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Abstract

Hydrogen metabolism plays a central role in sulfate-reducing bacteria of the *Desulfovibrio* genus and is based on hydrogenases that catalyze the reversible conversion of protons into dihydrogen. These metabolically versatile microorganisms possess a complex hydrogenase system composed of several enzymes of both [FeFe]- and [NiFe]-type that can vary considerably from one *Desulfovibrio* species to another. This review covers the molecular and physiological aspects of hydrogenases and H_2 metabolism in *Desulfovibrio* but focuses particularly on our model bacterium *Desulfovibrio fructosovorans*. The search of hydrogenase genes in more than 30 sequenced genomes provides an overview of the distribution of these enzymes in *Desulfovibrio*. Our discussion will consider the significance of the involvement of electron-bifurcation in H_2 metabolism.

Contents:

1. Introduction

1.1. The hydrogenases

Many prokaryotes are able to use molecular hydrogen (H_2) as a primary energy source or to produce H_2 in order to dissipate excess reducing power by reducing protons. Hydrogenases are the metalloenzymes that reversibly oxidize H_2, using various electron donors and acceptors, according to the reaction: $H_2 \rightleftarrows 2H^+ + 2e^-$ (equation 1)

They are very ancient enzymes and are widespread in bacterial and archaeal microorganisms (Greening et al., 2016; Vignais & Billoud, 2007; Vignais, Billoud, & Meyer, 2001). They can be classified into two main phylogenetically distinct classes, the [FeFe]- and [NiFe]-hydrogenases, according to the nature of the metal atoms at their active site (Vignais & Billoud, 2007; Vignais et al., 2001) (Figure 1). [NiFe]-hydrogenases are widely distributed among archaea and bacteria and [FeFe]-hydrogenases are present in bacteria and to a lesser extent in eukaryotic species. Despite the fact that [NiFe]- and [FeFe]-hydrogenases are phylogenetically unrelated, they share several features. In both types of hydrogenases, the metal atoms at the active sites are coordinated by inorganic carbon monoxide (CO) and cyanide (CN^-) ligands. They also have in common the presence of hydrophobic gas channels for the diffusion of H_2 towards or from the buried active site and a series of iron-sulfur clusters forming an electron transfer chain between the active site and the electron donor or acceptor at the surface of the enzyme (Cohen, Kim, King, Seibert, & Schulten, 2005; Fontecilla-Camps, 2007; Leroux et al., 2008; Montet et al., 1997; Nicolet, Piras, Legrand, Hatchikian, & Fontecilla-Camps, 1999; Peters et al., 2015). All the hydrogenases are reversibly or irreversibly inhibited by O_2 (Ghirardi, 2015; Stiebritz & Reiher, 2012).

[NiFe]-hydrogenases are minimally composed of two subunits: a large catalytic subunit and a small iron-sulfur cluster-containing subunit. Their active site is composed of Ni and Fe atoms bridged by a pair of Cys thiolates (Peters et al., 2015) (Figure 1). The maturation of [NiFe]-hydrogenases is a complex and species-specific process making it difficult to produce these enzymes heterologously. Biosynthesis and insertion of the [NiFe]-catalytic center involve the products of 6 *hyp* genes (Lacasse & Zamble, 2016) and a specific protease cleaves a peptide at the C-terminal end of the large subunit to finish the maturation process (Theodoratou, Huber, & Bock, 2005).

[FeFe]-hydrogenases are very efficient H_2-producing enzymes and show higher catalytic activity for H_2 evolution than [NiFe]-hydrogenases (Adams, 1990; Frey, 2002). One of the first tridimensional structure of an hydrogenase of this type to be determined was that of *Desulfovibrio desulfuricans* (Nicolet et al., 1999). Their catalytic sites, referred to H-clusters, consist of a unique [4Fe4S] cluster ligated by four cysteine residues linked to a unique organometallic di-iron subcluster (Figure 1) (Nicolet et al., 1999; Peters, Lanzilotta, Lemon, & Seefeldt, 1998). Besides the catalytic domain containing three evolutionarily conserved binding motifs for the H-cluster (Vignais et al., 2001), additional domains with accessory clusters can be present conferring a modular organization to [FeFe]-hydrogenases (Calusinska, Happe, Joris, & Wilmotte, 2010; Fontecilla-Camps, 2007; Poudel et al., 2016; Schwartz, Fritsch, & Friedrich, 2013). In green algae, such as *Chlamydomonas reinhardtii*, the simplest form of the [FeFe]-hydrogenase was identified consisting of the H-cluster domain only (Happe, Mosler, & Naber, 1994; Happe & Naber, 1993). One or two additional ferredoxin-type domains can be present, as in Clostridial monomeric hydrogenases, that contain four additional iron-sulfur clusters. In multimeric [FeFe]-hydrogenases, consisting of three or four subunits, the hydrogenase catalytic subunit is associated with subunits that generally contain iron-sulfur clusters. [FeFe]-hydrogenase maturation involves only three accessory proteins for the assembly of the 2Fe subcluster of the active site, HydE, HydF and HydG (Broderick et al., 2014;

Peters et al., 2015; Shepard et al., 2014). Active [FeFe]-hydrogenases can be heterologously produced, using either the maturation system of the host or by simultaneously express the maturases (Avilan et al., 2018; Gärtner, Lechno-Yossef, Cornish, Wolk, & Hegg, 2012; Sybirna et al., 2008)

1.2. Physiological role of hydrogenases in prokaryotes

In prokaryotes, the physiological role of hydrogenases is very diverse. In anaerobic microorganisms, H_2 is a terminal product in various types of fermentations, such as in the glucose-fermenting bacterium *Clostridium pasteurianum* (Therien et al., 2017), in the bacterium *Ruminococcus albus* (Y. Zheng, Kahnt, Kwon, Mackie, & Thauer, 2014) of the rumen flora, or in the thermophilic bacterium *Thermotoga maritima* (Schut & Adams, 2009). Fermentative H_2 production allows the recycling of the pool of reduced electron carriers produced during the fermentation process (reviewed in (Schwartz et al., 2013)). H_2 is also produced during the anaerobic oxidation of CO (Robb & Techtmann, 2018) or during N_2 fixation as a byproduct of the reaction catalysed by the nitrogenase (Bulen, Burns, & Lecomte, 1965). H_2 is widely used by microorganisms as an energy source and H_2-oxidation can occur in aerobic or anaerobic microorganisms. The Knallgas bacteria use H_2 as electron donor and O_2 as a terminal electron acceptor and fix CO_2. During methanogenesis, in which methane is formed from H_2 and CO_2 or partially reduced forms of carbon, several types of [NiFe]-hydrogenases are involved (Enzmann, Mayer, Rother, & Holtmann, 2018). H_2 is also used, under anaerobic conditions, to reduce a wide range of compounds such as iron, chlorinated compounds, fumarate, nitrate or sulfate (reviewed in (Schwartz et al., 2013; Vignais et al., 2001).

1.3. The genus *Desulfovibrio*

Sulfate-reducing microorganisms are widespread in anoxic habitats. They include both bacteria and archaea which are able to perform the dissimilatory reduction of sulfate, resulting in the production of hydrogen sulfide. *Desulfovibrio*, belonging to the class of deltaproteobacteria, is the most widely studied bacterial genus among the sulfate-reducing microorganisms. They are metabolically versatile and can couple oxidation of a variety of electron donors, such as lactate or pyruvate, to the reduction of sulfate. *Desulfovibrio* can also use electron acceptors other than sulfate for anaerobic respiration such as sulfur, nitrate, or fumarate (Lobo, Warren, & Saraiva, 2012; Miller & Wakerley, 1966; Thauer, Stackebrandt, & Hamilton, 2007; Voordouw, 1995). They can also reduce metal ions such as Fe(III) (Coleman, Hedrick, Lovley, White, & Pye, 1993), U(VI) (Lovley & Phillips, 1992) and Cr(VI) (Lovley & Phillips, 1994; Michel, Brugna, Aubert, Bernadac, & Bruschi, 2001) but these reduction processes are not coupled to growth. Moreover, some *Desulfovibrio* strains have the ability to ferment organic compounds such as pyruvate in the absence of sulfate or other electron acceptors (Hansen, 1993; Meyer et al., 2014; Singleton, 1993). Despite being considered obligate anaerobic organisms, *Desulfovibrio* species have been found to be tolerant to molecular oxygen and have developed different strategies to address the presence of O_2 (Dolla, Fournier, & Dermoun, 2006; Dolla, Kurtz, Teixeira, & Voordouw, 2007; Ramel et al., 2015). Many of them are able to reduce O_2 to water probably as a protective mechanism although there is no sustainable aerobic growth (Cypionka, 2000; Marschall, Frenzel, & Cypionka, 1993). Schoeffler et al. (2019) have recently obtained, by implementing O_2-driven experimental evolution, an evolved strain of *Desulfovibrio vulgaris* that is able to grow with energy derived from oxidative phosphorylation linked to oxygen reduction (Schoeffler et al., 2019).

The major part of this chapter will be devoted to the description of hydrogenases found in bacteria belonging to the genus *Desulfovibrio* and their physiological role in the hydrogen metabolism. The search for genes encoding hydrogenases in the genome of more than 30

Desulfovibrio strains available on the National Center for Biotechnology Information (NCBI) site gives an overview of the distribution of these enzymes in this genus (Table 1). Emphasis will be placed on the species *D. fructosovorans*, our model organism, which has a complex system of hydrogenases including multimeric [FeFe]-enzymes catalyzing flavin-based electron-bifurcation and a putative sensory-type [FeFe]-hydrogenase.

2. H$_2$ metabolism in *Desulfovibrio*

2.1. Versatility of the *Desulfovibrio* metabolism

Numerous genomes of *Desulfovibrio* species have been sequenced in the last years providing an overview of the hydrogen metabolism and more broadly of the energy metabolism of these bacteria (I. A. Pereira et al., 2011). Molecular hydrogen plays a central role in the energy metabolism of *Desulfovibrio* which can either use H$_2$ as an energy source or produce H$_2$ when growing fermentatively (Fauque et al., 1988; Rabus, Hansen, & Widdel, 2013). They also have the ability to live in stable syntrophic associations with H$_2$-scavenging methanogenic partners in the absence of sulfate with ethanol or lactate as electron donor (Bryant, Campbell, Reddy, & Crabill, 1977; Stolyar et al., 2007). Under these conditions, *Desulfovibrio* is the H$_2$-producing partner and the H$_2$-consuming partner keeps the H$_2$ partial pressure low to make possible the otherwise thermodynamically unfavorable oxidation of ethanol or lactate. The two partners are in a close contact and it was proposed that the H$_2$ produced is directly transferred from the producer to the H$_2$-scavenger (Brileya, Camilleri, Zane, Wall, & Fields, 2014; Conrad, Phelps, & Zeikus, 1985; Krumholz et al., 2015). This phenomenon, particularly important in anaerobic environments, was called interspecies hydrogen transfer (Schink & Stams, 2002; Wolin, 1976). More recently, tight cell-cell physical interactions between *Desulfovibrio* and *Clostridium* in a synthetic consortium

have been demonstrated, associated with an exchange of cytoplasmic molecules, allowing the survival of *D. vulgaris* in the absence of its nutrients. These physical interactions induce changes in the distribution of metabolic fluxes, and allow an important increase in H_2 production (Benomar et al., 2015).

2.2. Different models for the H_2 metabolism

The versatility of the H_2 metabolism in *Desulfovibrio* species is due to a complex hydrogenase system composed by several enzymes of both [FeFe]- and [NiFe]-type with different subunit composition and cellular localization and this system greatly varies in *Desulfovibrio* species (Table 1) (Morais-Silva et al., 2014; I. A. Pereira et al., 2011). In addition, some *Desulfovibrio* species can have [NiFeSe]-hydrogenases, a subfamily of the [NiFe]-hydrogenase with a selenocysteine on the active site (Figure 1). This multiplicity and diversity of hydrogenases confer to the bacteria the ability to quickly adapt their metabolism in response to the environmental changes but make difficult the elucidation of the physiological function of these enzymes (Caffrey et al., 2007; Casalot, De Luca, Dermoun, Rousset, & de Philip, 2002; Casalot, Valette, et al., 2002; Goenka, Voordouw, Lubitz, Gartner, & Voordouw, 2005; Keller & Wall, 2011; Malki et al., 1997; Morais-Silva, Santos, Rodrigues, Pereira, & Rodrigues-Pousada, 2013; Pohorelic et al., 2002). Indeed, the role in the energy metabolism of most of the hydrogenases in *Desulfovibrio* has still to be clarified.

Almost forty years ago, a model for the growth of *Desulfovibrio* with lactate as electron donor and sulfate as electron acceptor, called the "hydrogen cycling model", was proposed by Odom and Peck (Odom & Peck, 1981) (Figure 2) to explain the transient burst of H_2 observed in batch culture (Tsuji & Yagi, 1980). In this energy-conserving mechanism, lactate oxidation produces electrons and protons that are used by a cytoplasm-located hydrogenase to produce H_2. After the diffusion through the cytoplasmic membrane, H_2 is re-oxidized by a periplasmic

hydrogenase generating a proton gradient across the membrane that leads to ATP formation. The electrons generated by this oxidation are then returned to the cytoplasm thanks to the cytochrome c_3 network and several integral membrane complexes where they are used for sulfate reduction. This model requires the presence of both periplasmic and cytoplasmic hydrogenases. Since hydrogen cycling model publication, it has remained controversial and several studies have pointed out the non-essential character of the hydrogen cycling for the growth of *Desulfovibrio* (Fitz & Cypionka, 1989, 1991; Lupton, Conrad, & Zeikus, 1984; Morais-Silva et al., 2013; Pankhania, Gow, & Hamilton, 1986; Rabus et al., 2013). More recent models propose that two electron transfer routes from lactate to sulfate operate simultaneously: the pathway of the hydrogen cycling model including the transient production and consumption of H_2, and a second H_2-independent pathway involving a direct electron transfer from the donor to the acceptor (Figure 2) (Keller & Wall, 2011; Noguera, Brusseau, Rittmann, & Stahl, 1998; Sim et al., 2013). In the methanogenic archaeon *Methanosarcina barkeri*, Kulkarni et al. have very recently demonstrated, thanks to a series of hydrogenase mutants, the role of H_2 cycling in energy conservation and proposed, based on the common occurrence of both cytoplasmic and periplasmic hydrogenases, that this mechanism may be widespread in nature, especially among anaerobic microorganisms (Kulkarni, Mand, & Metcalf, 2018).

3. *Desulfovibrio fructosovorans*

Desulfovibrio fructosovorans is one of the most widely studied *Desulfovibrio* species. It can grow mixotrophically with H_2 as sole energy source and with acetate and CO_2 as carbon source, or heterotrophically with different carbon and energy source such as fructose, pyruvate or lactate, and sulfate as electron acceptor (Ollivier, Cord-Ruwisch, Hatchikian, & Garcia, 1988).

3.1. Fructose as a carbon source

D. fructosovorans differs from most other *Desulfovibrio* species by its ability to use fructose as a carbon source (Ollivier et al., 1988). Utilization of carbohydrates has been reported for a few other species of *Desulfovibrio* (Nielsen, Liesack, & Finster, 1999; Sass & Cypionka, 2004; Trinkerl, Breunig, Schauder, & König, 1990; Zellner, Messner, Kneifel, & Winter, 1989). Fructose can be metabolized and degraded to pyruvate by the Embden-Meyerhof-Parnas pathway of glycolysis for generation of energy and cellular biosynthesis. The *in silico* analysis of the draft genome of *D. fructosovorans* shows that all the enzymes of this pathway are present in this organism. In many bacteria, the uptake of sugar is mainly performed by the phosphotransferase system (PTS) that couples transport with sugar phosphorylation using phosphoenolpyruvate as phosphoryl donor (Barabote & Saier, 2005). PTS involves two cytosolic proteins, Enzyme I (EI) and Histidine protein (Hpr), as well as a sugar specific transporter called Enzyme II (EII) with three structurally different functional domains (IIA, IIB and the permease IIC) that can be fused in one molecule or separated in different chains. *D. fructosovorans* possesses at least 8 genes for PTS transport distributed in three different clusters positioned in the genome. One of these cluster contains a gene coding for a PTS EII (EIIBC) containing a permease specific for fructose, and another gene for a polypeptide containing EI, Hpr and the fructose-specific EIIA components (locus tags DesfrDRAFT_0726 and _0728). The two genes are separated by a gene coding for a 1-phosphofructokinase, enzyme that transforms fructose 1-phosphate into fructose 1,6-biphosphate (DesfrDRAFT_0727). The presence of fructose-specific permease EII and 1-phosphofructokinase in the same cluster suggests that fructose enters the cell as fructose 1-phosphate (Kornberg, 2001) and could be incorporated in the glycolysis pathway as fructose 1,6-biphosphate. Operons specific for fructose transport that include 1-phosphofructokinase are also found in *E. coli* and *Salmonella typhimurium* (Geerse, Izzo,

& Postma, 1989; Geerse, Ruig, Schuitema, & Postma, 1986). The other clusters of genes for PTS

lack permease and include proteins belonging to mannose/fructose/sorbose family.

3.2. Six different hydrogenases in *D. fructosovorans*

D. fructosovorans has a complex hydrogen metabolism and possesses 6 gene clusters

encoding six different hydrogenases, among them two are of [NiFe]-type and four are of [FeFe]-

type (Figure 3). Three of these hydrogenases, Hyn, Hyd and Hnd, have already been biochemically

and genetically studied. Hyn is a periplasmic heterodimeric [NiFe]-hydrogenase that was deeply

characterized in *D. fructosovorans*, especially at the molecular level. Hyd is a periplasmic

heterodimeric hydrogenase as well but of [FeFe]-type and Hnd is a cytoplasmic heterotetrameric

[FeFe]-hydrogenase, whose physiological role was already discussed more than 20 years ago (de

Luca, de Philip, Rousset, Belaich, & Dermoun, 1998; Malki et al., 1997). Only recently, we purified a

recombinant form of this enzyme and showed that it performs flavin-based electron-bifurcation

(Kpebe et al., 2018). The analysis of the sequence of the draft genome of *D. fructosovorans*

revealed the presence of three additional putative hydrogenases. One of them shows high

similarity with the energy-conserving, membrane-bound [NiFe]-hydrogenases Ech (Hedderich,

2004). The second one is very similar to the trimeric cytoplasmic [FeFe]-hydrogenase from

Thermotoga maritima that was the first hydrogenase demonstrated to be a flavin-based electron-

bifurcating hydrogenase using simultaneously a reduced ferredoxin and NADH as electron donors

(Schut & Adams, 2009). We called this putative enzyme from *D. fructosovorans* Hnt (H for

Hydrogenase, n for NA(D)P-dependent and t for trimeric). The third probable hydrogenase from *D.

fructosovorans* shows strong similarity with the putative sensory [FeFe]-hydrogenases HydS from

T. maritima (Chongdar et al., 2018) or the Hfs hydrogenase from *Thermoanaerobacterium

saccharolyticum* (Shaw, Hogsett, & Lynd, 2009). These three putative hydrogenases have never

been biochemically characterized in *D. fructosovorans*, even if the activity of a fourth hydrogenase

has been detected in the triple hydrogenase mutant deleted of Hyn, Hyd and Hnd (Casalot, De Luca, et al., 2002).The physiological role of these putative hydrogenases in the energy metabolism has yet to be elucidated.

Even if hydrogenases, especially those from the [FeFe]-type, are generally known to be highly O_2-sensitive (Orain et al., 2015), the three hydrogenases from *D. fructosovorans* that have been characterized so far (Hyn, Hyd and Hnd) remain active when purified under aerobic condition, as other hydrogenases from *Desulfovibrio* species. Indeed, these three hydrogenases can form O_2-protected inactive form: NiA and NiB states in the case of the [NiFe]-hydrogenase Hyn (Abou Hamdan, Liebgott, et al., 2012) and $Hox_{(inact)}$ state in the case of [FeFe]-hydrogenases Hnd and Hyd (Kpebe et al., 2018; Rodriguez-Macia et al., 2018). It should be noted that hydrogenases are not the only type of enzymes to have developed O_2-protection in *Desulfovibrio* species. The pyruvate:ferredoxin oxidoreductase (PFOR) from *D. africanus* was demonstrated to use a disulfide bond-dependent reversible mechanism of O_2-protection (Vita, Hatchikian, Nouailler, Dolla, & Pieulle, 2008). Moreover, this enzyme has an extension at the C-terminal end of one subunit containing a methionine that protects one of the iron-sulfur cluster from oxidative damage (Chabrière et al., 1999). The carbon monoxide dehydrogenase (CODH) from *D. vulgaris* was demonstrated to be less sensitive to O_2 than CODH from other bacteria such as *Carboxydothermus hydrogenoformans* (Merrouch et al., 2015).

4. Periplasmic hydrogenases

The genomes of *Desulfovibrio* species that we have probed for hydrogenase genes contain at least one and at most four soluble periplasmic hydrogenases (Table 1). These hydrogenases could be of [FeFe]-, [NiFe]- or [NiFeSe]-type. All *Desulfovibrio* species contain at least one copy of the Hyn enzyme (Table 1) (I. A. Pereira et al., 2011). *D. fructosovorans* contains, as already

mentioned, a [FeFe] and a [NiFe] periplasmic soluble hydrogenases but no gene was found for a [NiFeSe]-enzyme.

4.1. Molecular characterization of the periplasmic hydrogenases

4.1.1. The Hyd hydrogenase

The dimeric soluble [FeFe]-hydrogenase Hyd from *D. fructosovorans*, encoded by the two gene operon *hydAB*, is composed of HydA ,a large subunit of 46 kDa and HydB a small subunit of 13 kDa (Figure 3B) (Casalot et al., 1998). Its periplasmic localization was predicted by the presence of a TAT signal at the N-terminus of HydB (Casalot et al., 1998; Vignais & Billoud, 2007). This enzyme was partially purified (Casalot et al., 1998) and its hydrogen uptake activity was determined to be 17 000 U/mg of protein (1U= 1 μmol of H_2/min). No further characterization was performed on this enzyme. However, the two closely related Hyd hydrogenases from *D. desulfuricans* ATCC 7757 and *D. vulgaris* Hildenborough were deeply characterized using various spectroscopies and electrochemistry.

The enzymes from these two species were first purified in a native form (C. Hatchikian, Forget, Fernandez, Williams, & Cammack, 1992; Patil et al., 1988) from which most of the studies were performed. At present, Hyd from *D. desulfuricans* is produced in a recombinant and apo-form (lacking the H-cluster) in *E. coli* and then artificially maturated by the $(Et_4N)_2[Fe_2(adt)(CO)_4(CN)_2]$ complex (Birrell et al., 2016).

The structure of Hyd from *D. desulfuricans* was solved in 1999 by the group of Fontecilla-Camps (Nicolet et al., 1999) and was one of the first crystallographic structure determined for a [FeFe]-hydrogenase. It revealed the active site arrangement with a typical [4Fe4S] cluster, the H-cluster, bridged by a cysteine to a di-iron center with CO and CN$^-$ ligands and a dithiolate bridge between the two iron atoms. The large subunit contains the active site (H-cluster) and two additional [4Fe4S] clusters while the small subunit can be best described as a belt around the large subunit

(Figure 1). From this structure, proton and electron transfer pathways and gas channel were proposed.

Hyd from various *Desulfovibrio* species has a particular characteristic in that when it is isolated under air in an inactive form, it can be reactivated by reduction. When purified under aerobic conditions, the hydrogenase shows two different forms of the active site: an inactive "air-prepared" state, called $Hox_{(inact)}$ or $Hox_{(air)}$, which is O_2-insensitive and presents a specific Fourier Transform InfraRed spectroscopy (FTIR) signal and an active state, which is O_2-sensitive and can be obtained by reduction of the $Hox_{(inact)}$ state (Pierik et al., 1992). After the structure of Hyd was solved, different spectroscopies permit to further characterized the enzyme. The nature of the dithiolate bridge was identified by advanced EPR spectroscopy as an azadithiolate (Silakov, Wenk, Reijerse, & Lubitz, 2009). The mechanism of formation of $Hox_{(inact)}$ involving sulfide was proven by FTIR spectroscopy (Rodriguez-Macia et al., 2018).

Furthermore, the structure of Hyd hydrogenase from *D. desulfuricans* has provided the basis of several theoretical chemistry calculations, mainly by Density Functional Theory (DFT) in order to identify the dithiolate bridge of the active site, or to propose catalytic and inhibition mechanisms (Baffert et al., 2011; Dogaru, Motiu, & Gogonea, 2009; Fan & Hall, 2001; Greco, Bruschi, De Gioia, & Ryde, 2007). The catalytic properties of Hyd hydrogenases were studied both by standard biochemical methods (enzymatic activity assays followed by spectrophotometry) and by protein-film electrochemistry. Their H_2-uptake activity with methyl-viologen as artificial partner reaches 62200 U/mg for the *D. desulfuricans* enzyme and 50000 U/mg for the *D. vulgaris* enzyme while the H_2-production activity was determined to be 8200 U/mg for *D. desulfuricans* enzyme and 6900 U/mg for the *D. vulgaris* enzyme (Fauque et al., 1988; C. Hatchikian et al., 1992; Pierik et al., 1992). Using electrochemistry, it was determined that Hyd enzyme is reversibly inhibited by CO with an inhibition constant of 5 nM showing its high sensitivity to this gas. Hyd hydrogenases are also irreversibly inhibited by O_2 and NO with an O_2 inhibition rate constant of 1.8 s^{-1} mM^{-1} (Berlier et

al., 1987; Goldet et al., 2009). Furthermore, Hyd enzymes are inactivated at high potential (above

around 0 V/ESH) under anaerobic conditions (Goldet et al., 2009). The Km for H_2 was not

determined but was proposed to be around 100 µM (Goenka et al., 2005). This value is in the low

range of Km determined for other [FeFe]-hydrogenases, which are around 500 µM (Fourmond et

al., 2013).

These specific activity values indicate that Hyd hydrogenases are biased toward H_2-oxidation in

agreement with the physiological role proposed (see section 4.2). However, it is worth to note that

the experimental conditions used to determine the specific activities provide different driving-

force for the oxidation or reduction reaction, thus values cannot be directly compared. The Km

value for dihydrogen shows a low affinity of the enzyme for this substrate and this property

suggests that Hyd hydrogenase is important when the pressure of H_2 is high (Caffrey et al., 2007).

The high sensitivity to inhibitors of Hyd hydrogenase is not coherent with the versatility of

environments in which *Desulfovibrio* species can grow. However, the presence of an additional

small subunit and the formation of an O_2-insensitive state ($Hox_{(inact)}$) could protect the enzyme

during exposition to inhibitors and then allow reactivation of the hydrogenase when conditions

become favorable for its activity.

4.1.2. The Hyn hydrogenase

The dimeric soluble [NiFe]-hydrogenase from *D. fructosovorans* is encoded by the two

genes *hynA* and *hynB* (Figure 3B) which are part of an operon containing also a third gene

encoding the specific protease, HynC, involved in the maturation of the large subunit (Rousset,

Dermoun, Wall, & Belaich, 1993). HynA, the small subunit of 28.5 kDa, contains two [4Fe4S]-

clusters and one [3Fe4S]-cluster while HynB is the large subunit of 60 kDa that hosts the [NiFe]-

active site. Its periplasmic localization was predicted by the presence of a TAT signal on HynB

(Vignais & Billoud, 2007).

D. gigas Hyn hydrogenase was the first [NiFe]-hydrogenase purified and characterized in 1978 (Bell, Lee, Peck, & Gall, 1978; E. C. Hatchikian, Bruschi, & Le Gall, 1978). Its specific activity with artificial redox partners (methyl-viologen or benzyl-viologen) was determined to be 50.5 U/mg for H_2 oxidation and 90 U/mg for H_2 production. EPR spectroscopy proved the presence of a Ni atom in inactive O_2-protected states, named NiA and NiB (Moura et al., 1982; Teixeira et al., 1985). The structure of Hyn from *D. gigas* was first solved in 1995 but at a low resolution (Volbeda et al., 1995). Higher resolution of *D. vulgaris* Hyn enzyme and IR spectroscopy studies of *D. gigas* Hyn enzyme showed the presence of CO and CN^- ligands at the active site (Higuchi, Yagi, & Yasuoka, 1997; van der Spek et al., 1996).

The hydrogenase Hyn from *D. fructosovorans* was first characterized in 1990 (C. E. Hatchikian, Traore, Fernandez, & Cammack, 1990). The enzyme was determined to contain 11 iron atoms, 1 nickel atom and 12 inorganic sulfur atoms in close agreement with the prediction. Later, a Strep-tagged recombinant enzyme was homologously produced in *D. fructosovorans* and deeply characterized. EPR experiments confirmed the presence of a Ni(III) atom and iron-sulfur clusters. The enzyme purified under air-atmosphere present NiA and NiB EPR signatures characteristic of inactive forms of the enzyme that need reducing conditions (hydrogen or chemical reductant) to be converted into active enzyme. These states are re-formed upon exposure to O_2 or at high potential (above around -200 mV/ESH) under anaerobic conditions (Abou Hamdan et al., 2013). The structure of Hyn from *D. fructosovorans* was obtained in 1998 (pdb 1FRF) (Figure 1) (Rousset et al., 1998). It contains the [NiFe]- active site (Figure 1) in the large subunit HynB, one classical proximal [4Fe4S] cluster, one [3Fe4S] medial cluster and an unusual 3 cysteine/1 histidine residues coordinated-[4Fe4S] distal cluster in the small subunit HynA, the three clusters forming an electron transfer pathway. The specific activity of Hyn from *D. fructosovorans* with an artificial electron acceptor (methyl-viologen) was determined to be 205 U/mg of protein for H_2-uptake and 335 U/mg of protein for H_2-evolution (C. E. Hatchikian et al., 1990). However, the proton reduction

is strongly inhibited by H_2 (Fourmond et al., 2013) and the Km of Hyn for H_2 is 9 µM showing its high affinity for this substrate (Liebgott et al., 2010).

Several aspects of the catalysis were studied on Hyn from *D. fructosovorans*, using site-directed mutagenesis, biochemical, spectroscopic and electrochemical approaches: i) inter- and intramolecular electron transfers, ii) proton transfer, iii) gas diffusion and iv) reaction at the active site.

i) The rates of intermolecular and intramolecular electron transfers were deduced from the specific activity of enzyme variants around the iron-sulfur clusters of the electron transfer chain: the histidine ligand of the unusual distal [4Fe4S] cluster could be replaced by a glycine or a cysteine without impairing the cluster formation and the medial [3Fe4S] could be changed into a [4Fe4S] by the replacement of a proline by a cysteine (Dementin et al., 2011; Rousset et al., 1998). The specific activitiy of all the variants was affected at different extent (Dementin et al., 2006). These variations were interpreted in term of inter- and intramolecular electron transfer rate (Figure 4A).

ii) Mutagenesis of the glutamate 25 of HynB indicates that this amino acid is essential for proton transfer between the active site and the protein surface (Dementin et al., 2004). However, the entire proton pathway could not be determined because it may involve residues too close to the proximal iron-sulfur cluster to be mutated without modification of the electron transfer rate.

iii) Gas diffusion has been focused on because gas molecules such as CO or O_2 are inhibitors of the enzyme and inhibition by dioxygen is a major obstacle to the use of hydrogenases in biotechnological devices. Hyn was inhibited by CO with a rate constant of 63000 s^{-1} mM^{-1} and by O_2 with a rate constant of 32 s^{-1} mM^{-1} (Liebgott et al., 2010). The structure showed that two amino acids gate the access of gas to the active site. Gas diffusion and O_2 inhibition rate were determined for several variants mutated on these residues using direct electrochemistry (Dementin et al.,

2009; Leroux et al., 2008; Liebgott et al., 2011; Liebgott et al., 2010). Diverse strategies were followed: substitution by bulky amino acids with the idea of molecular sieve, substitution by reactive residues such as methionine or cysteine, substitution with charged residues (Figure 4B). This approach allowed to decrease the diffusion rate of gas into the protein and to improve the O_2 resistance of the enzyme.

iv)   The reaction at the active site and the catalytic bias (ratio of the catalytic rate constants of the forward *vs* backward reaction, i.e. proton reduction *vs* dihydrogen oxidation) could not be investigated by mutation around the active site because of the formation of an inactive enzyme. However, among the variants constructed, some shown catalytic properties that differ from the wild-type enzyme (Abou Hamdan, Dementin, et al., 2012). The bias is not mainly determined by redox potential of the active site but is tuned by the rate-limiting step. H_2 production rate is limited by H_2-diffusion in the gas channel whereas the electron-transfer rate limits the H_2-oxidaton by Hyn.

4.2. Role of periplasmic hydrogenases in the H_2 metabolism

A deletion mutant of the two structural genes *hydAB* was constructed in *D. fructosovorans* (Casalot, Valette, et al., 2002). When the bacterium is grown mixotrophically on H_2, acetate and CO_2, or with lactate as carbon source and sulfate, the deletion mutant and the wild-type strains show the same pattern of growth. When pyruvate is used in the presence of sulfate, the mutant grows three time slower than the wild-type but the molar growth yield relative to sulfate remains unchanged. However, when fructose is used for growth, both the rate and the molar growth yield of the mutant are affected compared to the wild-type (Casalot, Valette, et al., 2002). *hydAB* genes were also deleted in a single mutant depleted of one of the other two hydrogenases Hyn and Hnd. A triple mutant was also constructed by deletion of the genes encoding the three characterized hydrogenases, Hyd, Hyn and Hnd (Casalot, Valette, et al., 2002). The analysis of this series of

hydrogenase mutants indicated that the deletion of one enzyme might be compensated by another (Casalot, De Luca, et al., 2002; Casalot, Valette, et al., 2002) and this deletion strategy has not made possible the elucidation of the exact physiological role of the Hyd hydrogenase in the energy metabolism of *D. fructosovorans*.

hydAB genes are represented in most of *Desulfovibrio* species. Indeed, genes encoding a dimeric [FeFe] periplasmic hydrogenase are found in 27 of the 37 genomes of *Desulfovibrio* strains available on NCBI in which we have searched for hydrogenase genes (Table 1). *D. alaskensis* G20 even possesses two copies of *hydAB* genes (Table S1).

In *D. vulgaris*, multiple studies have indicated that the Hyd hydrogenase is involved in H_2 oxidation (Caffrey et al., 2007; Pohorelic et al., 2002), except the study by van den Berg et al., in which it was proposed a role of Hyd in H_2 production (van den Berg, van Dongen, & Veeger, 1991). A mutant strain lacking the Hyd enzyme grew less on H_2/ sulfate medium than the wild-type strain (Pohorelic et al., 2002). On lactate/sulfate medium, the growth yield was strongly affected in the mutant strain but it produced a larger amount of hydrogen, indicating that the role of Hyd is H_2 consumption. Comparison of the growth and of gene expression level pattern of a deletion strain of *D. vulgaris* lacking the Hyd hydrogenase on lactate-containing medium or in the presence of hydrogen at either 5 or 50% as electron donor for sulfate reduction, with the parental strain demonstrated that the [FeFe] periplasmic hydrogenase is involved in H_2 oxidation at high H_2 partial pressure in keeping with its low affinity for H_2 and its high specific activity (Caffrey et al., 2007).

The growth rate of a deletion mutant of the two structural genes *hynAB* of *D. fructosovorans* with hydrogen as sole energy source is similar to that of the wild-type but with a lag phase (Rousset, Dermoun, Chippaux, & Belaich, 1991). On fructose-, lactate- and pyruvate-containing medium and in the presence of sulfate, wild-type and deletant strains grew similarly

(Malki et al., 1997). Again, the analysis of single, double and triple hydrogenase mutant strains in *D. fructosovorans* indicated that the absence of one periplasmic hydrogenase can be compensated by another hydrogenase (Casalot, De Luca, et al., 2002; Malki et al., 1997). In *D. vulgaris*, results obtained by Caffrey et al. on mutant strains lacking periplasmic hydrogenases indicate that the [NiFeSe] periplamic hydrogenase is required for the growth when the partial pressure of H_2 is low, in accordance with the lower activity but the higher affinity for H_2 of this enzyme as compared to the [FeFe] Hyd enzyme (Caffrey et al., 2007).

Hyn has been shown in *D. gigas* to play a dominant role in the hydrogen metabolism because it is required for H_2 oxidation when the bacterium grows on H_2 as the only energy source but also for H_2 production when the bacterium ferments pyruvate (Morais-Silva et al., 2013). It is worth noting that *D. gigas* contains only two hydrogenases, a periplasmic Hyn and a membrane-bound cytoplasmically oriented Ech (Morais-Silva et al., 2014). Due to this unusually low number of hydrogenases for a *Desulfovibrio* species, it has been proposed that the hydrogen cycling contributes less to the energy yield in *D. gigas* as compared to other *Desulfovibrio* species (Morais-Silva et al., 2013).

Hyn hydrogenase is present in all *Desulfovibrio* species analyzed, even in two copies in *D. desulfuricans, D. fairfieldensis, D. gracilis, D. inopinatus*, *D. piger* and *D. vulgaris* (Table S1). [NiFeSe] hydrogenase (Figure 1) is a sub-class of Hyn hydrogenase in which a cysteine ligand to the active site is substituted by a seleno-cysteine (Baltazar et al., 2011). In addition, the medial cluster is a [4Fe4S]-cluster instead of a [3Fe4S]-cluster in the standard Hyn hydrogenase. One third of the *Desulfovibrio* species encodes for a [NiFeSe] hydrogenase (Table S1).

In summary, it seems clear that the main function of periplasmic hydrogenases in *Desulfovibrio* species is to oxidize hydrogen. They contribute to the overall energy metabolism of the cells. However, because of the redundancy of the periplasmic enzymes, their precise

physiological role is still to be clarified. The expression of each hydrogenase depends on the environmental conditions such as the availability of metal ions, in particular nickel and selenium (Valente et al., 2006), the level of O_2 (Fournier, Dermoun, Durand, & Dolla, 2004) or H_2 (Caffrey et al., 2007). In view of the Km value of Hyn and Hyd for H_2, Hyn could be efficient at low partial pressure of H_2 whereas Hyd is proposed to be required when the level of H_2 is high.

5. Membrane-bound hydrogenases

5.1. The Ech hydrogenase

In the genome of *D. fructosovorans*, we identified a six-gene cluster encoding a putative multisubunit membrane-bound [NiFe]-hydrogenase (Figure 3B). Sequence analysis of the deduced subunits revealed that this putative enzyme is of the Ech-type (Ech for Energy-Conserving Hydrogenase). The six subunits of Ech-type enzymes are closely related to subunits of the proton pumping complex I (NADH:quinone oxidoreductase) (Efremov & Sazanov, 2012; Hedderich, 2004; Hedderich & Forzi, 2005). Ech hydrogenases have been identified both in bacteria and archaea, and the most biochemically and genetically studied member of this hydrogenase family is the one from the methanogenic archaeon *Methanosarcina* (Kurkin, Meuer, Koch, Hedderich, & Albracht, 2002; Meuer, Bartoschek, Koch, Kunkel, & Hedderich, 1999; Meuer, Kuettner, Zhang, Hedderich, & Metcalf, 2002). This enzyme is membrane-bound but has its active site on the inner face of the cytoplasmic membrane (Hedderich & Forzi, 2005). Its redox physiological partner is a low-potential ferredoxin (Meuer et al., 1999) and it has been shown to be an H^+-translocating hydrogenase (Welte, Kratzer, & Deppenmeier, 2010) (Figure 5). In *Methanosarcina*, Ech is one of the key enzymes of the respiratory chain that contributes to electrochemical gradient which is the driving force for ATP-synthesis. In aceticlastic and methylotrophic methanogenesis, Ech couples

the reduction of protons to H_2 by reduced ferredoxin to the generation of an electrochemical proton potential (Figure 5A). Under autotrophic growth conditions, Ech catalyzes the reverse reaction and reduces ferredoxin with H_2, ferredoxin which is then used for CO_2 reduction or in biosynthetic reactions (Grahame & DeMoll, 1995; Meuer et al., 2002). This endergonic reduction is driven by the electrochemical proton potential (Forzi et al., 2005; Hedderich, 2004) (Figure 5B). It has recently been demonstrated that *Methanosarcina barkeri* is capable of energy conservation via H_2-cycling thanks to two H_2-producing cytoplasmic hydrogenases, among them is Ech, and a H_2-oxidizing periplasmic hydrogenase (Kulkarni et al., 2018). In *Thermoanaerobacter tengcongensis* (now *Caldanaerobacter subterraneus* subsp. *tengcongensis*), a 6-subunit ferredoxin-dependent Ech hydrogenase similar to the one from *M. barkeri*, has also been purified (Soboh, Linder, & Hedderich, 2004). The presence of a Ech-type hydrogenase was reported in *D. gigas* (Rodrigues, Valente, Pereira, Oliveira, & Rodrigues-Pousada, 2003).

As mentioned before, the gene cluster encoding a putative Ech-type hydrogenase in the genome of *D. fructosovorans* is composed of 6 genes. The amino acid sequence analysis of the six subunits showed that they share sequence identity with subunits of complex I (NADH:quinone oxidoreductase) and other Ech hydrogenases (Table S3). The 70-kDa protein encoded by *echA* is similar to EchA from *T. tengcongensis*, *M. barkeri* and *D. gigas* (Table S3). Sequence alignment of EchA from *D. fructosovorans* with the corresponding subunit of the Ech hydrogenase from *D. gigas*, *M. barkeri*, and *T. tengcongensis* shows the presence of a short N-terminal extension of 11 amino acids in *D. fructosovorans.* The presence of this short extension only in EchA from *D. fructosovorans* and the fact that the 12th amino acid is a methionine prompted us to propose a possible new start for the *echA* gene coding for a putative shorter 639-amino acid-containing protein with a theoretical molecular mass of 68.9 kDa. This subunit is predicted to contain 17 transmembrane helices. The following gene in the cluster, *echB*, codes for a putative membrane protein of 31 kDa, predicted to contain 6 transmembrane segments. The sequence of EchB shares

between 44 and 67 % identity with the sequence of EchB from *D. gigas*, *M. barkeri*, and *T. tengcongensis* (Table S3). EchA and EchB are thought to be involved in proton transport (Hedderich & Forzi, 2005; Welte & Deppenmeier, 2014). The *echC* gene encodes the conserved small [NiFe]-hydrogenase subunit EchC, a 15.5 kDa protein. As already pointed out by Hedderich and Forzi (Hedderich & Forzi, 2005), the small subunit of Ech hydrogenases is smaller than the corresponding subunits in standard [NiFe]-hydrogenases. The 4 cysteine residues possibly involved in the ligation of the only [4Fe4S] cluster are conserved (Rodrigues et al., 2003). The EchD subunit, encoded by the *echD* gene, is a 13.5 kDa protein with high identity to EchD from *D. gigas* (Table S3). Whereas in *D. gigas* EchD is proposed to contain a transmembranaire domain, in *D. fructosovorans*, no transmembranaire domain was predicted (Rodrigues et al., 2003).The *echE* gene encodes the large [NiFe]-hydrogenase subunit of 45.6 kDa, that shares between 48 to 66 % identity with EchE subunits from *D. gigas*, *M. barkeri*, and *T. tengcongensis* (Table S3). EchE from *D. fructosovorans* has an insertion of 50 amino acid residues as compared to EchE from *D. gigas* and *M. barkeri* (at the position 280). This insertion is predicted to be mainly in coil structure. The *echF* gene encodes a protein of 13.3 kDa. EchF shows two [4Fe4S]-cluster binding motifs and does not contain any transmembrane domain. The six-gene cluster has the same organization than the *ech* cluster in *D. gigas*, *M. barkeri* and *T. tengcongensis* (Figure 3B) (Künkel, Vorholt, Thauer, & Hedderich, 1998; Rodrigues et al., 2003; Soboh et al., 2004).

D. gigas was considered to be an interesting model to study the physiological role of hydrogenases in *Desulfovibrio* because it contains only two hydrogenases, Ech and Hyn. Construction of Δ*hyn* and Δ*ech* mutant strains showed that neither of the two hydrogenases are essential for the bacterial growth on lactate or pyruvate under respiratory conditions and it was concluded that the mechanism of H_2 cycling does not seem to be strictly essential for the *Desulfovibrio* genus (Morais-Silva et al., 2013). The presence of a Ech hydrogenase was revealed in

D. vulgaris by the sequence of the genome (Heidelberg et al., 2004). This ferredoxin-specific

hydrogenase is proposed to be bidirectional and may reduce ferredoxin for carbon fixation when

D. vulgaris grows with H_2 as an electron source or be involved in H_2 formation during growth on

pyruvate (I. A. Pereira et al., 2011; P. M. Pereira, He, Valente, et al., 2008). In *D. vulgaris*, the

expression of *ech* genes was found to be increased in biofilm cells as compared to planktonic cells
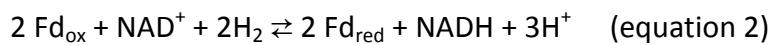
(Clark et al., 2012).

5.2. The Coo hydrogenase

The Coo hydrogenase, encoded by the *CooMKLXUH* operon, is an ion-translocating

complex closely related to the Ech hydrogenase belonging to the group of multisubunit

membrane-bound [NiFe]-hydrogenases, called energy-converting [NiFe]-hydrogenases (Hedderich

& Forzi, 2005). It uses reduced ferredoxin as electron donor. Whereas the genome of *D. vulgaris*

encodes both a Ech and a Coo hydrogenase, *cooMKLXUH* genes are not found in the genome of *D.*

fructosovorans (Table 1). In *D. vulgaris*, Coo hydrogenase is involved in H_2 production from lactate

during syntrophic growth whereas *coo* genes are down regulated during growth on H_2 (P. M.

Pereira, He, Valente, et al., 2008; Walker et al., 2009). Results obtained by Keller and Wall suggest

that the Coo hydrogenase is essential for growth on lactate-sulfate medium (Keller & Wall, 2011).

These authors proposed that, when concentration of H_2 is maintained low, Coo could be an

electron-bifurcating enzyme catalyzing the exergonic reduction of protons to form H_2 from the

reduced ferredoxin, coupled to the endergonic oxidation of menaquinol to produce H_2.

6. Cytoplasmic hydrogenases

6.1. Multimeric electron-bifurcating hydrogenases

6.1.1. Electron-bifurcating hydrogenases

Flavin-based electron-bifurcation, which is a novel mechanism of energy conservation in anaerobic microorganisms discovered by Wolfgang Buckel and Rudolph Thauer, couples an endergonic redox reaction to an exergonic redox reaction (Buckel & Thauer, 2013). Electron-bifurcating enzymes are as diverse as formate dehydrogenases, lactate dehydrogenases, transhydrogenases, heterodisulfide reductases or hydrogenases, to name a few. These enzymes have in common the presence of a flavin-harboring subunit that is thought to be the electron-bifurcation site and they catalyze a ferredoxin/flavodoxin dependent reaction (for recent reviews see (Baymann et al., 2018; Buckel & Thauer, 2018a, 2018b; Poudel et al., 2018). Flavin-based electron-bifurcating hydrogenases are trimeric or tetrameric cytoplasmic enzymes, of [FeFe]-type and harbor, in addition to the hydrogenase catalytic subunit, a [2Fe2S]-cluster subunit and a flavin- and FeS-containing $NAD(P)^+$-reducing subunit homologous to NuoF, a subunit of the complex I (Poudel et al., 2016) (Figure 6). They have been reported to bifurcate electrons derived from H_2 oxidation towards reduction of $NAD(P)^+$ and a ferredoxin (equation 2). Some of them have been shown to perform the reverse reaction and confurcate electron to produce H_2 (backward reaction of equation 2).

$$2\ Fd_{ox} + NAD^+ + 2H_2 \rightleftarrows 2\ Fd_{red} + NADH + 3H^+ \quad \text{(equation 2)}$$

where Fd_{ox} is an oxidized ferredoxin and Fd_{red} is a reduced ferredoxin.

The presence of the two or three additional subunits in addition to the hydrogenase catalytic subunit is proposed to be needed to confer the electron-bifurcation capability of the enzyme (Poudel et al., 2016). However, as illustrated by the example of the *Syntrophomonas wolfei* hydrogenase, the presence of these additional subunits does not mean necessary that the enzyme catalyses the electron-bifurcation (Losey, Mus, Peters, Le, & McInerney, 2017). The molecular mechanism of electron-bifurcation in still under debate. The most accepted hypothesis is that a

flavin with crossed-redox potential is the site for electron-bifurcation (Baymann et al., 2018). However, it was recently proposed that the H-cluster could be responsible for the electron-bifurcation reaction (Peters, Beratan, Bothner, et al., 2018; Peters, Beratan, Schut, & Adams, 2018). Further characterization, especially the resolution of the structure of multimeric hydrogenases, would help to decipher the electron-bifurcation mechanism of these enzymes.

The trimeric Hyd hydrogenase from *T. maritima* was the first flavin-based electron-bifurcating hydrogenase to be characterized 10 years ago (Schut & Adams, 2009; Verhagen, O'Rourke, & Adams, 1999). Since then, four other hydrogenases of this type, trimeric or tetrameric, have been isolated and characterized in the anaerobic bacteria *Acetobacterium woodii* (Schuchmann & Müller, 2012), *Moorella thermoacetica* (Wang, Huang, Kahnt, & Thauer, 2013), *Ruminococcus albus* (Y. Zheng et al., 2014), and the fourth being the Hnd hydrogenase from *D. fructosovorans* (Kpebe et al., 2018). In *Clostridium autoethanogenum*, an NADP-specific electron-bifurcating [FeFe]-hydrogenase forms an heptameric complex with a formate dehydrogenase (Wang, Huang, Kahnt, Mueller, et al., 2013). Sequences of these hydrogenase subunits share between 28 % and 64 % sequence identity (Tables S2). Figure 7 shows a comparison of gene clusters, subunit compositions and cofactor contents of electron-bifurcating hydrogenases that have been characterized to date including also the putative trimeric enzyme from *D. fructosovorans*. Tetrameric hydrogenases contain an extra subunit, the counterpart of HndB from *D. fructosovorans*, that does not seem essential for electron-bifurcation and its role and prosthetic group content is unclear (Poudel et al., 2016) (Figure 7). Indeed, the characterization of electron-bifurcating hydrogenases isolated up to this point, has not illuminated the impact of the subunit composition on the properties of the enzyme (reaction catalyzed, redox partner...).

6.1.2. Electron-bifurcating hydrogenases in *Desulfovibrio* species

Several but few *Desulfovibrio* species contain trimeric or tetrameric hydrogenases that are putative electron-bifurcating enzymes (Table 1). Only the tetrameric Hnd hydrogenase from *D. fructosovorans* has been isolated and characterized (Kpebe et al., 2018). Genes encoding such a tetrameric hydrogenase are not very widespread in *Desulfovibrio* and are found besides *D. fructosovorans*, only in seven *Desulfovibrio* species (*D. carbinolicus*, *D. magneticus*, D. sp. FW1012B, *D. sp.* DV, *D. alcoholivorans*, D. *sp.* TomC, D. *sp.* U5L) (Kpebe et al., 2018). Concerning the trimeric hydrogenase, only six *Desulfovibrio* species contain this type of enzyme (*D. carbinolicus*, *D. magneticus* RS-1, *D.* DV, *D. alcoholivorans*, *D. sp.* TomC and *D. fructosovorans*). It is worth noting that the six *Desulfovibrio* species that contain genes for a trimeric hydrogenase contain also genes for the tetrameric hydrogenase. In *D. fructosovorans,* two gene clusters code for multimeric [FeFe] hydrogenases: one for a putative trimeric enzyme and one for the tetrameric Hnd hydrogenase.

6.1.3. The tetrameric Hnd hydrogenase from *D. fructosovorans*

Hnd is a tetrameric cytoplasmic [FeFe]-hydrogenase (Figure 6A). The sequences of the four subunits HndA, B, C and D, encoded by the *hnd* operon (Figure 3B), have been known for more than 20 years and this enzyme has been previously described as an NADP-reducing enzyme (Malki et al., 1995). HndA (18.8 kDa) contains a [2Fe2S] cluster ligated by 4 conserved cysteine residues in its C-terminal thioredoxin-like domain and electron transfer between this cluster and the [2Fe2S] cluster of the N-terminal domain of HndD has been demonstrated (de Luca, Asso, Belaich, & Dermoun, 1998; Dermoun et al., 2002; Nouailler et al., 2006). HndB (13.8 kDa), whose function is still unknown, has been proposed to harbor a [2Fe2S]-cluster ligated by 3 cysteine and 1 serine residues (Dermoun et al., 2002). HndC, the NAD(P)$^+$-reducing subunit, a 51.7 kDa-protein, is predicted to contain binding sites for a flavin and for NAD(P) and to coordinate three [4Fe4S] clusters (Baymann et al., 2018; Kpebe et al., 2018; Malki et al., 1995). The hydrogenase catalytic

subunit HndD (63.6 kDa) is predicted to harbor one [2Fe2S]- and three [4Fe4S]-clusters in addition to the H-cluster (Figure 7). It shows 40% sequence identity with CpI, the well-characterized monomeric [FeFe]-hydrogenase from *C. pasteurianum* (Kpebe et al., 2018; Malki et al., 1995). It was recently shown that Hnd is an electron-bifurcating enzyme able to reduce simultaneously a ferredoxin and NAD$^+$ in the presence of H$_2$ (Kpebe et al., 2018). From the data obtained, a new mechanism was proposed in which the ferredoxin is recycled.

Unlike the other characterized electron-bifurcating hydrogenases, Hnd remains active after purification in the presence of dioxygen. Indeed, Hnd is able to form the O$_2$-protected Hox$_{(inact)}$ state of the active site, as the Hyd hydrogenase (Kpebe et al., 2018).

6.1.4. The trimeric Hnt hydrogenase from *D. fructosovorans*

The analysis of the draft genome of *D. fructosovorans* revealed the presence of a cluster of three genes encoding for a putative trimeric electron-bifurcating hydrogenase (Figure 3B), that we have named Hnt, which is highly similar to Hyd from *T. maritima* (Schut & Adams, 2009; Verhagen et al., 1999) and to other characterized multimeric hydrogenases (Figures 6B and 7, Tables 2). Amino-acid sequence analysis of HntA shows that this 24 kDa-subunit contains one [2Fe2S] cluster. HntC, a 67.4 kDa protein, is highly similar to the subunit HydB of the *T. mariti*ma enzyme, and harbors sequence motifs for coordination of NAD, flavin, one [2Fe2S]-clusters and three [4Fe4S]-clusters. It shares 49 % of identity with the HndC subunit from *D. fructosovorans* and a 120-amino acid N-terminal extension is present in the trimeric enzyme as compared to the tetrameric enzyme. HntD the hydrogenase catalytic subunit, the counterpart of HydA from *T. maritima*, is predicted to have a molecular weight of 73.9 kDa and to contain the H-cluster, three [4Fe4S]-clusters and two [2Fe2S]-clusters (Figure 6B). This subunit is highly similar to HndD (44 % of similarity) and possesses a 90-amino acid C-terminal extension as compared to HndD from *D. fructosovorans.* This putative trimeric hydrogenase Hnt has never been purified and biochemically

characterized in any *Desulfovibrio* species and no evidence concerning its possible physiological

role is available. As it was already mentioned for Hnd, putative Hnt-type hydrogenases are not

widespread in the *Desulfovibrio* genus since the presence of genes encoding this enzyme are

limited to only six species (Table 1). Moreover, *hnt* genes are only present in *Desulfovibrio*

genomes that contain further *hnd* genes meaning that Hnt hydrogenase is exclusively found in

Hnd-containing species. Because of its high similarity to trimeric electron-bifurcating hydrogenases

characterized so far (Tables S2), it is likely that *D. fructosovorans* Hnt hydrogenase catalyzes the

coupled reduction of ferredoxin and $NAD(P)^+$ by the oxidation of H_2 and/or the reverse reaction,

the coupled reduction of protons to H_2 with reduced ferredoxin and NAD(P)H (equation 2). In *C.*

autoethanogenum, the electron-bifurcating hydrogenase uses NADP instead of NAD (Wang,

Huang, Kahnt, Mueller, et al., 2013). In *D. fructosovorans*, NADP instead of NAD could be the

substrate of Hnt.

6.1.5. The physiological role of electron-bifurcating hydrogenases in *Desulfovibrio*

The role of these multimeric electron-bifurcating hydrogenases in *Desulfovibrio*

metabolism is unknown. In *Clostridia*, their implication in the energy metabolism has already been

discussed (Schuchmann, Chowdhury, & Muller, 2018). In *T. maritima*, *R. albus* and *M.*

thermoacetica, during growth on glucose containing-medium, both a ferredoxin and NAD^+ are

reduced and H_2 is produced using these reductants by the trimeric hydrogenase, in a concomitant

and energetically coupled way by electron confurcation (Schut & Adams, 2009; Wang, Huang,

Kahnt, & Thauer, 2013; Y. Zheng et al., 2014). In *M. thermoacetica*, during growth on H_2 and CO_2,

the trimeric hydrogenase possibly oxidizes H_2 and produces reduced ferredoxin, which is then

used for the CO_2 reduction to CO (Huang, Wang, Moll, & Thauer, 2012; Wang, Huang, Kahnt, &

Thauer, 2013). In *A. woodii*, the tetrameric hydrogenase produces NADH and a reduced ferredoxin

from H_2 in an electron-bifurcating process, which are used as reducing equivalents in the Wood-

Ljungdahl pathway for the CO_2 fixation (Schuchmann & Müller, 2012). The reduced ferredoxin fuels also the membrane Rnf complex allowing the re-oxidation of NADH and the generation of a transmembrane electrochemical gradient. The hydrogenase from *C. autoethanogenum* forms an heptameric complex with a formate dehydrogenase. This very complex enzyme containing putatively 19 iron-sulfur clusters catalyzes the reversible coupled reduction of ferredoxin and $NADP^+$ with H_2 or formate and the reversible formation of H_2 and CO_2 from formate. It is still unclear which reaction is catalyzed *in vivo* (electron-bifurcation or confurcation). It was also proposed that the hydrogenase function is to protect the cells from over-reduction when $NADP^+$ and ferredoxin get too reduced during growth on CO (Schuchmann et al., 2018; Wang, Huang, Kahnt, Mueller, et al., 2013). In *T. tengcongensis* and in *Thermoanaerobacterium saccharolyticum*, an heterotetrameric hydrogenase closely related to Hnd from *D. fructosovorans* is proposed to be involved in the release of excess reducing equivalents generated during fermentation. Indeed, this hydrogenase is involved in H_2-production during glucose fermentation (Shaw et al., 2009; Soboh et al., 2004).

Deletion of *D. fructosovorans hnd* genes encoding the hydrogenase subunit or the NADH-dehydrogenase subunit (Δ*hndD* or Δ*hndC*) is not lethal for the cells and *D. fructosovorans* can still grow using hydrogen as the sole energy source. During growth on fructose, pyruvate or lactate, the growth yield relative to sulfate is unchanged compared to the wild-type but the growth rates were affected, especially on pyruvate medium (by a factor of 3) (de Luca, de Philip, et al., 1998; Malki et al., 1997). Both the growth yield and growth rates are affected in the double mutant strain deleted from *hnd* and *hyn* genes. These studies have failed to establish the physiological role of Hnd due to a probable compensation mechanism between hydrogenases. However, the fact that the *hnd* deletion mutant produces more hydrogen than the wild-type strain when grown on a pyruvate medium containing a limiting amount of sulfate led us to propose that in these

conditions, the Hnd hydrogenase could possibly be involved in H_2 consumption (A. Kpebe and M. Brugna, unpublished results).

Nothing is yet known on Hnt metabolic role in *Desulfovibrio* species. Because it is always present in organisms in which Hnd is also present, it makes sense to hypothesize that these two enzymes are expressed in different culture conditions or that they are involved in different energy pathway with different redox partners (NAD^+ *vs* NADP).

6.2. H_2-sensing [FeFe]-hydrogenase

The presence of hydrogen can trigger the expression of some hydrogenase genes through a hydrogen sensing regulatory hydrogenase and two component signal transduction system. The well-known hydrogenase sensor of [NiFe]-type is that of *Ralstonia eutropha*. This sensor forms a complex with a histidine protein kinase, detects the H_2 level in the cellular environment and transmits the signal to a response regulator, which controls transcription of the hydrogenase genes (Friedrich, Buhrke, Burgdorf, & Lenz, 2005). This kind of [NiFe]-regulatory hydrogenase is not present in *Desulfovibrio* genomes that we have analyzed. However, among [FeFe]-hydrogenases, putative sensory type hydrogenases, called HydS (S for signaling) or Hfs, appear to have a regulatory role. In this type of hydrogenase, the hydrogenase domain containing a non-canonical H-cluster (Chongdar et al., 2018) is fused to a C-terminal PAS domain. This particular domain is the basis by which the sensory function was predicted (Greening et al., 2016). PAS domains are widely utilized for various signal transduction and sensory functions (Henry & Crosson, 2011).

In *D. fructosovorans*, we identified a three-gene cluster that contains a gene encoding a putative H_2-sensing hydrogenase of [FeFe]-type (Figure 3B and Figure 7). The first gene *hfsA* is

predicted to contain a [2Fe2S]-cluster. The 8.9 kDa-HfsA protein is 20 % identical to HfsA from *T. saccharolyticum*. *hfsB* encodes the monomeric putative sensing hydrogenase. The 63.8 kDa-protein HfsB shows 26 % identity with HydS from *T. maritima* and with HfsB from *T. saccharolyticum*. It has also 30% identity with the CpI hydrogenase from *C. pasteurianum*, 24% identity with the *Chlamydomonas reinhardtii* HydA1 hydrogenase and 26% with HndD subunit. A PAS sensory domain is present at the C-terminal end of the protein. However, in HfsB a conserved cysteine residue in typical [FeFe]-hydrogenases (cysteine 299 in CpI), essential for the activity which is supposed to be the first proton relay close to the active site, is replaced by an alanine at position 169 as it is the case in many HydS enzymes (Chongdar et al., 2018; Cornish, Gartner, Yang, Peters, & Hegg, 2011). HfsB is also predicted to contain three additional [4Fe4S]-clusters. The *hfsC* gene is annotated as a protein serine/threonine phosphatase. HfsC, a protein of 43.2 kDa, shows 31 % identity to *T. saccharolyticum* HfsC.

The HydS hydrogenase from *T. maritima* was the only putative H_2-sensing [FeFe]-hydrogenase to be purified and characterized (Chongdar et al., 2018). It was produced in *E. coli*, purified in an apo-form and then artificially maturated. It shows a low catalytic activity, both for H_2 oxidation and H_2 production. The reduction of the H-cluster is not associated with protonation of the azathiolate bridge, unlike for typical [FeFe]-hydrogenases. The results are consistent with the absence of the cysteine residue constituting the first proton relay during catalysis (Chongdar et al., 2018). The molecular basis of H_2 sensing by these enzymes and the way the signal is transferred need to be deciphered.

Eight *Desulfovibrio* species possess the gene cluster encoding a putative sensor [FeFe]-hydrogenase (*D. carbinolicus*, *D. magneticus*, *D.* sp. FW1012B, *D.* sp. DV, *D. alcoholivorans*, *D.* sp. TomC, *D.* sp. U5L and *D. fructosovorans*) (Table 1). It is worth noting that the presence of the

putative sensor hydrogenase is always correlated with the presence of Hnd hydrogenase genes. In addition, *Desulfovibrio* species that contain genes encoding a trimeric [FeFe]-hydrogenase have also *hfs* genes (Table 1). It is thus likely that these hydrogenases are linked in some way. The coexistence of Hfs and multimeric [FeFe]-hydrogenases is also found in other bacteria such as in *T. saccharolyticum*, *T. tengcongensis*, *T. maritima* and *Ruminococcus albus* (Chongdar et al., 2018; Pan, Menon, & Adams, 2003; Shaw et al., 2009; Soboh et al., 2004; Y. Zheng et al., 2014). Moreover, genes for a multimeric [FeFe]-hydrogenase or/and for a ferredoxin-dependent [FeFe]-hydrogenase and *hfs* genes can be part of the same gene cluster such as in *T. maritima* or in *R. albus* (Y. Zheng et al., 2014) (Figure 7). The Hfs/HydS hydrogenase could be involved in the regulation of the expression of the [FeFe]-hydrogenase genes. Zheng et al. have proposed that HydS/Hfs could sense the H_2 partial pressure via its hydrogenase domain, and transfer the signal via its PAS domain. The signal transduction cascade could involve the Ser/Thr protein kinase and phosphatase (Y. Zheng et al., 2014). Strain with a deletion of *hfs* genes in *T. saccharolyticum* exhibited a 95% reduction in H_2 production (Eminoglu et al., 2017; Shaw et al., 2009).

7. Regulation of hydrogenase genes in *Desulfovibrio*

Hydrogenase expression underlies specific regulation depending on their physiological role and growth conditions (reviews: (Greening & Cook, 2014; Kovacs et al., 2005). *Desulfovibrio* species contain at least two hydrogenases with different composition, localization and possible functions. A complex regulation must occur to coordinate the expression of these enzymes and the rapid response to environmental changes. Little is known about hydrogenase regulation in *Desulfovibrio* species. In bacteria, the presence of hydrogen can trigger the expression of some hydrogenases through a hydrogen sensing regulatory hydrogenase and two component signal transduction system. As it was already mentioned (section 6.2), some *Desulfovibrio* species harbor

a gene cluster for a putative H_2-sensing [FeFe]-hydrogenase whose mechanism has to be elucidated.

The hydrogenase gene expression is also regulated by the level of O_2 present in the cell environment. The expression of hydrogenase genes was investigated in *D. vulgaris* and was found to be dependent on the presence of O_2 or oxidative stress. The production of the periplasmic [FeFe]-hydrogenase was found to be up regulated in response to oxidative stress, oxygen exposed cells or increase of redox potential by chromate addition and a new function of the [FeFe]-hydrogenase in the protection against oxidative stress was proposed (Fournier et al., 2004). In 2008, Pereira et al. identified periplasmic [NiFeSe]-hydrogenase genes as up regulated and [NiFe]-hydrogenase genes (DVU2525 and DVU2526) as down regulated after oxygen exposure (P. M. Pereira, He, Xavier, et al., 2008). These results indicate that O_2 is involved in one way or another in the regulation of hydrogenases by a mechanism that has to be determined. Two transcription regulation factors have been shown to sense and respond to redox status of the cell and presence of oxygen, the Rex transcription factor (Ravcheev et al., 2012) and the Fnr anaerobic regulator (Kovacs et al., 2005).

Rex is a global transcription factor that senses and responds to the intra cellular ratio of [NADH]/[NAD$^+$] to regulate many genes involved in central metabolism and anaerobic fermentation. Rex orthologues were identified in *proteobacteria* in the delta subdivision, the *desulfovibrionales* species (Ravcheev et al., 2012). In *D. fructosovorans*, a *rex* gene (locus-tag DesfrDRAFT_2623) encodes a 24.8 kDa protein that shows 47 % identity with the Rex repressor from *T. maritima* whose crystallographic structure has been recently solved (Park, Jang, Joo, & Lee, 2018). In *T. saccharolyticum,* a Rex putative binding site in genes coding for the Ech and Hfs hydrogenases was identified suggesting that Rex may play an important role in *T. saccharolyticum* H_2 metabolism (T. Zheng, Lanahan, Lynd, & Olson, 2018). *rex* gene was also commonly found in the vicinity of [FeFe]-hydrogenase genes and thus may be involved in the regulation of [FeFe]-

hydrogenases in bacteria as a function of cellular redox status (Poudel et al., 2016). A comparative genomics approach in 11 taxonomic group of bacteria, identified hydrogenase genes as members of the Rex regulon with Rex-binding DNA motifs present in three groups of bacteria (*Thermotogales, Clostridiaceae and Thermoanaerobacterales*). However, hydrogenase genes of the 10 *Desulfovibrio* genomes analyzed do not belong to the Rex regulon (Ravcheev et al., 2012). Rex homologue in *D. vulgaris* was hypothesized to be involved in sulfate reduction (Christensen et al., 2015).

The anaerobic regulator Fnr (fumarate and nitrate reductase regulatory protein) is a member of the Crp/Fnr family of transcriptional regulators containing an N-terminal domain involved in triggering and a C-terminal DNA binding domain with a Helix-turn-Helix motif. The sensing region is adapted to respond to different effectors. Fnr and Fnr-like proteins commonly activate anaerobic expression of genes including the structural and/or maturation genes of hydrogenases in bacteria such as in *Thiocapsa roseopersicina, Rhizobium leguminosarum* or *E. coli* (Kovacs et al., 2005).

Several Crp/Fnr homologues have been identified in *Desulfovibrio* species, such as HcpR which has been proposed to have a protective role against nitrosative stress in *D. gigas* (da Silva et al., 2015; Rodionov, Dubchak, Arkin, Alm, & Gelfand, 2004). In 2012, Zhou et al. investigated the role of four different transcriptional regulators of the Crp/Fnr family in *D. vulgaris*. By analyzing knockout mutants, the authors suggested that the 4 global regulators play distinct roles in stress responses (Zhou et al., 2012). In *D. vulgaris*, a gene encoding CooA, a CO-sensing transcriptional activator of the Crp/Fnr family is present in the genome. Induction of the carbon monoxide dehydrogenase (CODH) gene is dependent on CO and on CooA, but the genes encoding the Coo hydrogenase are not under the control of this transcriptional regulator (Rajeev et al., 2012). In *D. fructosovorans*, neither genes for a Coo hydrogenase, nor for a complete CODH are present in the
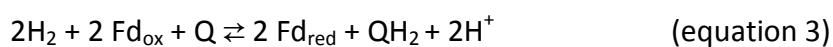
genome, whereas a gene whose function is not known encodes a protein showing high similarity with CooA from *D. vulgaris* (locus tag DesfrDRAFT_3232).

In addition to regulation at the gene level, the fact that genes coding for Histidine kinase-like proteins, Serine/Threonine kinases and Serine phophatases are found in the vicinity of genes for electron-bifurcating hydrogenases strongly suggests that the activity of these hydrogenases can be regulated by post-translational modifications. The tetrameric [FeFe]-hydrogenase from *Caldicellulosiruptor bescii* was shown, using mass spectrometry, to exist in two different forms, phosphorylated or dephosphorylated (Poudel et al., 2016).

8. Concluding remarks

Tables 1 and S1 summarize the hydrogenase content of 35 *Desulfovibrio* species. The number of hydrogenases varies from two to six per species. The periplasmic dimeric [NiFe]-hydrogenase is always present and membrane-bound [NiFe] Ech hydrogenase is present in almost 90 % of the *Desulfovibrio* genomes that we have analyzed. Only two strains do not contain any membrane-bound hydrogenases (Ech or Coo), *D. alaskensis* G20 and *D. aminophilus*. Soluble cytoplasmic hydrogenases are all of [FeFe]-type and are present only in less than one third of the analyzed *Desulfovibrio* genomes. Two *Desulfovibrio* species encode for a further [FeFe]-hydrogenase that is monomeric (*D. alaskensis* G20 and *D. piger*) (Table 1). This hydrogenase is predicted to contain two [4Fe4S]-clusters in addition to the H-cluster. None of these hydrogenases have been isolated so far. The hydrogenase combination found in our model bacterium *D. fructosovorans* is also found in *D. alcoholivorans*, *D. carbinolicus, D. magneticus* RS-1 and *D. sp.* DV. It is also important to note the co-occurrence of tetrameric [FeFe]-hydrogenases with putative H$_2$-sensing hydrogenases.

We recently characterized the first electron-bifurcating hydrogenase from a *Desulfovibrio* species (Kpebe et al., 2018). Flavin-based electron-bifurcation could be more important than previously thought in *Desulfovibrio* energy conserving mechanisms. Enzymes which could possibly catalyze flavin-based electron-bifurcation are largely represented in *Desulfovibrio* including hydrogenases, Nfn, Rnf,Hdr-flx, Qmo, just to name a few. Electron-bifurcation couples an endergonic to an exergonic redox reaction, allowing a non-thermodynamically favorable reaction to occur such as, for example, the reduction of a ferredoxin from H_2 or the oxidation of a quinol to produce H_2. Tri or tetrameric [FeFe]-hydrogenases, that are potentially electron-bifurcating enzymes, are found in only 9 over the 37 analyzed *Desulfovibrio* genomes. It is striking that, *Desulfovibrio* species that contain the Coo hydrogenase do not contain a multimeric [FeFe]-hydrogenase and species that contain multimeric [FeFe]-hydrogenase(s) do not contain a Coo hydrogenase (Table 1). It was proposed that the Coo hydrogenase, which is a flavoenzyme, could potentially confurcate electrons from a reduced ferredoxin and a quinol to produce H_2 (Keller & Wall, 2011). Genes encoding a complex I homolog (NADH:quinone oxidoreductase) have been found in the genome of some *Desulfovibrio* species (I. A. Pereira et al., 2011) and we found that a complex I homologue is always present in species encoding a tetrameric [FeFe]-hydrogenase. However, complex I homologue could also be present in organisms that do not have multimeric hydrogenase. In *D. fructosovorans*, a complex I homologue is encoded by a twelve-gene cluster (locus tags DesfrDRAFT_3136 to 3147). In mitochondria and bacteria, this complex catalyzes oxidation of NADH and reduction of quinone or the reverse reaction. Coo hydrogenase could catalyze, based on the hypothesis that it could be capable of electron-bifurcation, the reversible reaction:

$$2H_2 + 2\ Fd_{ox} + Q \rightleftarrows 2\ Fd_{red} + QH_2 + 2H^+ \qquad \text{(equation 3)}$$

where Q is a quinone, QH_2 a quinol, Fd_{ox} an oxidized ferredoxin and Fd_{red} a reduced ferredoxin. This reaction could also be catalyzed by a system combining a multimeric electron-bifurcating hydrogenase and a complex I homologue with the NAD^+/NADH couple as an intermediate (Figure 8). Mutually exclusive occurrence of *coo* genes and those for the electron-bifurcating hydrogenase/complex I system suggests that *Desulfovibrio* species might have developed two different strategies to take advantage of electron-bifurcation (Figure 8). This putative model has to be experimentally validated.

Although electron-bifurcation is possibly an important mechanism for hydrogen metabolism in *Desulfovibrio*, it is not essential, because more than one third of the analyzed species do not possess neither a multimeric [FeFe]-hydrogenase, nor a Coo hydrogenase (Table 1). Interestingly, the hydrogen-cycling mechanism has a non-essential character as well in *Desulfovibrio* since *D. alaskensis* G20 and *D. aminophilus* do not contain a cytoplasmic or cytoplasmically-oriented membrane-bound hydrogenase needed for this mechanism.

Legends:

Figure 1 : The three types of *Desulfovibrio* hydrogenases: [FeFe], [NiFe] and [NiFeSe]. Top panel: representation of the three active sites. Bottom panel: structure of the periplasmic [FeFe]-hydrogenase from *D. desulfuricans* (pdb 1HFE), the periplasmic [NiFe]-hydrogenase from *D.*

fructosovorans (pdb 1FRF), the periplasmic [NiFeSe]-hydrogenase from *D. vulgaris* Hildenborough (pdb 5JSH). Small and large subunits are represented as well as the active site and iron-sulfur clusters.

Figure 2: Schematic representation of the two electron-transfer pathways during growth of *Desulfovibrio* on lactate/sulfate medium: the hydrogen cycling pathway and a second H_2-independent pathway represented with dashed lines. Abbreviations: APS, adenosine 5'-phosphosulfate; Cyt, cytochrome; Fd, ferredoxin; Hase, hydrogenase; MK, menaquinone; PFO, pyruvate ferredoxin oxidoreductase. Adapted from (Keller & Wall, 2011; Sim et al., 2013).

Figure 3: Hydrogenase system of *D. fructosovorans* composed of six different enzymes. A: schematic representation of *D. fructosovorans* cell with the different hydrogenases and their localization. B: gene clusters encoding the six hydrogenases. Gene locus tags (DesfrDRAFT_) are indicated. The catalytic subunit containing either a [NiFe]-active site or a H-cluster is indicated for each enzyme.

Figure 4: Illustration of the molecular characterization of the HynAB [NiFe]-hydrogenase from *D. fructosovorans*. Left panel: modification of gas diffusion. A: enzyme structure with gas channel depicted in grey (pdb 1YQW). B and E: Insets showing the position of V74 and L122 residues located at the bottleneck of the gas channel in the vicinity of the active site, for the wild-type enzyme and a double methionine variant. C: CO concentration in the buffer as a function of time during the measurement of H_2-oxidation by the enzyme using direct electrochemistry after a transient exposure to CO. D and F: activity of the enzyme followed by direct electrochemistry for H_2 oxidation upon transient exposure to CO, for the wild-type enzyme and the double methionine variant (From (Baffert, dementin, Fourmond, & Léger, 2015)). Right panel: electron transfer chain

with the rate constants that determined the global kinetic of electron transfer. The P238 and H184 residues were mutated to modified the electron transfer kinetics. Abbreviations: AS, active site; medial, medial cluster of the electron-transfer chain. (From (Dementin et al., 2011)).

Figure 5: Schematic representation of Ech hydrogenase subunits and prosthetic groups. A: coupling of the reduction of protons to H_2 by the reduced ferredoxin to the generation of an electrochemical proton potential by Ech. B: reduction of the ferredoxin with H_2 driven by the electrochemical proton potential by Ech. Fd_{ox}: oxidized ferredoxin; Fd_{red}: reduced ferredoxin. (Adapted from (Welte & Deppenmeier, 2014)).

Figure 6: Schematic representation of the subunit compositions and prosthetic groups, predicted from amino-acid sequences, of (A) the cytoplasmic tetrameric Hnd electron-bifurcating hydrogenase and (B) the cytoplasmic trimeric Hnt hydrogenase from *D. fructosovorans*.

Figure 7: Gene clusters encoding the cytoplasmic trimeric or tetrameric electron-bifurcating [FeFe]-hydrogenases from *Desulfovibrio fructosovorans*, *Acetobacterium woodii*, *Termotoga maritima*, *Moorella thermoacetica* and *Ruminococcus albus* and the putative H_2-sensing [FeFe]-hydrogenase from *D. fructosovorans* and *T. maritima*. Subunit composition and predicted cofactor content of these enzymes are shown.

Figure 8: Simplified hydrogen metabolism model in *Desulfovibrio*, showing the hydrogen cycling and possible electron-transfer pathways involving flavin-based electron-bifurcation. The two boxes indicate the two possible species-specific systems using putatively electron-bifurcation: the Coo hydrogenase system and the cytoplasmic multimeric [FeFe]-hydrogenase/complex I system.

Abbreviations: Bif Hase; electron-bifurcating hydrogenase; Cyt, cytochrome; Fd, ferredoxin; Hase, hydrogenase; MK, menaquinone.

Table:

Table 1: Hydrogenase distribution in the *Desulfovibrio* genus. 37 genomes available on the National Center for Biotechnology Information (NCBI) site have been analyzed. Genes for formate hydrogen lyases have not been included in this study.

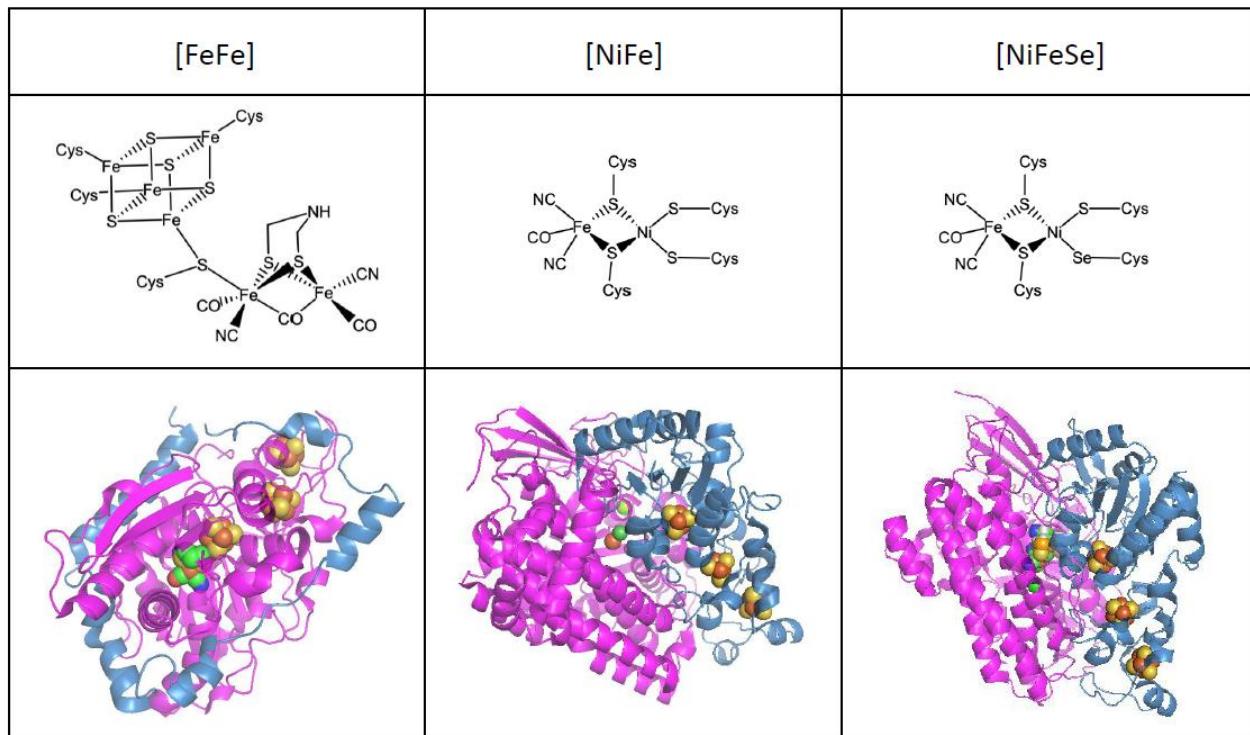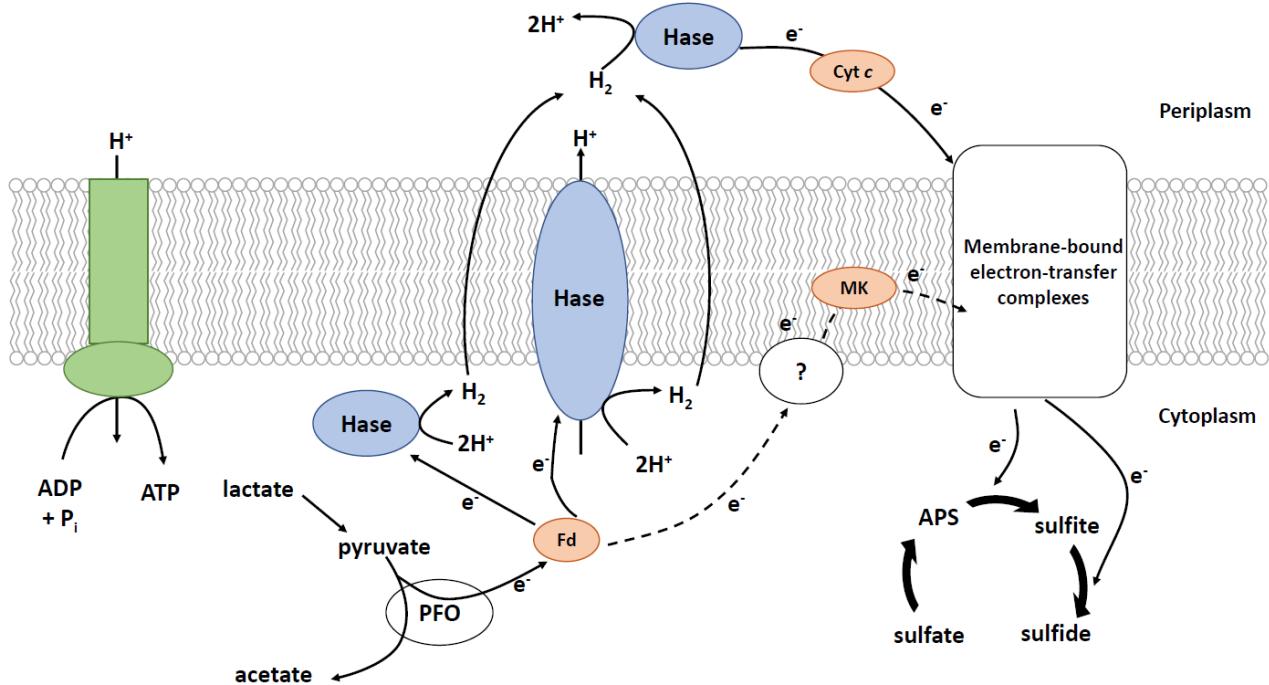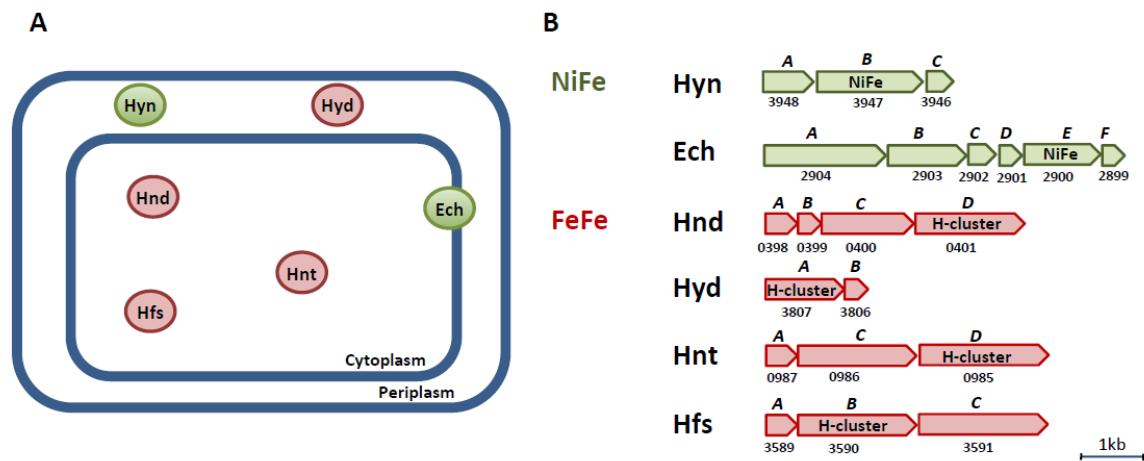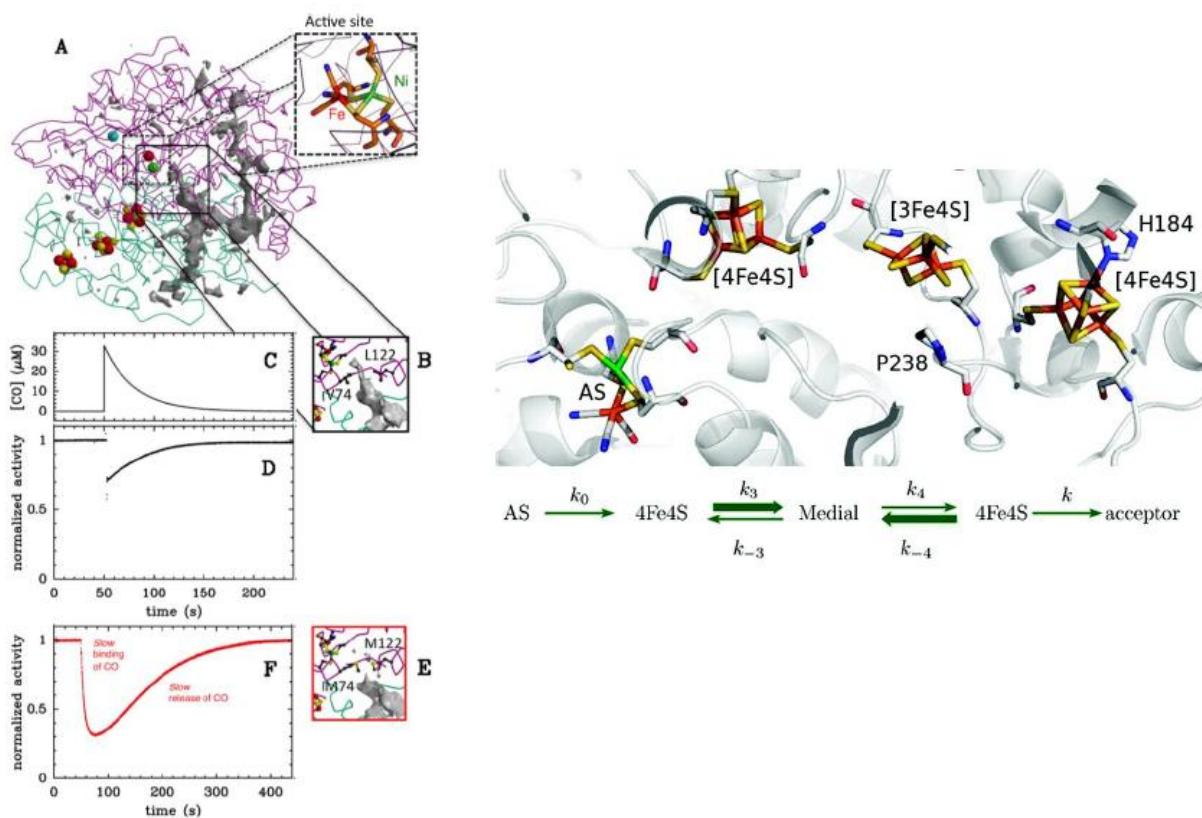| Species	Strain	periplasmic		Membrane		cytoplasmic				Total
		[NiFe(Se)]	[FeFe]	[NiFe] Ech	[NiFe] Coo	[FeFe] trimeric (bifurcating?)	[FeFe] tetrameric (bifurcating?)	[FeFe] sensor	[FeFe] monomeric	
D. africanus	Walvis bay	2		1						3
D. alaskensis	G20	2	2						1	5
D. alcoholivorans	DSM 5433	1	1	1		1	1	1		6
D. alkalitolerans	DSM 16529	1		1						2
D. aminophilus	DSM 12254	1	1							2
D. bastinii	DSM 16055	1	1	1	1					4
D. bizertensis	DSM18034	1	1	1	2					5
D. carbinolicus	DSM 3852	1	1	1		1	1	1		6
D. cuneatus	DSM 11391	1		1	1					3
D. desulfuricans	ATCC 27774	2	1	1	1					5
D. fairfieldensis	CCUG 45958	2	1	1	1					5
D. ferrireducens	DSM 16995	2	1	1						4
D. frigidus	DSM 17176	2		1						3
D. fructosovorans	JJ	1	1	1		1	1	1		6
D. gigas	DSM 1382	1		1						2
D. gracilis	DSM16080	2	1	1						4
D. hydrothermalis	DSM 14728	1	1	1	1					4
D. inopinatus	DSM 10711	2	1	1						4
D. legallii	KHC7	1		1	1					3
D. litoralis	DSM 11393	1			1					2
D. longus	DSM 6739	1	1	1						3
D. magneticus	RS-1	1	1	1		1	1	1		6
D. magneticus	Maddingley MBC34	1	1	1			1	1		5
D. mexicanus	DSM 13116	1	1	1	1					4
D. oxyclinae	DSM 11498	1		1						2
D. piger	FI 11049	3	1		1				1	6
D. putealis	DSM 16056	1	1	1	1					4
D. salexigens	DSM 2638	2	1	1						4
D. termitidis	HI1	2	1	1	1					5
D. vulgaris	Miyasaki	3		1	1					5
D. vulgaris	Hildenborough	3	1	1	1					6
D. zosterae	DSM 11974	2	1	1						4
D. sp.	A2	2		1	1					4
D. sp.	DV	1	1	1		1	1	1		6
D. sp.	FW1012B	1	1	1			1	1		5
D. sp.	TomC	1		1		1	1	1		5
D. sp.	U5L	1		1			1	1		4

Figure 1

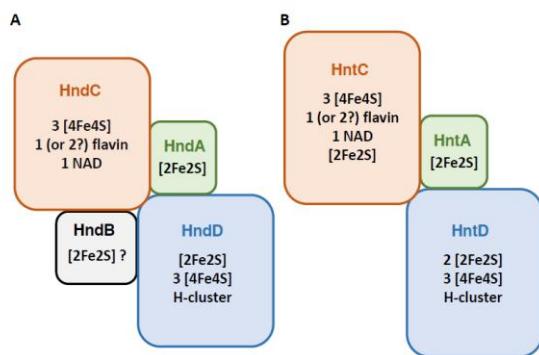| [FeFe]	[NiFe]	[NiFeSe]

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

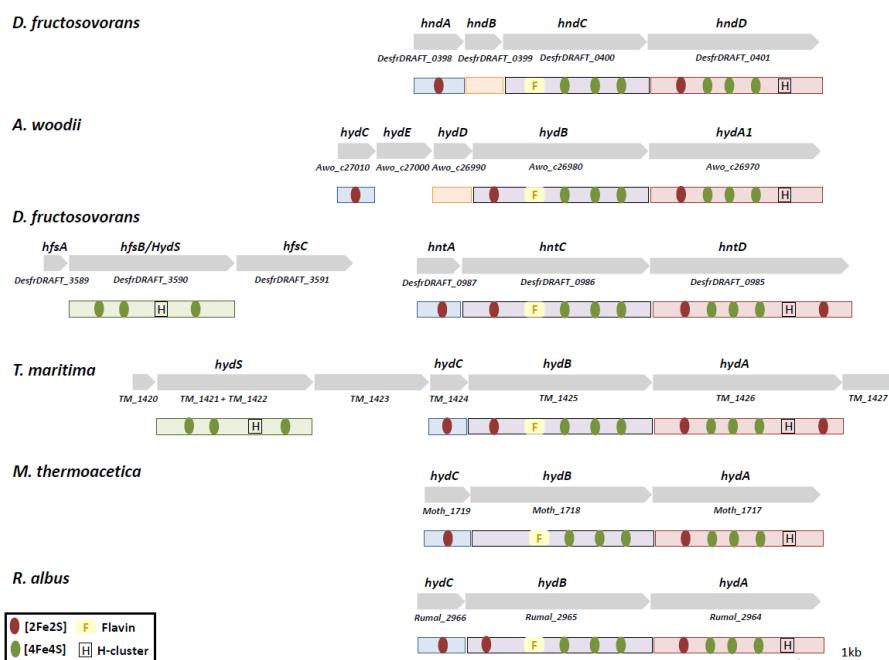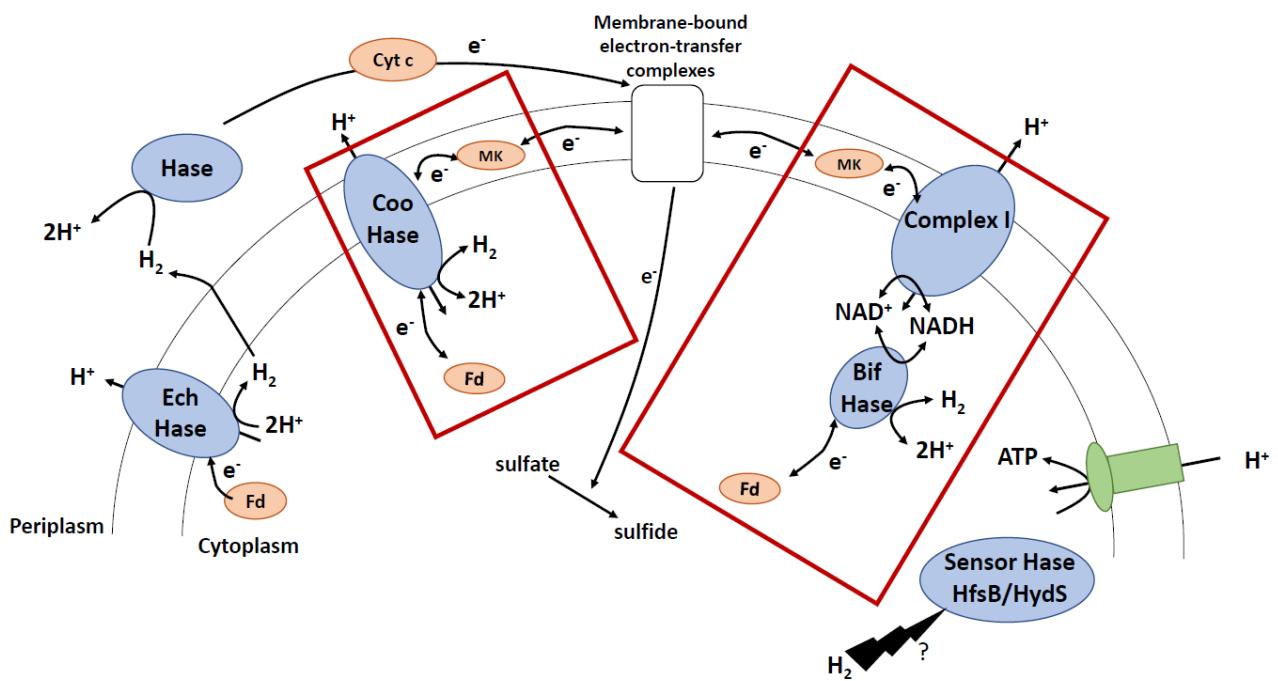Figure 8

Table S1 : Hydrogenase distribution in the *Desulfovibrio* genus. 37 genomes available on the National Center for Biotechnology Information (NCBI) site have been analyzed. The locus tag is indicated for each enzyme. Genes for formate hydrogen lyases have not been included in this study.

Species	Strain	Locus	periplasmic			Membrane		cytoplasmic			
			[NiFeSe]	[NiFe] Hyn	[FeFe] Hyd	[NiFe] Ech	[NiFe] Coo	[FeFe] trimeric	[FeFe] tetrameric	[FeFe] sensor	[FeFe] monomeric
D. africanus	Walvis bay	Desaf_	3130-1	3127-8		0581-2					
D. alaskensis	G20	Dde_	2134-5	2137-8	0081-2 and 2280-1						0725
D. alcoholivorans	DSM 5433	WP_		029459118-9	029459104-5	029460720-1		029460330	029460554	029460054	
D. alkalitolerans	DSM 16529	dsat_		1434-5		0916-7					
D. aminophilus	DSM 12254	WP_		027175155-6	027176560-1						
D. bastinii	DSM 16055	WP_		027179541-2	027179344-5	027178908 et 084407524	027179541-2				
D. bizertensis	DSM18034	SAMN02745702_		01379-80	02593-4	02102-3	00893 and 00953				
D. carbinolicus	DSM 3852	C3Y92_		13765-70	14580-85	19015-20		16550	00840	18630	
D. cuneatus	DSM 11391	WP_		027188523-4		027187945-6	027187553				
D. desulfuricans	ATCC 27774	Ddes_		1038-9 and 0835-7	1502-3	1669-70	1885				
D. fairfieldensis	CCUG 45958	AXF13_		05345-05350 and 03670-75-80	01430-35	09040-09045	11615				
D. ferrireducens	DSM 16995	SAMN05660337_	3449-50	2478-9	1948-9	3107-8					
D. frigidus	DSM 17176	WP_	031485205 and 084154232	031484057-8		031483086-8					
D. fructosovorans	JJ	DesfrDRAFT_		3947-8	3806-7	2900-1		0985	0401	3590	
D. gigas	DSM 1382	DGI_		2261-2		0037-8					
D. gracilis	DSM16080	SAMN02745704_		00190-1 and 00300-2	01506-7	00626-7					
D. hydrothermalis	DSM 14728	DESAM_		22353-4	22768-9	21228 and WP_081588419	22112-3				
D. inopinatus	DSM 10711	WP_	027182710-1	027185048 and 084448613	027183226-7	027182881 and 051261124					
D. legallii	KHC7	SAMN05192586_		102110-1		11129-30	11310				
D. litoralis	DSM 11393	SAMN02745728_		01296-7			00442				
D. longus	DSM 6739	WP_		022660650-1	022663178-9	022661458-9					
D. magneticus	RS-1	DMR_		15600-15610	12950-12960	02760-02770		07830	02480	03530	
D. magneticus	Maddingley MBC34	B193_		1452-3	0935-6	1129-30			3275	3730	
D. mexicanus	DSM 13116	SAMN04488503_		0994-5	1886-7	2353-4	0940				
D. oxyclinae	DSM 11498	WP_		018126001-2		018125730-1					
D. piger	FI 11049	DESPIGER_	00296-7	2408-9 and 02533-4	1380-1		2243				03092
D. putealis	DSM 16056	WP_		027191084-5	027191877-8	027190463-4	027191193				
D. salexigens	DSM 2638	Desal_	2049-50	1915-6	1332-3	3236-7					
D. termitidis	HI1	WP_	35064337 and 084559314	035064324-28	035064009-12	035066792-3	035070826				
D. vulgaris	Miyasaki	DvMF_	0273-4	0270-1 and 1731-3		1521-2	3189				
D. vulgaris	Hildenborough	DVU	1917-8	1921-2 and 2525-6	1769-70	0430-1	2291				
D. zosterae	DSM 11974	WP_	027722758 and 084147016	027722919-20	027722057-8	27723401-2				
D. sp.	A2	DA2_	0293-4	0289-90		1771-2	3754				
D. sp.	DV	DVDV_		3919-20	3242-3	1029-30		3984	3694	3300	
D. sp.	FW1012B	DFW101_		1509-?	1134-5	2235-6			2600	2206	
D. sp.	TomC	NY78_		1064-5		2642-3		3464	1499	2612	
D. sp.	U5L	DesU5LDRAFT_		3197-8		0128-9			0609	4122	

Tables S2: Amino acid sequence identity of subunits of characterized multimeric electron-bifurcating hydrogenases and the putative electron-bifurcating trimeric hydrogenase from *D. fructosovorans* Hnt, using ClustalW software. dfHnd locus tag: DesfrDRAFT_0398-0401; DfHnt locus tag: DesfrDRAFT_0985-0987; tmHyd locus tag: TM1424-1426; awHyd locus tag: Awo_c26970-27010; mtHyd locus tag: Moth_1717-1719; caHyt locus tag: CAETHG_2794-98; raHyd locus tag: Rumal_2964-66. df: *D. fructosovorans*; tm: *T. maritima*; aw: *A. woodii*; mt: *M. thermoacetica*; ca: *C. autoethanogenum*; ra: *R. albus*.

Table S2A :

	dfHndA	dfHntA	tmHydC	awHydC	mtHydC	caHytC	raHydC
dfHndA	100						
dfHntA	29	100					
tmHydC	32	44	100				
awHndB	33	36	38	100			
mtHydC	42	38	36.	39	100		
caHytC	30	33	41	35	35	100	
raHydC	45	40	38	45	44	37	100

Table S2B :

	dfHndB	awHydD
dfHndB	100	
awHydD	28	100

Table S2C :

	dfHndC	dfHntC	tmHydB	awHydB	mtHydB	caHytB	raHydB
dfHndC	100						
dfHntC	41	100					
tmHydB	43	51	100				
awHydB	53	48	51	100			
mtHydB	52	46	48	60	100		
caHytB	41	47	45	54	46	100	
raHydB	49	47	48	64	58	50	100

Table S2D :

	dfHndD	dfHntD	tmHydA	awHydA	mtHydA	caHytA	raHydA
dfHndD	100						
dfHntD	36	100					
tmHydA	35	40	100				
awHydA	50	36	35	100			
mtHydA	55	39	35	55	100		
caHytA	37	30	28	38	38	100	
raHydA	46	36	33	54	54	33	100

Table S3: Amino acid sequence identity of subunits of Ech hydrogenase from *D. fructosovorans* (df) with Ech hydrogenases from *T. tengcongensis* (tt) (now *Caldanaerobacter subterraneus* subsp. *tengcongensis*), *M. barkeri* (mb) and *D. gigas* (dg), using ClustalW software. dfEch locus tag: DesfrDRAFT_2899-2904; ttEch locus tag: TTE0123-0128; mbEch locus tag: Mbar_A0147-0152; dgEch locus tag: DGI_0034-9.

	dfEchA	dfEchB	dfEchC	dfEchD	dfEchE	dfEchF
ttEch	45	51	65	33	56	47
mbEch	38	44	55	29	48	36
dgEch	50	67	70	44	66	52

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