Chapter 3

Regulation of hydrogenase gene expression

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3.1. Hydrogenase regulation is guided by environmental factors and physiological requirements

Prokaryotes have the capacity to rapidly adapt to changes in their chemical environment. Proteins needed for growth are normally synthesized at levels sufficient to support maximal growth rate on a given substrate. In Nature, however, the cells are usually exposed to a mixture of nutrients, and complex regulatory networks guarantee a hierarchical utilization of the substrates, guided by metabolic efficiency.

The ability of microbes to take up or to evolve H_2 is usually a facultative trait. Therefore it is economically well designed that hydrogenases are predominantly formed only when the substrate is available, and that there are mechanisms of regulated gene expression. Nevertheless, a few specialists organisms (e.g. methanogens) whose metabolism is strictly adapted to H_2 activation are probably synthesizing hydrogenase constitutively. Little is known about gene regulation in archaea, extremophilic organisms such as *Aquifex aeolicus* or in obligate anaerobes, such as sulfate reducers. Regulated hydrogenase gene expression in these organisms, however, cannot be excluded *per se* since many of these specialized microbes harbour multiple isofunctional hydrogenases (see Chapter 2) which may be expressed differentially.

Methanococcus voltae, for example, contains four hydrogenases, two of which contain at the active site, in addition to nickel and Fe, Se. The Se-containing hydrogenases are formed constitutively, whereas the Se-free enzymes are synthesized only under Se depletion (Table 3.1). Likewise, two species of *Methanococcus* are able to use organic compounds for methanogenesis. Both strains contain multiple hydrogenases, and it has been observed that in the presence of acetate one of the hydrogenase operons is repressed (Table 3.1). Moreover, transcript analysis of the putative [Fe] hydrogenase gene *hydA* in *Clostridium acetobutylicum* revealed a fermentation-directed control. The level of *hydA* mRNA is high in cells from acidogenic or alcohologenic phosphate-limited continuous cultures but low when the cells undergo solventogenesis (Table 3.1).

Hydrogenase isoenzymes are also common among the metabolically more versatile bacteria (see Chapter 2). For instance, H_2 metabolism and isoenzyme composition in enteric bacteria, including *Escherichia coli* and *Salmonella typhimurium*, appear to be differentially regulated under the two modes of anaerobic life, fermentation and anaerobic respiration (Table 3.1). Furthermore, biosynthesis of the individual isoenzymes appears to be controlled at a global level by the quality of the carbon source,

Table 3.1 Examples of hydrogenase regulation in response to environmental and physiological factors[a]

Organism	*Type of hydrogenase*	*Relevant characteristics*	*Physiological function*	*Effectors and/or conditions affecting hydrogenase gene regulation*[b]	*Reference*[c]
Alcaligenes hydrogenophilus	2 [NiFe] hydrogenases	Cytoplasmic NAD reducing Membrane-bound cytochrome *b* reducing	Energy conservation	H_2, carbon and energy source limitation	1
Anabaena cylindrica sp. PCC7120	[NiFe] hydrogenase	Putative membrane-bound cytochrome *b* reducing	H_2 recycling during N_2 fixation	O_2, nitrogen limitation, heterocyst formation	2
Bradyrhizobium japonicum	[NiFe] hydrogenase	Membrane-bound cytochrome *b* reducing	Energy conservation H_2 recyling during N_2 fixation	H_2, O_2, nickel, carbon and energy source limitation	3
Clostridium acetobutylicum	[Fe] hydrogenase	Putative cytoplasmic, ferredoxin linked	H_2 production during fermentation	Fermentation pathway, phosphate limitation	4
Escherichia coli	[NiFe] hydrogenase 1/ 2	Membrane-bound cytochrome *b* reducing	H_2 uptake under anaerobic conditions H_2 production during fermentation	Formate, molybdenum, nitrate, carbon source limitation, low pH	5, 6
Escherichia coli	[NiFe] hydrogenase 3	Membrane-associated component of the formate hydrogen lyase complex	H_2 production during fermentation H_2 uptake under anaerobic conditions	Anaerobiosis, carbon source limitation, phosphate limitation, molybdenum, nitrate, formate	7, 8
Methanococcus voltae	2[NiFeSe] hydrogenases [NiFe] hydrogenases	F_{420} reducing F_{420} non-reducing F_{420} reducing F_{420} non-reducing	Methanogenesis Methanogenesis Methanogenesis Methanogenesis	Constitutive Constitutive Se Se	9

Table 3.1 Continued

Organism	*Type of hydrogenase*	*Relevant characteristics*	*Physiological function*	*Effectors and/or conditions affecting hydrogenase gene regulation*[b]	*Reference*[c]
Methanococcus mazeii	2[NiFe] hydrogenases	Membrane-bound cytochrome *b* reducing (Vht)	Methanogenesis	H_2/CO_2, methanol, acetate	10
		Membrane-bound cytochrome *b* reducing (Vho)	Methanogenesis	Constitutive	
Methanococcus barkeri	2[NiFe] hydrogenases	F_{420} reducing	Methanogenesis	H_2/CO_2, methanol, trimethylamine, acetate	11
Nostoc muscorum	2[NiFe] hydrogenases	Cytoplasmic NAD reducing	Energy conservation	Constitutive	12
		Membrane-bound cytochrome *b* reducing	H_2 recycling during N_2 fixation	Nitrogen limitation, light	
Pseudomonas hydrogenovora	[NiFe] hydrogenase	Membrane-bound cytochrone *b* reducing	Energy conservation	H_2	13
Ralstonia eutropha	[NiFe] hydrogenases	Cytoplasmic NAD reducing	Energy conservation	H_2, $\underline{O_2}$, nickel, carbon and energy source limitation,	14
		Membrane-bound cytochrome *b* reducing			
Rhizobium leguminosarum	[NiFe] hydrogenase	Membrane-bound cytochrome *b* reducing	H_2 recycling during N_2 fixation	$\underline{O_2}$, symbiosis	15
Rhodobacter capsulatus	[NiFe] hydrogenase	Membrane-bound cytochrome *b* reducing	Energy conservation	H_2, O_2	16, 17
			H_2 recycling during N_2 fixation		
Rhodospirillum rubrum	[NiFe] hydrogenase	Membrane-bound component of the CO dehydrogenase complex	H_2 production during CO oxidation	CO, $\underline{O_2}$	18

Notes

a Only those hydrogenases are listed which are investigated on the regulatory level.

b Effectors and/or conditions acting negatively are underlined.

c The list is restricted to the most relevant references: 1, Lenz *et al.* (1997); 2, Carrasco *et al.* (1995); 3 Durmowicz *et al.* (1998); 4, Gorwa *et al.* (1996); 5, Atlung *et al.* (1997); 6, Richard *et al.* (1999); 7, Rossman *et al.* (1991); 8, Rosental *et al.* (1995); 9, Sorgenfrei *et al.* (1997a); 10, Deppenmeier (1995); 11, Vaupel and Thauer (1998); 12, Axelsson *et al.* (1999); 13, Ohtuski *et al.* (1997); 14, Lenz and Friedrich (1998); 15, Brito *et al.* (1997); 16, Toussaint *et al.* (1997); 17, Dischert *et al.* (1999)); 18, Fox *et al.* (1996).

known as catabolite repression. This is exemplified by the case of *E. coli*, which contains four hydrogenase systems. Formate is the major effector directing the expression of genes coding for hydrogenase 3 in *E. coli*. This observation is entirely consistent with its role in formate hydrogen lyase-dependent H_2 evolution during fermentative growth (Table 3.1). The physiological function of the uptake hydrogenases 1 and 2 in *E. coli* is less well defined. Expression of the *hya* operon, coding for hydrogenase 1, is co-regulated with that of hydrogenase 3 which implies that an H_2-recycling process takes place during fermentative growth. The transcription factors that govern *hya* expression, however, are distinct from those instrumental in the control of the hydrogenase-3 operon (see Section 3.2). The *hya* operon is induced by formate and repressed by nitrate, its expression is strongly elevated by entry into the stationary phase pointing to a dependence on an alternative RNA polymerase σ subunit known as σ^S. In addition the transcriptional regulators AppY and ArcA influence the expression of *hya*. A fumarate respiration-linked H_2 uptake function is apparently assigned to hydrogenase 2 of *E. coli*. Consistent with this view is the fact that the hydrogenase 2 operon is controlled by the global regulator Fnr which directs the expression of genes involved in anaerobic respiration (Table 3.1).

A particularly interesting regulatory system has been uncovered in the cyanobacterium *Anabaena cylindrica* sp. strain PCC7120 (Table 3.1). Expression of the hydrogenase encoding *hupL* gene occurs during cellular differentiation to heterocysts which are specialized in N_2 fixation. Developmentally controlled gene rearrangments, resulting in the excision of a 10.5 kb DNA element from the *hupL* gene, encoding the large subunit of an NiFe uptake hydrogenase, lead to activation of hydrogenase gene expression. Coexpression of hydrogenase and nitrogenase genes has also been observed in other microbes such as *Nostoc muscorum, Rhizobium leguminosarum* and *Bradyrhizobium japonicum* (Table 3.1). Details of selected well-studied systems are depicted in Section 3.2.

Aerobic bacteria which often use H_2 as an alternative energy source express hydrogenase genes, with a few exceptions, when the substrate is provided (Table 3.1). How do these organisms recognize the presence of H_2, the smallest molecule on Earth? The underlying molecular mechanisms are subject of current research and will be discussed in Sections 3.2 and 3.3.

3.2. Diversity of hydrogenase operons and their transcriptional control

Analysis of the genomic arrangement of hydrogenase genes revealed that in most cases studied so far, the hydrogenase genes occur as tightly clustered functional units, or operons. These are also present also in methanogens as illustrated in Fig. 3.1. The proteobacterial species show complex hydrogenase gene clusters consisting of hydrogenase subunit genes and varying numbers of accessory genes (Figs 3.2–3.6) which code for functions involved in metal centre assembly, protein maturation and gene regulation (Chapter 4). Total genome sequences, now available for a few bacterial and archaeal species, revealed that the hydrogenase accessory genes may also be dispersed on the chromosome, e.g. in the cyanobacterium *Synechocystis* sp. PCC 6803, the hyperthermophilic bacterium *A. aeolicus* and the archaeon *Methanococcus jannaschii* (Bult *et al*. 1996; Deckertet *al*. 1998; Kaneko *et al*. 1996). It is too early to

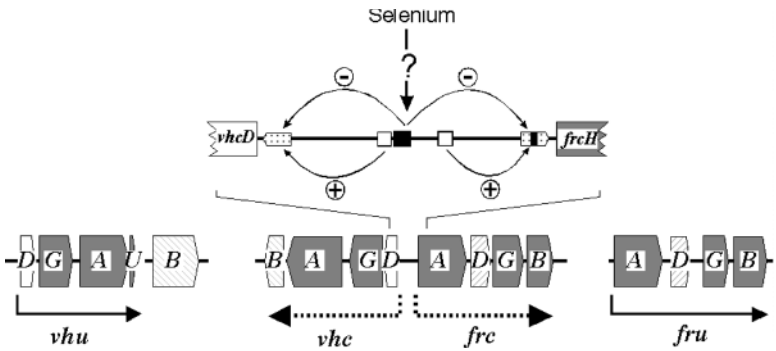

Figure 3.1 Se-controlled regulation of hydrogenase gene transcription in *M. voltae*. The intergenic regulatory region between the divergent *vhc* and *frc* operons is shown in a magnified form. Dotted boxes indicate the promoter regions. Open and solid boxes represent positive and negative regulatory elements, respectively. Hydrogenase structural genes are shaded in dark grey, accessory genes are hatched, genes involved in metallocentre assembly are dispersed over the chromosome and are not displayed. The functions of the *vhcD* and *vhuD* genes are unknown. Transcriptional start sites and the potential length of transcripts resulting from regulated and constitutive promoters, respectively, are indicated by dotted and solid arrows below the operons.

speculate if the difference in gene organization has any impact on hydrogenase gene expression and/or the activity of the resulting hydrogenase proteins. Therefore this section will focus only on those systems which have been well studied biochemically and genetically.

The arrangement of genes in an operon permits their coordinate expression by command of a principal regulator. Such a regulator normally binds to a promoter region, near the site of transcription initiation. Depending on the system, the regulator may exert a positive or a negative effect leading to induction or repression of transcription. The activity of the regulator may be modulated by low-molecular-weight compounds such as the substrate or a metabolite. For instance, the FhlA protein which controls the *hyc* (hydrogenase 3) operon in *E. coli* is modified by formate (Fig. 3.2) (Hopper *et al*. 1994). On the other hand, the regulators of hydrogenase operons might also be the target of a complex signal transduction cascade involving sensor protein(s) which recognize a given external or internal stimulus. These sensor proteins usually transmit the information by chemical modification of the regulator and thus direct its capacity to activate or to repress transcription (Section 3.3).

A major mechanism used by bacteria to respond to environmental changes is the so-called two-component regulatory system which uses phosphorylation/dephosphorylation as a means of information transfer (Hoch and Silhavy 1995). A standard two-component regulatory system consists of a response regulator and a sensor, a histidine protein kinase, which is able to autophosphorylate at the expense of ATP hydrolysis and to transfer its phosphoryl group to the response regulator. The sensor kinase may function also as a phosphatase, removing the phosphoryl group from the regulator. In these bifunctional cases, the direction of the sensor-mediated process is governed by the stimulus. Likewise, the phosphoryl group of the regulator may as

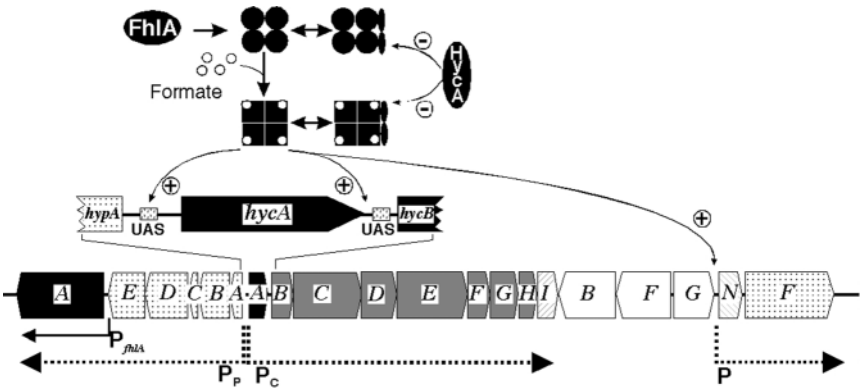

Figure 3.2 The formate regulon of *E. coli* hyc genes encoding structural components of the hydrogenase 3 are shaded in dark grey. Genes involved in metallocentre assembly (*hyp*) are shown in dotted boxes. Open boxes represent the cryptic *ascBFG* genes which form an operon for the degradation of β-glucosides. The hydrogenase-specific endoprotease gene *hycI* and the *hydN* gene are illustrated in hatched boxes. The regulatory genes (*hycA, fhlA*) and their respective products are marked in black. Not shown is *fdhF*, which encodes the seleno polypeptide of formate dehydrogenase H. FhlA binding sites (UAS) are indicated. Transcriptional start sites and the potential length of transcripts resulting from regulated and constitutive promoters, respectively, are indicated by dotted and solid arrows below the gene cluster.

well be hydrolysed by a distinct phosphatase, a potential extra component of a signal transduction system. Moreover, a possible extremely short half-life of the phosphorylated regulator is often observed (Hoch and Silhavy 1995).

Genes belonging to the superfamily of two-component regulatory systems have been identified in hydrogenase gene clusters of *B. japonicum* (Fig. 3.3), *Rhodobacter capsulatus* (Fig. 3.5) and *Ralstonia eutropha* (Fig. 3.6) (Dischert *et al.* 1999, Lenz and Friedrich 1998, Van Soom *et al.* 1999). Hydrogenase gene expression in *R. leguminosarum* is entirely adapted to symbiotic N_2 fixation and guided by the two master regulators of this control circuit, namely FnrN and NifA (Fig. 3.4) (Brito *et al.* 1997). The expression of the *hyc* operon in *E. coli* is coordinated with the expression of the formate hydrogen lyase complex (Sauter *et al.* 1992). Unlike standard two-component systems, the response regulator FhlA is not activated by phosphorylation but by binding formate as the effector molecule (Fig. 3.2). The two Se-sensitive operons in *M. voltae* are both positively and negatively controlled and the sites of regulation are well defined (Fig. 3.1) (Beneke *et al.* 1995); however, the trans-acting regulatory proteins remain to be elucidated. In the following, the regulation of six well-established hydrogenase systems is presented in more detail.

The methanogenic archaeon *M. voltae* harbours four hydrogenase operons (Chapter 2), two of which, *vhc* and *frc*, encode [NiFe] hydrogenases. The remaining two operons, *vhu* and *fru*, encode [NiFeSe] hydrogenases (Fig. 3.1, Table 3.1). The Se-containing hydrogenases are synthesized constitutively. Transcription of the *vhc* and *frc* operons only takes place under conditions of Se depletion, when the two Se-containing isoenzymes cannot be made in sufficient amounts (Berghofer *et al.* 1994).

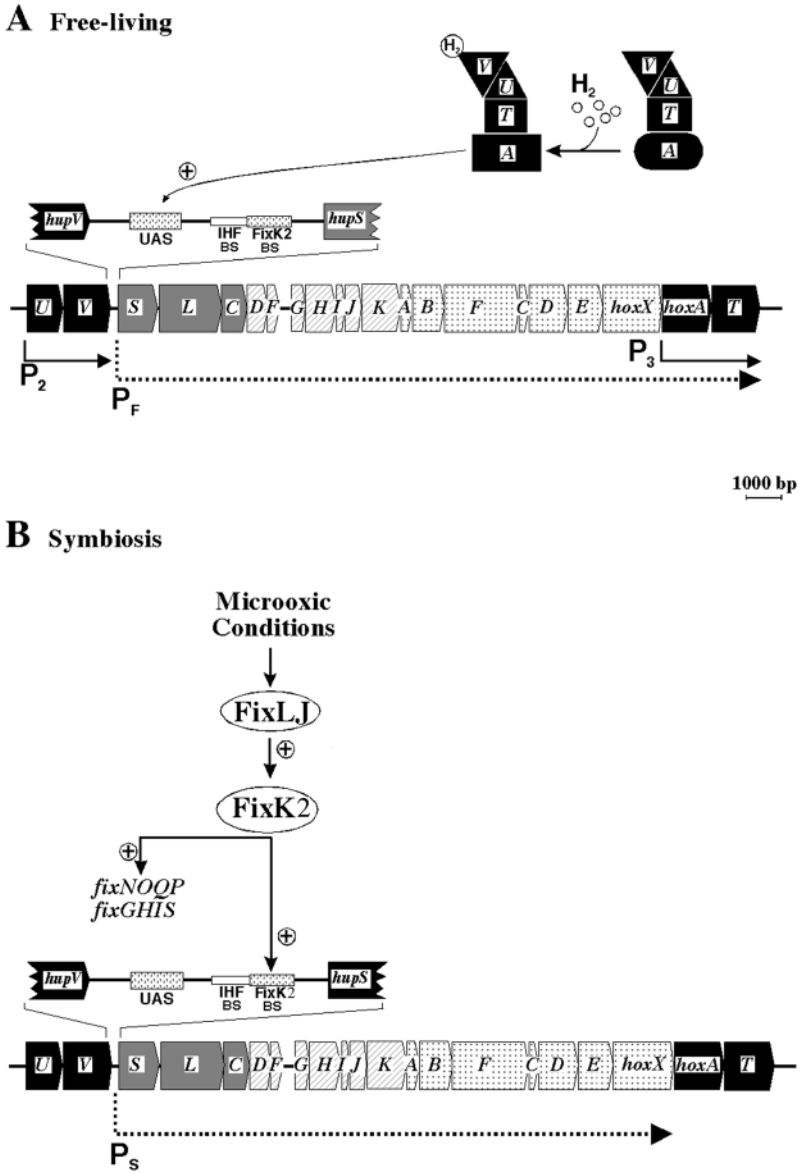

Figure 3.3 Hydrogenase gene expression in *B. japonicum* under free-living conditions (A) and during symbiosis (B). Hydrogenase structural genes (*hup*) are shaded in dark grey, accessory genes (*hup*) are hatched, genes involved in metallocentre assembly (*hyp*/*hox*) are shown in dotted boxes. The regulatory genes *(hox*/*hup*) and their respective products are marked in black. The *hupNOP* genes, of which *hupN* encodes a nickel permease (Chapter 4), are located 8.3 kb upstream of the *hupSL* genes and are not included in this figure. Putative FixK2 (FixK2 BS), IHF (IHF BS) and HoxA binding sites (UAS) are indicated. Transcriptional start sites and the potential length of transcripts resulting from regulated and constitutive promoters, respectively, are indicated by dotted and solid arrows below the gene cluster.

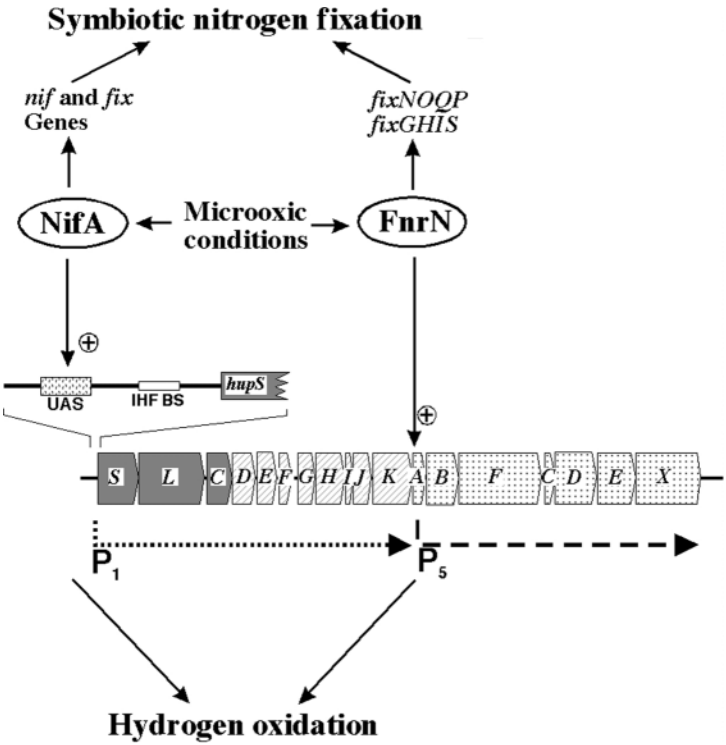

Figure 3.4 Symbiotic hydrogenase gene expression in *R. leguminosarum*. Hydrogenase structural genes (*hup*) are shaded in dark grey, accessory genes (*hup*) are illustrated in hatched boxes, genes involved in metallocentre assembly (*hyp*) are shown in dotted boxes. Putative IHF (IHF BS) and NifA binding sites (UAS) are indicated. Transcriptional start sites and the potential length of transcripts resulting from regulated and constitutive promoters, respectively, are indicated by dotted and solid arrows below the gene cluster.

A coordinated regulation of *vhc* and *fru* is facilitated by the genetic linkage of the two operons, which are separated by a relatively short 450 bp intergenic region. This region contains the promoters and sites implicated in positive and negative transcriptional regulation (Fig. 3.1) (Beneke *et al*. 1995). Mutational analysis has shown that a sequence located in the centre of the intergenic region is involved in negative regulation of both operons. Furthermore, there is evidence for an additional element within the promoter region of the *frc* operon which is involved in establishing the negative control of this gene group. Upstream of either operon, positive regulatory sites were identified that are necessary for full expression of the operons in the absence of Se. It is not yet understood how the signal 'Se deprivation' is transduced to the regulatory machinery. One possible explanation is that a negative regulator or a cofactor is a selenoprotein with a short half-life. Such a protein would not be actively synthesized when Se is depleted. An earlier hypothesis proposed that the

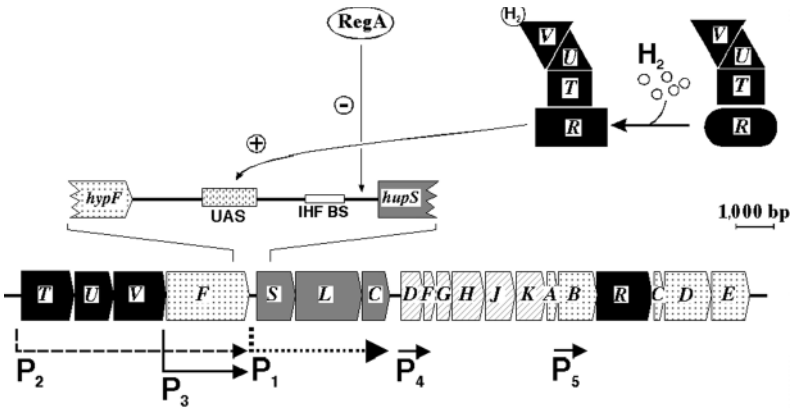

Figure 3.5 H_2-dependent hydrogenase gene expression in *R. capsulatus*. Hydrogenase structural *hupSLC* genes are shaded in dark grey, accessory *hup* genes are illustrated in hatched boxes, genes involved in Ni insertion are shown in dotted boxes. The regulatory *hupTUVR* genes and their respective products are marked in black. HupR (UAS) and IHF (IHF BS) binding sites are indicated. Transcriptional start sites and the potential length of transcripts resulting from regulated and constitutive promoters, respectively, are indicated by dotted and solid arrows below the gene cluster.

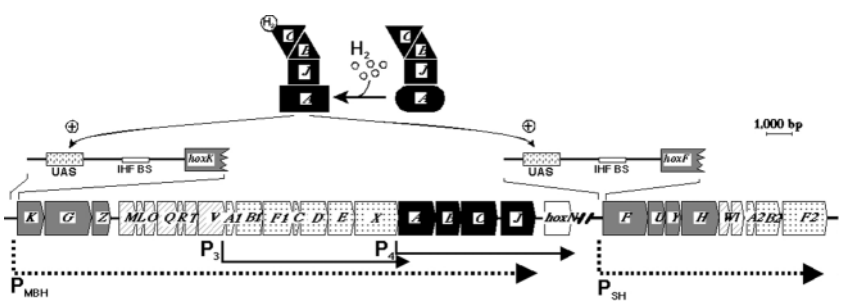

Figure 3.6 H_2-dependent expression of the hydrogenase regulon of *R. eutropha*. Hydrogenase structural genes (*hox*) are shaded in grey, MBH- and SH-specific accessory genes (*hox*) are illustrated in hatched boxes, genes involved in metallocentre assembly (*hyp*) are shown in dotted boxes. The regulatory genes (*hox*) and their respective products are marked in black. The *hoxN* gene encodes a nickel permease. Putative HoxA (UAS) and IHF (IHF BS) binding sites are indicated. Transcriptional start sites and the potential length of transcripts resulting from regulated and constitutive promoters, respectively, are indicated by dotted and solid arrows below the gene clusters.

smallest, Se-containing subunit (VhuU) of the Vhu hydrogenase acts as a regulatory factor. This assumption, however, is unlikely since a fusion of VhuU and the large subunit VhuG does not affect regulation (Pfeiffer *et al.* 1998).

The hydrogenase 3 of *E. coli* is part of the formate hydrogen lyase complex (FHL) whose corresponding genes are organized in the formate regulon (Fig. 3.2) (Rossmann *et al.* 1991). Hydrogenase maturation proteins are encoded by the

hypABCDE gene complex which is co-transcribed with the regulator gene *fhlA*. FhlA belongs to a subclass of response regulators whose activity is modulated by binding of an effector molecule. In the case of FhlA the effector is formate (Hopper *et al*. 1994). The *hyc* operon genes predominantly code for structural components of hydrogenase 3 with the exeption of HycA and HycI which code for a negative regulator and a maturation endopeptidase, respectively. A third transcriptional unit encodes HypF, a maturation component, and HydN whose function is unknown. FhlA complexed with formate binds to the upstream activator sequences (UAS) and activates the σ^{54}-dependent promoters P_P, P_C and P (Fig. 3.2). P_{fhlA}, on the other hand, is a weak constitutive promoter dependent on σ^{70}-containing RNA polymerase. Formate production by pyruvate formate lyase is the main signal for the onset of anaerobiosis. Formate binds to the regulator FhlA (possibly a tetramer), which in turn activates transcription from the UAS motifs upstream of P_P, P_C and P (Hopper *et al*. 1994). The transcription activation leads to an increase in FhlA. This autogenous control of FhlA is counteracted by the activity of HycA, an anti-activator which may function by direct interaction with FhlA (Sauter *et al*. 1992). Expression of the *hyc/fhl* genes leads to the degradation of formate and consequently to the decrease of the effector concentration. Hence, the Fhl system is well balanced by the two positively acting components, FhlA and formate production, and their negatively acting counterparts, HycA and formate degradation.

B. japonicum, the N_2-fixing symbiont of the soybean plant, synthesizes its membrane-bound uptake hydrogenase under two extremely different life styles: under free-living microaerobic conditions, in the presence of H_2, and during symbiosis in the root nodules. In free-living bradyrhizobia, hydrogenase gene transcription is induced by molecular H_2 provided that the O_2 concentration is low and trace amounts of nickel are available (Fig. 3.3A) (Black *et al*. 1994). Under these conditions HoxA, a response regulator of the NtrC family, is the major transcriptional factor. HoxA binds to UAS and activates transcription at the σ^{54}-dependent P_F promoter. Transcription activation is facilitated by the IHF protein (Black and Maier 1995). The activity of HoxA is proposed to be modulated by the histidine protein kinase HupT and the H_2 receptor HupUV. P_2 and P_3 are presumably housekeeping promoters which provide a low-level expression of the regulatory *hupUV* and *hoxAhupT* genes to establish the H_2-sensing apparatus under non-inducing conditions (Black *et al*. 1994; Van Soom *et al*. 1999). During symbiosis it is supposed that the hydrogenase improves the efficiency of N_2 fixation by recycling the H_2 which is released by the nitrogenase as a side product. Under these conditions hydrogenase operon expression is integrated into a complex regulatory network which coordinates the processes involved in symbiotic N_2 fixation (Fig. 3.3B). Transcription activation at the P_S promoter depends on the Fnr-like FixK2 protein (Durmowicz and Maier 1998). Expression of the *fixK2* gene is controlled by the response regulator FixJ whose activity is modulated by its cognate O_2-sensing histidine protein kinase FixL.

Transcription of hydrogenase genes of *R. leguminosarum* bv. viciae is co-regulated with N_2-fixation genes (*nif* and *fix*) and controlled by two global activators, NifA and FnrN, in response to the microaerobic conditions inside the legume nodules (Fig. 3.4) (Brito *et al*. 1997; Guttierrez *et al*. 1997). This environment ensures the expression of hydrogenase genes. The hydrogenase activity enables the bacteroids to take up H_2 evolved by nitrogenase (Brito 1997). Two major promoters, P_1 and P_5, have been

identified within the *R. leguminosarum* hydrogenase gene cluster. P_1 is a σ^{54}-dependent promoter responsible for the symbiotic activation of at least the *hupSLCD* operon, and likely for activation of the remaining *hup* genes as well. The symbiotic activation of P_1 is mediated by NifA, the N_2-fixation global transcription activator, and by the integration host factor (IHF). P_5 is an Fnr-type promoter responsible for the microaerobic expression of the *hypBFCDEX* genes independent of the symbiotic life style (Fig. 3.4). Unlike *B. japonicum*, *R. leguminosarum* is not able to synthesize its hydrogenase under free-living conditions since the transcriptional activator gene *hoxA*, which is located downstream of the *hypX* gene (not shown in Fig. 3.4) has been inactivated by accumulation of frameshift and deletion mutations (Brito *et al.* 1997).

The hydrogenase genes of the phototroph *R. capsulatus* are organized in several transcriptional units which are expressed in an H_2-dependent manner (Fig. 3.5). Two promoters have been unambiguously identified, P_1 upstream from the *hupSLC* structural gene operon and P_2 upstream from the *hupTUVhypF* operon (Elsen *et al.* 1996; Toussaint *et al.* 1997). The *hypF* gene can also be transcribed at a low level from its own promoter (P_3). The *hupDFGHJK* genes are separated from the *hupSLC* operon by a 200 bp intergenic region which contains a putative σ^{70}-dependent promoter (P_4). The *hupR* regulatory gene is transcribed together with the *hyp* genes from a promoter (P_5) localized in the *hypA* region (Fig. 3.5). The P_1 promoter which directs the transcription of the *hupSLC* operon is the σ^{70}-dependent type. Transcription activation requires the binding of two regulators. The positive regulator HupR binds to a specific palindromic sequence TTG-N_5-CAA localized at -160 to -150 nt upstream from the transcription start site (Dischert *et al.* 1999; Toussaint *et al.* 1997) and the histone-like IHF protein binds to an AT-rich sequence centred at nucleotide -87. The IHF protein of *R. capsulatus* is a global regulator which activates the *hupSLC* transcription without being essential (Toussaint *et al.* 1997). On the other hand, HupR is absolutely required to obtain transcription of the *hupSLC* operon. The P_1 promoter is activated in response to H_2 but negatively controlled by the global regulator RegA, which binds upstream from *hupS*, between the binding sites of IHF and RNA polymerase (Elsen *et al.* 2000). The P_2 promoter, although weakly expressed, is also regulated. It is almost inactive under autotrophic growth conditions. Thus, in *R. capsulatus*, hydrogenase synthesis is regulated in response to the presence of H_2 and organic substrates, through the activity of the H_2 sensor (HupUV) and the two-component signal transducing system (HupT/HupR) and in response to redox through the global regulatory system (RegB/RegA).

The genes for the membrane-bound (MBH) and the soluble hydrogenase (SH) of *R. eutropha* are arranged into two major operons on the indigenous megaplasmid pHG1 (Fig. 3.6). Both operons are preceded by σ^{54}-dependent promoters, designated as P_{MBH} and P_{SH}, respectively (Schwartz *et al.* 1998). Transcription initiation at the P_{MBH} and P_{SH} promoters depends on the transcription activator HoxA. Thus, the hydrogenase operons constitute a regulon. HoxA, which belongs to the NtrC subfamily of response regulators, receives signals from an H_2-dependent multicomponent signal transduction chain consisting of the H_2 sensor HoxBC and the histidine protein kinase HoxJ (see Section 3.2) (Lenz and Friedrich 1998). If H_2 is available, HoxA becomes activated. In the activated state HoxA binds at UAS and initiates transcription by contacting the σ^{54}-containing RNA polymerase. This protein–protein interaction leads to the formation of the open complex. Putative IHF binding sites

(IHF BS) in the MBH and SH promoter regions indicate that this process is stimulated by IHF (Zimmer *et al*. 1995). Two additional promoters of the σ^{70}-dependent type are located upstream of the *hyp* genes (P_3) and the regulatory genes (P_4), respectively, and provide a weak constitutive level of the corresponding gene products (Fig. 3.6). The Hyp proteins are not only required for cofactor insertion into the MBH and the SH but are also necessary for the activity of the H_2-sensing HoxBC protein (T. Buhrke and B. Friedrich, unpublished data). Thus, the low constitutive activity of the P_3 and P_4 promoters maintains the H_2-sensing signal transduction apparatus in a ready state under non-inducing conditions (Schwartz *et al*. 1998). This enables the cells to react instantaneously in the presence of molecular H_2. H_2-induced transcription from the P_{MBH} and P_{SH} promoters results in the synthesis of active MBH and SH (see also Chapter 2). Moreover, transcription from the strong MBH promoter augments *hox A* and most likely *hoxBCJ* expression under inducing conditions (Schwartz *et al*. 1998). Increased synthesis of HoxA in turn raises the transcription level from the P_{MBH} and P_{SH} promoters provided H_2 is present. This positive feedback loop leads to an adjustment of hydrogenase synthesis to the external concentration of molecular H_2.

3.3. Hydrogen sensing and signal transduction

3.3.1. R. capsulatus

To isolate genes and to study their function, it is common practice to isolate mutants with a given phenotype, e.g. mutants unable to synthesize hydrogenase. Complementation of the mutants by a gene bank then allows identification of the mutated gene(s). The study of the physiology of hydrogenase-deficient mutants (Hup^-) gave clues on the role of *hup/hyp* genes in hydrogenase synthesis and in H_2 metabolism of *R. capsulatus*. To identify hydrogenase regulatory mutants, the promoter of the structural *hupSL* hydrogenase genes was fused with the reporter gene *lacZ*. Subsequently the *hupS*::*lacZ* fusion, carried on a plasmid, was introduced in the wild-type strain B10 and in various Hup^- mutants. The *lacZ* gene encodes the β-galactosidase enzyme whose activity can easily be measured colorimetrically. Activation of hydrogenase gene expression was correlated with an increase of β-galactosidase activity indicating that *hupS*::*lacZ* expression was under the same control as the chromosomal *hupS* gene. The use of mutant strains has been instrumental in assigning genes responding to environmental signals.

Molecular H_2 was one of the first environmental factors shown to stimulate the synthesis of hydrogenase and that of β-galactosidase in cells containing the *hupS*::*lacZ* fusion (Fig. 3.7). High gene expression was observed under conditions that favour nitrogenase synthesis, e.g. anaerobiosis, light, malate-glutamate medium or nitrogen-limited medium. The highest level was obtained with cells grown in darkness in the presence of H_2 and O_2 (Colbeau and Vignais 1992) or in cells grown autotrophically with CO_2 as carbon source and H_2 as reductant.

3.3.1.1. H_2-specific signal transducing system

The H_2 signal transduction cascade involves an H_2 sensor (HupUV), the regulatory soluble [NiFe] hydrogenase and a two-component regulatory system (HupT/HupR).

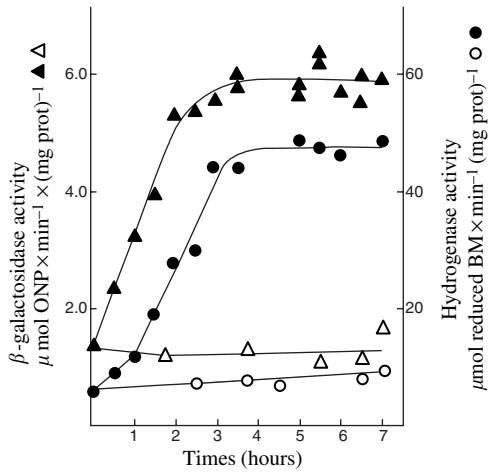

Figure 3.7 Increase in hydrogenase and β-galactosidase activities during growth with H_2 of B10 (pAC142) cells containing the hupS::*lacZ* fusion. ONP, *o*-nitrophenol; MB, methylene blue; prot, protein; solid symbols, H_2 added; open symbols, no H_2 added.

The response regulator HupR is a transcriptional activator necessary for H_2 induction of *hupSL* gene expression (Toussaint *et al*. 1997). HupR activity can be modified by phosphorylation of the aspartic residue in position 54 in the HupR protein. HupR activates *hupSL* transcription in the non-phosphorylated form, since HupR proteins mutated in the phosphoryl-accepting Asp54, or in which Asp54 has been deleted, have the same capacity to activate *in vivo hupSL* transcription in the absence of H_2, as wild-type HupR in the presence of H_2 (Table 3.2) (Dischert *et al*. 1999). The IHF protein (Toussaint *et al*. 1991) activates the *in vivo* transcription of the *hupSL* genes by bending DNA (Fig. 3.8) and facilitating the interaction of HupR, bound to upstream activating DNA sequences (UAS) of the promoter, with RNA polymerase bound at the transcription start site of *hupS*. The TTG-N_5-CAA binding site of HupR (Figs 3.9 and 3.10) is located upstream from the IHF site of the *hupS* promoter.

The HupT protein is a sensor histidine protein kinase. $HupT^-$ mutants have a higher hydrogenase activity than wild-type cells, i.e. they are derepressed for hydrogenase synthesis (Elsen *et al*. 1993). In other words, normally HupT exerts a negative control on hydrogenase synthesis unless H_2 is present (Fig. 3.11). The use of double *hupT, hupR* mutants has shown that HupT and HupR act in the same (H_2) signalling pathway (Toussaint *et al*. 1997). HupT and HupR communicate by trans-phosphorylation (Fig. 3.12) and form a two-component regulatory system. Since HupR is active in the non-phosphorylated form, it is proposed that HupT exerts its negative control on *hupSL* expression by phosphorylating the activator HupR (Dischert *et al*. 1999).

The presence of the inducer H_2 is not sensed by HupT but by the *hupUV*-encoded hydrogenase. Although very weak, the hydrogenase activity of HupUV could be detected. Figure 3.13 shows the hydrogen–deuterium (H–D) isotope exchange reaction

Table 3.2 Effect of mutations in H_2 signalling (HupR/HupTUV) or redox signalling (RegA/RegB) pathways on hydrogenase activity in *R. capsulatus*

Protein	*Function*	*Mutated protein*	*H_2ase activity*[a] $-H_2$	$+H_2$	*Features*
		wt	*7*	*45*	*H_2 dependent*
HupUV	H_2-sensing H_2ase	Hup(UV)Δ	58	48	H_2 independent Increased activity
HupT	Histidine protein kinase Autophosphorylable H_2 signal transmitter	HupTΔ	118	95	H_2 independent Increased activity
HupR	Response regulator Transcription activator Phosphorylated by HupT-P	HupR (wt)[b]	1	32	H_2 dependent
		HupRΔ	1	1	loss of activity
		$HupRD_{54}\Delta$	46	30	H_2 independent
		$HupRD_{54}R$	46	32	H_2 independent
IHF	DNA bending Global regulator	IHFα	4	17	H_2 dependent Non-essential activator
RegB	Histidine protein kinase Redox sensor	RegB	37	53	Global regulation
RegA	Response regulator Repressor Phosphorylated by RegB-P	RegA	34	59	Global regulation

Notes

a μmol methylene blue reduced × h^{-1} × mg $protein^{-1}$

b The $HupR^-$ mutant VBCl, in which the chromosomal *hupR* gene has been deleted (*hupR*Δ), was complemented with *hupR*-containing plasmids. HupR = wild-type protein; $HupRD_{54}\Delta$ = the codon for Asp_{54} in the *hupR* gene has been deleted; $HupRD_{54}R$ = the codon for Asp54 in the *hupR* gene has been replaced by a codon for arginine.

Strains were grown in mineral salt medium at 30°C aerobically in darkness with or without 10 per cent H_2. Hydrogenase was assayed directly in aliquots from the cultures at an OD_{660} of ca. 1.5.

catalysed by HupUV in the presence of D_2 (Vignais *et al.* 1997, 2000) and the formation of H_2 upon addition of reduced methyl viologen at pH 4. These are two partial reactions typically catalysed by hydrogenases. As for HupSL, formation of active *hupUV*-encoded hydrogenase requires the participation of *hyp* gene products (e.g. HypF) (Colbeau *et al.* 1998). Like the $HupT^-$ mutants, the $Hup(UV)^-$ mutants are derepressed for hydrogenase synthesis and there is no response to H_2 in the mutants (Elsen *et al.* 1996) (Fig. 3.11). The low level of *hupUV* gene expression is in keeping with a signalling function of the HupUV protein. HupUV is an H_2-signalling hydrogenase, which acts in concert with HupT (the respective mutants have the same phenotype). It is still unknown how HupUV and HupT communicate in response to H_2.

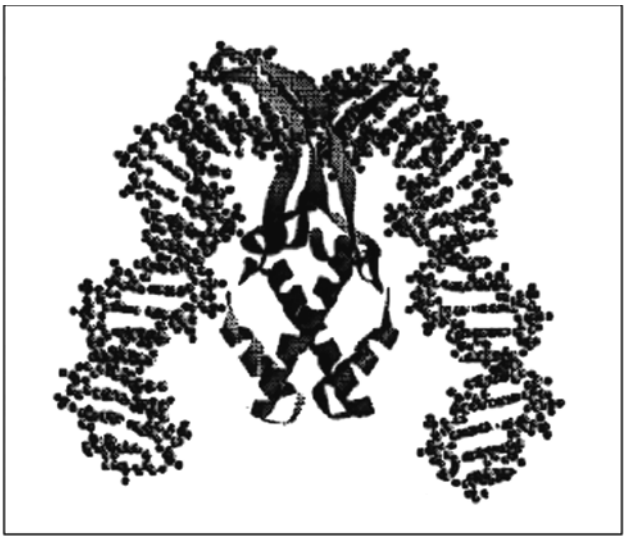

Figure 3.8 Ribbon view of the *R. capsulatus* IHF protein bound to its DNA binding site upstream from the *hupS* gene (cf. Toussaint *et al.* 1994).

3.3.1.2. Global redox control by RegB/RegA

Global regulators capable of integrating signals from nitrogen-, carbon- and H_2 metabolism have recently been identified in photosynthetic bacteria. The global regulator RegA, an anaerobic activator of photosynthesis which is phosphorylated by the histidine protein kinase RegB, is involved in negative control of hydrogenase gene expression in *R. capsulatus*. The global redox control by RegB/RegA is superimposed onto the specific control of hydrogenase synthesis in response to H_2, mediated by the HupT/HupR system (Table 3.2). The RegB/RegA system can also activate the synthesis of nitrogenase by increasing the expression of the transcriptional activator NifA, promoted by NtrC. Thus, there is a genetic link between the synthesis of uptake hydrogenase and of nitrogenase in *R. capsulatus* (Elsen *et al.* 2000).

3.3.2. R. eutropha

3.3.2.1. A multicomponent signal transduction system controls H_2-dependent hydrogenase gene expression in R. eutropha

The facultative chemolithoautotroph *R. eutropha*, a member of the β subgroup of proteobacteria, harbours two [NiFe] hydrogenases which are involved in energy conservation from H_2 (Chapter 2). Since H_2 is available at very low concentrations in aerobic environments where these bacteria thrive, the complex synthesis of the hydrogenases (Chapter 4) has to be efficiently regulated in response to H_2. H_2-dependent gene expression in *R. eutropha* is mediated by a complex multicomponent signal transduction pathway (Fig. 3.14) (Lenz and Friedrich 1998) whose individual components are listed in Table 3.3.

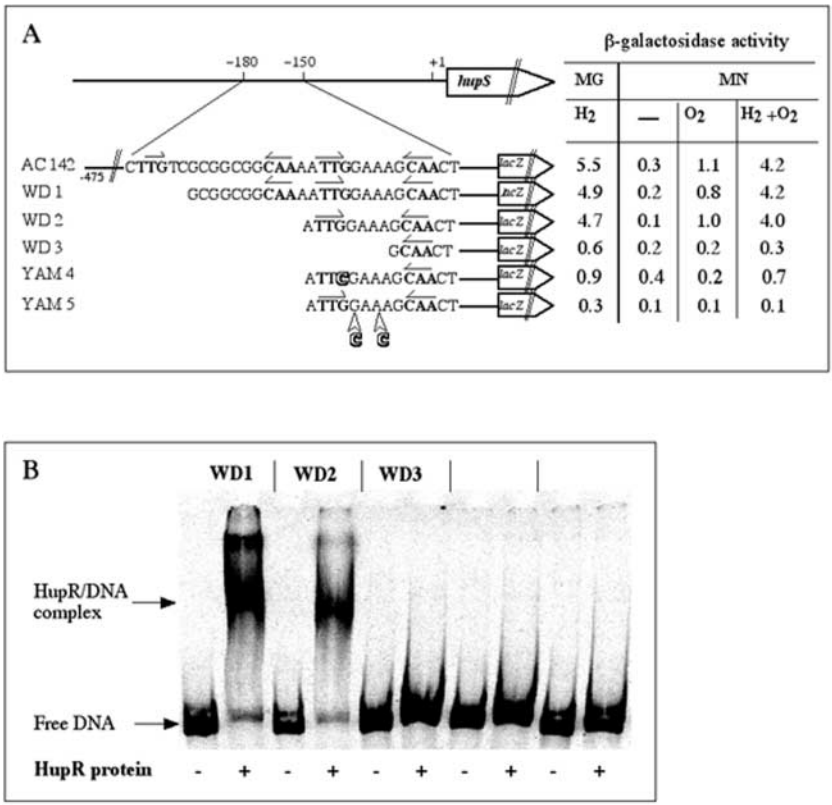

Figure 3.9 Identification of cis-regulatory sequences in the *hupS* promoter region. (A) *In vivo* experiments. DNA fragments encompassing the regulatory sequences of the *hupSL* promoter were progressively deleted, fused with the promoterless *lacZ* reporter gene (which encodes β-galactosidase), inserted in plasmids that were transferred to the wild-type strain B10. The β-galactosidase activities expressed in B10 cells grown in malate-glutamate (MG) or in malate-ammonia (MN) medium, in the presence or absence of H_2 or O_2, reflect the *in vivo* activity of the *hupSL* promoter. The table on the right shows that transcriptional activity is maximal in the presence of H_2 and requires the TTG-N5-CAA sequence, which is the binding site of the activator HupR. (B) *In vitro* experiments. The HupR protein was purified and used in electrophoretic mobility shift assays (EMSA). Only the DNA fragments containing the intact TTG-N5-CAA palindrome (WD1 and WD2, cf. (A)) form a complex with HupR. The complex HupR-DNA migrates more slowly in the electric field than free DNA (adapted from Toussaint *et al.* 1997).

R. eutropha harbours a third [NiFe] hydrogenase, denoted as the regulatory hydrogenase (RH), which enables the cells to sense the occurence of H_2 in the environment (Kleihues *et al.* 2000). The signal is subsequently transduced to a two-component regulatory system consisting of the soluble histidine protein kinase, HoxJ, and the response regulator, HoxA (Fig. 3.14). Although the molecular mechanism is yet unknown, preliminary experimental data indicate a complex formation between the RH and the kinase HoxJ (T. Buhrke and B. Friedrich, unpublished data). The communication

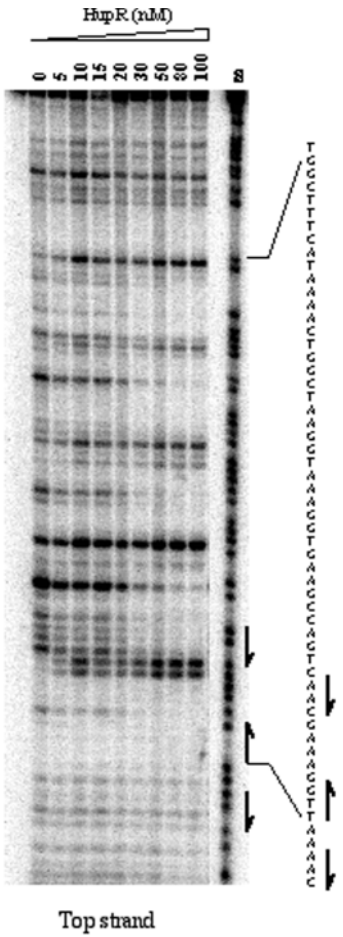

Figure 3.10 Identification of the HupR binding site at the *hupS* promoter by DNase footprinting analysis. DNA footprinting is a technique which detects the binding of proteins to DNA by their protection of the DNA from attack by various chemical agents. The disappearance of bands in the vertical lanes reveals the increasing protection afforded by increasing concentrations of the HupR protein shown in nM at the top of each lane. The arrows on the sequence, on the right of the figure, show the HupR binding site.

between histidine protein kinases and their cognate response regulators is principally based on phosphotransfer reactions (Hoch and Silhavy 1995). A phosphotransfer between HoxJ and HoxA was recently demonstrated *in vitro* using purified components (M. Forgber, O. Lenz and B. Friedrich, unpublished data).

The response regulator HoxA is crucial for transcription activation at the σ^{54}-dependent SH and MBH promoters (see Section 3.2 and Fig. 3.6) (Schwartz *et al.* 1998). Both HoxA and the H_2-sensing RH act positively, since a knockout of the two proteins abolished hydrogenase gene expression and hence prevent the MBH and SH from being synthesized (Fig. 3.15, Table 3.3)(Lenz and Friedrich 1998). Genetic and biochemical

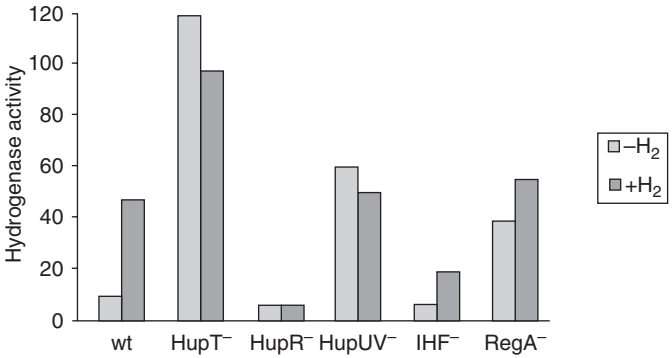

Figure 3.11 Hydrogenase activities in regulatory mutants of *R. capsulatus* affected in transcription of hydrogenase genes compared to the wild-type B10. Cells were grown aerobically (under air) in darkness in malate-ammonia medium at 30°C. Hydrogenase was assayed directly in aliquots from the cultures at an OD_{660} of approx. 1.5. The specific hydrogenase activity is given in μmol of methylene blue reduced per h per mg protein. The figure shows that the highest hydrogenase activities were observed in the $HupT^-$ mutant and the $Hup(UV)^-$ mutant, that those activities are independent of H_2, and that the transcription factor HupR is required for hydrogenase gene expression. In the $RegA^-$ mutant, hydrogenase activity was derepressed but still capable of stimulation by H_2. It is concluded that HupT and HupUV exert a negative control on hydrogenase synthesis, which can be antagonized by H_2. The transcription factors HupR and IHF activate hydrogenase gene expression while the global regulator RegA inhibits it.

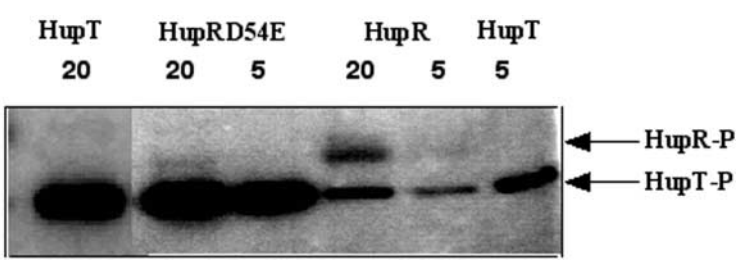

Figure 3.12 Phosphate transfer between the protein histidine kinase HupT and the response regulator HupR. HupT (7 pmol) was phosphorylated at 30°C with [γ-^{32}P]ATP for 5 min, before addition of wild-type (HupR) or mutated (HupR-D54E) HupR protein. Aliquots were withdrawn 5 min or 20 min after addition of HupR and analysed by SDS polyacrylamide gel electrophoresis. HupT20: HupT was phosphorylated for 20 min in the absence of HupR.

analysis revealed that the kinase HoxJ, on the other hand, has a primarily negative effect on hydrogenase gene expression. This negative control is released by the RH provided H_2 is available (Figs 3.14 and 3.15). The data obtained so far indicate that inactivation of HoxA results from its phosphorylation and that the non-phophorylated form of HoxA is active in transcription activation. This property puts the HoxJ/HoxA system

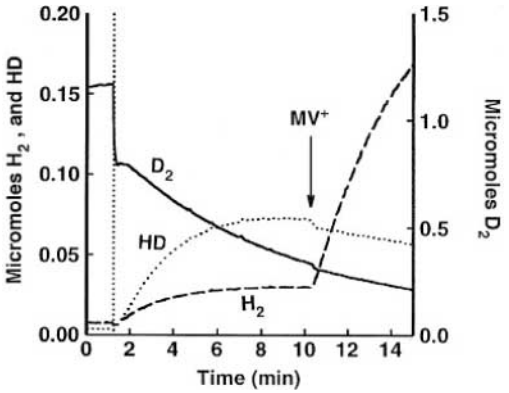

Figure 3.13 Time-course of H_2 and HD production and D2 consumption, in the D_2/H_2O system, catalysed by the *hupUV*-encoded hydrogenase. The HupUV activity was measured at pH 4 in extracts of the *hupSL* mutant JP91 containing plasmid pAC206, which encodes the *hupTUV* operon. The hydrogen–deuterium exchange was followed continuously in the aqueous phase by an on-line mass-spectrometric method. The reaction vessel, maintained under strict anaerobic conditions, was connected by a membrane inlet to the ion source of the mass spectrometer MM 8-80 (VG Instruments). The vertical dotted line indicates when the reaction vessel was closed and when the recording of concentrations of H_2 (....), HD (----) and D_2 (___) at masses 2, 3 and 4, respectively began. At the time indicated by the arrow, reduced methyl viologen (MV^+) was introduced in the medium.

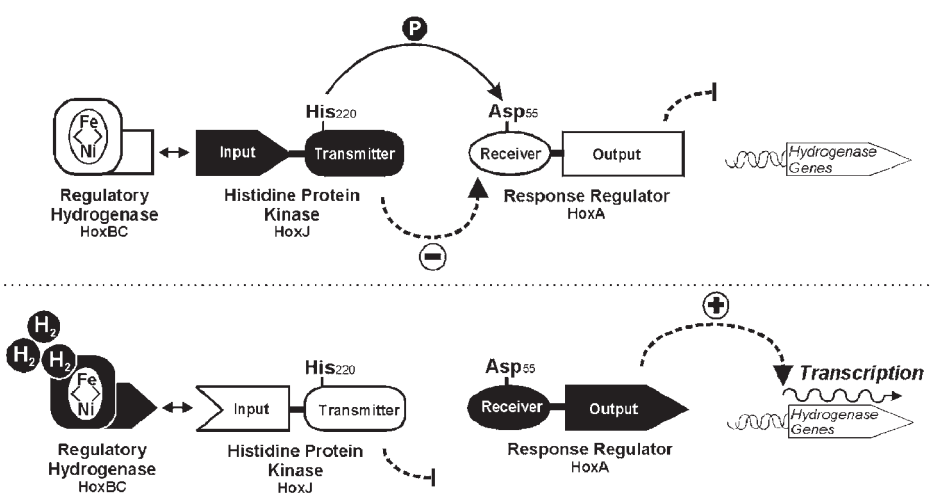

Figure 3.14 Model of H_2-dependent signal transduction in *R. eutropha*. Proteins which are regulatory active are highlighted in black. For details, see text.

apart from standard two-component regulatory systems (Lenz and Friedrich 1998). In compliance with this model is the observation that site-directed exchanges of the conserved phosphoryl acceptor residue Asp-55 in the receiver module of HoxA (Fig. 3.14) convert the system to an H_2-independent control. The HoxA variants

Table 3.3 Structural and functional properties of the hydrogenase regulatory proteins in *R. eutropha*

Protein	*Size (kDa)*	*Type*	*Function*	*Mutation/amino acid exchange*	*Effect on hydrogenase gene expression*
HoxB	36.5	RH, small subunit	[NiFe] hydrogenase active in H_2 oxidation and signal transduction	hoxBΔ	Abolished
HoxC	52.4	RH, large subunit		*hox*CΔ	Abolished
HoxJ*	51.2	Histidine protein kinase	Sensor kinase, ATP-dependent auto-phosphorylation	*hox*JΔ	H_2-independent
				HoxJG422S	H_2-independent
HoxA	53.6	Response regulator	DNA-binding protein, modified by phospho-rylation, activates transcription in response to H_2 and carbon supply	HoxAD55E	H_2-independent
				HoxAD55N	H_2-independent
				*hox*AΔ	Abolished
RpoN	54.8	σ^{54}	Alternative σ factor of the RNA polymerase, required for HoxA-dependent activation of transcription	*rpo*N::Tn5	Abolished

HoxAD55E and HoxAD55N are still capable of activating MBH and SH gene transcription at a high level. Thus, these mutants are constitutive so far as the H_2-dependent regulation is concerned; however, they still respond to a global carbon catabolite control (Lenz and Friedrich 1998). This control also depends on HoxA as the principal regulator and enables the cells to turn off hydrogenase gene expression when fast growth-supporting organic nutrients are available.

3.3.2.2. The H_2 sensor of R. eutropha *belongs to a distinct subclass of [NiFe] hydrogenases*

The RH protein of *R. eutropha* is a dimeric [NiFe] hydrogenase consisting of a large subunit (HoxC) which harbours the [NiFe] active site and a small FeS-containing subunit (HoxB). This conformation resembles the prototypic [NiFe] hydrogenases as found in *Desulfovibrio gigas* (Kleihues *et al*. 2000). Nevertheless, the RH as well as the isologous proteins implicated in H_2 sensing in other bacterial species such as *B. japonicum* and *R. capsulatus* exhibit characteristic structural features (Fig. 3.16) which are representatively discussed for the RH of *R. eutropha*. The large HoxC subunit of the RH is devoid of a C-terminal extension and terminates at a histidine residue which is located at the cleavage site of a specific endoprotease which usually removes the C-terminal peptide after metallocentre insertion (Chapter 4). Thus, the maturation of the regulatory hydrogenase seems to be less complex. The small subunit HoxB lacks an N-terminal Tat signal peptide which generally directs the transport

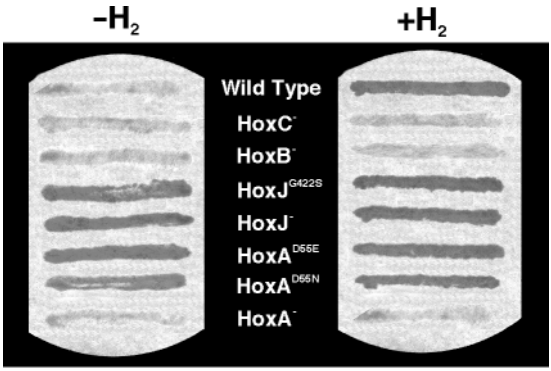

Figure 3.15 Hydrogenase synthesis in regulatory mutants of *R. eutropha*. Cells were grown on agar plates containing glycerol as the carbon source either in the presence or in the absence H_2. The cell material was transferred to filter paper and a triphenyl tetrazolium chloride-based hydrogenase activity staining was performed. Dark colour reflects activity of the MBH.

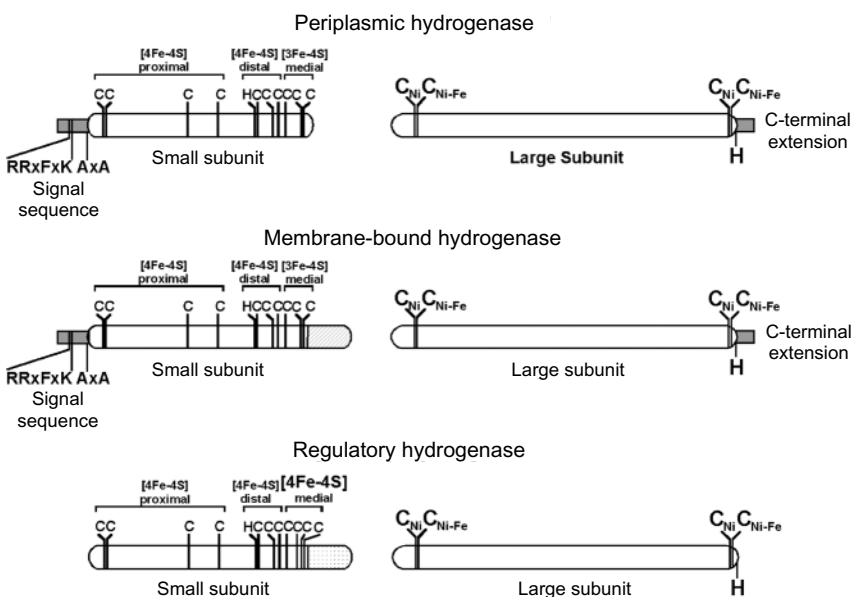

Figure 3.16 Primary structures of [NiFe] hydrogenases belonging to separate subclasses.

of periplasmic and MBH across the cytoplasmic membrane by the Tat system (Chapter 4). Since H_2 is a freely diffusible molecule and the cognate histidine protein kinase is of cytoplasmic nature (Lenz and Friedrich 1998), there is no need to anchor the RH to the membrane. Of great structural and functional importance is the C-terminal tail of HoxB. Such a tail is absent from the small subunits of periplasmic [NiFe] hydrogenases but usually present, although structurally distinct, in MBH (Fig. 3.16,

Chapter 2). Protein–protein interaction experiments revealed that this C-terminal domain of HoxB is necessary for the oligomerization of two RH dimers to form a tetramer and for contacting the histidine protein kinase HoxJ (T. Buhrke and B. Friedrich, unpublished results).

With the aid of a specifically designed overexpression system, the H_2 sensor of *R. eutropha* was characterized in more detail. The RH was identified as an Ni-containing hydrogenase which exhibits H_2-oxidizing activity in the presence of redox dyes (Fig. 3.17) (Kleihues *et al.* 2000). Both the hydrogenase activity and the regulatory function of the RH are Ni dependent. Fourier transform infrared spectroscopy (FTIR), electron paramagnetic resonance (EPR) and preliminary biochemical data revealed that the RH harbours an active site similar to that of prototypic [NiFe] hydrogenases including the two CN molecules and one CO molecule as ligands to the Fe (Pierik *et al.* 1998b). The catalytic activity of the RH is about two orders of magnitude lower than that of energy-generating hydrogenases. Furthermore, the RH activity is O_2 tolerant and insensitive towards CO (Pierik *et al.* 1998b). The unique structural and biochemical features of the RH assign this type of regulatory hydrogenase to a new subclass of [NiFe] hydrogenases (Kleihues *et al.* 2000).

3.4. Concluding remarks and perspectives

Hydrogenase gene regulation has to meet several requirements: (i) the environmental conditions to which the organisms are exposed, (ii) the physiological function of the hydrogenase in a given species, and (iii) the complex biosynthesis of [NiFe] hydrogenase which involves protein-assisted post-translational steps.

M. voltae responds to Se limitation by expressing two alternative Se-free [NiFe] hydrogenases. In this case Se acts as a regulatory signal of gene transcription, although the precise target is still unknown. *R. leguminosarum* takes advantage of hydrogenase activity during symbiotic N_2 fixation, hence it is conceivable that in this bacterium hydrogenase gene transcription is guided by the global N_2-fixation control and its major players NifA and FnrN. Unlike *R. leguminosarum, B. japonicum* uses its hydrogenase for energy generation also under free-living conditions. As a chemolithoautotroph it avoids the control by N_2, and adjusts hydrogenase gene expression to the availibility of H_2. This is recognized by *B. japonicum* and other aerobic H_2-oxidizing bacteria such as *R. capsulatus* and *R. eutropha* by a complex signal transduction chain consisting of an H_2-sensing [NiFe] hydrogenase, a histidine protein kinase and a response regulator.

The modular composition of [NiFe] hydrogenases allows their integration into various physiological pathways and frequently results in the occurrence of hydrogenase isoenzymes in a single cell. *E. coli* harbours four [NiFe] hydrogenases including H_2-evolving and H_2-consuming enzymes which are regulated separately. Hydrogenase 3 is a constituent of the formate hydrogen lyase system, its expression is governed by a σ^{54}-interacting response regulator whose activity is modulated by formate. The occurrence of multiple hydrogenases with distinct physiological functions in a single strain demands a high level of regulatory coordination between hydrogenase-specific and unrelated functions.

To obtain active hydrogenase positioned at a distinct cellular site the enzyme has to undergo multiple steps of protein-mediated maturation including [NiFe] cofactor

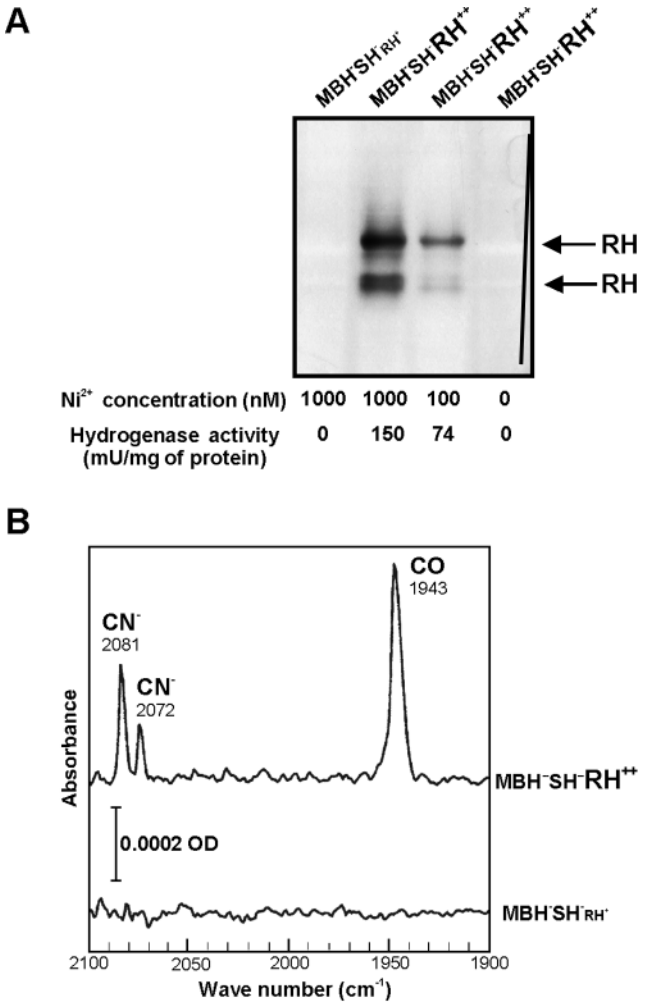

Figure 3.17 Catalytic and structural properties of the RH of *R. eutropha*. (A) Hydrogenase activity staining in gels, using phenazine methosulfate as the electron acceptor. Soluble extracts of cells devoid of the SH and the MBH, containing the RH at wild-type (RH^{+}) or overproduced level (RH^{++}) were separated by native PAGE. The cells were cultivated under various concentrations of $NiCl_2$. In parallel, hydrogenase activity was determined quantitatively by H2-dependent methylene blue reduction in soluble extracts. (B) FTIR spectra recorded for soluble extracts. The RH-overproducing strain shows the typical signals assigned to the diatomic ligands CN- and CO.

insertion, proteolysis and, if necessary, protein translocation. Thus, it is not surprising that the genes encoding the hydrogenase subunits are accompanied by hydrogenase-related accessory genes which form separate or extremely large transcriptional units. A fine tuning on the transcriptional and translational level is important to guarantee a balanced stoichiometric ratio of the various components.

We are at the very beginning of our understanding of the molecular background of hydrogenase regulation. The discovery of an [NiFe] hydrogenase which acts as an H_2 sensor, in concert with an unusual two-component regulatory system, has opened new insights into bacterial regulation and is an attractive model to be studied in more depth in the future. It is also desirable to learn more about the integration of hydrogenase control circuits into global regulatory networks which respond to redox, carbon, respiratory and nitrogen stimuli. Advances in functional genomics will hopefully offer novel tools to eludicate regulation of H_2 metabolism in organisms which are not yet accessible to genetic techniques.