pattern of *T. roseopersicina* (among the 10 codons preceding the GTG, 4 is preferred at 1-10 %

frequency in this strain). If *hynD* starts at this codon, the putative HynD enzyme consists of 156 amino acids (16.6 kDa) and the transposon is inserted into the *hynD* gene at the 107th bp of the 471 bp long gene. Thus, the *hypC$_2$* and the *hynD* genes are separated by 120 bp, and they are in opposite orientation. It should be noted that the C-terminal end of HynD was slightly shorter than those of its counterparts from other microorganisms.

3.1.3. Hydrogenase accessory genes from the partial genome sequence

Sequencing of the genome of *T. roseopersicina* BBS is in progress. Preliminary data mining revealed the presence of the *hypA* and *hypB* genes near each other. The conserved N-terminal end (MHEL) of the HypA protein was used during the genome search. The genes are separated by 336 bp, the *hypA* gene is 351 bp long (coding for the 116 aa HypA protein), the exact size of the *hypB* gene is not known yet, because the C-terminal end of the gene was not present on the clone sequenced. The 195 bp N-terminal region of the *hypB* was sequenced.

3. 2. Organisation and regulation of the auxiliary genes

3.2.1. Co-transcription of *hupK* and *hypC$_1$DE*

The *hupK* (*hoxV*) was separated from the *hypC$_1$* by 194 bp, the start codon of *hypD* was overlapping with the stop codon of *hypC$_1$* and *hypE* started 94 bp downstream from the stop codon of *hypD*. The distances between the *hupK*, *hypC$_1$D* and *hypE* genes are compatible with either an independent transcription of the *hupK*, *hypC$_1$D* and/or *hypE*, or all of these genes could be co-transcribed. In order to test this possibility, RT-PCR analysis was performed on total RNA isolated from *T. roseopersicina.* An mRNA species containing both the *hupK* and *hypC$_1$* genes was detected, which indicated a linked transcriptional regulation of these genes. The transcript, however, appeared very weak (Fig. 5.), and therefore, independent transcription had to be considered as well. The two possibilities were further examined in additional complementation experiments (Table 5A.). Two constructs were made in order to complement the strain carrying a *hypD*::Km mutation (M442). The two constructs differed from each other in the *hupK* gene and its regulatory region. One of them contained the *hupK-hypC$_1$DE* genes (pBRKCDE), and the other one contained only the *hypC$_1$DE* genes

(pBRCDE). The presence of the pleiotropic *hypE* gene in the constructs was necessary because of the possible polar effect of the transposon. (Separate RT-PCR experiments proved that the *hypD* and *hypE* genes were cotranscribed (data not shown)). Both constructions complemented the mutation in *hypD*::Km (Table 5A.), but the complementation was not complete in either case, it was significantly higher, when the construct with *hupK* was used (18 % without *hupK* and 43 % with *hupK*). These results suggest again the presence of two sets of regulatory elements, one between *hupK* and $hypC_1$, and one upstream from *hupK*. To some extent, it would explain the low complementation efficiency obtained in the $hypC_1$ complementation experiments, where the *hupK* was omitted from the complementing construct (see below, Table 5A.).

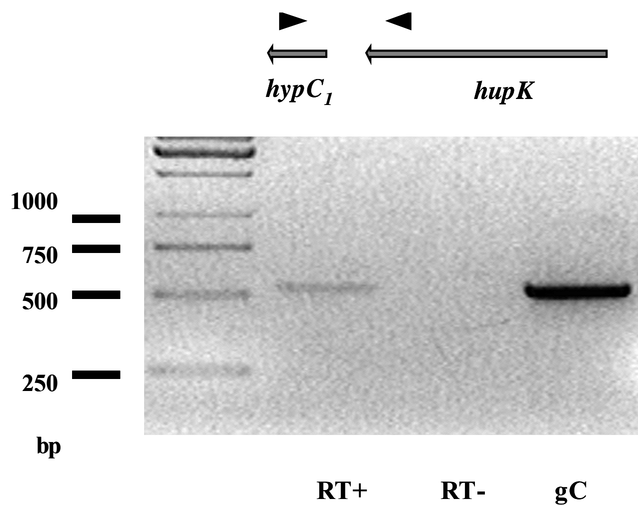


Figure 5. RT-PCR analysis of the co-transcription of the *hupK* and $hypC_1$ genes. M: marker, bp: base pairs, RT+: reverse transcription was made before PCR reaction, RT-: reverse transcriptase was omitted, gC: control PCR made on genomic DNA.

3.2.2. Characterization of the *hynD* gene

3.2.2.1. The *hynD*::Km transposon mutant strain

HynD is a processing endopeptidase-like protein. In the wild type *T. roseopersicina,* all hydrogenase activities, except that related to HynSL, could be eliminated by an appropriate heat treatment (30 min above 80°C). In accordance with the activity assays, only heat labile hydrogenase activity could be detected in a Δ*hynSL* mutant strain (GB11). Likewise, in the mutant, in which the *hynD* gene was disrupted by the Tn5 insertion (M4711), no heat stable hydrogenase activity was observable. A series of hydrogenase activity measurements were performed using the wild type cells, the *hynD*::Km (M4711), the Δ*hynSL* (GB11) mutants, and the complemented M4711 strain. Mutants lacking a functional *hynD* gene (M4711) or the heat stable [NiFe] hydrogenase, HynSL (GB11), showed the same behaviour in the activity

assays (Fig. 6.). Complementation of the *hynD* gene (pBRHynD) restored the heat stable HynSL hydrogenase activity to the level of the wild type control. Since the *in silico* analysis

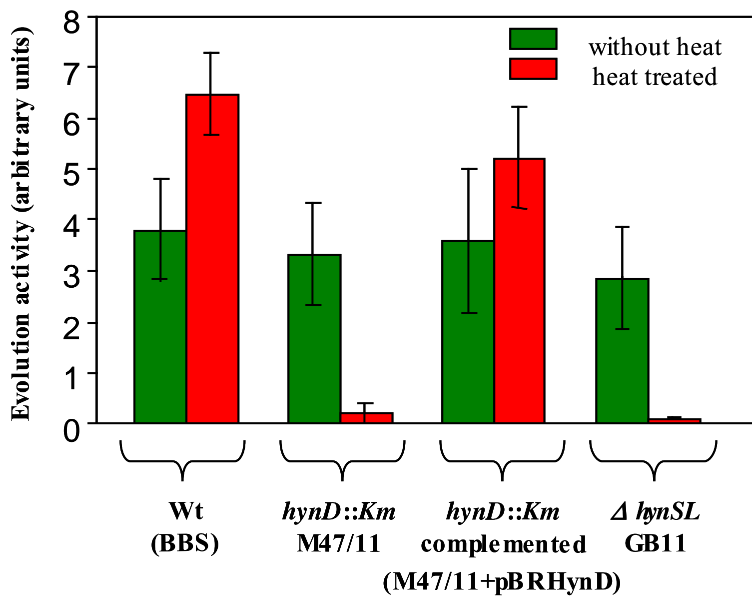


Figure 6. Hydrogen evolution activity of the wild type and the HynD mutant *T. roseopersicina* strains. The samples were or were not heat treated before the measurements. For the strains see Table 1. It should be noted, that HynSL is a thermophilic enzyme, i.e. its activity increases with temperature (at least up to 80°C). Therefore, heat treatment of the samples probably activates this hydrogenase, which explains the higher activity of the heat treated samples.

of the putative HynD gene product clearly predicted a [NiFe] hydrogenase processing endopeptidase, it was concluded that HynD is a protease carrying out the posttranslational modification of the C-terminus of the large subunit during the maturation of the thermophilic HynSL hydrogenase in *T. roseopersicina*.

3.2.2.2. Regulation of the *hynD* gene

FnrT mediated global anaerobic regulation plays an important role in the formation of active HynSL hydrogenase in *T. roseopersicina* (Á. T. Kovács et al., 2005). Reporter fusion constructs and *in vitro* transcription experiments proved that FnrT regulates HynSL expression by binding to two different FnrT-binding sites upstream from the Hyn structural genes. Since HynD was proven to be a HynL specific endopeptidase, and FnrT binding sites were found between the *hynD* and *hypC*$_2$ genes, it was plausible to assume that the expression of the HynD goes parallel with that of HynSL. Therefore the same methods were used to investigate whether the *hynD* accessory gene is also regulated by oxygen under the FnrT global regulator system. The mRNA level and p_{hynD} derived LacZ activity were measured in the wild type and FNRTM mutant grown anaerobically or under oxygen. No significant differences were detected in the mRNA level, or in β-galactosidase activity between the cultures (Table 3.).

		β-galactosidase activity (Miller Unit)		relative mRNA level (%)	
strain	phenotype	$-O_2$	$+O_2$	$-O_2$	$+O_2$
BBS	wild type	100.5 ±17.7	107.5 ±28.5	100 ±5.9	99.1 ±8.4
FNRTM	*fnrT*::ΩEm^r	110.3 ±7.5	118.6 ±12.1	112.6 ±12.6	83.1 ±9.2

Table 3. Expression of the *hynD* gene followed by β-galactosidase activities of *hynD-lacZ* fusion (pHYNDR) and relative *hynD* mRNA level with real-time RT-PCR in wild type and *fnrT*::ΩEm^r mutant strain under aerobic and anaerobic conditions.

3.2.3. Organisation and transcription of the *hupD* and *hoxW* genes

A single transcript is made from the *hoxEFUYHW* genes, which suggests that the protease-like *hoxW* gene is part of this operon and probably encodes for the specific endopeptidase of the soluble Hox hydrogenase (Fig. 7.). Similarly, a single mRNA is transcribed from the *hup* operon (*hupSLCDHI*) (J. Balogh et al., personal communication), thus the *hupD* gene product is formed together with the HupSL structural proteins.

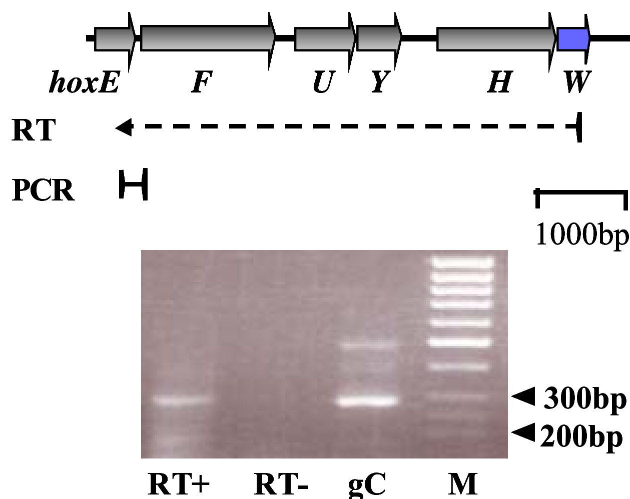


Figure 7. Reverse transcription-PCR analysis of the *hox* operon. Reverse transcription was initiated at the middle of the *hoxW* gene, while the PCR was performed with primers located in the *hoxE* and *hoxF* genes. Lane RT+, reverse transcriptase present in the reverse transcription reaction; lane RT-, reverse transcriptase not present in the reverse transcription reaction; lane M, marker; lane gC, genomic control.

3.3. Analysis of the roles of the accessory proteins

After the characterization of the transposon mutant strains and examination of the genomic organisation of the accessory genes found in *T. roseopersicina,* functional studies were started to get deeper insight into the *in vivo* roles of the unique accessory proteins. For this purpose the mutant strains, generated by the transposon mutagenesis, were not suitable in most cases, especially not in the cases of clustered genes because of the polar effect. More direct approaches, like deletion mutagenesis and protein-protein interaction studies were chosen to investigate the function of these gene products.

3.3.1. Deletion mutagenesis studies

Deletion mutagenesis provides a tool for investigating the importance of the accessory proteins in the assembly of the hydrogenase enzymes. This approach was used to investigate the specificities of the *hupK,* $hypC_1$, $hypC_2$, *hynD, hupD* and *hoxW* genes in *T. roseopersicina* (Fig. 8.). The technique gives information about the specific roles of the various gene products in the maturation of the hydrogenase iso-enzymes. It is particularly important in the case of our model organism, because this bacterium possesses at least three different hydrogenase enzymes. The direct method for testing the specificity of the auxiliary genes was the hydrogenase activity measurement, both in the direction of hydrogen evolution and uptake.

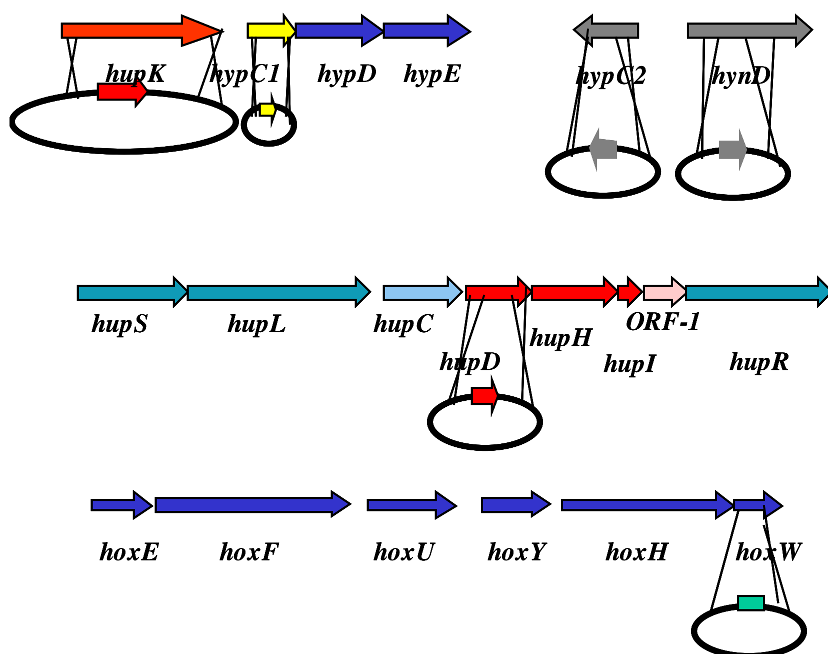


Figure 8. Deletions created in accessory genes (*hupK,* $hypC_1$, $hypC_2$, *hynD, hupD, hoxW)* in *T. roseopersicina*

3.3.1.1. Mutational analysis of the *hupK* gene

The role of the HupK (HoxV) in the maturation process of the [NiFe] hydrogenases is unknown. The *hupK* gene is not present in all bacteria harbouring [NiFe] hydrogenase enzymes; however strains possessing at least the Hup-type hydrogenase always contain this gene (*R. leguminosarum, B. japonicum, R. eutropha* (*hoxV*)*, A. chroococcum,* etc.). No *hupK*-type gene is present in *E. coli*. Conserved regions could be recognized at the N- and C-termini, while the middle portion of the proteins appeared variable. The highest homology was found at the C-terminus, and this region showed significant identity to the HupL (membrane-associated hydrogenase large subunit) proteins as well, although half of the conserved cysteines were missing (Imperial et al., 1993). In-frame deletion mutagenesis was used to determine the specificity of the HupK protein in *T. roseopersicina*. In the deletion construct the region of interest coded for 31 aa in the truncated 81 aa long HupK originated from the N-terminus, 37 aa from the C-terminus of the protein and 13 aa came from the multiple cloning site of the pK18*mobsacB* vector. The extensively shortened *hupK* derivative replaced the wild type gene in the BBS and Δ*hynSL* (GB11) strains. The physiological effects of the mutation on the hydrogenase enzyme activities were tested in H_2 uptake activity assays of each individual [NiFe] hydrogenase enzyme in *T. roseopersicina*. Approximately 90 % of both HynSL and HupSL activity was lost in comparison to the wild type (Table 4.). On the contrary, the soluble fraction retained almost all of its activity; around 75 % of Hox activity was detectable in the HupK deleted strain, with respect to the wild type. Homologous complementation with the *hupK* gene (pBRHupK) fully restored the hydrogenase activity of the cells (Table 5A.). This has further proven the selectivity of HupK, which is important for the formation of both functionally intact membrane-associated [NiFe] hydrogenases, but it is not involved in the maturation of the soluble Hox enzyme in this bacterium. It is to note, that the mutation of the *hupK* gene resulted in a remarkably good hydrogen producing strain, as only the Hox hydrogenase remained active, which works mainly in the direction of hydrogen evolution.

3.3.1.2. Mutants in the two HypC accessory proteins

The role of the putative HypC proteins was studied first by in-frame deletion mutagenesis in *T. roseopersicina*. Each of the *hypC* genes were deleted from the wild type,

the GB11 (HynSL minus) and GB1121 (HynSL and HupSL minus) genomes individually. In addition, a double *hypC* mutant strain was also generated from the wild type *T. roseopersicina* BBS (see Table 1). Hydrogenase activity assays, both in the uptake and in the evolution directions, were carried out both on membrane and soluble fractions of the various mutant strains. The absence of $HypC_1$ almost completely eliminated the activity of all [NiFe] hydrogenases: about 3-5 % of the activities of both membrane-bound hydrogenases (Hup and Hyn), and 10 % of the cytoplasmic (Hox) hydrogenase activity was detectable in the $\Delta hypC_1$ mutant (see Table 4).

Inactivated genes	Strain	Hyn activity	Hup activity	Hox activity
None (wild type)	BBS	100	100	100
hupK	DHKW426	7	12	76
hupK, *hynSL*	DHKG517	0	12	73
$hypC_1$	DC1B	3	5	10
$hypC_1$, *hynSL*	DC1G	0	5	12
$hypC_1$, *hynSL*, *hupSL*	DC1H	0	0	14
$hypC_2$	DC2B	9	11	6
$hypC_2$, *hynSL*,	DC2G	0	11	8
$hypC_2$, *hynSL*, *hupSL*	DC2H	0	0	6
$hypC_1$, $hypC_2$	DC12B	0	0	0
hynD, hupSL	DYDG2	0	0	96
hynD, hynSL	DYDG1	0	104	92
hynD, hynSL, hupSL	DYDG12	0	0	101
hupD, hynSL	DUDG1	0	0	89
hupD, hupSL	DUDG2	106	0	98
hupD, hynSL, hupSL	DUDG12	0	0	92
hoxW, hynSL, hupSL	DXDG12	0	0	0
hoxW, hynSL	DXDG1	0	95	0
hoxW, hupSL	DXDG2	104	0	0

Table 4. Hydrogenase activities of the wild type and in frame deletion mutant *T. roseopersicina* strains. H_2 uptake activities were measured on the membrane and soluble fractions respectively. The results are given in percentage activity compared to the wild type strain (100%). Experimental errors were within 10 %. For the description of the strains, see Table 1.

Homologous complementation with the $hypC_1$ gene (pBRC1, containing the $hypC_1$ upstream region) incompletely restored the activity: only 15 % of the wild type activity was

measurable (Table 5A.). The low complementation efficiency might be due either to the lack of the putative promoter preceding the *hupK* gene, or to the absence of the *hypD* gene in the complementing construct, i.e., an in frame deletion of *hypC*$_1$ might also have polar effect on the expression of *hypD* similar to *E. coli hypC* and *hypD* (M. Blokesch et al., personal communication). The mutation of the *hypC*$_2$ gene also affected all three hydrogenases, the HupSL and the HynSL activities decreased to 9-10 % and the soluble Hox hydrogenase retained only 6 % of its activity as compared to the wild type. Homologous complementation with the *hypC*$_2$ gene (pBRC2) was complete; the wild type Hup, Hyn and Hox activities of these [NiFe] hydrogenases were restored (Table 5A.). The results indicate that the two related putative proteins cannot replace one another in the maturation of the various hydrogenases.

A,

Complementing gene	Plasmid	*hupK*Δ, BBS (DHKW426)	*hypC*$_1$Δ, BBS (DC1B)	*hypC*$_2$Δ, BBS (DC2B)	*hypD*::Km (M442)
hupK	pBRHupK	96 ± 8	-	-	-
hypC$_1$	pBRC1	-	15 ± 4	-	-
hypC$_2$	pBRC2	-	0	98 ± 4	-
hypC$_1$*DE*	pBRCDE	-	-	-	18 ± 6
hupK, *hypC*$_1$*DE*	pBRKCDE	-	-	-	43 ±11

B,

Complementing gene	Plasmid	*hynD*Δ,GB21 (DYDG2)	*hupD*Δ, GB11 (DUDG1)	*hoxW*Δ, GB1121 (DXDG12)
hynD	pMHEYDCt	93 ± 6	-	-
hupD	pMHEUDCt	-	89 ± 11	-
hoxW	pMHEXDCt	-	-	107 ± 3

Table 5 (A, B). H_2 uptake activities in homologous complementation experiments. The results are given in percentage compared to the *T. roseopersicina* wild type strain. For the description of plasmids, see Table 1.

3.3.1.3. Specificity of the endopeptidases

Three endopeptidase-coding genes were identified in the *T. roseopersicina* genome. The transposon-containing M4711 strain was investigated in detail, and the results showed, that the *hynD* gene was responsible at least for the maturation of the Hyn stable hydrogenase. After repeating the experiments with separated membrane and soluble cell fractions of the

hynD::Km mutant strain, significant decrease in the activity of the soluble hydrogenase was also observed. To investigate the reasons of this phenomenon, mutant strains were created in all endopeptidase-coding genes. New, in frame deletion was created in the *hynD* gene in different hydrogenase mutant strains (ΔHyn, ΔHup, ΔHyn-ΔHup), and the results proved the clear specificity of the HynD protein for the Hyn enzyme (Table 4.).

Similar in frame deletions were made in the *hupD* gene using the same mutant backgrounds, the results showed that the product of the *hupD* gene plays a role in the maturation of the HupL large subunit exclusively (Table 4.).

The *hoxW* were disrupted by inserting an erythromycin antibiotic resistance cassette in the gene. Activity measurements showed that the Hox enzyme activity was selectively lost in these strains (Table 4.).

Homologous complementation experiments were performed in all cases, the activities of the appropriate hydrogenase enzymes were restored by the endopeptidases expressed from plasmids (Table 5B.). The applied broad host range vectors were not only constructed for the complementation experiments, they could be used for protein purification from homologous host as well.

3.3.2. Examination of the maturation process at protein level

Activity measurements of the various mutant strains resulted in useful information about the biosynthetic processes of the hydrogenase enzymes in *T. roseopersicina*, but the information provided by this method are limited. The maturation is a complex process with several protein participants, and the various steps of the process can be well defined both in time and regarding the players, the protein partners of a given step. The accessory proteins form different and dynamically changing complexes during the assembly of the large subunit. The protein-protein interactions can be followed using antibodies against the accessory proteins and the large hydrogenase subunit. Unfortunately, there were no antibodies available at the time of these experiments, although efforts are being made produce them at least against the three large subunits (HynL, HupL, HoxH). Alternative approaches had to be chosen in order to search for protein complexes, relationships between accessory and structural proteins.

3.3.2.1. Broad host range vectors for protein purification from homologous host

An alternative solution is to express and purify the proteins of interest from the original bacterial host by employing specific expression vectors. It is hard to find one that fulfils all the requirements needed for a particular study or organism. Existing vectors are complicated to redesign; moreover, it is laborious to change or add required properties because of the lack of sequence data, the large size, and often the need for several cloning steps.

A set of broad host range expression vectors was created in our lab (Fodor et al., 2004). The vectors are small and mobilizable, and their sequences are known. These vectors are useful for protein purification and for studying protein-protein interactions in a range of gram-negative bacteria, as the elements of the regulatory region can be changed easily as requested. Different fusion tags are available to help protein purification (six-His-tag, Strep-tagII, FLAG-tag, T7-tag). Promoter region of *T. roseopersicina crtD* gene was utilized to express proteins in this host organism.

First the HupK and the two HypC proteins were examined using this method in order to strengthen the number of valuable information available about them after the mutagenesis experiments.

3.3.2.2. Purification of the HupK accessory protein

The deletion mutagenesis studies of the HupK protein in *T. roseopersicina* showed that this protein was specific for the membrane-associated hydrogenases. This result fits well with the observations that this protein is present only in organisms containing at least one membrane-bound [NiFe] hydrogenase enzyme. In order to help the understanding of the exact role of HupK, interacting protein partners have to be found during the hydrogenase assembly. Our expression vector series proved to be useful in fulfilling this requirement. In the pB6HupK-Km construct the HupK protein is expressed with C-terminal affinity tag fusions (Strep-tagII and FLAG-tag) in the homologous host. The expression is promoted by the photosynthetic *crt* promoter, which is considered to be working constitutively in *T. roseopersicina* under the appropriate conditions (anaerobic growth under light). The pB6HupK-Km vector was conjugated into the Δ*hupK* deficient mutant strain (DHKW426), the complete restoration of the hydrogenase activity in the mutant indicated that the tagged HupK protein is actively expressed from the vector. The purification of the HupK protein on

M2 agarose column (containing anti-FLAG antibody) was successful (Fig. 9.), the tagged HupK protein appeared in the expected size range at ~43 kDa after PAGE. The protein band was analyzed by MALDI-TOF MS. Other bands were co-purified on the M2 agarose column, one of them (~62 kDa) was determined earlier by mass spectrometry, and it contained sequence elements of 60 kDa GroEL chaperonin (Fodor et al., 2004). Interestingly, at least half amount of the HupK proteins was observed in the membrane fraction. The soluble fraction also contained HupK, which can be the result of the cell disintegration during the fractionation if we assume that the HupK is loosely attached to the membrane. However, these results ascertain the membrane specific role of this accessory protein. Results of the various signal sequence predicting programs were not clear, the presence of a signal peptide at the N-terminal end of the HupK protein could not be excluded.

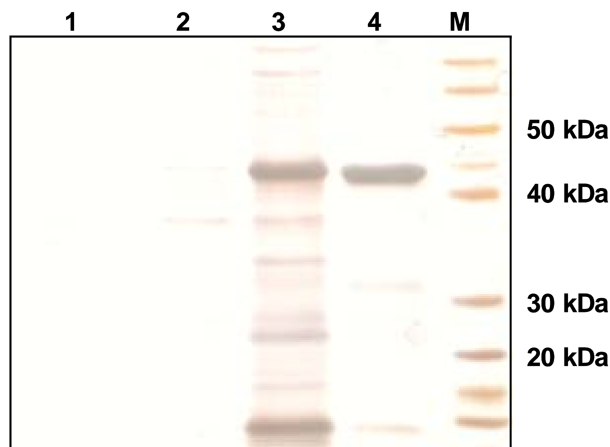


Figure 9. Purification of HupK from *T. roseopersicina.* Silver stained 10% SDS-PAGE of fractions after ANTI-FLAG M2 affinity purification from DHKW426 (1,2) or DHKW426\pB6HupK-Km (3,4). Lanes 1 and 3 are membrane, lanes 2 and 4 are soluble fractions. Intact HupK: 43 kDa

In order to identify intermediates of the maturation it is reasonable to stop the maturation process at definite points. One approach is the nickel starvation of the cells, which results in the accumulation of the intermediates because the Ni insertion into the active centre could not take place. Therefore the tagged HupK protein was purified (~43kDa) from Ni starved cells using mild conditions (Fig. 9). Bands co-purifying with HupK were observed, which did not appear in the acrylamide gel after the purification of the control strain (DHKW426). These bands were cut out and subjected to MALDI-TOF analysis. The bands of the sizes ~38kDa, ~28kDa, ~18kDa, ~15kDa contained different HupK fragments, degradation probably occurred during the purification. A ~35kDa protein band proved to be the PuhA protein, which is the H subunit of the photosynthetic reaction centre. The results of the database search of the PSD spectrum of MH^+= 1200.6 and 1660.9 proteins confirmed the expected QVLLPINFTR (188-197) and VATDFSIAEKDPDPR (134-148) sequences of PuhA of *T. roseopersicina*. Further experiments are needed to clear whether there is any HupK mediated relationship between the photosynthetic processes and the hydrogenase maturation.

Other approaches exist for identifying protein-protein interactions that take place during the hydrogenase biosynthesis. One can apply mutant strains lacking certain accessory proteins, which cause blocks during the maturation and accumulation of temporary complexes. Experiments are in progress using endopeptidase deficient strains.

3.3.2.3. Interacting partners of the HypC chaperones

Both the *ΔhypC₁* and *ΔhypC₂* strains lack most of the hydrogenase activities, which indicates that they share roles during the biosynthesis of all [NiFe] enzymes in *T. roseopersicina*. The same vector system, as in the case of HupK purification, was used for the examination of the HypC-s in *T. roseopersicina*. The pB6HypC1-Km and pB6HypC2-Km C-terminal tagged HypC constructs were conjugated into the *ΔhypC* mutant strains, the complementations proved the presence of active HypC proteins. The purification method and MS analysis were similar to that used for HupK.

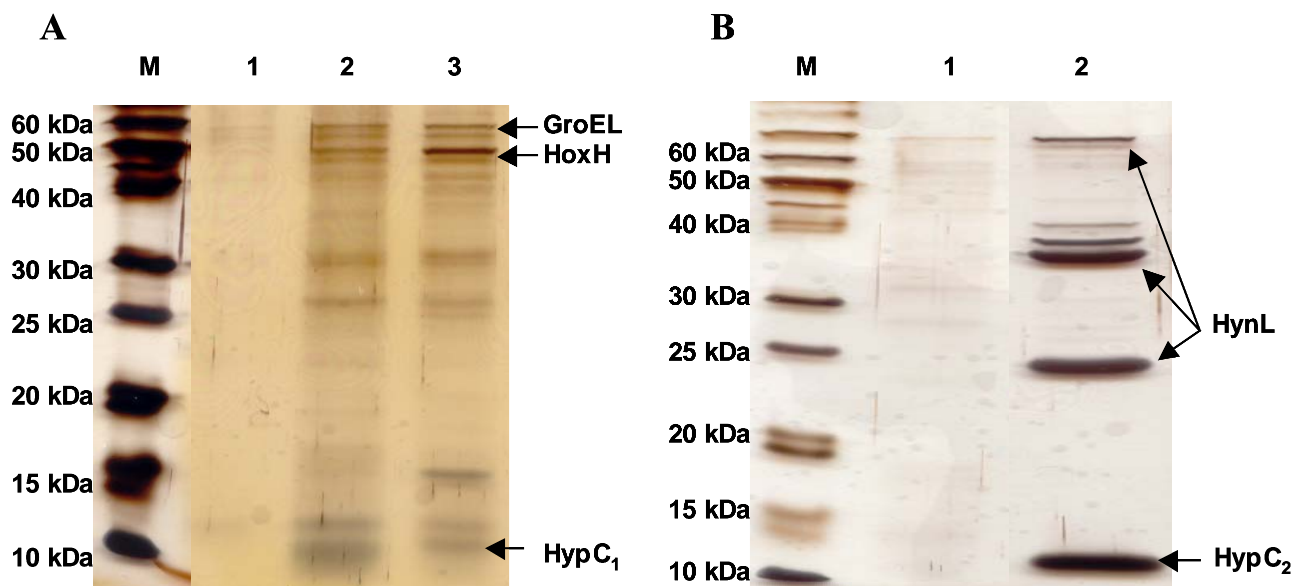


Figure 10. Expression and purification of $HypC_1$ (A) and $HypC_2$ (B) from *T. roseopersicina*. Silver stained 12% SDS-PAGE of fractions after ANTI-FLAG M2 affinity purification from DC1B (A1) or C100 (A2,3) and from DC2B (B1) or C200 (B2) .

The $HypC_2$ protein was shown to interact with the large subunit (HynL) of the Hyn hydrogenase (Fodor et al., 2004). In addition, to the intact HynL, other bands (~25 kDa, ~34 kDa, ~36kDa) corresponded the N- and C-terminus of the HynL protein were also identified

(Fig. 10.). The same degradation products occurred independently under the different growth and purification conditions.

The amount of $HypC_1$ protein present in the cells is small as compared to the $HypC_2$. Purification of $HypC_1$ using the same method and growth conditions as in the case of $HypC_2$ resulted in no specific protein complexes. Even the $HypC_1$ could not be detected. However, after the purification from cells cultured under Ni-free conditions, several bands were observed (Fig. 10.). The MALDI-TOF analysis showed that the large subunit of the soluble hydrogenase (HoxH) was in complex with $HypC_1$ during the maturation process. The database search of the mass spectrum of the ~50 kDa protein pointed to the presence of the HoxH protein with a probability value of 60%. Additional interacting proteins were identified from the same sample, the ~60kDa band represented the GroEL chaperonin again. The re-appearance of the GroEL can be explained by mechanisms independent from the hydrogenase maturation. The proper folding of many proteins *in vivo* requires the assistance of molecular chaperones, like GroEL (Venkatesh et al., 2004).

4. Discussion

Thiocapsa roseopersicina harbours at least 3 [NiFe] hydrogenase enzymes, two of them are attached to the membrane and one is located in the cytoplasm. Thus, it is intriguing and important to explore the functional relationship between the biosynthesis and maturation of the various hydrogenases. Mini Tn5 transposon mutagenesis was used to identify the hydrogenase accessory genes required for the maturation of the [NiFe] hydrogenase enzymes in this strain. Beside the previously identified *hypF* gene, detailed molecular investigation of the mutant strains resulted in the identification of one locus containing the *hupK*-$hypC_1DE$ accessory genes and another one, where the $hypC_2$, *hynD* genes were found. The organization of the accessory genes in this bacterium is unusual, as the corresponding genes are frequently organized into large gene clusters in other organisms (Friedrich and Schwartz 1993, Cammack et al., 2001). In order to examine the specificity of the auxiliary proteins, deletion mutant strains in all three hydrogenases were generated (Rákhely et al., 2004), and the effects of the deletions of the accessory genes were studied selectively via hydrogenase activity assay measurements. The physiological functions of the HupK, $HypC_1$, $HypC_2$ and the three endoproteases (HynD, HupD, HoxW) were investigated in detail by this mutagenesis method and additional information about their roles were obtained using protein-protein interaction studies.

In this chapter, first the results regarding the Δ*hupK* mutants is discussed, then the data collected from the mutagenesis studies of the endoproteases will be evaluated, finally the most intriguing results related to the HypC experiments will be considered.

4.1. Selectivity of HupK

The location of the *hupK* gene, upstream from the $hypC_1DE$, is somewhat surprising because this gene has been found in the *hup* operon of other organisms (Cammack et al., 2001). The role of HupK is ambiguous in the strains studied so far. In *R eutropha* deletion of the *hoxV* (*hupK*) gene reduced the activity of the membrane bound hydrogenase to 30 % compared to the wild type (Bernhard et al., 1996). To the contrary, inactivation of *hupK* led to the accumulation of the immature form of the inactive hydrogenase subunits in *R. leguminosarum* (Brito et al., 1994). Remarkably, this protein does not occur in all microbes

containing [NiFe] hydrogenase, hence the role of the HupK protein is still uncertain. It resembles the large subunit of the [NiFe] hydrogenases; therefore HupK has been suggested to function as a scaffolding protein during metal cofactor assembly. In *T. roseopersicina* the activities of both membrane associated [NiFe] hydrogenases (HynSL and HupSL) decreased dramatically in the absence of the HupK protein, whereas the soluble HoxEFUYH enzyme remained apparently unaffected. Purification of HupK protein from *T. roseopersicina* showed the interaction of this protein with the PuhA photosynthetic reaction centre subunit. Experiments are in progress to prove or disapprove the involvement of this protein in the hydrogenase maturation. No specific interaction between HupK and any other accessory proteins were detected to date, under a number of different growth conditions and mutant backgrounds have to be tested. Although this study did not uncover the precise function of HupK until now, this was the first demonstration that it made a selection among the various [NiFe] hydrogenases in the cell, and participated in the biosynthesis of the membrane bound ones. An important practical consequence of these studies is that the *hupK* deficient strains have notable hydrogen-producing capacity, where the hydrogen produced by a [NiFe] hydrogenase enzyme, namely the soluble Hox hydrogenase.

4.2. Endopeptidases of *T. roseopersicina*

The specific endopeptidases accomplish the last step in the maturation process of the large subunit of each [NiFe] hydrogenase, they cleave the C-terminal end of the precursor large subunit polypeptide, as soon as the [NiFe] heterobinuclear center with its diatomic ligands (Theodoratou et al. 2000, Cammack et al., 2001) has been successfully assembled and inserted into the active site of the enzyme. Three such endopeptidase-like genes were found in *T. roseopersicina*. The *hynD* gene showed a high level of homology to the *orf*-s encoding the specific endoproteases of the [NiFe] hydrogenases of other bacteria, although the gene was not in the vicinity of any hydrogenase structural genes. In the strain harbouring the Tn5 transposon inactivated *hynD* gene (M4711) no HynSL enzyme activity could be detected. In addition, only the activity of the HynSL was lost in the in frame deletion *hynD* mutant strain. The two types of mutations in the *hynD* gene strongly suggest its specificity for the HynSL enzyme during the maturation process. The identification and localization of the *hupD* and *hoxW* genes in the genome suggested that they might be specific for the Hup and Hox hydrogenases, respectively. The *hupD* gene was identified downstream from the *hupSLC* genes, while the *hoxW* gene was the last *orf* in the *hox* operon (*hoxEFUYHW*) coding for the

soluble hydrogenase. The specificity of the HupD protein was supported by in frame deletion mutagenesis, which clearly proved that the HupD protein is responsible only for the assembly of the HupSL enzyme. The disruption of the *hoxW* gene resulted in loss of only the Hox activity showing the specificity of this maturation endopeptidase for the soluble hydrogenase. Hyn, Hup and Hox activities were completely restored by *hynD, hupD* and *hoxW* homologous complementation experiments, respectively. The constructs created for homologous complementation experiments are also useful for affinity purification of the tagged proteins. These purifications are under way, the purified active endopeptidase proteins will be used in *in vitro* maturation experiments. The endopeptidase mutant strains provide an additional advantage: these strains probably accumulate various maturation complexes, because the assembly process is blocked at the last step in these strains. It means that introduced tagged accessory proteins other than the endoproteases can be used for detecting the accumulated maturation complexes. This feature will be used also in the case of the HupK and HypC purifications.

4.3. HypC hypothesis

HypC is a small, chaperone-like protein participating in two protein complexes and thus a dual function has been assigned to it. HypC interacted with the large subunit of the hydrogenase 3 (HycE) in *E. coli* (Magalon and Böck 2000) and it was shown to form a complex with the HypD protein (Blokesch and Böck 2002). In the model based on the observations in *E. coli*, first the HypC-HypD complex is formed, where the iron gets liganded with CO and 2 CN^- in a process involving HypF and HypE (and probably HypD) (Reissmann et al., 2003). Then the HypC, equipped with the Fe-CO-$(CN)_2$ complex is transferred to the HycE subunit with the concomitant dissociation of HypD. The pre-HycE-HypC complex is thought to be formed before the active site is assembled, based on two lines of evidence. First, *ΔhypC* strains have been found to produce nickel-free hydrogenase precursors. Second, the pre-HycE–HypC complex was formed in all *hyp* mutants other than *ΔhypC.* In addition, the presence of the carboxy-terminal extension of the precursor form was required *in vitro* for the complex to be formed (Magalon and Böck 2000). HypC selectively interacts with hydrogenase 3 and it can take over the functions of the homologous HybG in processing the hydrogenase 1 to some extent in *E. coli* but the HybG is not required for the maturation of HycE (Blokesch et al., 2001).

The molecular phenotype of HypC mutations is strikingly different in *T. roseopersicina*. In our case, both HypC proteins are important for the maturation of all three hydrogenases, i.e., both of them have a task in every case, even if they can partially substitute each other. Consequently, both HypC-s are truly pleiotropic accessory proteins in *T. roseopersicina*. The findings in the two bacteria can be assembled into a generalized [NiFe] hydrogenase maturation scheme if we assume that two HypC proteins are needed in the "HypC cycle". In our working hypothesis one HypC interacts with HypD, while the other one holds the pre-large subunit protein in an open conformation. Iron binding and ligation comes about on the HypC-HypD complex then this metal complex (possibly without the HypC protein) is transferred to the HypC-pre-large subunit complex formed independently (Fig. 11.). The HypC-s involved in the two separate steps can be the same proteins or homologous counterparts, which may have dissimilar affinities to the HypD and to the pre-large subunit of the [NiFe] hydrogenases. The difference in the affinity may determine the specificity of the various HypC chaperons. The purification of the HypC proteins from *T. roseopersicina* resulted in the identification of some important maturation complexes. $HypC_1$ interacts at least with HoxH,

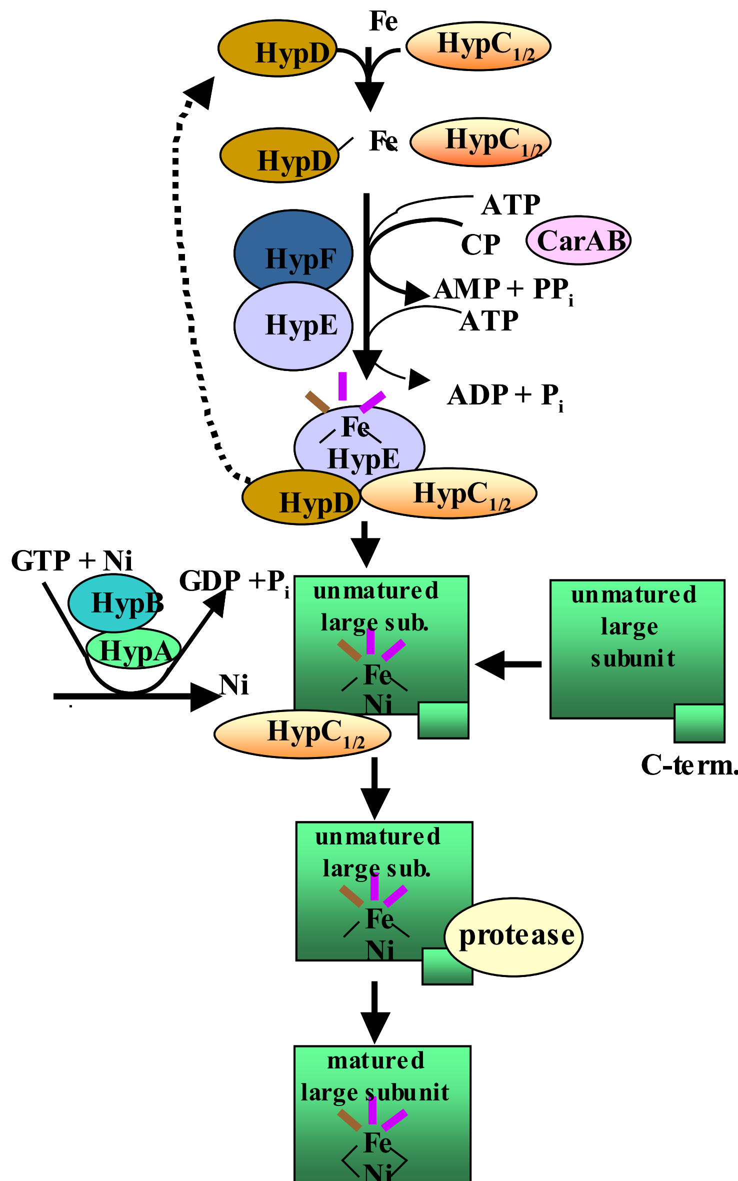


Figure 11. Model for the assembly of the hydrogenase large subunits in the phototrophic *T. roseopersicina*.

$HypC_2$ forms complex with HynL. It is in agreement with our model if both HypC-s are in complex also with HypD. The HypC-HypD complexes should be formed before the HypC-large subunit complexes dissociate. Further experiments with the HypC proteins have to be accomplished in *T. roseopersicina* in order to prove the exact role of both HypC-s in the maturation of the three different [NiFe] hydrogenase enzymes. Accessory protein mutant backgrounds (for example the endopeptidase mutant strains discussed above) have to be used to show the accumulated $HypC_1/_2$-HypD complexes.

There are at least two considerations, which are compatible with a "HypC cycle" involving two (iso)enzymes. On the one hand, all known HypC type proteins share the N-terminal highly conserved region (M-C-(L/I/V)-(G/A)-(L/I/V)-P) (Magalon and Böck 2000), which is the sequence element essential for the interaction with both target proteins. In our model, this interaction is made possible without a competition for the same binding site between the HypD and the pre-large subunit as only the iron complex is transferred from the HypC-HypD complex to the HypC-pre-large subunit assembly. On the other hand, it should be noted that there are two copies of the small chaperon-like protein in every [NiFe] hydrogenase containing microorganism studied in detail (even if it has only one hydrogenase!), e.g., in *E. coli* HypC and HybG (Blokesch et al., 2001), in *R. eutropha* HypC and HoxL (Bernhard et al., 1996, Dernedde et al., 1996), in *R. leguminosarum* (Rey et al., 1993), *R. capsulatus* and *B. japonicum* (Cammack et al., 2001) HypC and HupF, and in *T. roseopersicina* $HypC_1$ and $HypC_2$. Our model offers a function for both chaperons. Experimental evidences that support the cooperativity-based model are as follows. First, in *T. roseopersicina* both HypC proteins are required for the biosynthesis of each hydrogenase. A similar situation was observed in *R. eutropha,* where a mutation in either the *hypC* or in the homologous *hoxL* resulted in the dramatic reduction but not the complete loss of membrane-bound hydrogenase activity. Second, it was shown in *E. coli* that the HypC-preHycE complex existed on a HypD minus background (Blokesch and Böck 2002). This demonstrated the independent formation of the HypC-HypD and the HypC-preHycE complexes in *E. coli* as well. Third, the distinct affinity of the two chaperon-like proteins, HypC and HybG, to the target protein was demonstrated in *E. coli,* when both HybG and HypC proteins were expressed in HybG minus background and only the HybG-HypD complex was detectable, although this experiment was not evaluated quantitatively (Blokesch and Böck 2002). It should be noted that this is still only a working hypothesis, which can interpret the data obtained in various microbes, but further validation of the universal nature of the model is

necessary. Experiments to test this model and to identify further intermediates (mainly the $HypC_{1/2}$-HypD complexes) in the various *T. roseopersicina* mutants are in progress.

4.4. Prospects

Hydrogenase enzyme-based experimental biological hydrogen-producing systems exist, although the efficiency of these technologies is far from that required for economical, practical use. The most relevant features, which hydrogenases have to possess, are the functional activity and stability of the enzymes used in living or *in vitro* systems. *In vivo* systems use bacteria that are suitable to utilize various natural electron donors. *T. roseopersicina* has the capability to produce hydrogen both by its nitrogenase enzyme and using hydrogenase enzymes. Significant amount of hydrogen production was achieved by this phototrophic strain using both systems after modification of the assembly apparatus of the hydrogenase enzymes. The mutation of the *hypF* accessory gene abolished all hydrogenase activities in the *T. roseopersicina* cells, so the large amount of hydrogen produced by the nitrogenase is not consumed by the hydrogenases. Inactivation of hydrogen uptake activity of all [NiFe] hydrogenases in the *hypF* deficient mutant resulted in a 7-fold increase in hydrogen evolution capacity of *T. roseopersicina* under nitrogen fixing conditions (Fig. 12.) The *ΔhupK*

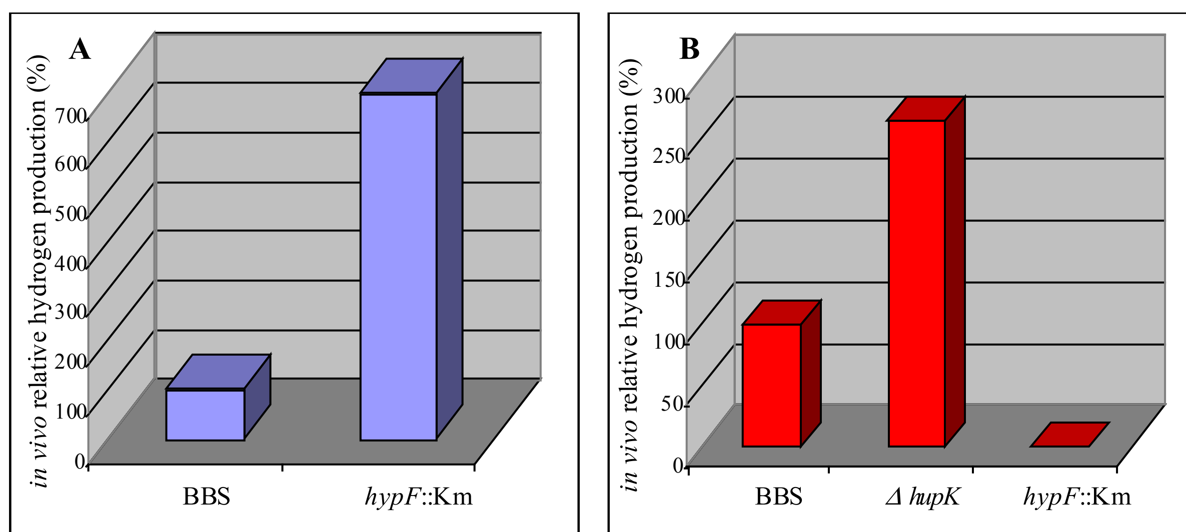


Figure 12. *In vivo* hydrogen production by various strains under nitrogen-fixing (**A**) and nitrogenase repressed (**B**) conditions. Experimental errors were within 20 %. For the description of the strains, see Table 1.

mutant strain displayed a remarkable H_2 production capacity due to the lack of uptake hydrogenase activities (HupSL and/or HynSL) and uninhibited expression of the bi-

directional HoxYH. Wild-type *T. roseopersicina* evolves little amount of H_2, therefore the hydrogen production of the *ΔhupK* mutant is a break-through in generation of H_2-producing bacteria via altering the molecular biology of proteins participating in H_2 metabolism. Therefore, the practical value of these studies is the demonstration that after understanding the details of the molecular biology of the metabolic processes, one can indeed "teach" bacteria and their enzyme-complexes to produce substantial amount of clean energy in a sustainable way.

The main goal of this work was to contribute to our understanding of the maturation process of the [NiFe] hydrogenase enzymes so that in the future one can alter the properties of the hydrogenase enzymes on demand. The current level of our knowledge is not enough to change freely the intrinsic stability and functional activity parameters of these important biotechnological catalysts, but it provides an indispensable foundation for the next generation structure-function studies and eventually leads to efficient biological hydrogen production technologies.

5. Summary

In summary, the behaviour of several accessory proteins was studied. These proteins are selectively or pleiotropically involved in the biosynthesis of the various [NiFe] hydrogenases in *T. roseopersicina*. The following statements could be made based on my results:

I. I identified several accessory proteins participating in the biosynthesis of the [NiFe] hydrogenases by using transposon mutagenesis. The analysis of the M442 mutant strain revealed the *hupK*- *hypC*$_1$-*hypD*-*hypE* genes. The transposon inserted into the *hynD* gene in the M1250 mutant strain, the *hypC*$_2$ gene was near the *hynD* in opposite orientation.

II. I have found the missing *hypA* and *hypB* basic accessory genes by searching in the partial genome sequence of *T. roseopersicina*.

III. I examined the regulation of some of the accessory genes. The *hypC*$_1$-*hypD* genes are transcribed together with the *hupK* gene according to the RT-PCR analysis, but independent transcription of *hypC*$_1$-*hypD* also exists, which was proven by homologous complementation experiments.

IV. The common transcription of the *hoxW* and *hupD* endopeptidase coding genes with the *hoxEFUYH* and *hupSLC* genes, respectively, was determined using RT-PCR. Although FnrT protein regulates the expression of the *hynSL* structural genes, its specific endopeptidase gene, *hynD*, is not regulated by this anaerobic regulator.

V. I created *ΔhupK* in frame deletion mutant strains in *T. roseopersicina* in order to examine the specificity and possible role of this protein. The mutation affected seriously the membrane associated [NiFe] hydrogenases, while the soluble hydrogenase retained almost all of its activity. Homologous complementation with the *hupK* gene restored all hydrogenase activities. HupK mutant strains of *T.*

roseopersicina became new and promising candidates for practical biohydrogen production systems.

VI. I mutated the *hypC*$_1$ (in frame deletion) and *hypC*$_2$ genes in several combinations regarding the hydrogenase background, which made possible the investigation of the specificity of these chaperon-like maturation peptides. In contrast to the *E. coli* model, both HypC-s have crucial roles during the maturation of all three [NiFe] hydrogenases in *T. roseopersicina*. The complementation experiments proved the involvement of the HypC-s in the observed effects.

VII. I performed the mutation analyses for the endopeptidase coding genes. In frame mutants were created in the cases of *hynD* and *hupD* genes, while an antibiotic resistance cassette was inserted into the *hoxW* gene. As I expected, the endopeptidases are strictly specific, the HynD cuts the C-terminal end of the HynL subunit, the HupL plays role only in the maturation of the HupL subunit, and the HoxW is necessary only for the assembly of the Hox hydrogenase.

VIII. I used the pMHE broad host range expression vector system, developed in our lab, for the purification of the accessory proteins from *T. roseopersicina*. I purified the tagged HupK and HypC proteins in their active form, and I have identified important maturation complexes. The HypC$_2$ interacts at least with the HynL, and direct contact was detected between HypC$_1$ and the large subunit of the soluble hydrogenase (HoxH).

IX. I set up a model for the maturation of [NiFe] hydrogenases in phototrophic sulfur bacteria, based on literature and my experimental data. The main discrepancy between this model and the *E. coli* model reported earlier was in the role of HypC-s. These chaperon-like proteins cannot substitute each other during the maturation process; I proposed distinct roles for HypC$_1$ and HypC$_2$.

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7. Publications

7.1. Publications covering the content of the thesis

Maróti, G., Fodor, B. D., Rákhely, G., Kovács Á. T., Arvani, S. and Kovács, K. L. (2003) Accessory proteins functioning selectively and pleiotropically in the biosynthesis of [NiFe] hydrogenases in *Thiocapsa roseopersicina*. *Eur. J. Biochem*. **270**:2218-2227.

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7.2. Other publications

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9. Összefoglalás (Summary in Hungarian)

Munkám során a [NiFe] hidrogenázok bioszintézisében résztvevő, úgynevezett kisegítő fehérjéket vizsgáltam, modell organizmusként a fotoszintetizáló, bíbor kénbaktériumot a *Thiocapsa roseopersicina*-t használva. A kisegítő gének egy része specifikus, míg másik csoportjuk minden, a sejtben megtalálható [NiFe] hidrogenáz enzim éréséhez nélkülözhetetlen. Kísérleteim eredményeit az alábbi pontokban foglalom össze:

I. Számos kisegítő fehérje génjét azonosítottam transzpozonos mutagenezissel. Az M442-es mutáns törzsben a *hupK*- *hypC*$_1$-*hypD*-*hypE* géneket találtam, míg az M1250-es törzsben a transzpozon a *hynD* génbe épült be. A *hynD* gén közelében, vele ellentétes orientációban egy másik, *hypC* jellegzetességeket mutató gént találtam, melyet *hypC*$_2$-nek neveztem el.

II. A jelenleg folyamatban lévő genomszekvenálás részleges adatait használva az ezidáig hiányzó *hypA* és *hypB* géneket megtaláltam.

III. A kisegítő gének szerveződését RT-PCR-rel vizsgáltam, ez alapján a *hypC*$_1$-*hypD* gének a *hupK* génnel közösen íródnak át, emellett azonban a *hypC*$_1$-*hypD* géneknek saját szabályozó régiója is van, melyet a homológ komplementációs kísérletek bizonyítottak.

IV. A *hupD* és a *hoxW* gének operonba szerveződését RT-PCR támasztotta alá, a *hupD* a *hupSLC* génekkel, a *hoxW* a *hoxEFUYH* génekkel alkot operont. A harmadik endopeptidáz kódoló gén, a *hynD*, az FNR anaerob szabályozó rendszer által nem regulált, annak ellenére, hogy a célfehérjéjét kódoló *hynSL* gének e rendszer ellenőrzése alatt állnak.

V. A *hupK* génben a leolvasási keretet megőrző deléciót hoztam létre a HupK fehérje vizsgálata céljából. A mutáció eredményeképpen a membránkötött hidrogenázok (Hyn, Hup) aktivitása minimálisra csökkent, míg a szolubilis hidrogenáz (Hox)

megőrizte teljes aktivitását. Az elvégzett homológ komplementációs kísérletek visszaállítottak minden hidrogenáz aktivitást a sejtben.

VI. A *hypC*$_1$ és *hypC*$_2$ génekben hasonló mutációkat hoztam létre különböző hidrogenázokban mutáns törzsekben a HypC fehérjék specifitásának vizsgálatára. Az *E. coli* hidrogenáz érési modelltől eltérően, *T. roseopersicina*-ban mindkét HypC fehérjére szükség van mindhárom hidrogenáz éréséhez. A homológ komplementációs kísérletek bizonyították, hogy a mutációk megfigyelt hatásai az elrontott gének hiányának a következményei.

VII. A mutációs analízist a három endopeptidázt kódoló gén esetében is elvégeztem. Ahogyan az várható volt, ezen gének termékei teljesen specifikusak egy-egy hidrogenáz összeszerelődési folyamatára. A HynD fehérje csak a HynL C-terminálisát hasítja le, a HupD csak a HupL éréséhez kell, míg a HoxW csak a Hox hidrogenáz összeszerelődésében játszik szerepet.

VIII. A kisegítő fehérjék homológ gazdából való tisztításához a laborunkban kifejlesztett expressziós vektorrendszert használtam. Az affinitás toldalékokkal ellátott HupK és HypC fehérjéket sikerült aktívan tisztítani, és néhány, az érés során létrejövő komplexet találtam. A HypC$_2$ fehérje kölcsönhat a stabil hidrogenáz nagy alegységével (HynL), a HypC$_1$ esetén pedig a szolubilis hidrogenáz nagy alegységével (HoxH) alkotott komplexet azonosítottam.

IX. Hidrogenáz érési modellt állítottam fel *T. roseopersicina*-ra, melyben az *E. coli* modelltől való lényegi eltérést a HypC fehérjék eltérő szerepe jelenti. Ezek a kisméretű fehérjék nélkülözhetetlenek mindhárom hidrogenáz bioszintéziséhez, és nem képesek egymást helyettesíteni.