

Metabolic engineering to enhance bacterial hydrogen production

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Summary

Hydrogen fuel is renewable, efficient and clean, and fermentative bacteria hold great promise for its generation. Here we use the isogenic *Escherichia coli* K-12 KEIO library to rapidly construct multiple, precise deletions in the *E. coli* genome to direct the metabolic flux towards hydrogen production. *Escherichia coli* has three active hydrogenases, and the genes involved in the regulation of the formate hydrogen lyase (FHL) system for synthesizing hydrogen from formate via hydrogenase 3 were also manipulated to enhance hydrogen production. Specifically, we altered regulation of FHL by controlling the regulators HycA and FhIA, removed hydrogen consumption by hydrogenases 1 and 2 via the *hyaB* and *hybC* mutations, and re-directed formate metabolism using the *fdnG*, *fdoG*, *narG*, *focA*, *fnr* and *focB* mutations. The result was a 141-fold increase in hydrogen production from formate to create a bacterium (BW25113 *hyaB hybC hycA fdoG/pCA24N-FhIA*) that produces the largest amount of hydrogen to date and one that achieves the theoretical yield for hydrogen from formate. In addition, the hydrogen yield from glucose was increased by 50%, and there was threefold higher hydrogen production from glucose with this strain.

Introduction

Hydrogen is a promising fuel as it has a higher energy content than oil (142 MJ kg⁻¹ for H₂ versus 42 MJ kg⁻¹ for oil) (Demirbas, 2002; Islam *et al.*, 2005). Most of the hydrogen now produced globally is by the process of steam reforming and the water–gas shift reaction (Yi and Harrison, 2005), or as a by-product of petroleum refining and chemicals production (Das and Vezirođlu, 2001). Use

of biological methods of hydrogen production should significantly reduce energy costs, as these processes do not require extensive heating (or extensive electricity as in electrolysis plants) (Das and Vezirođlu, 2001). Biological methods depend on hydrogenases that catalyse the reaction $2\text{H}^+ + 2\text{e}^- \leftrightarrow \text{H}_2$ (g) (Evans and Pickett, 2003). Hydrogen gas may be produced through either photosynthetic or fermentative processes, but fermentative hydrogen production is more efficient than photosynthetic ones (Yoshida *et al.*, 2005).

We chose to metabolically engineer *Escherichia coli* for hydrogen production as this is the best-characterized bacterium (Blattner *et al.*, 1997) (i.e. has well-established metabolic pathways) and it is one of the easiest strains to manipulate genetically. As a means to possibly help regulate internal pH (Böck and Sawers, 1996) and to regulate external pH by removing toxic formate, *E. coli* produces hydrogen from formate by hydrogenase 3 [encoded by *hycABCDEFGHI* (Sauter *et al.*, 1992; Bagramyan and Trchounian, 2003)] and formate dehydrogenase-H [encoded by *fdhF* (Axley *et al.*, 1990)] which are the key enzymes of the formate hydrogen lyase (FHL) system; these enzymes catalyse the reaction $\text{HCOO}^- + \text{H}_2\text{O} \leftrightarrow \text{H}_2 + \text{HCO}_3^-$ (Woods, 1936) (Fig. 1). *hycA* encodes a repressor for FHL (Sauter *et al.*, 1992), and *fhIA* encodes an essential activator of FHL (Schlensog *et al.*, 1994). Hence, the FHL may be manipulated to increase hydrogen by overexpression of *fhIA* (Yoshida *et al.*, 2005) and deletion of *hycA* (Penfold *et al.*, 2003; Yoshida *et al.*, 2005).

Whereas the FHL synthesizes hydrogen, hydrogen is consumed (Maeda *et al.*, 2007a) by *E. coli* hydrogenase 1 [*hyaB* encodes the large subunit (Menon *et al.*, 1990)] and hydrogenase 2 [*hybC* encodes the large subunit (Menon *et al.*, 1994)] (Fig. 1). There are also two additional formate dehydrogenases encoded by *fdnG* (α -subunit of formate dehydrogenase-N) and *fdoG* (α -subunit of formate dehydrogenase-O) which serve to consume formate (Rossmann *et al.*, 1991). Also, *focA* (Suppmann and Sawers, 1994) and *focB* (Andrews *et al.*, 1997) encode proteins that 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 formate dehydrogenase-N (Bertero *et al.*, 2003). In addition, FNR is a global DNA-binding transcriptional regulator which stimulates the transcription of many genes that are required for fermentation and anaerobic respiration (Salmon *et al.*, 2003), and the

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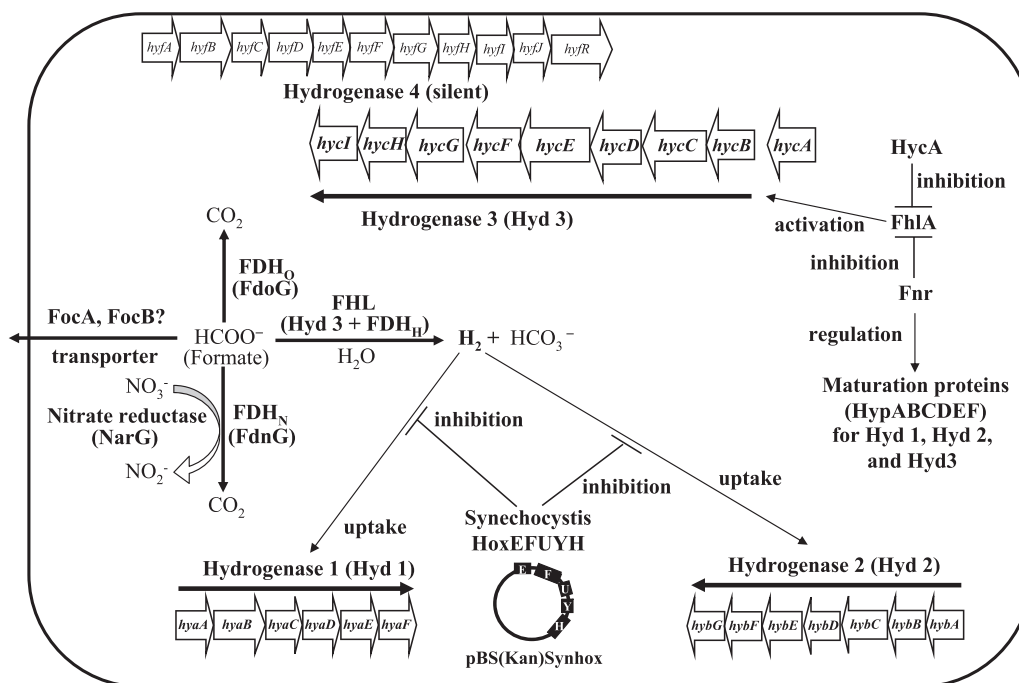


Fig. 1. Schematic of fermentative hydrogen production in *Escherichia coli*. Hydrogen is produced from formate by the formate hydrogen lyase (FHL) system [hydrogenase 3 and formate dehydrogenase-H (FDH_H)], which is activated by FhlA (that is regulated by Fnr) and repressed by HycA. Evolved hydrogen is consumed through the hydrogen uptake activity of hydrogenase 1 and hydrogenase 2. Formate is exported by FocA and/or FocB and is metabolized by formate dehydrogenase-N (FDH_N) which is linked with nitrate reductase A and formate dehydrogenase-O (FDH_O). Cyanobacterial hydrogenases (HoxEFUYH) derived from *Synechocystis* sp. PCC 6803 inhibit the activity of *E. coli* hydrogenase 1 and hydrogenase 2 resulting in enhanced hydrogen yield.

fnr mutation leads to threefold higher FhlA expression (Self and Shanmugam, 2000). Hence, hydrogen production should be increased by deleting *hyaB*, *hybC*, *fdnG*, *fdoG*, *focAB*, *fnr* and *narG*.

In the past, multiple mutations in a single strain have been introduced using different selection makers for each deleted gene (Lee *et al.*, 2005; Yoshida *et al.*, 2006). However, recently, an isogenic *E. coli* K-12 library containing all non-lethal deletion mutations (3985 genes) has been created (Keio collection) by the Genome Analysis Project in Japan (Baba *et al.*, 2006). This library allowed us to easily introduce multiple mutations into a single *E. coli* strain by combining a gene knockout step via P1 phage transduction and selection of antibiotic-resistant cells followed by an antibiotic resistance elimination step. Along with the ease of this process (each round of mutagenesis takes 2 days), the resulting deletion mutations are more stable for eliminating target genes compared with point mutations or frameshift mutations (reversion is far more difficult).

Here we show that multiple mutations may be introduced to a single strain for metabolic engineering to enhance hydrogen production. We create a quintuple mutant (BW25113 *hyaB hybC hycA fdoG/pCA24N-FhlA*) that produces 141 times more hydrogen by incorporating

the best of the pathway mutations *hyaB*, *hybC*, *focA*, *focB*, *fnr*, *narG*, *fdoG* and *fdnG* along with *fhIA* and *hycA*.

Results

Strategy and cell growth rates

Our strategy for metabolically engineering *E. coli* for enhanced hydrogen production consisted of (i) removing hydrogen uptake by inactivating hydrogenase 1 and 2 (by deleting *hyaB* and *hybC* respectively), (ii) manipulating the FHL regulatory proteins (by deleting a repressor, *hycA*, and by overexpressing an inducer, *fhIA*) and (iii) trying various combinations of mutations related to formate metabolism (*focA*, *focB*, *narG*, *fnr*, *fdnG* and *fdoG*). Our goal was to introduce mutations that did not make the cell less viable so specific growth rates were quantified after each mutation was added. For all cases, cell viability was not significantly affected in Luria–Bertani (LB) medium (Table 1) and this is in contrast to other approaches in which cell viability has been reduced [e.g. deleting the twin-arginine translocation system (Penfold *et al.*, 2006)]. However, the specific growth rates of some of the strains here were reduced in complex-formate medium (Table 2); for example, the specific growth rates of BW25113 *hyaB hybC hycA/pCA24N-FhlA*

Table 1. Effect of metabolic mutations on the aerobic specific growth rate in LB medium and on anaerobic hydrogen production from formate by *Escherichia coli* BW25113 in the closed system.

| Strain | Growth rate | | Hydrogen production ^a | |
|--|-----------------|----------|----------------------------------|----------|
| | h ⁻¹ | Relative | μmol mg-protein ⁻¹ | Relative |
| BW25113 | 1.6 ± 0.1 | 1 | 5 ± 2 | 1.0 |
| BW25113 <i>hyaB</i> | 1.42 ± 0.01 | 0.9 | 1.6 ± 0.0 | 0.3 |
| BW25113 <i>hybC</i> | 1.6 ± 0.1 | 1 | 7 ± 2 | 1.4 |
| BW25113 <i>hyaB hybC</i> | 1.6 ± 0.1 | 1 | 16 ± 6 | 3.2 |
| BW25113 <i>hyaB hybC hycA</i> | 1.4 ± 0.2 | 0.9 | 24 ± 7 | 4.8 |
| BW25113 <i>hyaB hybC hycA focA</i> | 1.6 ± 0.1 | 1 | 24 ± 5 | 4.8 |
| BW25113 <i>hyaB hybC hycA focB</i> | 1.58 ± 0.01 | 1 | 26 ± 5 | 5.2 |
| BW25113 <i>hyaB hybC hycA narG</i> | 1.46 ± 0.00 | 0.9 | 22 ± 7 | 4.4 |
| BW25113 <i>hyaB hybC hycA fnr</i> | 1.6 ± 0.2 | 1 | 5 ± 1 | 1.0 |
| BW25113 <i>hyaB hybC hycA fdnG</i> | 1.6 ± 0.1 | 1 | 14 ± 1 | 2.8 |
| BW25113 <i>hyaB hybC hycA fdoG</i> | 1.4 ± 0.1 | 0.9 | 53 ± 2 | 10.6 |
| BW25113 <i>hyaB hybC hycA fdnG fdoG</i> | 1.5 ± 0.1 | 0.9 | 49.9 ± 0.2 | 10.0 |
| BW25113 <i>hyaB hybC hycA fdoG focA</i> | 1.5 ± 0.2 | 0.9 | 48.0 ± 0.4 | 9.6 |
| BW25113 <i>hyaB hybC hycA focA focB</i> | 1.3 ± 0.2 | 0.8 | 12 ± 4 | 2.4 |
| BW25113 <i>hyaB hybC hycA focA narG</i> | 1.3 ± 0.1 | 0.8 | 19 ± 8 | 3.8 |
| BW25113 <i>hyaB hybC hycA focB narG</i> | 1.4 ± 0.1 | 0.9 | 31 ± 9 | 6.2 |
| BW25113 <i>hyaB hybC hycA focA focB narG</i> | 1.4 ± 0.3 | 0.9 | 36 ± 7 | 7.2 |
| BW25113 <i>hyaB hybC hycA focB fdnG</i> | 1.6 ± 0.1 | 1 | 0.6 ± 0.3 | 0.1 |
| BW25113 <i>hyaB hybC hycA focB fdoG</i> | 1.5 ± 0.1 | 0.9 | 1.1 ± 0.1 | 0.2 |
| BW25113 <i>hyaB hybC hycA focB fdnG fdoG</i> | 1.5 ± 0.1 | 1 | 0.9 ± 0.1 | 0.2 |
| BW25113/pCA24N | 1.46 ± 0.03 | 1 | 3 ± 2 | 1.0 |
| BW25113/pCA24N-FhIA | 1.47 ± 0.03 | 1 | 14.2 ± 0.4 | 4.7 |
| BW25113 <i>hyaB hybC/pCA24N-FhIA</i> | 1.47 ± 0.01 | 1 | 48 ± 3 | 16.0 |
| BW25113 <i>hyaB hybC hycA focB/pCA24N</i> | 1.44 ± 0.06 | 1 | 0.28 ± 0.06 | 0.1 |
| BW25113 <i>hyaB hybC hycA/pCA24N</i> | 1.39 ± 0.08 | 1 | 29 ± 5 | 9.7 |
| BW25113 <i>hyaB hybC hycA/pCA24N-FhIA</i> | 1.39 ± 0.02 | 1 | 55 ± 5 | 18.3 |
| BW25113 <i>hyaB hybC hycA focA/pCA24N-FhIA</i> | 1.4 ± 0.1 | 1 | 58 ± 12 | 19.3 |
| BW25113 <i>hyaB hybC hycA focB/pCA24N-FhIA</i> | 1.4 ± 0.2 | 1 | 59 ± 3 | 19.7 |
| BW25113 <i>hyaB hybC hycA narG/pCA24N-FhIA</i> | 1.38 ± 0.06 | 0.9 | 56 ± 11 | 18.7 |
| BW25113 <i>hyaB hybC hycA focB narG/pCA24N-FhIA</i> | ND | – | 48 ± 1 | 16.0 |
| BW25113 <i>hyaB hybC hycA focA focB narG/pCA24N-FhIA</i> | ND | – | 35 ± 12 | 11.7 |
| BW25113 <i>hyaB hybC hycA fdoG/pCA24N</i> | 1.5 ± 0.2 | 1 | 66 ± 1 | 22.0 |
| BW25113 <i>hyaB hybC hycA fdoG/pCA24N-FhIA</i> | 1.47 ± 0.03 | 1 | 79 ± 7 | 26.3 |

a. One hour in complex-formate medium.
ND, not determined.

and BW25113 *hyaB hybC hycA focB/pCA24N-FhIA* were reduced twofold compared with the wild-type strain. Also, the specific growth rate of BW25113 *hyaB hybC hycA fdoG/pCA24N-FhIA* was reduced 4.1-fold. These growth deficiencies did not impact the hydrogen closed/open assays in these strains as these experiments were conducted at turbidities of 1.3–2.5. The decrease in specific growth rates in complex-formate medium for these strains containing pCA24N-FhIA is probably due to FhIA-related toxicity as adding IPTG to increase FhIA expression leads to further decreases in growth (data not shown).

Hydrogenase deletions

To eliminate hydrogen uptake, the genes encoding the large subunits of hydrogenase 1 (*hyaB*) and hydrogenase 2 (*hybC*) were chosen to be inactivated as the active site of catalysis is located within each large subunit for these [NiFe]-hydrogenases (Forzi and Sawers, 2007). As expected, the double mutant (*hyaB hybC*) showed a significant decrease in hydrogen uptake activity (Maeda *et al.*,

2007a), and hydrogen production in the double mutant (*hyaB hybC*) was 3.2-fold higher than that in the wild-type strain in complex-formate medium after 1 h (Table 1). As there was only a 1.4-fold increase in hydrogen with the single mutation *hybC* and a 70% reduction in hydrogen with the single *hyaB* mutation, combining these mutations illustrates the importance of cumulative mutations.

HycA represses FHL by opposing *hyc* transcriptional activation by FhIA (Sauter *et al.*, 1992) (HycA may interact directly with the FhIA protein or prevent the binding of FhIA to activator sequences although the mechanism of regulation by HycA is unknown). To reveal whether an additional *hycA* mutation leads to enhanced hydrogen production, a triple mutant (*hyaB hybC hycA*) was constructed, and hydrogen production increased 4.8-fold compared with the wild type (Table 1).

Formate-related deletions

Escherichia coli has three pathways for eliminating formate produced by fermentation: (i) export of formate by

Table 2. Aerobic specific growth rates in complex-formate medium and anaerobic hydrogen production in complex-formate medium by metabolically engineered *Escherichia coli* strains using the low partial pressure assay.

| Strain | Description | Growth rate | | H ₂ production rate ^a | |
|---|---|-----------------|----------|---|----------|
| | | h ⁻¹ | Relative | μmol mg-protein ⁻¹ h ⁻¹ | Relative |
| BW25113/pCA24N | Wild type | 0.95 ± 0.01 | 1 | 0.8 ± 0.3 | 1 |
| BW25113/pCA24N-FhIA | Wild type + FhIA | ND | – | 7 ± 4 | 9 |
| BW25113 <i>hyaB hybC</i> /pCA24N-FhIA | Δ <i>hyaB</i> and Δ <i>hybC</i> (defective hydrogenases 1 and 2) + FhIA | ND | – | 57 ± 10 | 71 |
| BW25113 <i>hyaB hybC hycE</i> /pCA24N | Δ <i>hyaB</i> , Δ <i>hybC</i> and Δ <i>hycE</i> (defective hydrogenases 1, 2 and 3) | ND | – | 0.3 ± 0.03 | 0.4 |
| BW25113 <i>hyaB hybC hycA</i> /pCA24N-FhIA | Δ <i>hyaB</i> , Δ <i>hybC</i> and Δ <i>hycA</i> (defective hydrogenases 1 and 2 and defective FHL repressor) + FhIA | 0.42 ± 0.07 | 0.44 | 64 ± 3 | 80 |
| BW25113 <i>hyaB hybC hycA focB</i> /pCA24N-FhIA | Δ <i>hyaB</i> , Δ <i>hybC</i> , Δ <i>hycA</i> and Δ <i>focB</i> (defective hydrogenases 1 and 2, defective FHL repressor and defective putative formate transporter) + FhIA | 0.47 ± 0.07 | 0.49 | 61 ± 16 | 76 |
| BW25113 <i>hyaB hybC hycA fdoG</i> /pCA24N-FhIA | Δ <i>hyaB</i> , Δ <i>hybC</i> , Δ <i>hycA</i> and Δ <i>fdoG</i> (defective hydrogenases 1 and 2, defective FHL repressor and defective formate dehydrogenase-O) + FhIA | 0.23 ± 0.07 | 0.24 | 113 ± 12 | 141 |

a. Hydrogen production rate was calculated from 30 min incubation in complex-formate medium. ND, not determined.

the formate transporter FocA (Suppmann and Sawers, 1994) and its homologue FocB (Andrews *et al.*, 1997), (ii) degradation of formate by formate dehydrogenase-N coupling with nitrate reductase A and formate dehydrogenase-O which converts formate to CO₂; the electrons from formate oxidation are coupled to the respiratory electron transport chain which generates ATP (Wang and Gunsalus, 2003), and (iii) conversion of formate into hydrogen by FHL activity (through hydrogenase 3 and formate dehydrogenase-H). Hence, formate transport, formate dehydrogenase-N/nitrate reductase A activity and formate dehydrogenase-O activity may be deleted to enhance hydrogen production. Based on this strategy, *focA*, *focB*, *narG*, *fdnG* and *fdoG* mutations were introduced to the triple mutant (*hyaB hybC hycA*) to make five quadruple mutants (*hyaB hybC hycA focA*, *hyaB hybC hycA focB*, *hyaB hybC hycA narG*, *hyaB hybC hycA fdnG* and *hyaB hybC hycA fdoG*), and then hydrogen production was assayed. The additional *focB* mutation in *hyaB hybC hycA* led to a slight increase of hydrogen production; its quadruple mutant produced 5.2 times more hydrogen than the wild-type strain in complex-formate medium after 1 h; the *focA* mutation was not effective for producing more hydrogen. Deleting *fdoG* gene in a *hyaB hybC hycA* background led to a significant increase of hydrogen production as BW25113 *hyaB hybC hycA fdoG* cells produced 10.6-fold more hydrogen than the wild-type cells. In contrast, the other two quadruple strains (*hyaB hybC hycA fdnG* and *hyaB hybC hycA narG*) produced less hydrogen than the triple mutant (*hyaB hybC hycA*). Also, a *fnr* mutation was introduced to the triple

mutant (*hyaB hybC hycA*) to make BW25113 *hyaB hybC hycA fnr* as the *fnr* mutation activates expression of FhIA by threefold (Self and Shanmugam, 2000); unexpectedly, the *fnr* mutation in the *hyaB hybC hycA* background decreased hydrogen production (Table 1).

To further test the combination of the *focA*, *focB*, *narG*, *fdnG* and *fdoG* mutations, seven quintuple strains (*hyaB hybC hycA focA focB*, *hyaB hybC hycA focA narG*, *hyaB hybC hycA focB narG*, *hyaB hybC hycA focB fdnG*, *hyaB hybC hycA focB fdoG*, *hyaB hybC hycA fdnG fdoG* and *hyaB hybC hycA fdoG focA*) and two sextuple strains (*hyaB hybC hycA focA focB narG* and *hyaB hybC hycA focB fdnG fdoG*) were constructed and hydrogen production was assayed. Four quintuple mutants (*hyaB hybC hycA focA focB*, *hyaB hybC hycA focA narG*, *hyaB hybC hycA focB fdnG* and *hyaB hybC hycA focB fdoG*) and one sextuple (*hyaB hybC hycA focB fdnG fdoG*) had lower hydrogen production activity than triple mutant (*hyaB hybC hycA*); in particular, hydrogen production in BW25113 *hyaB hybC hycA focB fdnG*, *hyaB hybC hycA focB fdoG* and *hyaB hybC hycA focB fdnG fdoG* was lower than that in the wild-type strain, although each *focB* and *fdoG* gene was effective for producing more hydrogen in the triple mutation background (*hyaB hybC hycA*). On the other hand, BW25113 *hyaB hybC hycA focB fdnG* and *hyaB hybC hycA focA focB narG* significantly produced 6.2- and 7.2-fold higher hydrogen than the wild-type strain. In addition, hydrogen production in BW25113 *hyaB hybC hycA fdnG fdoG* was comparable to that in the quadruple strain (*hyaB hybC hycA fdoG*) whereas BW25113 *hyaB hybC hycA fdnG* had low hydrogen productivity.

FhIA overexpression

FhIA protein activates FHL by binding directly to the intergenic region between *hyc* and *hyp* operons or between the *hycA* and *hycB* genes (Schlensog *et al.*, 1994). To boost hydrogen productivity further, plasmid pCA24N-FhIA was added to the best eight of our recombinants, and hydrogen production was assayed (Table 1). The expression of *fhIA* in BW25113, BW25113 *hyaB hybC hycA* and BW25113 *hyaB hybC hycA fdoG* led to a 4.7-, 1.9- and 1.2-fold increase in hydrogen production (BW25113/pCA24N versus BW25113/pCA24N-FhIA, BW25113 *hyaB hybC hycA*/pCA24N-FhIA versus BW25113 *hyaB hybC hycA*/pCA24N and BW25113 *hyaB hybC hycA fdoG*/pCA24N-FhIA versus BW25113 *hyaB hybC hycA fdoG*/pCA24N). Ultimately, BW25113 *hyaB hybC hycA fdoG*/pCA24N-FhIA produced 26.3-fold more hydrogen than the wild-type strain (BW25113/pCA24N) in complex-formate medium after 1 h in the closed system.

Hydrogen production and yields with low partial pressure

As the accumulation of hydrogen in the headspace in the closed system will tend to reverse the hydrogen synthetic reaction, the hydrogen production for the best four strains was measured using an anaerobic system that maintained low hydrogen headspace pressure as shown in Fig. 2 and the results are shown in Table 2. Corroborating our hypothesis, BW25113 *hyaB hybC hycA fdoG*/pCA24N-FhIA produced 141 times more hydrogen than the wild-type strain with empty vector pCA24N whereas there was a 26-fold increase in the closed system. Similarly, hydrogen production in BW25113/pCA24N-FhIA, BW25113 *hyaB hybC*/pCA24N-FhIA, BW25113 *hyaB hybC hycA*/pCA24N-FhIA and BW25113 *hyaB hybC hycA fobC*/pCA24N-FhIA was 9-, 71-, 80- and 76-fold higher

than that in the wild-type strain (Table 2). As negative controls, autoclaved BW25113 *hyaB hybC hycA fdoG*/pCA24N-FhIA did not produce hydrogen, and BW25113 *hyaB hybC hycE*/pCA24N, which lacks an active hydrogenase 3, showed negligible hydrogen production that was 2.7-fold less than that of the wild-type cells (Table 2).

The hydrogen yield in BW25113 *hyaB hybC hycA fdoG*/pCA24N-FhIA was 1.15 ± 0.01 mol hydrogen/mol formate compared with 0.64 ± 0.01 mol hydrogen/mol formate for BW25113/pCA24N. This indicates that the metabolically engineered *E. coli* cells with five mutations (deletion of *hyaB*, *hybC*, *hycA* and *fdoG*, and overexpression of *fhIA*) more efficiently converts formate into hydrogen and that it reaches the theoretical yield of 1 mol hydrogen/mol formate (Woods, 1936).

Hydrogen from glucose

As it may be more practical to produce hydrogen from glucose rather than formate (Kraemer and Bagley, 2007), the hydrogen from complex-glucose medium was measured for the best strain BW25113 *hyaB hybC hycA fdoG*/pCA24N-FhIA in the low hydrogen partial pressure system (Fig. 2). Compared with the wild-type strain BW25113/pCA24N, BW25113 *hyaB hybC hycA fdoG*/pCA24N-FhIA produced 3.2-fold more hydrogen after 15 min (3.7 ± 0.1 versus 12 ± 1 $\mu\text{mol mg-protein}^{-1} \text{h}^{-1}$). Also, the hydrogen yield from glucose was increased by 50% compared with that in the original strain (0.47 ± 0.06 for BW25113/pCA24N versus 0.70 ± 0.02 mol H_2 /mol glucose for BW25113 *hyaB hybC hycA fdoG*/pCA24N-FhIA).

Discussion

In this work, we show that a single fermentative *E. coli* strain with four mutations, *hyaB hybC hycA fdoG*, and which overexpresses *fhIA*⁺ produces 141-fold more

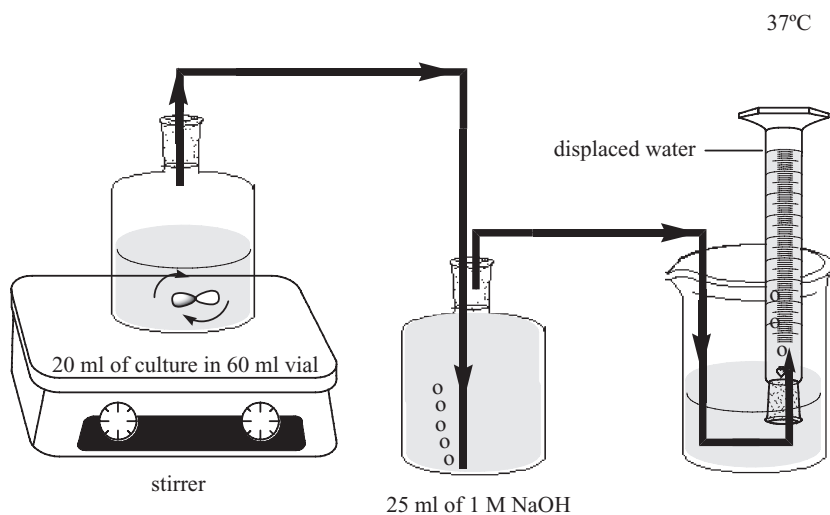


Fig. 2. Low-pressure, anaerobic hydrogen production in a simple batch reactor incubated at 37°C that allows the produced hydrogen to escape from the reactor vessel. The volume of hydrogen gas was measured after 30 min, and complex-formate medium was used.

hydrogen than the wild-type strain at a rate of $113 \mu\text{mol mg}^{-1} \text{h}^{-1}$ on a protein basis. This strain is also just as viable as the original strain in rich medium and none of the 26 new strains we created are significantly less viable than the wild-type strain in LB medium. Also, the metabolically engineered *E. coli* cells (BW25113 *hyaB hybC hycA fdoG/pCA24N-FhlA*) obtained the theoretical hydrogen yield (1 mol hydrogen/mol formate) (Woods, 1936) as a result of inactivating hydrogen consumption by hydrogenase 1 (*hyaB*) and hydrogenase 2 (*hybC*), activation of FHL by deleting the FHL repressor (*hycA*), overexpressing the FHL activator (*fhlA*), and inactivation of formate dehydrogenase-O (*fdoG*) to prevent formate consumption. Also, this best strain BW25113 *hyaB hybC hycA fdoG/pCA24N-FhlA* had 3.2 times higher initial hydrogen production than the wild-type cells in glucose medium and the metabolic engineering increased the hydrogen yield from glucose by 50%.

Three previous studies concerning enhanced hydrogen production in *E. coli* via fermentation have been reported. Deletion of the FHL repressor *hycA* and overexpression of *fhlA* increased hydrogen production by 2.8-fold from formate (Yoshida *et al.*, 2005). Deletion of the twin-arginine translocation system to inactivate hydrogenase 1, hydrogenase 2, formate dehydrogenase-N and formate dehydrogenase-O (strain does not transport these proteins to the periplasm) resulted in only twofold higher hydrogen production compared with wild-type cells from glucose and resulted in decreased viability (Penfold *et al.*, 2006). In addition, deleting lactate dehydrogenase (*ldhA*) and fumarate reductase (*frdBC*) resulted in 1.4-fold more hydrogen production relative to the wild-type strain from glucose (Yoshida *et al.*, 2006). Hence our approach appears to be more robust than these earlier *E. coli* methods. For non-*E. coli* strains, *Citrobacter* sp. Y19 ($65 \mu\text{mol mg}^{-1} \text{h}^{-1}$) (Oh *et al.*, 2003), *Rhodospseudomonas palustris* JA1 ($60 \mu\text{mol mg}^{-1} \text{h}^{-1}$) (Archana *et al.*, 2003), *R. palustris* P4 ($41 \mu\text{mol mg}^{-1} \text{h}^{-1}$) (Jung *et al.*, 1999) and *Klebsiella oxytoca* HP1 ($30 \mu\text{mol mg}^{-1} \text{h}^{-1}$) (Minnan *et al.*, 2005) have high maximum hydrogen activity; however, these organisms are more fastidious than *E. coli*, more difficult to engineer, and now produce less hydrogen than the engineered *E. coli* strain.

The 3.2-fold enhanced hydrogen production by deleting hydrogenase 1 and hydrogenase 2 (*hyaB* and *hybC*, Table 1) agrees well with our previous study that engineered *E. coli* cells expressing the cyanobacterial bidirectional hydrogenase (HoxEFUYH) derived from *Synechocystis* sp. PCC 6803 enhanced hydrogen yields by 41-fold by inhibiting the hydrogen uptake activity by hydrogenase 1 and hydrogenase 2 (Maeda *et al.*, 2007b). In contrast, inactivating FocA, the putative formate exporter, was not significant for producing more hydrogen although its inactivation leads to the accumulation of

formate (Suppmann and Sawers, 1994); instead, deletion of *focB* gene [*focB* is a homologue of *focA* (Andrews *et al.*, 1997)] was more effective although it only enhanced hydrogen production slightly (Table 1).

The three protein subunits of formate dehydrogenase-N (α from *fdnG*, β from *fdnH* and γ from *fdnI*) show high sequence similarity to those for formate dehydrogenase-O (*fdoG*, *fdoH* and *fdoI*) (Benoit *et al.*, 1998), and the three polypeptides for formate dehydrogenase-O were recognized by antibodies for formate dehydrogenase-N (Abaibou *et al.*, 1995); however, these two formate dehydrogenases have different cellular functions (Barker *et al.*, 2000). It has been reported that the deficiency of formate dehydrogenase-N leads to an accumulation of intracellular formate and activation of FHL pathway (Suppmann and Sawers, 1994); hence, mutating *fdnG* should be effective for enhanced hydrogen production. However, our results showed an additional mutation of *fdnG* in *hyaB hybC hycA fdnG* produced less hydrogen. This decrease may be due to enhanced formate consumption by formate dehydrogenase-O. As corroborating evidence, hydrogen production in the quintuple strain (*hyaB hybC hycA fdnG fdoG*) was comparable to that in BW25113 *hyaB hybC hycA fdoG* (Table 1). Therefore, these results indicate that consumption of formate that does not lead to hydrogen (formate hydrogenase-H is required for hydrogenase 3, Fig. 1) is primarily by formate dehydrogenase-O rather than formate dehydrogenase-N and the better route for increasing hydrogen production is through inactivation of formate dehydrogenase-O (deletion of *fdoG*).

Unexpectedly, the *fnr* mutation, which leads to threefold higher FhlA expression (Self and Shanmugam, 2000), decreased hydrogen production (Table 1); this may be due to a reduction in expression of the *hyp* operon (encodes maturation proteins for hydrogenases) due to the *fnr* mutation (Messenger and Green, 2003). The combination of both the *fdnG* and *fdoG* mutations with *hyaB hybC hycA* was even more deleterious; this argues for mathematical modelling to help understand the impact of the accumulated mutations on related metabolic pathways. Nonetheless, the method developed here to introduce multiple stable mutations in a single strain without reducing cell viability holds much promise for continued increases in hydrogen production using *E. coli* as well as promise for many other applications of pathway engineering where multiple mutations are required.

Experimental procedures

Bacterial strains, growth rates and total protein

Strains are shown in Table 3. *Escherichia coli* cells were initially streaked from -80°C glycerol stocks on LB agar

Table 3. Strains and plasmids used.

| Strains and plasmids | Genotype | Source |
|--|--|---------------------------|
| Strains | | |
| <i>Escherichia coli</i> BW25113 | <i>lacI^q rmB_{T14} ΔlacZ_{NJ16} hsdR514 ΔaraBAD_{AH33} ΔrhaBAD_{LD78}</i> | Yale CGSG Stock Center |
| <i>Escherichia coli</i> BW25113 Δ <i>hyaB</i> | BW25113 <i>hyaB</i> Km ^R ; defective in large subunit of hydrogenase 1 | Baba <i>et al.</i> (2006) |
| <i>Escherichia coli</i> BW25113 Δ <i>hybC</i> | BW25113 <i>hybC</i> Km ^R ; defective in large subunit of hydrogenase 2 | Baba <i>et al.</i> (2006) |
| <i>Escherichia coli</i> BW25113 Δ <i>focA</i> | BW25113 <i>hycA</i> Km ^R ; defective in repressor of FHL | Baba <i>et al.</i> (2006) |
| <i>Escherichia coli</i> BW25113 Δ <i>hycE</i> | BW25113 <i>hycE</i> Km ^R ; defective in large subunit of hydrogenase 3 | Baba <i>et al.</i> (2006) |
| <i>Escherichia coli</i> BW25113 Δ <i>focA</i> | BW25113 <i>focA</i> Km ^R ; defective in formate transporter | Baba <i>et al.</i> (2006) |
| <i>Escherichia coli</i> BW25113 Δ <i>focB</i> | BW25113 <i>focB</i> Km ^R ; defective in putative formate transporter | Baba <i>et al.</i> (2006) |
| <i>Escherichia coli</i> BW25113 Δ <i>narG</i> | BW25113 <i>narG</i> Km ^R ; defective in α -subunit of nitrate reductase A | Baba <i>et al.</i> (2006) |
| <i>Escherichia coli</i> BW25113 Δ <i>fnr</i> | BW25113 <i>fnr</i> Km ^R ; defective in FNR transcriptional dual regulator | Baba <i>et al.</i> (2006) |
| <i>Escherichia coli</i> BW25113 Δ <i>fdnG</i> | BW25113 <i>fdnG</i> Km ^R ; defective in α -subunit of formate dehydrogenase-N | Baba <i>et al.</i> (2006) |
| <i>Escherichia coli</i> BW25113 Δ <i>fdoG</i> | BW25113 <i>fdoG</i> Km ^R ; defective in α -subunit of formate dehydrogenase-O | Baba <i>et al.</i> (2006) |
| <i>Escherichia coli</i> BW25113 Δ <i>hyaB</i> Δ <i>hybC</i> | BW25113 <i>hyaB hybC</i> Δ <i>kan</i> ; defective in large subunit of hydrogenase 1 and hydrogenase 2 | This study |
| <i>Escherichia coli</i> BW25113 Δ <i>hyaB</i> Δ <i>hybC</i> Δ <i>hycA</i> | BW25113 <i>hyaB hybC hycA</i> Δ <i>kan</i> ; defective in large subunit of hydrogenase 1 and hydrogenase 2 and defective in repressor of FHL | This study |
| <i>Escherichia coli</i> BW25113 Δ <i>hyaB</i> Δ <i>hybC</i> Δ <i>hycE</i> | BW25113 <i>hyaB hybC hycE</i> Δ <i>kan</i> ; defective in large subunit of hydrogenase 1, hydrogenase 2 and hydrogenase 3 | This study |
| <i>Escherichia coli</i> BW25113 Δ <i>hyaB</i> Δ <i>hybC</i> Δ <i>hycA</i> Δ <i>focA</i> | BW25113 <i>hyaB hybC hycA focA</i> Δ <i>kan</i> ; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL and defective in formate transporter | This study |
| <i>Escherichia coli</i> BW25113 Δ <i>hyaB</i> Δ <i>hybC</i> Δ <i>hycA</i> Δ <i>focB</i> | BW25113 <i>hyaB hybC hycA focB</i> Δ <i>kan</i> ; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL and defective in putative formate transporter | This study |
| <i>Escherichia coli</i> BW25113 Δ <i>hyaB</i> Δ <i>hybC</i> Δ <i>hycA</i> Δ <i>narG</i> | BW25113 <i>hyaB hybC hycA narG</i> Δ <i>kan</i> ; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL and defective in α -subunit of nitrate reductase A | This study |
| <i>Escherichia coli</i> BW25113 Δ <i>hyaB</i> Δ <i>hybC</i> Δ <i>hycA</i> Δ <i>fnr</i> | BW25113 <i>hyaB hybC hycA fnr</i> Km ^R ; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL and defective in FNR transcriptional dual regulator | This study |
| <i>Escherichia coli</i> BW25113 Δ <i>hyaB</i> Δ <i>hybC</i> Δ <i>hycA</i> Δ <i>fdnG</i> | BW25113 <i>hyaB hybC hycA fdnG</i> Km ^R ; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL and defective in α -subunit of formate dehydrogenase-N | This study |
| <i>Escherichia coli</i> BW25113 Δ <i>hyaB</i> Δ <i>hybC</i> Δ <i>hycA</i> Δ <i>fdoG</i> | BW25113 <i>hyaB hybC hycA fdoG</i> Δ <i>kan</i> ; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL and defective in α -subunit of formate dehydrogenase-O | This study |
| <i>Escherichia coli</i> BW25113 Δ <i>hyaB</i> Δ <i>hybC</i> Δ <i>hycA</i> Δ <i>fdoG</i> Δ <i>focA</i> | BW25113 <i>hyaB hybC hycA fdoG focA</i> Δ <i>kan</i> ; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in α -subunit of formate dehydrogenase-O and defective in formate transporter | This study |
| <i>Escherichia coli</i> BW25113 Δ <i>hyaB</i> Δ <i>hybC</i> Δ <i>hycA</i> Δ <i>fdnG</i> Δ <i>fdoG</i> | BW25113 <i>hyaB hybC hycA fdoG fdnG</i> Km ^R ; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL and defective in α -subunit of formate dehydrogenase-N and formate dehydrogenase-O | This study |
| <i>Escherichia coli</i> BW25113 Δ <i>hyaB</i> Δ <i>hybC</i> Δ <i>hycA</i> Δ <i>focA</i> Δ <i>focB</i> | BW25113 <i>hyaB hybC hycA focA focB</i> Km ^R ; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL and defective in formate transporter and putative formate transporter | This study |

Table 3. cont.

| Strains and plasmids | Genotype | Source |
|---|--|-----------------------------------|
| <i>Escherichia coli</i> BW25113 Δ <i>hyaB</i> Δ <i>hybC</i> Δ <i>hycA</i> Δ <i>focA</i> Δ <i>narG</i> | BW25113 <i>hyaB hybC hycA focA narG</i> Km ^R ; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in formate transporter and defective in α -subunit of nitrate reductase A | This study |
| <i>Escherichia coli</i> BW25113 Δ <i>hyaB</i> Δ <i>hybC</i> Δ <i>hycA</i> Δ <i>focB</i> Δ <i>narG</i> | BW25113 <i>hyaB hybC hycA focB narG</i> Δ <i>kan</i> ; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in putative formate transporter and defective in α -subunit of nitrate reductase A | This study |
| <i>Escherichia coli</i> BW25113 Δ <i>hyaB</i> Δ <i>hybC</i> Δ <i>hycA</i> Δ <i>focA</i> Δ <i>focB</i> Δ <i>narG</i> | BW25113 <i>hyaB hybC hycA focA focB narG</i> Km ^R ; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in formate transporter and putative formate transporter and defective in α -subunit of nitrate reductase A | This study |
| <i>Escherichia coli</i> BW25113 Δ <i>hyaB</i> Δ <i>hybC</i> Δ <i>hycA</i> Δ <i>focB</i> Δ <i>fdnG</i> | BW25113 <i>hyaB hybC hycA focB fdnG</i> Km ^R ; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in putative formate transporter and defective in α -subunit of formate dehydrogenase-N | This study |
| <i>Escherichia coli</i> BW25113 Δ <i>hyaB</i> Δ <i>hybC</i> Δ <i>hycA</i> Δ <i>focB</i> Δ <i>fdoG</i> | BW25113 <i>hyaB hybC hycA focB fdoG</i> Δ <i>kan</i> ; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in putative formate transporter and defective in α -subunit of formate dehydrogenase-O | This study |
| <i>Escherichia coli</i> BW25113 Δ <i>hyaB</i> Δ <i>hybC</i> Δ <i>hycA</i> Δ <i>focB</i> Δ <i>fdnG</i> Δ <i>fdoG</i> | BW25113 <i>hyaB hybC hycA focB fdnG fdoG</i> Km ^R ; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in putative formate transporter and defective in α -subunit of formate dehydrogenase-N and formate dehydrogenase-O | This study |
| Plasmids | | |
| pCA24N | Empty vector; Cm ^R | Kitagawa <i>et al.</i> (2005) |
| pCA24N-FhIA | <i>pCA24N pT5-lac::fhIA</i> ; expresses FhIA derived from <i>Escherichia coli</i> | Kitagawa <i>et al.</i> (2005) |
| pCP20 | Ap ^R and Cm ^R plasmid with temperature-sensitive replication and thermal induction of FLP recombinase synthesis | Cherepanov and Wackernagel (1995) |

Km^R, Cm^R and Ap^R are kanamycin, chloramphenicol and ampicillin resistance respectively.

plates (Sambrook *et al.*, 1989) containing 100 μ g ml⁻¹ kanamycin (for those with chromosomal kanamycin resistance markers) and 30 μ g ml⁻¹ chloramphenicol (for those containing pCA24N-based plasmids) and incubated at 37°C. After growth on LB agar plates, a fresh single colony was cultured at 37°C with shaking at 250 r.p.m. (New Brunswick Scientific, Edison, NJ) in LB medium (Sambrook *et al.*, 1989), modified complex-glucose medium (Rachman *et al.*, 1997) to which 0.4 mg l⁻¹ (NH₄)₆Mo₇O₂₄ was added, or modified complex-formate medium in which formate (100 mM, Fisher Scientific, Fair Lawn, NJ) was substituted for glucose and 0.4 mg l⁻¹ (NH₄)₆Mo₇O₂₄ was added; 100 μ g ml⁻¹ kanamycin or 30 μ g ml⁻¹ chloramphenicol were also added where appropriate. The parent strain *E. coli* K-12 BW25113 was obtained from the Yale University CGSC Stock Center, and its isogenic deletion mutants (Keio collection) were obtained from the Genome Analysis Project in Japan (Baba *et al.*, 2006). Plasmids pCA24N (Kitagawa *et al.*, 2005) and pCA24N-FhIA were electroporated into hydrogen-overproducing *E. coli* strains (Table 3). Cell growth was measured using turbidity at 600 nm from 0.05 to 0.7 in LB medium and complex-formate medium under aerobic conditions, and total protein for *E. coli*

was 0.22 mg OD⁻¹ ml⁻¹ (Protein assay kit, Sigma Diagnostics, St Louis, MO).

Multiple chromosomal mutations

P1 transduction (Silhavy *et al.*, 1984) was performed in succession to knock out specific genes by selecting for the kanamycin-resistance gene that is transferred along with each chromosomal deletion that is available from the KEIO collection (Baba *et al.*, 2006). Each Keio deletion mutant is designed with the ability to eliminate the kanamycin-resistance selection marker by expressing the FLP recombinase protein from pCP20 (Cherepanov and Wackernagel, 1995) as each kanamycin-resistance gene is flanked by a FLP recognition target that is excised by FLP recombinase. Hence, plasmid pCP20 (Cherepanov and Wackernagel, 1995) was used as described previously (Datsenko and Wanner, 2000) to eliminate the kanamycin-resistance gene from each isogenic BW25113 mutant allele that was transferred to the chromosome via each P1 transduction so that the multiple mutations could be introduced into a single strain.

Hydrogen closed vial assay

Overnight, aerobic cultures (25 ml) were used to inoculate 75 ml of the complex-formate medium in 250 ml shake flasks, and these cultures were sparged for 5 min with nitrogen, sealed and incubated anaerobically at 37°C for 6 h. After 6 h the cultures were poured anaerobically into a 250 ml centrifuge tubes in an anaerobic glove box, and centrifuged (7350 g) for 10 min at 4°C. The supernatant was decanted in the glove box, and 20 ml of complex medium without formate was added, and then the cells were suspended to a turbidity of 1.3–2.5 at 600 nm. Sealed crimp-top vials (27 ml) were sparged for 5 min with nitrogen, and 9 ml of the cell suspension and 1 ml of 1 M formate were added to the bottles which were incubated at 37°C with shaking for 1 h. The amount of hydrogen generated in the headspace of the recombinant system was measured using a 50 µl aliquot by gas chromatography (GC) using a 6890N gas chromatograph as described previously (Maeda *et al.*, 2007b).

Hydrogen low partial pressure assay

Cells (30 ml) were prepared as above for the closed system, sparged, sealed in crimp-top vials (60 ml), 100 mM formate or 100 mM glucose was added, then the hydrogen gas was allowed to leave the headspace through a needle in the septum via tubing that directed the gas through 1 M NaOH [to remove carbon dioxide (Klibanov *et al.*, 1982)], and into an inverted graduated cylinder which was used to measure the volume of the gas (Fig. 2). The vials were incubated at 37°C with stirring for 30 min (formate) or 15 min (glucose), and hydrogen was assayed with the GC. For yield calculations, the vials were incubated for 16 h.

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References

- Abaibou, H., Pommier, J., Benoit, S., Giordano, G., and Mandrand-Berthelot, M.A. (1995) Expression and characterization of the *Escherichia coli* *fdo* locus and a possible physiological role for aerobic formate dehydrogenase. *J Bacteriol* **177**: 7141–7149.
- 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.
- Archana, A., Sasikala, C., and Ramana Ch, V. (2003) Augmentation of H₂ photoproduction in *Rhodospseudomonas palustris* by N-heterocyclic aromatic compounds. *Biotechnol Lett* **25**: 79–82.
- 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., and Trchounian, A. (2003) Structural and functional features of formate hydrogen lyase, an enzyme of mixed-acid fermentation from *Escherichia coli*. *Biochemistry (Mosc)* **68**: 1159–1170.
- Barker, H.C., Kinsella, N., Jaspe, A., Friedrich, T., and O'Connor, C.D. (2000) Formate protects stationary-phase *Escherichia coli* and *Salmonella* cells from killing by a cationic antimicrobial peptide. *Mol Microbiol* **35**: 1518–1529.
- Benoit, S., Abaibou, H., and Mandrand-Berthelot, M.A. (1998) Topological analysis of the aerobic membrane-bound formate dehydrogenase of *Escherichia coli*. *J Bacteriol* **180**: 6625–6634.
- Bertero, M.G., Rothery, R.A., Palak, M., Hou, C., Lim, D., Blasco, F., *et al.* (2003) Insights into the respiratory electron transfer pathway from the structure of nitrate reductase A. *Nat Struct Biol* **10**: 681–687.
- Blattner, F.R., Plunkett, G., 3rd, Bloch, C.A., Perna, N.T., Burland, V., Riley, M., *et al.* (1997) The complete genome sequence of *Escherichia coli* K-12. *Science* **277**: 1453–1474.
- Böck, A., and Sawers, G. (1996) Cellular and molecular biology. In *Escherichia coli and Salmonella*, 2nd edn. Neidhardt, F.C., Curtiss, J.R., II, Ingraham, J.L., Lin, E.C.C., Low, K.B., Magasanik, B., *et al.* (eds). Washington, DC, USA: ASM Press, pp. 262–282.
- Cherepanov, P.P., and Wackernagel, W. (1995) Gene disruption in *Escherichia coli*: Tc^R and Km^R cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* **158**: 9–14.
- Das, D., and Veziroğlu, T.N. (2001) Hydrogen production by biological processes: a survey of literature. *Int J Hydrogen Energy* **26**: 13–28.
- Datsenko, K.A., and Wanner, B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* **97**: 6640–6645.
- Demirbas, A. (2002) Fuel characteristics of olive husk and walnut, hazelnut, sunflower, and almond shells. *Ener Sour* **24**: 215–221.
- Evans, D.J., and Pickett, C.J. (2003) Chemistry and the hydrogenases. *Chem Soc Rev* **32**: 268–275.
- Forzi, L., and Sawers, R.G. (2007) Maturation of [NiFe]-hydrogenases in *Escherichia coli*. *Biometals* **20**: 565–578.
- Islam, M.N., Islam, M.N., Beg, M.R.A., and Islam, M.R. (2005) Pyrolytic oil from fixed bed pyrolysis of municipal solid waste and its characterization. *Renew Energy* **30**: 413–420.
- Jung, G.Y., Jung, H.O., Kim, J.R., Ahn, Y., and Park, S. (1999) Isolation and characterization of *Rhodospseudomonas palustris* P4 which utilizes CO with the production of H₂. *Biotechnol Lett* **21**: 525–529.
- Kitagawa, M., Ara, T., Arifuzzaman, M., Ioka-Nakamichi, T., Inamoto, E., Toyonaga, H., and Mori, H. (2005) Complete set of ORF clones of *Escherichia coli* ASKA library (a complete set of *E. coli* K-12 ORF archive): unique resources for biological research. *DNA Res* **12**: 291–299.

- Klibanov, A.M., Alberti, B.N., and Zale, S.E. (1982) Enzymatic synthesis of formic acid from H₂ and CO₂ and production of hydrogen from formic acid. *Biotechnol Bioeng* **24**: 25–36.
- Kraemer, J.T., and Bagley, D.M. (2007) Improving the yield from fermentative hydrogen production. *Biotechnol Lett* **29**: 685–695.
- Lee, S.J., Lee, D.Y., Kim, T.Y., Kim, B.H., Lee, J., and Lee, S.Y. (2005) Metabolic engineering of *Escherichia coli* for enhanced production of succinic acid, based on genome comparison and *in silico* gene knockout simulation. *Appl Environ Microbiol* **71**: 7880–7887.
- 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* (in press).
- Maeda, T., Vardar, G., Self, W.T., and Wood, T.K. (2007b) Inhibition of hydrogen uptake in *Escherichia coli* by expressing the hydrogenase from the cyanobacterium *Synechocystis* sp. PCC 6803. *BMC Biotechnol* **7**: 25.
- Menon, N.K., Robbins, J., Peck, H.D., Jr, Chatelus, C.Y., Choi, E.S., and Przybyla, A.E. (1990) Cloning and sequencing of a putative *Escherichia coli* [NiFe] hydrogenase-1 operon containing six open reading frames. *J Bacteriol* **172**: 1969–1977.
- 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.
- Messenger, S.L., and Green, J. (2003) FNR-mediated regulation of *hyp* expression in *Escherichia coli*. *FEMS Microbiol Lett* **228**: 81–86.
- Minnan, L., Jinli, H., Xiaobin, W., Huijuan, X., Jinzao, C., Chuannan, L., *et al.* (2005) Isolation and characterization of a high H₂-producing strain *Klebsiella oxytoca* HP1 from a hot spring. *Res Microbiol* **156**: 76–81.
- Oh, Y.-K., Seol, E.-H., Kim, J.R., and Park, S. (2003) Fermentative biohydrogen by a new chemoheterotrophic bacterium *Citrobacter* sp. Y19. *Int J Hydrogen Energy* **28**: 1353–1359.
- 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 Technol* **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.
- Rachman, M.A., Furutani, Y., Nakashimada, Y., Kakizono, T., and Nishio, N. (1997) Enhanced hydrogen production in altered mixed acid fermentation of glucose by *Enterobacter aerogenes*. *J Ferment Bioeng* **83**: 358–363.
- Rossmann, R., Sawers, G., and Böck, A. (1991) Mechanism of regulation of the formate-hydrogenlyase pathway by oxygen, nitrate, and pH: definition of the formate regulon. *Mol Microbiol* **5**: 2807–2814.
- Salmon, K., Hung, S.P., Mekjian, K., Baldi, P., Hatfield, G.W., and Gunsalus, R.P. (2003) Global gene expression profiling in *Escherichia coli* K12. The effects of oxygen availability and FNR. *J Biol Chem* **278**: 29837–29855.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning, A Laboratory Manual*. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press.
- 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.
- Schlensog, V., Lutz, S., and Böck, A. (1994) Purification and DNA-binding properties of FHLA, the transcriptional activator of the formate hydrogenlyase system from *Escherichia coli*. *J Biol Chem* **269**: 19590–19596.
- Self, W.T., and Shanmugam, K.T. (2000) Isolation and characterization of mutated FhIA proteins which activate transcription of the *hyc* operon (formate hydrogenlyase) of *Escherichia coli* in the absence of molybdate. *FEMS Microbiol Lett* **184**: 47–52.
- Silhavy, T.J., Berman, M.L., and Enquist, L.W. (1984) *Experiments with Gene Fusions*. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press.
- Suppmann, B., and Sawers, G. (1994) Isolation and characterization of hypophosphite-resistant mutants of *Escherichia coli*: identification of the FocA protein, encoded by the *pfl* operon, as a putative formate transporter. *Mol Microbiol* **11**: 965–982.
- Wang, H., and Gunsalus, R.P. (2003) Coordinate regulation of the *Escherichia coli* formate dehydrogenase *fdnGHI* and *fdhF* genes in response to nitrate, nitrite, and formate: roles for NarL and NarP. *J Bacteriol* **185**: 5076–5085.
- Woods, D.D. (1936) Hydrogenlyases: the synthesis of formic acid by bacteria. *Biochem J* **30**: 515–527.
- 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.