Microbial Biotechnology (2008) **1**(2), 107–125 doi:10.1111/j.1751-7915.2007.00009.x

Review

Metabolically engineered bacteria for producing hydrogen via fermentation

Gönül Vardar-Schara,[1]* Toshinari Maeda[2] and Thomas K. Wood[2,3,4]

[1]*Department of Molecular Biosciences and Bioengineering, University of Hawaii, 1955 East-West Road, Agricultural Sciences 218, Honolulu, HI 96822, USA.*
[2]*Artie McFerrin Department of Chemical Engineering,*
[3]*Department of Biology and* [4]*Zachry Department of Civil and Environmental Engineering, 220 Jack E. Brown Building, Texas A&M University, College Station, TX 77843-3122, USA.*

Summary

Hydrogen, the most abundant and lightest element in the universe, has much potential as a future energy source. Hydrogenases catalyse one of the simplest chemical reactions, $2H^+ + 2e^- \leftrightarrow H_2$, yet their structure is very complex. Biologically, hydrogen can be produced via photosynthetic or fermentative routes. This review provides an overview of microbial production of hydrogen by fermentation (currently the more favourable route) and focuses on biochemical pathways, theoretical hydrogen yields and hydrogenase structure. In addition, several examples of metabolic engineering to enhance fermentative hydrogen production are presented along with some examples of expression of heterologous hydrogenases for enhanced hydrogen production.

Fermentation versus photosynthesis for hydrogen

Hydrogen, the smallest biological substrate, has great potential as an alternative to limited fossil fuel resources (Das and Veziroglu, 2001). In addition to its higher energy content than fossil fuels (Chen *et al*., 2006), it is renewable if it is derived from renewable feedstocks (Hawkes *et al*., 2007), and the product of hydrogen oxidation is water; hence, the impact of hydrogen on the environment is not relevant (Das and Veziroglu, 2001).

Production of hydrogen by microorganisms is at ambient temperature and pressure; hence, it requires less energy compared with conventional thermal systems (steam methane reforming, 850°C, 25 bar) (Yi and Harrison, 2005) and compared with electrolytic processes. Microorganisms produce hydrogen via two main pathways: photosynthesis and fermentation. Photosynthesis is a light-dependent process, including direct biophotolysis, indirect biophotolysis and photo-fermentation, whereas, anaerobic fermentation, also known as dark fermentation, is a light-independent process (Benemann, 1996; Hallenbeck and Benemann, 2002). Photosynthetic hydrogen production is performed by photosynthetic microorganisms, such as algae (Benemann, 2000), photosynthetic bacteria (Matsunaga *et al*., 2000) and cyanobacteria (Dutta *et al*., 2005). Fermentative hydrogen production is conducted by fermentative microorganisms, such as strict anaerobes [*Clostridium* strains (Levin *et al*., 2006), thermophiles (van Niel *et al*., 2002), rumen bacteria (Nandi and Sengupta, 1998) and methanogens (Valentine *et al*., 2000)], facultative anaerobes [*Enterobacter* strains (Kumar and Das, 2000; Shin *et al*., 2007), *Escherichia coli* (Yoshida *et al*., 2007) and *Citrobacter* species (Vatsala, 1992)], or mixed cultures (Lay, 2000).

Compared with photosynthetic processes, fermentative hydrogen production generally yields two orders of magnitude higher rates, does not rely on the availability of light, utilizes a variety of carbon sources such as organic compounds, low-cost wastes, or insoluble cellulosic and cellobiose substrates, requires less energy, and is technically much simpler and more stable (Nandi and Sengupta, 1998; Levin *et al*., 2004; 2006; Chen *et al*., 2006; Kapdan and Kargi, 2006; Ust'ak *et al*., 2007). Although hydrogen production yields are usually higher with photosynthetic processes, oxygen is evolved during photosynthesis which inhibits the hydrogenase enzyme which is responsible for H_2 production (Nath and Das, 2004). In addition, fermentative microorganisms have rapid growth and are not affected by oxygen as much as the main process is anaerobic (any residual oxygen is rapidly consumed at the onset). Therefore, fermentative hydrogen production is more advantageous than the photosynthetic hydrogen production and appears to have more potential for practical applications (Das and Veziroglu, 2001).

Received 22 July, 2007; revised 30 September, 2007; accepted 3 October, 2007 . *For correspondence. E-mail gonul@hawaii.edu; Tel. (+1) 808 224 6451; Fax (+1) 808 956 3542.

Formate hydrogen lyase system

The formate hydrogen lyase (FHL) system is a multienzyme complex responsible for molecular hydrogen production from formate (Peck and Gest, 1957). Formate hydrogen lyase activity has been found in various bacteria such as *Salmonella typhimurium* (Chippaux *et al*., 1977; Barrett *et al*., 1984), *Klebsiella pneumoniae* (Steuber *et al*., 1999), *Rhodospirillum rubrum* (Schön and Voelskow, 1976); (Voelskow and Schön, 1980), *Methanobacterium formicicum* (Baron and Ferry, 1989), *E. coli*, and many other coli-aerogenes bacteria (Peck and Gest, 1957); however, the FHL complex of *E. coli* is the most studied and was discovered in 1931 (Stephenson and Stickland, 1931). The FHL system of *E. coli* is briefly reviewed here and more detailed information can be found in other reviews (Sawers, 1994; Sawers, 2005).

The *E. coli* genome (Hayashi *et al*., 2006) encodes four nickel-iron hydrogenases: hydrogenase-1 (Hyd-1) (Menon *et al*., 1991), hydrogenase-2 (Hyd-2) (Menon *et al*., 1994), hydrogenase-3 (Hyd-3) (Böhm *et al*., 1990) and hydrogenase-4 (Hyd-4) (Andrews *et al*., 1997). From these four hydrogenases, Hyd-3 is a part of the active anaerobic FHL complex and is encoded by the *hyc* operon (Fig. 1, Table 1) (Böhm *et al*., 1990; Sauter *et al*., 1992). Hyd-1 and Hyd-2 are known as uptake hydrogenases which catalyse hydrogen oxidation and are encoded by the *hya* (Menon *et al*., 1991) and *hyb* operons (Menon *et al*., 1994) respectively (Fig. 1, Table 1). Hyd-3 was first thought to only have hydrogen-producing activity (Bagramyan *et al*., 2002); however, Maeda and colleagues (2007a) recently showed that Hyd-3 has hydrogen uptake activity as well. Hence, Hyd-3 is a reversible hydrogenase, both producing and utilizing hydrogen although the synthesis reaction predominates. Hyd-4, encoded by the *hyf* operon (Fig. 1, Table 1), has high homology with the *hyc* operon and was first proposed to possess a second FHL complex (Andrews *et al*., 1997; Bagramyan *et al*., 2002). However, Self and colleagues (2004) later showed that the Hyd-4 did not replace the Hyd-3 in hydrogen production and the *hyf* operon is not expressed in *E. coli*, but can be activated in the presence

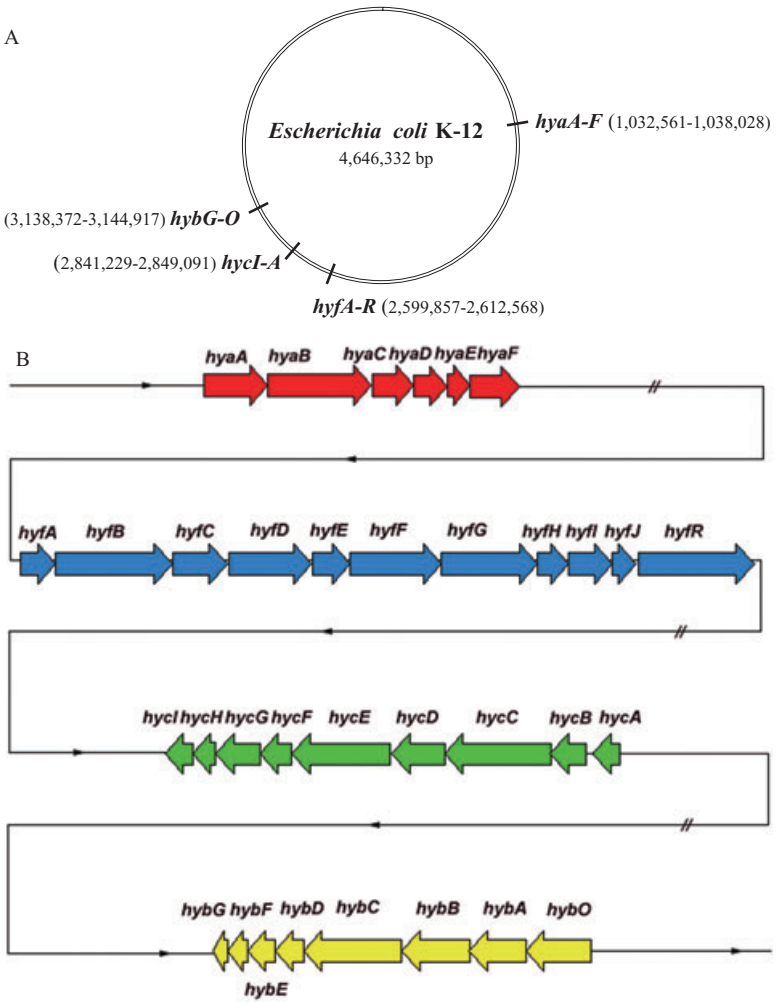

Fig. 1. A. Location of the four structural hydrogenase operons on the *E. coli* K12 chromosome (AC000091) (Hayashi *et al*., 2006). The values in brackets signify the locations of the respective genes on the genome map.
B. Organization of the genes of each hydrogenase operon in *E. coli*. Arrows indicate the direction of transcription. For details for each gene see Table 1 (Hayashi *et al*., 2006).

Table 1. Genes of four hydrogenase operons (*hya*, *hyb*, *hyc*, *hyf*) in *E. coli*.

Gene	Size, bp	Description
hyaA	372	Hydrogenase 1, small subunit
hyaB	597	Hydrogenase 1, large subunit
hyaC	235	Hydrogenase 1, *b*-type cytochrome subunit
hyaD	195	Protein involved in processing of HyaA and HyaB proteins
hyaE	132	Protein involved in processing of HyaA and HyaB proteins
hyaF	285	Protein involved in nickel incorporation into hydrogenase-1 proteins
hyfA	205	Hydrogenase 4, 4Fe-4S subunit
hyfB	672	Hydrogenase 4, membrane subunit
hyfC	315	Hydrogenase 4, membrane subunit
hyfD	479	Hydrogenase 4, membrane subunit
hyfE	216	Hydrogenase 4, membrane subunit
hyfF	526	Hydrogenase 4, membrane subunit
hyfG	555	Hydrogenase 4, subunit
hyfH	181	Hydrogenase 4, Fe-S subunit
hyfI	252	Hydrogenase 4, Fe-S subunit
hyfJ	137	Predicted processing element hydrogenase
hyfR	670	DNA-binding transcriptional activator, formate sensing
hycI	156	Protease involved in processing C-terminal end of HycE
hycH	136	Protein required for maturation of hydrogenase 3
hycG	255	Hydrogenase 3, small subunit
hycF	180	Formate hydrogenlyase complex Fe-S protein
hycE	569	Hydrogenase 3, large subunit
hycD	307	Hydrogenase 3, membrane subunit
hycC	608	Hydrogenase 3, membrane subunit
hycB	203	Hydrogenase 3, Fe-S subunit
hycA	153	Regulator of the transcriptional regulator FhlA
hybG	82	Hydrogenase 2 accessory protein
hybF	113	Protein involved with the maturation of hydrogenases 1 and 2
hybE	162	Hydrogenase 2-specific chaperone
hybD	164	Predicted maturation element for hydrogenase 2
hybC	567	Hydrogenase 2, large subunit
hybB	392	Predicted hydrogenase 2 cytochrome *b*-type component
hybA	328	Hydrogenase 2 4Fe-4S ferredoxin-type component
hybO	372	Hydrogenase 2, small subunit

of effector-independent FhlA (transcriptional activator of the FHL complex) mutant proteins (Self and Shanmugam, 2000; Self *et al*., 2001) or HyfR, which is an FhlA homologue for Hyd-4 (Skibinski *et al*., 2002).

The FHL complex of *E. coli* is responsible for the conversion of formate to CO_2 and H_2 under anaerobic conditions and in the absence of electron acceptors such as oxygen and nitrate (Axley *et al*., 1990). The active complex consists of seven proteins, six from the *hyc* operon (HycBCDEFG) (Böhm *et al*., 1990; Sauter *et al*., 1992) and formate dehydrogenase H (Fdh-H encoded by *fdhF*) (Axley *et al*., 1990). In addition, the FHL system is controlled by a transcriptional activator FhlA (Schlensog and Böck, 1990; Hopper *et al*., 1994), which is required for the transcription of *fdhF* and the *hyc* operon, and a negative transcriptional regulator, HycA (Sauter *et al*., 1992). Transcription of the FHL complex is dependent on the presence of formate, acidic pH and the σ^{54} factor (Hopper *et al*., 1994; Self and Shanmugam, 2000). In the presence of formate, FhlA is activated and induces the transcription of the FHL complex by σ^{54}-RNA polymerase after binding to the upstream-activating sequences. Molybdate also has a role in transcriptional control as a required second effector (Self and Shanmugam, 2000).

HycE (65 kDa) is the large subunit of Hyd-3 and contains the nickel-iron active site, whereas HycG (28 kDa) is the small subunit of Hyd-3 (Sauter *et al*., 1992; Sawers, 1994). HycB (22 kDa) and HycF (20 kDa) are iron-sulfur proteins of Hyd-3 which function as intermediate electron carrier proteins within the FHL complex (Table 1). HycC (64 kDa) and HycD (33 kDa) are membrane proteins which probably function as an electron transfer protein (Sauter *et al*., 1992; Sawers, 1994).

Fdh-H, encoded by the *fdhF* gene, is a 79 kDa cytoplasmic protein and contains selenocysteine, molybdenum and a [4Fe-4S] cluster at its active site (Axley *et al*., 1990; Boyington *et al*., 1997). Recently a new reaction mechanism was proposed for Fdh-H after re-analysing the crystal structure data and finding that a loop region close to the molybdenum active site was mistraced (PDB:2IV2) (Raaijmakers and Romão, 2006). It was shown that selenocysteine-140 is not a ligand for molybdenum hence is no longer bound to the metal after reduction of the enzyme with formate. The *E. coli* genome also encodes two other formate dehydrogenases, Fdh-O and Fdh-N, which can oxidize formate but are not a part of the FHL complex (Sawers, 2005). Fdh-N is induced in the presence of nitrate under anaerobic conditions and encoded by the *fdnGHI* operon, whereas Fdh-O is induced under aerobic as well as nitrate-respiring conditions and encoded by the *fdoGHI* operon. The crystal structure of the Fdh-N, a 600 kDa membrane protein, is also known (PDB:1KQF) (Jormakka *et al*., 2002).

Theoretical yield of hydrogen from glucose

The hydrogen yield is defined as the moles of H_2 produced per mole of substrate. Carbohydrates, mainly glucose, are the preferred substrates for fermentative hydrogen production. Starch, cellulose, as well as organic wastes can also be used as substrates. Many microorganisms such as *Enterobacter* sp., *Clostridium* sp. and *E. coli* are capable of producing hydrogen. Depending on the pathways used by the microorganisms and end-products, hydrogen yields may be variable (Levin *et al*., 2004; Nath and Das, 2004).

The fermentative route of hydrogen production starts with the conversion of glucose to pyruvate and NADH through glycolysis in both strict and facultative anaerobic bacteria (Hallenbeck, 2005) (Figs 2 and 3). In facultative

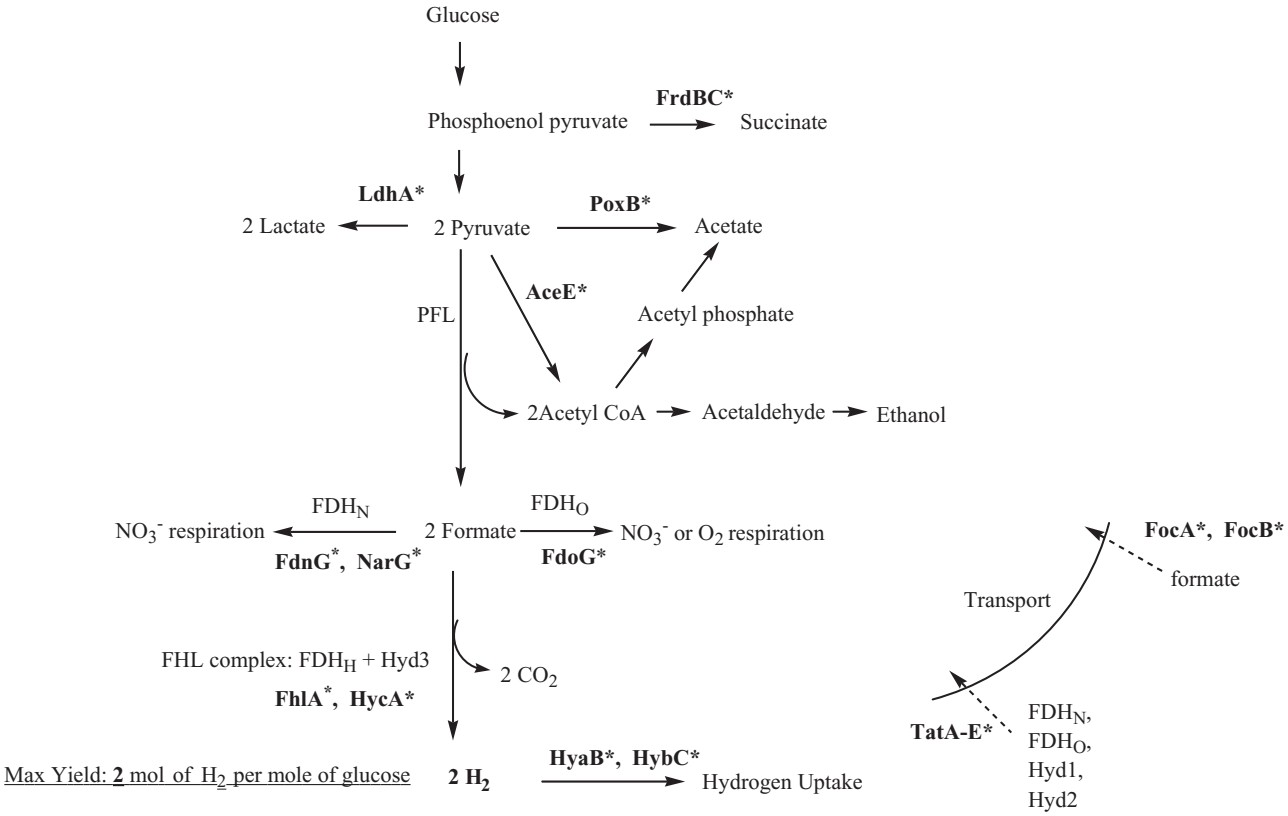

Fig. 2. Fermentative hydrogen production from glucose by *E. coli*, a well-studied facultative anaerobic bacterium. Hydrogen is produced through the action of the FHL complex. The maximum theoretical hydrogen yield is 2 mol of H_2 per mole of glucose. The glucose metabolic pathway yields succinate, lactate, acetate, ethanol and formate, as fermentation end-products. The proteins shown in bold with an asterisk have been studied through metabolic engineering in order to enhance the biohydrogen production. PFL, pyruvate formate lyase; FDH, formate dehydrogenase; FHL, formate hydrogen lyase; Hyd, hydrogenase; CoA, coenzyme A.

anaerobes, such as *E. coli* (Fig. 2), pyruvate is then converted to acetyl-CoA and formate, which is catalysed by pyruvate formate lyase (PFL). Hydrogen is produced from formate by the FHL complex. Because a maximum of two molecules of formate are produced from two pyruvate molecules, facultative anaerobic bacteria have a theoretical maximum yield of 2 mol of H_2 per mole of glucose. There are several factors that influence the yield, such as whether some of the pyruvate is converted to lactate; these competing paths for obtaining reducing power from pyruvate lower the yield (Hallenbeck, 2005).

In strict anaerobes, such as *Clostridium* sp. (Fig. 3), pyruvate is converted to acetyl-CoA and CO_2 through pyruvate ferredoxin oxidoreductase (PFOR), and this oxidation requires reduction of ferredoxin (Fd) (Hallenbeck, 2005; Kraemer and Bagley, 2007). Hydrogen is produced from the reduced Fd by the action of hydrogenase. This results with a maximum yield of 2 mol of H_2 per mole of glucose. Two additional moles of hydrogen can be produced from the NADH produced during glycolysis. In this step, NADH is oxidized by Fd reduction by NADH:ferredoxin oxidoreductase (NFOR). Again, hydrogen can be produced from the reduced Fd by hydrogenase. Overall, strict anaerobic bacteria have a theoretical maximum yield of 4 mol of H_2 per mole of glucose, which is the greatest theoretical yield possible. However, in practice, yields are lower, as the NADH oxidation by NFOR is inhibited under standard conditions and only proceeds at very low partial pressures of hydrogen (< 60 Pa) (Angenent *et al.*, 2004; Hallenbeck, 2005; Kraemer and Bagley, 2007). The relevant reactions are:

$$\text{PFOR: } 2\text{Pyruvate} + 2\text{CoA} + 2\text{Fd (oxidized)} \rightarrow 2\text{Acetyl-CoA} + 2\text{CO}_2 + 2\text{Fd (reduced)}$$

$$\text{NFOR: } 2\text{NADH} + 2\text{Fd (oxidized)} \rightarrow 2\text{NAD}^+ + 2\text{Fd (reduced)}$$

$$4\text{Fd (reduced)} \rightarrow 4\text{H}_2 \text{ (via hydrogenase)}$$

Products formed from acetyl-CoA such as acetate, butyrate, butanol, acetone, lactate or ethanol in strict anaerobes determine the theoretical yield of hydrogen (Chin *et al.*, 2003). One can obtain the greatest theoretical yield of hydrogen of 4 mol of H_2 per mole of glucose when acetate or acetone is the fermentation end-product:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$$

$$C_6H_{12}O_6 + H_2O \rightarrow CH_3COCH_3 + 3CO_2 + 4H_2$$

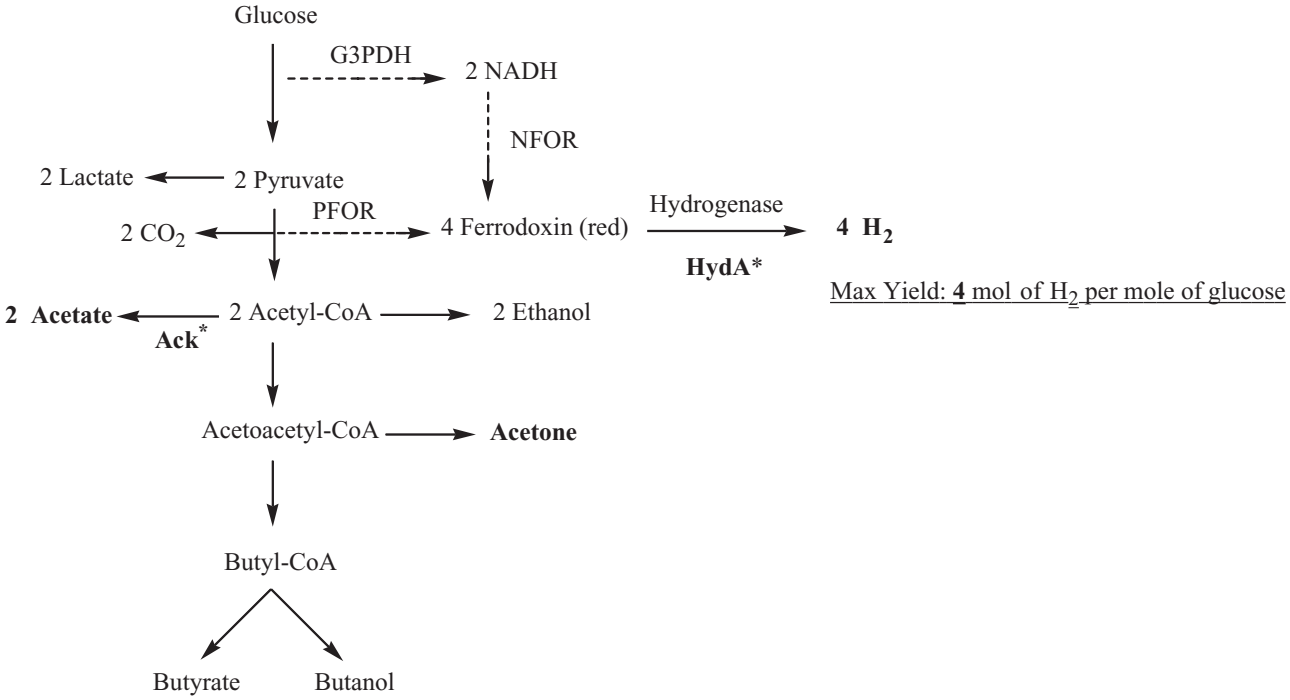

Fig. 3. Fermentative hydrogen production from glucose by *C. acetobutylicum*, a strict anaerobic bacterium. Hydrogen can be produced through the action of PFOR and NFOR. The maximum theoretical hydrogen yield is 4 mol of H_2 per mole of glucose, with acetate or acetone as the fermentation end-product. The glucose metabolic pathway results in lactate, acetate, ethanol, acetone, butanol and butyrate as fermentation end-products. The proteins shown in bold with an asterisk have been studied in *Clostridium* species through metabolic engineering in order to enhance biohydrogen production. G3PDH, glyceraldehyde-3-phosphate dehydrogenase; PFOR, pyruvate ferredoxin oxidoreductase; NFOR, NADH:ferredoxin oxidoreductase; NADH, nicotineamide-adenine dinucleotide; red, reduced.

When butyrate is the fermentation end-product, the maximum theoretical yield of hydrogen is 2 mol of H_2 per mole of glucose:

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$$

When alcohols are the end-products, lower yields of hydrogen are obtained as alcohols contain additional hydrogen atoms that have not been converted to hydrogen gas. Note that, the fermentation end-products and the resultant hydrogen yields vary based on the environmental conditions even within the same bacterium (Hawkes *et al.*, 2002).

A maximum yield of 4 mol of H_2 per mole of glucose is still low for practical applications and a two-stage process has been envisioned to obtain yields closer to the theoretical stoichiometric yield of 12 mol of H_2 per mole of glucose (Nath *et al.*, 2005; Hawkes *et al.*, 2007). Combining dark and photo-fermentation may be a possible way to achieve this goal; however, improvements in sunlight conversion efficiency (such as improvements in sunlight collection and light transfer systems) and photobioreactor development need to be performed to reduce the energy demand and to increase the productivity of the photo-fermentation step (Hallenbeck and Benemann, 2002; Akkerman *et al.*, 2003). In the integrated system, the first step, dark fermentation, produces fermentation end-products, which can then be converted to hydrogen by photo-fermentation. Theoretically, the maximum hydrogen yield of 12 mol of H_2 per mole of glucose may be obtained when glucose is converted to acetate as the terminal product through dark fermentation, which can then be converted into H_2 through photo-fermentation (Nath *et al.*, 2005):

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$$

$$2CH_3COOH + 4H_2O \rightarrow 4CO_2 + 8H_2$$

$$C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 12H_2 \text{ (overall)}$$

There are some examples in the literature in which the integrated systems were shown to improve the hydrogen yields; however, the yields were still far lower than the theoretical yield of 12 mol of H_2 per mole of glucose. *Enterobacter cloacae* strain DM11, a facultative anaerobe, produced 1.86 mol of H_2 from 1 mol of glucose through dark fermentation, and *Rhodobacter sphaeroides* strain O.U.001 produced 1.5–1.72 mol of H_2 from 1 mol of glucose through photo-fermentation. The hydrogen yield was shown to be higher with the integrated system compare to single-step fermentation (Nath *et al.*, 2005).

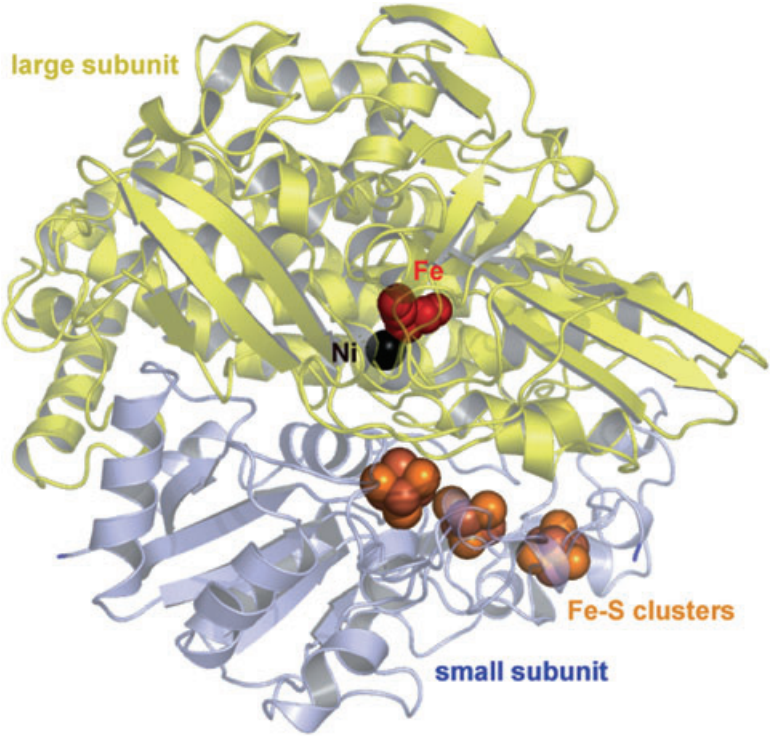

Fig. 4. Three-dimensional structure of [NiFe]-hydrogenase from *D. gigas* (PDB:2FRV). The large subunit which contains the Ni-Fe catalytic centre is shown in yellow. The small subunit which contains the Fe-S clusters is shown in blue. Metals and sulfur atoms are depicted as spheres. Colour scheme: nickel is black, carbonmonoxide-(dicyano) iron is red and Fe-S cluster is orange.

Another example of an integrated system resulting in higher hydrogen yields was shown by Kim and colleagues (2006). In the dark fermentation step, *Clostridium butyricum* produced hydrogen with a yield of 2.58 mol of H_2 per mole of glucose as well as intermediates such as formate, acetate, propionate and butyrate. These intermediates were then converted into 5.72 mol of H_2 by *R. sphaeroides* KD131, resulting in a total yield of 8.3 mol of H_2 from 1 mol of glucose.

The current status of mesophilic, continuous, dark, fermentative, hydrogen production using mixed microflora was reviewed by Hawkes and colleagues (2007). Possible second-stage processes to follow the dark fermentation stage include photo-fermentation, microbial fuel cells and anaerobic digestion; these second stages increase the hydrogen production yield, produce electricity or methane.

Theoretically, stoichiometric yields can be obtained under equilibrium conditions, meaning at very low partial pressures of hydrogen and very slow rates (Hallenbeck and Benemann, 2002). Under these conditions, which are not practical, Woodward and colleagues (2000) were able to achieve nearly complete conversion of glucose to H_2 and obtained a hydrogen yield of 11.6 mol of H_2 per mole of glucose 6-phosphate using pentose phosphate cycle enzymes combined with the hydrogenase from *Pyrococcus furiosus* that uses $NADP^+$ as the electron carrier (Woodward *et al.*, 2000).

Biochemical structure of hydrogenases

Hydrogenases, the enzymes responsible for the reversible conversion of molecular hydrogen into two protons and two electrons ($H_2 \leftrightarrow 2H^+ + 2e^-$), are complex metalloenzymes that can be classified into three groups based on the number and identity of the metals in their active sites: [NiFe]-, [FeFe]- and [Fe]-hydrogenases (Vignais *et al.*, 2001; Vignais and Colbeau, 2004). They are phylogenetically not related but still share common properties at their active sites; for example, they all contain Fe and CO as a ligand to the Fe atom.

The more common [NiFe]-hydrogenases consist of a small subunit of 30 kDa and a large subunit of 60 kDa which are tightly bound via a large, hydrophobic surface (Fig. 4) (Vignais *et al.*, 2001; Frey, 2002; Vignais and Colbeau, 2004). The small subunit contains up to three iron-sulfur clusters which mediate the electron transfer between the active site and electron acceptor (or donor) of hydrogenase, while the large subunit contains the nickel-iron active site in which the hydrogen activation occurs. The dinuclear active centre generally consists of a $(CysS)_2Ni(\mu\text{-}'O')(\mu\text{-}CysS)_2Fe(CN)_2(CO)$ structure where μ is the prefix for a bridging ligand (Fig. 5). The nickel group is coordinated to the protein via four thiol groups from cysteine residues. The Ni and Fe atoms are connected by two of these cysteine residues in both the active and inactive form of the enzyme and by an oxygen atom only

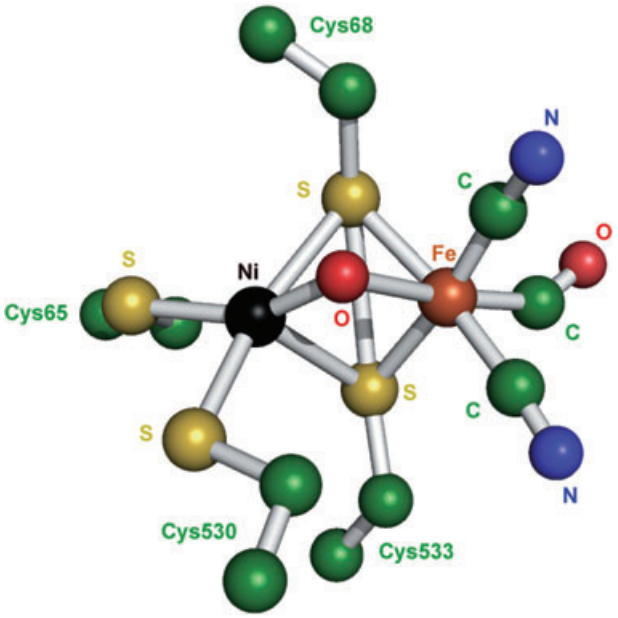

Fig. 5. Structure of the oxidized [NiFe]-hydrogenase active centre from *D. gigas* (PDB:2FRV). Colour scheme: nickel is black, iron is orange, oxygen is red, carbon is green and nitrogen is blue.

in the oxidized inactive form. In addition, one CO and two CN ligands are coordinated to the Fe atom. Hydrophobic channels connect the active centre with the protein surface and most probably mediate gas substrate and product transport. It has been suggested that a few oxygen-resistant hydrogenases have more narrow channels that serve to block oxygen transport to the active site (Vignais *et al*., 2001; Frey, 2002; Volbeda and Fontecilla-Camps, 2003; Vignais and Colbeau, 2004). The first crystal structure of a hydrogenase enzyme was the [NiFe]-hydrogenase from the sulfate-reducing bacterium *Desulfovibrio gigas* (PDB:1FRV, 2FRV) (Fig. 4) (Volbeda *et al*., 1995; 1996). Other known X-ray crystal structures of [NiFe]-hydrogenases were obtained from *Desulfovibrio vulgaris* Miyazaki F (PDB:1H2A, 1H2R) (Higuchi *et al*., 1997; 1999), *D. fructosovorans* (PDB:1FRF) (Rousset *et al*., 1998), *D. desulfuricans* ATCC 27774 (PDB:1E3D) (Matias *et al*., 1999) and *Desulfomicrobium baculatum* (PDB:1CC1) (Garcin *et al*., 1999).

The large subunits of [NiFe]-hydrogenases include *hyaB* for *E. coli* Hyd-1 (Andrews *et al*., 1997), *hybC* for *E. coli* Hyd-2, *hycE* for *E. coli* Hyd-3, *hyfG* for *E. coli* Hyd-4, HoxH for *Ralstonia eutropha* (Burgdorf *et al*., 2002), HoxH for *D. gigas* (Volbeda *et al*., 1995) and HoxH for *Synecocystis* sp. PCC 6803 (Tamagnini *et al*., 2002). We have aligned these hydrogenases using Vector NTI software to determine their identity (Table 2). The bioinformatics analysis shows that the large subunits of these hydrogenases have very low homology. For example, the large subunit of *E. coli* Hyd-3 has only 13.7% identity with HoxH of *D. gigas* whose crystal structure is known (Fig. 4). Modelling by homology to predict the structure of *E. coli* Hyd-3 using the structure of *D. gigas* is quite risky and may impede mutagenesis studies. This low homology illustrates the need for a crystal structure of *E. coli* Hyd-3.

The second class of [FeFe]-hydrogenases (also known as Fe-only hydrogenases) generally consist of a single catalytic subunit (Nicolet *et al*., 2000; Vignais *et al*., 2001; Frey, 2002; Vignais and Colbeau, 2004). The dinuclear active centre probably consists of a $(H_2O)(CO)(CN)Fe(\mu\text{-}CO)(\mu\text{-}NH(CH_2S^-)_2)Fe(CysS)(CN)(CO)$ overall structure. Both Fe atoms are connected by one CO and two S ligands of a dithiol-bridging ligand [a di(thiomethyl)amine molecule ($HN\text{-}CH_2S^-$) or propane dithiol] (Zilberman *et al*., 2007). Each Fe atom has a CO and a CN ligand. In addition, a [4Fe-4S] cluster is coordinated through the sulfur of a cysteine residue to one of the Fe atoms (Fig. 6) (Nicolet *et al*., 2000; Frey, 2002; Vignais and Colbeau, 2004; Albracht *et al*., 2006). The crystal structure of [FeFe]-hydrogenases from *Clostridium pasteurianum* (PDB:1FEH) (Peters *et al*., 1998) and *D. desulfuricans* ATCC 7757 (PDB:1HFE) (Nicolet *et al*., 1999) revealed the similarities between [NiFe]- and [FeFe]-hydrogenases

Table 2. Identity between hydrogenase large subunits derived from *Escherichia coli* (*E. coli*), *Ralstonia eutropha* H16 (*R. eutropha*), *Rhodoccus opacus* MR11 (*R. opacus*), *Synechocystis* sp. PCC 6803 (*Syn.* PCC 6803) and *Desulfovibrio gigas* (*D. gigas*).

	E. coli Hyd-1	*E. coli* Hyd-2	*E. coli* Hyd-3	*E. coli* Hyd-4	*R. eutropha* HoxH	*R. opacus* HoxH	*Syn.* PCC 6803 HoxH	*D. gigas* large subunit
E. coli Hyd-1		40.7%	13.9%	11.9%	18.8%	18.3%	16.1%	39.9%
E. coli Hyd-2	40.7%		13.8%	11.0%	17.3%	18.1%	19.0%	41.6%
E. coli Hyd-3	13.9%	13.8%		69.8%	13.1%	12.2%	12.4%	13.7%
E. coli Hyd-4	11.9%	11.0%	69.8%		12.7%	12.0%	13.0%	12.4%
R. eutropha HoxH	18.8%	17.3%	13.1%	12.7%		85.2%	42.7%	19.9%
R. opacus HoxH	18.3%	18.1%	12.2%	12.0%	85.2%		42.0%	19.8%
Syn. PCC 6803 HoxH	16.1%	19.0%	12.4%	13.0%	42.7%	42.0%		17.8%
D. gigas large subunit	39.9%	41.6%	13.7%	12.4%	19.9%	19.8%	17.8%	

Hyd-1, Hyd-2, Hyd-3, Hyd-4 are HyaB, HybC, HycE and HyfG of *E. coli* hydrogenases 1, 2, 3 and 4 respectively. Protein sequences for the large subunit or HoxH of hydrogenase were aligned by using a VectroNTI alignment software.

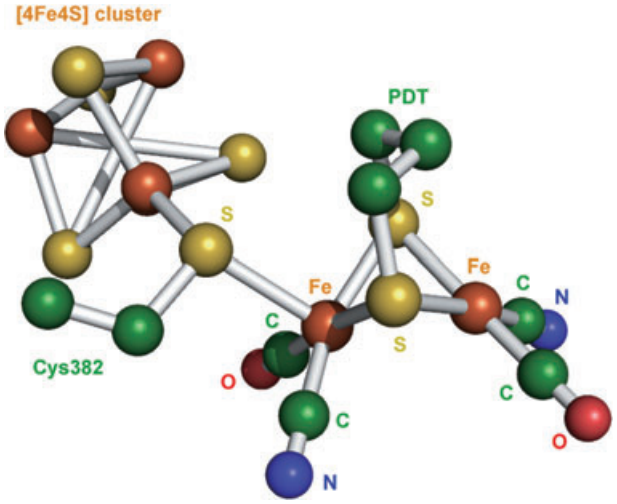

Fig. 6. Structure of the [FeFe]-hydrogenase active centre from *Desulfovibrio desulfuricans* ATCC 7757 (PDB:1HFE). The bridging CO that connects both Fe atoms and the water molecule that binds to the Fe atom can be viewed using the structure from *C. pasteurianum* (PDB:1FEH). Colour scheme is iron is orange, oxygen is red, carbon is green and nitrogen is blue. PDT, 1,3-propanedithiol.

such as the presence of iron-sulfur clusters for electron transport, a dinuclear metal centre with CO and CN ligands, and hydrophobic channels connecting the molecular surface to the active site (Volbeda *et al*., 1995; 1996; Montet *et al*., 1997).

The third class of [Fe]-hydrogenases (also known as iron-sulfur cluster-free hydrogenases or metal-free hydrogenases) are found in some methanogenic archaea (Zirngibl *et al*., 1990). These enzymes are also referred to as H_2-forming methylene-tetrahydromethanopterin dehydrogenase as the enzyme catalyses the reversible reduction of N^5,N^{10}-methenyl-tetrahydromethanopterin with H_2 to N^5,N^{10}-methylene-tetrahydromethanopterin and a proton (Thauer *et al*., 1996). Different from [NiFe]- and [FeFe]-hydrogenases, they do not catalyse the reversible conversion of molecular hydrogen into two protons and two electrons and contain neither Fe-S clusters nor nickel (Zirngibl *et al*., 1992). They contain a single Fe atom bound with two CO, one S and one or two N/O ligands (Korbas *et al*., 2006). Recently, the crystal structure of the apoenzyme of the iron-sulfur cluster-free hydrogenase from *Methanocaldococcus jannaschii* (PDB:2B0J) (Pilak *et al*., 2006) was determined. The crystal structure of the mature iron-sulfur cluster-free hydrogenase and its catalytic mechanism are still unknown.

Maturation proteins

The biosynthesis of [NiFe]-hydrogenases and [FeFe]-hydrogenases are distinct from each other; however, they share some common properties such as requiring guanine nucleotide-binding proteins (GTPases), with similar roles (Leach and Zamble, 2007). The biochemical pathways for the synthesis and incorporation of the $NiFe(CO)(CN)_2$ metal centre in [NiFe]-hydrogenases are briefly described here. By comparison, more progress needs to be made in understanding the biosynthesis of [FeFe]-hydrogenases.

The maturation steps of [NiFe] biosynthesis include (i) the biosynthesis of CO and CN, the diatomic ligands of the active site, (ii) insertion of the one CO and two CN ligands to the Fe atom, (iii) insertion of the $Fe(CN)_2CO$ complex into the hydrogenase, (iv) insertion of the Ni atom into the active site of hydrogenase, and (v) correct folding before delivery to the membrane (Forzi and Sawers, 2007). More detailed information can be found on the maturation steps of [NiFe]-hydrogenases in other review papers (Forzi and Sawers, 2007; Leach and Zamble, 2007).

There are many genes encoding accessory proteins that participate in the maturation of nickel-containing hydrogenases. For example, at least seven proteins are required for the maturation of Hyd-3 in *E. coli*, which is the most studied hydrogenase: six of the proteins are from the *hyp* (hydrogen pleiotropy) operon (*hypABCDEF*), and an endopeptidase is encoded by *hycI* (Blokesch *et al*., 2002). The function of these genes on the maturation process has been studied (Table 3); however, more research needs to be performed to understand the complete biochemical pathway of the active site synthesis.

The crystal structures of HypB from *M. jannaschii* (Gasper *et al*., 2006), HypC, HypD and HypE from *Thermococcus kodakaraensis* (Watanabe *et al*., 2007), HybD from *E. coli* (Fritsche *et al*., 1999), HypF N-terminal acylphosphatase domain (residues 1–91) from *E. coli* (Rosano *et al*., 2002), and the NMR solution structure of HycI from *E. coli* (Yang *et al*., 2007) are known (Table 3) and provide insight into their functions in maturation process.

Maturation of [FeFe]-hydrogenases may not require as many proteins as it only requires the biosynthesis and insertion of the catalytic iron-sulfur cluster known as the H-cluster. Initial studies on how [FeFe]-hydrogenases are biosynthesized showed that the HydE, HydF and HydG proteins are required (Table 3) (King *et al*., 2006). McGlynn and colleagues (2007) showed that the inactive hydrogenase structural protein (HydA) of *Clostridium saccharobutylicum* can be rapidly activated by the maturation proteins of *Clostridium acetobutylicum* which are expressed in concert (HydEFG) in *E. coli* (McGlynn *et al*., 2007). The group proposed that the HydE, HydF and HydG proteins form an H-cluster precursor, which is transferred to the inactive hydrogenase structural protein in order to active it.

Table 3. Maturation proteins and their functions.

Protein	Name	Function	PDB ID	Reference
HypA	Zn-containing protein	Maturation of the large subunit of Hyd-3 , nickel insertion along with SlyD, a peptidyl-prolyl *cis/trans*-isomerase that forms a complex with HypB	–	Hube *et al*. (2002); Atanassova and Zamble (2005); Zhang *et al*. (2005)
HypB	GTPase	Nickel liganding into hydrogenase large subunit	2HF9	Leach *et al*. (2005); Gasper *et al*. (2006)
HypC	Chaperone-like protein	Maturation of hydrogenase 3 by catalysing Fe insertion	2Z1C	Blokesch *et al*. (2001); Watanabe *et al*. (2007)
HypD	Fe/S protein	Possibly transferring the cyano group to the Fe atom or in the cyanation reaction	2ZID	Blokesch and Böck (2006); Watanabe *et al*. (2007)
HypF	Carbamoyl phosphate phosphatase	Catalyses the synthesis of the CN^- ligands of the active site iron of [NiFe]-hydrogenases using carbamoylphosphate as a substrate along with HypE	1GXT[a]	Rosano *et al*. (2002); Reissmann *et al*. (2003)
HypE	ATP-dependent dehydratase	Hydrogenase maturation protein	2Z1E, 2Z1F	Reissmann *et al*. (2003); Watanabe *et al*. (2007)
HybF	Zn-containing protein	Maturation of Hyd-1 and -2 (HypA homologous)	–	Hube *et al*. (2002)
HybG	Chaperone-like protein	Maturation of Hyd-1 and -2 (HypC homologous)	–	Blokesch *et al*. (2001)
HyaD	Endopeptidase	Maturation of Hyd-1	–	Forzi and Sawers (2007)
HybD	Endopeptidase	maturation of Hyd-2	1CFZ	Fritsche *et al*. (1999); Forzi and Sawers (2007)
HycI	Endopeptidase	Recognizes the Ni bound state of Hyd3, maturation of the large subunit of Hyd-3	2I8L	Forzi and Sawers (2007); Yang *et al*. (2007)
HydEHydG	*S*-adenosylmethionine enzymes	Required for the biosynthesis of [FeFe]-hydrogenases	–	King *et al*. (2006)
HydF	GTPase	Required for the biosynthesis of [FeFe]-hydrogenases	–	King *et al*. (2006)

a. The crystal structure of HypF N-terminal acylphosphatase domain (residues 1–91).
The crystal structure or the NMR solution structure is given by the Protein Data Bank identification number (PDB ID).

Metabolic engineering of fermentative systems

There are several examples of metabolic engineering applications to increase the fermentative or photosynthetic hydrogen production. Some of the examples concerning enhanced photosynthetic hydrogen production can be found elsewhere (Miyake *et al*., 1999; Vignais *et al*., 2006; Rey *et al*., 2007). Examples discussed here concern improving fermentative hydrogen production pathways.

Recombinant strains can be created through genetic and metabolic engineering, which leads to higher levels of hydrogen production. The hydrogen yield is suboptimal in many organisms containing uptake hydrogenases, as some of the produced hydrogen is consumed. Therefore, knocking out the genes encoding uptake hydrogenases is one way to enhance hydrogen production. Other ways to increase hydrogen production include overexpression of hydrogen-evolving hydrogenases, shutting down metabolic pathways that compete for hydrogen production, and overexpression of cellulases, hemicellulases and lignases that can maximize glucose availability (Levin *et al*., 2004; Nath and Das, 2004). Table 4 summarizes the H_2 production rates and yields for some of the recombinant and wild-type strains.

The metabolic pathway for the fermentation of glucose by *E. coli* is shown in Fig. 2. The fermentation products are hydrogen, acetate, ethanol, lactate, formate and some succinate (Clark, 1989). Although several microorganisms can produce hydrogen through dark fermentation, *E. coli* has many advantages, such as rapid growth and simple nutritional requirements. It is also easy to perform metabolic engineering to increase hydrogen production in *E. coli*, as *E. coli* is easily manipulated through genetic engineering. To enhance hydrogen production, recombinant strains of *E. coli* were developed having mutations in several genes, for example, in the large subunit of uptake Hyd-1 and -2 (*hyaB* and *hybC* respectively) (Maeda *et al*., 2007b,c), in lactate dehydrogenase (*ldhA*) (Bisaillon *et al*., 2006; Yoshida *et al*., 2006; Maeda *et al*., 2007c), in the FHL repressor (*hycA*) (Penfold *et al*., 2003; 2006; Yoshida *et al*., 2005; 2006; Maeda *et al*., 2007b,c), in the FHL activator (*fhlA*) (Yoshida *et al*., 2005; 2006; Bisaillon *et al*., 2006; Maeda *et al*., 2007b,c), in fumarate reductase (*frdBC*) (Yoshida *et al*., 2006; Maeda *et al*., 2007c), in the Tat system (*tatA–E*) (Penfold *et al*., 2003; 2006), in the alpha subunit of the formate dehydrogenase-N and -O (*fdnG* and *fdoG* respectively) (Maeda *et al*., 2007b,c), in the alpha subunit of nitrate reductase A (*narG*) (Maeda *et al*., 2007b,c), in pyruvate dehydrogenase (*aceE*) (Maeda *et al*., 2007c), in pyruvate oxidase (*poxB*) (Maeda *et al*., 2007c) and in proteins that transport formate (*focA* and *focB*) (Maeda *et al*., 2007b,c) (Fig. 2).

Table 4. Productivities and yields of recombinant and wild-type systems.

Strain	Productivity, μmol H_2 (mg protein)$^{-1}$ h^{-1}	Yield	Comments	Reference
E. coli BW25113 *hyaB hybC hycA fdoG fhlA*$^+$	113	1.2 mol of H_2 per mole of formate	Low partial pressure batch reactor using low cell density with complex-formate medium	Maeda *et al.* (2007b)
E. coli BW25113 *hyaB hybC hycA fdoG ldhA frdC aceE*	32	1.3 mol of H_2 per mole of glucose	Low partial pressure batch reactor with complex glucose	Maeda *et al.* (2007c)
E. coli TG1/pBS(Kan)Synhox	10	–	Hydrogen production from *E. coli* cells expressing cyanobacterium *Synechocystis* sp. PCC6803 *hoxEFUYH*	Maeda *et al.* (2007d)
E. coli SR15 (Δ*ldhA* Δ*frdBC*)	27	1.8 mol of H_2 per mole of glucose	Reactor equipped with pH sensor and ports for NaOH feed, gas exhaustion, substrate feed and sampling	Yoshida *et al.* (2006)
E. coli DADE (Δ*tatA–E*)	4.4	–	Reaction at 100 mM glucose	Penfold *et al.* (2006)
E. coli HD701 (Δ*hycA*)	5.7	–	Reaction at 100 mM glucose	Penfold *et al.* (2003)
E. coli SR13 (Δ*hycA fhlA*$^+$)	254	–	Reaction using high cell density and 25 mM formic acid	Yoshida *et al.* (2005)
E. coli SR14 (Δ*hycA fhlA*$^+$ Δ*ldhA* Δ*frdBC*)	288	–	Reaction by using a metabolite excretion system to remove excess metabolites from the medium	Yoshida *et al.* (2007)
E. coli BL21 *hydA*$^+$	5.6	3.1 mol of H_2 per mole of glucose	Hydrogen production with *E. coli* BL21 expressing the [Fe]-hydrogenase gene (*hydA*) from *E. cloacae* IIT-BT-08	Chittibabu *et al.* (2006)
E. coli HD701 (+invertase)	3.2	–	Hydrogen production from sucrose with *E. coli* HD701 strain expressing an invertase activity	Penfold and Macaskie (2004)
C. paraputrificum M-21 hydA$^+$	–	2.4 mol of H_2 per mole of GlcNAc	Cultured using GS medium with 1% GlcNAc as a carbon source	Morimoto *et al.* (2005)
C. tyrobutyricum (Δ*ack*)	–	2.2 mol of H_2 per mole of glucose	Fed-batch fermentations of glucose by free cells	Liu *et al.* (2006)
E. aerogenes AY-2 (double mutant)	6.8	1.1 mol of H_2 per mole of glucose	Reaction by self-flocculated cells in a packed-bed reactor in a minimal medium	Rachman *et al.* (1998)
E. cloacae IIT-BT-08 (double mutant)	–	3.4 mol of H_2 per mole of glucose	Mutant with lower alcohol dehydrogenase and butanediol dehydrogenase activity, reaction using immobilized bioreactor	Kumar *et al.* (2001)
E. cloacae IIT-BT-08 (wild-type)	59	6 mol of H_2 per mole of sucrose 2.2 mol of H_2 per mole of glucose	A Gram-negative hydrogen producing facultative anaerobe, reaction using sucrose at 36°C	Kumar and Das (2000)
E. asburiae SNU-1 (wild type)	76	0.4 mol of H_2 per mole of formate	A new fermentative hydrogen-producing bacterium isolated from a domestic landfill	Shin *et al.* (2007)
Citrobacter sp. Y19 (wild type)	65	2.5 mol of H_2 per mole of glucose	CO-dependent H_2 production	Oh *et al.* (2003)
Klesiella oxytoca HP1 (wild type)	30	3.2 mol of H_2 per mole of sucrose	Hydrogen-producing bacterial strain isolated from a hot spring	Minnan *et al.* (2005)
Rhodopseudomonas palustris JA1 (wild type)	60	–	A purple non-sulfur bacterium	Archana *et al.* (2003)
R. palustris P4 (wild type)	41	–	A photosynthetic bacterium isolated from an anaerobic wastewater	Jung *et al.* (1999)
Caldicellulosiruptor saccharolyticus (wild type)	21	6.6 mol of H_2 per mole of sucrose	Hydrogen-producing extreme thermophilic bacterium	van Niel *et al.* (2002)

GlcNAc, *N*-acetylglucosamine.

Yoshida and colleagues (2005) enhanced fermentative biohydrogen production from formic acid by overexpressing the FHL activator encoded by the *fhlA* and by inactivating the FHL repressor encoded by the *hycA* in *E. coli* K-12 strain W3110. The strain in which *fhlA* was overexpressed had 1.7-fold higher hydrogen production, whereas the *hycA*-inactivated strain had 1.2-fold higher hydrogen compared with the wild-type strain. The hydrogen production rate was 2.8-fold higher with both *fhlA* overexpressed and *hycA* inactivated (recombinant strain SR13). The *fhlA* overexpression and *hycA* inactivation caused a 6.5-fold increase in the formate dehydrogenase expression encoded by *fdhF* and a sevenfold increase in the Hyd-3 large subunit expression encoded by *hycE*. Further enhancement in hydrogen production rate from formic acid was achieved using the mutant strain SR13 under the high cell density [254 μmol (mg protein)$^{-1}$ h^{-1}] (Table 4) (Yoshida *et al*., 2005).

In another study by Yoshida and colleagues (2006), enhanced hydrogen yields from glucose was demonstrated by blocking the competing lactate (via deleting *ldhA*) and succinate (via deleting *frdBC*) production pathways. The maximum hydrogen yield was found to be 1.08 mol of H_2 per mole of glucose with the wild-type *E. coli* K-12 strain W3110, whereas the *E. coli* strain with *fhlA* overexpressed, *hycA* inactivated, and *ldhA* and *frdBC* deleted (SR14) had a maximum hydrogen yield of 1.87 mol of H_2 per mole of glucose. The maximum hydrogen yield with *ldhA* and *frdBC* deleted (SR15) was also around 1.82 mol of H_2 per mole of glucose; hence, *fhlA* overexpression and *hycA* inactivation did not cause a significant increase in hydrogen yield from glucose. Moreover, the hydrogen production rate from glucose was 1.4-fold higher with mutant strain SR15 [27 μmol (mg protein)$^{-1}$ h^{-1}] (Table 4) compared with the wild-type strain (Yoshida *et al*., 2006).

In a study by Penfold and colleagues (2003), the maximum hydrogen production rate with *hycA* inactivated in *E. coli* (strain HD701) was around twofold higher [5.7 μmol (mg protein)$^{-1}$ h^{-1}] (Table 4), compared with the wild-type strain MC4100 at a glucose concentration of 100 mM. The difference in rates between wild-type and the recombinant strain was better (up to 14-fold) at lower glucose concentrations. Increases in hydrogen production rates were similar when industrial waste water with high sugar content was used instead of glucose as a substrate (Penfold *et al*., 2003). Introducing an invertase gene, which is responsible for converting sucrose to glucose and fructose, enabled strain HD701 to utilize sucrose and produce hydrogen with a rate of 3.2 μmol (mg protein)$^{-1}$ h^{-1} (Table 4) (Penfold and Macaskie, 2004). Deletion of *tatC* to inactivate the twin arginine translocation (Tat) translocase in strain HD701 did not affect hydrogen production significantly probably because the saturated FHL system did not use the excess amount of formate (Penfold and Macaskie, 2004).

The Tat translocase of *E. coli* MC4100 transports two uptake hydrogenases, Hyd-1 and -2, and the two formate dehydrogenases, FdH-N and FdH-O, to the cell membrane (Penfold *et al*., 2006). The Tat system consists of the TatABCE proteins. Deletion of *tatC* (strain FTD701) or *tatA–E* (strain DADE) resulted in a twofold enhancement in hydrogen production rate compared with wild-type *E. coli* MC4100 [4.4 μmol (mg protein)$^{-1}$ h^{-1} with strain DADE and 2.2 μmol (mg protein)$^{-1}$ h^{-1} with wild type] (Table 4). Deletion of *hycA* (strain HD701) caused a 2.5-fold increase in hydrogen production rate compared with wild-type *E. coli* MC4100. Combining the *tatC* and *hycA* mutations did not improve hydrogen production further. Hence, inactivating the Tat system in *E. coli* is another way of producing a recombinant system with enhanced hydrogen productivity (Penfold *et al*., 2006).

In a study by Bisaillon and colleagues (2006), the effect of mutations in uptake hydrogenases (Hyd-1, Hyd-2) as well as in *ldhA* and *fhlA* of *E. coli* strain BW545 was investigated (Bisaillon *et al*., 2006). *Escherichia coli* strain JW135, which lacks uptake Hyd-1 and Hyd-2, showed a 37% increase in hydrogen production rate compared with wild-type strain BW545. Mutations in *ldhA* and in *fhlA* caused an 18% and 11% increase in hydrogen production, respectively, compared with the wild-type *E. coli* BW545, and a 47% increase was obtained with the double mutant (*ldhA* and *fhlA)*. Hydrogen yield approached 2 mol of H_2 per mole of glucose at low glucose concentrations with *E. coli* recombinant strain DJT135, which lacks Hyd-1 and Hyd-2, and has mutations in *ldhA* and *fhlA* (Bisaillon *et al*., 2006).

Maeda and colleagues (2007b,c) obtained the largest increase in hydrogen production to date by introducing various combinations of mutations in *E. coli* cells (Maeda *et al*., 2007b,c). To enhance hydrogen production from formate, the *hyaB* and *hybC* genes were deleted to remove the hydrogen uptake activity by Hyd-1 and -2; the *hycA* gene (FHL repressor) was also deleted and the *fhlA* gene (FHL activator) was overexpressed to change the regulation of the FHL complex. In addition, the alpha subunit of formate dehydrogenase-N encoded by *fdnG,* the alpha subunit of formate dehydrogenase-O encoded by *fdoG* and the alpha subunit of nitrate reductase A encoded by *narG* were deleted to inactivate formate consumption other than FHL. *focA* and *focB* deletions were also made to prevent export of formate (Maeda *et al*., 2007b). The quintuple mutant strain of BW25113 with *hyaB*, *hybC*, *hycA* and *fdoG* deleted and *fhlA* overexpressed was shown to be the best mutant strain, and its hydrogen production rate from formate [113 ± 12 μmol (mg protein)$^{-1}$ h^{-1}] (Table 4) was 141-fold higher compared with the wild-type strain BW25113. The

theoretical maximum hydrogen yield of 1 mol of H_2 per mole of formate was also achieved with the quintuple mutant (Maeda *et al*., 2007b). This paper is also noteworthy as the authors made 28 isogenic deletion mutants using a novel method involving successive P1 transformations and the *E. coli* Keio collection (Baba *et al*., 2006).

To increase the hydrogen production from glucose, Maeda and colleagues (2007c) studied four more mutations in addition to the nine mutations discussed above (Maeda *et al*., 2007c). The fumarate reductase encoded by *frdC* was deleted to prevent phosphoenol pyruvate from forming succinate (this should increase pyruvate for hydrogen), and to prevent pyruvate from forming anything but formate, the lactate dehydrogenase encoded by *ldhA*, the pyruvate dehydrogenase encoded by *aceE* and the pyruvate oxidase encoded by the *poxB* were deleted. The *E. coli* septuple mutant with *hyaB*, *hybC*, *hycA*, *fdoG*, *frdC*, *ldhA* and *aceE* deletions in strain BW25113 had the highest hydrogen production rate from glucose [4.6-fold higher compared with the wild-type strain BW25113, 32 $\pm$ 6 μmol (mg protein)$^{-1}$ h^{-1}] (Table 4). This septuple mutant also had a hydrogen yield of 1.3 mol of H_2 per mole of glucose compared with 0.65 mol of H_2 per mole of glucose with the wild-type BW25113 cells (Maeda *et al*., 2007c).

The strict anaerobe *Clostridium* sp. usually produces compounds such as acetate (acetic acid), butyrate (butyric acid), lactate, acetone, butanol and ethanol (Chin *et al*., 2003) (Fig. 3). Mutants of *Clostridium* sp. were also developed using metabolic and/or genetic engineering to enhance hydrogen production. Overexpression of its own *hydA* gene encoding a [Fe]-hydrogenase in *Clostridium paraputrificum* M-21 resulted in a 1.7-fold enhancement in hydrogen production from *N*-acetylglucosamine (GlcNAc) (2.4 mol of H_2 per mole of GlcNAc versus 1.4 mol of H_2 per mole of GlcNAc) (Table 4) (Morimoto *et al*., 2005). The improved hydrogen yield in recombinant *C. paraputrificum* M-21 was due to enhanced acetic acid production by overoxidation of NADH and drastic reduction of lactic acid production.

Another study showed that inactivation of *ack* encoding acetate kinase for acetate formation in *Clostridium tyrobutyricum* resulted in a 1.5-fold enhancement in hydrogen production from glucose (2.2 mol of H_2 per mole of glucose versus 1.4 mol of H_2 per mole of glucose) (Table 4) (Liu *et al*., 2006). The mutant also had 1.4-fold increase in hydrogenase activity.

One must note that, although the theoretical hydrogen yields are greater with strict anaerobes such as *Clostridium* sp., facultative anaerobes such as *E. coli* or *Enterobacter* sp. are more favourable for hydrogen production, as they are less sensitive to oxygen, able to recover their activities if accidentally exposed to oxygen, and have faster growth and hydrogen production rates (Shin *et al*., 2007).

Similar metabolic engineering studies have been performed using *Enterobacter* species as well. Blocking alcohol and some of the organic acid formation pathways in *E. cloacae* IIT-BT-08 (Kumar and Das, 2000) through mutagenesis resulted in enhanced hydrogen production from glucose (Kumar *et al*., 2001). An *E. cloacae* double mutant gave a hydrogen yield of 3.4 mol of H_2 per mole of glucose (Table 4), whereas the wild-type strain gave a yield of 2.2 mol of H_2 per mole of glucose. Improvement of hydrogen yields in the double mutant was due to the lower amounts of ethanol and butanediol production. Similarly, due to the lower amounts of ethanol and butanediol production with *Enterobacter aerogenes* double mutant, the hydrogen yield was enhanced by twofold (Rachman *et al*., 1997). *Enterobacter aerogenes* double mutant strain AY-2 had a hydrogen yield of 1.2 mol of H_2 per mole of glucose, whereas the wild-type strain (HU-101) had a yield of 0.56 mol of H_2 per mole of glucose in a batch cultures. In addition, the hydrogen production rate using a packed-bed reactor in a minimal medium was around twofold higher with mutant AY-2 [6.8 μmol (mg protein)$^{-1}$ h^{-1}] (Table 4), compared with wild-type HU-101 (Rachman *et al*., 1998).

Recombinant hydrogenases

One of the earliest studies for creating strains with recombinant hydrogenases was performed by Karube and colleagues (1983). Cloning and expressing the hydrogenase gene from *C. butyricum* in *E. coli* strain HK16 (Hyd$^-$, lacking the native hydrogenase activity) in CB medium resulted in a 3- and 3.5-fold enhancement in hydrogenase activity compared with wild-type *C. butyricum* and *E. coli* C600 (Hyd$^+$) respectively (Karube *et al*., 1983).

In another study, the hydrogen yield was enhanced when *hydA*, the [Fe]-hydrogenase gene from *E. cloacae* IIT-BT-08 was overexpressed in non-hydrogen-producing *E. coli* BL-21 as a glutathione–HydA fusion protein (Mishra *et al*., 2004; Chittibabu *et al*., 2006). The recombinant *E. coli* BL21 had a yield of 3.12 mol of H_2 per mole of glucose, which was higher than the wild-type strain *E. cloacae* IIT-BT-08. Moreover, the maximum hydrogen production rate was reported to be 5.6 μmol (mg protein)$^{-1}$ h^{-1} from glucose with the recombinant strain using a continuous immobilized whole-cell bioreactor in MYG medium (Table 4) (Chittibabu *et al*., 2006).

Recently, a cyanobacterial enzyme was successfully cloned and expressed in *E. coli* for the first time to improve hydrogen production (Maeda *et al*., 2007d). The hydrogen yield was enhanced up to 41-fold when the reversible hydrogenase encoded by *hoxEFUYH* from the cyanobacterium *Synechocystis* sp. PCC 6803 was cloned in *E. coli*. DNA microarrays were used to show that expression of the native *E. coli* hydrogenases was not

affected by the expression of the cyanobacterial HoxEFUYH, and a series of isogenic knockout mutants were used to discern that the enhanced hydrogen production was due to the inhibition of the native hydrogen uptake activity (by *E. coli* Hyd-1 and -2) by HoxEFUYH (Maeda *et al*., 2007d).

Reactor-based methods to increase hydrogen production

There are also non-metabolic engineering ways to increase fermentative hydrogen yields, such as heat treatment for mixed cultures to select for spore-forming bacteria in mixed cultures, sparging and operational controls (Nath and Das, 2004; Kraemer and Bagley, 2007). For example, gas sparging is usually beneficial for hydrogen production (Kraemer and Bagley, 2007), and we have found that using a low partial pressure system increases productivity with *E. coli* (Maeda *et al*., 2007b). In addition, sufficient mixing is beneficial for mass transfer of hydrogen from the fermentation to the headspace (Kraemer and Bagley, 2007).

Compounds other than formate and glucose stimulate hydrogen production by bacteria. For example, thiosulfate elevated hydrogen production by twofold for immobilized *E. coli* cells by limiting the consumption of glucose by metabolic paths other than those that produce hydrogen; a similar effect was observed by adding succinate (Nandi *et al*., 2001). The concentrations of phosphate (when growth limiting) also affect hydrogen yield (Bisaillon *et al*., 2006). In addition, low concentrations of nitrogen (less than 1 mM) are significant for enhancing hydrogen yields (reached 2 mol of H_2 per mole of glucose), and the hydrogen yield was dramatically decreased for high concentrations of ammonium (Bisaillon *et al*., 2006).

Yoshida and colleagues (2005) increased hydrogen production over two orders of magnitude to 254 μmol (mg protein)$^{-1}$ h^{-1} by using high-cell density and 25 mM formic acid (Table 4); they estimated that 1 kW of energy could be supplied with 2 l of bacteria producing hydrogen at this rate. Also, they improved hydrogen productivity from glucose by using a metabolite excretion system to remove excess metabolites from the medium; the maximum hydrogen production rate using *E. coli* SR14 (Δ*hycA* Δ*ldhA* Δ*frdBC* overexpressing *fhlA*) was 288 μmol (mg protein)$^{-1}$ h^{-1} at a dilution rate of 2 h^{-1} compared with 74 μmol (mg protein)$^{-1}$ h^{-1} in batch-mode incubation (Table 4) (Yoshida *et al*., 2007). Hence, reactor design is important for hydrogen production.

Perspectives

Metabolic engineering is clearly an important approach to improve fermentative hydrogen production, but further research and development are needed for converting cheap feedstocks to hydrogen. For example, hydrogen productivities are now such that reactors of reasonable size are required based on glucose and formate (2–500 l); however, if 24 mol of hydrogen per hour is required for 1 kW of electricity from a fuel cell (roughly enough to power a home) (Levin *et al*., 2004), then the annual cost of electricity if formate is used for the fermentation is ~$172 000 (assuming $12 per kilogram of formate and 1 mol of H_2 per mole of formate) and ~$6400 if glucose is used (assuming $0.22 per kilogram of glucose and 1.3 mol of H_2 per mole of glucose). Because the theoretical yield has been achieved with formate (Maeda *et al*., 2007b) and further increases in productivity will reduce the size of the reactor but not the cost of the reactants, then the main way to reduce these costs are to increase the yield from glucose or convert less expensive feedstocks into hydrogen.

Due to the complexity of the hydrogenases and their maturation proteins and due to the fact that 14% of the *E. coli* genome remains completely uncharacterized (14% is unknown, 32% is predicted by computational analysis and 54% is experimentally determined) (Riley *et al*., 2006; Willenbrock *et al*., 2006), unknown pathways need to be explored (rather than just deleting known pathways). One possible way to achieve this is by applying random chemical mutagenesis followed by the application of DNA microarrays to determine which paths are affected and to identify bottlenecks.

To determine which pathways need to be optimized for hydrogen production, linear programming could be conducted with a metabolic model. Metabolic flux analysis (MFA) is the technique by which flux distributions through metabolic pathways are either determined or predicted (Varma and Palsson, 1994; Stephanopoulos *et al*., 1998). Fluxes are calculated through the development of stoichiometric models of the metabolic reaction network. Flux balance analysis (FBA) is a variant of MFA, in which the goal is to find all the feasible flux distributions for an organism under prescribed conditions (Varma and Palsson, 1994; Schilling *et al*., 2000; Edwards *et al*., 2002; Reed and Palsson, 2003). The benefit of FBA is that it may be carried out at the genome scale with limited data and still provide insight into how the organism will behave (Edwards *et al*., 2002; Schilling *et al*., 2002; Price *et al*., 2004; Reed and Palsson, 2004).

More effort is also needed for creating recombinant strains expressing foreign hydrogenases. The cloned gene could be from an organism that produces an oxygen-insensitive hydrogenase such as *Rhodobacter capsulatus* (Vignais *et al*., 1997), *R. eutropha* (Burgdorf *et al*., 2005) or *Aquifex aeolicus* (Guiral *et al*., 2006) or it can be from *E. coli* itself. In addition, protein-engineering techniques, such as DNA shuffling (Canada *et al*., 2002),

may be used to create novel, oxygen-tolerant hydrogenases and hydrogenases with enhanced activity; however, an efficient high-throughput screening method is required to effectively screen thousands of colonies expressing hydrogenase variants. Along these lines, Nagy and colleagues (2007) successfully applied gene shuffling for the first time to create recombinant [FeFe]-hydrogenase libraries of *C. acetobutylicum* and *C. saccharobutylicum*; these enzymes have 67% protein identity. A single-stranded DNA approach was used on the *hyaA* sequences although no screening method was reported. By choosing 62 random clones, one mutant was found with a 30% increase in activity. Because it was necessary to coexpress the accessory proteins HydEFG to achieve activity of the *Clostridial* proteins in this heterologous host, it may be easier to shuffle native *E. coli* hydrogenases and make use of its native maturation proteins. This approach has been used successfully by T. Maeda and T.K. Wood who have increased activity of Hyd-3 of *E. coli* by over 30-fold using a hydrogen-sensitive membrane (Seibert *et al*., 1998) to detect hydrogen directly (unpublished). Another rapid screening method based on the spectrophotometric detection of the consumption of formate in *E. coli* has recently been developed (Maeda and Wood, submitted); this is an indirect method that relies on formate consumption being directly related to more active hydrogenases.

The latest project of J. Craig Venter Institute (Rockville, MD), which accumulates DNA from marine microorganisms, may also provide us with naturally engineered novel hydrogenases with interesting metabolic processes; the Venter team already has announced the discovery of a novel oxygen-insensitive hydrogenase (pers. comm.). Discovering novel hydrogenases and metabolic pathways through genetic engineering, high-throughput genomic sequencing, environmental genomics and/or metagenomic technologies (Handelsman, 2004; Riesenfeld *et al*., 2004; Binnewies *et al*., 2006) may also help to make biological hydrogen production more favourable, practical and commercially competitive.

Acknowledgements

This research was supported by DARPA (HR0011-06-1-0001). We thank Dr Ranjan Srivastava for his ideas on genome-scale modelling.

References

Akkerman, I., Janssen, M., Rocha, J.M.S., Reith, J.H., and Wijffels, R.H. (2003) Photobiological hydrogen production: photochemical efficiency and bioreactor design. In *Biomethane & Bio-hydrogen: Status and Perspectives of Biological Methane and Hydrogen Production*. Reith, J.H., Wijffels, R.H., and Barten, H. (eds). The Netherlands: Dutch Biological Hydrogen Foundation, pp. 124–145.

Albracht, S.P.J., Roseboom, W., and Hatchikian, E.C. (2006) The active site of the [FeFe]-hydrogenase from *Desulfovibrio desulfuricans*. I. Light sensitivity and magnetic hyperfine interactions as observed by electron paramagnetic resonance. *J Biol Inorg Chem* **11:** 88–101.

Andrews, S.C., Berks, B.C., McClay, J., Ambler, A., Quail, M.A., Golby, P., and Guest, J.R. (1997) A 12-cistron *Escherichia coli* operon (*hyf*) encoding a putative proton-translocating formate hydrogenlyase system. *Microbiology* **143:** 3633–3647.

Angenent, L.T., Karim, K., Al-Dahhan, M.H., Wrenn, B.A., and Domiguez-Espinosa, R. (2004) Production of bioenergy and biochemicals from industrial and agricultural wastewater. *Trends Biotechnol* **22:** 477–485.

Archana, A., Sasikala, C., and Ramana, C.V. (2003) Augmentation of H_2 photoproduction in *Rhodopseudomonas palustris* by *N*-heterocyclic aromatic compounds. *Biotechnol Lett* **25:** 79–82.

Atanassova, A., and Zamble, D.B. (2005) *Escherichia coli* HypA is a zinc metalloprotein with a weak affinity for nickel. *J Bacteriol* **187:** 4689–4697.

Axley, M.J., Grahame, D.A., and Stadtman, T.C. (1990) *Escherichia coli* formate-hydrogen lyase. Purification and properties of the selenium-dependent formate dehydrogenase component. *J Biol Chem* **265:** 18213–18218.

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., *et al*. (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* **2:** 2006.0008.

Bagramyan, K., Mnatsakanyan, N., Poladian, A., Vassilian, A., and Trchounian, A. (2002) The roles of hydrogenases 3 and 4, and the F_0F_1-ATPase, in H_2 production by *Escherichia coli* at alkaline and acidic pH. *FEBS Lett* **516:** 172–178.

Baron, S.F., and Ferry, J.G. (1989) Reconstitution and properties of a coenzyme F_{420}-mediated formate hydrogenlyase system in *Methanobacterium formicicum*. *J Bacteriol* **171:** 3854–3859.

Barrett, E.L., Kwan, H.S., and Macy, J. (1984) Anaerobiosis, formate, nitrate, and *pyrA* are involved in the regulation of formate hydrogenlyase in *Salmonella typhimurium*. *J Bacteriol* **158:** 972–977.

Benemann, J. (1996) Hydrogen biotechnology: progress and prospects. *Nat Biotechnol* **14:** 1101–1103.

Benemann, J.R. (2000) Hydrogen production by microalgae. *J Appl Phycol* **12:** 291–300.

Binnewies, T.T., Motro, Y., Hallin, P.F., Lund, O., Dunn, D., La, T., *et al*. (2006) Ten years of bacterial genome sequencing: comparative-genomics-based discoveries. *Funct Integr Genomics* **6:** 165–185.

Bisaillon, A., Turcot, J., and Hallenbeck, P.C. (2006) The effect of nutrient limitation on hydrogen production by batch cultures of *Escherichia coli*. *Int J Hydrogen Energy* **31:** 1504–1508.

Blokesch, M., and Böck, A. (2006) Properties of the [NiFe]-hydrogenase maturation protein HypD. *FEBS Lett* **580:** 4065–4068.

Blokesch, M., Magalon, A., and Böck, A. (2001) Interplay between the specific chaperone-like proteins HybG and

HypC in maturation of hydrogenases 1, 2, and 3 from *Escherichia coli*. *J Bacteriol* **183:** 2817–2822.
Blokesch, M., Paschos, A., Theodoratou, E., Bauer, A., Hube, M., Huth, S., and Böck, A. (2002) Metal insertion into NiFe-hydrogenases. *Biochem Soc Trans* **30:** 674–680.
Boyington, J.C., Gladyshev, V.N., Khangulov, S.V., Stadtman, T.C., and Sun, P.D. (1997) Crystal structure of formate dehydrogenase H: catalysis involving Mo, molybdopterin, selenocysteine, and an Fe_4S_4 cluster. *Science* **275:** 1305–1308.
Burgdorf, T., De Lacey, A.L., and Friedrich, B. (2002) Functional analysis by site-directed mutagenesis of the NAD^+-reducing hydrogenase from *Ralstonia eutropha*. *J Bacteriol* **184:** 6280–6288.
Burgdorf, T., Lenz, O., Buhrke, T., van der Linden, E., Jones, A.K., Albracht, S.P.J., and Friedrich, B. (2005) [NiFe]-hydrogenases of *Ralstonia eutropha* H16: modular enzymes for oxygen-tolerant biological hydrogen oxidation. *J Mol Microbiol Biotechnol* **10:** 181–196.
Böhm, R., Sauter, M., and Böck, A. (1990) Nucleotide sequence and expression of an operon in *Escherichia coli* coding for formate hydrogenlyase components. *Mol Microbiol* **4:** 231–243.
Canada, K.A., Iwashita, S., Shim, H., and Wood, T.K. (2002) Directed evolution of toluene *ortho*-monooxygenase for enhanced 1-naphthol synthesis and chlorinated ethene degradation. *J Bacteriol* **184:** 344–349.
Chen, X., Sun, Y., Xiu, Z., Li, X., and Zhang, D. (2006) Stoichiometric analysis of biological hydrogen production by fermentative bacteria. *Int J Hydrogen Energy* **31:** 539–549.
Chin, H.-L., Chen, Z.-S., and Chou, C.P. (2003) Fedbatch operation using *Clostridium acetobutylicum* suspension culture as biocatalyst for enhancing hydrogen production. *Biotechnol Prog* **19:** 383–388.
Chippaux, M., Pascal, M.-C., and Casse, F. (1977) Formate hydrogenlyase system in *Salmonella typhimurium* LT2. *Eur J Biochem* **72:** 149–155.
Chittibabu, G., Nath, K., and Das, D. (2006) Feasibility studies on the fermentative hydrogen production by recombinant *Escherichia coli* BL-21. *Process Biochem* **41:** 682–688.
Clark, D.P. (1989) The fermentation pathways of *Escherichia coli*. *FEMS Microbiol Rev* **63:** 223–234.
Das, D., and Veziroglu, T.N. (2001) Hydrogen production by biological processes: a survey of literature. *Int J Hydrogen Energy* **26:** 13–28.
Dutta, D., De, D., Chaudhuri, S., and Bhattacharya, S.K. (2005) Hydrogen production by Cyanobacteria. *Microb Cell Fact* **4:** 36.
Edwards, J.S., Covert, M., and Palsson, B. (2002) Metabolic modelling of microbes: the flux-balance approach. *Environ Microbiol* **4:** 133–140.
Forzi, L., and Sawers, R.G. (2007) Maturation of [NiFe]-hydrogenases in *Escherichia coli*. *Biometals* **20:** 565–578.
Frey, M. (2002) Hydrogenases: hydrogen-activating enzymes. *Chembiochem* **3:** 153–160.
Fritsche, E., Paschos, A., Beisel, H.-G., Böck, A., and Huber, R. (1999) Crystal structure of the hydrogenase maturating endopeptidase HybD from *Escherichia coli*. *J Mol Biol* **288:** 989–998.
Garcin, E., Vernede, X., Hatchikian, E.C., Volbeda, A., Frey, M., and Fontecilla-Camps, J.C. (1999) The crystal structure of a reduced [NiFeSe] hydrogenase provides an image of the activated catalytic center. *Structure* **7:** 557–566.
Gasper, R., Scrima, A., and Wittinghofer, A. (2006) Structural insights into HypB, a GTP-binding protein that regulates metal binding. *J Biol Chem* **281:** 27492–27502.
Guiral, M., Tron, P., Belle, V., Aubert, C., Léger, C., Guigliarelli, B., and Giudici-Orticoni, M.-T. (2006) Hyperthermostable and oxygen resistant hydrogenases from a hyperthermophilic bacterium *Aquifex aeolicus*: physicochemical properties. *Int J Hydrogen Energy* **31:** 1424–1431.
Hallenbeck, P.C. (2005) Fundamentals of the fermentative production of hydrogen. *Water Sci Technol* **52:** 21–29.
Hallenbeck, P.C., and Benemann, J.R. (2002) Biological hydrogen production; fundamentals and limiting processes. *Int J Hydrogen Energy* **27:** 1185–1193.
Handelsman, J. (2004) Metagenomics: application of genomics to uncultured microorganisms. *Microbiol Mol Biol Rev* **68:** 669–685.
Hawkes, F.R., Dinsdale, R., Hawkes, D.L., and Hussy, I. (2002) Sustainable fermentative hydrogen production: challenges for process optimisation. *Int J Hydrogen Energy* **27:** 1339–1347.
Hawkes, F.R., Hussy, I., Kyazze, G., Dinsdale, R., and Hawkes, D.L. (2007) Continuous dark fermentative hydrogen production by mesophilic microflora: principles and progress. *Int J Hydrogen Energy* **32:** 172–184.
Hayashi, K., Morooka, N., Yamamoto, Y., Fujita, K., Isono, K., Choi, S., *et al*. (2006) Highly accurate genome sequences of *Escherichia coli* K-12 strains MG1655 and W3110. *Mol Syst Biol* 2006.0007.
Higuchi, Y., Yagi, T., and Yasuoka, N. (1997) Unusual ligand structure in Ni-Fe active center and an additional Mg site in hydrogenase revealed by high resolution X-ray structure analysis. *Structure* **5:** 1671–1680.
Higuchi, Y., Ogata, H., Miki, K., Yasuoka, N., and Yagi, T. (1999) Removal of the bridging ligand atom at the Ni-Fe active site of [NiFe] hydrogenase upon reduction with H_2, as revealed by X-ray structure analysis at 1.4 Å resolution. *Structure* **7:** 549–556.
Hopper, S., Babst, M., Schlensog, V., Fischer, H.-M., Hennecke, H., and Böck, A. (1994) Regulated expression *in vitro* of genes coding for formate hydrogenlyase components of *Escherichia coli*. *J Biol Chem* **269:** 19597–19604.
Hube, M., Blokesch, M., and Böck, A. (2002) Network of hydrogenase maturation in *Escherichia coli*: role of accessory proteins HypA and HybF. *J Bacteriol* **184:** 3879–3885.
Jormakka, M., Törnroth, S., Byrne, B., and Iwata, S. (2002) Molecular basis of proton motive force generation: structure of formate dehydrogenase-N. *Science* **295:** 1863–1868.
Jung, G.Y., Jung, H.O., Kim, J.R., Ahn, Y., and Park, S. (1999) Isolation and characterization of *Rhodopseudomonas palustris* P4 which utilizes CO with the production of H_2. *Biotechnol Lett* **21:** 525–529.
Kapdan, I.K., and Kargi, F. (2006) Bio-hydrogen production from waste materials. *Enzyme Microb Tech* **38:** 569–582.

Karube, I., Urano, N., Yamada, T., Hirochika, H., and Sakaguchi, K. (1983) Cloning and expression of the hydrogenase gene from *Clostridium butyricum* in *Escherichia coli*. *FEBS Lett* **158:** 119–122.

Kim, M.-S., Baek, J.-S., Yun, Y.-S., Sim, S.J., Park, S., and Kim, S.-C. (2006) Hydrogen production from *Chlamydomonas reinhardtii* biomass using a two-step conversion process: anaerobic conversion and photosynthetic fermentation. *Int J Hydrogen Energy* **31:** 812–816.

King, P.W., Posewitz, M.C., Ghirardi, M.L., and Seibert, M. (2006) Functional studies of [FeFe] hydrogenase maturation in an *Escherichia coli* biosynthetic system. *J Bacteriol* **188:** 2163–2172.

Korbas, M., Vogt, S., Meyer-Klaucke, W., Bill, E., Lyon, E.J., Thauer, R.K., and Shima, S. (2006) The iron-sulfur cluster-free hydrogenase (Hmd) is a metalloenzyme with a novel iron binding motif. *J Biol Chem* **281:** 30804–30813.

Kraemer, J.T., and Bagley, D.M. (2007) Improving the yield from fermentative hydrogen production. *Biotechnol Lett* **29:** 685–695.

Kumar, N., and Das, D. (2000) Enhancement of hydrogen production by *Enterobacter cloacae* IIT-BT 08. *Process Biochem* **35:** 589–593.

Kumar, N., Ghosh, A., and Das, D. (2001) Redirection of biochemical pathways for the enhancement of H_2 production by *Enterobacter cloacae*. *Biotechnol Lett* **23:** 537–541.

Lay, J.-J. (2000) Modeling and optimization of anaerobic digested sludge converting starch to hydrogen. *Biotechnol Bioeng* **68:** 270–278.

Leach, M.R., and Zamble, D.B. (2007) Metallocenter assembly of the hydrogenase enzymes. *Curr Opin Chem Biol* **11:** 159–165.

Leach, M.R., Sandal, S., Sun, H., and Zamble, D.B. (2005) Metal binding activity of the *Escherichia coli* hydrogenase maturation factor HypB. *Biochemistry* **44:** 12229–12238.

Levin, D.B., Pitt, L., and Love, M. (2004) Biohydrogen production: prospects and limitations to practical application. *Int J Hydrogen Energy* **29:** 173–185.

Levin, D.B., Islam, R., Cicek, N., and Sparling, R. (2006) Hydrogen production by *Clostridium thermocellum* 27405 from cellulosic biomass substrates. *Int J Hydrogen Energy* **31:** 1496–1503.

Liu, X., Zhu, Y., and Yang, S.-T. (2006) Construction and characterization of *ack* deleted mutant of *Clostridium tyrobutyricum* for enhanced butyric acid and hydrogen production. *Biotechnol Prog* **22:** 1265–1275.

McGlynn, S.E., Ruebush, S.S., Naumov, A., Nagy, L.E., Dubini, A., King, P.W., *et al*. (2007) *In vitro* activation of [FeFe] hydrogenase: new insights into hydrogenase maturation. *J Biol Inorg Chem* **12:** 443–447.

Maeda, T., Sanchez-Torres, V., and Wood, T.K. (2007a) *Escherichia coli* hydrogenase 3 is a reversible enzyme possessing hydrogen uptake and synthesis activities. *Appl Microbiol Biotechnol* **76:** 1035–1042.

Maeda, T., Sanchez-Torres, V., and Wood, T.K. (2007b) Metabolic engineering to enhance bacterial hydrogen production. *Microb Biotechnol* [WWW document]. URL http://www.blackwell-synergy.com/toc/mbt/0/0.

Maeda, T., Sanchez-Torres, V., and Wood, T.K. (2007c) Enhanced hydrogen production from glucose by a metabolically-engineered *Escherichia coli*. *Appl Environ Microbiol* (in press).

Maeda, T., Vardar, G., Self, W.T., and Wood, T.K. (2007d) Inhibition of hydrogen uptake in *Escherichia coli* by expressing the hydrogenase from the cyanobacterium *Synechocystis* sp. PCC 6803. *BMC Biotechnol* **7:** 25.

Matias, P.M., Saraiva, L.M., Soares, C.M., Coelho, A.V., LeGall, J., and Carrondo, M.A. (1999) Nine-haem cytochrome *c* from *Desulfovibrio desulfuricans* ATCC 27774: primary sequence determination, crystallographic refinement at 1.8 and modelling studies of its interaction with the tetrahaem cytochrome c_3. *J Biol Inorg Chem* **4:** 478–494.

Matsunaga, T., Hatano, T., Yamada, A., and Matsumoto, M. (2000) Microaerobic hydrogen production by photosynthetic bacteria in a double-phase photobioreactor. *Biotechnol Bioeng* **68:** 647–651.

Menon, N.K., Robbins, J., Wendt, J.C., Shanmugam, K.T., and Przybyla, A.E. (1991) Mutational analysis and characterization of the *Escherichia coli hya* operon, which encodes [NiFe] hydrogenase 1. *J Bacteriol* **173:** 4851–4861.

Menon, N.K., Chatelus, C.Y., Dervartanian, M., Wendt, J.C., Shanmugam, K.T., Peck, H.D., Jr, and Przybyla, A.E. (1994) Cloning, sequencing, and mutational analysis of the *hyb* operon encoding *Escherichia coli* hydrogenase 2. *J Bacteriol* **176:** 4416–4423.

Minnan, L., Jinli, H., Xiaobin, W., Huijuan, X., Jinzao, C., Chuannan, L., *et al*. (2005) Isolation and characterization of a high H_2-producing strain *Klebsiella oxytoca* HP1 from a hot spring. *Res Microbiol* **156:** 76–81.

Mishra, J., Khurana, S., Kumar, N., Ghosh, A.K., and Das, D. (2004) Molecular cloning, characterization, and overexpression of a novel [Fe]-hydrogenase isolated from a high rate of hydrogen producing *Enterobacter cloacae* IIT-BT 08. *Biochem Biophys Res Commun* **324:** 679–685.

Miyake, J., Miyake, M., and Asada, Y. (1999) Biotechnological hydrogen production: research for efficient light conversion. *J Biotechnol* **70:** 89–101.

Montet, Y., Amara, P., Volbeda, A., Vernede, X., Hatchikian, E.C., Field, M.J., *et al*. (1997) Gas access to the active site of Ni-Fe hydrogenases probed by X-ray crystallography and molecular dynamics. *Nat Struct Biol* **4:** 523–526.

Morimoto, K., Kimura, T., Sakka, K., and Ohmiya, K. (2005) Overexpression of a hydrogenase gene in *Clostridium paraputrificum* to enhance hydrogen gas production. *FEMS Microbiol Lett* **246:** 229–234.

Nagy, L.E., Meuser, J.E., Plummer, S., Seibert, M., Ghirardi, M.L., King, P.W., *et al*. (2007) Application of gene-shuffling for the rapid generation of novel [FeFe]-hydrogenase libraries. *Biotechnol Lett* **29:** 421–430.

Nandi, R., and Sengupta, S. (1998) Microbial production of hydrogen: an overview. *Crit Rev Microbiol* **24:** 61–84.

Nandi, R., Dey, S., and Sengupta, S. (2001) Thiosulphate improves yield of hydrogen production from glucose by the immobilized formate hydrogenlyase system of *Escherichia coli*. *Biotechnol Bioeng* **75:** 492–494.

Nath, K., and Das, D. (2004) Improvement of fermentative hydrogen production: various approaches. *Appl Microbiol Biotechnol* **65:** 520–529.

Nath, K., Kumar, A., and Das, D. (2005) Hydrogen production by *Rhodobacter sphaeroides* strain O.U.001 using spent media of *Enterobacter cloacae* strain DM11. *Appl Microbiol Biotechnol* **68:** 533–541.

Nicolet, Y., Piras, C., Legrand, P., Hatchikian, C.E., and Fontecilla-Camps, J.C. (1999) *Desulfovibrio desulfuricans* iron hydrogenase: the structure shows unusual coordination to an active site Fe binuclear center. *Structure* **7:** 13–23.

Nicolet, Y., Lemon, B.J., Fontecilla-Camps, J.C., and Peters, J.W. (2000) A novel FeS cluster in Fe-only hydrogenases. *Trends Biochem Sci* **25:** 138–143.

van Niel, E.W.J., Budde, M.A.W., de Haas, G.G., van der Wal, F.J., Claassen, P.A.M., and Stams, A.J.M. (2002) Distinctive properties of high hydrogen producing extreme thermophiles, *Caldicellulosiruptor saccharolyticus* and *Thermotoga elfii*. *Int J Hydrogen Energy* **27:** 1391–1398.

Oh, Y.-K., Seol, E.-H., Kim, J.R., and Park, S. (2003) Fermentative biohydrogen production by a new chemoheterotrophic bacterium *Citrobacter* sp. Y19. *Int J Hydrogen Energy* **28:** 1353–1359.

Peck, H.D., Jr, and Gest, H. (1957) Formic dehydrogenase and the hydrogenlyase enzyme complex in coli-aerogenes bacteria. *J Bacteriol* **73:** 706–721.

Penfold, D.W., and Macaskie, L.E. (2004) Production of H_2 from sucrose by *Escherichia coli* strains carrying the pUR400 plasmid, which encodes invertase activity. *Biotechnol Lett* **26:** 1879–1883.

Penfold, D.W., Forster, C.F., and Macaskie, L.E. (2003) Increased hydrogen production by *Escherichia coli* strain HD701 in comparison with the wild-type parent strain MC4100. *Enzyme Microb Tech* **33:** 185–189.

Penfold, D.W., Sargent, F., and Macaskie, L.E. (2006) Inactivation of the *Escherichia coli* K-12 twin-arginine translocation system promotes increased hydrogen production. *FEMS Microbiol Lett* **262:** 135–137.

Peters, J.W., Lanzilotta, W.N., Lemon, B.J., and Seefeldt, L.C. (1998) X-ray crystal structure of the Fe-only hydrogenase (Cpl) from *Clostridium pasteurianum* to 1.8 Angstrom resolution. *Science* **282:** 1853–1858.

Pilak, O., Mamat, B., Vogt, S., Hagemeier, C.H., Thauer, R.K., Shima, S., *et al*. (2006) The crystal structure of the apoenzyme of the iron-sulfur cluster-free hydrogenase. *J Mol Biol* **358:** 798–809.

Price, N.D., Reed, J.L., and Palsson, B.O. (2004) Genome-scale models of microbial cells: evaluating the consequences of constraints. *Nat Rev Microbiol* **2:** 886–897.

Raaijmakers, H.C.A., and Romão, M.J. (2006) Formate-reduced *E. coli* formate dehydrogenase H: the reinterpretation of the crystal structure suggests a new reaction mechanism. *J Biol Inorg Chem* **11:** 849–854.

Rachman, M.A., Furutani, Y., Nakashimada, Y., Kakizono, T., and Nishio, N. (1997) Enhanced hydrogen producton in altered mixed acid fermentation of glucose by *Enterobacter aerogenes*. *J Ferm Bioeng* **83:** 358–363.

Rachman, M.A., Nakashimada, Y., Kakizono, T., and Nishio, N. (1998) Hydrogen production with high yield and high evolution rate by self-flocculated cells of *Enterobacter aerogenes* in a packed-bed reactor. *Appl Microbiol Biotechnol* **49:** 450–454.

Reed, J.L., and Palsson, B.O. (2003) Thirteen years of building constraint-based *in silico* models of *Escherichia coli*. *J Bacteriol* **185:** 2692–2699.

Reed, J.L., and Palsson, B.O. (2004) Genome-scale *in silico* models of *E. coli* have multiple equivalent phenotypic states: assessment of correlated reaction subsets that comprise network states. *Genome Res* **14:** 1797–1805.

Reissmann, S., Hochleitner, E., Wang, H., Paschos, A., Lottspeich, F., Glass, R.S., and Böck, A. (2003) Taming of a poison: biosynthesis of the NiFe-hydrogenase cyanide ligands. *Science* **299:** 1067–1070.

Rey, F.E., Heiniger, E.K., and Harwood, C.S. (2007) Redirection of metabolism for biological hydrogen production. *Appl Environ Microbiol* **73:** 1665–1671.

Riesenfeld, C.S., Schloss, P.D., and Handelsman, J. (2004) Metagenomics: genomic analysis of microbial communities. *Annu Rev Genet* **38:** 525–552.

Riley, M., Abe, T., Arnaud, M.B., Berlyn, M.K., Blattner, F.R., Chaudhuri, R.R., *et al*. (2006) *Escherichia coli* K-12: a cooperatively developed annotation snapshot-2005. *Nucleic Acids Res* **34:** 1–9.

Rosano, C., Zuccotti, S., Bucciantini, M., Stefani, M., Ramponi, G., and Bolognesi, M. (2002) Crystal structure and anion binding in the prokaryotic hydrogenase maturation factor HypF acylphosphatase-like domain. *J Mol Biol* **321:** 785–796.

Rousset, M., Montet, Y., Guigliarelli, B., Forget, N., Asso, M., Bertrand, P., *et al*. (1998) [3Fe-4S] to [4Fe-4S] cluster conversion in *Desulfovibrio fructosovorans* [NiFe] hydrogenase by site-directed mutagenesis. *Proc Natl Acad Sci USA* **95:** 11625–11630.

Sauter, M., Böhm, R., and Böck, A. (1992) Mutational analysis of the operon (*hyc*) determining hydrogenase 3 formation in *Escherichia coli*. *Mol Microbiol* **6:** 1523–1532.

Sawers, G. (1994) The hydrogenases and formate dehydrogenases of *Escherichia coli*. *Antonie Van Leeuwenhoek* **66:** 57–88.

Sawers, R.G. (2005) Formate and its role in hydrogen production in *Escherichia coli*. *Biochem Soc Trans* **33:** 42–46.

Schilling, C.H., Edwards, J.S., Letscher, D., and Palsson, B.O. (2000) Combining pathway analysis with flux balance analysis for the comprehensive study of metabolic systems. *Biotechnol Bioeng* **71:** 286–306.

Schilling, C.H., Covert, M.W., Famili, I., Church, G.M., Edwards, J.S., and Palsson, B.O. (2002) Genome-scale metabolic model of *Helicobacter pylori* 26695. *J Bacteriol* **184:** 4582–4593.

Schlensog, V., and Böck, A. (1990) Identification and sequence analysis of the gene encoding the transcriptional activator of the formate hydrogenlyase system of *Escherichia coli*. *Mol Microbiol* **4:** 1319–1327.

Schön, G., and Voelskow, H. (1976) Pyruvate fermentation in *Rhodospirillum rubrum* and after transfer from aerobic to anaerobic conditions in the dark. *Arch Microbiol* **107:** 87–92.

Seibert, M., Flynn, T., Benson, D., Tracy, E., and Gharardi, M. (1998) Development of selection and screening procedures for rapid identification of H_2-producing algal mutants with increased O_2 tolerance. In *Biohydrogen*. Zaborsky, O.R. (ed.). Plenum Publishing corporation, New York, N.Y. pp. 227–234.

Self, W.T., and Shanmugam, K.T. (2000) Isolation and characterization of mutated FhlA proteins which activate transcription of the *hyc* operon (formate hydrogenlyase) of *Escherichia coli* in the absence of molybdate. *FEMS Microbiol Lett* **184:** 47–52.

Self, W.T., Hasona, A., and Shanmugam, K.T. (2001) N-terminal truncations in the FhlA protein result in formate- and MoeA-independent expression of the *hyc* (formate hydrogenlyase) operon of *Escherichia coli*. *Microbiology* **147:** 3093–3104.

Self, W.T., Hasona, A., and Shanmugam, K.T. (2004) Expression and regulation of a silent operon, *hyf*, coding for hydrogenase 4 isoenzyme in *Escherichia coli*. *J Bacteriol* **186:** 580–587.

Shin, J.-H., Yoon, J.H., Ahn, E.K., Kim, M.-S., Sim, S.J., and Park, T.H. (2007) Fermentative hydrogen production by the newly isolated *Enterobacter asburiae* SNU-1. *Int J Hydrogen Energy* **32:** 192–199.

Skibinski, D.A.G., Golby, P., Chang, Y.-S., Sargent, F., Hoffman, R., Harper, R., *et al*. (2002) Regulation of the hydrogenase-4 operon of *Escherichia coli* by the σ^{54}-dependent transcriptional activators FhlA and HyfR. *J Bacteriol* **184:** 6642–6653.

Stephanopoulos, G.N., Aristidou, A.A., and Nielsen, J. (1998) *Metabolic Engineering: Principles and Methodologies*. San Diego, USA: Academic Press.

Stephenson, M., and Stickland, L.H. (1931) Hydrogenase: a bacterial enzyme activating molecular hydrogen. *Biochem J* **25:** 205–214.

Steuber, J., Krebs, W., Bott, M., and Dimroth, P. (1999) A membrane-bound NAD(P)$^+$-reducing hydrogenase provides reduced pyridine nucleotides during citrate fermentation by *Klebsiella pneumoniae*. *J Bacteriol* **181:** 241–245.

Tamagnini, P., Axelsson, R., Lindberg, P., Oxelfelt, F., Wünschiers, R., and Lindblad, P. (2002) Hydrogenases and hydrogen metabolism of cyanobacteria. *Microbiol Mol Biol Rev* **66:** 1–20.

Thauer, R.K., Klein, A.R., and Hartmann, G.C. (1996) Reactions with molecular hydrogen in microorganisms: evidence for a purely organic hydrogenation catalyst. *Chem Rev* **96:** 3031–3042.

Ust'ak, S., Havrland, B., Muñoz, J.O.J., Fernández, E.C., and Lachman, J. (2007) Experimental verification of various methods for biological hydrogen production. *Int J Hydrogen Energy* **32:** 1736–1741.

Valentine, D.L., Blanton, D.C., and Reeburgh, W.S. (2000) Hydrogen production by methanogens under low-hydrogen conditions. *Arch Microbiol* **174:** 415–421.

Varma, A., and Palsson, B.O. (1994) Metabolic flux balancing – basic concepts scientific and practical use. *Bio-Technol* **12:** 994–998.

Vatsala, T.M. (1992) Hydrogen production from (cane-molasses) stillage by *Citrobacter freundii* and its use in improving methanogenesis. *Int J Hydrogen Energy* **17:** 923–927.

Vignais, P.M., and Colbeau, A. (2004) Molecular biology of microbial hydrogenases. *Curr Issues Mol Biol* **6:** 159–188.

Vignais, P.M., Dimon, B., Zorin, N.A., Colbeau, A., and Elsen, S. (1997) HupUV proteins of *Rhodobacter capsulatus* can bind H_2: evidence from the H–D exchange reaction. *J Bacteriol* **179:** 290–292.

Vignais, P.M., Billoud, B., and Meyer, J. (2001) Classification and phylogeny of hydrogenases. *FEMS Microbiol Rev* **25:** 455–501.

Vignais, P.M., Magnin, J.-P., and Willison, J.C. (2006) Increasing biohydrogen production by metabolic engineering. *Int J Hydrogen Energy* **31:** 1478–1483.

Voelskow, H., and Schön, G. (1980) H_2 production of *Rhodospirillum rubrum* during adaptation to anaerobic dark conditions. *Arch Microbiol* **125:** 245–249.

Volbeda, A., and Fontecilla-Camps, J.C. (2003) The active site and catalytic mechanism of NiFe hydrogenases. *Dalton Trans* **21:** 4030–4038.

Volbeda, A., Charon, M.-H., Piras, C., Hatchikian, E.C., Frey, M., and Fontecilla-Camps, J.C. (1995) Crystal structure of the nickel-iron hydrogenase from *Desulfovibrio gigas*. *Nature* **373:** 580–587.

Volbeda, A., Garcin, E., Piras, C., de Lacey, A.L., Fernandez, V.M., Hatchikian, E.C., *et al*. (1996) Structure of the [NiFe] hydrogenase active site: evidence for biologically uncommon Fe ligands. *J Am Chem Soc* **118:** 12989–12996.

Watanabe, S., Matsumi, R., Arai, T., Atomi, H., Imanaka, T., and Miki, K. (2007) Crystal structures of [NiFe] hydrogenase maturation proteins HypC, HypD, and HypE: insights into cyanation reaction by thiol redox signaling. *Mol Cell* **27:** 29–40.

Willenbrock, H., Petersen, A., Sekse, C., Kiil, K., Wasteson, Y., and Ussery, D.W. (2006) Design of a seven-genome *Escherichia coli* microarray for comparative genomic profiling. *J Bacteriol* **188:** 7713–7721.

Woodward, J., Orr, M., Cordray, K., and Greenbaum, E. (2000) Enzymatic production of biohydrogen. *Nature* **405:** 1014–1015.

Yang, F., Hu, W., Xu, H., Li, C., Xia, B., and Jin, C. (2007) Solution structure and backbone dynamics of an endopeptidase HycI from *Escherichia coli*: implications for mechanism of the [NiFe] hydrogenase maturation. *J Biol Chem* **282:** 3856–3863.

Yi, K.B., and Harrison, D.P. (2005) Low-pressure sorption-enhanced hydrogen production. *Ind Eng Chem Res* **44:** 1665–1669.

Yoshida, A., Nishimura, T., Kawaguchi, H., Inui, M., and Yukawa, H. (2005) Enhanced hydrogen production from formic acid by formate hydrogen lyase-overexpressing *Escherichia coli* strains. *Appl Environ Microbiol* **71:** 6762–6768.

Yoshida, A., Nishimura, T., Kawaguchi, H., Inui, M., and Yukawa, H. (2006) Enhanced hydrogen production from glucose using *ldh*- and *frd*-inactivated *Escherichia coli* strains. *Appl Microbiol Biotechnol* **73:** 67–72.

Yoshida, A., Nishimura, T., Kawaguchi, H., Inui, M., and Yukawa, H. (2007) Efficient induction of formate hydrogen lyase of aerobically grown *Escherichia coli* in a three-step biohydrogen production process. *Appl Microbiol Biotechnol* **74:** 754–760.

Zhang, J.W., Butland, G., Greenblatt, J.F., Emili, A., and Zamble, D.B. (2005) A role for SlyD in the *Escherichia coli* hydrogenase biosynthetic pathway. *J Biol Chem* **280:** 4360–4366.

Zilberman, S., Stiefel, E.I., Cohen, M.H., and Car, R. (2007) Theoretical studies of [FeFe]-hydrogenase: infrared

fingerprints of the dithiol-bridging ligand in the active site. *Inorg Chem* **46:** 1153–1161.

Zirngibl, C., Hedderich, R., and Thauer, R.K. (1990) N^5,N^{10}-Methylenetetrahydromethanopterin dehydrogenase from *Methanobacterium thermoautotrophicum* has hydrogenase activity. *FEBS Lett* **261:** 112–116.

Zirngibl, C., Van Dongen, W., Schwörer, B., Von Bünau, R., Richter, M., Klein, A., and Thauer, R.K. (1992) H_2-forming methylenetetrahydromethanopterin dehydrogenase, a novel type of hydrogenase without iron-sulfur clusters in methanogenic archaea. *Eur J Biochem* **208:** 511–520.

