microbial biotechnology

Microbial Biotechnology (2012) **5**(2), 214–225 doi:10.1111/j.1751-7915.2011.00282.x

Minireview

Hydrogen production by recombinant *Escherichia coli* strains

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Summary

The production of hydrogen via microbial biotechnology is an active field of research. Given its ease of manipulation, the best-studied bacterium *Escherichia coli* has become a workhorse for enhanced hydrogen production through metabolic engineering, heterologous gene expression, adaptive evolution, and protein engineering. Herein, the utility of *E. coli* strains to produce hydrogen, via native hydrogenases or heterologous ones, is reviewed. In addition, potential strategies for increasing hydrogen production are outlined and whole-cell systems and cell-free systems are compared.

Introduction

Research on sustainable biogas production is increasing due to strong public interest in protecting the global climate and a growing demand for independency from scarce fossil energy sources (Kessel, 2000; Armaroli and Balzani, 2011). Hydrogen has tremendous potential because it is the most abundant element in the universe (Dunn, 2002), is renewable, efficient and clean (Hansel and Lindblad, 1998), and is utilized for fuel cells in portable electronics, power plants and internal combustion engines (Dunn, 2002). Among the existing renewable energy sources such as ethanol and algal diesel, hydrogen continues to be very attractive because of its various means of production, non-polluting nature and large energy content per mass (142 MJ kg^{-1} for H_2). In addition, it is estimated that the global energy system will shift from fossil fuels to hydrogen (Dunn, 2002) as well as methane (Alves *et al.*, 2009).

Currently, hydrogen is produced mainly by the water-gas shift reaction (Yi and Harrison, 2005), as a by-product of petroleum refining and chemical production (Das and Veziroglu, 2001), and by electrolysis of water (Armaroli and Balzani, 2011). However, methods such as electrolysis of water and thermocatalytic reformation of hydrogen-rich compounds (which are non-renewable resources) require higher energy input to generate hydrogen gas compared with biological means (Das and Veziroglu, 2001). The commercial production of hydrogen by electrolysis of water achieves an efficiency of 75%; however, this cost is currently several times higher than that produced from fossil fuels (Ewan and Allen, 2005). Also, hydrogen production dependent on fossil resources is clearly unsustainable, and all the operations associated with sequestration are energy intensive, costly, and potentially damaging to our environment (Edwards *et al.*, 2007).

In contrast, biological methods to produce hydrogen are environmentally more attractive because they utilize microorganisms to produce hydrogen from diverse renewable resources (Edwards *et al.*, 2007). Although there are several methods for biohydrogen production, biohydrogen may be produced through either photosynthetic or fermentative processes; in general, fermentative hydrogen production is more efficient than photosynthetic means (Yoshida *et al.*, 2005). Thus, to date, fermentative hydrogen production has been shown to be the most promising approach because it has the advantages of (i) higher hydrogen-production rates, (ii) independence from the availability of light, and (iii) a variety of carbon sources such as organic compounds, low-cost wastes, and cellulosic or cellobiose substrates (Vardar-Schara *et al.*, 2008).

Biological synthesis and uptake of hydrogen are catalysed by hydrogenases (Hyd), which catalyse the reaction $2H^+ + 2e^- \leftrightarrow H_2$ (g); the hydrogenases have been classified as [NiFe], [FeFe] or [Fe] enzymes based on the type of iron sequestered by the active site of the large subunit of Hyd. [FeFe] and [NiFe] hydrogenases have similar

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overall structures as the active site is located within a large subunit and electrons are delivered to this centre via iron–sulfur (Fe-S) centres located in the small subunits (Forzi and Sawers, 2007). [Fe] hydrogenases lack Fe-S clusters and are found only in a small group of methanogenic *archea* (Zirngibl *et al*., 1992). In general, these Hyd systems are complex requiring many maturation proteins (Vardar-Schara *et al*., 2008); for example, [NiFe] Hyd 3 of *E. coli* requires 10 maturation proteins (Forzi and Sawers, 2007), including HypABCDEF (metallochaperones for NiFe insertion) and SlyD (nickel insertion) (Drapal and Böck, 1998).

During the last 4 years, the number of research publications utilizing engineering strategies to increase hydrogen synthesis has been increasing. In this review, we summarize the research trends regarding enhanced bacterial hydrogen production by recombinant *Escherichia coli* strains redesigned through metabolic engineering, heterologous gene expression, adaptive evolution and protein engineering.

Utility of using *Escherichia coli* strains

Escherichia coli is a robust bacterium for developmental research based on genetic engineering because its whole genome sequence is available (Blattner *et al*., 1997) and its metabolic pathways are relatively well-established. Also, in general, cultivation, storage and genetic manipulation (e.g. plasmid purification and transformation) for *E. coli* are quite technically simpler than with other strains. Specifically, the KEIO Collection, an *E. coli* single deletion mutant library (Baba *et al*., 2006), is a powerful tool since it can be used to evaluate rapidly the importance of each nonlethal gene. Furthermore, since the kanamycin resistant (Km^R) gene in the knockout mutation can be eliminated by recombination between the flippase recognition target sites via the flippase recombinase protein produced from pCP20 (Cherepanov and Wackernagel, 1995), multiple deletion mutants may be rapidly constructed with little scarring by combining mutations of the Keio Collection via P1 transduction and selecting each mutation based on kanamycin resistance (Maeda *et al*., 2007a; 2008a). The only limitation to using P1 transduction to accumulate multiple deletions is that the mutations must not be too close. Similarly, multiple deletion strains may be made by sequential one-step inactivations (Datsenko and Wanner, 2000) followed by kanamycin gene-eliminating steps. In contrast, traditional methods require different selection makers (antibiotic resistant genes) to introduce multiple mutations into a single strain (Lee *et al*., 2005; Yoshida *et al*., 2006). This novel P1 transduction technique has facilitated metabolic engineering by allowing facile removal of many deleterious genes (Maeda *et al*., 2007a; 2008a,b). Furthermore, the ASKA Library is available, which allows each *E. coli* protein to be produced from a plasmid either with or without a fused GFP tag (Kitagawa *et al*., 2006).

Native *E. coli* hydrogenases

Escherichia coli has four native hydrogenases (Hyd 1, 2, 3 and 4). Hyd 1 and 2 have primarily hydrogen uptake activity (Ballantine and Boxer, 1986; King and Przybyla, 1999) [although one recent report indicates that Hyd 1 also has hydrogen synthesis activity under micro-aerobic conditions (Kim *et al*., 2010)], Hyd 3 is reversible with the synthesis rate greater than the uptake rate (Maeda *et al*., 2007a), and Hyd 4 is probably inactive (Self *et al*., 2004) although gene expression has been observed during biofilm formation (Herzberg *et al*., 2006; Domka *et al*., 2007). These four *E. coli* hydrogenases are classified as [NiFe] hydrogenases, and they contain two cyanide molecules and a carbon monoxide molecule at the active site (Blokesch *et al*., 2002); Hyd 1 is encoded by *hyaABCDEF* (Richard *et al*., 1999), Hyd 2 is encoded by *hybOABCDEFG* (Richard *et al*., 1999), Hyd 3 is encoded by *hycABCDEFGHI* (Bagramyan and Trchounian, 2003), and Hyd 4 is encoded by *hyfABCDEFGHI* (Andrews *et al*., 1997). Bioinformatics analysis for the large subunits of *E. coli* hydrogenases (*hyaB* for Hyd 1, *hybC* for Hyd 2, *hycE* for Hyd 3, and *hyfG* for Hyd 4) indicates that there is comparatively high homology between Hyd 1 and 2 and between Hyd 3 and 4 (Vardar-Schara *et al*., 2008). Given the hydrogen degradation and synthesis activity of these native hydrogenases, it is imperative that researchers utilizing *E. coli* for heterologous expression of hydrogenases work with mutants that lack native hydrogenase activity (Maeda *et al*., 2007a); for example, by using a *hyaB hybC hycE hyfG* mutant (T. Maeda and T.K. Wood, unpublished).

In *E. coli*, hydrogen is produced by the formate hydrogenlyase system (FHL) that consists of two enzymatic activities: a formate dehydrogenase-H [encoded by *fdhF* (Axley *et al*., 1990)] for producing $2H^+$, $2e^-$ and CO_2 from formate and Hyd 3 for synthesizing molecular hydrogen from $2H^+$ and $2e^-$ (Sawers *et al*., 1985). The FHL system may be used for the regulation of internal pH into the cells (Böck and Sawers, 1996) although this is far from clear. Regulation of FHL includes the HycA repressor (Sauter *et al*., 1992) and the FhlA activator (Schlensog *et al*., 1994). Two additional formate dehydrogenases are found in *E. coli* that consume formate: FDH_N (formate dehydrogenase-N) and FDH_O (formate dehydrogenase-O) (Rossmann *et al*., 1991). Also, FocA (Suppmann and Sawers, 1994) and FocB (Andrews *et al*., 1997) export formate, and nitrate reductase A (α-subunit encoded by *narG*) consumes formate by converting nitrate into nitrite by using electrons produced from

formate by FDH_N (Bertero *et al*., 2003). In addition, pyruvate dehydrogenase (AceE) and pyruvate oxidase (PoxB) consume pyruvate produced from glucose metabolism via the phosphoenolpyruvate-producing pathway (Angelides *et al*., 1979; Abdel-Hamid *et al*., 2001) as well as pyruvate produced glucose metabolism via the succinate-producing pathway (phosphoenolpyruvate to succinate) and lactate-producing pathway (pyruvate to lactate) (Manish *et al*., 2007). Thus, these well-established pathways motivated engineers to redirect the metabolic flux towards hydrogen production.

Metabolic engineering to enhance hydrogen production

Since hydrogen is produced through the reaction $HCOO^- + H_2O \leftrightarrow H_2 + HCO_3^-$ (Woods, 1936) by the FHL complex in well-known pathways, metabolic engineering from formate metabolism is possible using *E. coli* native hydrogenases. The first improvement to enhance hydrogen production from formate in *E. coli* was by inactivating the HycA repressor of FHL and by overproducing the FhlA activator of FHL; strain SR13 with both changes had sevenfold higher transcription of the FHL complex (e.g. *fdhF* and the *hyc* operon) and 2.8-fold higher hydrogen productivity than the wild-type strain (Yoshida *et al*., 2005) (see Table 1 for a comparison of the strains discussed in this review).

To increase hydrogen further, along with deleting *hycA* and overexpressing *fhlA*, hydrogen uptake activity was eliminated by deleting *hyaB*, the large subunit of Hyd 1 and *hybC*, the large subunit of Hyd 2 (Maeda *et al*., 2008a). In addition, to direct the formate metabolic flux towards hydrogen production, FDH_O which converts formate into CO_2 without producing hydrogen, was inactivated by deleting *fdoG* (Maeda *et al*., 2008a). The metabolically engineered strain with four mutations and one plasmid to produce activator FhlA had 141-fold higher hydrogen productivity than the wild-type strain and reached the theoretical yield (1 mol of hydrogen produced from 1 mol of formate) (Maeda *et al*., 2008a).

Since glucose is far less expensive than formate, hydrogen production from glucose was pursued based on the same principle and re-directing the metabolic flux of formate. As expected, inactivating hydrogen uptake via Hyd 1 and 2 (*hya* and *hyb*) and the FHL repressor (*hycA*) enhanced hydrogen production during glucose fermentation (Penfold *et al*., 2003; Yoshida *et al*., 2006; Maeda *et al*., 2007b; Turcot *et al*., 2008; Fan *et al*., 2009; Kim *et al*., 2009; Mathews *et al*., 2010). Hydrogen production was improved further by expressing an FhlA variant that carries a deletion of the N-terminal part of FhlA (Self *et al*., 2001; Turcot *et al*., 2008), whereas the original FhlA had a little effect for enhanced hydrogen production (Maeda *et al*., 2007b). The transcriptional activity for Hyd 3 (*hyc* operon) was increased twofold by the truncated FhlA, which is a formate-independent transcriptional activator (Self *et al*., 2001).

Escherichia coli metabolizes glucose to phosphoenolpyruvate, phosphoenolpyruvate to pyruvate, then pyruvate to formate via pyruvate formate lyase (Bagramyan and Trchounian, 2003); succinate and lactate are co-metabolites during glucose fermentation that are synthesized from phosphoenolpyruvate and pyruvate (Bagramyan and Trchounian, 2003) (Fig. 1). Therefore, pathways to produce succinate and lactate were silenced by deleting *ppc* to inactivate phosphoenolpyruvate carboxylase (Fan *et al*., 2009), by inactivating *frdABCD* encoding fumarate reductase (Yoshida *et al*., 2006; Maeda *et al*., 2007b; Kim *et al*., 2009; Mathews *et al*., 2010), by deleting *ldhA* encoding lactate dehydrogenase (Yoshida *et al*., 2006; Manish *et al*., 2007; Maeda *et al*., 2007b; Kim *et al*., 2009; Mathews *et al*., 2010), and by inactivating *aceE* encoding the component of pyruvate dehydrogenase (Maeda *et al*., 2007b). Furthermore, metabolic flux analysis revealed that the inactivation of lactate dehydrogenase and fumarate reductase directed most of the carbon from glucose to the glycolytic pathway leading to hydrogen production by the FHL system (Kim *et al*., 2009). Therefore, hydrogen productivity and hydrogen yield were improved by the disruption of these enzyme activities.

Other deleterious genes whose inactivation leads to enhanced hydrogen production include *iscR* and *tatABCDE* (Penfold *et al*., 2006; Akhtar and Jones, 2008a). Fe-S cluster assembly and incorporation into *E. coli* hydrogenases is an essential process, and IscR is a negative regulator for the iron sulfur cluster machinery; hence, inactivating IscR led to threefold increased hydrogen production probably by increasing the amount of active Hyd as well as by decreasing the sensitivity of Hyd to oxygen, which usually inactivates the Hyd function (Akhtar and Jones, 2008a). Hydrogen production has also been improved twofold by inactivating the twin-arginine translocation (TAT) system, which transports folded proteins across the cytoplasmic membrane (Penfold *et al*., 2006). The improvement may be due to inactivating formate dehydrogenase-N, formate dehydrogenase-O, Hyd 1 and Hyd 2, which are translocated by TAT system. In addition, NarL, a two-component regulator protein for the nitrate/nitrite response, and FocA encoding a formate transporter are deleterious for enhancing hydrogen production (Fan *et al*., 2009). In addition, producing Fnr, a DNA-binding transcriptional global regulator, was also effective in enhancing hydrogen production (Fan *et al*., 2009) in agreement with the fact that the deletion of *fnr* gene had a negative effect for hydrogen production (Maeda *et al*., 2007b). So far, the best metabolically modi-

Table 1. Comparison of *in vivo* hydrogen production by engineered *Escherichia coli* strains.

System	H_2 production rate (reported units)	H_2 production rate (converted units)	Substrate	Reference
Protein engineering				
Protein engineering of HycE (truncation) of *E. coli*	9 μmol H_2 (mg protein)$^{-1}$ h^{-1}	9 μmol H_2 (mg protein)$^{-1}$ h^{-1}	Formate	Maeda *et al.* (2008b)
Protein engineering of FhlA of *E. coli*	7 μmol H_2 (mg protein)$^{-1}$ h^{-1}	7 μmol H_2 (mg protein)$^{-1}$ h^{-1}	Formate	Sanchez-Torres *et al.* (2009)
Site-directed mutagenesis of HydA from *C. reinhardtii*	approximately 10 μmol H_2 ml^{-1}	19 μmol H_2 (mg protein)$^{-1}$ h^{-1}	Glucose	Agapakis *et al.* (2010)
Metabolic engineering through modifying multiple native genes in *E. coli*				
Inactivation of HycA and overexpression of FhlA	23.6 g H_2 l^{-1} h^{-1}	254 μmol H_2 (mg protein)$^{-1}$ h^{-1}	Formate	Yoshida *et al.* (2005)
Inactivation of HyaB, HybC, HycA, FdoG and overexpression of FhlA	113 μmol H_2 (mg protein)$^{-1}$ h^{-1}	113 μmol H_2 (mg protein)$^{-1}$ h^{-1}	Formate	Maeda *et al.* (2008a)
Inactivation of HycA, LdhA, FrdBC and overexpression of FhlA	13 mmol (g DCW)$^{-1}$ l^{-1} h^{-1}	26 μmol H_2 (mg protein)$^{-1}$ h^{-1}	Glucose	Yoshida *et al.* (2006)
Inactivation of HyaB, HybC, HycA, FdoG, FrdC, LdhA and AceE	32 μmol H_2 (mg protein)$^{-1}$ h^{-1}	32 μmol H_2 (mg protein)$^{-1}$ h^{-1}	Glucose	Maeda *et al.* (2007b)
Inactivation of Hyd 1, Hyd 2, LdhA and overexpression of truncated FhlA	5.3 mmol H_2 l^{-1} h^{-1}	24 μmol H_2 (mg protein)$^{-1}$ h^{-1} [a]	Glucose	Turcot *et al.* (2008)
Inactivation of HycA, HyaAB, HybBC, LdhA and FrdAB	31.3 mmol H_2 (g DCW)$^{-1}$ h^{-1}	63 μmol H_2 (mg protein)$^{-1}$ h^{-1}	Glucose	Kim *et al.* (2009)
Inactivation of HyaAB, HybABC, HycA, LdhA and FrdBC	1.9 mmol H_2 (g DCW)$^{-1}$ l^{-1} h^{-1}	1.5 μmol H_2 (mg protein)$^{-1}$ h^{-1}	Glucose	Mathews *et al.* (2010)
Production of Hyd 1	3 ml H_2 100 ml^{-1}	0.8 μmol H_2 (mg protein)$^{-1}$ h^{-1} [a]	Glucose + formate	Kim *et al.* (2010)
Inactivation of HycA and LacI	5.88 ml H_2 OD^{-1} h^{-1}	11 μmol H_2 (mg protein)$^{-1}$ h^{-1}	Cheese whey	Rosales-Colunga *et al.* (2010)
Adaptive evolution				
Chemical mutagenesis and adaptive evolution	22 μmol H_2 (mg protein)$^{-1}$	4 μmol H_2 (mg protein)$^{-1}$ h^{-1}	Glycerol	Hu and Wood (2010)
Heterologous gene expression				
Production of [Fe] hydrogenase from *E. cloacae*	0.96 mmol h^{-1}	14.5 μmol H_2 (mg protein)$^{-1}$ h^{-1} [a]	Glucose	Chittibabu *et al.* (2006)
Production of HoxEFUYH hydrogenase from *Synechocystis sp.* PCC 6803	22 ± 3 μmol H_2 (mg protein)$^{-1}$	4 μmol H_2 (mg protein)$^{-1}$ h^{-1}	Glucose	Maeda *et al.* (2007c)
Production of HoxEFUYH hydrogenase and the maturation proteins HypABCDEF and HoxW from *Synechocystis sp.* PCC 6803	8.4 μmol H_2 l^{-1}	0.004 μmol H_2 (mg protein)$^{-1}$ h^{-1}	Glucose	Wells *et al.* (2011)
Production of HydFEGA	429.3 nmol H_2 min^{-1} l^{-1}	0.12 μmol H_2 (mg protein)$^{-1}$ h^{-1} [a]	Glucose	Akhtar and Jones (2008a)
Production of HydFEGA and inactivation of IscR	1257.5 nmol H_2 min^{-1} l^{-1}	0.34 μmol H_2 (mg protein)$^{-1}$ h^{-1} [a]	Glucose	Akhtar and Jones (2008a)
Inactivation of IscR, production of HydFEGA hydrogenase from *C. acetobutylicum*, CpFdX ferredoxin from *C. pasterianum* and YdbK pyruvate–flavodoxin oxidoreductase from *E. coli*	9.6 mmol H_2 (g DCW)$^{-1}$ h^{-1}	19 μmol H_2 (mg protein)$^{-1}$ h^{-1}	Glucose	Akhtar and Jones (2009)
Production of HupSL hydrogenase from *Rhodobacter sphaeroides*	19.68 μL H_2 (ml culture^{-1}) h^{-1}	1.1 μmol H_2 (mg protein)$^{-1}$ h^{-1}	Glucose	Lee *et al.* (2010)
Inactivation of HycA and TatC and expression of the genes encoding ScrKYABR invertase from *Bacillus subtilis*	1.38 ml H_2 (mg DCW)$^{-1}$ l^{-1}	3.9 μmol H_2 (mg protein)$^{-1}$ h^{-1}	Sucrose	Penfold and Macaskie (2004)
Inactivation of IscR, production of HydFEGA hydrogenase from *C. acetobutylicum*, CpFdX ferredoxin from *C. pasterianum* and YdbK pyruvate–flavodoxin oxidoreductase from *E. coli*, and *amyE* from *B. subtilis*	30 μmol H_2 culture^{-1}	0.65 μmol H_2 (mg protein)$^{-1}$ h^{-1} [a]	Starch	Akhtar and Jones (2009)
Single gene knockout or expression				
Inactivation of HycA	NA	109 μmol H_2 (mg protein)$^{-1}$ h^{-1}	Formate	Yoshida *et al.* (2005)
Production of FhlA	7 μmol H_2 (mg protein)$^{-1}$ h^{-1}	7 μmol H_2 (mg protein)$^{-1}$ h^{-1}	Formate	Maeda *et al.* (2008a)
Inactivation of HycA	31 ml H_2 $(OD)^{-1}$ l^{-1}	6.3 μmol H_2 (mg protein)$^{-1}$ h^{-1}	Glucose	Penfold *et al.* (2003)
Inactivation of FocA	14.9 μmol H_2 (mg DCW)$^{-1}$	1.8 μmol H_2 (mg protein)$^{-1}$ h^{-1}	Glucose	Fan *et al.* (2009)
Inactivation of HybC	12.1 μmol H_2 (mg DCW)$^{-1}$	1.4 μmol H_2 (mg protein)$^{-1}$ h^{-1}	Glucose	Fan *et al.* (2009)
Inactivation of NarL	14.4 μmol H_2 (mg DCW)$^{-1}$	1.7 μmol H_2 (mg protein)$^{-1}$ h^{-1}	Glucose	Fan *et al.* (2009)
Inactivation of Ppc	11.2 μmol H_2 (mg DCW)$^{-1}$	1.3 μmol H_2 (mg protein)$^{-1}$ h^{-1}	Glucose	Fan *et al.* (2009)
Production of Fnr	6.2 μmol H_2 (mg DCW)$^{-1}$	3.1 μmol H_2 (mg protein)$^{-1}$ h^{-1}	Glucose	Fan *et al.* (2009)

a. Assuming cell turbidity is 1.

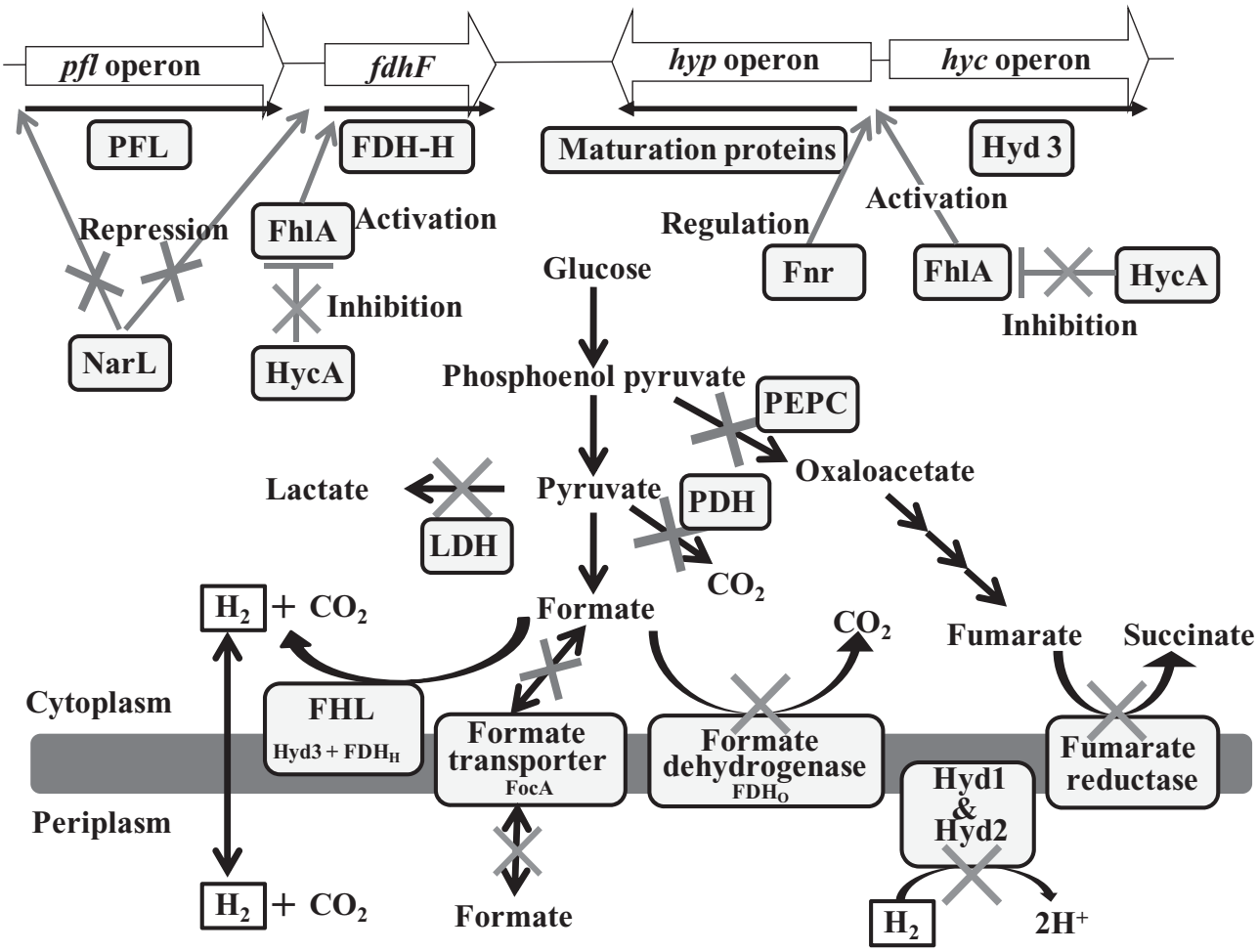

Fig. 1. Metabolic engineering strategies in *Escherichia coli*. Hydrogen is produced from formate through the formate hydrogenlyase system. Abbreviations: Hyd3, hydrogenase 3; FDH_H, formate dehydrogenate-H; FDH_O, formate dehydrogenase-O; Hyd1, hydrogenase 1; Hyd2, hydrogenase 2; LDH, lactate dehydrogenase; PDH, pyruvate dehydrogenase; PEPC, phosphoenolpyruvate carboxylase; and PFL, pyruvate formate lyase. The cross marks in the figure indicate inactivated genes.

fied strains for hydrogen production that incorporate the above concepts have either seven systems inactivated (*hyaB*, *hybC*, *hycA*, *fdoG*, *ldhA*, *frdC* and *aceE*) (Maeda *et al*., 2007b), five systems inactivated (*hyaAB*, *hybABC*, *hycA*, *ldhA* and *frdBC*) (Mathews *et al*., 2010) (*hycA*, *hya*, *hyb*, *ldhA* and *frdAB*) (Kim *et al*., 2009), or three systems inactivated (*hya*, *hyb* and *ldhA*) and a truncated FhlA produced (Turcot *et al*., 2008).

Recently, recombinant *E. coli* BL21 expressing HyaA and HyaB or HyaABCDEF of *E. coli* Hyd 1 actively produced hydrogen, and the hydrogen production was enhanced twofold by the addition of formate (Kim *et al*., 2010), implying that hydrogen synthesis by recombinant Hyd 1 could be similar to that by the FHL complex consisting of Hyd 3 and formate dehydrogenase-H. Also, an exhaustive search of all *E. coli* pathways for their impact on hydrogen production through screening the entire Keio mutant library (3985 isogenic mutants) using both glucose and formate as substrates was conducted by T. Maeda and T.K. Wood (unpublished), and several uncharacterized genes related to bacterial hydrogen production were identified. Hence, further improvements in hydrogen production are possible, and study of the four well-known native hydrogenases continues to provide surprises.

Adapted evolution to enhance hydrogen production from renewable resources

A recent trend for bacterial hydrogen production is to utilize renewable resources; for example, glycerol accumulates as a by-product during biodiesel manufacture (Sabourin-Provost and Hallenbeck, 2009), and the price of crude glycerol could be reasonable and sustainable (Willke and Vorlop, 2004). Hence, converting glycerol to hydrogen is attractive. However, the specific growth rate of wild-type *E. coli* on glycerol is quite low compared with

that with glucose, which results in low hydrogen productivity (Murarka *et al*., 2008). To overcome this limitation, an efficient glycerol-utilizing strain, HW2, was constructed. Starting with a strain harbouring the *frdC* mutation and by combining random chemical mutagenesis and adaptive evolution with selection based on growth on glycerol, a fivefold increase in growth on glycerol was achieved as well as a 20-fold increase in hydrogen synthesis (Hu and Wood, 2010). Ethanol production was also increased fivefold (Hu and Wood, 2010).

Metabolic engineering has also been performed to produce hydrogen from another renewable source, cheese whey, which contains a high concentration of lactose (Rosales-Colunga *et al*., 2010). In this study, hydrogen production from cheese whey was improved 20% by inactivating two proteins: HycA and LacI (LacI is a DNA-binding transcription factor that represses the transcription of the operon involved in the transport and catabolism of lactose).

Heterologous gene expression to enhance hydrogen production *in vitro*

Escherichia coli remains a robust host to clone hydrogen-related enzymes and then to utilize them *in vitro*. For example, an active cytoplasmic NADP-dependent Hyd derived from *Pyrococcus furiosus*, an anaerobic hyperthermophile, was expressed in *E. coli* by cloning the four structural genes encoding the Hyd and nine genes encoding maturation proteins, HypCDABEF, HycI, SlyD and ferredoxin oxidoreductase A (FrxA) under control of the promoter of the *E. coli hya* operon, which has the highest transcriptional activity among the *hya*, *hyb*, *hyc* and *hyp* operons (Sun *et al*., 2010). Additionally, a Hyd derived from *Desulfovibrio vulgaris* was successfully produced *in vitro* by cloning its structural genes (*hydAB*), its maturation proteins, and the *E. coli* iron–sulfur cluster operon (*hydEFG* and *ORF1-ORF2-iscS-iscU-iscA-hscB-hscA-fdx-ORF3* gene cluster) (Laffly *et al*., 2010).

Recombinant proteins NuoE and NuoF, which form the heterodimer of *Trichomonas vaginalis* hydrogenosome NADH dehydrogenase, were successfully expressed in *E. coli* and its reduced ferredoxin activity was characterized *in vitro* (Do *et al*., 2009). Also, a synthetic hydrogen-producing electron transfer circuit composed of heterologously expressed [FeFe]-Hyd, ferredoxin and pyruvate-ferredoxin oxidoreductase was successfully expressed and investigated *in vitro* to insulate an electron transfer pathway to produce hydrogen coupled to the breakdown of glucose (Agapakis *et al*., 2010). Similarly, a synthetic ferredoxin-dependent NAD(P)H:H_2 pathway model system was also constructed in *E. coli* cells and hydrogen accumulation was observed with a maximum partial hydrogen pressure equivalent to a biochemically effective intracellular NADPH/$NADP^+$ ratio of 13:1 (Veit *et al*., 2008). Also, an active recombinant [Fe-Fe]-type Hyd (HydA) of *Clostridium acetobutylicum* in *E. coli* BL21(DE3) was also achieved using three *Clostridium* maturation proteins (HydF, HydE and HydG); this heterologous expression resulted in hydrogen production *in vitro* (Akhtar and Jones, 2008b).

Heterologous gene expression to enhance hydrogen production *in vivo*

One of the earliest *E. coli* strains constructed with a recombinant Hyd was that which used a probable [FeFe] Hyd from *Clostridium butyricum* expressed in *E. coli* HK16 (this host is defective in native Hyd activity); there was roughly a threefold higher Hyd activity compared with both the wild-type *C. butyricum* and to *E. coli* C600 (Karube *et al*., 1983). A [Fe]-Hyd, HydA from *Enterobacter cloacae*, was also heterologously expressed as a glutathione-HydA fusion protein in *E. coli* BL21 (Mishra *et al*., 2004; Chittibabu *et al*., 2006), and the recombinant *E. coli* strain had a yield of 3.1 mol H_2 mol^{-1} glucose, which was higher than the *E. cloacae* wild-type strain. Other improvements in hydrogen production by expressing heterologous genes in *E. coli* cells were found by expressing the genes necessary for sucrose transport and metabolism in *E. coli* strains defective in the HycA FHL repressor and TAT system to promote hydrogen production from sucrose: *scrK* encoding an ATP-dependent fructokinase, *scrY* encoding a sucrose-specific porin of the outer membrane, *scrA* encoding enzyme II_{scr} of the phosphotransferase system for sucrose uptake, *scrB* encoding an intracellular β-D-fructofuranoside fructohydrolase, which catalyses the hydrolysis of sucrose 6-phosphate to β-D-fructose and α-D-glucose 6-phosphate, and *scrR* encoding the negative repressor of the *scr* regulon (Penfold and Macaskie, 2004).

The first cloning of a cyanobacterial [Ni-Fe]-Hyd, HoxEFUYH derived from *Synechocystis* sp. PCC 6803, yielded surprising results (Maeda *et al*., 2007c). The hydrogen yield from the resultant strain was enhanced 41-fold, and the results of a DNA microarray analysis demonstrated that the expression of the four hydrogenases of *E. coli* were not affected by the expression of the cyanobacterial HoxEFUYH. However, the mechanism for the large increase in hydrogen productivity was due to the unexpected finding that the heterologous Hyd inhibited hydrogen uptake activity through Hyd 1 and 2 rather than producing hydrogen (Maeda *et al*., 2007c). Recently, hydrogen production by this cyanobacterial [Ni-Fe]-Hyd was achieved at low rates in *E. coli* (Wells *et al*., 2011) by producing not just the five structural proteins HoxEFUYH but by also producing seven ORFs encoding the maturation proteins HypABCDEF and HoxW that are indepen-

dent of the *E. coli* FHL pathway for hydrogen production. This resulted in hydrogen production at a rate of 4 nmol H_2 (mg protein)$^{-1}$ h^{-1}.

Other improvements in bacterial hydrogen production through heterologous gene expression in *E. coli* were by overexpressing a [Fe-Fe]-Hyd, HydA derived from *Ethanoligenes harbinense* (Zhao *et al*., 2010), and a HupSL Hyd consisting of small and large subunits of Hyd isolated from *Rhodobacter sphaeroides* (Lee *et al*., 2010). Interestingly, hydrogen production by recombinant HupSL in *E. coli* was greater than in the original strain, *Rhodobacter sphaeroides* (Lee *et al*., 2010) in that hydrogen-producing activity of the Hyd was negligible (Koku *et al*., 2002).

Hydrogen production via the [Fe-Fe]-type Hyd (HydA) of *Clostridium acetobutylicum* was achieved *in vivo* in *E. coli* BL21(DE3) by expressing *E. coli* YdbK, a probable pyruvate–flavodoxin oxidoreductase, and *Clostridium pasteurianum* [4Fe-4S]-ferredoxin together with *Clostridium acetobutylicum* HydF, HydE, HydG and HydA (Akhtar and Jones, 2009). In a background that includes the *iscR* mutation, the hydrogen yield from glucose increased twofold, and hydrogen production from starch was also achieved by heterologously expressing *amyE* encoding an alpha-type amylase derived from *Bacillus subtilis* (Akhtar and Jones, 2009).

Protein engineering to enhance hydrogen production

Unlike metabolic engineering and heterologous-gene cloning strategies, protein engineering studies to improve Hyd enzyme function have not been developed extensively. The primary reason may be that there have been few high-throughput methods to readily measure Hyd activity (either directly or indirectly) since for protein engineering, a beneficial screening method is critical to find an improved Hyd variant among many candidate variants fabricated through error-prone PCR (epPCR), DNA shuffling, or saturation mutagenesis. There are several relatively easy methods to assay Hyd activity; for example, a facile method to detect formate concentrations consumed by the *E. coli* FHL by using potassium permanganate (Maeda and Wood, 2008) but this method is not a direct measure of Hyd activity. Also, there is a method to directly assay hydrogen gas by a chemical reaction (Bagramyan and Martirosov, 1989), but this method is not easily adapted to a high-throughput screen. Furthermore, many groups measure hydrogen uptake by methyl viologen (Krasna, 1979) and reason that Hyd enzymes are reversible so improvements in uptake should lead to improved hydrogen formation; however, this approach is not optimal since you usually get what you screen for and little else.

The best high throughput screens to date are (i) a chemochromic membrane, which detects hydrogen produced from single colonies via a blue-colour change, which is triggered by binding of hydrogen to a thin-film WO_3 sensor attached to the membrane (Seibert *et al*., 1999); and (ii) a high-throughput screening assay apparatus, which detects hydrogen directly based on the chemical reaction between a water-soluble tetrazolium colour indicator and hydrogen (Schrader *et al*., 2008). These tools should prove quite useful for protein engineering.

The first study to evolve a hydrogenase, [Fe-Fe] HydA from *Clostridia* sp., utilized DNA shuffling of the hydrogenases of *C. acetobutylicum* and *C. saccharobutylicum* (Nagy *et al*., 2007); however, no screening method was used and little improvement was obtained. The first protein engineering using a high-throughput screening method (via chemochromic membranes) evolved the large subunit of *E. coli* Hyd 3, HycE, by epPCR, DNA shuffling, and saturation mutagenesis; the best epPCR variant, epHycE95, had amino-acid changes S2T, Y50F, I171T, A291V, T366S, V433L, M444I and L523Q, which led to 17-fold better hydrogen productivity compared with wild-type HycE (Maeda *et al*., 2008b). DNA shuffling using the best three variants from epPCR created a HycE variant with a truncation at Y464, and the truncated HycE had 23-fold higher hydrogen production compared with wild-type HycE. Saturation mutagenesis at T366, which was identified via a common residue change in two epPCR variants, led to 30-fold higher activity compared with the wild-type HycE (Maeda *et al*., 2008b).

Hydrogen production has also been enhanced by protein engineering of FhlA, the FHL transcriptional activator of transcription of the *fdhF* (formate dehydrogenase-H), *hyc* (Hyd 3), *hyp* and *hydN-hypF* (maturation proteins) operons. Through random mutagenesis using epPCR over the whole gene as well as over the N-terminal region of FhlA, a variant was identified (Q11H, L14V, Y177F, K245R, M288K and I342) with ninefold increased hydrogen production (Sanchez-Torres *et al*., 2009). As expected, the mechanism by which the engineered FhlA variant improved hydrogen production was via increased transcription of all of the genes activated by FhlA (Sanchez-Torres *et al*., 2009).

Protein engineering of the [Fe-Fe]-type Hyd HydA1 from *Chlamydomonas reinhardtii* was performed using an *in vitro* screen (Stapleton and Swartz, 2010) based on hydrogen uptake (using methyl viologen). The HydA1 variants were constructed through a SIMPLEX (SIngle-Molecule PCR-Linked EXpression) method (Rungpragayphan *et al*., 2003), which enables variants to be synthesized *in vitro* directly from the epPCR products in a cell-free protein synthesis system. One variant with fourfold higher activity than the original enzyme was obtained (Stapleton and Swartz, 2010).

Lastly, site-directed mutagenesis of HydA from *C. reinhardtii* was performed to arrange the ferredoxin-binding surface of HydA (Agapakis *et al*., 2010). Based on a previous structural model for the interaction between Hyd and its cognate [2Fe-2S]-ferredoxin, four amino acids at the 2nd, 5th, 119th and 126th codons were selected and since ferredoxin is rich in negatively charged residues, these amino acids were replaced with lysines to confer a positive charge at the Hyd binding surface. As a result, it was demonstrated that the mutations in HydA, E5K and D126K, were important for enhanced hydrogen production (Agapakis *et al*., 2010).

Cell-free systems versus whole-cell systems for hydrogen production

To date, almost all bacterial hydrogen production is based on whole-cell systems; however, *in vitro* hydrogen production by cell-free systems has gained attention. In general, hydrogenases are sensitive to oxygen (Vincent *et al*., 2005) and this includes the *E. coli* hydrogenases (Glick *et al*., 1980). Exceptions are the hydrogenases from *Ralstonia eutropha* (Van der Linden *et al*., 2004) and *Aquifex aeolicus* (Pandelia *et al*., 2010), which are somewhat oxygen-resistant; hence, hydrogenase-related experiments are conducted under anaerobic conditions. Therefore, whole-cell systems are more robust since it is easier to recover hydrogenase activity with intact cells than via cell-free systems. The next problem to surmount in *in vitro* systems is the maturation of most hydrogenases (e.g. nickel insertion, iron–sulfur formation or C-terminus cleavage of hydrogenases). Therefore, many genes have to be expressed in an active state in cell-free systems. For example, the heterologous expression of a *Pyrococcus furiosus* Hyd required 13 genes (4 structural genes and 9 maturation genes) (Sun *et al*., 2010). Furthermore, the stability of complex hydrogenases and their maturation enzymes in cell-free systems needs to be addressed as well as the scale at which these systems may be operated (gram rather than nanogram quantities required). Lastly, purification of a large number of complex enzymes and cofactors like $NADP^+$ may not be cost effective.

In contrast to whole-cell systems, cell-free hydrogen production systems have the advantages that synthetic pathways to produce more hydrogen may be more readily conceived and their efficiencies are much greater. The first group to show such high efficiencies exist for cell-free systems utilized 11 enzymes from the pentose phosphate cycle along with a hydrogenase from *Pyrococcus furiosus* to generate 11.6 mol of hydrogen per mol glucose or 97% of the maximum stoichiometric yield (Woodward *et al*., 2000). Next, Zhang *et al*. (2007) extended this system by using 13 enzymes *in vitro* to convert starch into hydrogen using basically the same system with two additional enzymes to process glucan.

Perspectives

Microbial biotechnology is a promising approach to enhance bacterial hydrogen production (De Genève and Fernández, 2008; Ramos *et al*., 2008) as it should lead to biotechnology processes that utilize substrates efficiently, have high capacities, have fast sugar transport, tolerate inhibitors and end products, and have high metabolic fluxes and ultimately produce a single fermentation product such as hydrogen (Alper and Stephanopoulos, 2009). To date, there are several engineered *E. coli* strains that have overcome two major technical problems: (i) slow hydrogen production (Yoshida *et al*., 2005; Maeda *et al*., 2008a; Kim *et al*., 2009); and (ii) low hydrogen yield (Chittibabu *et al*., 2006; Yoshida *et al*., 2006; Maeda *et al*., 2008a), and these strains hold promise for further improvements in hydrogen production since there remain uncharacterized deleterious proteins as well as beneficial ones for hydrogen production in *E. coli*. To date, utilizing native *E. coli* hydrogenases has resulted in higher rates of hydrogen production (Table 1).

It has been estimated (Vardar-Schara *et al*., 2008) that the annual cost to maintain 1 kW of electricity generated from hydrogen may be ~ $172 000 when formate is used [assuming $12 per kg formate as 1 mol H_2 per mol formate) and may be ~ $4600 when glucose is used (assuming $0.22 per kg glucose and 1.8 mol H_2 per mol glucose (Mathews *et al*., 2010)]. However, the current annual cost for 1 kW of electricity in the residential sector is ~ $1000, which is generated mainly from fossil fuels (69%) (Booth and Spangler, 2011). Hence, a combination of increasing the hydrogen yield and reducing the cost of the substrate is required to obtain a 4.6-fold decrease in cost for hydrogen. Calculated another way, for using hydrogen as fuel for transportation, the US Department of Energy targeted a hydrogen cost goal by 2015 of $2.00–3.00 per gallon of gasoline equivalent (gge) (DOE, 2005). Considering that the energy content of 1 kg of H_2 is equivalent to 1 gallon of gasoline (DOE, 2005), the estimated feedstock cost to produce hydrogen is ~ $11 gge^{-1} [based on 1.8 mol H_2 per mol glucose (Mathews *et al*., 2010)]. Hence, again, about a fourfold reduction in cost is required. One way to reduce costs is to utilize low cost renewable resources for hydrogen production since feedstock costs account for 60% of overall costs for hydrogen production (Zhang, 2009). Since the maximum hydrogen yield in native *E. coli* is 2 mol of hydrogen from 1 mol of glucose (Hallenbeck and Ghosh, 2009), additional metabolic engineering is required to utilize by-products such as acetic acid to increase the hydrogen yield. In addition, protein engineering strategies (Maeda *et al*., 2008b) can continue to improve enzymes for

in vivo/*in vitro* biological hydrogen production and to stabilize hydrogenases (e.g. increase oxygen tolerance).

Given the current limitations of cell-free systems (enzyme and cofactor costs, enzyme instability, oxygen inactivation, requirements for maturation proteins, etc.), it appears whole-cell systems are more likely to be used in the near future to generate hydrogen. The proposal (Zhang, 2009) to utilize a minimal microbe (Glass *et al*., 2006) to produce a synthetic hydrogen-producing pathway or to create a hydrogen-producing strain by total synthesis of the whole genomic sequence as has been done previously (Gibson *et al*., 2008) may provide breakthroughs for bacterial hydrogen production using whole-cell systems in that it would allow one to design a synthetic system that uses all of the electron equivalents for hydrogen production in order to increase efficiency (i.e., the yield) of whole-cell systems. Additional engineering is also required to create cells to produce hydrogen from renewable resources such as kitchen garbage (containing mainly sugar substrates) and waste wood (targeting cellulosic substrate) since this is where these strains are mostly to be utilized first. Therefore, significant improvements in reaction rates as well as yields in engineered *E. coli* strains are expected.

Acknowledgements

This research was supported by the National Science Foundation (CBET-0753702) and the Japan Society for the Promotion of Science (22780070 and 21–7007).

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