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 FhlA, 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 fdoGpCA24N-FhlA) 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 Vezirog˘lu, 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 Vezirog˘lu, 2001). Biological methods depend on hydrogenases that catalyse the reaction 2H⁺ + 2e⁻ ↔ H₂ (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 hycABCDEFGH (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 HCOO⁻ + H₂O ↔ H₂ + HCO₃⁻ (Woods, 1936) (Fig. 1). hycA encodes a repressor for FHL (Sauter et al., 1992), and fhlA encodes an essential activator of FHL (Schlensog et al., 1994). Hence, the FHL may be manipulated to increase hydrogen by overexpression of fhlA (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
mutation leads to threefold higher FhlA expression (Self and Shanmugam, 2000). Hence, hydrogen production should be increased by deleting hyaB, hybC, 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 fhlA 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, fhlA) 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
and BW25113 hyaB hybC hycA focB/pCA24N-FhlA were reduced twofold compared with the wild-type strain. Also, the specific growth rate of BW25113 hyaB hybC hycA fdoG/pCA24N-FhlA 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-FhlA is probably due to FhlA-related toxicity as adding IPTG to increase FhlA 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 FhlA (Sauter et al., 1992) (HycA may interact directly with the FhlA protein or prevent the binding of FhlA 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

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

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

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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 hydroge-

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 | H₂ production rate* |
|-------------------------|---------------------------------------------------------|-------------|----------------------|
|                         |                                                         | h⁻¹         | (µmol mg-protein⁻¹ h⁻¹) | Relative |
| BW25113/pCA24N         | Wild type                                               | 0.95 ± 0.01 | 0.8 ± 0.3           | 1        |
| BW25113/pCA24N-FhIA    | Wild type + FhlA                                         | ND          | 7 ± 4                | 9        |
| BW25113 hyaB hybC hycA | ΔhyaB and ΔhybC (defective hydrogenases 1 and 2) + FhlA | ND          | 57 ± 10              | 71       |
| BW25113 hyaB hybC hycE | ΔhyaB, ΔhybC and ΔhycE (defective hydrogenases 1, 2 and 3) | ND          | 0.3 ± 0.03           | 0.4      |
| BW25113 hyaB hybC hycA | ΔhyaB, ΔhybC and ΔhycA (defective hydrogenases 1 and 2 and defective FHL repressor) + FhlA | 0.42 ± 0.07 | 61 ± 16              | 76       |
| BW25113 hyaB hybC hycA | ΔhyaB, ΔhybC, ΔhycA and ΔfocB (defective hydrogenases 1 and 2, defective FHL repressor and defective putative formate transporter) + FhlA | 0.47 ± 0.07 | 0.24                | 113 ± 12  |
| BW25113 hyaB hybC hycA | ΔhyaB, ΔhybC, ΔhycA and ΔfdoG (defective hydrogenases 1 and 2, defective FHL repressor and defective formate dehydrogenase-O) + FhlA | 0.23 ± 0.07 | 0.44                | 141      |

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

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FhlA overexpression

FhlA 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-FhlA was added to the best eight of our recombinants, and hydrogen production was assayed (Table 1). The expression of fhlA 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-FhlA, BW25113 hyaB hybC hycA/pCA24N-FhlA versus BW25113 hyaB hybC hycA/pCA24N and BW25113 hyaB hybC hycA fdoG/pCA24N-FhlA versus BW25113 hyaB hybC hycA fdoG/pCA24N). Ultimately, BW25113 hyaB hybC hycA fdoG/pCA24N-FhlA 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-FhlA 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-FhlA, BW25113 hyaB hybC hycA fdoG/pCA24N-FhlA and BW25113 hyaB hybC hycA fdoG/pCA24N-FhlA 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-FhlA 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-FhlA 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 fhlA) 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-FhlA in the low hydrogen partial pressure system (Fig. 2). Compared with the wild-type strain BW25113/pCA24N, BW25113 hyaB hybC hycA fdoG/pCA24N-FhlA produced 3.2-fold more hydrogen after 15 min (3.7 ± 0.1 versus 12 ± 1 μmol mg-protein⁻¹ 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 H₂/mol glucose for BW25113 hyaB hybC hycA fdoG/pCA24N-FhlA).

Discussion

In this work, we show that a single fermentative E. coli strain with four mutations, hyaB hybC hycA fdoG, and which overexpresses fhlA produces 141-fold more hydrogen...
hydrogen than the wild-type strain at a rate of 113 μmol mg⁻¹ 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-FhIA) 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 (hyaC), activation of FHL by deleting the FHL repressor (hyaA), overexpressing the FHL activator (fhA), and inactivation of formate dehydrogenase-O (fdoG) to prevent formate consumption. Also, this best strain BW25113 hyaB hybC hycA fdoG/pCA24N-FhIA 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 fhA 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 pro-duction than the wild-type cells in glucose medium and the metabolic engineering increased the hydrogen yield from glucose by 50%.

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 fdoG) 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 hybB hycA background (i.e. quadruple strain BW25113 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 production is through inactivation of formate dehydrogenase-O (deletion of fdoG).

Unexpectedly, the fnr mutation, which leads to threefold higher FhIA 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 | Genotype | Source |
|---------|----------|--------|
| Escherichia coli BW25113 | lac^+ rmB114 ΔlacZΔM15 hsdR514 ΔaraBAD△araC180 ΔaraBADΔaraC180 | Yale CGSG Stock Center |
| Escherichia coli BW25113 ΔhyaB | BW25113 hyaB Km^R^; defective in large subunit of hydrogenase 1 | Baba et al. (2006) |
| Escherichia coli BW25113 ΔhyaB | BW25113 hycA Km^R^; defective in repressor of FHL | Baba et al. (2006) |
| Escherichia coli BW25113 ΔhyaB | BW25113 hycA Km^R^; defective in repressor of FHL and defective in hydrogenase 1, hydrogenase 2 and hydrogenase 3 | Baba et al. (2006) |
| Escherichia coli BW25113 ΔfocA | BW25113 focA Km^R^; defective in formate transporter | Baba et al. (2006) |
| Escherichia coli BW25113 ΔfocB | BW25113 focB Km^R^; defective in putative formate transporter | Baba et al. (2006) |
| Escherichia coli BW25113 ΔnarG | BW25113 narG Km^R^; defective in α-subunit of nitrate reductase A | Baba et al. (2006) |
| Escherichia coli BW25113 Δfnr | BW25113 fnr Km^R^; defective in FNR transcriptional dual regulator | Baba et al. (2006) |
| Escherichia coli BW25113 ΔfdoC | BW25113 fdoG Km^R^; defective in α-subunit of formate dehydrogenase-N | Baba et al. (2006) |
| Escherichia coli BW25113 ΔfdoC | BW25113 fdoG Km^R^; defective in α-subunit of formate dehydrogenase-O | Baba et al. (2006) |
| Escherichia coli BW25113 ΔhyaB ΔhyaC ΔhyaA | BW25113 hycA Km^R^; defective in large subunit of hydrogenase 1 and hydrogenase 2 and defective in repressor of FHL | This study |
| Escherichia coli BW25113 ΔhyaB ΔhyaC ΔhyaA ΔfocA | BW25113 hycA Km^R^; defective in large subunit of hydrogenase 1 and hydrogenase 2 and defective in repressor of FHL and defective in formate transporter | This study |
| Escherichia coli BW25113 ΔhyaB ΔhyaC ΔhyaA ΔfocB | BW25113 hycA Km^R^; defective in large subunit of hydrogenase 1 and hydrogenase 2 and defective in repressor of FHL and defective in putative formate transporter | This study |
| Escherichia coli BW25113 ΔhyaB ΔhyaC ΔhyaA ΔnarG | BW25113 hycA Km^R^; defective in large subunit of hydrogenase 1 and hydrogenase 2 and defective in repressor of FHL and defective in α-subunit of nitrate reductase A | This study |
| Escherichia coli BW25113 ΔhyaB ΔhyaC ΔhyaA Δfnr | BW25113 hycA Km^R^; defective in large subunit of hydrogenase 1 and hydrogenase 2 and defective in repressor of FHL and defective in FNR transcriptional dual regulator | This study |
| Escherichia coli BW25113 ΔhyaB ΔhyaC ΔhyaA ΔfdoC | BW25113 hycA Km^R^; defective in large subunit of hydrogenase 1 and hydrogenase 2 and defective in repressor of FHL and defective in α-subunit of formate dehydrogenase-N | This study |
| Escherichia coli BW25113 ΔhyaB ΔhyaC ΔhyaA ΔfdoC | BW25113 hycA Km^R^; defective in large subunit of hydrogenase 1 and hydrogenase 2 and defective in repressor of FHL and defective in α-subunit of formate dehydrogenase-O | This study |
| Escherichia coli BW25113 ΔhyaB ΔhyaC ΔhyaA ΔfdoG | BW25113 hycA Km^R^; defective in large subunit of hydrogenase 1 and hydrogenase 2 and defective in repressor of FHL and defective in formate transporter and putative formate transporter | This study |
plates (Sambrook et al., 1989) containing 100 µg ml⁻¹ kanamycin (for those with chromosomal kanamycin resistance markers) and 30 µg 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 µg ml⁻¹ kanamycin or 30 µg 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 marker by expressing the FLP recombinase protein 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.

Table 3. cont.

| Strains and plasmids | Genotype | Source |
|----------------------|----------|--------|
| **Escherichia coli** | BW25113 ΔhyaB ΔhybC ΔhyCA ΔfocA ΔnarG | This study |
| BW25113 ΔhyaB ΔhybC ΔhyCA ΔfocA ΔnarG | This study |
| BW25113 ΔhyaB ΔhybC ΔhyCA ΔfocA ΔnarG | This study |
| BW25113 ΔhyaB ΔhybC ΔhyCA ΔfocA ΔnarG | This study |
| **Plasmids** | pCA24N | Empty vector; Cm⁹ |
| pCA24N::fhlA | pCA24N pT5-lac::fhlA; expresses FhlA derived from Escherichia coli |
| pCP20 | Ap⁹ and Cm⁹ plasmid with temperature-sensitive replication and thermal induction of FLP recombinase synthesis |

Km⁹, Cm⁹ and Ap⁹ are kanamycin, chloramphenicol and ampicillin resistance respectively.

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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 anaeroberically 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 (7500 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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Hydrogen from formate by engineering Escherichia coli

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