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Occurrence, Classification, and Biological Function of Hydrogenases: An Overview

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1. Introduction

If the atmosphere of the early Earth was hydrogen-rich,[1] it is reasonable to think that hydrogenases, the enzymes enabling cells to use molecular hydrogen, were probably "invented" during the earliest life on our planet. Not only are a wide variety of today's microorganisms able to use molecular hydrogen as an energy source by uptake hydrogenases, but prokaryotes are also endowed with the ability to produce H_2 and can potentially set up ecosystems powered by H_2 that can be independent from organic carbon and molecular oxygen, that is, from the products of photosynthesis. Indeed, it has been observed in an active deep-sea hydrothermal field in the Central Indian Ridge that geologically and abiotically derived hydrogen and carbon dioxide can support hydrogen-driven subsurface microbial communities forming an ecosystem called "HyperSLIME" (for *hyper*thermophilic *s*ubsurface *li*thoautotrophic *m*icrobial *e*cosystem).[2] The issue of whether hydrogen-driven communities (SLIME) can exist and persist independently of the products of photosynthesis is of great interest, not only with regard to the nature of primitive life on Earth but also in the search for life on other planetary bodies.[3] The atmosphere of Mars is rich in photochemically produced H_2 and CO,[4] both gases that are used by a large number of various organisms on Earth. Recent work with CO-oxidizing bacteria has shown that several Bacteria and Archaea can grow autotrophically at the expense of CO with release of H_2 as end-product.[5−7]

To study hydrogenases three main approaches have been used. The biochemical approach was the first one; it led to the isolation of enzyme proteins and the determination of their catalytic properties. The genetic approach was the second one; it resulted in the identification of a large number of hydrogenase structural genes and of the accessory genes involved in the synthesis of [NiFe]-hydrogenases, by the end of the 1980s. The structural studies of hydrogenase crystals have then permitted the identification of structural domains, sometimes found in separate subunits; part of the gene sequences encoding such domains were subsequently used to identify putative hydrogenase genes in the newly sequenced genomes. However, because similar domains are present in a variety of proteins with different catalytic activities,[8] this procedure may lead to erroneous conclusions.

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Paulette M. Vignais (born 1928) received her diploma of chemical engineering from the Ecole Nationale Supérieure de Chimie de Paris (ENSCP) and her undergraduate degree in chemistry from the Sorbonne in 1952. She undertook graduate studies at the Pasteur Institute, Paris. With her husband, Pierre V. Vignais, she began in 1954 to study oxidative phosphorylation in mitochondria in the laboratory of Sir Hans Krebs in Oxford, U.K. In 1957, she obtained her Ph.D. in biochemistry from the University of Paris. She has been a postdoctoral fellow in the laboratories of I. Zabin (UCLA, Los Angeles,CA) (1957–1958), K. Linderstrøm-Lang (Carlsberg Laboratory, Cophenhagen) (1958), and A. L. Lehninger (Johns Hopkins University, Baltimore, MD) (1962–1963). She settled in Grenoble in 1963 where Pierre V. Vignais created the laboratory of Biochemistry in the Research Center of the CEA (Commissariat à l'Energie Atomique). After the energy crisis of 1973, she decided to study the production of H_2 by microorganisms. After a year spent in the School of Botany in Oxford (F. R. Whatley) (1975–1976) and a stay at the University of Missouri—Columbia (J. D. Wall) (1981), she obtained the position of Director of Research at the CNRS and created the Laboratory of Microbial Biochemistry, where the role of nitrogenase and hydrogenase in the photosynthetic bacterium *Rhodobacter capsulatus* has been extensively studied at the physiological, biochemical, and genetic levels. She is now emeritus Director of Research at CNRS.

Bernard Billoud was born in 1964. His Ph.D. work was an experimental study in developmental molecular biology. His interest then turned toward in silico biology, and he has worked since 1995 in the "Atelier de BioInformatique" at University "Pierre et Marie Curie" (Paris). He has been involved in software development for identifying RNA secondary structure patterns and has proposed a way to use them in phylogenetic analysis. He presently works on micro-RNAs and their role in biotic stress in plants. He is still interested in evolution, which is a key issue in the study of functional relationships in very ancient and widespread protein families, such as hydrogenases. He also teaches computer science (algorithmics, programming), biocomputing (computer methods in sequence analysis, phylogeny, origins of life), and genomics.

Phylogenetic analyses, based on sequence alignments of catalytic subunits of hydrogenases,[9,10a] have led to the identification of three phylogenetically distinct classes of proteins, the [NiFe]-hydrogenases, the [FeFe]-hydrogenases, and the iron−sulfur-free hydrogenases, initially called metal-free and now renamed [Fe]-hydrogenases.[10b] Most hydrogenases are found in microorganisms belonging to the Archaea and the Bacteria domains of life, but a few are present in Eukarya as well (reviewed in refs 10−13). The genes necessary for the biosynthesis, maturation, and processing of [NiFe]-hydrogenases have been identified and their products characterized biochemically and functionally (reviewed in ref 14−18). On the other hand, proteins necessary for the biosynthesis of [FeFe]-hydrogenases have only recently been identified and studied.[19−21] Microbial genome sequences have provided a significant body of additional hydrogenase sequence data and contribute to the understanding of hydrogenase distribution and evolution.

Typically, hydrogenases are modular enzymes; after frequent gene exchange and reshuffling during the course of evolution, hydrogenase proteins appear to have been created like a brick-assembling game. In particular, two types of enzyme complexes, the respiratory NAD(P)H ubiquinone oxidoreductase (or complex I) and some multimeric hydrogenases, share several homologous subunits. To correctly assign those subunits to either one of the two complexes identified by their structural genes in sequenced genomes, that is, to distinguish orthologues (genes evolved by vertical descent via speciation) from paralogues (genes related via duplication),[22,23] we have taken into account not only the gene content (that evolves more slowly than gene order) but also the gene co-occurrence in the structural operons.[24−27] Most of the hydrogenase genes have evolved by normal vertical transmission, although some horizontal gene transfers from archaea to bacteria or between bacteria[28−30] and from bacteria to anaerobic protists[13] have been pointed out.

The occurrence of domain families and of changes in domain partnerships in the course of evolution is one of the difficulties found for annotating the sequenced genomes. Another difficulty is the presence, in the same organism, of several hydrogenase genes with (quasi-) identical sequences. To date, these similar genes are assumed to carry out similar functions, but further analyses may disclose differences in their activity and/or regulation. Finally, it must be taken into account that the first hydrogenase genes identified were named according to the context of that time. These names may be useful to follow the history of hydrogenase research and, in some cases, it is reasonable to keep the nomenclature currently used and understood by the specialists of the field. The situation is different for newly sequenced genomes of species from which no hydrogenase protein has as yet been isolated. This is why, it is hoped that the effort of classification made in this review will be useful to those who are annotating newly sequenced genomes.

The aim of this review is not to recall the historical steps that have led to the discovery of hydrogenases in a broad variety of prokaryotes and give a detailed account of the work of a vast number of contributors. Studies dealing with the biodiversity of H_2 metabolism, the species in which H_2 metabolism has been investigated, the occurrence, function, and evolution of different hydrogenases and the genes that encode them have been reported in several recent reviews,[10−18,31−35] a book,[36] and journal special issues.[37,38] The purpose is rather to provide a reliable source of information regarding the wide distribution of hydrogenases in various taxa and facilitate the retrieving of hydrogenase gene sequences from databases. Some features common to groups

of hydrogenases are highlighted to provide some insights into the evolutionary events that led to the biodiversity of these enzymes.

2. Occurrence and Diversity of Hydrogenases in Nature

2.1. Evolutionary Relationship of Living Organisms

2.1.1. Universal Tree of Life

The determination of molecular sequences and the concept that sequences could be used to relate organisms[39,40] have revolutionized our views on microbial diversity. To construct a phylogenetic tree, all of the sequences of interest from different organisms, which are encoded by homologous genes, are aligned. The pairwise differences scored on such a multiple alignment can be considered to be a kind of a measure of the evolutionary distance between the gene products. Only changes in nucleotide (protein) sequences are taken into account, not the time required to bring about such changes as the evolutionary clock is not constant in different lineages.[41] A phylogenetic tree constructed from a set of genes is expected to represent the evolutive history of these genes, but not necessarily the descent of the organisms that contain these genes. Indeed, phenomena such as convergence or horizontal transfer (gene exchange between species living at the same time) can lead to considerable differences between the reconstructed histories of genes versus taxa. It is usually believed that such events do not occur within rRNA genes, which are thus considered as good markers for the long-term organism evolution. By comparing ribosomal RNA (rRNA) sequences, Carl Woese established a molecular sequence-based phylogenetic tree that could be used to relate all organisms and reconstruct the history of life.[41,42] With this approach, Woese et al.[43] established in 1990 that there are two distinct lines of prokaryotic descent, the bacterial one and a newly identified one comprising the archaebacteria. The three primary lines of evolutionary descent are now termed "domains"; they consist of the Eukarya (eukaryotes), those organisms that contain a nucleus; the Bacteria (formerly called eubacteria); and the Archaea (initially called archaebacteria), which are both prokaryotic, that is, they contain organisms with no nuclear membrane. It is not yet clear how these domains originated and what the evolutionary relationships among them are.[44] In Figure 1, a community of primitive organisms freely exchanging their genes[45−47] is shown at the origin of a common ancestor (called the Last Universal Cellular Ancestor, LUCA) from which two lineages diverged, one leading to Bacteria and the other to a common ancestor of Archaea and Eukarya.[43] LUCA was proposed to have an RNA genome.[41,48] A new theory, called the three viruses, three domains theory,[49,50] posits that viruses played a major role in early life evolution. According to that theory, each cellular domain originated independently from the fusion of an RNA cell and a large DNA virus. Because DNA genomes can be replicated more faithfully than RNA genomes,[51] the viral-induced transformation of an RNA cell into a DNA cell would have been accompanied by a drastic drop in the rate of protein evolution for all proteins that were previously encoded by RNA genes. The DNA cells and their descendants able to accumulate genes in larger genomes would have rapidly outcompeted contemporary lineages of RNA cells.

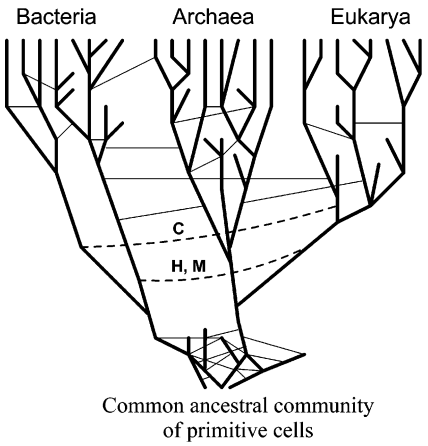

Figure 1. Schematic representation of the universal tree of life as determined from comparative ribosomal RNA sequencing. The tree shows the three primary groupings of organisms forming three phylogenetic domains, two of which (bacteria and archaea) contain only prokaryotic representatives. The web of thin lines between primitive cells and connecting bifurcating branches represents horizontal gene transfer leading to stable inheritance. Dashed thick lines indicate the endosymbiotic events that led to the emergence of hydrogenosomes (H) and mitochondria (M) in eukaryotic cells and of chloroplasts (C) in plant cells. Short branches represent lines of descent that became extinct.

2.1.2. Terminology

Formerly, a mode of bacterial grouping was based on growth requirements. In 1946, at a Cold Spring Harbor Symposium, a committee reexamined the terminology used to specify the growth type (quoted by Brock and Schlegel[52]). The committee[53] stipulated that it is essential to distinguish between two aspects of cellular nutrition: the source of energy and the source of carbon. The proposed terminology emphasized energy source and electron donor. In relation to energy, two broad groups of organisms were recognized, those using light, called *phototrophs*, and those using chemical energy, called *chemotrophs*. The organisms that use chemical energy provided by inorganic electron donors (H_2, H_2S, ...) are called *lithotrophs* in contrast to *organotrophs* (e.g., fermentative bacteria) which use organic material. Some organisms can use CO_2 as sole carbon source; they are *autotrophs*; those that use organic substrates as carbon source are *heterotrophs*. For the heterotrophs (most bacteria, animals), organic substrates are usually both the source of energy and the source of carbon.

The photosynthetic organisms that use inorganic oxidizable substrates (water, H_2S, H_2) as electron donors and require light energy for growth are *photolithotrophs*. They are usually autotrophic, that is, *photolithoautotrophs*. Examples are green plants, green and purple sulfur bacteria, and cyanobacteria. The *chemolithotrophs* derive energy from the oxidation of inorganic electron donors in the dark, whereas *chemoorganotrophs* derive energy from the oxidation of organic compounds.

The anaerobes derive energy by photosynthetic electron transport phosphorylation (e.g., green sulfur bacteria) or, for example, sulfur reduction to H_2S (anaerobic sulfur reducers; *Thermoproteus*), sulfate reduction to H_2S (sulfate reducers), nitrate reduction (denitrifiers), CO_2 reduction to methane (methanogens), and CO_2 reduction to acetate (acetogens), respectively.

A widespread mechanism of energy conservation is called *respiration*. Initially, respiration was understood as the vital

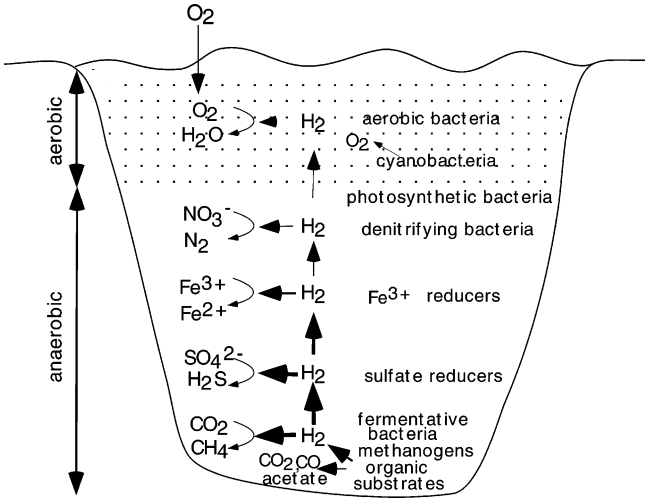

Figure 2. Anaerobic and aerobic bacterial metabolism in an aquatic stratified system as can be found in a lagoon, pond, or flooded soil. The scheme illustrates the vertical distribution of different redox reactions catalyzed by communities of microorganisms capable of producing or consuming H_2. The redox potential, more negative at the bottom of the anaerobic fluid, increases upward and is positive in the aerobic phase near the surface of water in contact with air. The vertical arrows emphasize the decreasing H_2 flux from bottom to top of the stagnant water. Adapted from Conrad[54] and Cammack[108].

process that sustains life in the presence of oxygen, as opposed to fermentation, which sustains life in the absence of O_2. Oxygen respiration is universally found in Eukarya and Prokarya. It is now known that respiration, which involves electron transfer between redox components of a respiratory chain located in a membrane coupled to a vectorial proton transfer across the membrane, can also occur in the absence of O_2, in the presence of alternative electron acceptors such as nitrate, fumarate, Fe^{3+}, or sulfate. Thus, *aerobic respiration* uses O_2 as terminal electron acceptor, whereas *anaerobic respiration* implies other terminal electron acceptors such as sulfate, fumarate, or nitrate, the type of respiration being then referred to more specifically as sulfate respiration, fumarate respiration, or nitrate respiration, respectively. The most recent edition of *The Prokaryotes* (Springer, 2006) provides numerous illustrations of the diversity of energy transduction systems used by existing microorganisms.

2.2. Hydrogen as an Energy Source

Hydrogen gas is often referred to as an energy vector by chemists and technologists. In nature, H_2 is for many bacteria an energy source, the highest yield of chemical energy being provided by the oxidation of H_2 by O_2. H_2 oxidation in anaerobic and aerobic environments implicates hydrogenase enzymes as catalysts. H_2 is considered as a trace gas as very little is released into the atmosphere, H_2 formed geologically and biologically being rapidly consumed in situ by the various microbial communities that it encounters. As illustrated in Figure 2, which shows some of the chemical reactions occurring in stagnant water where molecular hydrogen is being produced, there is a flow of H_2 from the site where fermentative bacteria excrete H_2 as a waste product to a hierarchy of bacteria stratified according to the redox potential at which the bacteria are able to oxidize H_2. In the sediment, clostridia are involved in the fermentation of organic matter releasing H_2 and CO_2; in rice fields anaerobic degradation of polysaccharides can be syntrophically coupled to methanogens and homoacetogens (cf. Conrad[54,55]). H_2 is the central source of reducing power for the formation of methane produced by methanogenic archaea in anoxic soils and sediments. The presence of alternative electron acceptors (sulfate, Fe^{3+}, nitrate) changes the microbial community structure. Sulfate reducers, such as species of *Desulfovibrio*, use H_2 to reduce sulfate to sulfide. The family of Fe^{3+} reducers, such as *Geobacter* species, which predominate in a wide diversity of sedimentary environments, use oxides of Fe^{3+} to oxidize H_2 under anoxic conditions; they can also use nitrate and oxygen as alternative electron acceptors. Microbiological and geochemical evidence suggests that Fe^{3+} reduction may have been the first form of microbial respiration, although the capacity for Fe^{3+} reduction appears to have evolved several times as phylogenetically distinct Fe^{3+} reducers have different mechanisms for Fe^{3+} reduction.[56] H_2 is oxidized with nitrate as oxidant by denitrifying bacteria. Near the surface where the conditions are aerobic due to contact with the air and O_2 production in the light by cyanobacteria, aerobic bacteria use O_2 to oxidize H_2 to water. The energy yielded by the oxidation of H_2 by the various oxidants shown in Figure 2 is recovered in the form of ATP by the chemiosmotic mechanism of oxidative phosphorylation. Figure 2 represents a general scheme of possible chemical reactions. The contribution of the various groups of organisms will depend on the availability of nutrients and electron acceptors; for example, in coastal lagoons, denitrification is often limited by the availability of nitrate, whereas sulfate reduction is enhanced by the large amounts of sulfate originating from seawater (20−25 mM), and methanogenesis is generally quite negligible in coastal environment (reviewed in ref 57).

2.3. Diversity of Species Able To Metabolize H_2

Most of the organisms able to metabolize H_2 are prokaryotes belonging to the Bacteria and Archaea domains of life. They include fermentative organisms, photosynthetic prokaryotes, aerobes, anaerobes, autotrophs, heterotrophs, etc. Some lower eukaryotes able to evolve H_2 contain [FeFe]-hydrogenase(s); they contain hydrogenosomes instead of mitochondria as do parasitic protozoas (e.g., *Trichomonas vaginalis*) and anaerobic fungi (e.g., *Neocallimastix frontalis*) or chloroplasts such as unicellular green algae (e.g., *Chlamydomonas* and *Scenedesmus*). The diversity of organisms in which hydrogen metabolism has been studied or hydrogenase genes have been identified is shown in Tables 1 and 2. The tables also provide the taxonomic classification of the organisms according to the TAXONOMY database.[58] A **species** is a prokaryote having a 16S ribosomal RNA sequence differing by more than 3% from that of all other organisms (i.e., the sequence is less than 97% identical to any other sequence); it is usually defined from the characterization of several strains or clones (although see ref 59). A group of species is collected into a **genus**, groups of genera are collected into **families**, families into **orders**, orders into **classes**, classes into **phyla**, and so on up to the highest level taxon, the **domain** (or kingdom). The simplified classification proposed by Margulis[60] comprising the taxa, Prokarya (bacteria) and Eukarya (symbiosis-derived nucleated organisms), subdivided into subtaxa, Protoctista, Animalia, Fungi, and Plantae, has not yet been adopted. The domain Bacteria groups the vast majority of known prokaryotes including all those of medical relevance and most of those known to be of environmental significance.

Table 1. Taxonomy of Organisms Containing [NiFe]-Hydrogenase Genes and Those in Which a [NiFe]-Hydrogenase Activity Has Been Characterized[i]

TaxID	superkingdom / phylum / class / order / genus / species / subspecies	[Fe]	[NiFe]								[FeFe]
			1	2a	2b	3a	3b	3c	3d	4	A	
2	Bacteria		179	24	26		24	13	38	56		+
976	Bacteroidetes		1						1			+
117743	Flavobacteria		1									
200644	Flavobacteriales		1									
237	Flavobacterium		1									
986	*Flavobacterium johnsoniae*		1									
376686	*Flavobacterium johnsoniae UW101*		1									
200643	Bacteroidetes (class)								1			+
171549	Bacteroidales								1			+
49894	Acetomicrobium								1			
49896	*Acetomicrobium flavidum*								1			
1090	Chlorobi		7				8					
191410	Chlorobia		7				8					
191411	Chlorobiales		7				8					
1091	Chlorobium		4				5					
1092	*Chlorobium limicola*		1				1					
290315	*Chlorobium limicola*		1				1					
1096	*Chlorobium phaeobacteroides*		2				2					
331678	*Chlorobium phaeobacteroides BS1*		1				1					
290317	*Chlorobium phaeobacteroides DSM 266*		1				1					
84205	*Chlorobium ferrooxidans*		1				1					
377431	*Chlorobium ferrooxidans DSM 13031*		1				1					
337090	*Chlorobium chlorochromatii*						1					
340177	*Chlorobium chlorochromatii CaD3*						1					
1099	Pelodictyon		2				1					
1100	*Pelodictyon luteolum*		1									
319225	*Pelodictyon luteolum DSM 273*		1									
34090	*Pelodictyon phaeoclathratiforme*		1				1					
324925	*Pelodictyon phaeoclathratiforme BU-1*		1				1					
1101	Prosthecochloris		1				1					
1102	*Prosthecochloris aestuarii*		1				1					
290512	*Prosthecochloris aestuarii DSM 271*		1				1					
256319	Chlorobaculum						1					
1097	*Chlorobaculum tepidum WH 8501*						1					
1117	Cyanobacteria			13					12			
1118	Chroococcales			3					5			
1129	Synechococcus								3			
32046	*Synechococcus elongatus*[a]								2			
1140	*Synechococcus elongatus PCC 7942*								1			
269084	*Synechococcus elongatus PCC 6301*								1			
32049	*Synechococcus sp. PCC 7002*								1			
1142	Synechocystis								2			
1143	*Synechocystis sp.*								1			
1148	*Synechocystis sp. PCC 6803*								1			
28070	Gloeothece			1								
44473	*Gloeothece membranacea*			1								
197232	*Gloeothece sp. ATCC 27152*			1								
43988	Cyanothece			1								
43989	*Cyanothece sp.*			1								
263510	Crocosphaera			1								
263511	*Crocosphaera watsonii*			1								
165597	*Crocosphaera watsonii*			1								
1150	Oscillatoriales			3					1			
1205	Trichodesmium			1								
1206	*Trichodesmium erythraeum*			1								

Table 1 (Continued)

203124	*Trichodesmium erythraeum IMS101*		1						
28073	Lyngbya		2			1			
118322	*Lyngbya aestuarii*		1						
158786	*Lyngbya majuscula*		1			1			
197229	*Lyngbya majuscula CCAP 1446/4*		1			1			
1161	Nostocales		7			5			
1163	Anabaena		4			3			
1167	*Anabaena sp.*		1						
1172	*Anabaena variabilis*		2			3			
240292	*Anabaena variabilis ATCC 29413*		2			2			
213767	*Anabaena siamensis*		1						
213768	*Anabaena siamensis TISTR8012*		1						
1177	Nostoc		3			2			
103690	*Nostoc sp. PCC 7120*		1			1			
272131	*Nostoc punctiforme*		1						
63737	*Nostoc punctiforme PCC 73102*		1						
350813	*Nostoc sp.*		1			1			
1212	Prochlorales					1			
1222	Prochlorothrix					1			
1223	*Prochlorothrix hollandica*					1			
1224	Proteobacteria	137	6	26	10	10	20	43	+
1236	Gammaproteobacteria	59	1	3	5		5	28	+
72273	Thiotrichales	1							
28884	Hydrogenovibrio								
28885	*Hydrogenovibrio marinus*							x[539]	
933	Thiomicrospira	1							
39765	*Thiomicrospira crunogena*	1							
317025	*Thiomicrospira crunogena XCL-2*	1							
72274	Pseudomonadales	4			1				
286	Pseudomonas	1							
39439	*Pseudomonas hydrogenovora*	1							
352	Azotobacter	3			1				
353	*Azotobacter chroococcum*	1							
355	*Azotobacter chroococcum str. mcd 1*	1							
354	*Azotobacter vinelandii*	2			1				
354	*Azotobacter vinelandii ATCC 13705 / OP1 / DSM 366 / NCIB 11614 / LMG 3878 / UW*	1							
322710	*Azotobacter vinelandii AvOP*	1			1				
91347	Enterobacteriales	33						24	
544	Citrobacter	1							
546	*Citrobacter freundii*	1							
561	Escherichia	10						8	
562	*Escherichia coli*	10						8	
362663	*Escherichia coli 536*	2						1	
562	*Escherichia coli K12*	2						2	
83334	*Escherichia coli O157:H7*	2						3	
217992	*Escherichia coli O6*	2						1	
364106	*Escherichia coli UTI89*	2						1	
590	Salmonella	11						5	
591	*Salmonella choleraesuis*	2						1	
601	*Salmonella typhi*	3						2	
602	*Salmonella typhimurium*	3						1	
54388	*Salmonella paratyphi*	3						1	
28901	*Salmonella enterica*								
90371	*Salmonella enterica serovar Typhimurium*							x[494]	
620	Shigella	10						10	
621	*Shigella boydii*	2						2	
300268	*Shigella boydii Sb227*	2						2	
622	*Shigella dysenteriae*	2						2	
300267	*Shigella dysenteriae Sd197*	2						2	
623	*Shigella flexneri*	4						4	
373384	*Shigella flexneri 5*	2						2	

Table 1 (Continued)

624	*Shigella sonnei*	2				2		
300269	*Shigella sonnei Ss046*	2				2		
122277	Pectobacterium	1				1		
29471	*Pectobacterium atrosepticum*	1				1		
570	Klebsiella							
573	*Klebsiella pneumoniae*					1[217a]	x[217]	
547	Enterobacter							
548	*Enterobacter aerogenes*							
	Enterobacter aerogenes HU-101						x[540]	
550	*Enterobacter cloacae*							
	Enterobacter cloacae DM11						x[541]	
	Enterobacter cloacae IIT-BT-08						x[548]	
118969	Legionellales				3			
445	Legionella				3			
446	*Legionella pneumophila*				3			
272624	*Legionella pneumophila subsp. pneumophila str. Philadelphia 1*				1			
297245	*Legionella pneumophila str. Lens*				1			
297246	*Legionella pneumophila str. Paris*				1			
135613	Chromatiales	4		2		2		
1056	Thiocapsa	2		1		1		
1058	*Thiocapsa roseopersicina*	2		1		1		
85072	Allochromatium	1				1		
1049	*Allochromatium vinosum*[b]	1				1		
133193	Alkalilimnicola	1		1				
351052	*Alkalilimnicola ehrlichei*	1		1				
187272	*Alkalilimnicola ehrlichei MLHE-1*	1		1				
212109	Lamprobacter							
	Lamprobacter modestohalophilus						x[542]	
135618	Methylococcales	1				1		
413	Methylococcus	1				1		
414	*Methylococcus capsulatus*	1				1		
135619	Oceanospirillales	1	1	1	1	1		
965	Oceanospirillum	1	1	1	1			
207954	*Oceanospirillum sp.*	1	1	1	1			
158481	Hahella					1		
158327	*Hahella chejuensis*					1		
349521	*Hahella chejuensis KCTC 2396*					1		
135622	Alteromonadales	11				1	1	+
22	Shewanella	11						+
24	*Shewanella putrefaciens*	1						
319224	*Shewanella putrefaciens CN-32*	1						
56812	*Shewanella frigidimarina*	1						
318167	*Shewanella frigidimarina NCIMB 400*	1						
60478	*Shewanella amazonensis*	1						
326297	*Shewanella amazonensis SB2B*	1						
60480	*Shewanella sp. MR-4*	1						+
60481	*Shewanella sp. MR-7*	1						
62322	*Shewanella baltica*	2						
325240	*Shewanella baltica OS155*	1						
399599	*Shewanella baltica OS195*	1						
70863	*Shewanella oneidensis*	1						+
94122	*Shewanella sp.*	1						+
323850	*Shewanella sp.*	1						
351745	*Shewanella sp.*	1						
67572	Psychromonas					1	1	
314282	*Psychromonas sp.*						1	
357794	*Psychromonas ingrahamii*					1		
357804	*Psychromonas ingrahamii 37*					1		
135623	Vibrionales	2					3	
657	Photobacterium	1					2	
74109	*Photobacterium profundum*						1	

Table 1 (Continued)

314280	*Photobacterium profundum 3TCK*					1		
121723	*Photobacterium sp.*	1				1		
662	Vibrio	1				1		
145288	*Vibrio angustum*	1				1		
314292	*Vibrio angustum S14*	1				1		
135625	Pasteurellales	2						
713	Actinobacillus	1						
715	*Actinobacillus pleuropneumoniae*							
209841	*Actinobacillus pleuropneumoniae serovar 7*					x[543]		
67854	*Actinobacillus succinogenes*	1						
339671	*Actinobacillus succinogenes 130Z*	1						
75984	Mannheimia	1						
157673	*Mannheimia succiniciproducens*	1						
221988	*Mannheimia succiniciproducens MBEL55E*	1						
28211	Alphaproteobacteria	21	3	16		2	5	+
356	Rhizobiales	13	1	9			2	+
6	Azorhizobium	1		1				
7	*Azorhizobium caulinodans*	1		1				
279	Xanthobacter	1		1				
280	*Xanthobacter autotrophicus*	1		1				
78245	*Xanthobacter autotrophicus Py2*	1		1				
374	Bradyrhizobium	5	1	2				
375	*Bradyrhizobium japonicum*	2		1				
192180	*Bradyrhizobium sp. UPM1029*	1						
192183	*Bradyrhizobium sp. UPM1167*	1						
288000	*Bradyrhizobium sp.*	1	1	1				
379	Rhizobium	1						
384	*Rhizobium leguminosarum*	1						
387	*Rhizobium leguminosarum bv. viciae*	1						
1073	Rhodopseudomonas	4		4			2	+
1076	*Rhodopseudomonas palustris*	4		4			2	+
316055	*Rhodopseudomonas palustris BisA53*	1		1				+
316056	*Rhodopseudomonas palustris BisB18*	1		1			2	
316057	*Rhodopseudomonas palustris BisB5*	1		1				
40136	Oligotropha	1		1				
40137	*Oligotropha carboxidovorans*	1		1				
204441	Rhodospirillales	3	1	1		1	3	
522	Acidiphilium	1						
524	*Acidiphilium cryptum*	1						
349163	*Acidiphilium cryptum JF-5*	1						
1081	Rhodospirillum	1					3	
1085	*Rhodospirillum rubrum*	1					3	
269796	*Rhodospirillum rubrum ATCC 11170*	1					2	
13134	Magnetospirillum	1	1	1		1		
84159	*Magnetospirillum magneticum*	1	1	1		1		
342108	*Magnetospirillum magneticum AMB-1*	1	1	1		1		
204455	Rhodobacterales	5		6		1		
265	Paracoccus	1		1				
266	*Paracoccus denitrificans*	1		1				
318586	*Paracoccus denitrificans PD1222*	1		1				
1060	Rhodobacter	3		4		1		
1061	*Rhodobacter capsulatus B10*	1		1		1		
1063	*Rhodobacter sphaeroides*	2		3				
272943	*Rhodobacter sphaeroides 2.4.1*	1		1				
74030	Roseovarius	1		1				
314265	*Roseovarius sp.*	1		1				
204457	Sphingomonadales		1					
165697	Sphingopyxis		1					
117207	*Sphingopyxis alaskensis*		1					
28216	Betaproteobacteria	10	1	6	1	7		
32003	Nitrosomonadales					1		
35798	Nitrosospira					1		

Table 1 (Continued)

1231	*Nitrosospira multiformis*					1			
323848	*Nitrosospira multiformis ATCC 25196*					1			
80840	Burkholderiales	7		5		5			
507	Alcaligenes	1		1					
516	*Alcaligenes hydrogenophilus*	1		1					
28065	Rhodoferax	1		1		1			
192843	*Rhodoferax ferrireducens*	1		1		1			
338969	*Rhodoferax ferrireducens DSM 15236*	1		1		1			
28067	Rubrivivax	1							
28068	*Rubrivivax gelatinosus*[c]	1							
32008	Burkholderia	1		2		2			
36873	*Burkholderia xenovorans*					1			
266265	*Burkholderia xenovorans LB400*					1			
60552	*Burkholderia vietnamiensis*	1		1					
269482	*Burkholderia vietnamiensis G4*	1		1					
95486	*Burkholderia cenocepacia*			1		1			
331272	*Burkholderia cenocepacia HI2424*			1		1			
106589	Cupriavidus	3		1		2			
106590	*Cupriavidus necator*[d]	2		1		1			
119219	*Cupriavidus metallidurans*	1				1			
266264	*Ralstonia metallidurans CH34*	1				1			
119069	Hydrogenophilales	1			1				
919	Thiobacillus	1			1				
36861	*Thiobacillus denitrificans*	1			1				
292415	*Thiobacillus denitrificans ATCC 25259*	1			1				
206389	Rhodocyclales	2	1	1		1			
73029	Dechloromonas	2	1	1		1			
259537	*Dechloromonas aromatica*	2	1	1		1			
159087	*Dechloromonas aromatica RCB*	2	1	1		1			
28221	Deltaproteobacteria	28			3	10	5	9	+
29	Myxococcales	1				1			
161492	Anaeromyxobacter	1				1			
161493	*Anaeromyxobacter dehalogenans*	1				1			
290397	*Anaeromyxobacter dehalogenans 2CP-C*	1				1			
69541	Desulfuromonadales	6			2	2	2	4	+
890	Desulfuromonas								
891	*Desulfuromonas acetoxidans*								
28168	*Desulfuromonas acetoxidans DSM 684*							x[544]	
18	Pelobacter							3	+
29543	*Pelobacter propionicus*							3	+
338966	*Pelobacter propionicus DSM 2379*							3	+
28231	Geobacter	6			2	2	2	1	
28232	*Geobacter metallireducens*	1				1	1		
269799	*Geobacter metallireducens GS-15*	1				1	1		
35554	*Geobacter sulfurreducens*	2				1	1		
316067	*Geobacter sp.*				1				
351604	*Geobacter uraniumreducens*	3			1			1	
351605	*Geobacter uraniumreducens Rf4*	3			1			1	
213115	Desulfovibrionales	16						5	+
872	Desulfovibrio	14						5	+
876	*Desulfovibrio desulfuricans*	5							+
207559	*Desulfovibrio desulfuricans G20*	3							+
878	*Desulfovibrio fructosovorans*	1							+
879	*Desulfovibrio gigas*	1						1	
881	*Desulfovibrio vulgaris*	7						4	+
882	*Desulfovibrio vulgaris subsp. vulgaris str. Hildenborough*	3						2	+
883	*Desulfovibrio vulgaris (strain Miyazaki)*	1							
391774	*Desulfovibrio vulgaris subsp. vulgaris*	3						2	+
898	Desulfomicrobium	1							
899	*Desulfomicrobium baculatum*[e]	1							
41707	Lawsonia	1							

Table 1 (Continued)

29546	*Lawsonia intracellularis*	1						
363253	*Lawsonia intracellularis PHE/MN1-00*	1						
213118	Desulfobacterales	2			1	1		+
109168	Desulfotalea	2			1	1		+
84980	*Desulfotalea psychrophila*	2			1	1		+
213462	Syntrophobacterales	1		1	4	1		+
29526	Syntrophobacter	1			4	1		+
119484	*Syntrophobacter fumaroxidans*	1			4	1		+
335543	*Syntrophobacter fumaroxidans MPOB*	1			4	1		+
43773	Syntrophus			1				+
316277	*Syntrophus aciditrophicus*			1				+
56780	*Syntrophus aciditrophicus SB*			1				+
262489	*delta proteobacterium*	2			2	1		
29547	Epsilonproteobacteria	18	1				1	
213849	Campylobacterales	18	1				1	
194	Campylobacter	10						
195	*Campylobacter coli*	2						
306254	*Campylobacter coli RM2228*	2						
197	*Campylobacter jejuni*	4						
195099	*Campylobacter jejuni RM1221*	2						
201	*Campylobacter lari*	2						
306263	*Campylobacter lari RM2100*	2						
28080	*Campylobacter upsaliensis*	2						
306264	*Campylobacter upsaliensis RM3195*	2						
209	Helicobacter	6						
210	*Helicobacter pylori*	3						
85963	*Helicobacter pylori J99*	1						
357544	*Helicobacter pylori HPAG1*	1						
212	*Helicobacter acinonychis*	1						
382638	*Helicobacter acinonychis Sheeba*	1						
32025	*Helicobacter hepaticus*	2						
843	Wolinella	1					1	
844	*Wolinella succinogenes*	1					1	
39766	*Thiomicrospira denitrificans*	1	1					
326298	*Thiomicrospira denitrificans ATCC 33889*	1	1					
162171	Magnetococcus	1		1	1			
156889	*Magnetococcus sp.*	1		1	1			
377315	Mariprofundus					1		
314344	*Mariprofundus ferrooxydans*					1		
314345	*Mariprofundus ferrooxydans PV-1*					1		
1239	Firmicutes	14					9	+
186801	Clostridia	14					9	+
53433	Halanaerobiales	1						+
32636	Halothermothrix	1						+
31909	*Halothermothrix orenii*	1						+
373903	*Halothermothrix orenii H 168*	1						+
68295	Thermoanaerobacteriales						3	+
1754	Thermoanaerobacter						2	+
119072	*Thermoanaerobacter tengcongensis*						2	+
44260	Moorella						1	+
1525	*Moorella thermoacetica*						1	+
264732	*Moorella thermoacetica ATCC 39073*						1	+
186802	Clostridiales	13					6	+
1485	Clostridium	2					2	+
1488	*Clostridium acetobutylicum*	1						+
1515	*Clostridium thermocellum*						1	+
203119	*Clostridium thermocellum ATCC 27405*						1	+
1520	*Clostridium beijerinckii*	1						+
290402	*Clostridium beijerincki NCIMB 8052*	1						+
66219	*Clostridium phytofermentans*						1	+
357809	*Clostridium phytofermentans ISDg*						1	+
36853	Desulfitobacterium	9					2	+
36854	*Desulfitobacterium dehalogenans*	1						

Table 1 (Continued)

49338	*Desulfitobacterium hafniense*	8				2	+
138119	*Desulfitobacterium hafniense Y51*	4				1	+
272564	*Desulfitobacterium hafniense*	4				1	+
44000	Caldicellulosiruptor					1	+
44001	*Caldicellulosiruptor saccharolyticus*					1	+
351627	*Caldicellulosiruptor saccharolyticus DSM 8903*					1	+
129957	Carboxydothermus	1				1	
129958	*Carboxydothermus hydrogenoformans*	1				1	
246194	*Carboxydothermus hydrogenoformans Z-2901*	1				1	
191373	Pelotomaculum	1					+
110500	*Pelotomaculum thermopropionicum*	1					+
370438	*Pelotomaculum thermopropionicum SI*	1					+
57723	Acidobacteria	3			1		
204432	Acidobacteria (class)	1					
204433	Acidobacteriales	1					
204669	*Acidobacteria bacterium Ellin345*	1					
332159	Solibacteres	2			1		
332160	Solibacterales	2			1		
332162	Solibacter	2			1		
332163	*Solibacter usitatus*	2			1		
234267	*Solibacter usitatus Ellin6076*	2			1		
200783	Aquificae	2	1				
187857	Aquificae (class)	2	1				
32069	Aquificales	2	1				
2713	Aquifex	2	1				
63363	*Aquifex aeolicus*	2	1				
2714	*Aquifex pyrophilus*					x^{545}	
939	Hydrogenobacter						
940	*Hydrogenobacter thermophilus strain TK-6*					x^{139}	
200795	Chloroflexi	4	1	3	2	3	+
32061	Chloroflexi (class)	1	1		2		
32064	Chloroflexales	1	1		2		
1107	Chloroflexus		1		1		
1108	*Chloroflexus aurantiacus*		1		1		
324602	*Chloroflexus aurantiacus J-10-fl*		1		1		
120961	Roseiflexus	1			1		
357808	*Roseiflexus sp.*	1			1		
301297	Dehalococcoidetes	3		3		3	+
61434	Dehalococcoides	3		3		3	+
61435	*Dehalococcoides ethenogenes*	1		1		1	+
243164	*Dehalococcoides ethenogenes 195*	1		1		1	+
216389	*Dehalococcoides sp.*	1		1		1	+
255470	*Dehalococcoides sp. CBDB1*	1		1		1	+
201174	Actinobacteria	11	3	6	2		+
1760	Actinobacteria (class)	11	3	6	2		+
2037	Actinomycetales	11	3	6	2		
1716	Corynebacterium	1					
1717	*Corynebacterium diphtheriae*	1					
1763	Mycobacterium	4	1	3	1		
110539	*Mycobacterium vanbaalenii*	1	1		1		
350058	*Mycobacterium vanbaalenii PYR-1*	1	1		1		
164756	*Mycobacterium sp.*	1		1			
164757	*Mycobacterium sp.*	1		1			
189918	*Mycobacterium sp.*	1		1			
1827	Rhodococcus	1		1	1		
37919	*Rhodococcus opacus*				1		
101510	*Rhodococcus sp. RHA1*	1		1			
1854	Frankia	4	2	1			
1859	*Frankia alni*	1	1				
326424	*Frankia alni ACN14A*	1	1				
106370	*Frankia sp. CcI3*	1	1	1			
298653	*Frankia sp.*	2					

Table 1 (Continued)

1883	Streptomyces		1					
33903	*Streptomyces avermitilis*		1					
28048	Acidothermus				1			
28049	*Acidothermus cellulolyticus*				1			
351607	*Acidothermus cellulolyticus 11B*				1			
203682	Planctomycetes						1	
203683	Planctomycetacia						1	
112	Planctomycetales						1	
380738	Candidatus Kuenenia						1	
174633	*Candidatus Kuenenia stuttgartiensis*						1	
2157	Archaea		11	19	7	14	28	
28889	Crenarchaeota		2				2	
183924	Thermoprotei		2				2	
2266	Thermoproteales		1				2	
2268	Thermofilum		1				2	
2269	*Thermofilum pendens*		1				2	
368408	*Thermofilum pendens Hrk 5*		1				2	
2281	Sulfolobales		1					
12914	Acidianus		1					
2283	*Acidianus ambivalens*[546]		1					
28890	Euryarchaeota		9	19	7	14	26	
183925	Methanobacteria			2		3	5	
2158	Methanobacteriales			2		3	5	
2172	Methanobrevibacter							
39441	*Methanobrevibacter arboriphilus*[588,589]	+						
2173	*Methanobrevibacter smithii*[590]	+						
2179	Methanothermus					1		
2180	*Methanothermus fervidus*[65b, 590]	+				1		
2316	Methanosphaera			1		1	1	
2317	*Methanosphaera stadtmanae*			1		1	1	
339860	*Methanosphaera stadtmanae DSM 3091*			1		1	1	
145260	Methanothermobacter			1		1	4	
145263	*Methanothermobacter marburgensis*							
79929	*Methanothermobacter marburgensis*[f,65a,591]	+				1		x [202]
145262	*Methanothermobacter thermautotrophicus*[g]			1		1	4	
187420	*Methanothermobacter thermautotrophicus str.DeltaH*			1		1	2	
187420	*Methanothermobacter thermautotrophicus*[h,592,593]	+						
145261	*Methanothermobacter wolfeii*[65a]	+						
183939	Methanococci			6		5	5	
2182	Methanococcales			6		5	5	
2184	Methanococcus			4		4	3	
42879	*Methanococcus aeolicus*[i]	+						
2187	*Methanococcus vannielii*[594]	+						
2188	*Methanococcus voltae*[65b]	+		2		2		
39152	*Methanococcus maripaludis*[595,596]	+		2		2	3	
196118	Methanocaldococcus			2		1	2	
2190	*Methanocaldococcus jannaschii*[597,598]	+		2		1	2	
2189	*Methanotorris igneus*[65b]	+						
155862	Methanothermococcus							
2186	*Methanothermococcus thermolithotrophicus*[599]	+						
183968	Thermococci				7		5	
2258	Thermococcales				7		5	
2260	Pyrococcus				5		4	
2261	*Pyrococcus furiosus*				2		1	
29292	*Pyrococcus abyssi*				2		2	
53953	*Pyrococcus horikoshii*				1		1	
2263	Thermococcus				2		1	
	Thermococcus celer							x[547]
2265	*Thermococcus litoralis*				1			
311400	*Thermococcus kodakarensis*				1		1	

Table 1 (Continued)

69014	*Thermococcus kodakarensis KOD1*			1	1	
183980	Archaeoglobi		1		1	
2231	Archaeoglobales		1		1	
2233	Archaeoglobus		1		1	
2234	*Archaeoglobus fulgidus*		1		1	
183988	Methanopyri			2	2	1
68985	Methanopyrales			2	2	1
2319	Methanopyrus			2	2	1
2320	*Methanopyrus kandleri*[65a]	+		2	2	1
224756	Methanomicrobia		7	7	1	9
2191	Methanomicrobiales			2	1	5
2202	Methanospirillum			1		3
2203	*Methanospirillum hungatei*			1		3
323259	*Methanospirillum hungatei JF-1*			1		3
45989	Methanoculleus			1	1	2
2198	*Methanoculleus marisnigri*			1	1	2
368407	*Methanoculleus marisnigri JR1*			1	1	2
94695	Methanosarcinales		7	5		4
2207	Methanosarcina		7	5		4
2208	*Methanosarcina barkeri*		2	3		3
269797	*Methanosarcina barkeri str. fusaro*		2	2		2
2209	*Methanosarcina mazei*		3	1		1
2214	*Methanosarcina acetivorans*		2	1		
351160	*uncultured methanogenic archaeon RC-I*		1	2	2	1

[a] S*ynechococcus elongatus* was formerly called *Anacystis nidulans*. [b] Formerly *Chromatium vinosum*. [c] Formerly *Rhodocyclus gelatinosus*, *Rhodopseudomonas gelatinosa*. [d] Formerly *Ralstonia eutropha*, *Alcaligenes eutrophus*. *R. eutropha* was first reclassified in a novel genus, *Wautersia* gen. nov.[537] It was later demonstrated[538] that *Wautersia eutropha*, the type species of the genus *Wautersia*, is a later synonym of *Cupriavidus necator*, the type species of the genus *Cupriavidus*. In conformity with the Rules of the International Code of Nomenclature of Bacteria, the new name of *R. eutropha* is therefore *Cupriavidus necator*. It is used in the tables but not in the text. [e] Formerly *Desulfovibrio baculatus*. [f] Formerly *Methanobacterium thermoautotrophicum* strain Marburg. [g] Formerly *Methanobacterium thermoautotrophicum*. [h] S. Shima, Max-Planck Institute for Terrestrial Microbiology, Marburg, Germany, unpublished results (personal communication). [i] TaxID is the identifier in the taxonomy database (when available). Values on the right (columns 1−4) indicate number of enzymes (i.e., one for each dimeric complex comprising a small and a large subunit) in each group (see text). Species in which a hydrogenase activity has been detected, but no gene yet sequenced, have an "x" in column A (for activity). Taxa in which an [FeFe]-hydrogenase is also known to be present have a "+" in the last column.

From the number of [NiFe]- and [FeFe]-hydrogenase gene sequences, given on the right of Tables 1 and 2, it can be seen that many species contain several [NiFe]-hydrogenases and that some of them contain both an [FeFe]- and one or several [NiFe]-hydrogenases. The classification of [NiFe]-hydrogenases into four groups established earlier[10a] has been confirmed in the present study.

The evolutionary relationship between various organisms containing at least one hydrogenase is illustrated schematically in Figures 3 and 4.

Figure 3 indicates the hydrogenase distribution in major subdivisions (phyla) of the *Bacteria* and shows that the *Proteobacteria* are particularly well represented. Genome sequencing projects on microorganisms of economical interest have uncovered a large number of additional hydrogenase sequences and also the presence of different types of hydrogenase in single species. [FeFe]-hydrogenases are mainly present in Gram-positive bacteria (*Firmicutes*) and in species belonging to the γ and δ divisions of the *Proteobacteria*. Among the *Archaea* (Figure 4), methanogenic species in the phylum *Euryarchaeota* prevail (mesophilic and moderately thermophilic methanogens are the best studied *Archaea*). The phylum *Crenarchaeota* of the *Archaea* contains hyperthermophiles from terrestrial volcanic habitats (e.g., *Sulfolobus solfataricus*) and submarine volcanic habitats (e.g., *Pyrodictium*, *Pyrolobus*). Figures 3 and 4 allow firm conclusions about the distribution of the [NiFe]- and [FeFe]-hydrogenases.

3. Classification of Hydrogenases

3.1. Hydrogenase Enzymes

The key enzyme involved in the metabolism of H_2 is hydrogenase. The enzyme catalyzes the simplest chemical reaction: $2H^+ + 2e^- \leftrightarrows H_2$. The reaction is reversible, and its direction depends on the redox potential of the components able to interact with the enzyme. In the presence of H_2 and an electron acceptor, it will act as a H_2 uptake enzyme; in the presence of an electron donor of low potential, it may use the protons from water as electron acceptors and release H_2. The first classification of these enzymes was based on the identity of specific electron donors and acceptors, namely, NAD (hydrogenases of EC class 1.12.1.12), cytochromes (class 1.12.2.1), coenzyme F_{420} (class 1.12.99.1), or ferredoxins (class 1.18.99.1).

Most of the known hydrogenases are iron−sulfur proteins with two metal atoms at their active site, either a Ni and an Fe atom (in [NiFe]-hydrogenases)[61,62] or two Fe atoms (in [FeFe]-hydrogenases).[63,64] A different type of hydrogenase,

Table 2. Taxonomy of Organisms Containing [FeFe]-Hydrogenase Genes and Those in Which [FeFe]-Hydrogenase Activity Has Been Characterized (Column A)[a]

TaxID	superkingdom / kingdom / phylum / class / order / genus / species / subspecies	[FeFe]	A	[NiFe]
2	Bacteria	118		+
976	Bacteroidetes	4		+
200643	Bacteroidetes (class)	4		+
171549	Bacteroidales	4		+
816	Bacteroides	4		
817	*Bacteroides fragilis*	2		
272559	*Bacteroides fragilis* NCTC 9343	1		
818	*Bacteroides thetaiotaomicron*	2		
1224	Proteobacteria	36		+
1236	Gammaproteobacteria	7		+
135622	Alteromonadales	7		+
22	Shewanella	7		+
60480	*Shewanella* sp.	2		+
70863	*Shewanella oneidensis*	2		+
94122	*Shewanella* sp.	1		+
256839	*Shewanella decolorationis*	2		
91347	Enterobacteriales			
547	Enterobacter			
550	*Enterobacter cloacae*			
	Enterobacter cloacae IIT-BT 08[548]	1	x	
28211	Alphaproteobacteria	2		+
356	Rhizobiales	2		+
1073	Rhodopseudomonas	2		+
1076	*Rhodopseudomonas palustris*	2		+
258594	*Rhodopseudomonas palustris* CGA009	1		+
316055	*Rhodopseudomonas palustris* BisA53	1		+
28221	Deltaproteobacteria	27		+
69541	Desulfuromonadales	2		+
18	Pelobacter	2		+
19	*Pelobacter carbinolicus*	1		
338963	*Pelobacter carbinolicus* DSM 2380	1		
29543	*Pelobacter propionicus*	1		+
338966	*Pelobacter propionicus* DSM 2379	1		+
213115	Desulfovibrionales	20		+
872	Desulfovibrio	20		+
876	*Desulfovibrio desulfuricans*	8		+
207559	*Desulfovibrio desulfuricans* G20	6		+
878	*Desulfovibrio fructosovorans*	3		+
881	*Desulfovibrio vulgaris*	9		+
882	*Desulfovibrio vulgaris subsp.vulgaris*(Hildenborough)	3		+
884	*Desulfovibrio vulgaris subsp. oxamicus* (Monticello)	2		
391774	*Desulfovibrio vulgaris subsp. vulgaris*	3		+
213118	Desulfobacterales	2		+
109168	Desulfotalea	2		+
84980	*Desulfotalea psychrophila*	2		+
213462	Syntrophobacterales	3		+
29526	Syntrophobacter	2		+
119484	*Syntrophobacter fumaroxidans*	2		+
335543	*Syntrophobacter fumaroxidans* MPOB	2		+
43773	Syntrophus	1		+
316277	*Syntrophus aciditrophicus*	1		+
56780	*Syntrophus aciditrophicus* SB	1		+
1239	Firmicutes	69		+
186801	Clostridia	69		+
53433	Halanaerobiales	4		+
32636	Halothermothrix	4		+

Table 2 (Continued)

31909	*Halothermothrix orenii*	4	+
373903	*Halothermothrix orenii* H 168	4	+
68295	Thermoanaerobacteriales	4	+
1754	Thermoanaerobacter	2	+
1757	*Thermoanaerobacter ethanolicus*	1	
340099	*Thermoanaerobacter ethanolicus* ATCC 33223	1	
119072	*Thermoanaerobacter tengcongensis*	1	+
44260	Moorella	2	+
1525	*Moorella thermoacetica*	2	+
264732	*Moorella thermoacetica* ATCC 39073	2	+
186802	Clostridiales	61	+
862	Syntrophomonas	4	
863	*Syntrophomonas wolfei*	4	
335541	*Syntrophomonas wolfei subsp. wolfei*	4	
906	Megasphaera	1	
907	*Megasphaera elsdenii*	1	
1485	Clostridium	37	+
1488	*Clostridium acetobutylicum*	2	+
1496	*Clostridium difficile*	3	
272563	*Clostridium difficile* 630	3	
1501	*Clostridium pasteurianum*	1	
1502	*Clostridium perfringens*	12	
1503	*Clostridium perfringens* 13 / Type A	4	
195103	*Clostridium perfringens* ATCC 13124	4	
289380	*Clostridium perfringens* SM101	4	
1513	*Clostridium tetani*	2	
1515	*Clostridium thermocellum*	4	+
203119	*Clostridium thermocellum* ATCC 27405	3	+
1520	*Clostridium beijerinckii*	5	+
290402	*Clostridium beijerincki*	5	+
29363	*Clostridium paraputrificum*	1	
36745	*Clostridium saccharoperbutylacetonicum*	1	
66219	*Clostridium phytofermentans*	4	+
357809	*Clostridium phytofermentans* ISDg	4	+
169679	*Clostridium saccharobutylicum*	1	
350688	*Clostridium* sp.	1	
1562	Desulfotomaculum	4	
59610	*Desulfotomaculum reducens*	4	
349161	*Desulfotomaculum reducens* MI-1	4	
1730	Eubacterium	1	
1731	*Eubacterium acidaminophilum*	1	
28063	Heliobacillus	1	
28064	*Heliobacillus mobilis*	1	
36853	Desulfitobacterium	8	+
49338	*Desulfitobacterium hafniense*	8	+
138119	*Desulfitobacterium hafniense* Y51	4	+
272564	*Desulfitobacterium hafniense*	4	+
44000	Caldicellulosiruptor	1	+
44001	*Caldicellulosiruptor saccharolyticus*	1	+
351627	*Caldicellulosiruptor saccharolyticus* DSM 8903	1	+
114627	Alkaliphilus	2	
208226	*Alkaliphilus metalliredigenes*	2	
293826	*Alkaliphilus metalliredigenes* QYMF	2	
191373	Pelotomaculum	2	+
110500	*Pelotomaculum thermopropionicum*	2	+
370438	*Pelotomaculum thermopropionicum* SI	2	+
200795	Chloroflexi	3	+
301297	Dehalococcoidetes	3	+
61434	Dehalococcoides	3	+
61435	*Dehalococcoides ethenogenes*	1	+
243164	*Dehalococcoides ethenogenes* 195	1	+
216389	*Dehalococcoides* sp.	1	+
255470	*Dehalococcoides* sp. CBDB1	1	+

Table 2 (Continued)

200918	Thermotogae	2		
188708	Thermotogae (class)	2		
2419	Thermotogales	2		
2335	Thermotoga	2		
2336	*Thermotoga maritima*	2		
201174	Actinobacteria	2		+
1760	Actinobacteria (class)	2		+
2733	Symbiobacterium	2		
2734	*Symbiobacterium thermophilum*	2		
203691	Spirochaetes	2		
203692	Spirochaetes (class)	2		
136	Spirochaetales	2		
157	Treponema	2		
158	*Treponema denticola*	2		
2759	Eukaryota	22		
4751	Fungi	3		
4761	Chytridiomycota	3		
29006	Neocallimastigales	3		
4756	Neocallimastix	2		
4757	*Neocallimastix frontalis*	2		
4821	Piromyces	1		
73868	*Piromyces* sp. E2	1		
5740	Giardia	2		
5741	*Giardia intestinalis*	2		
184922	*Giardia lamblia* ATCC 50803	1		
5758	Entamoeba	5		
5759	*Entamoeba histolytica*	5		
294381	*Entamoeba histolytica* HM-1:IMSS	3		
33090	Viridiplantae	7		
3041	Chlorophyta	7		
3166	Chlorophyceae	7		
3069	Chlorococcales			
44649	Chlorococcum			
56200	*Chlorococcum littorale*[549]		x	
3042	Chlamydomonadales	4		
3052	Chlamydomonas	4		
3054	*Chlamydomonas moewusii*	1		
3055	*Chlamydomonas reinhardtii*	3		
35491	Sphaeropleales	3		
3087	Scenedesmus	3		
3073	*Chlorella fusca*	1		
3088	*Scenedesmus obliquus*	2		
3152	Prasinophyceae			
3160	Platymonas			
3161	*Platymonas subcordiformis*[550]		x	
33829	Spirotrichea	2		
33830	Armophorida	2		
70074	Nyctotherus	2		
70075	*Nyctotherus ovalis*	2		
39709	Spironucleus	1		
103874	Spironucleus barkhanus	1		
285690	Trichomonada	2		
37104	Trichomonadida	2		
5721	Trichomonas	2		
5722	*Trichomonas vaginalis*	2		

[a] Last column indicates concomitant presence of [NiFe]-hydrogenase in the species.

discovered in some methanogens,[65] functions as H_2-forming *m*ethylenetetrahydromethanopterin *d*ehydrogenase, abbreviated Hmd (EC 1.12.99.4). The Hmd enzyme, which contains no Fe-S cluster and no Ni, was initially referred to as "metal-free" hydrogenase; it was later renamed iron−sulfur-cluster-free hydrogenase or [Fe]-hydrogenase.[10b]

At this time, the sequences of altogether ca. 450 hydrogenases are available. These data confirm that despite their

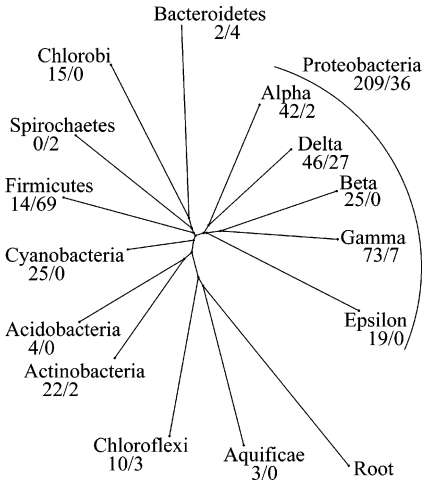

Figure 3. Phylogenetic tree of bacteria. The tree is derived from 16S ribosomal RNA sequences (data obtained from the European ribosomal RNA database http://www.psb.ugent.be/rRNA/index.html). The evolutionary distances are not to scale. Sequences from *Eukaryota* and *Archaea* were used for the root. Numbers at the ends of the branches represent the number of hydrogenase genes known in species of that group. The figure on the left of the slash represents the number of [NiFe]-hydrogenases and the one on the right the number of [FeFe]-hydrogenases (see Tables 1−3). The proteobacteria were formerly called purple bacteria (http://www.cme.msu.edu/RDP/).

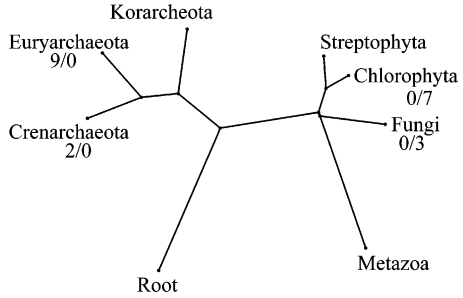

Figure 4. Phylogenetic tree of Archaea and Eukarya. The tree is derived from 16S ribosomal RNA sequences (data obtained from the European ribosomal RNA database http://www.psb.ugent.be/rRNA/index.html). Three phyla have been identified in the domain of the Archaea: the Euryarchaeota, which contains methanogenic and extremely halophilic prokaryotes; the Crenarchaeota, which consists of both hyperthermophiles and cold-dwelling species; and the Korarchaeota, which are, as far as is known, hyperthermophiles. The evolutionary distances are not to scale. Sequences from bacteria were used for the root. Numbers at the ends of the branches represent the number of hydrogenase genes known in species of that group. The figure on the left of the slash represents the number of [NiFe]-hydrogenases and the one on the right the number of [FeFe]-hydrogenases. No [FeFe]-hydrogenase has as yet been found in the Archaea, and no [NiFe]-hydrogenase has been found in the Eukarya (see Tables 1−3).

conspicuous diversity in many respects (size, quaternary structure, electron donors and acceptors) hydrogenases consist of three phylogenetically distinct classes, the [NiFe]-, the [FeFe]-, and the [Fe]-hydrogenases, each characterized by a distinctive functional core that is conserved within each class[10a,b,13] (this paper). This core consists of the subunits or domains that accommodate the catalytic site and that are minimally required for structure and function. Metal content and sequence similarity is thus a reliable classification criterion. The [Fe]-hydrogenases being restricted to some methanogens, their phylogeny cannot be adequately discussed, and therefore only the [NiFe]- and [FeFe]-hydrogenases are considered in some detail in this review.

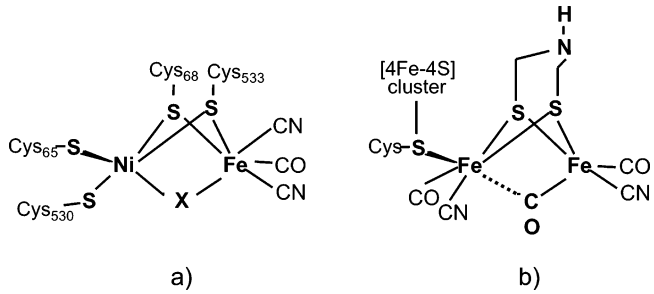

Figure 5. Schematic structure of the active site of [NiFe]- and [FeFe]-hydrogenases. (A) [NiFe]-hydrogenase in the oxidized inactive form;[61,62,70−76] the bridging ligand X has been proposed to be O^{2-}, OH^-, OH_2, or SO. In the reduced active form X = H^-. (B) [FeFe]-hydrogenase: the Fe_2-S_3 subsite of the H-cluster with a bridging di(thiomethyl)amine unit. The diatomic ligand, CO, is seen in a bridging position in *C. pasteurianum* hydrogenase (CpI),[63] whereas in *D. desulfuricans* (DdH) it appears to be rather asymmetrically bound to Fe_2 (distal to the [4Fe-4S] cluster).[64,105,551]

3.1.1. Hmd or [Fe]-Hydrogenases

The Hmd enzyme discovered in *Methanothermobacter marburgensis*[65a] has been the most extensively studied hydrogenase of this type. It catalyzes an intermediary step in CO_2 reduction with H_2 to methane,[65b,66] that is, the reversible reduction of methenyltetrahydromethanopterin (methenyl-H_4MPT^+) with H_2 to methylene-H_4MPT and H^+. Hmd is essential only under growth conditions of nickel limitation,[66] where the F_{420}-reducing [NiFe]-hydrogenase (Frh) is no longer synthesized (cf. Figure 11). Hmd is composed of two identical subunits (38 kDa), encoded by a monocistronic gene, and contains two iron per homodimer but no iron−sulfur cluster.[65] It has been identified in a dozen methanogenic species (Table 1). Hmd does not function as a purely organic catalyst as initially thought;[65b] its activity depends on an iron-containing cofactor.[67,68] The crystal structure of its apoenzyme has been recently published.[69] In short, the Hmd enzymes differ from the [NiFe]- and [FeFe]-hydrogenases not only by the primary and tertiary structures but also by the fact that the iron, required for enzyme activity, is not redox active. Associated with a specific cofactor, they have catalytic properties different from those described for [NiFe]- and [FeFe]-hydrogenases; in particular, they do not catalyze the reversible reaction: $2H^+ + 2e^- \leftrightarrows H_2$.

3.1.2. [NiFe]-Hydrogenases

The most numerous and best studied class of hydrogenases have been the [NiFe]-hydrogenases from the domain of *Bacteria*. The core enzyme consists of an $\alpha\beta$ heterodimer with the large subunit (α-subunit) of ca. 60 kDa hosting the bimetallic active site and the small subunit (β-subunit) of ca. 30 kDa hosting the Fe-S clusters (the size of the small and large subunits is smaller in multimeric hydrogenases; cf. Figure 6). Crystal structures of *Desulfovibrio* hydrogenases[61,62,70−73] have revealed the general fold and the nature of the binuclear NiFe active site (Figure 5a); they have shown that the two subunits interact extensively through a large contact surface and form a globular heterodimer. The bimetallic NiFe center is deeply buried in the large subunit; it is coordinated to the protein by four cysteines. The presence of three non-protein ligands, 1 CO and 2 CN^-, bound to the Fe atom[70,74,75] or SO, CO and CN^-[62,76] (Figure 5a) was revealed by infrared spectroscopy. The FTIR and EPR properties of the NiFe center of the cytoplasmic NAD-reducing hydrogenase of *Ralstonia eutropha* (formerly *Al-*

Group	Function		Length
1	Membrane-bound H_2 uptake hydrogenases	S	268 - 552
		L	428 - 633
2a	Cyanobacterial uptake hydrogenases	S	284 - 384
		L	416 - 547
2b	H_2-sensing hydrogenases	S	258 - 347
		L	472 - 496
3a	F_{420}-reducing hydrogenases	S	216 - 298
		L	370 - 469
3b	Bifunctional (NADP) hydrogenases	S	237 - 282
		L	412 - 458
3c	Methyl-Viologen-reducing hydrogenases	S	287 - 366
		L[a]	418 - 496
3d	Bidirectional NAD(P)-linked hydrogenases	S	160 - 209
		L	471 - 507
4	Membrane-bound H_2 evolving hydrogenases	S	135 - 277
		L	358 - 588

Group	Large subunit pattern
1	L1 [EGMQS]**R**x**C**[GR][IV]Cxxx[HT]xxx[AGS]x(0,4)[VANQD]
	L2 [AFGIKLMV][HMR]xx[HR][AS][AFLY][DN]PC[FILMV]xC[AGS]xH
2a	L1 PR[AIV]CGICx(1,3)Hx(0,2)Lxx[AST]
	L2 Vx[KR]S[FHY]DxCxVC[ST][TV][HK]
2b	L1 PR[IV]CGICS[IV][AS]Q[GS]xA
	L2 H[IV]VRSFDPCMVCT[AV]H
3a	L1 R[FIV]CG[ILV]C[PQ]x[APT]H[ACGT]x[AS][AGS]
	L2 R[ACS]YD[IP]C[AILV][AS]Cx(2,3)Hx[ILMV]
3b	L1 R[IV]C[AGS][FIL]Cxxx[HY]xx[AST][ANS]xx[AS][AILV]
	L2 R[ANS][FHY]DPCISC[AS][ATV]H
3c[b]	L1 Px[FILV][TV][ADPST]x[IV]CG[IV]CxxxHxx[AC][AS]xxA
	L2 E[FMV][AGLV][FIV]Rx[FY]DPCx[AS]C[AS][ST]Hx[AILV]
3d	L1 Ex[APV]xxxxRxCG[IL]Cxx[AS]Hx[IL][ACS][AGS][AGNSV][KR][ATV]xD
	L2 DPC[IL]SC[AS][AST]**H**[ASTV]x[AG]xx[APV]
4	L1 C[GS][ILV]C[AGNS]xxH
	L2 [DE][PL]Cx[AGST]Cx[DE][RL]

Figure 6. Characterization of [NiFe]-hydrogenase groups. L1 and L2 signatures are derived from [NiFe]-hydrogenase amino acid sequences of each group shown in Table 3. Patterns were determined as described under section 3.3 and are presented in PROSITE format:[552−554] brackets include the residues occurring at a single position in the set of sequences, and "x" means "any amino acid". In addition, residues in bold type occur in more than 80% of the sequences. In group 1, one exceptionally long S sequence (813 aa, Q31DZ6) or short L sequence (267 aa, Q57PA0) and in group 3c, S (491 aa, Q2IH66), were not included to determine the average size of subunits. [a]In the Vhu enzymes, the L1 pattern is found in VhuA and L2 is in the complementary VhuU subunit. [b]Full-length proteins only were taken into account, excluding VhuA subunits because the ~50 C-terminal amino acids are provided by an additional VhuU subunit.

caligenes eutrophus now renamed *Cupriavidus necator*) suggested the presence of two additional CN^- ligands, with one CN^- bound to Ni, so that the structure of the active site may be $Ni(CN)Fe(CN)_3(CO)$.[77]

The small subunit contains up to three linearly arranged cubane Fe-S clusters of the [4Fe-4S] type, which conduct electrons between the H_2-activating center and the physiological electron acceptor (or donor) of hydrogenase. The small subunit of the [NiFeSe]-hydrogenases from *Desulfomicrobium baculatum*[78] and *Desulfovibrio vulgaris* Hildenborough[79] (HysBA) and that of the F_{420}-reducing [NiFeSe]-hydrogenase (Fru) of *Methanococcus voltae*[80] contain indeed three [4Fe-4S] clusters, whereas standard *Desulfovibrio* [NiFe]-hydrogenases have a [3Fe-4S] cluster with a relatively high redox potential in the median position between the proximal and the distal [4Fe-4S] cluster. The [4Fe-4S] cluster that is proximal to the active site (within 14 Å) is "essential" to H_2 activation.[61,81] Hydrophobic channels linking the active site to the surface of the molecule have been suggested to facilitate gas access to the active site.[71,81,82] The crystallographic structure of *D. desulfuricans* ATCC 27774 [NiFe]-hydrogenase has revealed that the [4Fe-4S] cluster nearest the NiFe center has been modified by the loss of one sulfur atom and inclusion of three oxygen atoms [4Fe-3S-3O].[73] A [Fe-S] cluster organization different from the canonical one found in the first two three-dimensional structures published of *Desulfovibrio* [NiFe]-hydrogenases has been reported for the regulatory hydrogenase HoxBC of *R. eutropha*. According to the analysis of iron EXAFS spectra, the small subunit seems to harbor two [2Fe-2S] clusters and a 4Fe species, which may be a [4Fe-3S-3O] cluster.[83] Alignments of the full amino acid sequences of the small and large subunits have shown that the two subunits evolved conjointly.[10a]

In *Proteobacteria*, the genes that encode H_2-uptake hydrogenases are clustered. These clusters comprise the structural genes (generally labeled L for large subunit and S for small subunit), accessory genes for maturation and the insertion of Ni, Fe, CO, and CN^- at the active site of the heterodimer, and in some cases also regulatory genes that control expression of the structural genes. The biosynthesis of *Escherichia coli* hydrogenase-3 has been extensively studied by the group of A. Böck[16,84,85] (reviewed in refs 10a, 14, 16−18, 86, and 87). It begins by the synthesis of the large subunit (HycE) as a precursor protein (pre-HycE) with an extension at the carboxyl terminus (32 amino acids). After insertion of the metallocenter, an endopeptidase removes the C-terminal extension from the precursor of the large subunit.[88,89] After proteolysis, the large subunit is then capable of binding to the small subunit. Because hydrogenase gene clusters from various species encode homologous proteins, it is inferred that analogous biosynthetic mechanisms operate in the various organisms containing those clusters. The correspondence of these genes, designated differently in different organisms, can be found in refs 10a and 14. It should be noted that even though hydrogenase operons are well conserved and exhibit a high degree of similarity, each *cis*-acting maturation system is specific to the corresponding structural gene products. Thus, the precursor of the large subunit of *E. coli* hydrogenase-3 is processed by the HycI endopeptidase, whereas that of hydrogenase-2 is processed by HybD.[90] This specificity may explain why in some cases hydrogenases cannot be matured when produced in heterologous hosts (there are examples in the literature demonstrating heterologous expression of [NiFe]-hydrogenases).

3.1.3. [FeFe]-Hydrogenases

Unlike [NiFe]-hydrogenases composed of at least two subunits, many [FeFe]-hydrogenases are monomeric and consist of the catalytic subunit only, although dimeric, trimeric, and tetrameric enzymes are also known.[10a,91,92] The smallest [FeFe]-hydrogenases (ca. 45−48 kDa) have been found in green algae.[93−98] This type of enzyme is found in anaerobic prokaryotes, such as clostridia and sulfate reducers,[99−101] and in lower eukaryotes[102,103] (reviewed in refs 10a, 12, 13, and 104). [FeFe]-hydrogenases are the only type of hydrogenase to have been found in eukaryotes, and they are located exclusively in organelles, that is, in chloroplasts or in hydrogenosomes.

The catalytic subunits of [FeFe]-hydrogenases, in contrast to those of Ni-containing enzymes, vary considerably in size. Besides the conserved domains of ca. 350 residues containing the active site (H-cluster),[99] they often comprise additional domains, which accommodate Fe-S clusters. The H-cluster consists of a binuclear [FeFe] center bound to a [4Fe-4S] cluster by a bridging cysteine belonging to the protein. Nonprotein ligands, CN^- and CO, are attached to the iron atoms of the binuclear Fe center[63,64,91,105] (Figure 5b). The Fe atoms also share two bridging sulfur ligands of a small five-atom

molecule, possibly a di(thiomethyl)amine molecule, HN−(CH_2−S^-)$_2$.[92] The Fe atom distal to the [Fe_4-S_4] cluster (Fe2) has a vacant coordination site which is occupied by carbon monoxide, a competitive inhibitor, in the CO-inhibited form of the enzyme; it is therefore thought to be the position where dihydrogen or hydride binds during enzyme turnover. A single hydrophobic channel that runs from the molecular surface to the active site and points at Fe2 was detected in the structures of the [FeFe]-hydrogenase from *D. desulfuricans* ATCC 7757[64] and the hydrogenase I from *Clostridium pasteurianum*,[63,92] but molecular dynamics investigation has indicated that H_2 is also able to diffuse through a number of alternative routes within the enzyme molecule.[106] Similarly to [NiFe]-hydrogenases, a plausible proton pathway has been proposed for [FeFe]-hydrogenases.[63,92] The chemical synthesis of the H-cluster framework of [FeFe]-hydrogenase has been achieved through linking of a di-iron subsite to a [4Fe-4S] cluster.[107]

3.2. Assays of Hydrogenase Activity

The methods used to assay hydrogenase activity are based on the enzyme ability to catalyze H_2 evolution and H_2 oxidation, interconversion of *para*- and *ortho*-H_2, and deuterium or tritium exchange reactions with H^+ (in the absence of electron donors or acceptors). Oxidation of H_2 can be associated with the reduction of a dye, measurable by spectrophotometry; to afford interaction of hydrogenase with exogenous electron acceptors, whole cells are usually permeabilized by a detergent (e.g., Triton X-100 or CTAB). Production or consumption of H_2 can be measured amperometrically, using a Clark-type electrode, or manometrically or by gas chromatography with a thermal conductivity detector. Isotope exchange, using tritium gas or tritiated water, can be measured by radioactive counting. Exchange with deuterium can be detected by mass spectrometry. These different assay methods, summarized by Cammack,[108] have been described in the *Methods in Enzymology* series;[109] Vignais[110] has more specifically described and discussed proton−deuterium (H/D) exchange measurements. Direct bioelectrocatalysis by hydrogenases adsorbed on carbon black electrodes, first used by Berezin and co-workers,[111] permits one to correlate the anodic current with H_2 oxidation and the cathodic current observed at negative potentials with H_2 evolution.[112] Studies dealing with the electrochemistry of hydrogenases are described in detail by F. A. Armstrong et al. in this issue.

To correctly test their activity, hydrogenases have to be reactivated as, in the oxidized aerobic state, most hydrogenases are inactive. Whereas [FeFe]-hydrogenases are irreversibly inactivated by O_2, [NiFe]-hydrogenases can be reactivated by reduction (H_2, dithionite) to become catalytically competent. The oxidized forms of the enzyme produce distinct EPR signals (Ni-A and Ni-B states), whereas the fully reduced states (Ni-S and Ni-R) are EPR silent or EPR visible (Ni-C).[108] The hydrogenase activation process has been linked to the removal of the additional bridging ligand at the active site.[72,113] Upon reductive activation, the ligand (X), a hydroxo or oxygen species (Figure 5a), leaves by protonation to water[114] and the Ni ion is reduced from Ni-(III) to Ni(II) to yield the EPR-silent intermediate Ni-S. Protein film voltammetry has been used to define the sensitivity of hydrogenase to O_2[115] and to CO.[116]

The use of hydrogen isotopes (deuterium, tritium) enables detection of the splitting of the hydrogen molecule at the active site and study of the mechanism of enzyme action.[117] From the study of isotope exchange and *para*-H_2 to *ortho*-H_2 (spin nuclear isomers) conversion reaction, it has been concluded that hydrogenase catalyzes heterolytic splitting of hydrogen with formation of an intermediate enzyme hydride.[118] If D_2 gas is used, the splitting of the D_2 molecule results in the formation of a deuteron (D^+) and a deuteride (D^-). In the absence of an electron donor or acceptor, the back reaction, in the presence of excess protons from the solvent, leads to the formation of HD. Overall, there is no electron transfer. Electron acceptors, if present, compete with H^+ for the hydride intermediate so that the exchange reaction is lowered and may even be abolished. The H/D exchange reaction was used more than 20 years ago to monitor hydrogenase activation of *Alcaligenes eutrophus* (*R. eutropha*)[119] and *Desulfovibrio*[120] [NiFe]-hydrogenases. It was concluded that the process involves two successive steps: (a) a slow nonreductive step probably consisting in the removal of the oxygen species from the active center and (b) a fast reductive step linked to the reduction of the enzyme by H_2 or a reductant (dithionite). These two steps for hydrogenase anaerobic activation have been demonstrated by the H/D exchange reaction with *Dm. baculatum*,[121] *D. fructosovorans*,[121] and *Synechocystis* PCC 6308[121] [NiFe]-hydrogenases. In the case of *Synechocystis* bidirectional [NiFe]-hydrogenase, reactivation was brought about by either NADH or NADPH. The H/D exchange reaction provides an ideal assay for determining the activity of the enzyme active site alone even in systems as complex as whole microorganisms. Measurements of the H/D exchange reaction in cells of the photosynthetic bacterium *Rhodobacter capsulatus* have demonstrated, for the first time, that the regulatory HupUV protein could catalyze H/D exchange, and thus bind H_2, a prerequisite for a H_2 detector, and that HupUV is a true hydrogenase.[122] It has been used to discuss the hydrogenase catalytic cycle with hydrogenases isolated from *Thiocapsa roseopersicina*[123] and *Azotobacter vinelandii*[124] and to demonstrate the insensitivity to oxygen of the HupUV H_2-sensing regulatory hydrogenase from *R. capsulatus*.[121]

The H_2-forming, iron−sulfur-cluster-free hydrogenase, Hmd, catalyzes the reversible conversion of N^5,N^{10}-methylenetetrahydromethanopterin (CH_2═H_4MPT) to N^5,N^{10}-methenyltetrahydromethanopterin (CH≡H_4MPT^+) and dihydrogen. The formation of H_2, HD, and D_2 by Hmd isolated from *Methanobacterium thermoautotrophicum* strain Marburg (now called *Methanothermobacter marburgensis*) was studied in experiments in which either the methylene group of CH_2═H_4MPT or water was deuterium labeled.[125] The results indicated that Hmd catalyzes the transfer of a hydrogen, most likely a hydride, from the methylene group of CH_2 = H_4-MPT to a proton of water with formation of HD (50%). Evidence has been presented that HD is not an intermediate in the formation of dihydrogen.[126] Although Hmd is considered to be a novel type of hydrogenase, it does not catalyze the reversible oxidation of H_2 and does not catalyze the H/D exchange in the absence of the substrate (CH≡H_4MPT^+).

3.3. [NiFe]-Hydrogenases: Classification

Sequence comparisons of the large subunits of [NiFe]-hydrogenases revealed two very conserved regions surrounding the two pairs of cysteine ligands of the NiFe center, near the N and C termini of the sequence. The L1 and L2 patterns obtained in 2001[10a] have been updated by alignment of all

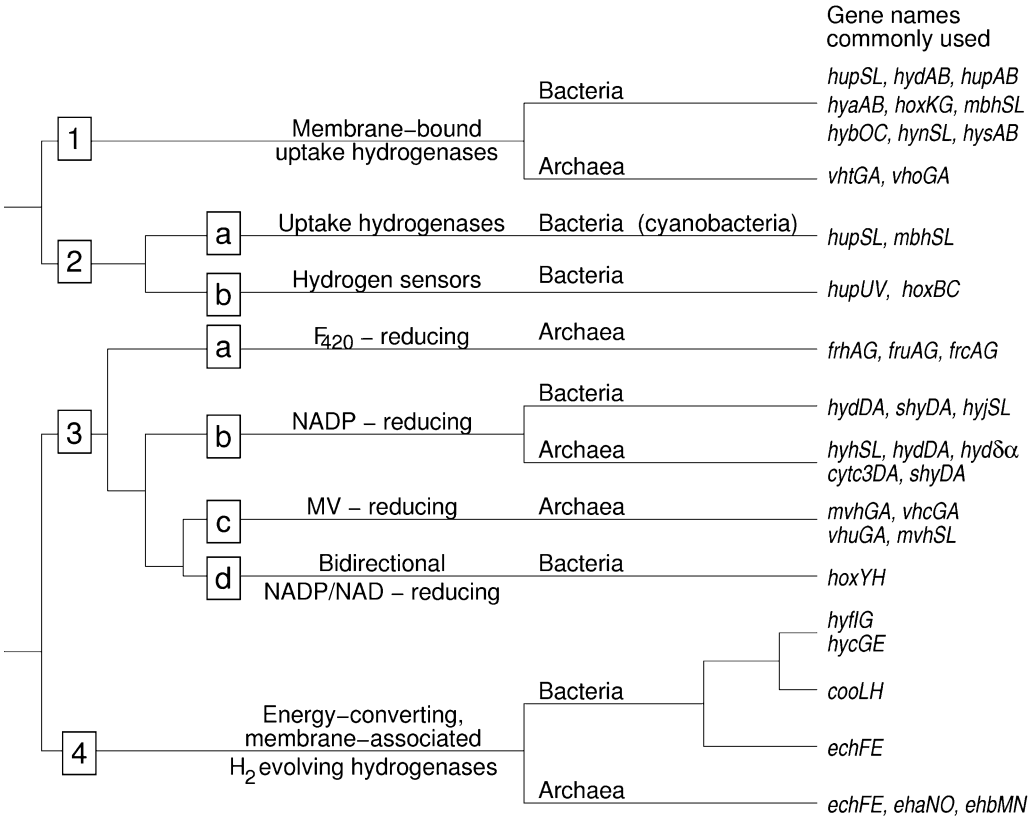

Figure 7. Schematic representation of the phylogenetic tree of [NiFe]-hydrogenases based on the complete sequences of the small and the large subunits (the same tree was obtained with each type of subunit), originally established by ref 10a.

the sequences now available. The complete sequences (i.e., those having two occurrences of the CxxC pattern) were submitted to PRATT[127,128] to obtain an initial pair of patterns common to all sequences of the same group. The two best patterns surrounding CxxC were retained as a starting point. Then, optimized patterns were obtained by successive searches in the Uniprot/KB database release 8.9[129] using ps_scan,[130,131] and refinement by hand until the intersection of their respective sets of responding sequences did not contain any false positives (Figure 6).

Each group is fully characterized by a pair of patterns. Some additional proteins could also be retrieved. They were discarded from our study either because they were sequence fragments or because they corresponded to duplicate entries nearly identical to the retained sequences. In the case of Vhu enzymes, the VhuA subunit bears the N-terminal pattern, whereas the VhuU subunit contains the C-terminal pattern. In only two cases did we find proteins with a divergent amino acid in a position encompassed in one of the characteristic patterns. They are K instead of H in O66988 (L2a of *Aquifex aeolicus*) and L instead of R in Q1PZL4 (L4 of *Candidatus Kuenenia*). These exceptions were not taken into account in the presented patterns. It is noteworthy that allowing these additional residues in the patterns did not result in the inclusion of any other protein into the set of positive sequences. These patterns define groups of [NiFe]-hydrogenases (Figure 7; Table 3), which are consistent with full sequence alignments and the cellular functions of the enzymes. At the end of the L2 pattern, in most of the cases, there is a conserved histidine residue, which is the endopeptidase cleavage site at the C terminus of the large subunit. At variance, the typical feature of group 4 hydrogenases is the presence of an arginine residue at the position of the conserved histidine (R was replaced by an L in only three sequences) as was demonstrated for *E. coli* HycE[89] (Figure 6). Proteolytic cleavage of the carboxyl terminus of the large subunit precursor is the final step in [NiFe]-hydrogenase biosynthesis;[14] it liberates a short polypeptide and triggers a conformational change, resulting in the closure of the bridge between the two metals of the NiFe center by the most C-terminally located cysteine residue and the formation of the complete hetero-binuclear center[90,132] (Figure 5a). The mature large subunit can then be assembled with the mature small subunit to form the functional heterodimer. C-terminal endopeptidases are specific; for example, HycI cleaves the precursor of *E. coli* hydrogenase-3 (Hyc), and HybD is specific for the maturation of *E. coli* hydrogenase-2 (Hyb). This specificity has also been observed for the hydrogenase-specific C-terminal endopeptidases in cyanobacteria.[133] The H_2 sensor proteins, called HupUV or HoxBC, group 2b, and also Ech from *M. barkeri*, Coo from *R. rubrum*, and Coo from *C. hydrogenoformans* lack the carboxyl-terminal extension cleaved in the precursor form of [NiFe]-hydrogenase large subunits. Thus, no protease is needed for maturation and the mechanism of interaction with a hydrogenase-specific chaperone needs to be assessed. *R. rubrum* (strain ATCC 11170) contains two hydrogenases belonging to group 4, one of the Coo type and one of the Hyc type. Accordingly, the large subunit CooH (Q2RUG9) ends at the conserved R, whereas HycE (Q2RXM4), which contains an R at the conserved position, has an extension of 32 amino acids ending also by an R.

3.3.1. Uptake [NiFe]-Hydrogenases (Group 1)

The membrane-bound respiratory hydrogenases perform respiratory hydrogen oxidation linked to quinone reduction. They link the oxidation of H_2 to the reduction of anaerobic electron acceptors, such as NO_3^-, SO_4^{2-}, fumarate, or CO_2

Table 3. Catalytic Subunits of [NiFe]- and [FeFe]-Hydrogenases and Their Classification[c]

Rmq	organism	length[a]	group[b]	AC	annotation
	Acetomicrobium flavidum DSM20663	179	S3d	Q59113	
	Acetomicrobium flavidum DSM20663	475	L3d	Q59114	
T	*Acidianus ambivalens* DSM3772, and Lei 10	420	S1	Q8NKV6	hydS
	Acidianus ambivalens DSM3772, and Lei 10	628	L1	Q8NKV3	hydL
T	*Acidiphilium cryptum* JF-5	373	S1	Q2DD49	AcryDRAFT_1977
	Acidiphilium cryptum JF-5	598	L1	Q2DD50	AcryDRAFT_1976
	Acidobacteria bacterium Ellin345	401	S1	Q1IIR3	Acid345_4237
	Acidobacteria bacterium Ellin345	563	L1	Q1IIR0	Acid345_4240
	Acidothermus cellulolyticus 11B	277	S3b	Q2DXJ9	AcelDRAFT_1301
	Acidothermus cellulolyticus 11B	431	L3b	Q2DXK0	AcelDRAFT_1300
T	*Actinobacillus succinogenes* 130Z	381	S1	Q3EIF8	AsucDRAFT_1393
	Actinobacillus succinogenes 130Z	569	L1	Q3EIG1	AsucDRAFT_1390
T	*Alcaligenes hydrogenophilus*	363	S1	P33375	hupS
	Alcaligenes hydrogenophilus	621	L1	P33374	hupL
	Alcaligenes hydrogenophilus M50	344	S2b	P94154	hoxB
	Alcaligenes hydrogenophilus M50	485	L2b	P94155	hoxC
T	*Alkalilimnicola ehrlichei* MLHE-1	371	S1	Q0A716	Mlg_2029
	Alkalilimnicola ehrlichei MLHE-1	595	L1	Q0A717	Mlg_2028
	Alkalilimnicola ehrlichei MLHE-1	339	S2b	Q0A734	Mlg_2011
	Alkalilimnicola ehrlichei MLHE-1	482	L2b	Q0A735	Mlg_2010
	Alkaliphilus metalliredigenes QYMF	582	LFe	Q3C9E8	AmetDRAFT_2889
	Alkaliphilus metalliredigenes QYMF	591	LFe	Q3C5M2	AmetDRAFT_1287
	Allochromatium vinosum	314	S1	Q5XQ37	hydS
	Allochromatium vinosum	576	L1	Q4KVK0	hydL
	Allochromatium vinosum	180	S3d	Q2KJQ3	hoxY
F	*Allochromatium vinosum*	349	L3d	Q2KJQ2	hoxH
	Anabaena siamensis TISTR8012	320	S2a	Q4G6A7	hupS
	Anabaena siamensis TISTR8012	531	L2a	Q84GM3	hupL
	Anabaena sp. PCC 7120	320	S2a	Q44215	hupS
	Anabaena sp. PCC 7120	531	L2a	Q44216	hupL
	Anabaena variabilis ATCC 29413	320	S2a	Q9ZAK3	hupS
	Anabaena variabilis ATCC 29413	531	L2a	Q9ZAK2	hupL
	Anabaena variabilis ATCC 29413	320	S2a	Q3M493	Ava_4596
	Anabaena variabilis ATCC 29413	531	L2a	Q3M494	Ava_4595
	Anabaena variabilis ATCC 29413	205	S3d	Q44515	hoxY
	Anabaena variabilis ATCC 29413	487	L3d	Q44517	hoxH
	Anabaena variabilis ATCC 29413	181	S3d	Q3M430	Ava_4659
	Anabaena variabilis ATCC 29413	487	L3d	Q3M428	Ava_4661
S	*Anabaena variabilis* IAM M58	181	S3d	Q9AJB7	hoxY
T	*Anaeromyxobacter dehalogenans* 2CP-C	374	S1	Q2IN73	Adeh_0481
	Anaeromyxobacter dehalogenans 2CP-C	577	L1	Q2IN69	Adeh_0478
	Anaeromyxobacter dehalogenans 2CP-C	491	S3c	Q2IH66	Adeh_4163
	Anaeromyxobacter dehalogenans 2CP-C	494	L3c	Q2IH67	Adeh_4162
T	*Aquifex aeolicus* VF5	353	S1	O66894	mbhS1
	Aquifex aeolicus VF5	633	L1	O66895	mbhL1
T	*Aquifex aeolicus* VF5	349	S1	O67095	mbhS2
	Aquifex aeolicus VF5	564	L1	O67092	mbhL2
	Aquifex aeolicus VF5	284	S2a	O66987	mbhS3
	Aquifex aeolicus VF5	416	L2a	O66988	mbhL3
T	*Archaeoglobus fulgidus* ATCC 49558/VC-16/DSM 4304/JCM 9628/ NBRC 100126	353	S1	O28890	AF_1381
	Archaeoglobus fulgidus ATCC 49558/VC-16/DSM 4304/JCM 9628/ NBRC 100126	569	L1	O28891	AF_1380
	Archaeoglobus fulgidus ATCC 49558/VC-16/DSM 4304/JCM 9628/ NBRC 100126	293	S3c	O28898	AF_1373
	Archaeoglobus fulgidus ATCC 49558/VC-16/DSM 4304/JCM 9628/ NBRC 100126	458	L3c	O28899	AF_1372
T	*Azorhizobium caulinodans* ORS571	360	S1	Q6PTB6	hupS
	Azorhizobium caulinodans ORS571	604	L1	Q6PTB5	hupL
	Azorhizobium caulinodans ORS571	340	S2b	Q6PTB9	hupU
	Azorhizobium caulinodans ORS571	490	L2b	Q6PTB8	hupV
T	*Azotobacter chroococcum* str. mcd1	354	S1	P18190	hupA
	Azotobacter chroococcum str. mcd1	601	L1	P18191	hupL
T	*Azotobacter vinelandii* ATCC 13705/OP1/DSM 366/NCIB 11614/ LMG 3878/UW	358	S1	P21950	hoxK
	Azotobacter vinelandii ATCC 13705/OP1/DSM 366/NCIB 11614/ LMG 3878/UW	602	L1	P21949	hoxG
T	*Azotobacter vinelandii* AvOP	358	S1	Q4IUP9	AvinDRAFT_1758
	Azotobacter vinelandii AvOP	602	L1	Q4IUQ0	AvinDRAFT_1759
	Azotobacter vinelandii AvOP	256	S3b	Q4J217	AvinDRAFT_7309
	Azotobacter vinelandii AvOP	429	L3b	Q4J218	AvinDRAFT_7310
	Bacteroides fragilis NCTC 9343	489	LFe	Q5L986	BF3662
	Bacteroides fragilis YCH46	489	LFe	Q64PE7	BF3892

Table 3 (Continued)

Rmq	organism	length[a]	group[b]	AC	annotation
	Bacteroides thetaiotaomicron ATCC 29148/DSM 2079/ NCTC 10582/E50/VPI-5482	482	LFe	Q8A6P3	BT_1834
	Bacteroides thetaiotaomicron ATCC 29148/DSM 2079/ NCTC 10582/E50/VPI-5482	588	LFe	Q8ABI6	BT_0124
T	*Bradyrhizobium japonicum* USDA 110	363	S1	P12635	hupA
	Bradyrhizobium japonicum USDA 110	596	L1	P12636	hupB
T	*Bradyrhizobium japonicum* USDA 110	363	S1	Q9ANR0	hupS
	Bradyrhizobium japonicum USDA 110	596	L1	Q9ANQ9	hupL
	Bradyrhizobium japonicum USDA 110	338	S2b	Q45254	hupU
	Bradyrhizobium japonicum USDA 110	479	L2b	Q45255	hupV
T	*Bradyrhizobium* sp. BTAi1	363	S1	Q35NX2	BradDRAFT_6907
	Bradyrhizobium sp. BTAi1	596	L1	Q35NX1	BradDRAFT_6908
	Bradyrhizobium sp. BTAi1	320	S2a	Q35L02	BradDRAFT_5525
	Bradyrhizobium sp. BTAi1	532	L2a	Q35L01	BradDRAFT_5526
	Bradyrhizobium sp. BTAi1	329	S2b	Q35NX4	BradDRAFT_6905
	Bradyrhizobium sp. BTAi1	479	L2b	Q35NX3	BradDRAFT_6906
T	*Bradyrhizobium* sp. UPM1029 Z89	363	S1	Q1KZV7	hupS
	Bradyrhizobium sp. UPM1029 Z89	596	L1	Q1KZV6	hupL
T	*Bradyrhizobium* sp. UPM1167 M5	366	S1	Q1KZX5	hupS
	Bradyrhizobium sp. UPM1167 M5	596	L1	Q1KZX4	hupL
	Burkholderia cenocepacia HI2424	340	S2b	Q4LG95	Bcen2424DRAFT_0064
F	*Burkholderia cenocepacia* HI2424	274	L2b	Q4LG94	Bcen2424DRAFT_0065
	Burkholderia cenocepacia HI2424	188	S3d	Q4LGC7	Bcen2424DRAFT_0024
F	*Burkholderia cenocepacia* HI2424	237	L3d	Q4LGC8	Bcen2424DRAFT_0023
T	*Burkholderia vietnamiensis* G4	411	S1	Q4BRM6	Bcep1808DRAFT_7155
	Burkholderia vietnamiensis G4	618	L1	Q4BRM7	Bcep1808DRAFT_7154
	Burkholderia vietnamiensis G4	345	S2b	Q4BRP2	Bcep1808DRAFT_7140
	Burkholderia vietnamiensis G4	485	L2b	Q4BRP3	Bcep1808DRAFT_7139
	Burkholderia xenovorans LB400	193	S3d	Q13HK9	Bxe_C0530
	Burkholderia xenovorans LB400	504	L3d	Q13HK8	Bxe_C0531
	Caldicellulosiruptor saccharolyticus DSM 8903	154	S4	Q2ZDW0	CsacDRAFT_2370
F	*Caldicellulosiruptor saccharolyticus* DSM 8903	83	L4	Q2ZEI5	CSACDRAFT_2574
	Caldicellulosiruptor saccharolyticus DSM 8903	579	LFe	Q2ZJ38	CsacDRAFT_1631
T	*Campylobacter coli* RM2228	379	S1	Q4HHS0	CCO0675
	Campylobacter coli RM2228	571	L1	Q4HHS1	CCO0676
S	*Campylobacter coli* RM2228	499	S1	Q4HEP6	CCO1507
T	*Campylobacter jejuni* NCTC 11168	379	S1	Q0P8Y9	hydA
	Campylobacter jejuni NCTC 11168	571	L1	Q0P8Z0	hydB
S	*Campylobacter jejuni* NCTC 11168	497	S1	Q0P8L5	hydA2
T	*Campylobacter jejuni* RM1221	360	S1	Q5HTJ6	hydA
	Campylobacter jejuni RM1221	571	L1	Q5HTJ7	hydB
S	*Campylobacter jejuni* RM1221	497	S1	Q5HT18	CJE1586
	Campylobacter lari RM2100	339	S1	Q4HKM2	CLA1080
	Campylobacter lari RM2100	571	L1	Q4HKM1	CLA1081
S	*Campylobacter lari* RM2100	544	S1	Q4HLR4	CLA0777
T	*Campylobacter upsaliensis* RM3195	379	S1	Q4HQM5	CUP0084
	Campylobacter upsaliensis RM3195	571	L1	Q4HQM6	CUP0085
S	*Campylobacter upsaliensis* RM3195	539	S1	Q4HR27	CUP1342
	Candidatus Kuenenia stuttgartiensis	262	S4	Q1PZL3	hycG
	Candidatus Kuenenia stuttgartiensis	531	L4	Q1PZL4	hycE
T	*Carboxydothermus hydrogenoformans* Z-2901	354	S1	Q3ABV6	CHY_1546
	Carboxydothermus hydrogenoformans Z-2901	475	L1	Q3ABV7	CHY_1545
	Carboxydothermus hydrogenoformans Z-2901	143	S4	Q3AB34	cool
	Carboxydothermus hydrogenoformans Z-2901	360	L4	Q3AB37	cooH
	Chlamydomonas moewusii SAG 24.91	458	LFe	Q56UD8	hydA1
	Chlamydomonas reinhardtii 21gr	497	LFe	Q9FYU1	hyd1
	Chlamydomonas reinhardtii 21gr, and Cc425	505	LFe	Q8VZZ0	hydA2
	Chlamydomonas reinhardtii SE	505	LFe	Q6T533	None
	Chlorella fusca	436	LFe	Q8VX03	hydA
	Chlorobaculum tepidum ATCC 49652/DSM 12025/TLS	255	S3b	Q8KB96	hydD
	Chlorobaculum tepidum ATCC 49652/DSM 12025/TLS	424	L3b	Q8KB95	hydA
	Chlorobium chlorochromatii CaD3	247	S3b	Q3AU05	Cag_0244
	Chlorobium chlorochromatii CaD3	426	L3b	Q3AU04	Cag_0245
T	*Chlorobium ferrooxidans* DSM 13031	361	S1	Q0YQ62	CferDRAFT_0349
	Chlorobium ferrooxidans DSM 13031	572	L1	Q0YQ63	CferDRAFT_0348
	Chlorobium ferrooxidans DSM 13031	251	S3b	Q0YRW6	CferDRAFT_1020
	Chlorobium ferrooxidans DSM 13031	424	L3b	Q0YRW5	CferDRAFT_1021
T	*Chlorobium limicola* DSM 245	361	S1	Q44R92	ClimDRAFT_2251
	Chlorobium limicola DSM 245	572	L1	Q44R93	ClimDRAFT_2250
	Chlorobium limicola DSM 245	252	S3b	Q44QJ9	ClimDRAFT_2180
	Chlorobium limicola DSM 245	424	L3b	Q44QJ8	ClimDRAFT_2181
T	*Chlorobium phaeobacteroides* BS1	363	S1	Q4AM64	Cphamn1DRAFT_2778
	Chlorobium phaeobacteroides BS1	572	L1	Q4AM63	Cphamn1DRAFT_2779
	Chlorobium phaeobacteroides BS1	254	S3b	Q4AJW0	Cphamn1DRAFT_1759

Table 3 (Continued)

Rmq	organism	length[a]	group[b]	AC	annotation
	Chlorobium phaeobacteroides BS1	439	L3b	Q4AJV9	Cphamn1DRAFT_1760
T	*Chlorobium phaeobacteroides* DSM 266	360	S1	Q43JF7	Cpha266DRAFT_2299
	Chlorobium phaeobacteroides DSM 266	572	L1	Q43JF6	Cpha266DRAFT_2300
	Chlorobium phaeobacteroides DSM 266	254	S3b	Q43IL9	Cpha266DRAFT_2142
	Chlorobium phaeobacteroides DSM 266	424	L3b	Q43IL8	Cpha266DRAFT_2143
	Chloroflexus aurantiacus J-10-fl	323	S2a	Q3E285	CaurDRAFT_0071
	Chloroflexus aurantiacus J-10-fl	545	L2a	Q3E284	CaurDRAFT_0072
	Chloroflexus aurantiacus J-10-fl	177	S3d	Q3E4S8	CaurDRAFT_0835
	Chloroflexus aurantiacus J-10-fl	485	L3d	Q3E4S7	CaurDRAFT_0836
T	*Citrobacter freundii*	375	S1	Q46045	hyaA
	Citrobacter freundii	597	L1	Q46046	hyaB
	Clostridium acetobutylicum ATCC 824/DSM 792/JCM 1419/ LMG 5710/VKM B-1787	291	S1	Q9AMN6	hupS
	Clostridium acetobutylicum ATCC 824/DSM 792/JCM 1419/ LMG 5710/VKM B-1787	428	L1	Q9AMN5	hupL
	Clostridium acetobutylicum ATCC 824/DSM 792/JCM 1419/ LMG 5710/VKM B-1787	582	LFe	Q59262	hydA
	Clostridium acetobutylicum ATCC 824/DSM 792/JCM 1419/ LMG 5710/VKM B-1787	450	LFe	Q97E85	CA_C3230
	Clostridium beijerincki NCIMB 8052	291	S1	Q2WRL7	CbeiDRAFT_3284
	Clostridium beijerincki NCIMB 8052	486	L1	Q2WRL8	CbeiDRAFT_3283
	Clostridium beijerincki NCIMB 8052	644	LFe	Q2WI78	CbeiDRAFT_0272
	Clostridium beijerincki NCIMB 8052	449	LFe	Q2WK96	CbeiDRAFT_0932
	Clostridium beijerincki NCIMB 8052	461	LFe	Q2WUD6	CbeiDRAFT_3964
	Clostridium beijerincki NCIMB 8052	567	LFe	Q2WVX8	CbeiDRAFT_4712
	Clostridium difficile 630	461	LFe	Q180F8	hydA
	Clostridium difficile 630	478	LFe	Q180A2	CD3258
	Clostridium difficile 630	593	LFe	Q180Q5	hymC
	Clostridium paraputrificum	582	LFe	Q6F4C7	hydA
	Clostridium pasteurianum ATCC 6013/DSM 525/NCIB 9486/ VKM B-1774/W5	574	LFe	P29166	none
	Clostridium perfringens 13/type A	449	LFe	Q8XNQ6	CPE0276
	Clostridium perfringens 13/type A	572	LFe	Q9RHU8	hydA
	Clostridium perfringens 13/type A	490	LFe	Q8XHB0	CPE2575
	Clostridium perfringens ATCC 13124	449	LFe	Q0TUF9	CPF_0270
	Clostridium perfringens ATCC 13124	490	LFe	Q0TM76	CPF_2900
	Clostridium perfringens ATCC 13124	572	LFe	Q0TMV5	hydA
	Clostridium perfringens ATCC 13124	696	LFe	Q0TS68	CPF_1076
	Clostridium perfringens NCTC 8237	572	LFe	Q9ZNE4	hydA
	Clostridium perfringens SM101	449	LFe	Q0SWA8	CPR_0261
	Clostridium perfringens SM101	490	LFe	Q0SPY1	CPR_2579
	Clostridium perfringens SM101	572	LFe	Q0SQK1	hydA
	Clostridium perfringens SM101	696	LFe	Q0SUE5	CPR_0938
	Clostridium phytofermentans ISDg	144	S4	Q1FP26	CphyDRAFT_3348
	Clostridium phytofermentans ISDg	359	L4	Q1FP28	CPHYDRAFT_3346
	Clostridium phytofermentans ISDg	567	LFe	Q1FJL3	CphyDRAFT_2333
	Clostridium phytofermentans ISDg	484	LFe	Q1FJL6	CphyDRAFT_2330
	Clostridium phytofermentans ISDg	644	LFe	Q1FHS1	CphyDRAFT_0997
	Clostridium phytofermentans ISDg	582	LFe	Q1FFT8	CphyDRAFT_0772
	Clostridium saccharobutylicum P262	574	LFe	Q59261	hydA
	Clostridium saccharoperbutylacetonicum N1-4	562	LFe	Q5MIB2	HupA
	Clostridium sp. OhILAs	567	LFe	Q1F047	ClosDRAFT_0965
	Clostridium tetani Massachusetts/E88	448	LFe	Q899J2	CTC_00184
	Clostridium tetani Massachusetts/E88	494	LFe	Q891G1	CTC_02417
F	*Clostridium thermocellum* ATCC 27405	579	LFe	Q9XC55	hydA
	Clostridium thermocellum ATCC 27405	145	S4	Q4CDJ8	CtheDRAFT_1259
	Clostridium thermocellum ATCC 27405	359	L4	Q4CDJ6	CtheDRAFT_1261
	Clostridium thermocellum ATCC 27405	644	LFe	Q4CDI0	CtheDRAFT_1275
	Clostridium thermocellum ATCC 27405	566	LFe	Q4CGI4	CtheDRAFT_2180
	Clostridium thermocellum ATCC 27405	582	LFe	Q4CDK8	CtheDRAFT_1129
T	*Corynebacterium diphtheriae* ATCC 700971/NCTC 13129/ biotype gravis	418	S1	Q6NIU4	DIP0672
	Corynebacterium diphtheriae ATCC 700971/NCTC 13129/ biotype gravis	581	L1	Q6NIU3	DIP0673
	Crocosphaera watsonii WH 8501	320	S2a	Q4BUZ6	CwatDRAFT_0515
	Crocosphaera watsonii WH 8501	531	L2a	Q4BUZ7	CwatDRAFT_0516
T	*Cupriavidus necator* ATCC 17699/H16/DSM 428/NCIB 10442/ Stanier 337	360	S1	P31892	hoxK
	Cupriavidus necator ATCC 17699/H16/DSM 428/NCIB 10442/ Stanier 337	617	L1	P31891	hoxG
	Cupriavidus necator ATCC 17699/H16/DSM 428/NCIB 10442/ Stanier 337	209	S3d	P22319	hoxY
	Cupriavidus necator ATCC 17699/H16/DSM 428/NCIB 10442/ Stanier 337	487	L3d	P22320	hoxH

Table 3 (Continued)

Rmq	organism	length[a]	group[b]	AC	annotation
	Cupriavidus necator H16	351	S1	Q7WXQ4	PHG064
	Cupriavidus necator H16	603	L1	Q7WXQ3	PHG065
	Cupriavidus necator H16 PLASMID = megaplasmid pHG1	347	S2b	P95603	hoxB
	Cupriavidus necator H16 PLASMID = megaplasmid pHG1	485	L2b	P95604	hoxC
	Cyanothece sp. ATCC 51142	320	S2a	Q0ZA87	hupS
	Cyanothece sp. ATCC 51142	531	L2a	Q0ZA86	hupL
T	*Dechloromonas aromatica* RCB	363	S1	Q478L5	Daro_3989
	Dechloromonas aromatica RCB	598	L1	Q478L6	Daro_3988
	Dechloromonas aromatica RCB	394	S1	Q478N0	Daro_3974
	Dechloromonas aromatica RCB	570	L1	Q478N3	Daro_3971
	Dechloromonas aromatica RCB	311	S2a	Q47C13	Daro_2888
	Dechloromonas aromatica RCB	505	L2a	Q47C12	Daro_2889
	Dechloromonas aromatica RCB	333	S2b	Q478P3	Daro_3961
	Dechloromonas aromatica RCB	472	L2b	Q478P4	Daro_3960
	Dechloromonas aromatica RCB	182	S3d	Q47HE4	Daro_0981
	Dechloromonas aromatica RCB	487	L3d	Q47HE3	Daro_0982
	Dehalococcoides ethenogenes 195	354	S1	Q3ZA87	DET0111
	Dehalococcoides ethenogenes 195	526	L1	Q3ZA88	DET0110
	Dehalococcoides ethenogenes 195	312	S3c	Q3Z8U3	DET0614
	Dehalococcoides ethenogenes 195	479	L3c	Q3Z8U2	DET0615
	Dehalococcoides ethenogenes 195	155	S4	Q3Z861	DET0862
	Dehalococcoides ethenogenes 195	359	L4	Q3Z856	DET0867
	Dehalococcoides ethenogenes 195	573	LFe	Q3ZA52	DET0147
	Dehalococcoides sp. BAV1	354	S1	Q2DW85	DehaBAV1DRAFT_1259
	Dehalococcoides sp. BAV1	526	L1	Q2DW84	DehaBAV1DRAFT_1260
	Dehalococcoides sp. BAV1	312	S3c	Q2DUV4	DehaBAV1DRAFT_0364
	Dehalococcoides sp. BAV1	479	L3c	Q2DUV5	DehaBAV1DRAFT_0363
	Dehalococcoides sp. BAV1	155	S4	Q2DW10	DehaBAV1DRAFT_0779
	Dehalococcoides sp. BAV1	359	L4	Q2DW05	DehaBAV1DRAFT_0784
	Dehalococcoides sp. BAV1	573	LFe	Q2DWB9	DehaBAV1DRAFT_1225
	Dehalococcoides sp. CBDB1	354	S1	Q3ZWL4	hupS
	Dehalococcoides sp. CBDB1	526	L1	Q3ZWL5	hupL
	Dehalococcoides sp. CBDB1	312	S3c	Q3ZWZ2	cbdbA596
	Dehalococcoides sp. CBDB1	479	L3c	Q3ZWZ1	cbdbA597
	Dehalococcoides sp. CBDB1	155	S4	Q3ZXK1	echC
	Dehalococcoides sp. CBDB1	359	L4	Q3ZXP4	cbdbA850
	Dehalococcoides sp. CBDB1	573	LFe	Q3ZWM9	hymC
T	δ-proteobacterium MLMS-1	299	S1	Q1NRB7	MldDRAFT_4185
	δ-proteobacterium MLMS-1	504	L1	Q1NRB8	MldDRAFT_4184
T	δ-proteobacterium MLMS-1	331	S1	Q1NST6	MldDRAFT_0299
	δ-proteobacterium MLMS-1	515	L1	Q1NM23	MldDRAFT_2844
	δ-proteobacterium MLMS-1	366	S3c	Q1NJF2	MldDRAFT_2071
	δ-proteobacterium MLMS-1	496	L3c	Q1NJF1	MldDRAFT_2072
	δ-proteobacterium MLMS-1	362	S3c	Q1NPA6	MldDRAFT_3745
	δ-proteobacterium MLMS-1	496	L3c	Q1NPA7	MldDRAFT_3744
	δ-proteobacterium MLMS-1	176	S3d	Q1NQY4	MldDRAFT_4737
	δ-proteobacterium MLMS-1	473	L3d	Q1NQY3	MldDRAFT_4738
T	*Desulfitobacterium dehalogenans*	362	S1	Q9RPI3	hydA
	Desulfitobacterium dehalogenans	516	L1	Q9RPI2	hydB
T	*Desulfitobacterium hafniense* DCB-2	359	S1	Q191Z4	Dhaf_2515
	Desulfitobacterium hafniense DCB-2	570	L1	Q191Z3	Dhaf_2516
T	*Desulfitobacterium hafniense* DCB-2	362	S1	Q194H4	Dhaf_1985
	Desulfitobacterium hafniense DCB-2	518	L1	Q194H5	Dhaf_1984
T	*Desulfitobacterium hafniense* DCB-2	316	S1	Q192I6	Dhaf_2431
	Desulfitobacterium hafniense DCB-2	517	L1	Q192I7	Dhaf_2430
T	*Desulfitobacterium hafniense* DCB-2	375	S1	Q18R74	Dhaf_0608
	Desulfitobacterium hafniense DCB-2	465	L1	Q18R73	Dhaf_0609
	Desulfitobacterium hafniense DCB-2	155	S4	Q18YX3	DHAF_3332
	Desulfitobacterium hafniense DCB-2	507	L4	Q18YX2	Dhaf_3333
	Desulfitobacterium hafniense DCB-2	1150	LFe	Q18XD7	Dhaf_4051
	Desulfitobacterium hafniense DCB-2	425	LFe	Q18R81	Dhaf_0601
	Desulfitobacterium hafniense DCB-2	454	LFe	Q18RP8	Dhaf_0459
T	*Desulfitobacterium hafniense* DCB-2	527	LFe	Q18T66	Dhaf_1708
T	*Desulfitobacterium hafniense* Y51	359	S1	Q24VB5	DSY2238
	Desulfitobacterium hafniense Y51	573	L1	Q24VB4	DSY2239
T	*Desulfitobacterium hafniense* Y51	319	S1	Q24VQ2	DSY2101
	Desulfitobacterium hafniense Y51	517	L1	Q24VQ3	DSY2100
T	*Desulfitobacterium hafniense* Y51	374	S1	Q24ZF7	DSY0796
	Desulfitobacterium hafniense Y51	478	L1	Q24ZF8	DSY0795
T	*Desulfitobacterium hafniense* Y51	362	S1	Q24 × 54	DSY1599
	Desulfitobacterium hafniense Y51	518	L1	Q24 × 55	DSY1598
	Desulfitobacterium hafniense Y51	155	S4	Q24ST9	hycG
	Desulfitobacterium hafniense Y51	554	L4	Q24ST8	hycE
	Desulfitobacterium hafniense Y51	425	LFe	Q24ZF0	DSY0803

Table 3 (Continued)

Rmq	organism	length[a]	group[b]	AC	annotation
	Desulfitobacterium hafniense Y51	460	LFe	Q24PC7	DSY4326
T	*Desulfitobacterium hafniense* Y51	555	LFe	Q24N91	DSY4712
	Desulfitobacterium hafniense Y51	1150	LFe	Q24Z17	DSY0936
T	*Desulfomicrobium baculatum* DSM 1743	315	S1	P13063	none
	Desulfomicrobium baculatum DSM 1743	513	L1	P13065	none
T	*Desulfotalea psychrophila* LSv54/DSM 12343	364	S1	Q6AQS0	hynB
	Desulfotalea psychrophila LSv54/DSM 12343	566	L1	Q6AQR9	hynA
	Desulfotalea psychrophila LSv54/DSM 12343	300	S1	Q6ARY6	DP0160
	Desulfotalea psychrophila LSv54/DSM 12343	499	L1	Q6ARY7	DP0159
	Desulfotalea psychrophila LSv54/DSM 12343	313	S3c	Q6API3	DP1012
1CxxC	*Desulfotalea psychrophila* LSv54/DSM 12343	456	L3c	Q6API2	DP1013
	Desulfotalea psychrophila LSv54/DSM 12343	207	S3d	Q6AL35	DP2211
	Desulfotalea psychrophila LSv54/DSM 12343	471	L3d	Q6AL34	DP2212
	Desulfotalea psychrophila LSv54/DSM 12343	471	LFe	Q6AR16	DP0479
	Desulfotalea psychrophila LSv54/DSM 12343	483	LFe	Q6AKL7	DP2379
	Desulfotomaculum reducens MI-1	429	LFe	Q2D600	DredDRAFT_2292
T	*Desulfotomaculum reducens* MI-1	520	LFe	Q2CZF6	DredDRAFT_3054
	Desulfotomaculum reducens MI-1	593	LFe	Q2D1M4	DredDRAFT_0478
	Desulfotomaculum reducens MI-1	659	LFe	Q2D1M7	DredDRAFT_0475
S	*Desulfovibrio desulfuricans* ATCC 27774/DSM 6949	268	S1	P13061	hydA
T	*Desulfovibrio desulfuricans* G20	321	S1	Q9AM33	hynB
1CxxC	*Desulfovibrio desulfuricans* G20	554	L1	Q9AM32	hynA
T	*Desulfovibrio desulfuricans* G20	123	SFe	Q9AM35	hydB
	Desulfovibrio desulfuricans G20	421	LFe	Q9AM36	hydA
	Desulfovibrio desulfuricans G20	294	S1	Q30ZG5	Dde_2134
	Desulfovibrio desulfuricans G20	488	L1	Q30ZG4	Dde_2135
T	*Desulfovibrio desulfuricans* G20	317	S1	Q30ZG2	Dde_2137
	Desulfovibrio desulfuricans G20	568	L1	Q30ZG1	Dde_2138
T	*Desulfovibrio desulfuricans* G20	321	S1	Q30UU8	Dde_3755
	Desulfovibrio desulfuricans G20	554	L1	Q30UU7	Dde_3756
T	*Desulfovibrio desulfuricans* G20	113	SFe	Q30Z19	Dde_2280
	Desulfovibrio desulfuricans G20	439	LFe	Q30Z18	Dde_2281
	Desulfovibrio desulfuricans G20	483	LFe	Q314X0	Dde_0725
	Desulfovibrio desulfuricans G20	458	LFe	Q315X0	Dde_0475
T	*Desulfovibrio desulfuricans* G20	123	SFe	Q317L3	Dde_0082
	Desulfovibrio desulfuricans G20	421	LFe	Q317L4	Dde_0081
	Desulfovibrio fructosovorans	585	LFe	Q46508	none
T	*Desulfovibrio fructosovorans* ATCC 49200/DSM 3604/ VKM B-1801/JJ	313	S1	P18187	hydA
	Desulfovibrio fructosovorans ATCC 49200/DSM 3604/ VKM B-1801/JJ	563	L1	P18188	hydB
T	*Desulfovibrio fructosovorans* DSM 3604	124	SFe	O08312	hydB
	Desulfovibrio fructosovorans DSM 3604	421	LFe	O08311	hydA
	Desulfovibrio gigas	288	S1	P12943	hydA
	Desulfovibrio gigas	550	L1	P12944	hydB
	Desulfovibrio gigas	147	S4	Q7WT80	echC
	Desulfovibrio gigas	358	L4	Q7WT78	echE
T	*Desulfovibrio vulgaris* str. Miyazaki	317	S1	P21853	hydA
	Desulfovibrio vulgaris str. Miyazaki	567	L1	P21852	hydB
	Desulfovibrio vulgaris Hildenborough	606	LFe	Q46606	hydC
T	*Desulfovibrio vulgaris* subsp. *oxamicus* str. Monticello	124	SFe	P13628	hydB
	Desulfovibrio vulgaris subsp. *oxamicus* str. Monticello	421	LFe	P13629	hydA
T	*Desulfovibrio vulgaris* subsp. *vulgaris* DP4	317	S1	Q0EP66	DvulDRAFT_2627
1CxxC	*Desulfovibrio vulgaris* subsp. *vulgaris* DP4	488	L1	Q0EP67	DvulDRAFT_2626
T	*Desulfovibrio vulgaris* subsp. *vulgaris* DP4	324	S1	Q0ELC9	DvulDRAFT_1771
	Desulfovibrio vulgaris subsp. *vulgaris* DP4	549	L1	Q0ELC8	DvulDRAFT_1772
T	*Desulfovibrio vulgaris* subsp. *vulgaris* DP4	317	S1	Q0EP69	DvulDRAFT_2624
	Desulfovibrio vulgaris subsp. *vulgaris* DP4	566	L1	Q0EP70	DvulDRAFT_2623
	Desulfovibrio vulgaris subsp. *vulgaris* DP4	144	S4	Q0EJG4	DvulDRAFT_1451
	Desulfovibrio vulgaris subsp. *vulgaris* DP4	366	L4	Q0EJG1	DvulDRAFT_1454
	Desulfovibrio vulgaris subsp. *vulgaris* DP4	157	S4	Q0EK45	DvulDRAFT_1676
	Desulfovibrio vulgaris subsp. *vulgaris* DP4	358	L4	Q0EK43	DvulDRAFT_1678
T	*Desulfovibrio vulgaris* subsp. *vulgaris* DP4	123	SFe	Q0ENS7	DvulDRAFT_2757
	Desulfovibrio vulgaris subsp. *vulgaris* DP4	606	LFe	Q0ENS8	DvulDRAFT_2756
	Desulfovibrio vulgaris subsp. *vulgaris* DP4	421	LFe	Q0ENS6	DvulDRAFT_2758
T	*Desulfovibrio vulgaris* subsp. *vulgaris* str. Hildenborough	317	S1	Q06173	hynB1
	Desulfovibrio vulgaris subsp. *vulgaris* str. Hildenborough	566	L1	Q72AS0	hynA-1
T	*Desulfovibrio vulgaris* subsp. *vulgaris* str. Hildenborough	324	S1	P61429	hynB2
	Desulfovibrio vulgaris subsp. *vulgaris* str. Hildenborough	549	L1	Q728S7	hynA-2
T	*Desulfovibrio vulgaris* subsp. *vulgaris* str. Hildenborough	317	S1	Q72AS4	hysB
	Desulfovibrio vulgaris subsp. *vulgaris* str. Hildenborough	510	L1	Q72AS3	hysA
	Desulfovibrio vulgaris subsp. *vulgaris* str. Hildenborough	157	S4	Q72EY6	DVU_0432
	Desulfovibrio vulgaris subsp. *vulgaris* str. Hildenborough	358	L4	Q72EY8	DVU_0430
	Desulfovibrio vulgaris subsp. *vulgaris* str. Hildenborough	144	S4	Q729R1	DVU_2288

Table 3 (Continued)

Rmq	organism	length[a]	group[b]	AC	annotation
	Desulfovibrio vulgaris subsp. *vulgaris* str. Hildenborough	366	L4	Q729Q8	DVU_2291
T	*Desulfovibrio vulgaris* subsp. *vulgaris* str. Hildenborough	123	SFe	P07603	hydB
	Desulfovibrio vulgaris subsp. *vulgaris* str. Hildenborough	421	LFe	P07598	hydA
	Desulfovibrio vulgaris subsp. *vulgaris* str. Hildenborough	606	LFe	Q72B67	hydC
	Entamoeba histolytica	468	LFe	Q9GTX0	none
	Entamoeba histolytica HM-1:IMSS	468	LFe	Q51EJ9	9.t00061
	Entamoeba histolytica HM-1:IMSS	472	LFe	Q50YQ4	131.t00027
	Entamoeba histolytica HM-1:IMSS	504	LFe	Q511D6	103.t00038
	Entamoeba histolytica HM-1:IMSS	504	LFe	Q869B1	none
T	*Escherichia coli* 536	372	S1	Q0TDB3	ECP_3083
	Escherichia coli 536	567	L1	Q0TDB6	ECP_3080
T	*Escherichia coli* 536	372	S1	Q0TJ89	ECP_0977
	Escherichia coli 536	597	L1	Q0TJ88	ECP_0978
	Escherichia coli 536	255	S4	Q0TEF8	ECP_2682
	Escherichia coli 536	569	L4	Q0TEF6	ECP_2684
T	*Escherichia coli* K12	372	S1	P69741	hyb0
	Escherichia coli K12	566	L1	P0ACE0	hybC
T	*Escherichia coli* K12	372	S1	P69739	hyaA
	Escherichia coli K12	597	L1	P0ACD8	hyaB
	Escherichia coli K12	255	S4	P16433	hycG
	Escherichia coli K12	569	L4	P16431	hycE
	Escherichia coli K12	252	S4	P77668	hyfI
	Escherichia coli K12	555	L4	P77329	hyfG
T	*Escherichia coli* O157:H7	372	S1	P69743	hyb0
	Escherichia coli O157:H7	566	L1	P0ACE1	hybC
T	*Escherichia coli* O157:H7	372	S1	Q8XC39	hyaA
	Escherichia coli O157:H7	597	L1	Q8XC37	hyaB
	Escherichia coli O157:H7	252	S4	Q8XBB8	hyfI
	Escherichia coli O157:H7	569	L4	Q8X833	hycE
	Escherichia coli O157:H7	252	S4	Q7ABN7	ECs3351
	Escherichia coli O157:H7	569	L4	Q7ABB8	ECs3577
	Escherichia coli O157:H7	255	S4	Q8X838	hycG
	Escherichia coli O157:H7	571	L4	Q8XBC0	hyfG
T	*Escherichia coli* O6 O6:H1	372	S1	P69742	hyb0
	Escherichia coli O6 O6:H1	567	L1	Q8CVQ8	hybC
T	*Escherichia coli* O6 O6:H1	372	S1	P69740	hyaA
	Escherichia coli O6 O6:H1	597	L1	Q8FJ64	hyaB
	Escherichia coli O6 O6:H1	255	S4	Q8FEM3	hycG
	Escherichia coli O6 O6:H1	569	L4	Q8CVS6	hycE
T	*Escherichia coli* UTI89	372	S1	Q1R6Z8	UTI89_C3419
	Escherichia coli UTI89	567	L1	Q1R701	hybC
T	*Escherichia coli* UTI89	381	S1	Q1RDP0	hyaA
	Escherichia coli UTI89	597	L1	Q1RDN9	hyaB
	Escherichia coli UTI89	255	S4	Q1R7Y0	hycG
	Escherichia coli UTI89	569	L4	Q1R7X8	hycE
	Eubacterium acidaminophilum	578	LFe	Q93SF7	hymC
T	*Flavobacterium johnsoniae* UW101	375	S1	Q1XS80	FjohDRAFT_2102
	Flavobacterium johnsoniae UW101	578	L1	Q1XS81	FjohDRAFT_2101
	Frankia alni ACN14A	359	S1	Q0RN51	hupS1
	Frankia alni ACN14A	597	L1	Q0RN50	hupL1
	Frankia alni ACN14A	320	S2a	Q0RPQ0	hupS2
	Frankia alni ACN14A	537	L2a	Q0RPQ1	hupL2
	Frankia sp. CcI3	354	S1	Q2JBM6	Francci3_1941
	Frankia sp. CcI3	597	L1	Q2JBM5	Francci3_1942
	Frankia sp. CcI3	323	S2a	Q2JE33	Francci3_1077
	Frankia sp. CcI3	535	L2a	Q2JE34	Francci3_1076
	Frankia sp. CcI3	278	S3b	Q2J4E9	Francci3_4497
	Frankia sp. CcI3	430	L3b	Q2J4F0	Francci3_4496
T	*Frankia* sp. EAN1pec	400	S1	Q3W6M0	Franean1DRAFT_3144
	Frankia sp. EAN1pec	573	L1	Q3W6M1	Franean1DRAFT_3143
	Frankia sp. EAN1pec	351	S1	Q3W427	Franean1DRAFT_2067
	Frankia sp. EAN1pec	596	L1	Q3W428	Franean1DRAFT_2066
T	*Geobacter metallireducens* GS-15	379	S1	Q39QC9	Gmet_3332
	Geobacter metallireducens GS-15	566	L1	Q39QD0	Gmet_3331
	Geobacter metallireducens GS-15	316	S3c	Q39QE2	Gmet_3319
	Geobacter metallireducens GS-15	473	L3c	Q39QE1	Gmet_3320
	Geobacter metallireducens GS-15	180	S3d	Q39WM2	Gmet_1112
	Geobacter metallireducens GS-15	476	L3d	Q39WM1	Gmet_1113
	Geobacter sp. FRC-32	247	S3b	Q0YHC7	GeobDRAFT_1311
	Geobacter sp. FRC-32	425	L3b	Q0YHC8	GeobDRAFT_1310
T	*Geobacter sulfurreducens* ATCC 51573/DSM 12127/PCA	361	S1	Q74GX1	GSU0123
	Geobacter sulfurreducens ATCC 51573/DSM 12127/PCA	564	L1	Q74GX2	GSU0122
T	*Geobacter sulfurreducens* ATCC 51573/DSM 12127/PCA	367	S1	Q74F27	GSU0782
	Geobacter sulfurreducens ATCC 51573/DSM 12127/PCA	560	L1	Q74F24	GSU0785

Table 3 (Continued)

Rmq	organism	length[a]	group[b]	AC	annotation
	Geobacter sulfurreducens ATCC 51573/DSM 12127/PCA	316	S3c	Q74AF8	GSU2418
	Geobacter sulfurreducens ATCC 51573/DSM 12127/PCA	473	L3c	Q74AF7	GSU2419
	Geobacter sulfurreducens ATCC 51573/DSM 12127/PCA	195	S3d	Q749M3	hoxY
	Geobacter sulfurreducens ATCC 51573/DSM 12127/PCA	478	L3d	Q749M4	hoxH
T	*Geobacter uraniumreducens* Rf4	377	S1	Q2DLI6	GuraDRAFT_1653
	Geobacter uraniumreducens Rf4	569	L1	Q2DLI7	GuraDRAFT_1652
T	*Geobacter uraniumreducens* Rf4	378	S1	Q2DR37	GuraDRAFT_3052
	Geobacter uraniumreducens Rf4	560	L1	Q2DR34	GuraDRAFT_3055
T	*Geobacter uraniumreducens* Rf4	373	S1	Q2DNV6	GuraDRAFT_2494
	Geobacter uraniumreducens Rf4	566	L1	Q2DNV7	GuraDRAFT_2493
	Geobacter uraniumreducens Rf4	251	S3b	Q2DLA6	GuraDRAFT_2090
	Geobacter uraniumreducens Rf4	425	L3b	Q2DLA5	GuraDRAFT_2091
	Geobacter uraniumreducens Rf4	147	S4	Q2DSU9	GuraDRAFT_4042
	Geobacter uraniumreducens Rf4	359	L4	Q2DR95	GURADRAFT_3339
	Giardia intestinalis	474	LFe	Q9BKJ3	none
	Giardia lamblia ATCC 50803 WB C6	474	LFe	Q7QXP8	none
	Gloeothece sp. PCC 6909	320	S2a	Q841J8	hupS
	Gloeothece sp. PCC 6909	531	L2a	Q841J7	hupL
	Hahella chejuensis KCTC 2396	187	S3d	Q2SQP7	HCH_00106
	Hahella chejuensis KCTC 2396	490	L3d	Q2SQP8	HCH_00105
	Halothermothrix orenii H 168	339	S1	Q2AI39	HoreDRAFT_1956
	Halothermothrix orenii H 168	484	L1	Q2AI40	HoreDRAFT_1955
	Halothermothrix orenii H 168	456	LFe	Q2AFL4	HoreDRAFT_1054
	Halothermothrix orenii H 168	570	LFe	Q2AFM5	HoreDRAFT_1043
	Halothermothrix orenii H 168	578	LFe	Q2AE40	HoreDRAFT_0394
	Halothermothrix orenii H 168	666	LFe	Q2AG82	HoreDRAFT_1681
	Helicobacter acinonychis Sheeba	385	S1	Q17XT5	hyaA
	Helicobacter acinonychis Sheeba	578	L1	Q17XT4	hyaB
T	*Helicobacter hepaticus* ATCC 51449/3B1	386	S1	Q7VK36	hyaA
	Helicobacter hepaticus ATCC 51449/3B1	576	L1	Q7VK35	hyaB
S	*Helicobacter hepaticus* ATCC 51449/3B1	552	S1	Q7VJP5	HH_0198
	Helicobacter pylori ATCC 700392/26695	384	S1	O25348	HP_0631
	Helicobacter pylori ATCC 700392/26695	578	L1	O25349	HP_0632
	Helicobacter pylori HPAG1	384	S1	Q1CTP1	HPAG1_0614
	Helicobacter pylori HPAG1	578	L1	Q1CTP0	HPAG1_0615
	Helicobacter pylori J99	384	S1	Q9ZLK5	hyaA
	Helicobacter pylori J99	578	L1	Q9ZLK4	hyaB
	Heliobacillus mobilis	606	LFe	Q1MSH5	hydA
T	*Lawsonia intracellularis* PHE/MN1-00	418	S1	Q1MR83	hyaA
	Lawsonia intracellularis PHE/MN1-00	602	L1	Q1MR82	hyaB
	Legionella pneumophila str. Lens	261	S3b	Q5WTY5	lpl2388
	Legionella pneumophila str. Lens	430	L3b	Q5WTY6	lpl2387
	Legionella pneumophila str. Paris	261	S3b	Q5 × 260	lpp2533
	Legionella pneumophila str. Paris	430	L3b	Q5 × 261	lpp2532
	Legionella pneumophila subsp. *pneumophila* str. Philadelphia 1	261	S3b	Q5ZSP9	lpg2468
	Legionella pneumophila subsp. *pneumophila* str. Philadelphia 1	430	L3b	Q5ZSQ0	hydA
	Lyngbya aestuarii CCY 9616	320	S2a	Q2EJS8	hupS
	Lyngbya aestuarii CCY 9616	537	L2a	Q2EJS7	hupL
	Lyngbya majuscula CCAP 1446/4	320	S2a	Q846P7	hupS
	Lyngbya majuscula CCAP 1446/4	537	L2a	Q846P6	hupL
	Lyngbya majuscula CCAP 1446/4	182	S3d	Q6JB18	hoxY
	Lyngbya majuscula CCAP 1446/4	476	L3d	Q6JB17	hoxH
T	*Magnetococcus* sp. MC-1	376	S1	Q3XNS7	Mmc1DRAFT_1094
	Magnetococcus sp. MC-1	567	L1	Q3XNT0	Mmc1DRAFT_1091
	Magnetococcus sp. MC-1	335	S2b	Q3XNT9	Mmc1DRAFT_1083
	Magnetococcus sp. MC-1	484	L2b	Q3XNU0	Mmc1DRAFT_1082
	Magnetococcus sp. MC-1	282	S3b	Q3XRU7	Mmc1DRAFT_2192
	Magnetococcus sp. MC-1	434	L3b	Q3XRU8	Mmc1DRAFT_2191
T	*Magnetospirillum magneticum* AMB-1	376	S1	Q2W6S1	amb1650
	Magnetospirillum magneticum AMB-1	567	L1	Q2W6S4	amb1647
	Magnetospirillum magneticum AMB-1	384	S2a	Q2W8A6	amb1115
	Magnetospirillum magneticum AMB-1	512	L2a	Q2W8A7	amb1114
	Magnetospirillum magneticum AMB-1	341	S2b	Q2W5X 8	amb1943
	Magnetospirillum magneticum AMB-1	481	L2b	Q2W5X9	amb1942
	Magnetospirillum magneticum AMB-1	183	S3d	Q2W1S6	amb3395
	Magnetospirillum magneticum AMB-1	496	L3d	Q2W1S5	amb3396
T	*Mannheimia succiniciproducens* MBEL55E	392	S1	Q65PY8	hyaA
	Mannheimia succiniciproducens MBEL55E	569	L1	Q65PZ2	hyaB
	Mariprofundus ferrooxydans PV-1	160	S3d	Q0EZV1	SPV1_09568
	Mariprofundus ferrooxydans PV-1	486	L3d	Q0EZV0	SPV1_09573
	Megasphaera elsdenii ATCC25940	484	LFe	Q9RGN3	hydA
	Methanocaldococcus jannaschii ATCC 43067/DSM 2661/JAL-1/ JCM 10045/NBRC 100440	230	S3a	Q60340	frhG

Table 3 (Continued)

Rmq	organism	length[a]	group[b]	AC	annotation
	Methanocaldococcus jannaschii ATCC 43067/DSM 2661/JAL-1/ JCM 10045/NBRC 100440	415	L3a	Q60338	frhA
	Methanocaldococcus jannaschii ATCC 43067/DSM 2661/JAL-1/ JCM 10045/NBRC 100440	216	S3a	Q58136	MJ0726
	Methanocaldococcus jannaschii ATCC 43067/DSM 2661/JAL-1/ JCM 10045/NBRC 100440	298	L3a	Q58137	MJ0727
	Methanocaldococcus jannaschii ATCC 43067/DSM 2661/JAL-1/ JCM 10045/NBRC 100440	288	S3c	Q58591	vhuG
	Methanocaldococcus jannaschii ATCC 43067/DSM 2661/JAL-1/ JCM 10045/NBRC 100440	418	L3c	Q58592	vhuA
	Methanocaldococcus jannaschii (*Methanococcus jannaschii*) ATCC 43067/DSM 2661/AL-1/JCM 10045/NBRC 100440	50		P81335	vhuU
	Methanocaldococcus jannaschii ATCC 43067/DSM 2661/JAL-1/ JCM 10045/NBRC 100440	148	S4	Q57936	MJ0516
	Methanocaldococcus jannaschii ATCC 43067/DSM 2661/JAL-1/ JCM 10045/NBRC 100440	380	L4	Q57935	MJ0515
	Methanocaldococcus jannaschii ATCC 43067/DSM 2661/JAL-1/ JCM 10045/NBRC 100440	151	S4	Q58758	MJ1363
	Methanocaldococcus jannaschii ATCC 43067/DSM 2661/JAL-1/ JCM 10045/NBRC 100440	377	L4	Q58433	MJ1027
S	*Methanococcus maripaludis* JJ	147	S4	O50252	fhl7
	Methanococcus maripaludis S2/LL	228	S3a	Q6LXG7	fruG
	Methanococcus maripaludis S2/LL	414	L3a	Q6LXG9	fruA
	Methanococcus maripaludis S2/LL	242	S3a	Q6LZ11	frcG
	Methanococcus maripaludis S2/LL	410	L3a	Q6LZ09	frcA
	Methanococcus maripaludis S2/LL	300	S3c	Q6LZ07	vhcG
	Methanococcus maripaludis S2/LL	471	L3c	Q6LZ06	vhcA
	Methanococcus maripaludis S2/LL	288	S3c	Q6LWL4	vhuG
	Methanococcus maripaludis S2/LL	418	L3c	Q6LWL5	vhuA
	Methanococcus maripaludis S2/LL	44		P0C1V5	vhuU
	Methanococcus maripaludis S2/LL	156	S4	Q6LX91	ehaN
	Methanococcus maripaludis S2/LL	375	L4	Q6LX90	ehaO
	Methanococcus maripaludis S2/LL	147	S4	Q6LWT4	ehbM
	Methanococcus maripaludis S2/LL	375	L4	Q6LY40	ehbN
	Methanococcus voltae DSM 1537/PS	243	S3a	Q00393	frhG
	Methanococcus voltae DSM 1537/PS	398	L3a	Q00390	frhA
	Methanococcus voltae DSM 1537/PS	228	S3a	Q00397	fruG
	Methanococcus voltae DSM 1537/PS	411	L3a	Q00394	fruA
	Methanococcus voltae DSM 1537/PS	287	S3c	Q00409	vhuG
	Methanococcus voltae DSM 1537/PS	420	L3c	Q00407	vhuA
	Methanococcus voltae DSM 1537/PS	43		Q00410	vhuU
	Methanococcus voltae DSM 1537/PS	306	S3c	Q00406	vhcG
	Methanococcus voltae DSM 1537/PS	474	L3c	Q00404	vhcA
	Methanoculleus marisnigri JR1	298	S3a	Q0YCM9	MemarDRAFT_1797
	Methanoculleus marisnigri JR1	455	L3a	Q0YCN1	MemarDRAFT_1795
	Methanoculleus marisnigri JR1	305	S3c	Q0Y7I6	MemarDRAFT_0071
	Methanoculleus marisnigri JR1	455	L3c	Q0Y7I5	MemarDRAFT_0072
	Methanoculleus marisnigri JR1	151	S4	Q0Y9V8	MemarDRAFT_0808
	Methanoculleus marisnigri JR1	362	L4	Q0Y9V9	MemarDRAFT_0807
	Methanoculleus marisnigri JR1	160	S4	Q0YAS3	MemarDRAFT_1088
	Methanoculleus marisnigri JR1	359	L4	Q0YAS5	MEMARDRAFT_1086
	Methanopyrus kandleri AV19/DSM 6324/JCM 9639/NBRC 100938	252	S3a	Q8TWV1	MK0930
	Methanopyrus kandleri AV19/DSM 6324/JCM 9639/NBRC 100938	416	L3a	Q8TWV0	MK0931
	Methanopyrus kandleri AV19/DSM 6324/JCM 9639/NBRC 100938	238	S3a	Q8TXX1	MK0538
	Methanopyrus kandleri AV19/DSM 6324/JCM 9639/NBRC 100938	370	L3a	Q8TXX2	MK0537
	Methanopyrus kandleri AV19/DSM 6324/JCM 9639/NBRC 100938	305	S3c	Q8TYW2	MK0179
	Methanopyrus kandleri AV19/DSM 6324/JCM 9639/NBRC 100938	434	L3c	Q8TYW3	MK0178
	Methanopyrus kandleri AV19/DSM 6324/JCM 9639/NBRC 100938	42		P0C1V6	MK0177
	Methanopyrus kandleri AV19/DSM 6324/JCM 9639/NBRC 100938	304	S3c	Q8TYM9	MK0267
	Methanopyrus kandleri AV19/DSM 6324/JCM 9639/NBRC 100938	482	L3c	Q8TYN0	MK0266
	Methanopyrus kandleri AV19/DSM 6324/JCM 9639/NBRC 100938	162	S4	Q8TY42	ehaN
	Methanopyrus kandleri AV19/DSM 6324/JCM 9639/NBRC 100938	409	L4	Q8TY43	ehaO
T	*Methanosarcina acetivorans* ATCC 35395/DSM 2834/JCM 12185/C2A	410	S1	Q8TRM9	vhtG
	Methanosarcina acetivorans ATCC 35395/DSM 2834/JCM 12185/C2A	596	L1	Q8TRM8	vhtA
T	*Methanosarcina acetivorans* ATCC 35395/DSM 2834/JCM 12185/C2A	383	S1	Q8TRN4	vhtG
	Methanosarcina acetivorans ATCC 35395/DSM 2834/JCM 12185/C2A	595	L1	Q8TRN3	vhtA
	Methanosarcina acetivorans ATCC 35395/DSM 2834/JCM 12185/C2A	262	S3a	Q8TS30	frhG
	Methanosarcina acetivorans ATCC 35395/DSM 2834/JCM 12185/C2A	456	L3a	Q8TS32	frhA
	Methanosarcina barkeri Fusaro DSM 804	259	S3a	O33163	Frh
	Methanosarcina barkeri Fusaro DSM 804	456	L3a	O33161	Frh
	Methanosarcina barkeri Fusaro DSM 804	156	S4	O59654	echC
	Methanosarcina barkeri Fusaro DSM 804	358	L4	O59656	echE
T	*Methanosarcina barkeri* str. fusaro	411	S1	Q46BG0	Mbar_A1841
	Methanosarcina barkeri str. fusaro	596	L1	Q46BG1	Mbar_A1840

Table 3 (Continued)

Rmq	organism	length[a]	group[b]	AC	annotation
T	*Methanosarcina barkeri* str. fusaro	386	S1	Q46BF4	Mbar_A1847
	Methanosarcina barkeri str. fusaro	591	L1	Q46BF5	Mbar_A1846
	Methanosarcina barkeri str. fusaro	258	S3a	P80491	frhG
	Methanosarcina barkeri str. fusaro	455	L3a	P80489	frhA
	Methanosarcina barkeri str. fusaro	274	S3a	Q46A79	Mbar_A2289
	Methanosarcina barkeri str. fusaro	456	L3a	Q46A81	Mbar_A2287
S	*Methanosarcina barkeri* str. fusaro	163	S4	Q469S1	Mbar_A2455
	Methanosarcina barkeri str. fusaro	156	S4	Q46G57	Mbar_A0150
F	*Methanosarcina barkeri* str. fusaro	358	L4	Q46G59	MBAR_A0148
T	*Methanosarcina mazei* Go1	403	S1	Q8PV03	MM_2175
	Methanosarcina mazei Go1	596	L1	Q8PV02	MM_2176
T	*Methanosarcina mazei* Go1	383	S1	Q50225	vhtG
	Methanosarcina mazei Go1	591	L1	Q50226	vhtA
T	*Methanosarcina mazei* Go1	383	S1	Q50248	vhoG
	Methanosarcina mazei Go1	591	L1	Q50249	vhoA
	Methanosarcina mazei Go1	287	S3a	Q8PSN6	MM_3043
	Methanosarcina mazei Go1	455	L3a	Q8PSN4	MM_3045
	Methanosarcina mazei Go1	156	S4	Q8PUL2	echC
	Methanosarcina mazei Go1	358	L4	Q8PUL0	echE
	Methanosphaera stadtmanae DSM 3091	256	S3a	Q2NES2	frhG
	Methanosphaera stadtmanae DSM 3091	406	L3a	Q2NES4	frhA
	Methanosphaera stadtmanae DSM 3091	305	S3c	Q2NI07	mvhG
	Methanosphaera stadtmanae DSM 3091	476	L3c	Q2NI05	mvhA
	Methanosphaera stadtmanae DSM 3091	150	S4	Q2NED8	ehbM
	Methanosphaera stadtmanae DSM 3091	383	L4	Q2NED9	ehbN
	Methanospirillum hungatei JF-1	262	S3a	Q2FTG7	Mhun_2330
	Methanospirillum hungatei JF-1	469	L3a	Q2FTG5	Mhun_2332
	Methanospirillum hungatei JF-1	150	S4	Q2FLL5	Mhun_2104
	Methanospirillum hungatei JF-1	361	L4	Q2FLL4	Mhun_2105
	Methanospirillum hungatei JF-1	148	S4	Q2FL38	Mhun_1743
	Methanospirillum hungatei JF-1	359	L4	Q2FL36	MHUN_1745
	Methanospirillum hungatei JF-1	145	S4	Q2FU30	Mhun_2588
	Methanospirillum hungatei JF-1	409	L4	Q2FTW4	Mhun_2590
	Methanothermobacter thermautotrophicus Delta H	235	S3a	P19498	frhG
	Methanothermobacter thermautotrophicus Delta H	404	L3a	P19496	frhA
	Methanothermobacter thermautotrophicus Delta H	307	S3c	Q50782	mvhG
	Methanothermobacter thermautotrophicus Delta H	472	L3c	Q50783	mvhA
	Methanothermobacter thermautotrophicus Delta H	148	S4	O27307	MTH1239
	Methanothermobacter thermautotrophicus Delta H	381	L4	O27306	MTH1238
	Methanothermobacter thermautotrophicus Delta H	148	S4	O26497	MTH397
	Methanothermobacter thermautotrophicus Delta H	370	L4	O26498	MTH398
	Methanothermobacter thermautotrophicus Marburg	148	S4	Q9V2X8	ehbM
	Methanothermobacter thermautotrophicus Marburg	376	L4	Q9V2X7	ehbN
	Methanothermobacter thermautotrophicus Marburg	148	S4	Q9UXP5	ehaN
	Methanothermobacter thermautotrophicus Marburg	370	L4	Q9UXP4	ehaO
F	*Methanothermus fervidus*	127	S3c	Q49178	mvhG
	Methanothermus fervidus	472	L3c	Q49179	mvhA
T	*Methylococcus capsulatus* Bath/NCIMB 11132	349	S1	Q8RJI7	hupS
	Methylococcus capsulatus Bath/NCIMB 11132	597	L1	Q8RJI6	hupL
	Methylococcus capsulatus Bath/NCIMB 11132	180	S3d	Q603S4	MCA2726
	Methylococcus capsulatus Bath/NCIMB 11132	494	L3d	Q60CJ2	MCA0114
	Moorella thermoacetica ATCC 39073	252	S4	Q2RGG6	Moth_2184
	Moorella thermoacetica ATCC 39073	574	L4	Q2RGG4	Moth_2186
	Moorella thermoacetica ATCC 39073	460	LFe	Q2RHA6	Moth_1883
	Moorella thermoacetica ATCC 39073	573	LFe	Q2RHS0	Moth_1717
	Mycobacterium sp. JLS	351	S1	Q1U3G8	MjlsDRAFT_5002
	Mycobacterium sp. JLS	598	L1	Q1U3G7	MjlsDRAFT_5003
	Mycobacterium sp. JLS	252	S3b	Q1TZM0	MjlsDRAFT_4064
	Mycobacterium sp. JLS	430	L3b	Q1TZL9	MjlsDRAFT_4065
	Mycobacterium sp. KMS	351	S1	Q1TED3	MkmsDRAFT_1999
	Mycobacterium sp. KMS	598	L1	Q1TED4	MkmsDRAFT_1998
	Mycobacterium sp. KMS	252	S3b	Q1TFE0	MkmsDRAFT_2428
	Mycobacterium sp. KMS	430	L3b	Q1TFD9	MkmsDRAFT_2429
	Mycobacterium sp. MCS	351	S1	Q1BA32	Mmcs_2144
	Mycobacterium sp. MCS	598	L1	Q1BA33	Mmcs_2143
	Mycobacterium sp. MCS	252	S3b	Q1B6G0	Mmcs_3417
	Mycobacterium sp. MCS	430	L3b	Q1B6G1	Mmcs_3416
	Mycobacterium vanbaalenii PYR-1	351	S1	Q25UN7	MvanDRAFT_1369
1CxxC	*Mycobacterium vanbaalenii* PYR-1	532	L1	Q25UN9	MvanDRAFT_1367
	Mycobacterium vanbaalenii PYR-1	321	S2a	Q25UT8	MvanDRAFT_1320
	Mycobacterium vanbaalenii PYR-1	536	L2a	Q25UT7	MvanDRAFT_1321
	Mycobacterium vanbaalenii PYR-1	205	S3d	Q261H9	MvanDRAFT_5545
	Mycobacterium vanbaalenii PYR-1	489	L3d	Q261I0	MvanDRAFT_5544
	Neocallimastix frontalis	636	LFe	Q8TFP2	HydL2

Table 3 (Continued)

Rmq	organism	length[a]	group[b]	AC	annotation
F	*Neocallimastix frontalis* L2	389	LFe	Q86ZE7	Hyd
	Nitrosospira multiformis ATCC 25196	182	S3d	Q2Y8F1	Nmul_A1672
	Nitrosospira multiformis ATCC 25196	493	L3d	Q2Y8F0	Nmul_A1673
	Nostoc punctiforme PCC73102	320	S2a	O68306	none
	Nostoc punctiforme PCC73102	531	L2a	O68307	none
	Nostoc sp. PCC 7120	320	S2a	Q7A2H6	all0688
	Nostoc sp. PCC 7120	531	L2a	Q8YZ11	hupL
	Nostoc sp. PCC 7120	181	S3d	Q8YYT2	hoxY
	Nostoc sp. PCC 7120	483	L3d	Q8YYT0	hoxH
	Nostoc sp. PCC 7422	320	S2a	Q3C1T9	hupS
	Nostoc sp. PCC 7422	531	L2a	Q3C1T8	hupL
	Nostoc sp. PCC 7422	181	S3d	Q3C1T4	hoxY
	Nostoc sp. PCC 7422	482	L3d	Q3C1T3	hoxH
	Nyctotherus ovalis	1198	LFe	Q5DM85	HDG
F	*Nyctotherus ovalis*	1206	LFe	O96948	None
T	*Oceanospirillum* sp. MED92	358	S1	Q2BLI3	MED92_11104
	Oceanospirillum sp. MED92	602	L1	Q2BLI4	MED92_11099
	Oceanospirillum sp. MED92	317	S2a	Q2BJK8	MED92_09456
	Oceanospirillum sp. MED92	500	L2a	Q2BJK9	MED92_09451
	Oceanospirillum sp. MED92	330	S2b	Q2BN60	MED92_03288
F	*Oceanospirillum* sp. MED92	386	L2b	Q2BN59	MED92_03293
	Oceanospirillum sp. MED92	251	S3b	Q2BRA3	MED92_07386
	Oceanospirillum sp. MED92	428	L3b	Q2BRA2	MED92_07391
T	*Oligotropha carboxidovorans* OM5	360	S1	O33405	hoxS
	Oligotropha carboxidovorans OM5	603	L1	O33406	hoxL
	Oligotropha carboxidovorans OM5	258	S2b	Q6LB89	hoxB
	Oligotropha carboxidovorans OM5	484	L2b	Q6LB90	hoxV
T	*Paracoccus denitrificans* PD1222	361	S1	Q3PJ19	PdenDRAFT_3945
	Paracoccus denitrificans PD1222	597	L1	Q3PJ20	PdenDRAFT_3944
	Paracoccus denitrificans PD1222	329	S2b	Q3PJ16	PdenDRAFT_3948
	Paracoccus denitrificans PD1222	477	L2b	Q3PJ17	PdenDRAFT_3947
T	*Pectobacterium atrosepticum* SCRI 1043/ATCC BAA-672	377	S1	Q6D7V0	hybO
	Pectobacterium atrosepticum SCRI 1043/ATCC BAA-672	564	L1	Q6D7U7	hybC
	Pectobacterium atrosepticum SCRI 1043/ATCC BAA-672	259	S4	Q6D7T6	hyfI
	Pectobacterium atrosepticum SCRI 1043/ATCC BAA-672	578	L4	Q6D7T4	hyfG
	Pelobacter carbinolicus DSM 2380	598	LFe	Q3A1L6	Hyd
	Pelobacter propionicus DSM 2379	144	S4	Q3G8L4	PproDRAFT_3513
	Pelobacter propionicus DSM 2379	409	L4	Q3G8L2	PPRODRAFT_3515
	Pelobacter propionicus DSM 2379	179	S4	Q3FYN2	PPRODRAFT_0597
	Pelobacter propionicus DSM 2379	557	L4	Q3FYM9	PproDRAFT_0600
	Pelobacter propionicus DSM 2379	164	S4	Q3G5A7	PproDRAFT_2591
	Pelobacter propionicus DSM 2379	409	L4	Q3G5A5	PPRODRAFT_2593
	Pelobacter propionicus DSM 2379	601	LFe	Q3G7B5	PproDRAFT_3331
T	*Pelodictyon luteolum* DSM 273	362	S1	Q3B2X6	Plut_1446
	Pelodictyon luteolum DSM 273	572	L1	Q3B2X5	Plut_1447
T	*Pelodictyon phaeoclathratiforme* BU-1	362	S1	Q3VLH0	PphaDRAFT_2215
	Pelodictyon phaeoclathratiforme BU-1	572	L1	Q3VLH1	PphaDRAFT_2214
	Pelodictyon phaeoclathratiforme BU-1	258	S3b	Q3VQC2	PphaDRAFT_0759
	Pelodictyon phaeoclathratiforme BU-1	424	L3b	Q3VQC1	PphaDRAFT_0760
T	*Pelotomaculum thermopropionicum* SI	346	S1	Q1X4F4	none
	Pelotomaculum thermopropionicum SI	482	L1	Q1X4F5	none
T	*Pelotomaculum thermopropionicum* SI	548	LFe	Q1WWT1	none
	Pelotomaculum thermopropionicum SI	624	LFe	Q1X1Z8	none
	Photobacterium profundum 3TCK	277	S4	Q1Z848	P3TCK_26867
	Photobacterium profundum 3TCK	584	L4	Q1Z850	P3TCK_26857
	Photobacterium sp. SKA34	378	S1	Q2C4Z7	SKA34_13055
	Photobacterium sp. SKA34	567	L1	Q2C4Z8	SKA34_13050
	Photobacterium sp. SKA34	277	S4	Q2C1Q5	SKA34_09563
	Photobacterium sp. SKA34	577	L4	Q2C1Q7	SKA34_09553
F	*Piromyces* sp. E2	555	LFe	Q8TG63	none
	Prochlorothrix hollandica ACC 15-2	178	S3d	O05930	hoxY
	Prochlorothrix hollandica ACC 15-2	482	L3d	O05932	hoxH
T	*Prosthecochloris aestuarii* DSM 271	364	S1	Q3VUY4	PaesDRAFT_1547
	Prosthecochloris aestuarii DSM 271	572	L1	Q3VUY3	PaesDRAFT_1548
	Prosthecochloris aestuarii DSM 271	254	S3b	Q3VWY8	PaesDRAFT_2204
	Prosthecochloris aestuarii DSM 271	458	L3b	Q3VWY7	PaesDRAFT_2205
T	*Pseudomonas hydrogenovora* 38846	363	S1	Q51860	hupS
	Pseudomonas hydrogenovora 38846	622	L1	Q51862	hupL
	Psychromonas ingrahamii 37	433	S3d	Q1FZ33	PingDRAFT_3293
	Psychromonas ingrahamii 37	499	L3d	Q1FZ32	PingDRAFT_3294
	Psychromonas sp. CNPT3	261	S4	Q1ZGP1	PCNPT3_00361
	Psychromonas sp. CNPT3	577	L4	Q1ZGN9	PCNPT3_00371
	Pyrococcus abyssi GE5/Orsay	261	S3b	Q9V0C4	PYRAB08660
	Pyrococcus abyssi GE5/Orsay	428	L3b	Q9V0C5	PYRAB08650

Table 3 (Continued)

Rmq	organism	length[a]	group[b]	AC	annotation
	Pyrococcus abyssi GE5/Orsay	241	S3b	Q9V044	PYRAB09540
	Pyrococcus abyssi GE5/Orsay	415	L3b	Q9V043	PYRAB09550
	Pyrococcus abyssi GE5/Orsay	170	S4	Q9V0R6	PYRAB07230
	Pyrococcus abyssi GE5/Orsay	426	L4	Q9V0R8	PYRAB07210
	Pyrococcus abyssi GE5/Orsay	271	S4	Q9UYN6	PYRAB14710
	Pyrococcus abyssi GE5/Orsay	588	L4	Q9UYN4	PYRAB14730
	Pyrococcus furiosus ATCC 43587/DSM 3638/JCM 8422/Vc1	261	S3b	Q59669	Hyd
	Pyrococcus furiosus ATCC 43587/DSM 3638/JCM 8422/Vc1	428	L3b	Q59670	Hyd
	Pyrococcus furiosus ATCC 43587/DSM 3638/JCM 8422/Vc1	237	S3b	Q9P9M5	shyD
	Pyrococcus furiosus ATCC 43587/DSM 3638/JCM 8422/Vc1	412	L3b	Q9P9M4	shyA
	Pyrococcus furiosus ATCC 43587/DSM 3638/JCM 8422/Vc1	167	S4	Q8U0Z8	PF1432
	Pyrococcus furiosus ATCC 43587/DSM 3638/JCM 8422/Vc1	427	L4	Q8U0Z6	PF1434
	Pyrococcus horikoshii OT3	266	S3b	O59013	PH1292
	Pyrococcus horikoshii OT3	429	L3b	O59011	PH1294
	Pyrococcus horikoshii OT3	173	S4	O59104	PH1434
	Pyrococcus horikoshii OT3	427	L4	O59107	PH1437
T	*Ralstonia metallidurans* CH34	364	S1	Q1LNU3	Rmet_1298
	Ralstonia metallidurans CH34	619	L1	Q1LNU4	Rmet_1297
	Ralstonia metallidurans CH34	209	S3d	Q1LN69	Rmet_1524
	Ralstonia metallidurans CH34	488	L3d	Q1LN68	Rmet_1525
T	*Rhizobium leguminosarum* bv. *viviae* 128c53	360	S1	P18637	hupS (hupA)
	Rhizobium leguminosarum bv. *viviae* 128c53	596	L1	P18636	hupL (hupB)
T	*Rhodobacter capsulatus* ATCC 33303/B10	358	S1	P15283	hupS (hupA)
	Rhodobacter capsulatus ATCC 33303/B10	597	L1	P15284	hupL (hupB)
	Rhodobacter capsulatus ATCC 33303/B10	332	S2b	Q52695	hupU
	Rhodobacter capsulatus ATCC 33303/B10	476	L2b	O86457	hupV
S	*Rhodobacter capsulatus* ATCC 33303/B10	503	L3d	Q9XBW8	hoxH
T	*Rhodobacter sphaeroides* 2.4.1	369	S1	Q3J0L8	hupS
	Rhodobacter sphaeroides 2.4.1	596	L1	Q3J0L7	hupL
	Rhodobacter sphaeroides 2.4.1	330	S2b	Q3J0M1	hupU
	Rhodobacter sphaeroides 2.4.1	475	L2b	Q3J0M0	hupV
S	*Rhodobacter sphaeroides* 2.4.1	330	S2b	Q53163	hupU1
T	*Rhodobacter sphaeroides* RV	369	S1	O86467	hupS
	Rhodobacter sphaeroides RV	596	L1	O86468	hupL
	Rhodobacter sphaeroides RV	330	S2b	O86466	hupU
	Rhodobacter sphaeroides RV	475	L2b	Q53164	hupV
	Rhodococcus opacus 1b	209	S3d	P72306	hoxY
	Rhodococcus opacus 1b	488	L3d	P72307	hoxH
	Rhodococcus sp. RHA1	351	S1	Q0S7U7	RHA1_ro04603
	Rhodococcus sp. RHA1	597	L1	Q0S7U6	RHA1_ro04604
	Rhodococcus sp. RHA1	261	S3b	Q0SKR6	RHA1_ro00034
	Rhodococcus sp. RHA1	422	L3b	Q0SKR7	RHA1_ro00033
	Rhodoferax ferrireducens DSM 15236	393	S1	Q21R17	Rfer_4088
	Rhodoferax ferrireducens DSM 15236	568	L1	Q21R14	Rfer_4091
	Rhodoferax ferrireducens DSM 15236	340	S2b	Q21QY5	Rfer_4120
	Rhodoferax ferrireducens DSM 15236	496	L2b	Q21QY4	Rfer_4121
	Rhodoferax ferrireducens DSM 15236	188	S3d	Q21RP8	Rfer_3856
	Rhodoferax ferrireducens DSM 15236	507	L3d	Q21RP9	Rfer_3855
T	*Rhodopseudomonas palustris* BisB18	374	S1	Q20ZX9	RPC_3772
	Rhodopseudomonas palustris BisB18	597	L1	Q20ZY0	RPC_3771
	Rhodopseudomonas palustris BisB18	333	S2b	Q20ZX6	RPC_3775
	Rhodopseudomonas palustris BisB18	480	L2b	Q20ZX7	RPC_3774
	Rhodopseudomonas palustris BisB18	246	S4	Q20XP6	RPC_4568
	Rhodopseudomonas palustris BisB18	571	L4	Q20XP4	RPC_4570
	Rhodopseudomonas palustris BisB18	144	S4	Q20XV9	RPC_4504
	Rhodopseudomonas palustris BisB18	361	L4	Q20XW2	RPC_4501
T	*Shewanella frigidimarina* NCIMB 400	378	S1	Q3NNK1	SfriDRAFT_1340
	Shewanella frigidimarina NCIMB 400	567	L1	Q3NNK2	SfriDRAFT_1339
T	*Shewanella oneidensis* MR-1	378	S1	Q8CVD3	hoxK
	Shewanella oneidensis MR-1	567	L1	Q8EF87	hyaB
	Shewanella oneidensis MR-1	106	SFe	Q8EAI1	hydB
	Shewanella oneidensis MR-1	410	LFe	Q8EAI2	hydA
T	*Shewanella putrefaciens* CN-32	378	S1	Q2ZP63	Sputcn32DRAFT_0511
	Shewanella putrefaciens CN-32	567	L1	Q2ZP62	Sputcn32DRAFT_0512
T	*Shewanella* sp. ANA-3	378	S1	Q36FL4	Shewana3DRAFT_2723
	Shewanella sp. ANA-3	567	L1	Q36FL3	Shewana3DRAFT_2724
	Shewanella sp. ANA-3	410	LFe	Q364V4	Shewana3DRAFT_4172
T	*Shewanella* sp. MR-4	378	S1	Q0HJ71	Shewmr4_1822
	Shewanella sp. MR-4	567	L1	Q0HJ72	Shewmr4_1821
	Shewanella sp. MR-4	106	SFe	Q0HF48	Shewmr4_3251
	Shewanella sp. MR-4	410	LFe	Q0HF49	Shewmr4_3250
T	*Shewanella* sp. MR-7	378	S1	Q0HUR2	Shewmr7_2155
	Shewanella sp. MR-7	567	L1	Q0HUR1	Shewmr7_2156
T	*Shewanella* sp. PV-4	378	S1	Q33TI8	ShewDRAFT_1285

Table 3 (Continued)

Rmq	organism	length[a]	group[b]	AC	annotation
	Shewanella sp. PV-4	568	L1	Q33TI7	ShewDRAFT_1286
T	*Shewanella* sp. W3-18-1	378	S1	Q2WXF3	Sputw3181DRAFT_0449
	Shewanella sp. W3-18-1	567	L1	Q2WXF2	Sputw3181DRAFT_0450
T	*Shigella boydii* Sb227	372	S1	Q31X24	SBO_2866
	Shigella boydii Sb227	567	L1	Q31X21	hybC
T	*Shigella boydii* Sb227	372	S1	Q31YN4	hyaA
	Shigella boydii Sb227	597	L1	Q31YN5	hyaB
	Shigella boydii Sb227	252	S4	Q31Y01	hyfI
	Shigella boydii Sb227	571	L4	Q31Y03	hyfG
	Shigella boydii Sb227	255	S4	Q31X83	hycG
	Shigella boydii Sb227	569	L4	Q31X85	hycE
T	*Shigella dysenteriae* Sd197	372	S1	Q32HT5	hyaA
	Shigella dysenteriae Sd197	568	L1	Q32HT4	hyaB
T	*Shigella dysenteriae* Sd197	372	S1	Q32C63	SDY_3076
	Shigella dysenteriae Sd197	567	L1	Q32C60	hybC
	Shigella dysenteriae Sd197	252	S4	Q32D78	hyfI
	Shigella dysenteriae Sd197	571	L4	Q32D79	hyfG
Sp	*Shigella dysenteriae* Sd197	569	L4	Q32CK9	hycE
	Shigella flexneri 5 8401	354	S1	Q0T0Q0	SFV_3050
	Shigella flexneri 5 8401	567	L1	Q0T0Q3	hybC
T	*Shigella flexneri* 5 8401	362	S1	Q0T664	hyaA
	Shigella flexneri 5 8401	597	L1	Q0T663	hyaB
Sp	*Shigella flexneri* 5 8401	252	S4	Q0T230	hyfI
	Shigella flexneri 5 8401	255	S4	Q0T1E6	hycG
	Shigella flexneri 5 8401	569	L4	Q0T1E8	hycE
T	*Shigella flexneri* ATCC 700930/2457T/serotype 2a	372	S1	Q83Q63	SF3044
	Shigella flexneri ATCC 700930/2457T/serotype 2a	566	L1	P0ACE2	hybC
T	*Shigella flexneri* ATCC 700930/2457T/serotype 2a	372	S1	Q83RW9	hyaA
	Shigella flexneri ATCC 700930/2457T/serotype 2a	597	L1	P0ACD9	hyaB
	Shigella flexneri ATCC 700930/2457T/serotype 2a	252	S4	Q83QL9	hyfI
	Shigella flexneri ATCC 700930/2457T/serotype 2a	569	L4	Q83QF5	hycE
F	*Shigella flexneri* ATCC 700930/2457T/serotype 2a	39	S4	Q7UBT7	HYCF
	Shigella flexneri ATCC 700930/2457T/serotype 2a	569	L4	Q7UBT6	hycE
T	*Shigella sonnei* Ss046	372	S1	Q3YXN6	SSO_3142
	Shigella sonnei Ss046	567	L1	Q3YXN9	hybC
T	*Shigella sonnei* Ss046	372	S1	Q3Z3E9	hyaA
	Shigella sonnei Ss046	597	L1	Q3Z3E8	hyaB
	Shigella sonnei Ss046	252	S4	Q3YZ63	hyfI
	Shigella sonnei Ss046	571	L4	Q3YZ65	hyfG
	Shigella sonnei Ss046	255	S4	Q3YYE2	hycG
	Shigella sonnei Ss046	565	L4	Q3YYE0	hycE
	Solibacter usitatus Ellin6076	355	S1	Q43Z07	AcidDRAFT_4002
	Solibacter usitatus Ellin6076	598	L1	Q43Z08	AcidDRAFT_4001
T	*Solibacter usitatus* Ellin6076	382	S1	Q44B09	AcidDRAFT_7269
	Solibacter usitatus Ellin6076	566	L1	Q44B06	AcidDRAFT_7272
	Solibacter usitatus Ellin6076	176	S3d	Q43RZ4	AcidDRAFT_2090
	Solibacter usitatus Ellin6076	474	L3d	Q43RZ3	AcidDRAFT_2091
	Sphingopyxis alaskensis RB2256	320	S2a	Q1J423	Sala_3197
	Sphingopyxis alaskensis RB2256	547	L2a	Q1J422	Sala_3198
	Spironucleus barkhanus ATCC50380	467	LFe	Q9GTP1	none
	Streptomyces avermitilis ATCC 31267/DSM 46492/JCM 5070/ NCIMB 12804/NRRL 8165	362	S1	Q93HH6	hydA
	Streptomyces avermitilis ATCC 31267/DSM 46492/JCM 5070/ NCIMB 12804/NRRL 8165	594	L1	Q93HH5	hydB
	Symbiobacterium thermophilum T/IAM 14863	456	LFe	Q67J76	STH3293
	Symbiobacterium thermophilum T/IAM 14863	596	LFe	Q67JF9	STH3209
	Synechococcus elongatus PCC 6301 1402-1	184	S3d	P94158	hoxY
	Synechococcus elongatus PCC 6301 1402-1	476	L3d	P94159	hoxH
	Synechococcus elongatus PCC 7942	184	S3d	Q31K33	Synpcc7942_2556
	Synechococcus elongatus PCC 7942	476	L3d	Q31K34	Synpcc7942_2555
	Synechococcus sp. PCC 7002	188	S3d	Q8KX26	hoxY
	Synechococcus sp. PCC 7002	474	L3d	Q8KX24	hoxH
	Synechocystis sp. PCC 6803	182	S3d	P74021	hoxY
	Synechocystis sp. PCC 6803	474	L3d	Q79A10	hoxH
	Synechocystis sp. PCC 6803	182	S3d	P74021	hoxY
	Synechocystis sp. PCC 6803	474	L3d	P74018	hoxH
T	*Syntrophobacter fumaroxidans* MPOB	312	S1	Q3N6H0	SfumDRAFT_3271
	Syntrophobacter fumaroxidans MPOB	546	L1	Q3N6G(	SfumDRAFT_3272
	Syntrophobacter fumaroxidans MPOB	300	S3c	Q3MXR0	SfumDRAFT_0477
	Syntrophobacter fumaroxidans MPOB	482	L3c	Q3MXR1	SfumDRAFT_0476
	Syntrophobacter fumaroxidans MPOB	324	S3c	Q3N421	SfumDRAFT_2528
1CxxC	*Syntrophobacter fumaroxidans* MPOB	469	L3c	Q3N422	SfumDRAFT_2527
	Syntrophobacter fumaroxidans MPOB	309	S3c	Q3N1L9	SfumDRAFT_1616
1CxxC	*Syntrophobacter fumaroxidans* MPOB	449	L3c	Q3N1L8	SfumDRAFT_1617

Table 3 (Continued)

Rmq	organism	length[a]	group[b]	AC	annotation
	Syntrophobacter fumaroxidans MPOB	312	S3c	Q3N2R4	SfumDRAFT_1699
1CxxC	*Syntrophobacter fumaroxidans* MPOB	449	L3c	Q3NR5	SfumDRAFT_1698
	Syntrophobacter fumaroxidans MPOB	184	S3d	Q3MYG8	SfumDRAFT_0631
	Syntrophobacter fumaroxidans MPOB	479	L3d	Q3MYG7	SfumDRAFT_0632
	Syntrophobacter fumaroxidans MPOB	417	LFe	Q3MXY8	SfumDRAFT_0499
	Syntrophobacter fumaroxidans MPOB	574	LFe	Q3MXZ2	SfumDRAFT_0495
T	*Syntrophomonas wolfei* subsp. *wolfei* Goettingen	135	SFe	Q0AVN1	Swol_1926
	Syntrophomonas wolfei subsp. *wolfei* Goettingen	387	LFe	Q0AVN2	Swol_1925
	Syntrophomonas wolfei subsp. *wolfei* Goettingen	563	LFe	Q0AU79	Swol_2436
	Syntrophomonas wolfei subsp. *wolfei* Goettingen	574	LFe	Q0AY73	Swol_1017
	Syntrophus aciditrophicus SB	253	S3b	Q2LYD8	SYNAS_01250
	Syntrophus aciditrophicus SB	441	L3b	Q2LYD7	SYNAS_01260
	Syntrophus aciditrophicus SB	605	LFe	Q2LSB7	SYNAS_10950
	Thermoanaerobacter ethanolicus ATCC 33223	58‘	LFe	Q3CJE2	Teth39DRAFT_0375
	Thermoanaerobacter tengocongensis DSM 15242/JCM 1107/ NBRC 100824/MB4	155	S4	Q8RDB6	NuoB
	Thermoanaerobacter tengcongensis DSM 15242/JCM 11007/ NBRC 100824/MBR	360	L4	Q8RDB4	NuoD
	Thermoanaerobacter tengcongensis DSM 15242/JCM 11007/ NBRC 100824/MBR	247	S4	Q8R9B7	TTE1698
	Thermoanaerobacter tengcongensis DSM 15242/JCM 11007/ NBRC 100824/MBR	576	L4	Q8R9B5	HycE
	Thermoanaerobacter tengcongensis DSM 15242/JCM 11007/ NBRC 100824/MBR	581	LFe	Q8RBC8	NuoG
	Thermococcus kodakarensis KOD1	264	S4b	Q8NKS3	hydD
	Thermococcus kodakarensis KOD1	428	L3b	Q8NKS2	hydA
	Thermococcus kodakarensis KOD1	176	S4	Q5JHU7	TK2089
	Thermococcus kodakarensis KOD1	426	L4	Q5JIL3	TK2091
	Thermococcus litoralis DSM 4573	263	S3b	Q9UW@7	hydD
	Thermococcus litoralis DSM 4573	426	L3b	Q9UWQ6	hydA
T	*Thermofilum pendens* Hrk 5	423	S1	Q0Y596	TpenDRSFT_0048
	Thermofilum pendens Hrk 5	596	L1	Q0Y597	TpenDRAFT_0047
	Thermofilum pendens Hrk 5	142	S4	Q0Y632	TpenDRAFT_0799
	Thermofilum pendens Hrk 5	537	L4	Q0Y631	TpenDRAFT_0800
	Thermofilum pendens Hrk 5	254	S4	Q0Y439	TpenDRAFT_1638
	Thermofilum pendens Hrk 5	567	L4	Q0Y441	TPENDRAFT_1636
	Thermotoga maritima ATCC 43578/MSB8/DSM 3109/JCM10099	608	LFe	Q9WY44	TM_0201
	Thermotoga maritima ATCC 43578/MSB8/DSM 3109/JCM10099	645	LFe	O52683	hydA
	Thiobacillus dentrificans ATCC 25259	360	S1	Q3SJ39	Tbd_1368
	Thiobacillus dentrificans ATCC 25259	596	L1	Q3SJ42	Tbd_1375
	Thiobacillus dentrificans ATCC 25259	267	S3b	Q3SJE9	Tbd_1262
	Thiobacillus dentrificans ATCC 25259	433	L3b	Q3SJE8	Tbd_1263
	Thiocapsa roseopersicina	360	S1	Q56359	hupS
	Thiocapsa roseopersicina	596	L1	Q56360	hupL
	Thiocapsa roseopersicina BBS	369	S1	O51820	hydS
	Thiocapsa roseopersicina BBS	576	L1	O51823	hydL
	Thiocapsa roseopersicina BBS	331	S2b	Q3MKP0	hupU
	Thiocapsa roseopersicina BBS	481	L2b	Q3MKN9	hupV
	Thiocapsa roseopersicina BBS	180	S3d	Q6XQK2	hoxY
	Thiocapsa roseopersicina BBS	475	L3d	Q6XQK1	hoxH
	Thiomicrospira crunogena XCL-2	813	S1	Q31DZ5	Tcr_2038
	Thiomicrospira crunogena XCL-2	568	L1	Q31DZ6	Tcr_2037
	Thiomicrospira denitrificans ATCC 33889	383	S2	Q30QL8	Tmden_1436
	Thiomicrospira denitrificans ATCC 33889	577	L1	Q30QL9	Tmden_1435
	Thiomicrospira denitrificans ATCC 33889	293	S2a	Q30QL6	Tmden_1438
	Thiomicrospira denitrificans ATCC 33889	417	L2a	Q30QL7	Tmden_1437
	Treponema denticola ATCC 35405/CIP 103919/DSM 14222	493	LFe	Q73N78	TDE_1277
	Treponema denticola ATCC 35405/CIP 103919/DSM 14222	596	LFe	Q73MB6	TDE_1593
	Trichodesmium erythaeum IMS101	320	S2a	Q10Z53	Tery_3369
	Trichodesmium erythaeum IMS101	534	L2a	Q10Z54	Tery_3368
	Trichomonas vaginalis	449	LFe	Q27096	TvhydB
	Trichomonas vaginalis ATCC 30001	468	LFe	Q27094	TvhydA
S	uncultured methanogenic archaeon RC-I	388	S1	Q0W5V7	vhtG
	uncultured methanogenic archaeon RC-I	233	S3a	Q0W2B2	frhG
	uncultured methanogenic archaeon RC-I	417	L3a	Q0W2B4	frhA
	uncultured methanogenic archaeon RC-I	232	S3a	Q0W2X8	frhG
	uncultured methanogenic archaeon RC-I	410	L3a	Q0W2X7	frhA
	uncultured methanogenic archaeon RC-I	305	S3c	Q0W6J6	mvhG
	uncultured methanogenic archaeon RC-I	471	L3c	Q0W6J7	mvhA
	uncultured methanogenic archaeon RC-I	306	S3c	Q0W6U9	mvhG
	uncultured methanogenic archaeon RC-I	467	L3c	Q0W6U1	mvhA
	uncultured methanogenic archaeon RC-I	135	S4	Q0W6T1	echC
	uncultured methanogenic archaeon RC-I	486	L4	Q0W6T4	echE
	Vibrio angustum S14	378	S1	Q1ZT18	VAS14_03798

Table 3 (Continued)

Rmq	organism	length[a]	group[b]	AC	annotation
	Vibrio angustum S14	567	L1	Q1ZT17	VAS14_03803
	Vibrio angustum S14	277	S4	Q1ZUC1	VAS14_14269
	Vibrio angustum S14	584	L4	Q1ZUB9	VAS14_14279
T	*Wolinella succinogenes* DSMZ 1740	354	S1	P31884	hydA
	Wolinella succinogenes DSMZ 1740	575	L1	P31883	hydB
	Wolinella succinogenes DSMZ 1740	276	S4	Q7M874	nuoB
	Wolinella succinogenes DSMZ 1740	579	L4	Q7M872	nuoD
	Xanthobacter autotrophicus Py2	370	S1	Q26JY8	XautDRAFT_0612
	Xanthobacter autotrophicus Py2	604	L1	Q26JY9	XautDRAFT_0611
	Xanthobacter autotrophicus Py2	339	S2b	Q26N48	XautDRAFT_1557
	Xanthobacter autotrophicus Py2	485	L2b	Q26N49	XautDRAFT_1556

[a] Length = number of amino acids. [b] Group refers to the classification schematized in Figure 7, e.g. S1, L1 = small and large subunit, respectively, of a [NiFe]-hydrogenase of group 1. LFe represents either a monomeric [FeFe]-hydrogenase or the H-cluster-containing subunit. [c] Column Rmq reads as follows: 1CxxC = large subunit sequence containing only one CxxC instead of two; F = fragment; S = single protein, i.e., a [NiFe] large subunit without a known small subunit partner, or a small subunit without a known large subunit partner; Sp = single protein where the other subunit is a pseudo-gene; T = sequence containing a twin-arginine pattern (RRxFxK) within the 100 first amino acids. The annotation column shows the explicit annotation in Uniprot, the gene names are not always those currently used in the literature.

(anaerobic respiration) or to O_2 (aerobic respiration), with recovery of energy in the form of a protonmotive force. They are connected to the quinone pool of the respiratory chain in the membrane by a third subunit, a di-heme cytochrome *b*, which, together with the hydrophobic C terminus of the small subunit, anchors the hydrogenase dimer to the membrane. Linked to redox components of potentials higher than that of the H_2/H^+ couple, they serve to consume H_2 and are called (H_2) uptake hydrogenases (generally termed Hup); they have been mainly studied in Proteobacteria (recent reviews in refs 17, 134, and 135) (cf. Figure 3). The prototype, the hydrogenase of *Wolinella succinogenes*,[136] encoded by the *hydABC* genes is shown in Figure 8a. The hyperthermophilic, hydrogen-oxidizing bacterium *Aquifex aeolicus* contains three hydrogenases recently characterized biochemically.[137,138] Two of them, hydrogenases I and II, are connected to the membrane by a membrane-integral cytochrome *b*. Whereas hydrogenase I is rather involved in a hydrogen−oxygen pathway, hydrogenase II, isolated as a multiprotein complex with a sulfur reductase, appears to be involved in sulfur respiration.[138] The membrane-bound [NiFe]-hydrogenase isolated from the extreme thermophilic hydrogen-oxidizing bacterium *Hydrogenobacter thermophilus* strain TK-6,[139] a member of the Aquificaceae family, probably also belongs to group 1.

Other members of group 1, such as the Hyn enzyme from *Thiocapsa roseopersicina*,[140] the periplasmic *Desulfovibrio* hydrogenase able to interact with low-potential *c*-type cytochromes and a transmembrane redox protein complex encoded by the *hmc* operon,[141] and *E. coli* hydrogenase-2, encoded by the *hybOABCDEFG* operon,[142] present a slightly different structure. *E. coli* hydrogenase-2 is predicted to be a large tetrameric complex consisting of the large (HybC) and the small (HybO) subunits associated with two other subunits, an Fe-S-containing periplasmic subunit (HybA) and an integral membrane protein (HybB)[143] (Figure 8b). *E. coli* hydrogenase-2 (Hyb) has been shown to function as a respiratory uptake enzyme at low potential[144] as Hyb from *Geobacter sulfurreducens*.[145] Some *Desulfovibrio* species, for example, *D. vulgaris* Hildenborough[79] and *Desulfomicrobium baculatum* (formerly *Desulfovibrio baculatus*), contain a [NiFeSe]-hydrogenase (HysSL). In the Se-containing hydrogenase of *Dm. baculatum*, the SeCys is a ligand to Ni;[78] at the 5′ end of the gene encoding the large subunit, there is a TGA codon for insertion of selenocysteine at a position homologous to the TGC codon for cysteine.[146]

A similar membrane-bound uptake hydrogenase (VhoGA) has been identified in the methanogenic archaeon *Methanosarcina mazei* Gö1.[147] Its third subunit (VhoC) (also a cytochrome *b*), donates the electrons from H_2 to the membrane electron carrier methanophenazine, which shuttles electrons in the membrane, as do quinones in bacteria, between Vho and heterodisulfide reductase (Hdr) (Figure 8c) for the reductive cleavage by Hdr of the heterodisulfide CoM−S−S−CoB formed in the release of methane (see Figure 11). In *M. mazei*, the Vho hydrogenase and the heterodisulfide reductase may associate to form a complex known as H_2:heterodisulfide oxidoreductase, which may provide an energy-conserving proton pump.[147]

The uptake hydrogenases are characterized by the presence of a long signal peptide (ca. 35−50 amino acid residues) at the N terminus of their small subunit. The signal peptide contains a conserved [DENST]RRxFxK motif recognized by a specific protein translocation pathway designated the membrane targeting and translocation (Mtt)[148] or twin-arginine translocation (Tat)[149] pathway. It serves as signal recognition to target the fully folded heterodimer to the membrane and the periplasm[10a,150−158] (Figure 9). Several hydrogenases of group 1, *E. coli* hydrogenases-1 and -2[143,149,159] and the membrane-bound hydrogenase of *W. succinogenes*[160] and of *R. eutropha*[161] have been shown to be exported by this so-called hitchhiker mechanism of cotranslocation of the two subunits.

Tat signal peptides have a tripartite structure (much like classical Sec signal peptides) comprising a polar "n-region", a relatively hydrophobic "h-region", and a polar "c-region". The conserved [DENST]RRxFxK twin-arginine motif is always located at the boundary between the n- and h-regions.[150] The n-region varies in size and amino acid composition (Figure 9). The c-region contains an AxA cleavage site (or an acceptable variation) and often proline residues located between the h-region and the AxA motif. The proline residue could facilitate signal peptidase recognition of the cleavage site by acting as a "helix breaker"[150] (Figure 9). The conserved AxA amino acid motif (or an acceptable variation) is the recognition site for type I signal peptidase (LepB in *E. coli*). A method has been developed (TatP) to discriminate Tat signal peptides from cytoplasmic proteins carrying a similar motif, as well as from Sec signal peptides.[162] (The TatP prediction server is available as a public Web server at http://www.cbs.dtu.dk/services/TatP/.) A potential cleavage site of the Tat signal peptide is also

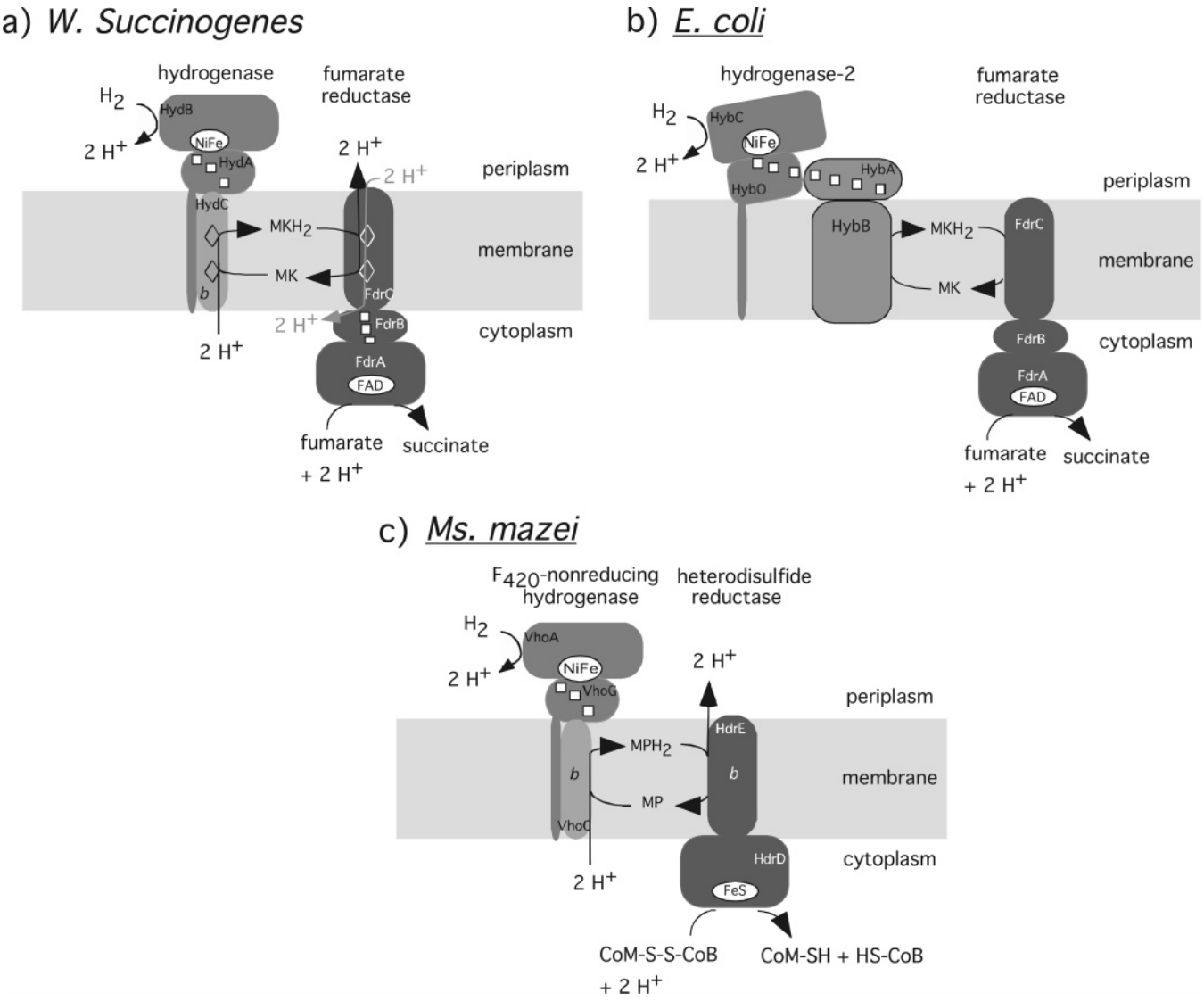

Figure 8. Examples of respiratory [NiFe]-hydrogenases of group 1. (a, b) Hypothetical mechanism of fumarate respiration with H_2, in *Wolinella succinogenes* (a) and in *Escherichia coli* (b). (a) Electron and proton tranfer in the membrane of *W. succinogenes* according to the "E pathway hypothesis".[555] The HydC protein of the hydrogenase forms four transmembrane helices; the heme *b* groups are shown as diamonds. The menaquinone binding site is close to the distal heme *b* group, near the cytoplasmic side of the membrane.[556] [4Fe-4S] and [3Fe-S] clusters are represented by squares, and the [2Fe-2S] cluster is represented by a rectangle. (b) In *E. coli*, hydrogenase-2 donates electrons to heme-less fumarate reductase. Unlike trimeric uptake hydrogenases with a membrane integral cytochrome *b* as third subunit, *E. coli* hydrogenase-2 is heterotetrameric; instead of cytochrome *b* it comprises the AB heterodimeric core, a "16Fe" ferredoxin (HybA) most closely related to the periplasmically oriented HmcB protein from *Desulfovibrio vulgaris*[557] and HybB most closely related to the HmcC protein from *D. vulgaris* and predicted to comprise 10 transmembrane helices.[143] (c) Trimeric F_{420}-nonreducing hydrogenase (Vho) from *Methanosarcina mazei* Gö1, with a cytochrome *b* subunit that acts as the primary electron acceptor of the core hydrogenase, is shown to interact with the heterodisulfide reductase via methanophenazine (MP), the membrane integral electron carrier connecting protein complexes of the respiratory chain of *Ms. mazei*. The scheme shows that the membrane integral cytochrome *b* subunit accepts two protons from the cytoplasm for the reduction of MP and that the overall reaction leads to the production of two scalar protons[147] (adapted from Deppenmeier[35]). Reproduced with permission from ref 135 (Figure 2). Copyright 2007 Springer Science and Business Media, Springer-Verlag.

predicted. This software is thought to be the most accurate to date, but it remains possible to find cases of the predicted cleavage site not corresponding to the experimentally determined site, especially in recently characterized species, which were not included in the training set when the program was developed. Tat systems show a substrate−Tat component specificity and a species specificity,[157] also suggested by the alignments of Figure 9.

The twin-arginine translocation (Tat) pathway is a system with the unique ability to export proteins in a fully folded conformation, in particular, cofactor-containing proteins.[151−153,157] It is structurally and mechanistically similar to the delta-pH-dependent pathway used to import chloroplast proteins into the thylakoid.[157,158,163,164] The energy for Tat transport is provided by the transmembrane proton electrochemical gradient;[165,166] a H^+/protein antiporter mechanism may account for the direct utilization of protons from the gradient.[167] Homologues of Tat proteins are found in many bacteria, chloroplasts, and *Archaea*.[151,157,168,169] The Tat system is of bacterial origin.[157] In *E. coli*, the Tat translocation apparatus (or "translocon") is formed by the integral membrane proteins TatA, TatB, and TatC. The TatB and TatC proteins form a large (∼600 kDa) and equimolecular complex in the membrane.[170] TatC provides the primary recognition site for the signal peptide of the Tat substrate, which once bound is adjacent to the TatB protein.[171]

Type	AC	Gene	Species	Signal sequence	Mature	Length
[NiFe]	P21950	*hoxK*	*Azotobacter vinelandii*	MSRLETFYDVMRRQGITRRSFLKYCSLTAAALGLGPAFAPRIAHA	METKP ...	45
[NiFe]	P18190	*hupA*	*Azotobacter chroococcum*	MSQLETXYDVMRRQGITRRSFLKYCSLTGRPCLGPTFAPQIAHA	METRP ...	44
[NiFe]	P15283	*hupS*	*Rhodobacter capsulatus*	MSDIETFYDVMRRQGITRRSFMKSVRSPQHVLGLGPSFVPKIGEA	METKP ...	45
[NiFe]	O86467	*hupS*	*Rhodobacter sphaeroides*	MPQIETFYDVMRRQGITRRSFIKYCSLTAAALGLGPSFVPRIAHA	METKP ...	45
[NiFe]	P31892	*hoxK*	*Cupriavidus necator*	MVETFYEVMRRQGISRRSFLKYCSLTATSLGLGPSFLPQIAHA	METKP ...	43
[NiFe]	P17633	*hupS*	*Rhodocyclus gelatinosus*	METFYEVMRRQGISRRSFLKYCSLTATSLGLAPSFVPQIAHA	METKP ...	42
[NiFe]	O33405	*hoxS*	*Oligotropha carboxidovorans*	MTPTETFYEVMRRQGVTRRSFLKFCSLTATALGLGPAYTSEIAHA	METKP ...	45
[NiFe]	Q56359	*hupS*	*Thiocapsa roseopersicina*	MPTTETYYEVMRRQGITRRSFLKFCSLTATALGLSPTFAGKIAHA	METKP ...	45
[NiFe]	P12635	*hupS1*	*Bradyrhizobium japonicum*	MGAATETFYSVIRRQGITRRSFHKFCSLTATSLGLGPLAASRIANA	LETKP ...	46
[NiFe]	Q9ANR0	*hupS2*	*Bradyrhizobium japonicum*	MGDATETFYGVIRRQGITRRSFLKFCSFTAASLGLGASSIAHA	LETKP ...	43
[NiFe]	Q2RV83	*hupS*	*Rhodospirillum rubrum*	MGETETFYEVIRRQGISRRGFLKFCGVTAAGLGLGAGGAARIAQA	LETKP ...	45
[NiFe]	P69739	*hyaA*	*Escherichia coli*	MNNEETFYQAMRRQGVTRRSFLKYCSLAATSLGLGAGMAPKIAWA	LENKP ...	45
[NiFe]	P13063	*hysB*	*Desulfomicrobium baculatum*	MSLSRREFVKLCSAGVAGLGISQIYHPGIVHA	MTEGA ...	32
[NiFe]	Q9AM33	*hynB*	*Desulfovibrio desulfuricans*	MPNGNRFDALKMTVGTREVSRRDFMKFCGVMATFLGLGPAFAPQIAHA	LMTKK ...	48
[NiFe]	P31884	*hydA*	*Wolinella succinogenes*	MLEEKGIERRDFMKWAGAMTAMLSLPATFTPLTAKA	AELAD ...	36
[NiFe]	O51820	*hynS*	*Thiocapsa roseopersicina*	MAARNPTDKTLGESLRERGVSRRGFLKFCAATASMMALPPSMAPAIA	AALEQ ...	47
[NiFe]	P21853	*hydA*	*Desulfovibrio vulgaris*	MKISIGLGKEGVEERLAERGVSRRDFLKFCTAIAVTMGMGPAFAPEVARA	LMGPR ...	50
[NiFe]	Q06173	*hynB*	*Desulfovibrio vulgaris* Hildenborough	MRFSVGLGKEGAEERLARRGVSRRDFLKFCTAIAVTMGMGPAFAPEVARA	LTGSR ...	50
[NiFe]	Q30ZG2	*Dde_2137*	*Desulfovibrio desulfuricans* G20	MKFSVGLGKEGAEERLASRGVSRRDFLKFCSTVAVAMGMGPAFAPEVARA	LTSGK ...	50
[NiFe]	P18187	*hydA*	*Desulfovibrio fructosovorans*	MNFSVGLGRMNAEKRLVQNGVSRRDFMKFCATVAAAMGMGPAFAPKVAE	ALTAK ...	49
[NiFe]	P69741	*hyb0*	*Escherichia coli*	MTGDNTLIHSHGINRRDFMKLCAALAATMGLSSKAAA	EMAES ...	37
[FeFe]	O08312	hydB	*Desulfovibrio fructosovorans*	MSILATTRRGFMKTACVLTGGALIGLRLTSKAVA	AAKQL ...	34
[FeFe]	P13628	hydB	*Desulfovibrio vulgaris* Monticello	MQIVNLTRRGFLKAACVVTGGALISIRMTGKAVA	AAKQL ...	34
[FeFe]	P07603	hydB	*Desulfovibrio vulgaris* Hildenborough	MQIASITRRGFLKVACVTTGAALIGIRMTGKAVA	AVKQI ...	34
[FeFe]	Q0ENS7	DRAFT_2757	*Desulfovibrio vulgaris* DP4	MQIASITRRGFLKVACVTTGAALIGIRMTGKAVA	AVKQI ...	34
[FeFe]	Q317L3	Dde_0082	*Desulfovibrio desulfuricans* G20	MSIAAFTRRQFLKAGCMACGAAIVGIRFTGKALA	AVKQV ...	34
[FeFe]	Q9AM35	hydB	*Desulfovibrio desulfuricans*	MSIAAFTRRQFLKGGCMACGAAIVGIRFTGKALA	AVKQV ...	34
[FeFe]	Q0AVN1	Swol_1926	*Syntrophomonas wolfei* Goettingen	MKLFHESEGITRRQFFKGAGMLTMAAVISGVFA	KFGFD ...	33
[FeFe]	Q30Z19	Dde_2280	*Desulfovibrio desulfuricans* G20	MSRLGTVSRRGFIKLAGFAAGYAVFGFNMARQACA	ATLEF ...	35
[FeFe]	Q18T66	Dhaf_1708	*Desulfitobacterium hafniense* DCB-2	MESKAGKGSNLSRRSFLKFAGGAGIAGASLSLTGCGQ	PLTPA ...	44
[FeFe]	Q24N91	DSY4712	*Desulfitobacterium hafniense* Y51	MMMQLKHPFQSGFQQQSCKRHTKKVVVDMESKAGKGSNLSRRSFLKFAGGAGIAGA	SLSLT ...	56
[FeFe]	Q2CZF6	DRAFT_3054	*Desulfotomaculum reducens* MI-1	MQNQQEGKDKQKQITRRGFLKMMGGIGLTGITATIAGCSTDPA	GGKGW ...	43

Figure 9. Examples of twin-arginine motif in signal peptides that function in [NiFe]- and [FeFe]-hydrogenase transport. The presented sequences of group 1 [NiFe]-hydrogenases are those for which a cleavage site has been experimentally determined, but for the [FeFe]-hydrogenases the cleavage site is putative. The N terminus amino acid sequences of the precursors are presented with their Tat signal aligned and emphasized by gray shading. The sequences are ordered according to their similarity (evaluated by the ClustalW guide tree). The predicted length of the signal peptide indicated on the right includes the first translated methionine residue.

Thylakoid orthologues of *E. coli* TatC (cpTatC) and TatB (Hcf106) have been shown to interact with different regions of the signal peptide.[172] TatA forms in the membrane a separate homo-oligomeric ring-shaped structure from 450 to 750 kDa in size[173−175] supposed to be the protein-conducting channel.[150,152] It is recruited by the TatBC complex loaded with the redox cofactor-containing substrate to form the translocase and stabilizes it.[176] After translocation of the mature protein, the temporary translocase disassembles into its components, TatA and TatB−TatC. Complex cofactor-containing Tat substrates acquire their redox cofactors prior to export from the cell and require correct assembly before transport can proceed. A folding quality-control mechanism intrinsic to the export process has the ability to recognize the folded state of a substrate protein and to reject unfolded proteins.[177,178] Substrate-specific accessory proteins prevent improperly assembled substrates from interacting with the Tat transporter.[152] Some Tat signal peptides operate in tandem with cognate binding chaperones to coordinate the assembly and transport of complex enzymes.[179] Two-hybrid experiments have demonstrated that *E. coli* HyaE interacts specifically with the precursor form of HyaA, the hydrogenase-1 β-subunit, and that HybE interacts specifically with HybO, the β-subunit precursor of hydrogenase-2.[180] The authors[180] have proposed that HyaE and HybE are hydrogenase-specific chaperones acting at a "proofreading" stage in hydrogenase assembly. According to a model of proofreading mediated by twin-arginine signal-peptide binding chaperones,[151] binding of the chaperone to the signal peptide masks the twin-arginine motif and prevents targeting of the apoprotein to TatBC. Following successful cofactor insertion, the signal-binding chaperone is displaced. The export-ready precursor can then associate with TatBC and enter the Tat transport cycle leading to protein export. The paradigm proofreading chaperone is *E. coli* TorD, which coordinates maturation and export of the respiratory enzyme trimethylamine *N*-oxide reductase (TorA). TorD has been shown to bind tightly and with exquisite specificity to the TorA twin-arginine signal peptide in vitro.[181] TorD belongs to a class of nucleotide-binding proteins; its affinity is enhanced by initial signal peptide binding. It has been proposed[181] that GTP governs signal peptide binding-and-release cycles during Tat proofreading. The folding proofing feature of the Tat pathway is of interest for biotechnological applications: for example, as TorD coexpression with a TorA signal peptide fused to the green fluorescent protein (GFP) markedly enhances export of the fusion protein,[182] it should be possible to enhance translocation efficiency of valuable Tat secreted proteins, including hydrogenases.

3.3.2. Cyanobacterial Uptake [NiFe]-Hydrogenases and H_2 Sensors (Group 2)

Two features distinguish the hydrogenases belonging to group 2 from those of group 1: (1) The small subunit of group 2 enzymes does not contain a signal peptide at its N terminus; accordingly these hydrogenases are not exported but remain in the cytoplasm. (2) There are numerous identical deletions in the primary amino acid sequences of both group 2a and group 2b small subunits compared to those of group 1. More specifically, taking as reference the amino acid sequence of *C. necator* HoxB (group 2b), the small subunits of uptake hydrogenases (group 1) show a one amino acid deletion at position 34, two deletions of two amino acids each with an interval of seven amino acids at positions 106 and 115, one deletion of 13 amino acids at position 165, and one deletion of 7 amino acids at position 324; on the other hand, there is one insertion of four amino acids at position 255 and of one amino acid at position 289.

Group 2a includes the cyanobacterial uptake hydrogenases (called HupSL). They are linked to the occurrence of nitrogenase[183] and induced under N_2 fixing conditions.[31] Studies on cyanobacterial hydrogenases, their distribution, their physiological functions, their evolution, and their use in the photoproduction of biohydrogen have been reviewed.[31,32,183−185] Group 2a also includes the third hydrogenase from *Aquifex aeolicus*, a member of the Aquificales, the very early branching order of the Bacteria. This soluble enzyme has been proposed to provide reductant to the reductive TCA cycle for CO_2 fixation.[137]

Group 2b comprises the regulatory hydrogenases, called HupUV or HoxBC. They function as H_2 sensors in the regulatory cascade that controls the biosynthesis of some proteobacterial uptake hydrogenases in response to H_2. They have been studied in *Bradyrhizobium japonicum*,[186] *R. eutropha*,[83,187−189,266] *R. capsulatus*,[121,122,190−193] *T. roseopersicina*,[194] and *Rhodopseudomonas palustris*.[195] Their role in signal transduction has been reviewed recently.[17,196−200] These H_2-sensing hydrogenases have the interesting property of being insensitive to oxygen, in contrast to the majority of hydrogenases. A possible reason is that, in these regulatory hydrogenases, the main gas channel leading to the NiFe active site is too narrow, due to the presence of amino acids bulkier than in standard [NiFe]-hydrogenases and that molecular oxygen cannot reach the active site and inactivates it.[71] This hypothesis has been confirmed by site-directed mutagenesis of *R. capsulatus* HupUV[193] and of the homologous *R. eutropha* HoxBC[189] hydrogenases. Replacement of two bulky amino acids by smaller ones enlarged the gas channel leading to the active site and yielded mutant derivatives sensitive to O_2. Thus, it is the inacessibility of O_2 to the active site of the regulatory hydrogenases that permits the latter to remain operative in the presence of molecular oxygen. The *R. eutropha* H_2 sensor presents an interesting structural feature that may contribute also to its O_2 insensitivity; its small subunit does not appear to contain the canonical three Fe-S clusters but rather two [2Fe-2S] clusters and a 4Fe species, which may be a [4Fe-3S-3O] cluster.[83]

3.3.3. Bidirectional Heteromultimeric Cytoplasmic [NiFe]-Hydrogenases (Group 3)

In group 3, the dimeric hydrogenase module is associated with other subunits able to bind soluble cofactors, such as cofactor 420 (F_{420}, 8-hydroxy-5-deazaflavin), NAD, or NADP. They are termed bidirectional because, physiologically, they function reversibly and can thus reoxidize the cofactors under anaerobic conditions by using the protons of water as electron acceptors. Many members of this group are found in the *Archaea*. They include the trimeric F_{420}-reducing hydrogenases, the tetrameric bifunctional hydrogenases of hyperthermophiles, able to reduce S^0 to H_2S in vitro and to use NADPH as electron donor,[201] and the F_{420}-non-reducing hydrogenases (Mvh) (Figure 7). The physiological role of the Mvh hydrogenase from *Methanothermobacter marburgensis* is to provide reducing equivalents for heterodisulfide reductase.[202] In *Methanosarcina mazei*, the energy-conserving electron transfer from H_2 involves a [NiFe]-hydrogenase, a *b*-type cytochrome, and $F_{420}H_2$ dehydrogenase. The $F_{420}H_2$ dehydrogenase, encoded by the *fpo* genes, is a redox-driven proton pump sharing similarities with

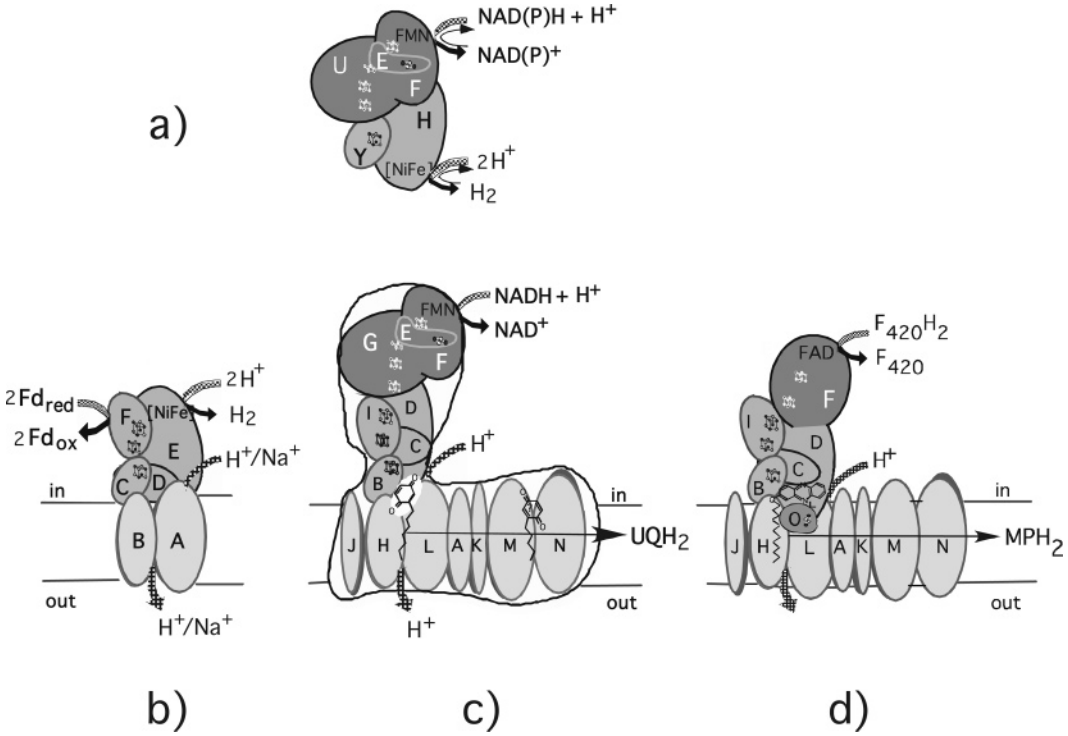

Figure 10. Models of [NiFe]-hydrogenases from groups 3 and 4 and of $F_{420}H_2$ dehydrogenase compared with that of complex I from *R. capsulatus* (c) (adapted from refs 419, 558, and 559). The [4Fe-4S] and [2Fe-2S] clusters are shown in the appropriate subunits. (a) Bidirectional Hox hydrogenase from *Synechocystis* encoded by *hoxEFUYH*; (b) Ech hydrogenase from *Methanosarcina barkeri*, encoded by the *echABCDEF* genes. Redox titrations at different pH values demonstrated that the proximal cluster (in the EchC subunit) and one of the clusters in the EchF subunit have a pH-dependent midpoint redox potential,[560] a result which supports the hypothesis that the Fe-S clusters are involved in an electron-transfer driven proton-pumping unit (adapted from ref 34). (d) $F_{420}H_2$ dehydrogenase from *Methanosarcina mazei* encoded by the *fpoA-O* genes. $F_{420}H_2$ dehydrogenase can couple the transfer of about two protons/$2e^-$[203] (adapted from ref 35). Reproduced with permission from ref 135 (Figure 4). Copyright 2007 Springer Science and Business Media, Springer-Verlag.

the proton-translocating NADH:quinone oxidoreductase of respiratory chains[203] (reviewed in refs 35 and 204) (Figure 10d). The role in methanogenesis of the above-mentioned enzymes is illustrated in Figure 11.

Bidirectional NAD(P)-linked hydrogenases are also found in bacteria and cyanobacteria. The first NAD-dependent [NiFe]-hydrogenase was isolated from *R. eutropha* and found to be activatable by NADPH.[205] It is expressed from a megaplasmid.[206] It was described up to now as a tetrameric enzyme, consisting of the HoxYH dimer (hydrogenase moiety) and the HoxFU dimer (NADH-dehydrogenase moiety). A new high molecular weight form of the enzyme has recently been isolated;[207] it comprises two additional HoxI subunits. Whereas the tetrameric form can be activated only by NADH, the hexameric form can be activated also by NADPH. This suggests that HoxI provides a binding site for NADPH. Besides, the NiFe center of the *R. eutropha* NAD^+-dependent [NiFe]-hydrogenase contains four cyanide groups and one carbon monoxide molecule, one cyanide group being bound to the Ni.[208] Removal of the Ni-bound cyanide group results in inactivation of the enzyme by oxygen, indicating that it is responsible for the O_2 insensitivity of the enzyme. Homologous enzymes were later discovered in cyanobacteria[209−211] and recently in the photosynthetic bacteria *T. roseopersicina*[212] and *Allochromatium vinosum*.[213] These latter bidirectional hydrogenases are pentameric, made of the hydrogenase moiety (HoxYH) and the diaphorase moiety (HoxFUE) (Figure 10a). The HoxFUE subunits are homologous to subunits of complex I of mitochondrial and bacterial respiratory chains and contain NAD(P), FMN, and Fe-S binding sites (Figure 10c; Table 4) (reviewed in refs 10a, 17, 31, 32, 135, and 214). The NAD(P)-dependent [NiFe]-hydrogenase of the cyanobacterium *Synechocystis* PCC6803 is sensitive to O_2; H_2 production by anaerobic cells maintained in the dark ceases rapidly in the light when O_2 is generated photosynthetically.[215] The transient H_2 outburst observable upon re-illumination of cells, due probably to the increase in NAD(P)H concentration in response to photosystem I activity,[215] illustrates the proposal[216] that the bidirectional hydrogenase functions as an electron valve for the disposal of low-potential electrons generated at the onset of illumination. In *Klebsiella pneumoniae*, a membrane-bound $NAD(P)^+$-reducing [NiFe]hydrogenase provides reduced pyridine nucleotides during citrate fermentation without the involvement of membrane potential (hence, not by reverse electron flow);[217] it remains to be assessed if this hydrogenase belongs to group 3. Concerning *Geobacter sulfurreducens*, a member of the family Geobacteraceae of δ-Proteobacteria, examination of its genome indicated that *G. sulfurreducens* can produce four [NiFe]-hydrogenases: two periplasmically oriented, membrane-bound hydrogenases, Hya and Hyb, and two cytoplasmic hydrogenases, Mvh and Hox. The large and small subunits of Mvh and Hox appear to be related to archaeal and cyanobacterial hydrogenases, respectively.[30]

3.3.4. H_2-Evolving, Energy-Conserving, Membrane-Associated Hydrogenases (Group 4)

The multimeric enzymes (six subunits or more) of group 4 reduce protons from water to dispose of excess reducing equivalents produced by the anaerobic oxidation of C_1 organic compounds of low potential, such as carbon monoxide or formate. *E. coli* hydrogenase-3, the prototype of this group, encoded by the *hyc* operon, is part of the formate

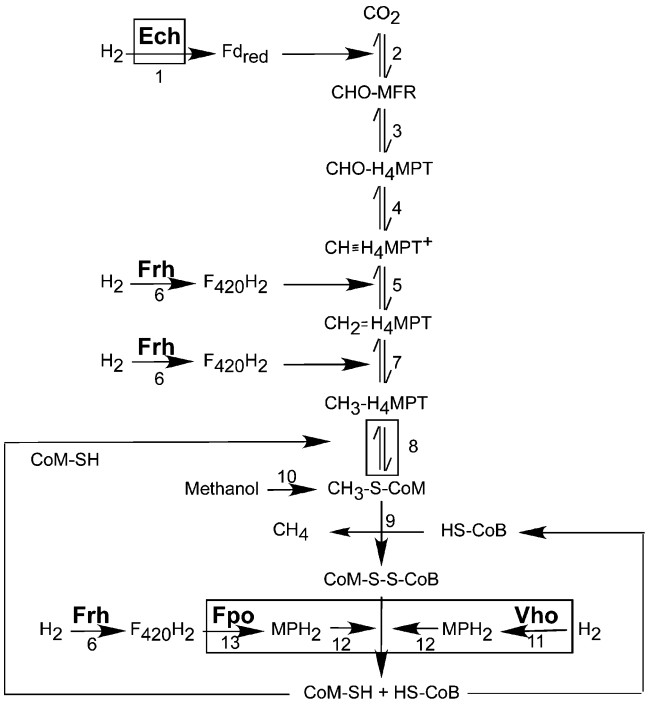

Figure 11. Pathway of methanogenesis from $CO_2 + H_2$ and from methanol in *Methanosarcina* species. Reactions catalyzed by membrane-bound energy-transducing enzyme complexes are boxed. Abbreviations: $F_{420}H_2$, reduced form of coenzyme F_{420}; Fd_{red}, reduced form of ferredoxin; MFR, methanofuran; H_4MPT, tetrahydromethanopterin; HS-CoM, coenzyme M; HS-CoB, coenzyme B; MPH_2, reduced form of methanophenazine. Enzymes: 1, Ech, Ech hydrogenase; 2, formylmethanofuran dehydrogenase; 3, formyl-MFR:H_4MPT formyl transferase; 4, methenyl-H_4MPT cyclohydrolase; 5, methylene-H_4MPT dehydrogenase; 6, Frh, F_{420}-reducing hydrogenase; 7, methylene-H_4MPT reductase; 8, methyl-H_4MPT: HS-CoM methyltransferase; 9, methyl-CoM reductase; 10, soluble methyltransferases; 11, Vho, F_{420}-nonreducing hydrogenase; 12, heterodisulfide reductase; 13, Fpo, $F_{420}H_2$ dehydrogenase. Each type of hydrogenase with its specific electron acceptor (ferredoxin for Ech, cofactor F_{420} for Frh, and methanophenazine for Vho) is highlighted (adapted from ref 35). Reproduced with permission from ref 135 (Figure 7). Copyright 2007 Springer Science and Business Media, Springer-Verlag.

hydrogenlyase complex (FLH-1) (encoded by *hycBCDEFGHI*),[218] which metabolizes formate to H_2 and CO_2.[16,219] Its biosynthesis has been elucidated by the group of A. Böck.[16,18] At another locus, the *hyf* operon of *E. coli* encodes a putative 10-subunit hydrogenase complex (hydrogenase-4).[220] Seven genes of the *hyf* operon (*hyfABCGHIJ*) encode homologues of seven Hyc subunits of hydrogenase-3. Three additional genes (*hyfD*, *hyfE*, and *hyfF*) that have no counterpart in the Hyc complex are capable of encoding integral membrane proteins, two of them sharing similarities with subunits that play a crucial role in proton translocation and energy coupling in the NADH:quinone oxidoreductase (complex I). Up to now, no Hyf-derived hydrogenase or formate dehydrogenase activity could be detected, and no Ni-containing protein corresponding to HyfG, the large subunit of hydrogenase-4, was observed.[221] However, phylogenetic trees of this group of enzymes (cf. Figure 14) show that several newly sequenced genomes do contain genes that are likely *hyf* genes homologues. This hypothesis is supported by the similarities observed for the surrounding genes, which probably belong to the same operon.

The CO-induced hydrogenase of the photosynthetic bacterium *Rhodospirillum rubrum* (CooLH) is another member of this group. Together with CO-dehydrogenase, it oxidizes CO to CO_2 with concomitant production of H_2, and allows *R. rubrum* to grow in the dark with CO as sole energy source. Because CO dehydrogenase is a peripheral membrane protein, it has been proposed that CooLH constitutes the energy coupling site.[5,222a] The Fe-S protein CooF is required to mediate electron transfer between the CO dehydrogenase and the CO-induced hydrogenase.[222b] *E. coli* hydrogenase-3 (HycGE) and *R. rubrum* CooLH are labile enzymes; the exact number of their subunits is still unknown. The CO-linked hydrogenase of *Rubrivivax gelatinosus*, able to oxidize CO with H_2 production and energy recovery in the form of a membrane potential, is also probably of the same type of enzyme.[223] A homologous enzyme complex was isolated from the thermophilic Gram-positive bacterium *Carboxydothermus hydrogenoformans*[224] (reviewed in refs 34 and 225). It comprises a Ni-containing CO-dehydrogenase (CooS), an electron-transfer protein containing four [4Fe-4S] clusters (CooF), and a membrane-bound [NiFe]-hydrogenase composed of four hydrophilic subunits and two membrane integral subunits (CooL,X,U,H and CooM,K), which couple the conversion of CO to CO_2 and H_2 to energy conservation.

The majority of hydrogenases assigned to group 4 have been found in the *Archaea* (Table 1). They include *Methanosarcina barkeri*,[226−228] *Methanothermobacter marburgensis*,[229] and *Pyrococcus furiosus*.[230,231] They have been shown to couple H_2 evolution and energy conservation (recent reviews in refs 17, 34, 135, and 225). The Ech hydrogenase found in the methanogenic archaeon *M. barkeri* has been thoroughly studied biochemically and genetically.[226−228] It is encoded by the *echABCDEF* operon, and the isolated enzyme is an integral membrane protein composed of six subunits corresponding to the *ech* products. A schematic representation of the Ech hydrogenase is shown in Figure 10b. The use of a *M. barkeri* mutant lacking Ech hydrogenase (Δ*ech*) revealed that this enzyme is absolutely required for the reduction of CO_2 to formylmethanofuran by H_2. Ech catalyzes the reduction of a low-potential ferredoxin by H_2, and the reduced ferredoxin serves as electron donor for the synthesis of formylmethanofuran[228] (Figure 11). The authors suggested that the thermodynamically unfavorable reduction of ferredoxin by H_2 is coupled to the consumption of a membrane ion gradient, the Ech hydrogenase functioning as an ion pump.

The hyperthermophilic archaeon *P. furiosus* contains two cytoplasmic H_2-evolving hydrogenases (I and II),[232,233] members of group 3, and a membrane-bound hydrogenase (Mbh), a member of group 4, encoded by a 14-gene operon[234] termed *mbh* (either *mbh1−14*[230] or *mbhA−N*[231]). Four gene products of this operon share similarities with subunits of complex I (Table 4). These multimeric membrane-bound hydrogenase complexes comprise transmembrane subunits homologous to complex I subunits involved in proton pumping and energy coupling and appear to be able to couple the oxidation of a carbonyl group (originating from formate, acetate, or carbon monoxide) with the reduction of protons to H_2 (reviewed in ref 34). Indeed, Mbh from *P. furiosus* was shown to couple electron transfer from reduced ferredoxin to both proton reduction and proton translocation, that is, to couple the production of H_2 to ATP synthesis.[235]

A membrane-bound Ech [NiFe]-hydrogenase sharing similarities with *M. sarcina* Ech has recently been identified in *Desulfovibrio gigas*.[236] From phylogenetic analyses, Calteau et al.[29] concluded that the *ech* operon found in *D.*

Table 4. Relationships between Complex I and NDH-1 Subunits and Subunits of Selected [NiFe]-Hydrogenases and of F_{420} Dehydrogenase

	bovine[561]	*Synechocystis*[212,562]		*E. coli*[563] or *R. capsulatus*[564]	*P. denitrificans*[565]	*E. coli*[566]	*M. barkeri*[566]	*R. rubrum*[5,222a]	*P. furiosus*[230a]	*Ms. mazei*[203]
	complex I	NDH-1	HoxEFUYH H_2ase	NDH-1	NDH-1	Hyc H_2ase	Ech H_2ase	Coo H_2ase	Mbh H_2ase	Fpo
hydrophilic NADH-oxidizing module	9 kDa									
	24 kDa		HoxE	NuoE	Nqo2					
	51 kDa		HoxF	NuoF	Nqo1					
	75 kDa		HoxU[b]	NuoG	Nqo3					
subunits of the connecting module				NuoCD (*E. c.*)[c]		HycE				
	30 kDa	NdhJ		NuoC (*R. c.*)	Nqo5	N-ter HycE	EchD			FpoC
	49 kDa	NdhH	HoxH	NuoD (*R. c.*)	Nqo4	C-ter HycE	EchE	CooH	Mbh12	FpoD
	20 kDa (PSST)	NdhK	HoxY	NuoB	Nqo6	HycG	EchC	CooL	Mbh10	FpoB
	23 kDa (TYKY)	NdhI		NuoI	Nqo9	HycF	EchF	CooX	Mbh14	FpoI
	39 kDa									
	18 kDa									
	13 kDa B									FpoO
					(Nqo15[d])					
intrinsic membrane hydrophobic subunits	39 kDa									
	NDI	NdhA		NuoH	Nqo8	HycD	EchB	CooK	Mbh13	FpoH
	ND2	NdhB		NuoN	Nqo14	HycC[e]	EchA[e]	N-ter CooM[d]	Mbh8	FpoN
	ND3	NdhC		NuoA	Nqo7					FpoA
	ND4	NdhD		NuoM	Nqo13	HycC[e]	EchA[e]	N-ter CooM[d]		FpoM
	ND4L	NdhE		NuoK	Nqo11					FpoK
	ND5	NdhF		NuoL	Nqo12	HycC[e]	EchA[e]	N-ter CooM[d]		FpoL
	ND6	NdhG		NuoJ	Nqo10					FpoJ

[a] *P. furiosus* genome database (http://comb5−156.umbi.umd.edu/). [b] Sequence similarities between HoxU and N-ter NuoG. [c] NuoC and NuoD are fused in *E. coli*. [d] Nqo15 in *Thermus thermophilus*.[568] [e] NuoL, NuoM, and NuoN are homologous to one particular class of Na^+/H^+ antiporters.[569] (Reproduced from Vignais[135] *Hydrogenases and H$^+$-reduction in primary energy conservation* (Table 1) with kind permission of Springer Science and Business Media, Springer-Verlag, Berlin, Germany.) Relationships between complex I and NDH-1 subunits and subunits of selected [NiFe]-hydrogenases and of $F_{420}H_2$ dehydrogenase.

gigas[236] and in the hyperthermophile *Thermoanaerobacter tengcongensis*[237] was probably acquired by horizontal gene transfer from an archaebacterium belonging to the *Methanosarcina* clade. Similarly, they suggested that the 13-gene operon found in the genome of *Thermotoga maritima*, the putative products of which resemble a Mbh hydrogenase, was probably transmitted from an archaebacterium belonging to the *Pyrococcus* group.

3.4. [FeFe]-Hydrogenases

[FeFe]-hydrogenases are found in anaerobic prokaryotes known to produce H_2, such as clostridia and sulfate reducers, in some anaerobic eukaryotes, in anaerobic fungi and ciliates, in trichomonads, and in some green algae (Tables 1 and 2) (reviewed in refs 10a, 12, 13, 99, 101, and 104). Recently, components of an [FeFe]-hydrogenase have been found associated with formate dehydrogenases from *Eubacterium acidaminophilum*.[238] Whereas [NiFe]-hydrogenases tend to be involved in H_2 consumption, [FeFe]-hydrogenases are usually involved in H_2 production. However, the periplasmic [FeFe]-hydrogenase of *D. vulgaris* Hildenborough has been demonstrated to function as an uptake hydrogenase.[239] Production of that periplasmic enzyme is up-regulated in response to oxidative stress and a new function, protection against oxidative stress, has been proposed for the periplasmic [FeFe]-hydrogenase of *D. vulgaris* Hildenborough.[240] The periplasmic [FeFe]-hydrogenase from *D. desulfuricans* ATCC 7757[241] shares complete sequence identity with the *D. vulgaris* Hildenborough [FeFe]-hydrogenase.[242] It consists of a small subunit (HydB, 13.5 kDa) bearing a Tat signal peptide at its N terminus (Figure 9) and a large subunit (HydA, 46 kDa) that undergoes a carboxy-terminal processing involving the removal of a 24 amino acid long peptide,

Name	Pattern	Occurrences
FeFe_P1	[296] [FILT][ST][SCM]**C**[CS]**P**[AGSMIV][FWY] [303]	172
FeFe_P2	[352] [FILV][MGTV]**PC***xx**K**[DKQRS]x[EV] [361]	290
FeFe_P3	[495] **ExMxC***xx**GC***xx**G**[AGP] [507]	203

Figure 12. Characteristic sequence signatures within the H-cluster domain of [FeFe]-hydrogenases. The P1, P2, and P3 signatures have been derived from sequences listed in Table 2 and are written using the PROSITE format (see legend to Figure 6). In addition, the bold letters represent fully conserved residues and cysteine ligands of the H-cluster are starred. The edges of the three segments have been numbered according to the *C. pasteurianum* sequence.[63,570]

in agreement with the three-dimensional structure of the enzyme.[64] The authors[241] suggested that the C-terminal processing of the large subunit is involved in the export of the protein to the periplasm.

Alignments of the complete sequences of [FeFe]-hydrogenases showed that the most conserved parts of the H-cluster domain are three segments encompassing the cysteine ligands of the metal site (Figure 5). The three characteristic sequence signatures within the H-cluster domain derived earlier[10a] were first used to identify [FeFe]-hydrogenase sequences from the database. They were then optimized by successive rounds of refinement using PRATT[127,128] and ps_scan.[130,131] A set of three characteristic patterns (P1, P2, and P3) was obtained (Figure 12). Each of these patterns can be found in proteins that are not [FeFe]-hydrogenases, but any sequence bearing the three patterns does belong to the [FeFe]-hydrogenase class (Table 5).

In some [FeFe]-hydrogenases additional [4Fe-4S] and [2Fe-2S] clusters are postulated to be present because of their

Table 5. Catalytic Subunits of [FeFe]-Hydrogenases[a]

taxon	length		AC
Alkaliphilus metalliredigenes QYMF	582		Q3C9E8
Alkaliphilus metalliredigenes QYMF	591		Q3C5M2
Bacteroides fragilis	489		Q5L986
Bacteroides fragilis NCTC 9343 YCH46	489		Q64PE7
Bacteroides thetaiotaomicron ATCC 29148/DSM 2079/ NCTC 10582/E50/VPI-5482	482		Q8A6P3
Bacteroides thetaiotaomicron ATCC 29148/DSM 2079/ NCTC 10582/E50/VPI-5482	588		Q8ABI6
Caldicellulosiruptor saccharolyticus DSM 8903	579		Q2ZJ38
Chlamydomonas moewusii SAG 24.91	458		Q56UD8
Chlamydomonas reinhardtii 21gr	497		Q9FYU1
Chlamydomonas reinhardtii 21gr, and Cc425	505		Q8VZZ0
Chlamydomonas reinhardtii SE	505		Q6T533
Chlorella fusca	436		Q8VX03
Clostridium acetobutylicum ATCC 824/DSM 792/JCM 1419/ LMG 5710/VKM B-1787	450		Q97E85
Clostridium acetobutylicum ATCC 824/DSM 792/JCM 1419/ LMG 5710/VKM B-1787	582		Q59262
Clostridium beijerincki NCIMB 8052	449		Q2WK96
Clostridium beijerincki NCIMB 8052	461		Q2WUD6
Clostridium beijerincki NCIMB 8052	567		Q2WVX8
Clostridium beijerincki NCIMB 8052	644		Q2WI78
Clostridium difficile 630	461		Q180F8
Clostridium difficile 630	478		Q180A2
Clostridium difficile 630	593		Q180Q5
Clostridium paraputrificum	582		Q6F4C7
Clostridium pasteurianum ATCC 6013/DSM 525/NCIB 9486/ VKM B-1774/W5	574		P29166
Clostridium perfringens 13/type A	449		Q8XNQ6
Clostridium perfringens 13/type A	490		Q8XHB0
Clostridium perfringens 13/type A	572		Q9RHU8
Clostridium perfringens ATCC 13124	449		Q0TUF9
Clostridium perfringens ATCC 13124	490		Q0TM76
Clostridium perfringens ATCC 13124	572		Q0TMV5
Clostridium perfringens ATCC 13124	696		Q0TS68
Clostridium perfringens NCTC 8237	572		Q9ZNE4
Clostridium perfringens SM101	449		Q0SWA8
Clostridium perfringens SM101	490		Q0SPY1
Clostridium perfringens SM101	572		Q0SQK1
Clostridium perfringens SM101	696		Q0SUE5
Clostridium phytofermentans ISDg	484		Q1FJL6
Clostridium phytofermentans ISDg	567		Q1FJL3
Clostridium phytofermentans ISDg	582		Q1FFT8
Clostridium phytofermentans ISDg	644		Q1FHS1
Clostridium saccharobutylicum P262	574		Q59261
Clostridium saccharoperbutylacetonicum N1-4	562		Q5MIB2
Clostridium sp. OhILAs	567		Q1F047
Clostridium tetani Massachusetts/E88	448		Q899J2
Clostridium tetani Massachusetts/E88	494		Q891G1
Clostridium thermocellum ATCC 27405	566		Q4CGI4
Clostridium thermocellum ATCC 27405	579	F	Q9XC55
Clostridium thermocellum ATCC 27405	582		Q4CDK8
Clostridium thermocellum ATCC 27405	644		Q4CDI0
Dehalococcoides ethenogenes 195	573		Q3ZA52
Dehalococcoides sp.	573		Q3ZWM9
Dehalococcoides sp. CBDB1 BAV1	573		Q2DWB9
Desulfitobacterium hafniense DCB-2	425		Q18R81
Desulfitobacterium hafniense DCB-2	454		Q18RP8
Desulfitobacterium hafniense DCB-2	527		Q18T66
Desulfitobacterium hafniense DCB-2	1150		Q18XD7
Desulfitobacterium hafniense Y51	425		Q24ZF0
Desulfitobacterium hafniense Y51	460		Q24PC7
Desulfitobacterium hafniense Y51	555		Q24N91
Desulfitobacterium hafniense Y51	1150		Q24Z17
Desulfotalea psychrophila LSv54/DSM 12343	471		Q6AR16
Desulfotalea psychrophila LSv54/DSM 12343	483		Q6AKL7
Desulfotomaculum reducens MI-1	429		Q2D600
Desulfotomaculum reducens MI-1	520		Q2CZF6
Desulfotomaculum reducens MI-1	593		Q2D1M4
Desulfotomaculum reducens MI-1	659		Q2D1M7
Desulfovibrio desulfuricans G20	421		Q9AM36
Desulfovibrio desulfuricans G20	421		Q317L4
Desulfovibrio desulfuricans G20	439		Q30Z18
Desulfovibrio desulfuricans G20	458		Q315X0

Table 5 (Continued)

taxon	length		AC
Desulfovibrio desulfuricans G20	483		Q314X0
Desulfovibrio fructosovorans	585		Q46508
Desulfovibrio fructosovorans DSM 3604	421		O08311
Desulfovibrio vulgaris Hildenborough	606		Q46606
Desulfovibrio vulgaris subsp. *oxamicus* str. Monticello DP4	606		Q0ENS8
Desulfovibrio vulgaris subsp. *vulgaris*	421		P07598
Desulfovibrio vulgaris subsp. *vulgaris*	606		Q72B67
Desulfovibrio vulgaris subsp. *vulgaris* str. Hildenborough	421		P13629
Desulfovibrio vulgaris subsp. *vulgaris* str. Hildenborough DP4	421		Q0ENS6
Entamoeba histolytica	468		Q9GTX0
Entamoeba histolytica HM-1:IMSS	468		Q51EJ9
Entamoeba histolytica HM-1:IMSS	472		Q50YQ4
Entamoeba histolytica HM-1:IMSS	504		Q511D6
Entamoeba histolytica HM1:IMSS	504		Q869B1
Eubacterium acidaminophilum	578		Q93SF7
Giardia intestinalis	474		Q9BKJ3
Giardia lamblia ATCC 50803 WB C6	474		Q7QXP8
Halothermothrix orenii H 168	456		Q2AFL4
Halothermothrix orenii H 168	570		Q2AFM5
Halothermothrix orenii H 168	578		Q2AE40
Halothermothrix orenii H 168	666		Q2AG82
Heliobacillus mobilis	606		Q1MSH5
Megasphaera elsdenii ATCC25940	484		Q9RGN3
Moorella thermoacetica ATCC 39073	460		Q2RHA6
Moorella thermoacetica ATCC 39073	573		Q2RHS0
Neocallimastix frontalis	636		Q8TFP2
Neocallimastix frontalis L2	389	F	Q86ZE7
Nyctotherus ovalis	1198		Q5DM85
Nyctotherus ovalis	1206	F	O96948
Pelobacter carbinolicus DSM 2380	598		Q3A1L6
Pelobacter propionicus DSM 2379	601		Q3G7B5
Pelotomaculum thermopropionicum SI	548		Q1WWT1
Pelotomaculum thermopropionicum SI	624		Q1X1Z8
Piromyces sp. E2	555	F	Q8TG63
Rhodopseudomonas palustris ATCC BAA-98/CGA009	619		Q6NDH4
Rhodopseudomonas palustris BisA53	619		Q370P7
Scenedesmus obliquus	449		Q9AR66
Scenedesmus obliquus wild type D3	403	F	Q9AU60
Shewanella decolorationis S12	410		Q27PY7
Shewanella oneidensis MR-1	410		Q8EAI2
Shewanella sp. ANA-3	410		Q364V4
Shewanella sp. MR-4	410		Q0HF49
Spironucleus barkhanus ATCC50380	467		Q9GTP1
Symbiobacterium thermophilum T/IAM 14863	456		Q67J76
Symbiobacterium thermophilum T/IAM 14863	596		Q67JF9
Syntrophobacter fumaroxidans MPOB	417		Q3MXY8
Syntrophobacter fumaroxidans MPOB	574		Q3MXZ2
Syntrophomonas wolfei subsp. *wolfei* Goettingen	387		Q0AVN2
Syntrophomonas wolfei subsp. *wolfei* Goettingen	563		Q0AU79
Syntrophomonas wolfei subsp. *wolfei* Goettingen	574		Q0AY73
Syntrophus aciditrophicus SB	605		Q2LSB7
Thermoanaerobacter ethanolicus ATCC 33223	581		Q3CJE2
Thermoanaerobacter tengcongensis DSM 15242/JCM 11007/ NBRC 100824/MB4	581		Q8RBC8
Thermotoga maritima ATCC 43589/MSB8/DSM 3109/JCM 10099	608		Q9WY44
Thermotoga maritima ATCC 43589/MSB8/DSM 3109/JCM 10099	645		O52683
Treponema denticola ATCC 35405/CIP 103919/DSM 14222	493		Q73N78
Treponema denticola ATCC 35405/CIP 103919/DSM 14222	596		Q73MB6
Trichomonas vaginalis	449		Q27096
Trichomonas vaginalis ATCC 30001	468		Q27094

[a] All sequences contain the three P1, P2, and P3 motifs shown in Figure 12; when a protein is annotated as a fragment, its length is followed by F.

primary sequence similarity to the [FeFe]-hydrogenase of the bacterium *C. pasteurianum* for which the three-dimensional structure is known[63] (Figure 13).

Many of the listed bacterial [FeFe]-hydrogenases (Table 5) have been characterized biochemically, and their genes have also been cloned and characterized at the molecular level. In eukaryotes, the genes are located in the nucleus, whereas the enzyme is localized to organelles (chloroplast or hydrogenosome) of endosymbiotic origin. In the green algae, *Chlamydomonas reinhardtii*,[96,98,243] *Scenedesmus obliquus*,[94,95] and *Chlorella fusca*,[97] the enzyme is located in the chloroplast stroma and is linked via ferredoxin to the photosynthetic electron transport chain.[94,244,245] It functions as an electron "valve" that enables the algae to survive under anaerobic conditions.[246] Hydrogenosomes are peculiar organelles that supply ATP to the cell and make molecular

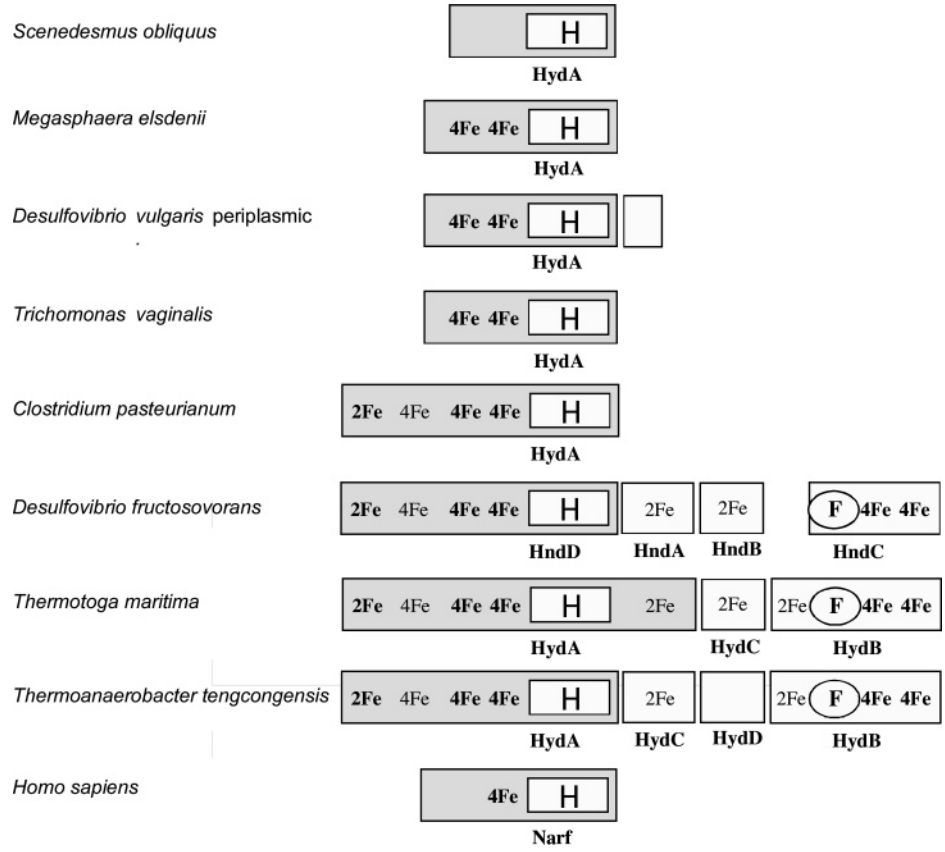

Figure 13. Schematic representation of the modular structure and domain organization of [FeFe]-hydrogenases: comparison with the Fe-hydrogenase-like Narf protein. The sequences (also listed in Table 3) are from *S. obliquus*,[94] *M. elsdenii*,[101] *D. vulgaris* (Hildenborough),[242] *T. vaginalis*,[571] *C. pasteurianum*,[570] *D. fructosovorans*,[572] *T. maritima*,[573] *T. tengcongensis*,[237] and *H. sapiens* (HeLa) (Narf).[574] The domains are inferred from comparisons of sequences and structures.[63,64] They are not drawn to scale. Symbols: H, H-cluster; **2Fe**, [2Fe-2S] plant ferredoxin; 2Fe, [2Fe-2S] NuoE-like; **4Fe**, [4Fe-4S] cluster; 4Fe, $(Cys)_3$His-ligated [4Fe-4S]; F, FMN and NADP binding site. Names of gene products under the boxes representing the subunit are those used in the literature. The catalytic subunit is gray-shaded. The monomeric hydrogenases interact with ferredoxins or flavodoxins, and the periplasmic dimeric *Desulfovibrio* enzyme interacts with low-potential cytochrome c_3. The three multimeric hydrogenases shown in the lower part of the figure interact with $NADP^+$; they belong to the Hnd subgroup.

hydrogen in the process.[247] They are found in various unrelated eukaryotes, such as anaerobic flagellates, chytridiomycete fungi, and ciliates. The presence of [FeFe]-hydrogenases in these lower eukaryotes has often been deduced from the DNA sequences of complete genes. Sequences encoding [FeFe]-hydrogenases are also found in anaerobic eukaryotes lacking hydrogenosomes, such as *Entamoeba histolytica*, *Spironucleus barkhanus*,[104] and *Giardia intestinalis*,[248,249] where the hydrogenase is localized in the cytoplasm. The distribution of [FeFe]-hydrogenases among contemporary eukaryotes, their structural diversity, and their evolutionary relationships have been reviewed recently.[12,13,104,250]

The [FeFe]-hydrogenase-like sequences found in the genomes of higher aerobic eukaryotes including the human genome bring evidence of a common ancestry with [FeFe]-hydrogenases. The proteins termed Narf (*n*uclear prelamin *A r*ecognition *f*actor) show similarity to [FeFe]-hydrogenases, especially with respect to conservation of residues implicated in the coordination of a putative H-cluster. Narf-like genes are present in the genomes of a variety of eukaryotes[10a,13,104] (Table 6) including the smallest eukaryote genome sequenced so far, that of the obligately intracellular microscopiridian parasite *Encephalitozoon cuniculi*.[251a] Published data on the Narf1 protein suggest the presence of two [4Fe-4S] clusters and the absence of the 2Fe catalytic moiety. Accordingly, the Narf1-type proteins display no hydrogenase activity.[251b]

4. Biosynthesis of Hydrogenases

4.1. Biosynthesis of [NiFe]-Hydrogenases

In *Proteobacteria*, the genes that encode H_2-uptake hydrogenases are clustered. These clusters comprise the structural genes (generally labeled L for large subunit and S for small subunit) and accessory genes for maturation and the insertion of Ni, Fe, CO, and CN^- at the active site of the heterodimer. In some organisms, the hydrogenase gene cluster also comprises regulatory genes that control the expression of the structural genes. The maturation of hydrogenase follows a complex pathway, which involves at least seven auxiliary proteins, the products of the so-called *hyp* genes, namely, HypA, HypB, HypC, HypD, HypE, and HypF, and an endopeptidase. This set of proteins directs the synthesis and incorporation of the metal center into the large subunit, controls the fidelity of insertion of the correct metal, maintains a folding state of the protein competent for metal addition, and allows protein conformational changes for internalization of the assembled metal center. The gene/protein designations used for homologous proteins in various microorganisms are available in refs 10a and 14. The best-studied hydrogenase maturation system is the one involved in the biosynthesis of *E. coli* hydrogenase-3, deciphered by the group of Böck, and summarized in numerous recent reviews.[14,16−18,86,87,252,253] The iron atoms at the active site of

Table 6. [FeFe]-Hydrogenase-like Sequences Containing One or Two of the P Motifs[a]

patterns		taxon	length		AC	gene	annotation
P2		*Acidobacteria bacterium* Ellin345	397		Q3K7N9	Pfl_4478	
	P3	*Alkaliphilus metalliredigenes* QYMF	100	F	Q3C8E0	AmetDRAFT_2268	
P2		*Alkaliphilus metalliredigenes* QYMF	569		Q3C7C3	AmetDRAFT_2186	
P2	P3	*Anaplasma marginale* str. St. Maries TU502	560		Q5CGG4	Chro.10029	H
	P3	*Arabidopsis thaliana*	203		Q8GXY2	none	
P2	P3	*Arabidopsis thaliana*	474		Q94CL6	none	N
P2	P3	*Aspergillus fumigatus* Af293/CBS 101355/FGSC A1100	597		Q4WQ87	Afu4g11960	H
P2		*Bradyrhizobium japonicum* MAFF303099	146		Q98AX4	mll5816	
P2	P3	*Burkholderia pseudomallei* 1710b SN15	632		Q0UM75	SNOG_07139	
P2	P3	*Caenorhabditis briggsae* AF16	452		Q60RJ4	CBG21318	
P2	P3	*Caenorhabditis elegans* Bristol N2	457		Q9N392	Y54H5A.4	
P2	P3	*Caenorhabditis elegans* Liverpool	478		Q16ML2	AaeL_AAEL012261	
P2	P3	*Campylobacter upsaliensis* CBS 148.51	586		Q2HEF1	CHGG_01403	
P1		*Campylobacter upsaliensis* CBS 148.51	942		Q2HCY8	CHGG_01916	
P2	P3	*Campylobacter upsaliensis* SB210	488		Q22NP0	TTHERM_00198090	H
P2	P3	*Candidatus Kuenenia stuttgartiensis* PEST	479	F	Q7PWB8	ENSANGG00000004952	
P2		*Carboxydothermus hydrogenoformans* Z-2901	732		Q3ABV5	CHY_1547	
P2	P3	*Carboxydothermus hydrogenoformans* Z-2901 RS	618		Q1E736	CIMG_01627	
P2	P3	*Chlorobium chlorochromatii* CaD3 NIH2624	599		Q0CR17	ATEG_03867	
P2		*Chlorobium ferrooxidans* DSM 13031	261		Q0YUQ9	CferDRAFT_2151	
P2	P3	*Clostridium beijerincki* NCIMB 8052	496		Q2WME5	CbeiDRAFT_2269	H
P2	P3	*Clostridium difficile* 630	498		Q18A86	CD0894	H
P2		*Clostridium difficile* 630	509		Q18A87	CD0893	H
P2		*Clostridium phytofermentans* ISDg	577		Q1FJL8	CphyDRAFT_2328	H
P2	P3	*Clostridium* sp. OhILAs	578		Q1EVZ7	ClosDRAFT_0034	H
P2		*Clostridium thermocellum* ATCC 27405	556		Q4CGI0	CtheDRAFT_2176	H
P2	P3	*Desulfotomaculum reducens* MI-1	462		Q2D663	DredDRAFT_2236	H
P2		*Desulfotomaculum reducens* MI-1	500		Q2D377	DredDRAFT_1510	H
P2		*Desulfotomaculum reducens* MI-1	573		Q2CXB9	DredDRAFT_2383	
P2	P3	*Desulfotomaculum reducens* MI-1	594		Q2D1D0	DredDRAFT_0870	
P2	P3	*Dictyostelium discoideum* AX4	522		Q54F30	DDBDRAFT_0189262	
P2	P3	*Drosophila melanogaster*	296		Q5LJW9	CG17683	
P2	P3	*Drosophila melanogaster*	430		Q7PLS3	CG17683	
P2	P3	*Drosophila melanogaster*	473		Q5LJX0	CG17683	
P2	P3	*Drosophila melanogaster* Berkeley	477		Q8SYS7	CG17683	
P2	P3	*Emericella nidulans* FGSC 4	636		Q5B748	AN3632.2	
	P3	*Entamoeba histolytica*	105		Q5DCU1	none	
P2	P3	*Entamoeba histolytica* Ankara	666		Q4UCR4	TA05450	N
P2		*Entamoeba histolytica* GB-M1	365		Q8SVJ2	ECU05_0970	H
	P3	*Entamoeba histolytica* HM-1:IMSS	102		Q50YQ3	131.t00028	H
P2	P3	*Entamoeba histolytica* Iowa type II	560		Q8IS95	cgd1_190	H
P2	P3	*Entamoeba histolytica* Muguga	664		Q4N0Y8	TP03_0164	H
P2		*Entamoeba histolytica* Muguga	1084		Q4MZF5	TP03_0565	
	P3	*Gibberella zeae* 927/4 GUTat10.1	475		Q389R3	Tb10.406.0260	H
P2		*Gibberella zeae* 927/4 GUTat10.1	769		Q381N3	Tb11.01.7160	
	P3	*Gibberella zeae* CL Brener	474		Q4D686	Tc00.1047053503583.90	H
	P3	*Gibberella zeae* CL Brener	474		Q4D679	Tc00.1047053504625.60	H
	P3	*Gibberella zeae* Friedlin	642		Q4QJI0	LmjF05.0230	H
P2	P3	*Gibberella zeae* PH-1/NRRL 31084	577		Q4IQN3	FG00475.1	H
P2	P3	*Halothermothrix orenii* H 168	491		Q2AG55	HoreDRAFT_1707	H
P2	P3	*Halothermothrix orenii* H 168	571		Q2AG58	HoreDRAFT_1704	
P2	P3	*Halothermothrix orenii* H 168	584		Q2AG86	HoreDRAFT_1677	
P2		*Halothermothrix orenii* H 168	877		Q2AFM2	HoreDRAFT_1046	H
P2		*Homo sapiens*	213		Q3T1K9	Nicn1	
P2	P3	*Homo sapiens*	374		Q9H6J8	none	
P2	P3	*Homo sapiens*	476		Q9H6Q4	NARFL	
P2	P3	*Homo sapiens*	476		Q5BK18	Narfl	
P2	P3	*Homo sapiens*	476	F	Q53GC6	none	
P2	P3	*Homo sapiens*	525		Q96S10	NARFL	
P2	P3	*Homo sapiens* C57BL/6J TISSUE = colon	476		Q9D320	Narfl	
P2	P3	*Homo sapiens* C57BL/6J TISSUE = cortex	476		Q8BRR3	Narfl	
P2	P3	*Homo sapiens* C57BL/6J TISSUE = head	476		Q9CXS6	Narfl	
P2		*Homo sapiens* C57BL/6J TISSUE = kidney	213		Q3TFI4	Nicn1	
P2	P3	*Homo sapiens* C57BL/6J TISSUE = whole body	492		Q3ULM7	Narfl	
P2	P3	*Homo sapiens* C57BL/6NCr TISSUE = hematopoietic stem cell	476		Q7TMW6	Narfl	
P2		*Homo sapiens* CZECHII	333		Q5QKN3	8430426H19Rik	
P2		*Homo sapiens* FVB/N TISSUE = liver	213		Q9CQM0	Nicn1	
P2	P3	*Kluyveromyces lactis* ATCC 8585/CBS 2359/ DSM 70799/NRRL Y-1140/WM37	469		P53998	LET1	
P1		*Magnetospirillum magneticum* AMB-1	246		Q49W07	SSP1908	
	P3	*Medicago truncatula*	130		Q1S1X2	MtrDRAFT_AC148609g36v1	H
P2		*Medicago truncatula*	438		Q1S1X3	MtrDRAFT_AC148609g35v1	H
P2	P3	*Medicago truncatula*	478		Q2P9S0	gollum	

Table 6 (Continued)

patterns		taxon	length		AC	gene	annotation
P2	P3	*Medicago truncatula*	479		Q93YF9	none	N
P2		*Moorella thermoacetica* ATCC 39073	748		Q2RHS4	Moth_1713	
P2		*Moorella thermoacetica* ATCC 39073	752		Q2RHA0	Moth_1889	
	P3	*Neurospora crassa*	120	F	Q9P809	none	
P2	P3	*Neurospora crassa* 521	827		Q4PAR1	UM02802.1	
P2	P3	*Neurospora crassa* 74-OR23-1A/FGSC 987	581		Q7SGW5	NCU03204.1	
P2		*Neurospora crassa* ATCC 2001/CBS 138/ IFO 0622/NRRL Y-65	551		Q6FP07	CAGL0J07590g	
	P3	*Neurospora crassa* JEC21	650		Q5KB85	CNI03410	H
P2		*Neurospora crassa* SC5314	549		Q5AMS5	CaO19.12040	
P2	P3	*Neurospora crassa* SC5314	609		Q5APK7	NAR1	
P2	P3	*Oryza sativa*	476		Q8W303	OSJNBa0069E14.4	H
P2		*Pelobacter carbinolicus* DSM 2380	583		Q3A3I3	Pcar_1833	H
P2	P3	*Pelobacter carbinolicus* DSM 2380	585		Q3A430	Pcar_1633	H
P2	P3	*Pelobacter carbinolicus* DSM 2380	585		Q3A458	Pcar_1605	H
P2		*Pelotomaculum thermopropionicum* SI	578		Q1X3H0	none	H
P2	P3	*Pongo pygmaeus*	476		Q5RF36	DKFZp469G0432	
P1		*Pseudomonas hydrogenovora*	234		Q2R8E5	LOC_Os11g12470	
P1		*Pseudomonas hydrogenovora*	372	F	Q0ITP3	Os11g0231400	
P1		*Pseudomonas hydrogenovora*	435		Q53MD0	LOC_Os11g12470	
P2	P3	*Pseudomonas hydrogenovora*	476		Q10CV7	Os03g0748700	
	P3	*Rhodobacter sphaeroides* 2.4.1 B-3501A	650		Q55MV8	CNBH3260	
P2		*Saccharomyces cerevisiae* ATCC 204508/S288c	491		P23503	NAR1	N
P2	P3	*Schizosaccharomyces pombe* ATCC 38366/972	538		Q9Y7N7	SPCC1450.10c	
P2	P3	*Schizosaccharomyces pombe* GS115	438	F	Q5J882	none	
P2	P3	*Shewanella baltica* X514	580		Q0ESG0	Teth514DRAFT_0931	H
P2	P3	*Synechococcus* sp. PCC 7002 ATCC 10895/NRRL Y-1056/CBS 109.51	451		Q75E78	ABL205C	
P2	P3	*Tetraodon nigroviridis*	479		Q4RJI7	GSTENG00033416001	
P2	P3	*Thermoanaerobacter ethanolicus* ATCC 33223	577		Q3CI75	Teth39DRAFT_0175	
P2	P3	*Thermoanaerobacter tengcongensis* DSM 15242/ CM 11007/NBRC 100824/MB4	581		Q8RBW1	NapF	H
P2		*Thermotoga maritima* ATCC 43589/MSB8/ DSM 3109/JCM 10099	301		Q9X1D8	TM_1421	H
P2	P3	*Yarrowia lipolytica* ATCC 36239/CBS 767	545		Q6BUI4	DEHA0C11418g	
P2	P3	*Yarrowia lipolytica* CLIB 122/E 150	491		Q6CFR3	YALI0B04532g	
P2		*Yarrowia lipolytica* CLIB 122/E 150	505		Q6C2G2	YALI0F08151g	
P2		*Yarrowia lipolytica* CLIB 122/E 150	564		Q6CF61	YALI0B10021g	
P2	P3	*Yarrowia lipolytica* RIB 40/ATCC 42149	607		Q2UJY8	AO090003001020	N

[a] The pattern column shows the occurrence of one or two of the three patterns defined as characteristic of true [FeFe]-hydrogenases (see Figure 12). Protein length followed by F indicates a protein annotated as a fragment. The annotation column shows the explicit annotation in Uniprot when available: H = hydrogenase; N = Narf or Narf-like. Other proteins are annotated as "hydrogenase-like" or various other descriptive terms.

hydrogenases are linked to the nonbiological ligands, carbon monoxide and cyanide. Carbamoylphosphate has been shown to be the educt for the synthesis of the CN ligands of the NiFe metal center,[252,254,255] which requires the activity of two hydrogenase maturation proteins: HypF, a carbamoyltransferase, and HypE, which receives the carbamoyl moiety to its COOH-terminal cysteine to form an enzyme−thiocarbamate. HypE dehydrates the *S*-carbamoyl moiety to yield the enzyme thiocyanate, which can donate CN to iron.[256,257] HypE and HypF form a dynamic complex with HypC and HypD; CN is transferred to HypC−HypD and then attached to the iron atom of the NiFe site.[258] Conserved cysteine residues in the HypD protein are proposed to play a role in the maturation process.[259] The biosynthetic route for carbon monoxide to the NiFe active site is different from that for cyanide.[85] The products of the *hupGHIJ* operon have been shown recently to be involved in the maturation of the HupS hydrogenase subunit of *Rhizobium leguminosarum* uptake hydrogenase.[260]

[NiFe]-hydrogenases are found in organisms endowed with physiological attributes allowing their growth under very diverse environmental conditions: autotrophic or heterotrophic, in the light or in darkness, aerobically or anaerobically. Many metabolically versatile bacteria having several hydrogenase isoenzymes (Table 3) are differentially regulated according their lifestyle (reviewed in refs 17, 32, 33, 185, 200, and 261). The control of hydrogenase synthesis represents a means to quickly and efficiently respond to changes in the environment and in particular to new energy demands. It is exerted at the transcription level. Transcriptional control involves usually one or several two-component regulatory systems, which may act either positively or negatively. In response to a specific signal, the first component, a sensor histidine kinase, autophosphorylates at a conserved histidine residue and then transphosphorylates the cognate response regulator transcription factor at a conserved aspartate residue that activates or represses gene expression when phosphorylated by the sensor kinase.[262,263] Hydrogenase synthesis responds to several types of signals.

Molecular hydrogen, which is also the substrate, activates hydrogenase expression in aerobic bacteria (e.g., *R. eutropha*), in photosynthetic bacteria (e.g., *R. capsulatus*, *R. sphaeroides*, *R. palustris*), or in free-living *Rhizobia* (e.g., *B. japonicum*). The *H_2-specific regulatory system* comprises a hydrogen-sensing regulatory hydrogenase (HupUV/HoxBC) and a two-component signal transduction system, the histidine protein kinase HupT/HoxJ, and the response regulator HupR/HoxA. This system has been particularly well studied in *R. capsulatus*,[190,192,193,199,264,265] *R. eutropha*,[83,187,189,196,197,266,267] and very recently in *R. palustris*.[195]

In all of these bacteria, the regulatory cascade responding to H_2 uses the same elements: the H_2 signal is detected by the H_2 sensor (HupUV/HoxBC) and transmitted to the histidine kinase (HupT/HoxJ); it is transduced by phosphotransfer between the histidine kinase and the response regulator (HupR/HoxA) and integrated at the promoter of the structural genes of hydrogenase by the response regulator. However, in the absence of the H_2 sensor, whereas in *R. capsulatus* hydrogenase synthesis is derepressed,[190,199,268] in *B. japonicum*, *R. eutropha*, and *R. palustris*[195,197,266] there is no synthesis of the membrane-bound uptake hydrogenase. In *T. roseopersicina*, the components of the H_2-regulatory system (HupUV, HupT, and HupR) are present, but expression of the structural *hupSL* hydrogenase genes is not affected by the presence or absence of H_2.[194]

Carbon monoxide can support anaerobic growth of *R. rubrum.* CO-dependent growth relies on a CO oxidation system encoded by the *coo* genes organized in two CO-regulated transcriptional units. The *coo* regulon comprises CooS, an O_2-sensitive CO dehydrogenase, and CooLH, a CO-induced, CO-tolerant hydrogenase. Expression of the *coo* genes depends upon the activity of the CooA (*CO-o*xidation *a*ctivator) transcription factor (recently reviewed in refs 269−272). CooA is a homodimer in which each monomer contains a *b*-type heme and senses CO under anaerobic conditions.[273] Actually, CooA senses both the redox state of the cell and CO, for only the reduced form of the heme Fe (reduced at about −300 mV[274]) can bind CO. CO binding stabilizes a conformation of the dimeric protein that allows sequence-specific DNA binding and activation of transcription. The crystal structure of *R. rubrum* Fe(II)CooA has been solved[275] and the preliminary one of *Carboxydothermus hydrogenoformans* CooA reported.[276]

Molecular oxygen negatively regulates the synthesis of most hydrogenases, which usually require strict anaerobiosis or microaerobiosis for optimal synthesis. The sensing of low O_2 concentrations involves global regulatory proteins homologous to the *E. coli* Fnr protein. The *E. coli* anaerobic regulator Fnr (for *f*umarate *n*itrate *r*eduction) is a cytoplasmic O_2-responsive regulator with a sensory and a regulatory DNA-binding domain. Fnr activates the transcription of genes involved in anaerobic respiratory pathways while it represses the expression of genes involved in aerobic energy generation.[277] The protein binds as a dimer to an Fnr consensus sequence of dyad symmetry, TTGAT-N_4-ATCAA. Fnr activity depends on the presence of a $[4Fe\text{-}4S]^{2+}$ cluster converted rapidly to a more O_2-stable $[2Fe\text{-}2S]^{2+}$ cluster in the presence of O_2.[278] It is the O_2 lability of the $[4Fe\text{-}4S]^{2+}$ cluster that makes of Fnr an O_2 sensor.[279−282] In *E. coli*, Fnr binds and activates in anaerobiosis the *hyp* operon and thus affects indirectly hydrogenase synthesis. In *Rhizobia*, Fnr homologues, which regulate hydrogenase synthesis, are either Fnr-like (such as FixK1 in *B. japonicum* or FnrN in *R. leguminosarum*) or FixK-like (such as FixK2 in *B. japonicum*). FixK-like proteins lack the N-terminal region of Fnr for the binding of the [4Fe-4S] cluster. The main difference between Fnr-like and FixK-like regulators is therefore at the level of the redox control. The FixK-like proteins, which lack the redox-sensitive cysteines, are activated by an associated O_2-sensitive two-component system, FixLJ. In *B. japonicum*, under symbiotic conditions, O_2 signal transduction is organized along two regulatory cascades involving the activators FixK2 and NifA (nitrogen fixation activator).[283] In *R. leguminosarum* nodules, hydrogenase transcription is co-regulated with that of nitrogenase and controlled by NifA and FnrN in response to low O_2 concentrations. NifA activates directly hydrogenase gene expression by binding to an upstream activating sequence of the promoter region of the *hupSL* genes.[284] The Fnr homologue, FnrT, found in *T. roseopersicina*, induces anaerobic expression of the heat-stable membrane-associated HynSL hydrogenase[285] (see reviews in refs 17, 200, and 261 for additional references).

Redox regulation was first studied in *E. coli*. In *E. coli*, the synthesis of hydrogenases-1 and -2 depends on the global two-component regulatory system ArcB/ArcA.[277] Under anaerobic conditions ArcB, a tripartite membrane-associated sensor kinase, autophosphorylates and transphosphorylates the global transcriptional regulator ArcA. ArcA-phosphate is the active form that represses target genes of aerobic metabolism and activates genes of anaerobic metabolism. Quinones are redox signals for the Arc system. Oxidized forms of quinone electron carriers act as direct negative signals and inhibit autophosphorylation of ArcB during aerobiosis, thus providing a link between the respiratory chain and gene expression.[286,287] By oxidizing H_2 and generating low-potential electrons used by energy-consuming processes, such as carbon dioxide and dinitrogen fixation, hydrogenases participate in cellular redox metabolism. A global two-component signal transduction system, called RegB/RegA in *R. capsulatus* and PrrB/PrrA in *R. sphaeroides*, is implicated in the redox control of the above-mentioned processes[288] (reviewed in refs 289 and 290). It has been shown recently that a periplasmic loop between the transmembrane helices 3 and 4 of RegB contains a ubiquinone binding site. This domain was suggested to be responsible for sensing the redox state of the ubiquinone pool and subsequently controlling RegB autophosphorylation.[291] In *R. capsulatus*, RegB−RegA exerts a negative control on hydrogenase synthesis; the global regulation by RegB−RegA is superimposed on the H_2 regulation.[290,292] In *R. palustris*, the homologous RegS−RegR two-component regulatory system also represses hydrogenase gene expression. In contrast to *Rhodobacter*, RegSR does not play a pivotal role in global gene regulation in *R. palustris*.[195]

Formate Regulation. Optimal expression of the *hyc* operon, coding for *E. coli* hydrogenase-3, requires anaerobiosis, the absence of nitrate, and acidic pH. All of these factors act at the transcriptional level by regulating the level of formate. The *hyc* operon belongs to the formate regulon regulated by the transcriptional regulator FhlA.[293] FhlA shares homology with regulators of the NtrC family in its central and C-terminal domains but differs in possessing an extended N-terminal domain lacking the aspartate residue, which is the site of phosphorylation of response regulators. Thus, FhlA is not activated by phosphorylation but by binding an effector molecule, formate. It promotes a strong and specific binding to specific sequences of DNA. FhlA is a homotetramer, which binds to and activates the *hyc*, *hyp*, *fhlF*, and *hypF* promoters.[294,295] Thus, the regulator FhlA controls the expression of the structural and accessory genes of hydrogenase. The *hyf* operon, which can encode a putative hydrogenase-4 in *E. coli*, was found to resemble the *hyc* operon in being induced under anaerobic conditions by formate at low pH; purified HyfR, the homologue of FhlA, was found to specifically interact with the *hyf* promoter/operator region.[221]

Induction under N Limitation. In some N_2-fixing prokaryotes, hydrogenase is co-regulated with nitrogenase. The transcription of *R. leguminosarum* uptake hydrogenase (HupSL) has been shown to be directly controlled by the global regulator NifA.[284] Induction of a HupL transcript in *Nostoc* strains was observed after a shift from non-nitrogen-fixing conditions to N_2-fixing conditions.[296] Expression of the bidirectional NAD(P)-dependent hydrogenase in the cyanobacterium *Gloeocapsa alpicola* CALU 743 (*Synechocystis* PCC 6308) is increased in nitrate-limiting growth conditions.[297]

Sulfur and Selenium Regulation. The hyperthermophilic archaeon *P. furiosus* can grow on maltose either in the absence of elemental sulfur S^0 (it then produces H_2 as an end-product instead of H_2S) or in the presence of S^0. The effect of S^0 on the level of gene expression in *P. furiosus* cells was investigated with the use of DNA microarrays.[234] Subunits associated with the three hydrogenases characterized in *P. furiosus* (two cytoplasmic, hydrogenases I and II, and one membrane-bound) were found to be strongly down-regulated by S^0 (an indication that these hydrogenases are probably not directly involved in S^0 reduction). The effect of sulfur in the regulation of *P. furiosus* hydrogenases was further demonstrated by showing that the presence of S^0 in the growth medium resulted in decreases in specific activities of the three hydrogenases, each by an order of magnitude.[298] The nature of the enzyme system that reduces S^0 and the mechanism by which S^0 affects hydrogenase gene expression in *P. furiosus* are still unknown.

A regulation by selenium has been described in *Methanococcus voltae*, which encodes two pairs of [NiFe]-hydrogenases. One hydrogenase of each pair contains a selenocysteine in the active site, whereas the other one is selenium-free. The Se-free [NiFe]-hydrogenases, Vhc and Frc, are produced only upon Se deprivation[299,300] from the two *vhc* and *frc* transcription units, linked by a common 453 bp intergenic region subject to negative and positive regulation.[301] A protein binding to a negative regulatory element involved in the regulation of the two operons has been found to be a LysR-type regulator and named HrsM (*h*ydrogenase gene *r*egulator, *s*elenium-dependent in *M. voltae*).[302] In *hrsM* knockout mutants, the *vhc* and *frc* operons are constitutively transcribed in the presence of selenium.[302]

D. vulgaris Hildenborough contains the periplasmic-facing [FeFe]-, [FeNi]-, and [FeNiSe]-hydrogenases, encoded by the *hydBA*, *hynBA*, and *hysBA* genes, respectively. These periplasmic hydrogenases are translocated by the Tat system (cf. Figure 9). They have a similar physiological role in H_2 oxidation but are differently expressed in response to element availability. Inclusion of Se in the growth medium leads to a strong repression of the [FeFe]- and [NiFe]-hydrogenases and a strong increase in the [NiFeSe]-hydrogenase that is not detected in the absence of Se. Ni also leads to increased formation of the [NiFe]-hydrogenase, except for growth with H_2, when its synthesis is very high even without Ni added to the medium.[303]

Ni-Specific Regulation. In *E. coli*, the nickel-specific transport system, encoded by the *nikABCDE* operon,[304] is a member of the ABC transporter family and provides Ni^{2+} ions for the anaerobic biosynthesis of hydrogenases.[86,305] In the presence of excess nickel, expression of the *nik* operon is transcriptionally repressed by the Ni-responsive repressor NikR,[306] a protein of the ribbon−helix−helix family of transcription factors[307,308] having an affinity for nickel that responds to DNA binding.[309] NikR is a direct sensor of nickel ions.[310,311] A NikR orthologue, present in *Pyrococcus horikoshii* (PhNikR), has been crystallized and structurally characterized.[312,313] In *B. japonicum* the HypB protein, a nickel-binding GTPase necessary for incorporation of Ni into the hydrogenase apoprotein, carries out also a nickel storage/sequestering function; it may relay the Ni signal to regulatory proteins controlling hydrogenase synthesis.[314,315] Not clear either is the mechanism by which Ni regulates hydrogenase transcription in *R. leguminosarum*,[316] in *Nostoc* strains,[317] or how Hmd hydrogenase is induced and F_{420}-reducing hydrogenase completely repressed in *M. marburgensis*, under nickel limitation.[318]

4.2. Biosynthesis of [FeFe]-Hydrogenases

It is only recently that accessory genes necessary for the biosynthesis of [FeFe]-hydrogenases have been identified, when it was discovered that two novel radical *S*-adenosylmethionine (SAM) proteins were required for the assembly of the active site of *C. reinhardtii* hydrogenases.[19] Random insertional mutants having their *hydEF* gene inactivated were incapable of assembling an active [FeFe]-hydrogenase. In the *C. reinhardtii* genome, the *hydEF* gene is adjacent to another hydrogenase-related gene, *hydG*. Both HydE and HydG belong to the radical *S*-adenosylmethionine (commonly designated "radical SAM") superfamily of proteins;[319] their radical-SAM domains contain the conserved motif Cx_3Cx_2C, with additional motifs in the C-terminal ends that are characteristic of [Fe-S] cluster-binding sites.[320] Radical SAM proteins generate a radical species by reductive cleavage of *S*-adenosylmethionine through an [Fe-S] center to catalyze reactions involved in cofactor biosynthesis, metabolism, and synthesis of deoxyribonucleotides.[319] The HydF maturation protein contains at its N-terminal end conserved GTP-binding motifs suggesting that it belongs to the GTPase protein family.[19] The anaerobically reconstituted HydE and HydG proteins from *Thermotoga maritima* are indeed able to reductively cleave SAM when reduced by dithionite, confirming that they are radical SAM enzymes,[321] and HydF from *T. maritima* is a GTPase with an iron−sulfur cluster.[21] Anaerobic coexpression of the *C. reinhardtii hydEF*, *hydG*, and *hydA1* genes in *E. coli* resulted in the formation of an active HydA1 enzyme.[19] [Fe-Fe]-hydrogenases with high specific activities were obtained in *Clostridium acetobutylicum* by homologous and heterologous overexpression of the *hydA* gene from *C. acetobutylicum*, *C. reinhardtii*, and *S. obliquus*, respectively.[322] Because the *C. acetobutylicum hydE*, *hydF*, and *hydG* clones are more stable in *E. coli* than their *C. reinhardtii* homologues, an efficient biosynthetic system has been developed in *E. coli* by expression cloning of the *hydE*, *hydF*, and *hydG* genes from *C. acetobutylicum*. An active [FeFe]-hydrogenase was obtained with the fully functional maturation proteins and the N-terminally deleted *C. acetobutylicum* HydA and *C. pasteurianum* HydA, that is, with the catalytic H-cluster-containing domain only.[20] Consistent with the role of radical *S*-adenosylmethionine enzymes involved in the production of active [FeFe]-hydrogenases, a mechanistic scheme for hydrogenase H-cluster biosynthesis has been presented in which both carbon monoxide and cyanide ligands can be derived from the decomposition of a glycine radical[323a] (see also ref 323b). In his survey of [FeFe]-hydrogenase genes present in sequenced genomes, Meyer[13] pointed out that whereas [FeFe]-hydrogenase maturases are present in hydrogeno-

some-containing protists (*T. vaginalis*),[324] they are absent in hydrogenosome-less protists (*G. lamblia* and *E. histolytica*) and in the α-proteobacteria *R. palustris* and *R. rubrum*.

4.3. Biosynthesis of [Fe-S] Clusters

Pioneering studies of the biosynthesis of nitrogenase, which is encoded by the *nif* genes, led to the identification of proteins involved in [Fe-S] cluster assembly (reviewed in refs 325−327). The NifS and NifU proteins of *Azotobacter vinelandii* were originally found to be necessary for the synthesis of both components of the nitrogenase enzyme, each of which contains [Fe-S] clusters. It was later shown that NifS is a homodimeric pyridoxal-phosphate-dependent enzyme, with a cysteine (Cys_{325}) at its active site, that cleaves L-cysteine as a substrate to form alanine and an enzyme-bound cysteine-persulfide, the proposed activated form of sulfur that is ultimately used for [Fe-S] cluster assembly.[328,329] NifS belongs to a class of proteins [IscS, CsdB (now called SufS), CSD (now called CsdA)] having cysteine desulfurase activity.[330,331] Several of them have been analyzed by crystallography.[332−335] NifU is a modular, homodimeric protein that provides a molecular scaffold for the NifS-directed formation of [Fe-S] clusters. The NifU protein comprises three domains, the N- and C-terminal domains and a central domain with a redox-active $[2Fe\text{-}2S]^{2+,+}$ cluster per monomer, which is stable and is designated the "permanent" cluster. A second type of [2Fe-2S] cluster, highly labile, is assembled on NifU when it is co-incubated with NifS, Fe^{2+}, and cysteine. This second, labile cluster type, designated a "transient" cluster, is ultimately destined for nitrogenase [Fe-S] cluster formation.[336] The N-terminal domain of NifU is related to a family of [Fe-S] cluster biosynthetic scaffolds designated IscU (U-type) (see below), and the C-terminal domain exhibits sequence similarity to a second family of proposed [Fe-S] cluster biosynthetic scaffolds designated Nfu. Both scaffolding domains of NifU are separately competent for in vitro maturation of nitrogenase component proteins, although the N-terminal domain appears to have a dominant function.[337] Results obtained with full-length NifU and truncated forms involving only the N-terminal domain or the central and C-terminal domains have demonstrated sequential assembly of labile $[2Fe\text{-}2S]^{2+}$ and $[4Fe\text{-}4S]^{2+}$ clusters in the U-type N-terminal scaffolding domain and the assembly of $[4Fe\text{-}4S]^{2+}$ clusters in the Nfu-type C-terminal scaffolding domain. $[4Fe\text{-}4S]^{2+}$ clusters preassembled on either the N- or C-terminal domains were rapidly transferred to the apo nitrogenase Fe protein. In *A. vinelandii*, NifU and NifS required for the maturation of *nif*-specific [Fe-S] proteins cannot functionally replace the *isc*-gene products used for the maturation of other [Fe-S] proteins.[338] However, the Nif type system is not restricted to N_2 fixing organisms; in *Helicobacter pylori* that do not fix nitrogen, there is good evidence that a Nif-like system is necessary for generalized maturation of [Fe-S] proteins.[339]

Because the inactivation of either *nifS* or *nifU* only decreased, but did not eliminate, nitrogenase activity, non-*nif* genes that encode proteins similar in structure and function to NifS and NifU were sought. The *iscS* and *iscU* genes ("*isc*" for *i*ron−*s*ulfur *c*luster formation) were found in the *iscRSUA-hscBA-fdx* gene cluster within the *A. vinelandii* genome.[340] The *iscR* gene encodes a [2Fe-2S]-containing transcription factor, a negative regulator of the expression of all genes contained within the *isc* region.[341] The *isc* region is widely conserved among most bacteria. Homologues of proteins encoded by the *isc* gene cluster are also present in eukaryotic organisms.[342−347] All of the products of the *isc* operon are involved in [Fe-S] cluster biogenesis. IscS and NifS bear a great deal of primary sequence similarity, in particular between the respective active site cysteine and pyridoxal-phosphate binding regions. In *E. coli*, IscS activity is necessary for the mobilization of S for the maturation of various cofactors and proteins.[348−352] The crystal structure of IscS has been determined.[353] IscU is a truncated version of NifU, containing the N-terminal domain of NifU.[336] IscU provides molecular scaffolds for the IscS-mediated assembly of [Fe-S] clusters.[354,355] The mechanism of [Fe-S] cluster assembly involves the formation of an IscS−IscU complex[356,357] in which a covalent disulfide bond is formed between a conserved cysteine residue (Cys_{328}) of IscS and Cys_{63} of IscU.[331,353,358] The transient [Fe-S] clusters in IscU are subsequently transferred to target proteins.[359,360] IscA was suggested to function as an alternative scaffold for [Fe-S] cluster assembly, as IscA, like IscU, can host a transient [2Fe-2S] cluster.[361−363] Because the crystal structure of IscA[364] revealed the presence of a well-ordered fold in contrast to the highly mobile secondary structural elements within IscU,[365−367] the two proteins may not have equivalent function. Indeed, it was shown recently[368] that [Fe-S] cluster-loaded IscU can transfer its cluster to apoIscA, whereas the reverse reaction (transfer of [Fe-S] cluster from holoIscA to apoIscU) is not possible, suggesting that IscU is the primary cluster assembly factory where [Fe-S] clusters are preassembled and that IscA is the second one, where preassembled clusters transit before transfer to target apo-acceptor. In *Synechocystis*, it is cystine rather than cysteine that is the source of activated S,[332,369−371] and the activated species is free cysteine-persulfide rather than a cysteine persulfide residue bound to an active-site enzyme.

Although it is clearly established that sulfur in [Fe-S] clusters is provided by cysteine desulfurases (NifS, IscS, CsdA, SufS, or yeast Nfs1p) via desulfurization of L-cysteine, the iron donor is essentially unknown. It has been reported that human frataxin, present in the mitochondrial matrix, may act as the iron donor for [Fe-S] assembly in ISU, a human IscU homologue.[372] Human apofrataxin can bind up to six or seven iron atoms. Holofrataxin then mediates the transfer of iron to the nucleation sites for [2Fe-2S] cluster formation on ISU. Similarly, the yeast frataxin homologue Yfh1 has been shown to physically interact with the core [Fe-S] cluster assembly complex, composed of the scaffold protein Isu1 and the cysteine desulfurase Nfs1 (the orthologue of the bacterial cysteine desulfurase IscS[373]), and to be involved in the de novo [Fe-S] cluster synthesis on Isu1.[374] This suggests that frataxin might play a role in iron loading of Isu1. Although IscU was reported to bind mononuclear iron,[336,354,375,376] association of an [Fe-S] cluster with the homologous yeast protein Isu1p, rather than mononuclear iron, was deduced by Mülenhoff et al.[377] IscA, which can bind iron with an apparent iron association constant of 3.0×10^{19} M^{-1},[378] has been proposed to act as an iron donor for [Fe-S] clusters in *E. coli*. The iron-loaded IscA can provide iron for the assembly of transient [Fe-S] clusters in IscU in the presence of IscS and L-cysteine,[379,380] under aerobic conditions.[381a] The precise function of IscA, as a scaffold protein or an iron donor, is still unknown because many IscA proteins were isolated directly with an Fe-S cluster. That is the case for IscA from *Thermosynechococcus elongatus*, the structure of which was recently solved,[381b] and

IscA from *Synechocystis*[381c] and *Acidithiobacillus ferrooxidans*.[381d] Another iron donor for the assembly of [2Fe-2S] clusters in the scaffold IscU has been identified in *E. coli* as the CyaY protein, the bacterial orthologue of frataxin.[382] CyaY was shown to interact specifically with IscS without formation of an intermolecular disulfide bridge between the two proteins and to bind Fe^{3+} (up to 8 Fe^{3+}/polypeptide chain) with an iron association constant of higher than 1.0×10^{17} M^{-1}. The proposed mechanism for the formation of [2Fe-2S] in IscU with Fe^{3+}-loaded CyaY as iron donor implies, in the first step, transfer of the sulfur atom from L-cysteine to IscS to generate a persulfide, in a second step, upon reduction, iron liberation and transfer from CyaY to IscS, generating protein-bound cysteine−sulfur−sulfur iron species, followed by transfer of FeS to IscU.[382] Studies of the interactions of IscA, CyaY with IscS and IscU will help to elucidate whether delivery of iron[376] or sulfur[356,357] is the first step in [Fe-S] cluster assembly in IscU or if iron and sulfur can be transmitted together to IscU as suggested.[382] The products of *hsc*A and *hsc*B genes ("hsc", *h*eat *s*hock *c*ognate) similar to the molecular chaperones DnaK and DnaJ, respectively, appear to be intimately involved in [Fe-S] cluster assembly in the IscU scaffold.[383−385] The yeast chaperone homologues, Ssq1p and Jac1p, form a functional unit in [Fe-S] protein biogenesis but, instead of being involved in de novo [Fe-S] cluster assembly on Isu1p, the chaperone system would be more likely required for the dislocation of a preassembled [Fe-S] cluster from Isu1.[377]

Other genes playing a role in [Fe-S] cluster formation have been identified in *E. coli*, namely, the *sufABCDSE* operon (*suf* for mobilization of *sul*fur).[386] SufS, like IscS, exhibits cysteine desulfurase activity, whereas SufA shares sequence similarity with IscA, including the three conserved cysteines involved in [Fe-S] cluster assembly. However, there is no homologue of *iscU* or *hscBA* in the *suf* operon. The Suf proteins, all located in the cytosol, form a third bacterial system for the assembly of [Fe-S] clusters.[387] The ISC and SUF systems comprise in common a cysteine desulfurase (sulfur donor) and scaffold proteins (sulfur and iron acceptors); they differ by the presence of a pair of heat shock-like chaperones present only in ISC and, in SUF, by the presence of an unorthodox ATP-binding cassette (ABC)-like component, the function of which is still unknown. ISC is present in eubacteria and most eukaryotes and SUF is found in bacteria, archaea, plants, and parasites.[388] ISC appears to be the housekeeping [Fe-S] cluster assembly system,[389] whereas SUF is specifically adapted to synthesize [Fe-S] clusters in harsh environmental conditions such as oxidative stress and iron starvation.[388−390] Actually, both the *isc* and the *suf* operons are induced during exposure to hydrogen peroxide (H_2O_2) and the iron chelator 2,2′-dipyridyl. Regulation of the *isc* operon is mediated by IscR, which in the [2Fe-2S] bound form serves as a repressor of *iscRSUA* gene expression under anaerobic conditions; under oxidative stress conditions, the demetalated form derepresses the *isc* operon and directly activates the *suf* operon.[391] Induction of the *suf* operon in response to oxidative stress requires the transcription factors OxyR and IHF[389,392,393] and, in response to iron starvation, the global regulatory protein called Fur.[386,394,395] The DNA binding site of these regulators has been determined.[389,393] The three-dimensional structure of the SufA,[396] SufC,[397] SufD,[398] and SufE[399] proteins has been determined. The mechanisms of [Fe-S] cluster assembly by the SUF machinery have been reviewed recently.[327,388,390] SufE enhances the cysteine desulfurization activity of SufS up to 50-fold.[400,401] There is direct transfer of the sulfur atom from the cysteine persulfide of SufS to the single invariant cysteine residue of SufE;[402] this transpersulfuration is probably at the origin of the cysteine desulfurase enhancement. The crystal structure of *E. coli* SufE shows that the persulfide-forming cysteine occurs at the tip of a loop; despite lack of sequence homology, the core of SufE shows strong structural similarity to IscU, and the sulfur-acceptor site in SufE coincides with the location of the cysteine residues mediating [Fe-S] cluster assembly in IscU.[403] SufE interacts with SufB for sulfur transfer to SufB that can act as a novel site for [FeS] cluster assembly in the Suf system. The interaction occurs only if SufC is present.[404] In *E. coli* and *Erwinia chrysanthemi*, SufA is a scaffold protein on which [FeS] clusters are transiently assembled before being inserted into the target apoprotein.[388,390,400,405] The molecular mechanism of FeS assembly on *E. coli* SufA has been recently discussed by Sendra et al.[405] Sulfur is provided by the activity of the SufES complex, but the source of iron remains unknown. In cyanobacteria, it is Nfu and not SufA or IscA that is the essential [Fe-S] cluster scaffold protein. Instead of being involved in generalized [Fe-S] cluster assembly, SufA and IscA have been proposed to play regulatory roles in iron homeostasis and the sensing of redox stress in cyanobacteria.[406] In *Synechocystis* sp. strain PCC 6803, the *sufR* gene (sll0088) functions as a repressor of the *sufBCDS* operon. The SufR protein harbors an [Fe-S] cluster. A null *sufR* mutant exhibits derepression of the *suf* operon under conditions of oxidative or iron stress.[407] In *E. coli*, the sulfur-generating system referred to as CSD, which involves CsdA−CsdE cysteine desulfurase, also contributes to [Fe-S] cluster biogenesis in vivo.[408]

Homologues to some SUF proteins have been discovered in the plant *Arabidopsis thaliana*.[409−412] The SUF system is specific for the plastid and is therefore of symbiotic origin. *A. thaliana* chloroplasts contain a NifS-like cysteine desulfurase (AtCpNifS) with low activity. Addition of CpSufE increases CpNifS activity over 40-fold and the affinity of the enzyme for cysteine.[413] CpIscA has been proposed to serve as scaffold in chloroplast [Fe-S] cluster assembly.[414] Features of the plastidic machinery for [Fe-S] cluster assembly have been reviewed recently.[415,416] In contrast to other SUF proteins, AtSufE localizes to plastids and mitochondria interacting with the plastidic AtSufS and mitochondrial AtNifS1 cysteine desulfurases; AtSufE acts as an activator of plastidic and mitochondrial cysteine desulfurases in *Arabidopsis*.[411]

5. Hydrogenases and the Origins of Cells

How can our understanding of the origin, structure, evolution, and function of hydrogenases in present-day organisms, including eukaryotes, provide insight into the early evolution of nucleated cells? Sequence similarities between hydrogenases and the energy-converting NADH-ubiquinone oxidoreductase of mitochondria and bacteria, also known as respiratory complex I, have been emphasized in many papers.[10a,17,34,135,417−421] Not only [NiFe]-hydrogenases (Figure 10; Table 4) but also [FeFe]-hydrogenases (Figure 13) have subunits or [Fe-S] cluster-containing domains homologous to complex I subunits. It has been proposed[419] that the [NiFe] active site of hydrogenase was reorganized into a quinone-reduction site carried by the NuoB−NuoD dimer in complex I and a hydrophobic subunit such as

NuoH[422] (Figure 10). Homology between hydrogenases and complex I is found not only among electron-transferring modules but also in proton-pumping modules. According to Mathiesen and Hägerhäll,[423] the last common ancestor of complex I and the membrane-bound [NiFe]-hydrogenases of group 4 contained the NuoKLMN subunit module (cf. Figure 10 and Table 4).

A prominent role of hydrogenase in the origin of the eukaryotic cell has been proposed in two new hypotheses, the hydrogen hypothesis[424] and the syntrophic hypothesis.[425] These two hypotheses represent a paradigm shift[426] from the endosymbiosis theory for the origin of mitochondria and chloroplasts, revived by Margulis.[427] The two hypotheses posit that a metabolic symbiosis (syntrophy) between a methanogenic archaebacterium and a proteobacterium able to release H_2 in anaerobiosis was the first step in eukaryogenesis.[428] The hydrogen hypothesis[424,429] proposes that an anaerobic heterotrophic α-*Proteobacterium* producing H_2 and CO_2 as waste products formed a symbiotic metabolic association (syntrophy) with a strictly anaerobic, autotrophic archaebacterium, possibly a methanogen dependent on H_2. The intimate relationship over long periods of time allowed the symbiont and the host to co-evolve and become dependent on each other. In an anaerobic environment the symbiont either was lost, as in type I amitochondriate eukaryotes, or became a hydrogenosome, that is, a hydrogen-generating and ATP-supplying organelle, as in type II amitochondriate eukaryotes.[247] By further evolution, the host lost its autotrophic pathway and its dependence on H_2 and the endosymbiont adopted a more efficient aerobic respiration to become the ancestral mitochondrion. Thus, the eukaryotic cell would have emerged as the result of endosymbiosis between two prokaryotes, an H_2-dependent, autotrophic archaebacterium (the host) and an H_2- and ATP-producing eubacterium (the symbiont), the common ancestor of mitochondria and hydrogenosomes. The syntrophy hypothesis for the origin of eukaryotes, proposed at the same time and independently,[425] is based on similar metabolic consideration (interspecies hydrogen transfer), but the latter authors speculated that the organisms involved in syntrophy with methanogenic *Archaea* belonged to the δ-*Proteobacteria* (ancestral sulfate-reducing myxobacteria) (it was also suggested that a second anaerobic symbiont was involved in the origin of mitochondria).

The two hypotheses, based on energy metabolism considerations,[424,425] suggest an anaerobic energy metabolism for the origin of the proto-mitochondrial symbiosis and posit that the origins of the heterotrophic organelle (the symbiont) and the origins of the eukaryotic lineage are identical. The complete genome sequences for many oxygen-respiring mitochondria and for some bacteria lead to the conclusion that mitochondria descend from α-proteobacteria,[430,431] and a wealth of data indicate that mitochondria and hydrogenosomes share a common ancestry.[424,430,432,433] The work of many laboratories (reviewed in refs 430, 434, and 435) has shown that hydrogenosomes are in fact anaerobic forms of mitochondria. One of the debated questions is to know whether hydrogenosomes are relics of the ancestral endosymbiont or are biochemically modified mitochondria that have lost the capacity for oxidative phosphorylation, gained the capacity to make hydrogen, and evolved several times as adaptations of mitochondria to anaerobic environments.[250,436−442] It has been shown recently that *Trichomonas* hydrogenosomes contain the NADH module of mitochondrial complex I, which can reduce ubiquinone and also ferredoxin, the electron carrier used for hydrogen production. Recruitment of complex I subunits for H_2 production was taken as evidence that mitochondria and hydrogenosomes are aerobic and anaerobic homologues of the same endosymbiotically derived organelle.[443] Furthermore, in the hydrogenosomes of the anaerobic ciliate *Nyctotherus ovalis*, which thrives in the hindgut of cockroaches, a rudimentary genome can encode components of a mitochondrial electron transport chain.[444] Those proteins are homologous with counterparts from aerobic ciliates. The production of H_2, the presence in the genome of genes encoding respiratory chain components and biochemical features characteristic of anaerobic mitochondria, identify for the authors[444] the *N. ovalis* organelle as a missing link between mitochondria and hydrogenosomes. On the other hand, phylogenetic analyses indicate that neither of the proteins Ndh51 and Ndh24 of the hydrogenosomal complex I-like has a common origin with mitochondrial homologues; this conclusion argues against a vertical origin of trichomonad hydrogenosomes from the proto-mitochondrial endosymbiont.[445]

Eukaryotic organelles contain only [FeFe]-hydrogenases. The source of an ancestral [FeFe]-hydrogenase is not resolved; its presence in eukaryotes may reflect an early lateral gene transfer from a eubacterium. The plastidial [FeFe]-hydrogenases appear to have a non-cyanobacterial origin, because cyanobacteria, the progenitors of chloroplasts, contain only [NiFe]-hydrogenases and no [FeFe]-hydrogenases[10a,32,183,185] (this review). Possibly, the hydrogenase of the original endosymbiont has been replaced by an [FeFe]-hydrogenase of non-cyanobacterial origin, encoded by the host nucleus. A phylogenetic analysis of eukaryotic [FeFe]-hydrogenases[12,13,104] (see below) suggests a polyphyletic origin of these enzymes, implying an acquisition by lateral gene transfer from different prokaryotic sources or by symbiosis with a clostridium or δ-proteobacterium.[13] On the other hand, the [FeFe]-hydrogenases from green algae emerge as a monophyletic group with hydrogenosomal [FeFe]-hydrogenases from microaerophilic protists[12,13] (see section 6).

Mitochondria do not contain [FeFe]-hydrogenase but have kept a key enzyme, cysteine desulfurase (called IscS or Nfs1), which performs a crucial role in cellular [Fe-S] protein maturation[342,343,446] and appears to have originated from the ancestor endosymbiont. The capacity to synthesize [Fe-S] clusters is the essential biosynthetic process performed by mitochondria (recent reviews in refs 344 and 447). A novel protein of the mitochondrial matrix, termed Isd11, forms a stable complex with Nfs1, the mitochondrial cysteine desulfurase,[373] and is essential in [Fe-S] cluster biogenesis in mitochondria.[448,449] Isd11, highly conserved from yeast to human, is unique to eukaryotes but functions closely with the α-proteobacterium-derived cysteine desulfurase IscS. According to Richards and van der Giezen[450] the eukaryotic invention of Isd11 as a functional partner to IscS implies a single shared α-proteobacterial endosymbiotic ancestry for all eukaryotes; the α-proteobacterial endosymbiotic event would have occurred before the last common ancestor of all eukaryotes appeared. The intestinal pathogen *Giardia intestinalis* and the human genitourinary parasite *Trichomonas vaginalis*, representatives of early diverging eukaryotic lineages, are eukaryotes without standard mitochondria but contain mitochondrial type [Fe-S] cluster (Isc) assembly proteins located to mitosomes in *Giardia* and hydrogenos-

omes in *Trichomonas*. The capacity of the [Fe-S] cluster assembly of *Giardia* mitosomes[451] and of *Trichomonas* hydrogenosomes[452] supports also the conclusion that the process is inherited from the proteobacterial ancestor of mitochondria. The presence and development of [Fe-S] clusters during evolution underscore the role that iron and sulfide are postulated to have played at the origin of life.[453,454] The hypothesis[453,454] favors a single origin of life with the emergence of a non-free-living universal ancestor confined in structured Fe-S precipitates at a warm submarine seepage site.

6. Evolutionary Relationships between Hydrogenases

Hydrogenases display modular structures with a large diversity of quaternary structure and size of the catalytic subunit (Figure 6), in particular in the case of [FeFe]-hydrogenases (Figure 13). Besides, most of the subunits and domains other than the H-cluster domain of [FeFe]-hydrogenases have counterparts in other redox proteins, for example, ferredoxins and NADH-ubiquinone oxidoreductase (Figures 10 and 13; Table 4). This diversity witnesses the widespread swapping of redox protein modules among energy-conserving systems that occurred during evolution. Within the framework of hydrogenase biodiversity and evolution, the focus was put on features that are very well conserved within either the [NiFe]- or [FeFe-hydrogenases, which represent an example of convergent evolution.

6.1. Phylogeny of [NiFe]-Hydrogenases

Phylogenetic trees of [NiFe]-hydrogenases were derived from amino acid alignments of the sequence of entire subunits. Simplified trees of the small and large subunits are shown in Figure 14, parts A and B, respectively. Only hydrogenases for which complete sequences of both subunits were available have been included in the trees.

The same types of groupings were produced by the small and large subunits, respectively. These groupings are consistent with the functional classes defined under section 3. It is therefore very likely that the four classes have been individualized as distinct genes before the separation of the main phyla, that is, in the earliest steps of cellular evolution on Earth. The deepest branchings, however, do not appear in the same order in the two trees. Yet, as these nodes correspond to very ancient events, they are not expected to be accurately reconstructed. On the contrary, the four groups are all very robust clades. The subgroups of groups 2 and 3 are less sustained by bootstrap values. In some cases, a single sequence is responsible for a significant lowering of its subgroup bootstrap value. This is the case for *A. aeolicus* MbhL3 (subgroup 2a), *M. voltae* VhcAG (subgroup 3c), and *C. necator* HoxYH (subroup 3d). The fact that both subunits follow so similar evolutionary schemes indicates that these enzymes have consisted of two tightly associated subunits for most, and probably all, of their evolutionary history.

The tree does not exactly reflect the relative distribution of enzymes in each subgroup. In *Proteobacteria* of medical or environmental interest (Table 1) the sequences are very closely related to each other and do not provide much additional information from the viewpoint of evolution. The uptake hydrogenases from cyanobacteria belong to group 2, together with the H_2-sensors. As mentioned earlier (section 3.3.2), proteins of groups 2a and 2b are characterized by several conserved deletions (place and size of deletions) with respect to group 1 enzymes, suggesting that they probably evolved from a common ancestor, but their function diverged depending on the host organism. Because the *Archaea* have no representatives in group 2, the emergence of group 2 hydrogenases appears to have occurred within the *Bacteria* after the divergence of the domains *Archaea* and *Bacteria*. These lineages are unequally represented in the four [NiFe]-hydrogenase groups: most archeal sequences map within groups 3 and 4, whereas bacterial ones belong mainly to groups 1 and 2. However, genome sequencing has now uncovered the presence of bacterial enzymes belonging to groups 3 and 4 as well. The prototype of enzymes from group 4 is *E. coli* hydrogenase-3, but many hydrogenases assigned to group 4 share similarities with the Ech enzyme found recently in the *Archaea* or are of the Hyf-type, the fourth *E. coli* hydrogenase that has not yet been proven to be functional in *E. coli*. Coppi[30] was the first to recognize that several of the proteins that map in group 4 are not really [NiFe]-hydrogenases because they lack the typical CxxC motifs containing the cysteine residues present at the N and C terminus of the large subunit; she proposed to designate them Ehr, *E*ch-*h*ydrogenase-*r*elated. Proteins devoid of a CxxC pattern in the large subunit (EhrL) are listed in Table 7 (EhrS for the putative corresponding small subunit).

Hydrogenase sequences from photosynthetic prokaryotes are found in each of the four groups of [NiFe]-hydrogenases (Figure 15). Besides the uptake enzymes of group 1 and those of group 2 (cyanobacterial uptake ones and H_2-sensing), genome sequencing has also disclosed the presence of group 3 enzymes in phototrophic green sulfur bacteria of the genus *Chlorobium* and *Pelodictyon* (group 3b), in green non-sulfur bacteria of the class *Chloroflexi* (group 3c), in *Cyanobacteria*, and in phototrophic bacteria of the order *Chromatiales* (*T. roseopersicina* and *Allochromatium vinosum*) (group 3d). As already noted,[183] the uptake and bidirectional hydrogenases of *Chloroflexus aurantiacus* cluster with those of cyanobacteria (Figure 15), and it was suggested that a *Chloroflexus*-like bacterium might have been the ancestor of *C. aurantiacus* and cyanobacteria.[183] In group 4, the CooLH hydrogenase from the purple bacterium *R. rubrum*, associated with CO dehydrogenase, has been the best studied. This type of hydrogenase is apparently also present in *R. palustris* and *R. gelatinosus*. Our results are in agreement with the recently published phylogenetic analysis of the hydrogenases of all five major groups of photosynthetic bacteria (heliobacteria, green non-sulfur bacteria, green sulfur bacteria, photosynthetic proteobacteria, and cyanobacteria).[183]

6.2. Phylogeny of [FeFe]-Hydrogenases

The variety of the size of the catalytic subunit and of the quaternary structure of [FeFe]-hydrogenases precludes the use of full-length sequences for tree construction (Figure 16). The aligned residues used in the tree-building procedure were not restricted a priori. However, most of the regions outside the H-cluster are too divergent to retain a valuable phylogenetical signal: among the 155 informative characters retained by the GBlocks procedure,[585] 133 do belong to the H-cluster, which spans from residues 210 to 574 in the *C. pasteurianum* sequence.[63,570] Moreover, the three patterns defined in Figure 12, which are included in the H-cluster, also belong to the set of informative residues.

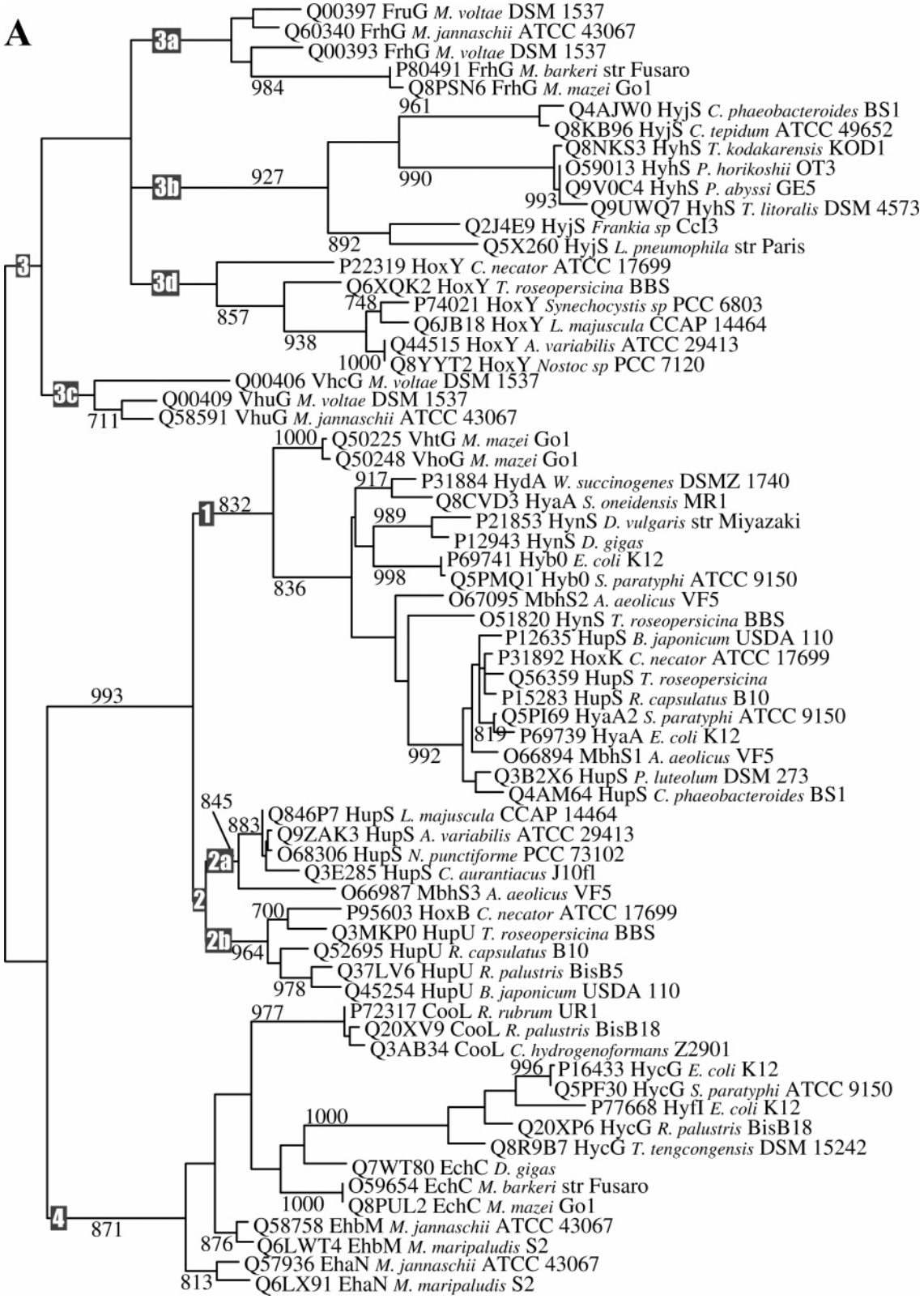

Figure 14. (1 of 2)

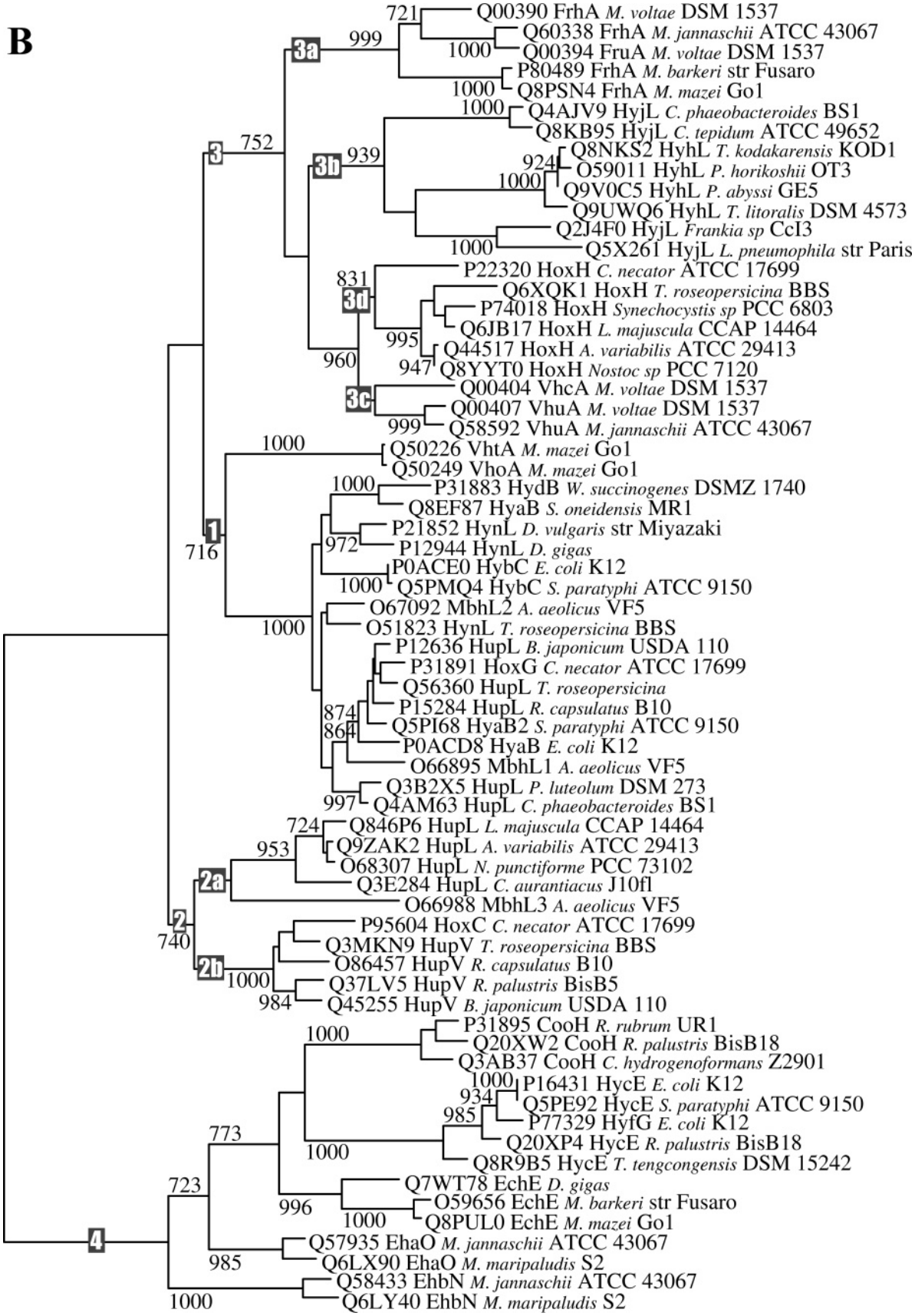

Figure 14. Simplified phylogenetic tree of [NiFe]-hydrogenases constructed with full-length enzymes from small (A) and large (B) subunits of subgroup representatives. The alignments made with Clustal W[584] were manually improved, and informative characters were selected with Gblocks.0.91b.[585] Trees were computed with PhyML[586] using the bootstrap procedure with 1000 replicates and then displayed and printed with NJPLOT.[587] The same method was used to construct all of the phylogenetic trees presented in this review. Branch lengths along the horizontal axis reflect the degree of relatedness of the sequences. New gene symbols have been assigned for some sequences, on the basis of the similarity level with the closest sequences in the tree, which belong to well-identified enzymes. In subgroup 3b, the new term *hyjSL* is proposed for the *Chlorobi* genes to distinguish them from the *hyhSL* archeal genes. The group numbers are those defined in 2001.[10a] The nodes are displayed so that the corresponding small and large subnits can be read in the same top-down order.

Table 7. Ech-Hydrogenase-like (Ehr) Sequences[a]

organism	length	group	AC	annotation	proposed annotation
Acidiphilium cryptum JF-5	476	L4	Q2D7H1	AcryDRAFT_0193	ehrL
Acidothermus cellulolyticus 11B	499	L4	Q2E2H0	AcelDRAFT_0345	ehrL
Bradyrhizobium japonicum USDA 110	177	S4	Q89GK1	hycG	ehrS
Bradyrhizobium japonicum USDA 110	503	L4	Q89GK2	blr6343	ehrL
Burkholderia pseudomallei 1710b	567	L4	Q3JMD7	hyfG	ehrL
Burkholderia pseudomallei K96243	551	L4	Q63L65	BPSS1143	ehrL
Burkholderia thailandensis E264	559	L4	Q2T5T8	BTH_II1265	ehrL
Burkholderia xenovorans LB400	177	S4	Q13JW7	Bxe_B0324	ehrS
Burkholderia xenovorans LB400	515	L4	Q13JW8	Bxe_B0325	ehrL
Burkholderia xenovorans LB400	522	L4	Q13II9	Bxe_C0175	ehrL
Dehalococcoides ethenogenes 195	171	S4	Q3Z682	DET1570	ehrS?
Dehalococcoides ethenogenes 195	526	L4	Q3Z681	DET1571	ehrL
Dehalococcoides sp. BAV1	171	S4	Q2DUH7	DehaBAV1DRAFT_0412	ehrS?
Dehalococcoides sp. BAV1	526	L4	Q2DUH6	DehaBAV1DRAFT_0413	ehrL
Dehalococcoides sp. CBDB1	171	S4	Q3ZW32	hycG	ehrS?
Dehalococcoides sp. CBDB1	526	L4	Q3ZW31	hycE	ehrL
Geobacter metallireducens GS-15	244	S4	Q39SF9	Gmet_2596	ehrS1
Geobacter metallireducens GS-15	506	L4	Q39SF8	Gmet_2597	ehrL1
Geobacter metallireducens GS-15	262	S4	Q39YR2	Gmet_0369	ehrS2
Geobacter metallireducens GS-15	502	L4	Q39YR0	Gmet_0371	ehrL2
Geobacter sulfurreducens ATCC 51573/DSM 12127/PCA	79	S4	Q74F65	GSU0744	ehrS
Geobacter sulfurreducens ATCC 51573/DSM 12127/PCA	505	L4	Q74F66	GSU0743	ehrL
Leptospira interrogans 56601/serogroup icterohemeorrhagiae/ erovar lai	466	L4	Q8EYD9	hycE	ehrL
Leptospira interrogans serovar Copenhageni Fiocruz L1−130	466	L4	Q72LX0	hycE	ehrL
Methanosarcina acetivorans ATCC 35395/DSM 2834/CM 12185/C2A	170	S4	Q8THY5	MA_4373	ehrS
Methanosarcina acetivorans ATCC 35395/DSM 2834/CM 12185/C2A	545	L4	Q8THY6	MA_4372	ehrL
Methanosarcina mazei ATCC BAA-159/DSM 3647/Goe1/ o1/JCM 11883/OCM 88	170	S4	Q8PY02	MM_1064	ehrS
Methanosarcina mazei ATCC BAA-159/DSM 3647/Goe1/ o1/JCM 11883/OCM 88	530	L4	Q8PY03	MM_1063	ehrL
Methanospirillum hungatei JF-1	170	S4	Q2FKT6	Mhun_1817	ehrS?
Methanospirillum hungatei JF-1	519	L4	Q2FKT5	Mhun_1818	ehrL
Mycobacterium bovis ATCC BAA-935/AF2122/97	492	L4	Q7U2V6	hycE	ehrL
Mycobacterium tuberculosis ATCC 25618/H37Rv	159	S4	O53627	Rv0082	ehrS
Mycobacterium tuberculosis ATCC 25618/H37Rv	492	L4	Q10884	hycE	ehrL
Nocardioides sp. JS614 JS614	567	L4	Q3H216	NocaDRAFT_3178	ehrL
Ralstonia metallidurans CH34	171	S4	Q1LE98	Rmet_4666	ehrS
Ralstonia metallidurans CH34	514	L4	Q1LE97	Rmet_4667	ehrL
Rhodopseudomonas palustris	174	S4	Q21AT2	RPC_0934	ehrS
Rhodopseudomonas palustris	502	L4	Q21AT3	RPC_0933	ehrL
Rhodopseudomonas palustris BisA53	173	S4	Q36YT7	RPEDRAFT_2016	ehrS
Rhodopseudomonas palustris BisA53	502	L4	Q36YT6	RPEDRAFT_2017	ehrL
Rhodopseudomonas palustris BisB5	211	S4	Q37ED2	RPDDRAFT_0358	ehrS
Rhodopseudomonas palustris BisB5	503	L4	Q37ED3	RPD_3851	ehrL
Sulfolobus solfataricus ATCC 35092/DSM 1617/JCM 11322/P2	391	L4	Q97ZA7	hycE	ehrL
Thermoplasma volcanium ATCC 51530/DSM 4299/IFO 15438/CM 9571/GSS1	389	L4	Q978D6	TV1481	ehrL
uncultured methanogenic archaeon RC-I	157	S4	Q0W3I3	echC	ehrS
uncultured methanogenic archaeon RC-I	524	L4	Q0W2B9	hycE	ehrL

[a] All large subunits are characterized by the absence of any CxxC pattern. The group of the closest relative sequences is indicated. It is proposed to name the genes ehrS and ehrL in keeping with Coppi's proposal.[30].

The main features that emerge from the tree are the following. Bacterial sequences from different genera do not segregate well. Clostridial sequences are present in all subgroups even in the clade formed by the eukaryotic ones (green algae and microaerophilic protists). However, a well-separated group in which Clostridiales predominate, also noted by Meyer,[13] is characterized by a 100% bootstrap support. The previously defined clade,[12] including the enzymes from *Trichomonas*, *Entamœba*, *Giardia*, *Spironucleus*, *Scenedesmus*, *Chlorella*, *Chlamydomonas*, and *Neocallimastix*, is here found to also encompass sequences from Clostridiales and Thermotogales. The [FeFe]-hydrogenase-like proteins (Narf) of aerobic eukaryotes form a clear separate branch, used here as an outgroup. The [FeFe]-hydrogenase genes found in the genome of *Dehalococcoides ethenogenes* strain 195[455] (*Chloroflexi*) and of *R. palustris* (Table 2) cluster with those from *Desulfovibrio* (Figure 16). Because *R. palustris* does not appear to contain the necessary

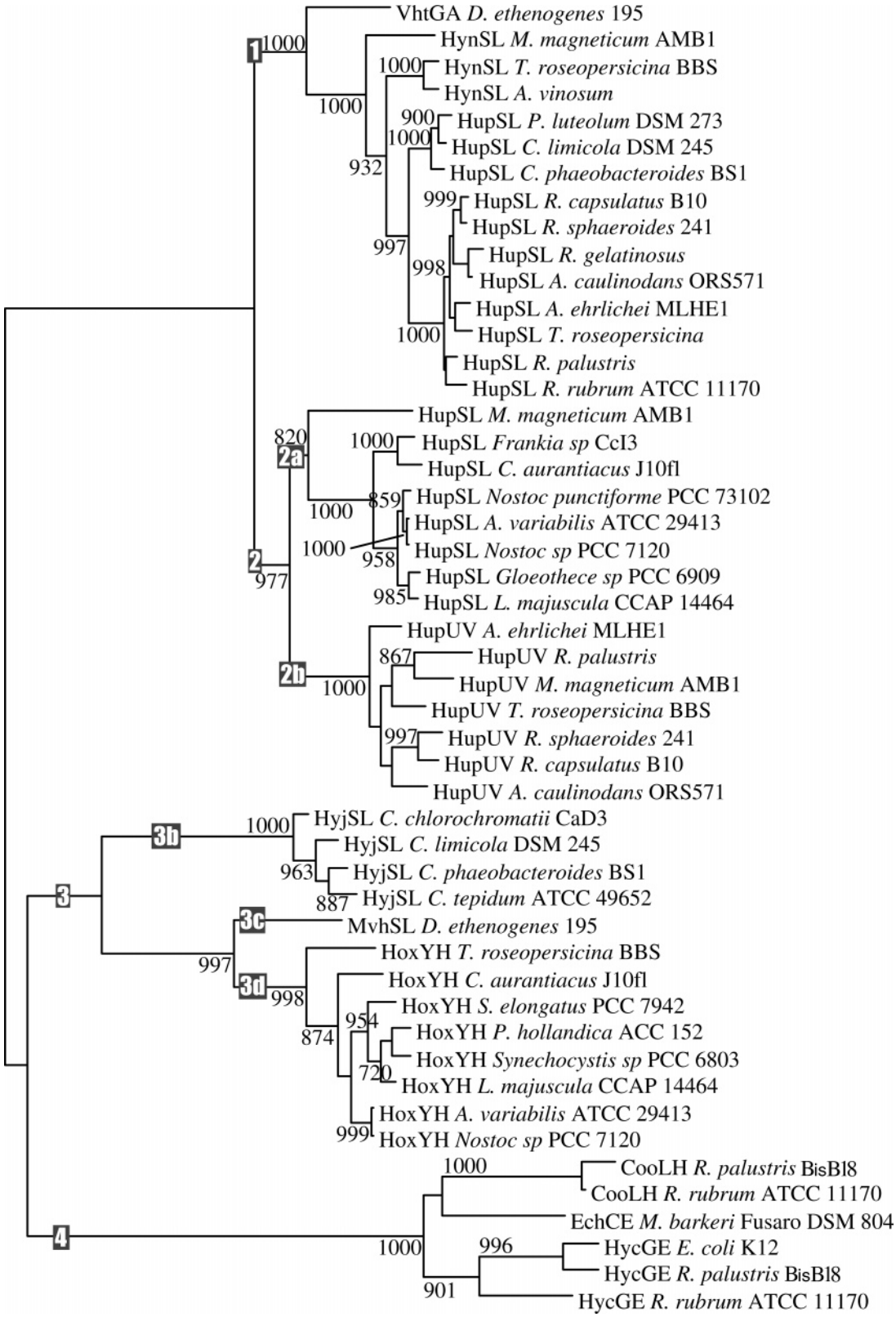

Figure 15. Phylogenetic tree of [NiFe]-hydrogenases present in photosynthetic prokaryote representatives. The complete sequences of the two subunits were separately aligned and filtered with Gblocks and then clustered before phylogenetic analysis as described in the legend to Figure 14. To make the figure more easily readable, not all of the sequences listed in Table 3 and found in phototrophs were used; in particular, all of the sequences annotated as draft were discarded. In group 4, the Hyc enzyme from *E. coli* and the Ech enzyme from *M. barkeri* were included as markers. The new term HyjSL is proposed for the [NiFe]-hydrogenases of *Chlorobi* to distinguish them from the archeal HyhSL enzymes, which belong also to subgroup 3b. The names HupUV for the proteins of *A. ehrlichei* (Q0A734, Q0A735) and CooLH (Q20XV9, Q20XW2) and HycGE (Q20XP6, Q20XP4) for those of *R. palustris* are proposed due to the similarity level with the closest sequences in the tree, which belong to well-identified enzymes.

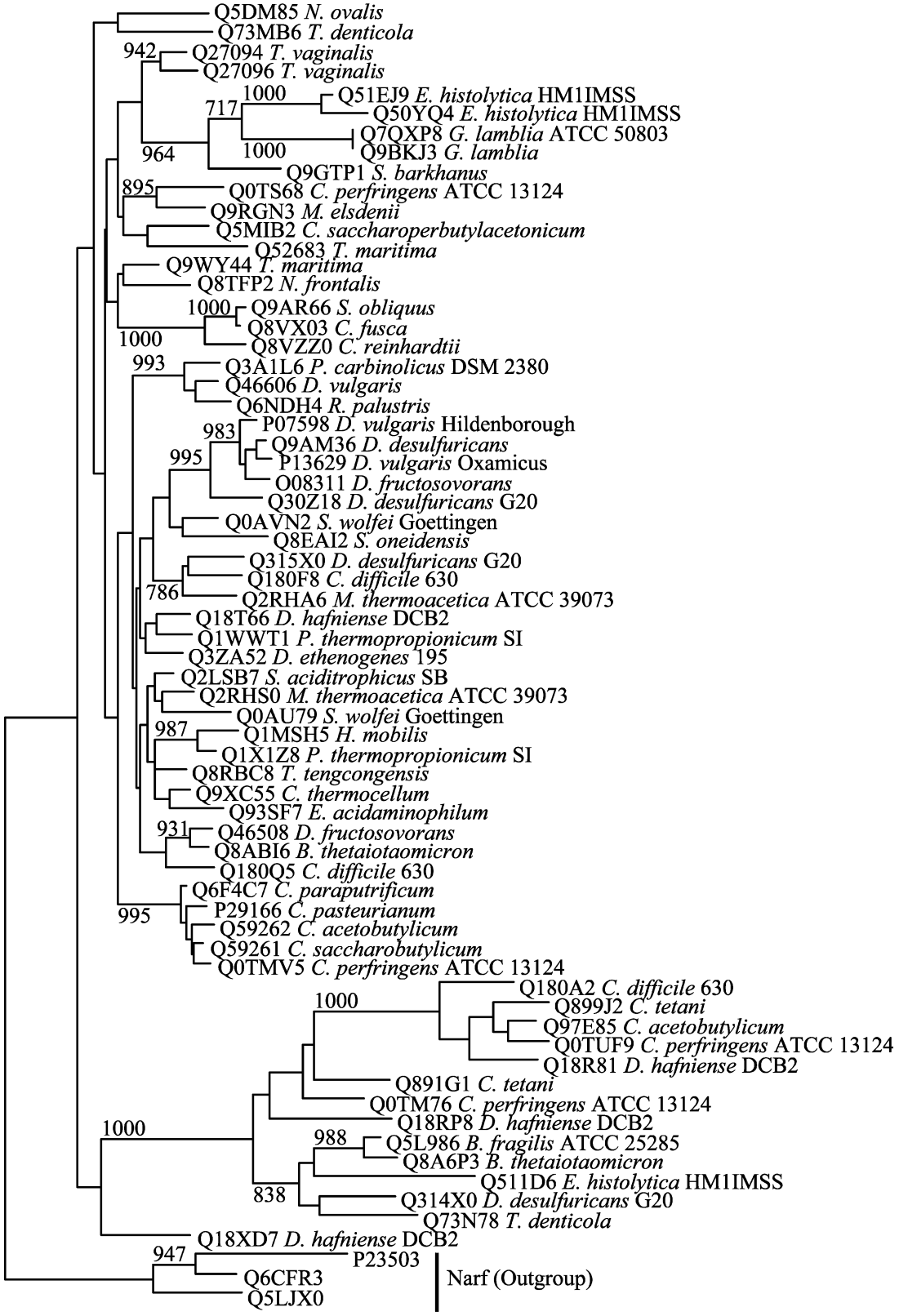

Figure 16. Phylogenetic tree derived from sequence alignments of the catalytic subunit of representatives of [FeFe]-hydrogenases. The tree was computed as described in the legend to Figure 14. To make the figure more easily readable, not all of the sequences listed in Table 5 were used; in particular, all of the sequences annotated as draft were discarded, and often only one strain representative of a species was analyzed. Three [FeFe]-hydrogenase-like sequences were used as outgroup. It was proposed earlier[10a] to name HydA the hydrogenase protein containing the H domain.

maturases,[13] the enzyme may have been acquired by horizontal gene transfer without the maturases or before the emergence of maturases[13] and is probably not functional. The same remark applies to *R. rubrum*, which does not contain the *hydE*, *hydF*, and *hydG* genes necessary for maturation.[13] Furthermore, the putative [FeFe]-hydrogenase of *R. rubrum*

(AC Q2RXN0) contains two mismatches in pattern 1, two mismatches in P2, and one mismatch in P3 (cf. Figure 12); this is why it was not included in Table 5.

7. Roles of Hydrogenases in Nature

7.1. Methanogenesis

The formation of methane is one of the most important ecological processes on Earth.[456] Methanogens obtain most or all of their energy for growth from the process of methanogenesis, considered to be an anaerobic respiration (reviewed in refs 34, 35, 225, and 457). Strictly anaerobic archaea of the genus *Methanosarcina* derive their metabolic energy from the conversion to methane of a restricted number of C_1 compounds and acetate.[457] This capacity is of great ecological importance because acetate is the precursor of 60% of the methane produced on Earth; thus, these organisms contribute significantly to the production of this greenhouse gas, for example, in rice paddies.[55] The pathway of methane formation from $CO_2 + H_2$ via the CO_2-reducing pathway, or from methanol, is shown in Figure 11. Three types of [NiFe]-hydrogenases identified recently[34,35] (and Hmd under Ni limitation[66]) are involved in these two systems in which either H_2 or $F_{420}H_2$ is used as electron donor and the heterodisulfide CoM−S−S−CoB as electron acceptor (hence the term "disulfide respiration" proposed by Hedderich and Whitman[458]). In acetoclastic methanogenesis, Ech couples the oxidation of reduced ferredoxin (arising from the oxidation of the carbonyl group of acetate) to the production of H_2. Methanophenazine (MP) acts in the membrane of the methanogen as the quinone in respiratory chains of bacteria and mitochondria. It can be reduced with H_2, by the F_{420}-non-reducing VhoAG hydrogenase via its VhoC third subunit, which interacts with MP (Figure 8c), or with $F_{420}H_2$ by the $F_{420}H_2$dehydrogenase (FpoDH), a multimeric complex encoded by the *fpo* genes, having subunits homologous to subunits of complex I (Table 4; Figures 10d and 11). Intrinsic membrane subunits of the Ech hydrogenase and Fpo dehydrogenase catalyze redox-driven proton translocation that generates a protonmotive force and hence energy recovery during methanogenesis (Figure 11). The heterodisulfide reductase (HdrED) receives electrons from the reduced form of methanophenazine, MPH_2 (Figure 8c). Each partial reaction, the reduction of MP by H_2 or $F_{420}H_2$ and the reduction of CoM−S−S−CoB by MPH_2, is coupled to the translocation of $2H^+/2e^-$. H^+-Translocation in both reactions can occur via a redox-loop mechanism, whereas $F_{420}H_2$ dehydrogenase is thought to function as a proton pump.[147,203]

7.2. Nitrogen Fixation

Nitrogen fixation (the reduction of dinitrogen to ammonia) is another important biochemical process taking place on Earth. Dinitrogen reduction is carried out by nitrogenase, a complex enzyme that requires anaerobicity, ATP, and low potential reductant (ferredoxin or flavodoxin) to function. It is an intrinsic property of nitrogenase to evolve H_2 during N_2 reduction.[459] In the absence of N_2, the entire activity of the enzyme is devoted to the reduction of protons to hydrogen. Because the energy required for electrons to reduce protons is the same as that for them to reduce dinitrogen, evolution of H_2 represents a waste of energy for the cell. Hydrogen evolution is a general phenomenon associated with nitrogen fixation by *Rhizobium* bacteroids,[460] and its extent during nitrogen reduction is a major factor affecting the efficiency of nitrogen fixation by agronomically important legumes.[460] From the observations that efficiency is increased by the possession of an uptake hydrogenase and that H_2 inhibits N_2 reduction, Dixon[461] postulated that hydrogenase could support N_2 fixation in aerobic organisms by (1) acting as an O_2 scavenger to protect nitrogenase from inhibition by O_2, (2) preventing inhibition of N_2 reduction by H_2 generated by nitrogenase, and (3) recycling H_2 produced by nitrogenase to provide reducing power. Nitrogenase−hydrogenase interrelationships were then observed in a variety of nitrogen fixing organisms, such as Rhizobia,[462] *Azotobacter chroococcum*,[463] cyanobacteria,[464,465] and photosynthetic bacteria.[466−468] Symbiotic hydrogenase activity in *Bradyrhizobium* sp. (*Vigna*) results in increase in nitrogen content in *Vigna unguiculata* plants and in plant yield.[469] However, despite the beneficial effect on plant productivity, only a limited number of strains from several genera of Rhizobiaceae can express a hydrogenase system that allows partial or full recycling of H_2 evolved by nitrogenase.[470,471] Phylogenetic analysis of *hup* genes indicates distinct evolutionary origins for hydrogenase genes in *Rhizobia*.[471] In *R. leguminosarum* bv viciae, hydrogenase genes are uncommon and their sequence highly conserved, suggesting that they were acquired recently.[472] Expression of uptake hydrogenase genes in *R. leguminosarum* in symbiosis with peas is directly activatable by the nitrogen fixation regulator NifA; thus, in that case, hydrogenase and nitrogenase are co-regulated at the genetic level.[284] In *R. capsulatus*, expression of uptake hydrogenase and nitrogenase is co-regulated by the RegB−RegA two-component regulatory system.[292] In cyanobacteria, the uptake hydrogenase is present only in N_2 fixers.[183,185] In the heterocystous cyanobacterium *Nostoc* sp. PCC 73102, the *hup* genes are transcribed in cells grown under N_2 fixing but not under non-N_2-fixing conditions.[473]

7.3. Bioremediation

The presence of chlorinated compounds in nature results from the development in the past decades of solvents, pesticides, cooling agents, etc., by the chemical industry. The solvent tetrachloroethene (perchloroethylene, PCE) is a common groundwater pollutant. Highly toxic and suspected to be a human carcinogen, it is non-biodegradable by aerobes but can be reductively dechlorinated under anaerobic conditions by natural microbial communities. Some anaerobic bacteria have the capacity to use chlorinated compounds as electron acceptors and make the synthesis of ATP during the dechlorination process. This respiratory process has been termed "dehalorespiration"[474] to indicate that the dehalogenation process is coupled to ATP synthesis via a chemiosmotic mechanism. Many microorganisms can use H_2 (or formate) as electron donor for reductive dehalogenation. The dehalorespiratory chain proposed for *Dehalobacter restrictus*[474] is of the type described for fumarate respiration in *W. succinogenes* (Figure 8a): it comprises a periplasmically oriented uptake hydrogenase linked to a membrane-bound cytochrome *b* channeling the electrons from H_2, via menaquinone, to a membrane-embedded PCE reductive dehalogenase. The microorganisms capable of reductive dechlorination belong to the *Bacteria*; several of them are related to sulfate or sulfur reducers of the δ- and ϵ-subgroups of the *Proteobacteria*. Examples of anaerobic bacteria capable of dechlorination with H_2 as electron donor are given

Table 8. Anaerobic Bacteria Capable of Reductive Dechlorination with H_2 as Electron Donor[a]

organism	dechlorinated compounds[b]	electron donor[b]
Desulfomonile tiedjei[575]	PCE, $TCEH_2$, 3-chlorobenzoate	H_2, formate
Desulfitobacterium chlororespirans[576]	2,4,6-trichlorophenol, 3-chloro-4-hydroxy-phenylacetate	H_2, formate, pyruvate
Desulfitobacterium dehalogenans[577]	PCE, 2,4,6-trichlorophenol	H_2, formate
Dehalobacter restrictus[578]	PCE, TCE	H_2
isolate TEA[579]	PCE, TCE	H_2
Dehalospirillum multivorans (now *Sulfospirillum multivorans*)[580,581]	PCE, TCE	H_2, formate, pyruvate
Dehalococcoides ethenogenes[475]	PCE, TCE, DCE, chloroethene	H_2
Desulfitobacterium hafniense strain TCE1[582,583]	PCE	H_2, pyruvate, lactate

[a] Adapted from Holliger et al.[474] [b] Incomplete selection. PCE, tetrachloroethene; TCE, trichloroethene, DCE, dichloroethene

in Table 8. The bacterium *Dehalococcoides ethenogenes* strain 195, affiliated with the *Chloroflexi* (green nonsulfur bacteria), was the first organism to be isolated that is capable of dechlorinating PCE and trichloroethene (TCE) past dichloroethene (DCE) to vinyl chloride and the nontoxic ethene.[475] Its metabolism is very specialized because only H_2 as an electron donor and chlorinated compounds as electron acceptors can support growth. In accordance with this, the sequence of its genome has revealed the presence of 17 putative reductive dehalogenases and 5 hydrogenase complexes.[455] The metabolic capacity of this organism may have evolved fairly recently, because pollution of groundwater by chloroethenes has been significant only during the past 50 years. Analysis of the genome suggests that many of the special genes may have been acquired by lateral gene transfer. A hybrid bioinorganic catalyst obtained via reduction of Pd(II) to Pd(0) onto the surface cells of *D. desulfuricans* at the expense of H_2 has been used for dehalogenation of chlorinated aromatic compounds.[476] Palladized biomass, supplied with formate or H_2 as an electron donor, catalyzed the dehalogenation of 2-chlorophenol and polychlorinated biphenyls. Finally, the prospect of recovering energy from H_2 evolved during fermentation of organic wastes by the use of hydrogenase electrodes and converting it through fuel cells has been presented.[477]

Microbial reduction of toxic heavy metals contributes to the remediation of metal-containing industrial wastes.[478,479] Bacterial hydrogenases have been exploited to remove heavy metals from solution by reduction to less soluble metal species.[480] *E. coli* and *D. desulfuricans* reduce Tc(VII) with formate or hydrogen as electron donors.[481] The reaction is catalyzed by the formate hydrogenlyase complex of *E. coli* (that comprises hydrogenase-3) and is associated with a periplasmic hydrogenase activity in *D. desulfuricans* [also shown to reduce uranium (VI)[482]]. The bioreduction of Pd-(II) by *D. desulfuricans* cells results in the deposition of cell-bound Pd(0) nanoparticles that are ferromagnetic and have a high catalytic activity.[480] Biomass of *D. desulfuricans* has been used to recover Au(III) as Au(0) from waste electronic leachate as well as Pd(II) and Cu(II)[483] and to reduce Cr-(VI), a carcinogen and mutagen, to less environmentally problematic Cr(III).[484] The periplasmic [NiFe]-hydrogenase of *D. fructosovorans* performs Tc(VII) reduction either in situ or in the isolated form.[485] Cell suspensions of the hyperthermophile *Pyrobaculum islandicum* can reduce at 100 °C with hydrogen as electron donor the following metals: U(VI), Tc(VII), Cr(VI), Co(III), Fe(III), and Mn(IV).[486] The phototrophic bacteria *T. roseopersicina* and *Lamprobacter modestohalophilus* and their hydrogenases have been shown to reduce Ni(II), Pt(IV), Pd(II), or Ru(III) to their metallic forms under an H_2 atmosphere.[487] The dissimilatory Fe(III)- and U(VI)-reducing family Geobacteraceae can grow utilizing hydrogen or acetate as an electron donor.[56,488] Their metabolic activities can influence the cycling of organic matter and minerals in the subsurface of the Earth[56,488] and play a crucial role in bioremediation of both organic and metal contamination.[489] In *G. sulfurreducens* that predominates in Fe(III)-reducing sedimentary environments the uptake Hyb hydrogenase is required for hydrogen-dependent reduction of Fe(III).[145]

7.4. Pathology

Pathogenic *Helicobacter* species, *Helicobacter pylori* and *H. hepaticus*, can respire H_2 through a respiratory [NiFe]-hydrogenase that has a high affinity for H_2 (apparent K_m of 2.5 μM).[490] H_2 is produced in the large intestine of animals as a byproduct of carbohydrates fermentation, and it was demonstrated that H_2 concentrations in live mouse stomach[491] or the livers of live mice[492] are over 20 times as much as the apparent whole-cell K_m for hydrogen. A hydrogenase mutant strain of *H. pylori* is much less efficient in its colonization of mice; thus, H_2 is an energy-yielding substrate that can facilitate the maintenance of the gastric pathogen.[491] In the case of *H. hepaticus*, a causative agent of chronic hepatitis and hepatocellular carcinoma in mice, mutants inactivated in the *hyaB* gene are deficient in hydrogen-supported amino acid uptake and in causing liver lesions in mice.[493] Similarly, in the enteric pathogen *Salmonella enterica* serovar *Typhimurium* the three putative membrane-associated H_2-oxidizing hydrogenases have been shown to contribute to the virulence of the bacterium in a typhoid fever mouse model.[494] Partial complementation of the triple mutant (by reintroduction of one of the uptake hydrogenases on a plasmid) rendered the mutant capable of oxidizing H_2 and restored the virulence capacity.[494] The importance of H_2 use by enteric bacteria for growth within a mammal makes uptake [NiFe]-hydrogenases a virulence factor.[495] One way to fight against H_2-utilizing [NiFe]-hydrogenases is to prevent import of Ni into the cell. Consumption of Mg^{2+}, formerly used to relieve pain from gastritis and peptic ulcers, may restrain Ni^{2+} entry into the cells via the Mg^{2+}-transporter (Mg^{2+} competitively inhibits Ni transport by the Mg^{2+}-transporter). It represents a means to reduce hydrogenase biosynthesis. Another way to render inefficient uptake hydrogenase(s) is to inactivate the Tat transport process. Tat proteins are good targets for antimicrobial drugs because they are not present in mammalian cells.[496] The protozoan parasite *Entamoeba histolytica* causes colitis and liver abscesses. *E. histolytic*a HM-1:IMSS is a virulent strain. An *E. histolytica* DNA microarray consisting of 2110 genes has been used to assess transcriptional differences between the virulent and nonvirulent strains (or species).[497] Genes encoding [FeFe]-hydrogenase were among the 29 genes that had decreased

expression in the nonvirulent strains/species *E. histolytica* HM-1:IMSS.[497]

7.5. Biohydrogen Production

Molecular hydrogen produced from renewable sources (biomass, water, organic wastes) either biologically or photobiologically is called "biohydrogen". Biohydrogen can be produced by both types of hydrogenases and also by the nitrogenase enzyme, which functions as an H_2-evolving hydrogenase (not covered here). Potential applications of photosynthetic and fermentative microorganisms in the generation of H_2 by direct biophotolysis, indirect biophotolysis, photofermentations, and dark fermentations have often been reviewed,[31,33,185,244,246,498−511] and two special issues of the *International Journal of Hydrogen Energy* are devoted to the subject.[37,38] The identified potentially critical factors have been discussed.[185,499,506−513] Fermentative mesophilic bacteria (such as clostridia) or thermophiles (e.g., *Pyrococcus*) have a real potential.[513] Fermentative and photosynthetic bacteria have been experimented in a combined dark and photofermentation process that achieved complete degradation of the substrate (glucose) and then higher yields of H_2.[514] When produced by fermentation, H_2 is contaminated by various gases (H_2S, CH_4), which have to be eliminated for use in fuel cells; when produced from water by oxygenic phototrophs (cyanobacteria and green algae), O_2 is the contaminant.

Photobiological production of H_2 gas linked to photosynthetic water oxidation means recovery of energy from light and water, two sources of renewable energy widely distributed and plentiful. In *Scenedesmus obliquus*[94,95] or *C. reinhardtii*[96,244,515] the electrons originating from water or provided by fermentative metabolism are transferred to PSI in the light via the plastoquinone pool. In turn, PSI reduces a [2Fe-2S] ferredoxin, the physiological electron donor to [FeFe]-hydrogenase. In cyanobacteria, the soluble NAD(P)-dependent bidirectional [NiFe]-hydrogenase is using protons to reoxidize the pyridine nucleotides reduced during dark anaerobic metabolism.[516,517] In the cyanobacterium *Synechocystis* PCC 6803, the bidirectional hydrogenase produces significant amounts of H_2 in the dark, in anaerobiosis,[33,215] the rate of H_2 production being higher in the presence of fermentative substrates such as glucose. A NDH-1 mutant of *Synechocystis*, impaired in CO_2 uptake and CO_2 fixation, was shown to produce H_2 in the light using electrons gained by water photolysis.[215]

Although much progress has been made in the elucidation of gene expression, structure, and regulation of the key hydrogenase enzymes, no practical and economically competitive process for the continuous production of biological H_2 has, as yet, been put on the market. One of the difficulties is due to the fact that H_2 output represents an energy loss for the cell and that microbial metabolic network has evolved for rationalization of energy use and optimization of specific growth rate. By the use of recombinant DNA techniques one may try to restructure metabolic networks to improve the production of H_2. However, it is difficult to predict how genetic perturbations will affect complex cellular responses. Genetic manipulation has been applied to increase the flux of electrons reaching the H_2-producing catalyst (nitrogenase) in *R. capsulatus*;[518] metabolically engineered *R. sphaeroides* strains, PHA^- and Hup^- mutants, were constructed to prevent the competition of H_2 photoproduction with polyhydroxyalkanoate (PHA) accumulation by inactivating the PHA synthase and with H_2 recycling by abolishing the uptake hydrogenase;[519] metabolic manipulation has been used to maintain a metabolic state with low O_2 production to induce H_2-evolving [FeFe]-hydrogenase in *C. reinhardtii* chloroplasts.[97,520−525] H_2 recycling by uptake hydrogenase, an efficient means of the cell to recoup the energy lost in the form of H_2, has to be counteracted for increasing H_2 production efficiency. Targeted inactivation of uptake hydrogenase structural and accessory genes by genetic engineering has led to an increase in H_2 production by photosynthetic bacteria[519,526−528] and cyanobacteria.[529−533]

O_2 sensitivity of hydrogenases is one of the main difficulties encountered for the use of those enzymes in H_2 production. To develop a water splitting system that can produce H_2 under aerobic conditions, it is important to understand the reasons for O_2 sensitivity. Some hydrogenases are O_2-tolerant: the soluble NAD-dependent hydrogenase of *R. eutropha*, which contains a modified metallocenter with two additional CN^- ligands,[77] is one example; the H_2 sensors with narrow gas channels[189,193] is another example. *Rubrivivax gelatinosus* also contains a hydrogenase tolerant to O_2.[6] This hydrogenase, linked to a CO oxidation pathway, was shown to produce H_2 using electrons from reduced ferredoxin of a cyanobacterial source. If the hydrogenase can use the host electron donor, then a cyanobacterial recombinant system may be expected to be able to mediate H_2 production from water photolysis.[507]

The [FeFe]-hydrogenases are enzymes of high turnover but, besides their high sensitivity to O_2, they are also light sensitive.[534] This may pose an additional problem for their biotechnological use in photosynthetic organisms such as algae. Now that the genes for [FeFe]-hydrogenase biosynthesis [H cluster) have been identified (section 4.2), including the system(s) for [Fe-S] cluster assembly (section 4.3), these highly performing enzymes can be expressed in various hosts.[20,322,535,536]

8. Concluding Comments

Hydrogenases are a structurally and functionally diverse group of enzymes, and phylogenetic analyses have led to the identification of several phylogenetically distinct groups and subgroups that form the basis of a coherent system of classification. The large number of hydrogenase gene sequences has been augmented by whole genome sequencing, which has revealed the presence of these enzymes in a wide variety of organisms including pathogens and of multiple hydrogenases in several species of the *Bacteria* and *Archaea*. Postgenomic analysis (transcriptome, proteome, metabolome) has and will be essential to elucidating the metabolic roles of these enzymes and the regulation of their biosynthesis and activity. The mechanisms of [NiFe]-hydrogenase biosynthesis are the best understood; those for the biosynthesis of [FeFe]-hydrogenases have just been disclosed. There are still open questions that have to be addressed, for example, the biosynthesis of diatomic ligands and their incorporation, the mechanisms of reaction, and the mode of [Fe-S] cluster assembly. Biochemical and regulation studies are no longer restricted to the uptake [NiFe]-hydrogenases of *Proteobacteria*, but have recently been extended to other types of hydrogenases and other microorganisms, in particular to *Archaea*.

The existence of multiple hydrogenases within a living organism allows the organism to best meet its energy need. The main role of hydrogenases is clearly the oxidation of H_2 or the reduction of protons, coupled to energy-conserving

electron-transfer chain reactions, which allows energy to be obtained either from H_2 or from the oxidation of substrates of lower potential. These energy-conserving reactions are generally restricted to the prokaryotes, but are widely distributed among the bacterial and archaeal domains of life. In the past decade, additional roles have been revealed. Thus, the so-called H_2-sensor hydrogenases are involved in regulating the biosynthesis of uptake [NiFe]-hydrogenases in response to H_2, their substrate. Bidirectional hydrogenases may interact with respiratory electron transport chains and act as electron "valves" to control the redox poise of the respiratory chain at the level of the quinone pool. This is essential to ensure the correct functioning of the respiratory chain in the presence of excess reducing equivalents, particularly in photosynthetic microorganisms. An additional finding concerns some hydrogenases that were originally thought to play a purely fermentative role, but which are now known to be involved in membrane-linked energy conservation through the generation of a transmembrane protonmotive force. The H_2 uptake hydrogenases appear to play a major role in nature in the bioremediation of chlorinated compounds and have been exploited for remediation of toxic heavy metals. These uptake hydrogenases have been recently identified as a serious virulence factor in pathogenic bacteria and parasites.

The current interest in H_2 as an alternative to fossil fuels has led to a resurgence of interest in the biological production of H_2, and research into hydrogenases will clearly play a major role in this area. Structural studies of hydrogenases will be important in directing protein engineering, for example, in rendering these enzymes O_2-tolerant. Identification of factors, linked to the protein environment of the active site and indispensable for the stability and high efficiency of the enzyme, will contribute to the development of synthetic chemical systems able to mimick the active site metallocenter. Studies of H_2 metabolism and regulation will also be important in engineering microorganisms at the cellular level to maximize H_2 production. The isolation of novel H_2-producing organisms will also be a priority. Prokaryotic biodiversity is much greater than previously thought, and whole phylogenetic groupings exist, which have never been cultivated. Given the importance of H_2 metabolism among microorganisms generally, it can be anticipated that many of these so-far uncultivated species will contain hydrogenases and that novel types of hydrogenases and H_2 metabolism remain to be discovered.

This overview has pointed out some of the ways elaborated by living organisms to use molecular hydrogen as an energy source and an energy carrier. These examples can teach us how to use this renewable and environmentally friendly source of energy (no greenhouse gas produced by H_2 oxidation) if our civilization is to be the H_2 civilization. As the visionary French writer Jules Verne wrote "...water will one day be employed as fuel, hydrogen and oxygen which constitute it, used singly or together, will furnish an inexhaustible source of heat and light, of an intensity of which coal is not capable, ... we shall heat and warm ourselves with water... Water will be the coal of the future." [*L'île mystérieuse*" (1874).]

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