ESCHERICHIA COLI ENHANCED HYDROGEN PRODUCTION, GENOME-WIDE SCREENING FOR EXTRACELLULAR DNA, AND INFLUENCE OF GGDEF PROTEINS ON EARLY BIOFILM FORMATION

A Dissertation

by

VIVIANA SANCHEZ TORRES

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2010

Major Subject: Chemical Engineering

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Approved by:

Chair of Committee,	Thomas K. Wood
Committee Members,	Arul Jayaraman
	Michael J. Benedik
	Zhilei Chen
Head of Department,	Michael Pishko

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ABSTRACT

Escherichia coli Enhanced Hydrogen Production, Genome-wide Screening for Extracellular DNA, and Influence of GGDEF Proteins on Early Biofilm Formation.

(December, 2010)

Viviana Sanchez Torres, B.S., Universidad Industrial de Santander

Chair of Advisory Committee: Dr. Thomas K. Wood

Escherichia coli is the best characterized bacterium; it grows rapidly, and it is easy to manipulate genetically. An increased knowledge about the physiology of this model organism will facilitate the development of engineered *E.coli* strains for applications such as production of biofuels and biofilm control. The aims of this work were the application of protein engineering to increase *E. coli* hydrogen production, the identification of the proteins regulating extracellular DNA production (eDNA), and the evaluation of the effect of the proteins synthesizing the signal 3'-5'-cyclic diguanylic acid (c-di-GMP) on biofilm formation.

The *Escherichia coli* hydrogen production rate was increased 9 fold through random mutagenesis of *fhlA*. Variant FhlA133 (Q11H, L14V, Y177F, K245R, M288K, and I342F) enhances hydrogen production by increasing transcription of the four transcriptional units regulated by FhlA. The amino acid replacements E363G and L14G in FhlA increased hydrogen production 6 fold and 4 fold, respectively.

The complete *E. coli* genome was screened to identify proteins that affect eDNA production. The *nlpI*, *yfeC*, and *rna* mutants increased eDNA production and the *hns* and *rfaD* mutants decreased eDNA production. Deletion of *nlpI* increases eDNA 3 fold while overexpression of *nlpI* decreases eDNA 16 fold. Global regulator H-NS is required for eDNA

with *E. coli* since deletion of *hns* abolished eDNA production while overexpression of *hns* restored eDNA to 70% of the wild-type levels. Our results suggest that eDNA production in *E. coli* is related to direct secretion.

Deletions of the genes encoding the diguanylate cyclases YeaI, YedQ, and YfiN increased swimming motility and eDNA as expected for low c-di-GMP levels. However, contrary to the current paradigm, early biofilm formation increased dramatically for the *yeaI* (30 fold), *yedQ* (12 fold), and *yfiN* (18 fold) mutants. Hence, our results suggest that c-di-GMP levels should be reduced for initial biofilm formation because motility is important for initial attachment to a surface.

DEDICATION

To my parents, Luz Alba and Luis Hernando, and my brother Andrés

To the memory of my grandmother Leticia Sanchez

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CHAPTER I

INTRODUCTION

1.1 Background and motivation

Escherichia coli is the best-characterized bacterium (18); however, the function of 11% of its genes is still unknown (133). The information available about the *E. coli* gene functions, metabolic pathways and its easy genetic manipulation (43) have promoted the use of this bacterium for many industrial and pharmaceutical applications. For example, *E. coli* has been the host for expression of heterologous proteins for therapeutic use, such as human insulin (51) and human growth hormone (157), and metabolic engineering of *E. coli* has been used for the production of valuable products (7, 82, 83, 93).

Microbial fermentation is a potential method for large-scale hydrogen production because it requires less energy than conventional thermal systems and electrolytic processes (176). Since *E. coli* produces hydrogen by fermentation and the enzymes and genes related to *E. coli* hydrogen production are known (19), it is an attractive microorganism to engineer for hydrogen production. Previously, we successfully enhanced *E.coli* hydrogen production and yield from formate (93) and glucose (91) through metabolic engineering and performed the first protein engineering of a hydrogenase for increased hydrogen production (94).

Biofilms are surface-attached microbial communities enclosed in a matrix (32) and are likely to be used to generate hydrogen. Approximately 99% of all bacteria in natural ecosystems are found in biofilms (146), and 80% of all bacterial infections are caused from bacteria living as biofilms (31). Biofilms also generate problems for industrial systems by causing corrosion, fouling in pipes, degradation of industrial coatings, and spoiled foods and pharmaceuticals (117).

This dissertation follows the style of Applied and Environmental Microbiology.

However, biofilms can also be beneficial for applications such as bioremediation (161), inhibition of corrosion (65), control of plant pathogens (105), waste water treatment (108), and the production of chemicals (127). In order to control biofilms it is important to know the genes and conditions promoting biofilm formation and biofilm dispersal.

The biofilm matrix is composed of polysaccharides, proteins, lipids, and extracellular DNA (eDNA) (45). eDNA has a structural role interconnecting cells in the biofilm (184). eDNA present in biofilms of the pathogen *Pseudomonas aeruginosa* promotes neutrophils proinflammatory responses (48). Neutrophils produce eDNA forming extracellular structures called NETs where pathogenic microorganisms are trapped and killed by antimicrobial proteins (25). Hence, the study of eDNA is important to control bacterial infections.

3'-5'-cyclic diguanylic acid (c-di-GMP) is an intracellular second messenger that controls several phenotypes related to biofilm formation. High levels of c-di-GMP stimulate the production of exopolysaccharides and bacterial aggregation, while inhibit motility and eDNA. Hence, the current paradigm is that c-di-GMP acts as switch between the motile planktonic and sessile biofilm lifestyles (58). c-di-GMP is synthesized by diguanylate cyclases characterized by the GGDEF domain (58) and degraded by phosphodiesterases characterized by EAL or HD-GYP domains (150). *E. coli* has 29 proteins with GGDEF/EAL domains (162) that have not been completely characterized.

1.2 Research objectives, importance, and novelty

This study seeks to improve the current knowledge about the physiology of the model organism *E. coli* which facilitates the development of engineered *E. coli* strains for industrial applications and the design of strategies to control bacterial biofilms. The specific aims were:

- Enhance *E. coli* hydrogen production by protein engineering of the transcriptional activator FhlA
- Increase the knowledge of FhlA regulation by comparing via whole-transcriptome analysis the gene expression of strains producing wild-type and engineered FhlA
- Identify the genes controlling the release of eDNA in *E. coli* via genome-wide screening for eDNA
- Identify the mechanism of *E. coli* eDNA production by measuring cell lysis and membrane vesicles production of the mutants with the highest impact on eDNA
- Evaluate the effect of the 12 *E. coli* GGDEF-only proteins on biofilm formation and motility
- Evaluate the effect on eDNA of GGDEF proteins altering biofilm formation

The engineering of *E. coli* to increase hydrogen production will facilitate the application of microbial hydrogen as an energy carrier since enhancements in hydrogen production rates will decrease the size of the reactor required to produce hydrogen (176). Random mutagenesis was applied for FhlA previously but not with the aim of increasing hydrogen production. The whole-transcriptome analysis of cells producing the variant FhlA133, which increases hydrogen production, vs. cells producing wild-type FhlA revealed new genes related to hydrogen production. This indicates that whole-transcriptome analyses of strains with increased hydrogen production vs. the wild-type strain are useful tools for the identification of new genes and metabolic pathways related to hydrogen production.

A genome-wide screening for eDNA has not been performed before. Genes controlling eDNA were rapidly identified by high-throughput screening based in the intensity of a fluorescence signal and the use of the KEIO library of single deletion mutants (9). A similar strategy may be used to study genes regulating other *E. coli* phenotypes. This is the first study that explores the mechanism of eDNA production in *E. coli*.

c-di-GMP is a signal controlling different phenotypes related to biofilm formation. However, the influence of c-di-GMP in early biofilm formation has not been studied before. This research is significant since the control of the different steps of biofilm formation is important for therapeutic and industrial applications of biofilms. The characterization of proteins related with the turnover of c-di-GMP is important to identify target proteins to engineer for biofilm control. In this study we describe phenotypes controlled by the previously uncharacterized protein YeaI and describe new phenotypes (early biofilm formation and eDNA) altered by YedQ and YfiN.

CHAPTER II

LITERATURE REVIEW*

2.1 *Escherichia coli*

The Gram-negative bacterium *Escherichia coli* is the main nonpathogenic facultative anaerobe of the human gastrointestinal tract (107). However, there are some *E. coli* strains that are pathogenic causing enteric, urinary, pulmonary, or central nervous system infections (18). *E. coli* is one of the best-studied organisms in molecular biology (9). The genome sequence of the laboratory strain *E. coli* K-12 MG1655 was determined in 1997 (18). The MG1655 chromosome contains 4464 genes (133); of these, about 11% encode proteins of unknown function, 7% correspond to phage genes and IS elements, and 3.5% encode RNAs (133).

2.2 *E. coli* hydrogen production

There are two primary pathways for microbial hydrogen production: photosynthesis and fermentation (28). Photosynthetic processes have higher yields than fermentative processes; but, of magnitude less than fermentative ones (86), and, for fermentative reactors, a variety of biomass feedstocks may be used (70). During the fermentation of sugars under anaerobic

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"Metabolic engineering to enhance bacterial hydrogen production" by Toshinari Maeda, Viviana Sanchez-Torres, and Thomas K. Wood, 2008, Microbial Biotechnology 1:30-39, Copyright 2008, Blackwell Publishing, doi: 10.1111/j.1751-7915.2007.00003.x.
"Enhanced hydrogen production from glucose by metabolically-engineered *Escherichia coli*" by Toshinari Maeda, Viviana Sanchez-Torres, and Thomas K. Wood, 2007, Applied Microbiology and Biotechnology 77:879-890, Copyright 2007, Springer-Verlag, doi: 10.1007/s00253-007-1217-0.
"Protein engineering of hydrogenase 3 to enhance hydrogen production" by Toshinari Maeda, Viviana Sanchez-Torres, and Thomas K. Wood, 2007, Applied Microbiology and Biotechnology 79:77-86, Copyright 2008, Springer-Verlag, doi: 10.1007/s00253-008-1416-3.

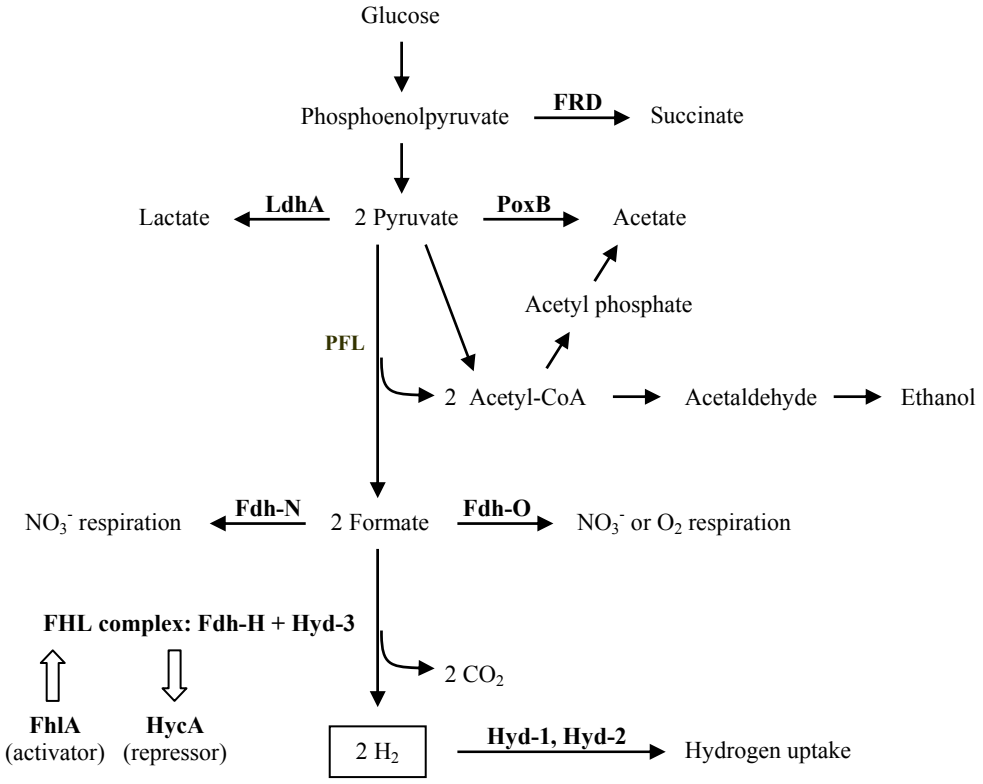

Fig. 2.1. Fermentative hydrogen production from glucose by *E. coli*. Through glucose fermentation, hydrogen is produced from formate by the FHL complex. The maximum theoretical hydrogen yield is 2 mol of H_2 per mol of glucose and 1 mol of H_2 per mol of formate (176). The fermentation pathway yields succinate, lactate, acetate, ethanol, and formate, as fermentation end-products. The key proteins are shown in bold. FRD, fumarate reductase; PFL, pyruvate formate lyase; Fdh, formate dehydrogenase; FHL, formate hydrogen lyase; Hyd, hydrogenase. (Adapted from Vardar-Schara et. al. (176)).

conditions, *E. coli* produces molecular hydrogen from formate (Fig. 2.1) by the formate hydrogen lyase complex (FHL).

The structural components of the FHL complex are formate dehydrogenase-H encoded by *fdhF* (8) which converts formate into $2H^+$, $2e^-$, and CO_2, hydrogenase 3 (Hyd-3) encoded by the *hycE* (large subunit) and the *hycG* (small subunit), which is a NiFe hydrogenase (148) that synthesizes molecular hydrogen (147), and the electron transfer proteins encoded by *hycBCDF* (148) (Fig. 2.2). The FHL complex also requires the protease HycI (137), the putative electron carrier HydN (96), and the maturation proteins HycH (147), HypF (96), and HypABCDE (64); all of these proteins constitute the formate regulon.

FhlA is the transcriptional activator of the formate regulon; it requires formate to activate transcription from -12/-24 promoter regions by the σ^{54}-RNA polymerase complex. It is not known if FhlA can activate transcription of other genes that are not part of the formate regulon since a whole-transcriptome analysis has not been performed. FhlA is the last gene of the *hyp* operon and is transcribed from three promoters, the *hyp* promoter (P_{hyp}), a FNR dependent promoter located within *hypA* (P_{hypB}), and its own weak constitutive promoter. FhlA is a protein composed of three domains: the N-domain (amino acids 1 to 381) (104) is responsible for binding formate (84), the central domain (amino acids 388 to 617) (104) is responsible of ATP hydrolysis and activation of transcription once formate is bound to the N-domain (84), and the C-terminal domain (amino acids 618 to 692) (104) contains a helix-turn-helix motif responsible for DNA binding (151).

HycA is a repressor that counteracts FhlA activity (147). The mechanism of repression of FHL by HycA is not completely known; previous studies demonstrated that HycA inhibits the transcription of the *hyc* (84) and *hyp* operons (147) but its effect in the transcription of the other

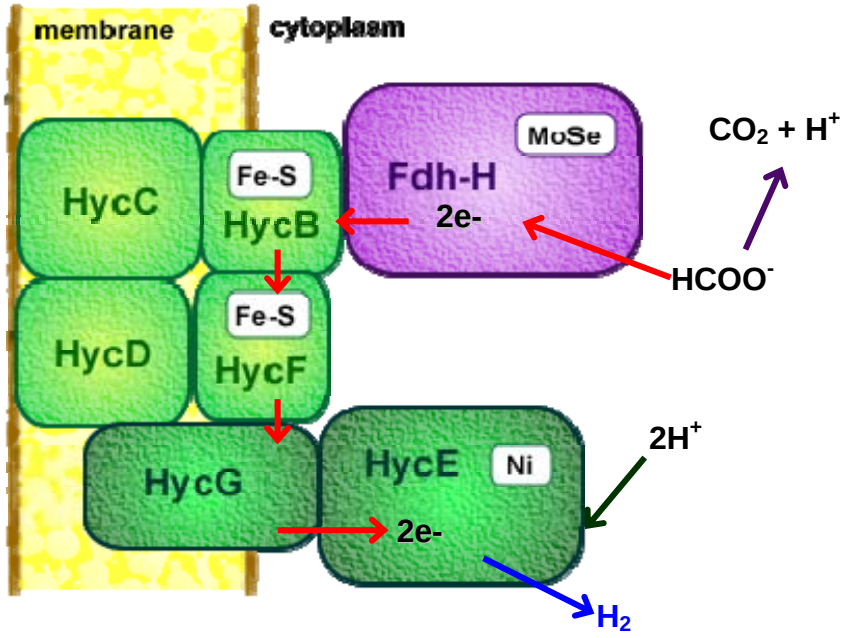

Fig. 2.2. Putative model for the organization of the components of FHL complex. The red arrows indicate the transport of electrons. (Adapted from Sauter. et. al. (147)).

units of FHL (*fdhF* and *hydN-HypF*) has not been studied and it is not known if this repressor affects the transcription of other genes that are not part of FHL.

2.2.1 E. coli hydrogenase 3 is a reversible enzyme possessing hydrogen uptake and synthesis activities

E. coli possesses four nickel-iron hydrogenases: hydrogenase-1 (Hyd-1) (100) encoded by the *hya* operon, hydrogenase-2 (Hyd-2) (99) encoded by the *hyb* operon, hydrogenase-3 (Hyd-3) (23) encoded by *hyc* operon, and hydrogenase-4 (Hyd-4) (3) encoded by the *hyf* operon. Under anaerobic conditions and in the absence of electron acceptors such as oxygen and nitrate (8), Hyd-3 produces H_2 through the FHL complex while Hyd-1 and Hyd-2 function as uptake hydrogenases catalyzing H_2 oxidation (75). Hyd-4, encoded by the *hyf* operon (3) is not expressed in *E. coli*.

Previously, it was reported that *E. coli* hydrogenase 3 has only hydrogen production activity (10, 102); however, the [NiFe] hydrogenases of other bacteria such as *Synechocystis* sp. PCC 6803 (4) and *Desulfovibrio gigas* (36) are reported as reversible enzymes possessing hydrogen synthesis and uptake activities. We evaluated the hydrogen uptake and synthesis activity of hydrogenase 3 independent of the other two active *E. coli* hydrogenases (Hyd-1 and Hyd-2); we found that Hyd-3 has a significant hydrogen uptake activity (compare 24 ± 9 nmol H_2 min^{-1} mg-$protein^{-1}$ for BW25113 *hyaB hybC* with 9 ± 1 nmol H_2 min^{-1} mg-$protein^{-1}$ for BW25113 *hyaB hybC hycE* (92)) and corroborated that Hyd-3 is the primary hydrogenase responsible for hydrogen synthesis in *E. coli* (92). Recently, it was reported that Hyd-1 (74) and Hyd-2 (172) can also function as H_2 synthesizing enzymes under conditions where the FHL complex was inactive; hence, Hyd-1, Hyd-2, and Hyd-3 are reversible enzymes with H_2 uptake and production activity.

2.2.2 Metabolic engineering to increase E. coli hydrogen production

We sought to increase the hydrogen production rate and yield by directing the metabolism from glucose and formatetoward hydrogen formation through the accumulation of multiple deletions of those genes responsible for competitive reactions or that remove formate. We increased the hydrogen production rate 141 fold and achieved the theoretical yield from formate (1 mol H_2/mol formate) by constructing the strain BW25113 *hyaB hybC hycA fdoG*/pCA24N-*fhlA*^{2} (93); we also enhanced the hydrogen production rate from glucose 4.6 fold and the yield 2 fold by constructing the strain BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE*^{3} (91). To construct the strains, we used the BW25113 isogenic deletion library (Keio collection) to perform multiple rounds of P1 transduction, followed by selection on kanamycin plates and elimination of the kanamycin resistance marker using the FLP recombinase of pCP20.

2.2.2.1 Rapid gene knock-out

This is a novel strategy for metabolic engineering to enhance hydrogen production; it has many advantages such as only one antibiotic is used, the addition of a new deletion takes only two days, the mutations are stable, the cell viability is not significantly affected, and it is possible to continue adding mutations to the same strain. A schematic of the general method used to accumulate deletions in a single strain to increase the hydrogen production is shown on Fig. 2.3. To increase the hydrogen production rate and yield from formate and glucose by directing the metabolic flux towards hydrogen, we proceeded with the following strategy: (i) removal of hydrogen uptake by deleting the large subunit of Hyd-1 (*hyaB*) and Hyd- 2 (*hybC*), (ii) deletion of the FHL repressor *hycA,* (iii) once the *hyaB hybC hycA* strain was constructed, to enhance the hydrogen production from formate we explored additional deletions of the formate transporters *focA* and *focB* (to prevent formate export), (iv) inactivation of the formate

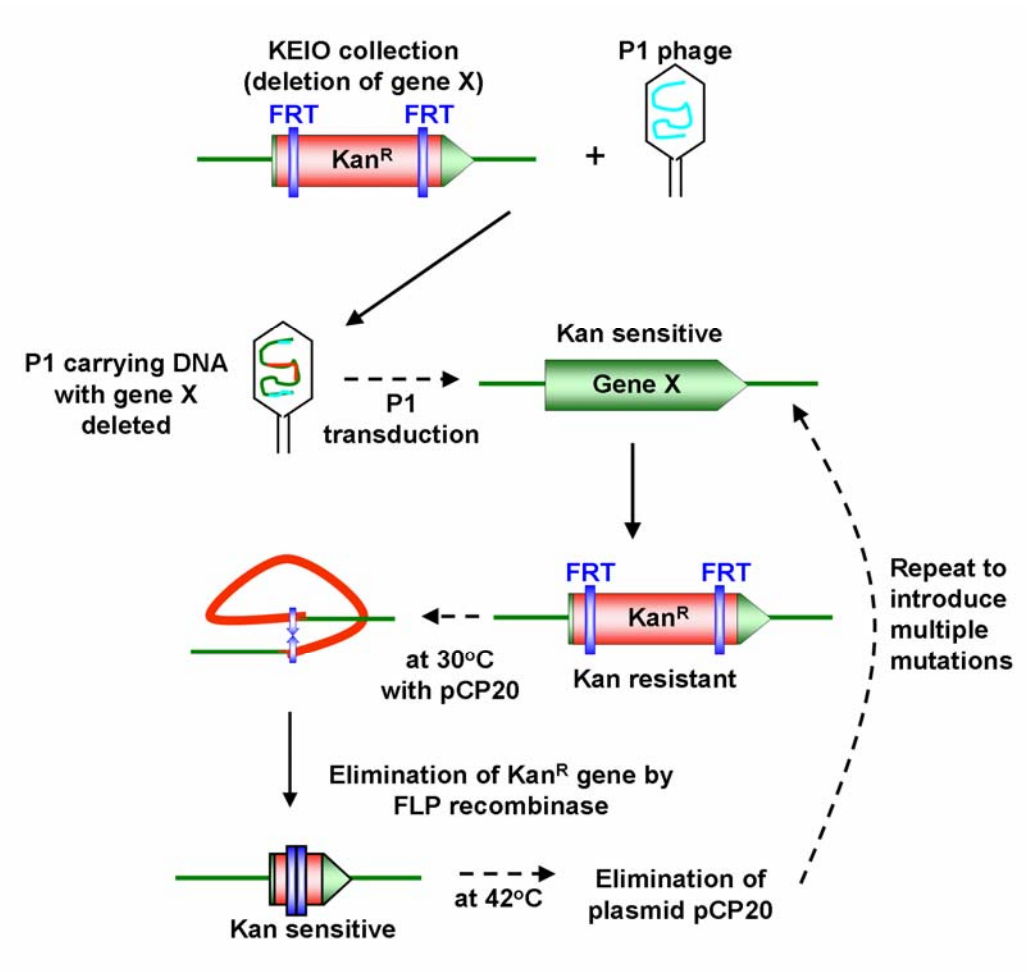

Fig. 2.3. Rapid gene knock-out. The first step is the construction of a P1 phage carrying the DNA surrounding the Kan^R marker of the Keio strain of the gene that we want to delete; then, this phage is used to infect the metabolically-engineered strain and transfer the deletion by homologous recombination. The Kan^R gene is removed from the chromosome of the metabolically-engineered strain using the FLP recombinase of pCP20 at 30°C. After elimination of the plasmid pCP20 at 42°C, the new strain may be subject to new deletions. (Based on Baba et al. (9))

consumption by formate dehydrogenase-N (*fdnG* and *narG*) and formate dehydrogenase-O (*fdoG*), (v) deletion of *fnr* because it increases *fhlA* expression, and (vi) overexpression of the activator *fhlA* using pCA24N-*fhlA*. For the hydrogen production from glucose, we explored the same mutations used for the metabolic engineering from formate plus inactivation of succinate synthesis (*frdC*), lactate synthesis (*ldhA*), and pyruvate consumption (*aceE* and *poxB*).

2.2.3 Protein engineering of hydrogenase 3 to enhance E. coli hydrogen production

We sought to increase the *E. coli* hydrogen production rate by evolving through random mutagenesis the large subunit of Hyd-3 (HycE). We used three different techniques for the mutagenesis of *hycE*. First, we performed error-prone polymerase chain reaction (epPCR) of the whole *hycE* using plasmid pBS(Kan)HycE (92) as a template, pBS(Kan) as vector, and BW25113 *hyaB hybC hycE* which is defective in active hydrogenases as host. Second, we performed DNA shuffling using the mutants with enhanced hydrogen production activity isolated from the HycE epPCR library as template. Third, we performed saturation mutagenesis at position T366 because this position was mutated in two of the variants with enhanced hydrogen production obtained from the epPCR library.

2.2.3.1 Screening

We used chemochromic membranes (GVD Corp., Cambridge, MA) as a high-throughput screening method for the epPCR, DNA shuffling, and saturation mutagenesis HycE libraries. This is a novel screening method that directly detects the hydrogen produced by the bacteria (Figure 2.4).

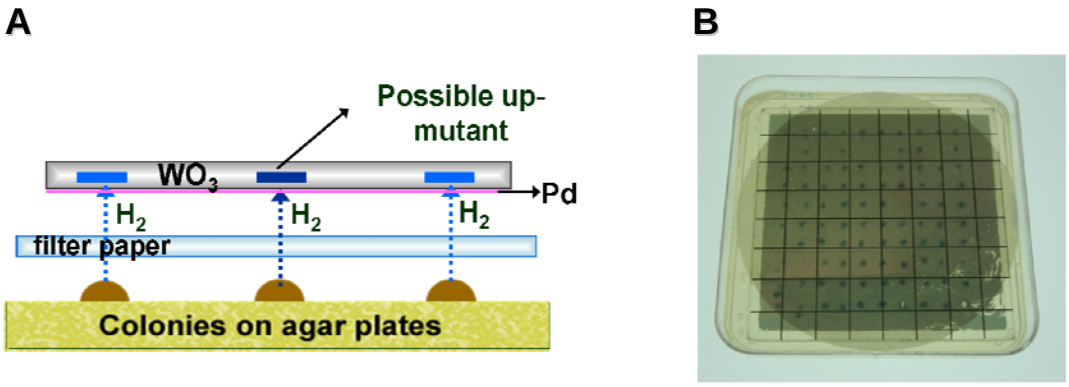

Fig. 2.4. Chemochromic membranes for hydrogen screening. (A) The chemochromic membrane is placed over filter paper that covers a complex-formate agar plate with colonies incubated anaerobically; the hydrogen produced by the colonies go through a catalytic layer of palladium and then reacts in a film of WO_3 producing blue color. The colonies with higher blue intensity are selected as possible up-mutants. (B) Screening using chemochromic membranes.

2.2.3.2 Hydrogen production by HycE variants

The hydrogen production by BW25113 *hyaB hybC hycE* expressing wild-type HycE or re-electroporated HycE variants via pBS(Kan)-HycE was evaluated by gas chromatography. Using epPCR, we isolated 7 variants that increased the hydrogen production rate from formate up to 17-fold relative to wild-type HycE; using DNA shuffling, we found that a 74 amino acid C-terminal truncation that increased the hydrogen production rate 23-fold; and using saturation mutagenesis, we found that a 204 amino acid truncation increases the hydrogen production rate 30-fold (Table 2.1). Unexpectedly, the best hydrogen producing mutants are truncated proteins defective Cys531 and Cys534 which are important residues for the active site of Ni-Fe hydrogenases such as the hydrogenase of *D. gigas* (179).

2.3 Biofilm formation

Biofilms are communities of cells enclosed in a self-produced polymeric matrix attached to a surface; biofilms are highly structured since cells form clusters separated by open water channels that provide nutrients to the cells that are deep in the complex structure (166). Biofilm formation gives advantages to the bacterial cells such as increased tolerance to stress, antibiotics, and host immunological defenses (34). The biofilm matrix is composed by polysaccharides, proteins, lipids, and nucleic acids (45).

2.4 Extracellular DNA (eDNA)

eDNA is an important part of the biofilm matrix of many Gram-positive (55, 132, 171, 178) and Gram-negative bacteria (57, 81, 184) working as a cell-to-cell interconnecting compound (81). eDNA has been also detected in planktonic cultures of different bacteria (40, 110).

Table 2.1. Hydrogen production by HycE variants.

HycE variants	HycE aa changes[b]	H_2 production rate[a]	
		μmol mg-protein^{-1} h^{-1}	fold-change
wild-type	wild-type HycE	0.3 ± 0.4	1
epHycE17	F297L, L327Q, E382K, L415M, A504T, D542N	2.1 ± 0.5	7
epHycE21	Q32R, V112L, G245C, F409L	4.6 ± 0.2	15
epHycE23-2	D210N, I271F, K545R	2.3 ± 0.6	8
epHycE39	I333F, K554*	2 ± 1	7
epHycE67	S2P, E4G, M314V, T366S, V394D, S397C	4 ± 2	13
epHycE70	D202V, K492*	3.2 ± 0.1	11
epHycE95	S2T, Y50F, I171T, A291V, T366S, V433L, M444I, L523Q	5.0 ± 0.6	17
shufHycE1-9	Y464*	6.8 ± 0.5	23
satHycE12T366	T366*	9 ± 4	30
satHycE19T366	T366*	8 ± 1	27

[a] The rate was calculated from hydrogen production after 5 h in complex-formate medium
[b] Asterisk (*) indicates a truncation

The most common mechanism of eDNA release is cell lysis (1, 115, 126, 165). Membrane vesicles (MVs) released from the outer membrane of Gram-negative bacteria (184) are also present in the biofilm matrix (153); MVs was proposed as mechanism for eDNA release (184) since they contain DNA and enzymes that promote lysis (68). eDNA release can also occur by secretion from intact cells as has been reported in *Bacillus subtilis*, associated with spores formation (24), and in *Neisseria meningitidis,* through a genetic island conferring a type IV secretion system (54).

eDNA release regulated by quorum-sensing during late-log phase culture has been reported in *P. aeruginosa* (1) and in *Streptococcus pneumoniae* (165). We also have reported that eDNA levels are inversely related to 3'-5'-cyclic diguanylic acid (c-di-GMP) in *P. aeruginosa* (174). eDNA is dependent on the presence of genes related to purine synthesis in *Bacillus cereus* (178).

2.5 Regulation of *E. coli* biofilm formation

Biofilm formation occurs following a developmental sequence (111). First, bacteria move in the liquid culture and reach a surface where cells are reversibly attached due to attracting and repulsing forces between the bacteria and the surface (134); some cells have a strong adhesion to the surface enhanced by the secretion of exopolymeric materials becoming irreversibly attached (53). These cells can be redistributed on the surface by pilus-mediated twitching motility (112). Through cell division and the recruitment of cells from the bulk fluid, cells aggregate forming mature biofilms; the extracellular polymeric substances interconnect the cells (166). Finally cells disperse from the biofilm and return to a motile state (71); dispersal allows cells to find other conditions that are more favorable than the biofilm and let the cell colonize other habitats (71).

The mechanisms regulating biofilm formation are complex since the products of different genes are required at different times in the biofilm development (134). Swimming motility is important for the initial contact between cells and the surface (124), type 1 fimbriae are required for the attachment to a surface (124), and curli fimbriae (30) and the conjugation pilus promote cell-surface and cell-cell interactions (128). While the biofilm is growing, the outer membrane protein Antigen 43 encoded by *flu* promotes cell-cell adhesion and inhibits motility (175). Extracellular polysaccharides (e.g., β-1,6-N-acetyl-D-glucosamine polymer, colonic acid, and cellulose) are important for biofilm maturation since they provide structural support for the biofilm (167) while cell surface polysaccharides (lipopolysaccharides, capsules) are important for interaction of bacteria with the environment (134). For biofilm dispersal, swimming motility is required (71).

2.5.1 Regulation of E. coli biofilm formation by c-di-GMP

c-di-GMP is a second messenger whose synthesis is performed by diguanylate cyclases (harboring GGDEF domains) and its degradation is performed by phosphodiesterases (harboring EAL or HD-GYP domains) (162). *E.coli* K-12 has 12 proteins with a GGDEF domain (AdrA, YdaM, YcdT, YddV, YdeH, YeaP, YeaI, YeaJ, YedQ, YfiN, YliF, and YneF) (Fig. 2.5), 10 proteins with an EAL domain (Rtn, YcgF, YcgG, YdiV, YhjH, YjcC, YlaB, YliE, YoaD, and YahA), and 7 proteins with both GGDEF and EAL domains (YciR, Dos, YegE, YfeA, YfgF, YhdA, and YhjK) (162).

c-di-GMP increases biofilm formation in Gram-negative bacteria by stimulating the production of adhesion factors and by repressing motility (67). The levels of GGDEF and EAL proteins are controlled by transcriptional and posttranscriptional mechanisms. The diguanylate cyclase YdaM activates transcription of the curli regulator CsgD thus promoting the synthesis of

curli fimbriae. YdaM activity is counteracted by the action of YciR (GGDEF + EAL) that functions as a phosphodiesterase. The global regulator H-NS promotes curli formation by inducing *ydaM* expression while repressing *yciR* (182).

The GGDEF protein YddV also promotes curli formation by activating transcription of the *csgBAC* operon (encoding curli structural subunits) but not by affecting the expression of *csgD*. The phosphodiesterases Dos (GGDEF + EAL) counteracts the activity of YddV (169). The GGDEF protein YedQ promotes cellulose synthesis in the commensal strain *E. coli* 1094 independently of CsgD (33). The carbon storage regulator CsrA binds to the mRNA of *ycdT* and *ydeH* inhibiting the synthesis of the corresponding GGDEF proteins (67).

Motility is reduced by c-di-GMP through the action of YcgR. YcgR with bound c-di-GMP interacts with MotA (21), FliG, and FliM (118) reducing the flagella motor speed thus breaking flagella movement. The PilZ domain of YcgR is the region responsible of c-di-GMP binding (140). Deletion of the gene encoding the phosphodiesterase YhjH impairs motility (c-di-GMP should increase); motility of the *yhjH* mutant was partially restored by single deletions of the genes encoding YegE (GGDEF + EAL, probably acting as diguanylate cyclase), and the GGDEF proteins YedQ, YfiN, and YddV (21).

Fig. 2.5. Alignment of *E. coli* proteins with a GGDEF domain. The sequences were obtained from Ecogene (139) and only the fragment of the sequence close to the catalytic site was analyzed using COBALT (116). The conserved residues are highlighted in yellow; the "A-site" corresponds to the active site and the "Ï-site" is the site where c-di-GMP binds acting as an allosteric inhibitor. The GG[D/E]EF motif is conserved except for YeaI.

CHAPTER III

PROTEIN ENGINEERING OF THE TRANSCRIPTIONAL ACTIVATOR FHLA TO ENHANCE HYDROGEN PRODUCTION IN *ESCHERICHIA COLI* *

3.1 Overview

Escherichia coli produces H_2 from formate via the formate hydrogenlyase (FHL) complex during mixed acid fermentation; the FHL complex consists of formate dehydrogenase H (encoded by *fdhF*) for forming $2H^+$, $2e^-$, and CO_2 from formate and hydrogenase 3 (encoded by *hycGE*) for synthesizing H_2 from $2H^+$ and $2e^-$. FHL protein production is activated by the σ^{54} transcriptional activator FhlA, which activates transcription of *fdhF* and the *hyc*, *hyp*, and *hydN-hypF* operons.

Here, through random mutagenesis using error-prone PCR over the whole gene, as well as over the *fhlA* region encoding the first 388 amino acids of the 692-amino-acid protein, we evolved FhlA to increase H_2 production. The amino acid replacements in FhlA133 (Q11H, L14V, Y177F, K245R, M288K, and I342F) increased hydrogen production 9 fold, and the replacements in FhlA1157 (M6T, S35T, L113P, S146C, and E363K) increased hydrogen production 4 fold.

Saturation mutagenesis at the codons corresponding to the amino acid replacements in FhlA133 and at position E363 identified the importance of position L14 and of E363 for the increased activity; FhlA with replacements L14G and E363G increased hydrogen production (4 fold and 6 fold, respectively) compared to FhlA. Whole-transcriptome and promoter reporter

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*Reprinted with permission from "Protein engineering of the transcriptional activator FhlA to enhance hydrogen Production in *Escherichia coli*" by Viviana Sanchez-Torres, Toshinari Maeda, and Thomas K. Wood, 2009, Applied & Environmental Microbiology 75: 5639-5646, Copyright 2009, American Society for Microbiology, doi:10.1128/AEM.00638-09. V. Sanchez-Torres performed all of the experiments except the construction of the JW098 strain.

constructs revealed that the mechanism by which the FhlA133 changes increase hydrogen production is by increasing transcription of all of the genes activated by FhlA (the FHL complex). With FhlA133, transcription of P_{fdhF} and P_{hyc} is less sensitive to formate regulation, and with FhlA363 (E363G), P_{hyc} transcription increases but P_{hyp} transcription decreases and hydrogen production is less affected by the repressor HycA.

3.2 Introduction

Hydrogen is a promising fuel, since it can be produced from renewable sources (56) and its combustion does not produce pollutants, such as CO, CO_2, and SO_2, like conventional fossil fuels (103). To create a sustainable energy system based on hydrogen, improvements in hydrogen production are required to make it competitive with fossil fuels (103). It is important to note that the cost of new infrastructure to transport hydrogen may be avoided if hydrogen can be generated at the end user's location rather than at a central production facility (192).

Microbial fermentation is a potential method for large-scale hydrogen production (35), and there are two primary means of microbial hydrogen production: photosynthesis and fermentation. Fermentative reactors have the advantage that waste biomass (70) may be used as a feedstock. In addition, reactors with fermentative bacteria are considered more practical than those with photosynthetic bacteria, as photosynthetic systems require reactors with large surface areas (16) and have hydrogen production rates orders of magnitude lower than those of fermentative bacteria (86).

The hydrogen required to power a home using a 1-kW hydrogen fuel cell is 24 mol H_2/h (86). If hydrogen is produced by fermentation of glucose, the annual cost of the glucose is approximately $6,400 (176). To decrease the cost, it is necessary to increase the yield or use less expensive feedstocks (176). The hydrogen yield may be increased by utilizing additional

fermentation end products, such as acetate, succinate, and lactate, to produce hydrogen. To power a home, the required size of the reactor for hydrogen production by fermentation of glucose or formate is approximately 500 liters. This size may be reduced by increasing the hydrogen production rate (176).

Escherichia coli is an attractive fermentative microorganism to engineer for hydrogen production because the majority of enzymes and genes related to hydrogen production are known (19) and it is easy to manipulate genetically (43). Under anaerobic conditions, *E. coli* produces hydrogen from formate through the reaction $HCOO^- + H_2O \leftrightarrow H_2 + HCO_3^-$, which is catalyzed by the formate hydrogenlyase (FHL) complex (188). The structural components of the FHL complex are formate dehydrogenase H, encoded by *fdhF* (8), which converts formate to $2H^+$, $2e^-$, and CO_2; hydrogenase 3 (Hyd-3), encoded by *hycE* (large subunit) and *hycG* (small subunit), which is reported to be a NiFe hydrogenase (148) that synthesizes molecular hydrogen from $2H^+$ and $2e^-$ (147); and the electron transfer proteins encoded by *hycBCDF*, which are thought to shuttle electrons between formate dehydrogenase H and Hyd-3 (148). An active FHL complex also requires the protease HycI (137), the putative electron carrier HydN (96), and the maturation proteins HycH (147), HypF (96), and HypABCDE (64).

The FHL complex has at least two regulators, FhlA and HycA. FhlA, the product of the last gene of the *hyp* operon (151) (Fig. 3.1), is the transcriptional activator of the *fdhF* gene and the *hyc*, *hyp*, and *hydN-hypF* operons, which form the formate regulon (79). In addition to the FhlA-dependent promoter P_{hyp}, *fhlA* is also transcribed RNA polymerase complex. FhlA with bound formate (62) binds to the upstream activating sequences (UAS) located about 100 bp upstream of the transcriptional start site of *fdhF* (17) and to *hydN-hypF* (96), in the region between the divergently transcribed *hyp* and *hyc* operons for activation of *hyc*, and in the intergenic region between *hycA* and *hycB* for the activation of *hyp* (152) (Fig. 3.1). Intracellular

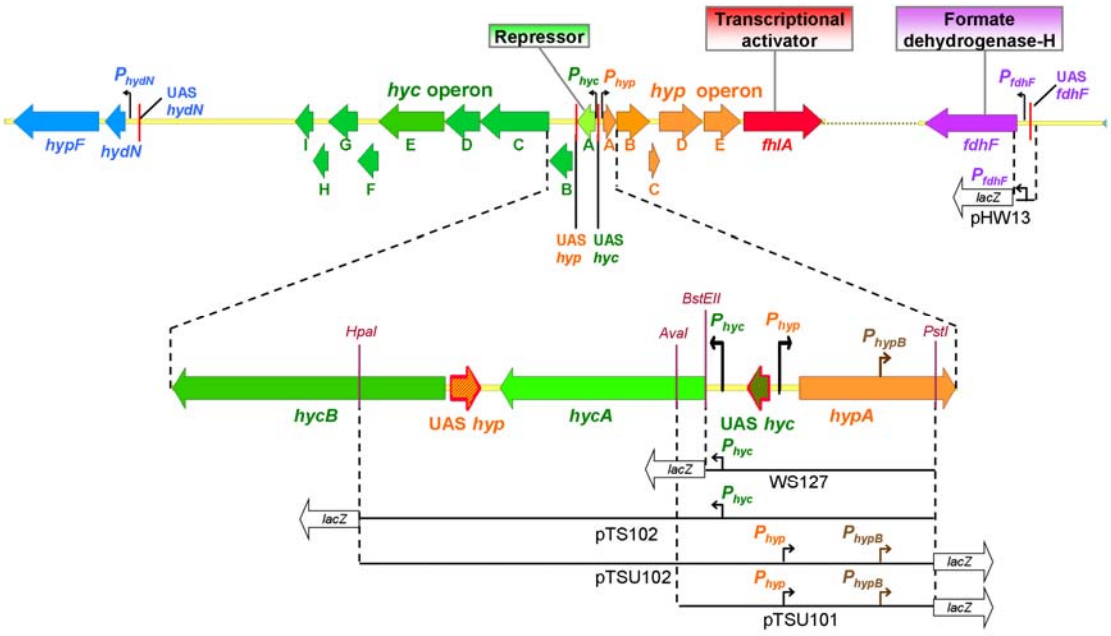

Fig. 3.1. Physical map of the transcriptional units activated by FhlA. Coding regions are represented by block arrows, –12/–24 promoters are indicated by black right-angled arrows, the FNR-dependent promoter P*hypB* is indicated by brown right-angled arrows, and the UAS where FhlA binds (17, 96, 152) are shown by a red-hatched arrow and red boxes. The fragments present in the *lacZ* reporter fusions used for the transcriptional studies are indicated in black (the *lacZ* gene is not drawn to scale).

molybdate is required for transcription of *fdhF* and *hyc* (136). Also, for maximum transcription of *hyc*, the integration host factor must bind between the UAS and the promoter of the *hyc* operon (61). FhlA, as a member of the σ^{54} family, has a structure composed of three domains (104, 158). The N domain (amino acids 1 to 381) (104) is responsible for binding formate and oligomerization as a tetramer (84); it is very large, and its sequence does not show similarity to other σ^{54} regulators (151). The central domain (amino acids 388 to 617) (104) is responsible for ATP hydrolysis once formate is bound to the N domain; this reaction is essential for the formation of the open complex of RNA polymerase with DNA, which leads to transcription initiation (62). This region is not influenced by formate and is thought to interact with the RNA polymerase-σ^{54} complex (84). The C-terminal domain (amino acids 618 to 692) (104) contains a helix-turn-helix motif responsible for DNA binding (151).

Most of the previous studies to enhance *E. coli* hydrogen production have focused on metabolic engineering (119, 120, 191); for example, we achieved a 141-fold enhancement with the *hyaB*, *hybC*, *hycA*, and *fdoG* mutations coupled with overexpression of *fhlA*$^+$ using formate as the substrate (93), and a 4.6-fold enhancement was achieved with the *hyaB*, *hybC*, *hycA*, *fdoG*, *frdC*, *ldhA*, and *aceE* mutations using glucose as the substrate (91). In contrast, protein engineering has not been used extensively to increase hydrogen production, although we recently reported that hydrogen production may be increased 30-fold by using error-prone PCR (epPCR), DNA shuffling, and saturation mutagenesis of *hycE* (the large subunit of Hyd-3) (94). In this work, we sought to increase hydrogen production by *E. coli* through epPCR and saturation mutagenesis of *fhlA*.

3.3 Materials and methods

3.3.1 Bacterial strains, growth, and total protein

The *E. coli* strains and plasmids used in this study are listed in Table 3.1; all strains were grown at 37°C. Overnight cultures were made from fresh, single colonies using either Luria-Bertani medium (142), modified complex medium (93) without formate, or modified complex-formate medium with 20 mM formate. Antibiotics were used to maintain plasmids, as well as to select the host, and were used at the following concentrations: ampicillin at 100 μg/ml, chloramphenicol (Cm) at 30 μg/ml, kanamycin (Km) at 100 μg/ml, and spectinomycin at 50 μg/ml. The total protein concentration was 0.22 mg ml^{-1} (turbidity at 600 nm)$^{-1}$ (177). JW0098 (Δ*oxyS*) was constructed via P1 transduction (160) by selecting for Cm resistance that was transferred along with the *oxyS* deletion from *E. coli* K-12 GSO35 (2). For each strain from the Keio collection, the deletion of the target gene was verified by two PCRs (Table 3.2). First, to determine if the wild-type allele was deleted, a PCR using a primer upstream of the target gene and a primer inside the coding region of the target gene was performed. Second, to verify that the Km resistance gene was inserted at the target locus, a PCR using a primer upstream of the target gene and a primer inside the coding region of the Km resistance gene was performed. The deletions in strains JW0098 and MW1002 were also verified by PCR.

3.3.2 Random mutagenesis of fhlA

The *fhlA* gene from plasmid pASKA2701 (76) under the control of the *p*T*5-lac* promoter was mutated by epPCR as described previously (44) using 50 pmol of each primer (FhlAfront and FhlArev) (Table 3.2), 0.5 mM $MnCl_2$, and a 3-min extension time. The epPCR product was

Table 3.1. Strains and plasmids used in this study.

Strains and plasmids	Genotype; description[a]	Source
E. coli strains		
BW25113	F$^-$ *Δ(araD-araB)567 ΔlacZ4787*(*::*rrnB-3) LAM$^-$ *rhp-1 Δ(rhaD-rhaB)568 hsdR514*; parental strain for the Keio collection.	Yale Coli Genetic Stock Center
JW2701-1	BW25113 *ΔfhlA735::kan* Km^R; lacks the gene which encodes the FHL complex activator	(9)
JW0833-1	BW25113 *ΔgrxA750::kan* Km^R; lacks the gene which encodes glutaredoxin 1	(9)
JW0599-1	BW25113 *ΔahpF745::kan* Km^R; lacks the gene which encodes alkyl hydroperoxide reductase, subunit F	(9)
JW0098	BW25113 *ΔoxyS2::cm* Cm^R; lacks the gene which encodes oxidative stress regulator OxyS	This study
MW1002	BW25113 *ΔhyaB887 ΔhybC867 ΔfhlA735::kan* Km^R; lacks the gene that encodes the large subunit of uptake hydrogenases 1 and 2 and lacks the gene which encodes the FHL complex activator	(92)
WS127	*cysC43 srl-300: :Tn10 thr-1 leu-6 thi-1 proA2 galK2 ara-14 xyl-5 mtl-1 lacY1 his-4 argE3 rpsL31 tsx-33* Δ*(srl-fhlA)* Δ*lac* Δ*mod* λWS1Δ*gal mod*$^+$ Ap^R; λWS1 harbors the *hyc* UAS and P_{hyc}*::lacZ.*	(156)
Plasmids		
pCA24N	*lacI*^{q}, Cm^R; cloning vector	(76)
pASKA2695[b]	pCA24N $P_{T5\text{-}lac}$*::hycA* Cm^R; encodes wild-type HycA	(76)
pASKA2701[b]	pCA24N $P_{T5\text{-}lac}$*::fhlA* Cm^R; encodes wild-type FhlA	(76)
pVSC133	pCA24N $P_{T5\text{-}lac}$*::fhlA133* Cm^R; encodes FhlA with Q11H, L14V, Y177F, K245R, M288K, I342F	This study
pVSC14	pCA24N $P_{T5\text{-}lac}$*::fhlA14* Cm^R; encodes FhlA with L14G	This study
pVSC1157	pCA24N $P_{T5\text{-}lac}$*::fhlA1157* Cm^R; encodes FhlA with M6T, S35T, L113P, S146C, E363K	This study
pVSC363	pCA24N $P_{T5\text{-}lac}$*::fhlA363* Cm^R; encodes FhlA with E363G	This study
pVLT35	RSFl0l0 *lacI*$^q P_{tac}$ Sm^R/Sp^R*;* broad-host-range cloning vector	(37)
pVSV2701	pVLT35 P_{tac}*::fhlA* Sm^R/Sp^R; encodes wild-type FhlA	This study
pVSV133	pVLT35 P_{tac}*::fhlA133* Sm^R/Sp^R; encodes FhlA with Q11H, L14V, Y177F, K245R, M288K, I342F	This study
pVSV363	pVLT35 P_{tac}*::fhlAE363G* Sm^R/Sp^R; encodes FhlA with E363G	This study
pHW13	P_{fdhF}*::lacZ* Ap^R	(181)

Table 3.1. (continued)

Strains and plasmids	Genotype; description[a]	Source
pTSU101	P_{hyp}*::lacZ* Km^R Ap^R*;* harbors the *hyc* UAS	(152)
pTS102	P_{hyc}*::lacZ* Km^R Ap^R; harbors the *hyc* UAS*, hycA* and *hyp* UAS	(152)
pTSU102	P_{hyp}*::lacZ* Km^R Ap^R*;* harbors the *hyc* UAS*, hycA* and *hyp* UAS	(152)

[a] Km^R, Cm^R, Ap^R, Sm^R, Sp^R are kanamycin, chloramphenicol, ampicillin, streptomycin, and spectinomycin resistance, respectively.
[b] The plasmids from the ASKA collection were named as pASKAnumber where the number indicates the JW locus-tag of the ORF that is inserted.

Table 3.2. Primers used for epPCR, saturation mutagenesis, DNA sequencing, qRT-PCR and verification of the relevant deletions in the strains used.

Primer	Purpose	Sequence[a,b]
FhlAfront	epPCR of *fhlA* and the *fhlA* region encoding the N-domain of FhlA	5'-CACCGATCGCCCTTCCCAACAGTTGC-3'
FhlArev	epPCR of *fhlA*	5'-CATCCGCTTACAGACAAGCTGTGACC-3'
FhlAN	epPCR of the *fhlA* region encoding the N-domain of FhlA	5'-GAGATTATGGATCGCACGGGCAATCAGC-3'
FhlAfQ11	Saturation mutagenesis at position Q11 of FhlA	5'-CCGATGAGTGATCTCGGANNSCAAGGGTTGTTCGACATCAC-3'
FhlArQ11		5'-GTGATGTCGAACAACCCTTGSNNTCCGAGATCACTCATCGG-3'
FhlAfL14	Saturation mutagenesis at position L14 of FhlA	5'-GATCTCGGACAACAAGGGNNSTTCGACATCACTCGGAC-3'
FhlArL14		5'-GTCCGAGTGATGTCGAASNNCCCTTGTTGTCCGAGATC-3'
FhlAfY177	Saturation mutagenesis at position Y177 of FhlA	5'-GTCGTTAACAATGTCGACNNSGAGTTGTTATGCCGGGAACGCG-3'
FhlArY177		5'-CGCGTTCCCGGCATAACAACTCSNNGTCGACATTGTTAACGAC-3'
FhlAfK245	Saturation mutagenesis at position K245 of FhlA	5'-CTCCACTCACTATCTTGATNNSCAGCATCCCGCCCACGAAC-3'
FhlArK245		5'-GTTCGTGGGCGGGATGCTGSNNATCAAGATAGTGAGTGGAG-3'
FhlAfM288	Saturation mutagenesis at position M288 of FhlA	5'-CCCTATGAACGCNNSTTGTTCGACACCTGGGGCAACC-3'
FhlArM288		5'-GGTTGCCCCAGGTGTCGAACAASNNGCGTTCATAGGG-3'
FhlAfI342	Saturation mutagenesis at position I342 of FhlA	5'-CAGATTGCCGAACGTGTGGCANNSGCTGTCGATAACGC-3'
FhlArI342		5'-GCGTTATCGACAGCSNNTGCCACACGTTCGGCAATCTG-3'
FhlAfE363	Saturation mutagenesis at position E363 of FhlA	5'-GAAAGAACGGCTGGTTGATNNSAACCTCGCCCTGACCG-3'
FhlArE363		5'-CGGTCAGGGCGAGGTTSNNATCAACCAGCCGTTCTTTC-3'
SfhlA1	DNA Sequencing	5'-GCCCTTTCGTCTTCACCTCGAG-3'
SfhlA2		5'-GAGTTCAATCGTCTGCAAAC-3'
SfhlA3		5'-TCGCCTATCAGGAAATCCATCG-3'
SfhlA4		5'-ACTACGCGAGCGTCCGGAAG-3'
GrxAf	qRT-PCR *grxA*	5'-GTTGCCCTTACTGTGTGCGTGC-3'
GrxArev	qRT-PCR *grxA* and verification of JW0833-1	5'-GTATAGCCGCCGATATGTTGCTG-3'
AhpFf	qRT-PCR *ahpF*	5'-TACTCGCTCTCTTGCCACAACTGC-3'

Table 3.2. (continued)

Primer	Purpose	Sequence[a,b]
AhpFrev	qRT-PCR *ahpF* and verification of JW0599-1	5'-CGATTTCAGTCAACGTCATGCGG-3'
HycEf	qRT-PCR *hycE*	5'-GACCACCGATGCTGAAACCTACG-3'
HycErev		5'-GCGATGGACGTAGAACAGACGGTAG-3'
HypDf	qRT-PCR *hypD*	5'-ATGCCGACCACCGCTATCACTC-3'
HypDrev		5'-GCCGATAACCATACTGACGTGACCC-3'
FdhFf	qRT-PCR *fdhF*	5'-GCGATACTTCAGATGCCGATCAGG-3'
FdhFrev		5'-TCATCGAACGGCAAGAGTAGTGACC-3'
HydNf	qRT-PCR *hydN*	5'-ATTGGTTGCCGTACCTGTGAAGTAGC-3'
HydNrev		5'-GATAGCACCATTCGGGCAGACG-3'
RrsGf	qRT-PCR *rrsG* (housekeeping gene)	5'-TATTGCACAATGGGCGCAAG-3'
RrsGr		5'-ACTTAACAAACCGCTGACTT-3'
FhlAup	Verification of JW2701-1 and MW1002	5'-CACCGATCGCCCTTCCCAACAGTTGC-3'
FhlAdown		5'-CATCCGCTTACAGACAAGCTGTGACC-3'
Kanrev	Verification of JW2701-1, JW0833-1, JW0599-1, and MW1002	5'-GAGATTATGGATCGCACGGGCAATCAGC-3'
GrxAfront	Verification of JW0833-1	5'-GCCAGTAACAGGGAGTCGCTTACC -3'
AhpFfront	Verification of JW0599-1	5'-CAGGGTATCATCCAGGCAATCGAAG-3'
OxySfront	Verification of JW0098	5'-ATGCCACCAGGTACTCAAGATCACG-3'
OxySrev		5'-TGCAATCGTGCCTCGACAAGCGTG-3'
HyaB-F	Verification of MW1002	5'-AGACGTCATAACCAGCAACCTAC-3'
HyaB-R		5'-AAGACCGACATCAGGAAATAGC-3'
HybC-F		5'-GTTAAGCTGACCAACACCATCAG-3'
HybC-R		5'-CCATCGAGGATCTCAACATAATCC-3'

[a] N is A, T, G, or C and S is G or C.
[b] The positions mutated by saturation mutagenesis are underlined.

cloned into pASKA2701 using the MfeI and HindIII restriction enzymes with Antarctic phosphatase (New England Biolabs, Beverly, MA) treatment of the vector; the ligation mixture was electroporated (85) into strain JW2701-1 (Δ*fhlA*) (9) (complementation of the *fhlA* deletion by pASKA2701 was reported by us previously (92)). For epPCR of the *fhlA* region encoding the N domain of FhlA, the conditions were the same as described above, but primers FhlAfront and FhlAN (Table 3.2) were used with a 2-min extension time. The enzymes used for the cloning were MfeI and BsrGI.

3.3.3 Saturation mutagenesis

A QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to perform saturation mutagenesis of *fhlA* at all of the mutated codons of *fhlA133* (Table 3.3) and at the codon corresponding to E363, which is mutated in *fhlA1157* (Table 3.3), using pASKA2701 as a template. The DNA primers (Table 3.2) contained NNS at the target codon (N is A, G, C, or T, and S is G or C) to allow the substitution of all 20 amino acids using the 32 possible codons (26); the constructed plasmids were electroporated (85) into JW2701-1, and at least 300 colonies (80) were screened for enhanced hydrogen production using chemochromic membranes.

3.3.4 Hydrogen screening

Chemochromic membranes (GVD Corp., Cambridge, MA) formed by a thin film of WO_3 covered with a catalytic layer of palladium, were used to detect hydrogen gas, by a colorimetric response, from colonies grown anaerobically (154). These membranes were used to identify mutants with enhanced hydrogen production due to mutations in *fhlA* generated by epPCR and saturation mutagenesis as described previously (94). Modified complex (93) agar plates containing 20 mM formate and Cm were used for screening; isopropyl β-D-1-

Table 3.3. Hydrogen production by *E. coli* BW25113 *fhlA*/pCA24N-*fhlA* mutants after 0.5 h of anaerobic incubation in modified-complex 20 mM formate medium.

Strains	*fhlA* allele	FhlA aa changes	H_2 production rate[e]		
			n[a]	µmol mg protein^{-1} h^{-1}	Relative
JW2701-1 (pASKA2701)	*fhlA*	-	24	0.8 ± 0.3	1
JW2701-1 (pVSC133)	*fhlA133*[b]	Q11H, L14V, Y177F, K245R, M288K, I342F	5	7 ± 2	9
JW2701-1 (pVSC14)	*fhlAL14*[c]	L14G	4	3.5 ± 0.5	4
JW2701-1 (pVSC1157)	*fhlA1157*[d]	M6T, S35T, L113P, S146C, E363K	4	2.9 ± 0.5	4
JW2701-1 (pVSC363)	*fhlA363*[c]	E363G	5	5 ± 1	6

[a] Number of independent cultures
[b] Obtained via epPCR of whole *fhlA*
[c] Obtained via saturation mutagenesis
[d] Obtained via epPCR of the *fhlA* region encoding the N-domain of FhlA
[e] The values are the average ± standard deviation

thiogalactopyranoside (IPTG) was not added because overexpression of *fhlA* by adding IPTG is not beneficial for hydrogen production (91).

3.3.5 Hydrogen assay

For all of the mutants with enhanced hydrogen production that were identified with the chemochromic membranes, hydrogen production was quantified using anaerobic cells. Overnight aerobic cultures (25 ml) in modified complex medium (93) supplemented with 20 mM formate and Cm, as well as uninoculated modified complex medium supplemented with 20 mM formate and Cm, were sparged for 5 min with nitrogen to remove oxygen. Sealed crimped-top vials (27 ml) were also sparged for 2 min with nitrogen. Inside an anaerobic glove box, 9 ml of sparged uninoculated modified complex medium and 1 ml of sparged overnight culture were added to each vial. The amount of hydrogen generated in the headspace was measured after 0.5 h of anaerobic incubation at 37°C by gas chromatography using a 6890N gas chromatograph (Agilent Technologies Inc., Santa Clara, CA) as described previously (95). The work in the anaerobic glove box took about 36 min; therefore, at the time of analysis (listed as 0.5 h of incubation), the cells had been anaerobic for over 1 h.

3.3.6 Cloning of fhlA alleles

To study the impacts of the beneficial mutations on the transcription of the FhlA-controlled loci using compatible plasmids, the *fhlA*, *fhlA133*, and *fhlA363* alleles were cloned from plasmids pASKA2701, pVSC133, and pVSC363 into plasmid pVLT35 (37). The plasmids harboring the *fhlA* alleles were digested with XhoI and HindIII, and pVLT35 was digested with SalI and HindIII (New England Biolabs, Beverly, MA). The DNA fragments were ligated after

the digested pVLT35 was treated with Antarctic phosphatase (New England Biolabs, Beverly, MA) and were electroporated into JW2701-1 (9).

3.3.7 Hydrogen production with overexpression of hycA and with isogenic mutants

The hydrogen production of the JW2701-1 strains harboring the pVLT35-derived plasmids pVSV2701, pVSV133, and pVSV363 (carrying the *fhlA*, *fhlA133*, and *fhlA363* alleles, respectively) with and without pASKA2695 ($hycA^+$) was evaluated with a hydrogen assay (*hycA* and *fhlA* were expressed constitutively). Hydrogen production by JW0833-1 (Δ*grxA*), JW0599-1 (Δ*ahpF*), and JW0098 (Δ*oxyS*) (Table 3.1) was also evaluated with a hydrogen assay. At least three independent cultures of each strain were assayed.

3.3.8 Hydrogen uptake assay

pVSC133 and pASKA2701 were electroporated into MW1002, a strain that lacks activity of the uptake hydrogenases Hyd-1 and Hyd-2, as well as chromosomal *fhlA*. Hydrogen uptake activity by Hyd-3 was assayed in modified complex medium with 20 mM formate, as described previously (95), by measuring the increase in absorbance that results from reducing colorless, oxidized methyl viologen to a purple product ($MV^{2+} + 1/2H_2 \rightarrow MV^+ + H^+$). Two independent cultures of each strain were evaluated.

3.3.9 Transcription of the fdhF gene and hyc and hyp operons

To explore the mechanism by which the FhlA variants enhance hydrogen production, transcription of the *hyc*, *hyp*, and *fdhF* promoters was evaluated using a β-galactosidase assay in strains lacking *fhlA* in the chromosome. For the *hyc* promoter (P_{hyc}) and the *hyp* promoter (P_{hyp}), two *lacZ* fusion systems were studied: one set included the *hyc* UAS (strain WS127 (156) for P_{hyc}

and pTSU101 (152) for P_{hyp}) (Table 3.1), and the other set included the *hyc* UAS, *hycA*, and the *hyp* UAS (pTS102 (152) for P_{hyc} and pTSU102 (152) for P_{hyp}) (Table 3.1). Thus, the transcriptional activation levels of *hyp* in the presence of one or two FhlA binding regions could be compared. pHW13 (181), which harbors a P_{fdhF}::*lacZ* fusion, was used for the *fdhF* promoter (P_{fdhF}). The DNA fragments in these *lacZ* fusion systems are shown in Fig. 3.1.

Plasmids pASKA2701, pVSC133, and pVSC363 (harboring the *fhlA* alleles) were electroporated into strain WS127, which lacks *fhlA* and contains the chromosomal *lacZ* reporter harboring the *hyc* UAS and P_{hyc}, whereas the *lacZ* reporter plasmids to study the P_{hyc}, P_{hyp}, and P_{fdhF} promoters were electroporated into JW2701-1 strains harboring plasmids pVSV2701, pVSV133, and pVSV363. For the β-galactosidase assay, cells were prepared in the same manner as for the hydrogen assay using appropriate antibiotics, and enzyme activity was conducted as described previously (187).

3.3.10 Whole-transcriptome analysis

To investigate why strains with FhlA133 produce more hydrogen, whole-transcriptome analysis was performed. JW2701-1(pVSC133) and JW2701-1(pASKA2701) were cultured as for the hydrogen assay, and total RNA was isolated with the RNeasy kit (Qiagen, Inc., Valencia, CA) as described previously (131) using a bead beater. *E. coli* GeneChip Genome 2.0 arrays (part no. 511302; Affymetrix, Inc., Santa Clara, CA) were used; they contained 10,208 probe sets for open reading frames, rRNA, tRNA, and 1,350 intergenic regions for four *E. coli* strains (MG1655, CFT073, O157:H7-Sakai, and O157:H7-EDL933). cDNA synthesis, fragmentation, end terminus biotin labeling, and hybridization were performed as described previously (52). Background values, noise values, and scaling factors for the two arrays were comparable, and the intensities of polyadenosine RNA controls were used to monitor the labeling process. For each

binary microarray comparison of differential gene expression, if the gene with the higher transcription rate did not have a consistent transcription rate based on the 11 probe pairs (a detection *P* value of less than 0.05), the genes were discarded. A gene was considered differentially expressed when the *P* value for comparing two chips was less than 0.05 (to ensure that the change in gene expression was statistically significant and that false positives arose at less than 5%) and when the expression ratio was higher (1.2-fold) than the standard deviation for all K-12 genes of the microarrays (1.2-fold) (130).

3.3.11 qRT-PCR

To validate the whole-transcriptome analysis data, the transcription of g*rxA*, *ahpF*, *hycE*, *hypD*, *fdhF*, and *hydN* was quantified using quantitative real-time reverse transcription PCR (qRT-PCR) (12) with the RNA samples used for the whole-transcriptome analysis. The housekeeping gene *rrsG* (16S rRNA) was used to normalize the expression data. Three technical replicates were performed for each gene using the StepOne Real-Time PCR system (Applied Biosystems, Foster City, CA) and the Power SYBR green RNA-to-C_T 1-Step Kit (Applied Biosystems, Foster City, CA). The primers for qRT-PCR are given in Table 3.2. The expression ratios for the genes analyzed were calculated according to the $2^{-\Delta\Delta CT}$ method (87).

3.3.12 Plasmid isolation, SDS-PAGE, and DNA sequencing

Plasmids were isolated using the QIAprep Spin Miniprep kit (Qiagen, Inc., Valencia, CA). The formation of recombinant proteins under the conditions used for the hydrogen assay was analyzed with standard Laemmli discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12%) (142). A dideoxy chain termination method (145) with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Wellesley,

MA) was used to determine the nucleotide changes in the *fhlA* alleles; the primers used for sequencing are given in Table 3.2.

3.3.13 Microarray data accession number

The expression data for the whole-transcriptome analysis of JW2701-1(pVSC133) and JW2701-1(pASKA2701) have been deposited in the NCBI Gene Expression Omnibus (GEO) (15) and are accessible as GSE13902.

3.4 Results

3.4.1 Isolation of mutants with enhanced H_2 production

To increase hydrogen production and to better understand the transcription activation of the genes of FHL by FhlA, epPCR was used to construct a random-mutagenesis library of *fhlA*. We screened 2,200 colonies using the chemochromic membranes in a host that lacks *fhlA* in the chromosome (JW2701-1). Using the hydrogen assay, we identified variant FhlA133, which allows JW2701-1(pVSC133) to have a 9-fold higher hydrogen production rate than JW2701-1(pASKA2701) (Table 3.3).

The FhlA133 variant has six amino acid changes (Q11H, L14V, Y177F, K245R, M288K, and I342F) in the N-terminal domain, which motivated us to focus on mutagenesis of *fhlA* only in the N-terminal domain (the *fhlA* region coding for the first 388 amino acids of FhlA). Thus, a second epPCR library was constructed targeting only this region, and an additional 4,400 colonies were screened from this new library. From this screening, variant FhlA1157 (expressed via pVSC1157), which has five amino acid changes (M6T, S35T, L113P, S146C, and E363K), was identified as causing a 4-fold increase in hydrogen production (Table 3.3).

3.4.2 Saturation mutagenesis

To identify which amino acid replacements in FhlA133 are important for enhanced hydrogen production, saturation mutagenesis of *fhlA* was performed on each of the six mutated codons in *fhlA133* that corresponded to Q11, L14, Y177, K245, M288, and I342. For each position, at least 300 colonies were screened to ensure, with a probability of 99.99%, that all possible codons were utilized (80). Only the mutation encoding L14G (expressed in pVSC14) resulted in an increase in the hydrogen production rate (4 fold) (Table 3.3); therefore, position L14 of FhlA is important for controlling hydrogen production.

Saturation mutagenesis was also performed at the codon corresponding to E363 of FhlA, since the replacement E363K was identified in FhlA1157 and because the E363K amino acid replacement increases P_{hyc} transcription approximately 3-fold in the presence of 30 mM formate (34-fold without formate) and decreases the impact of formate (79). FhlA363 (E363G) was identified from the hydrogen screen with JW2701-1, and this replacement caused hydrogen production rates 6-fold higher than that of the strain with FhlA.

Plasmids harboring each of four mutated *fhlA* alleles found through epPCR and saturation mutagenesis of *fhlA* (*fhlA133*, *fhlA1157*, *fhlA14*, and *fhlA363*) were isolated and reelectroporated into JW2701-1; hydrogen production was assayed to confirm that the mutations in the plasmid were responsible for the higher hydrogen production rates (Table 3.3). In addition, enhanced hydrogen production by the mutants harboring *fhlA133* and *fhlA363* was confirmed for a third time with the hydrogen assay after the *fhlA*, *fhlA133*, and *fhlA363* alleles were cloned into pVLT35 (data not shown). Hence, consistent data were obtained demonstrating that the beneficial mutations in *fhlA* were directly related to enhanced hydrogen production. SDS-PAGE of the cell lysates from JW2701-1 expressing the *fhlA* alleles from pASKA2701, pVSC133, pVSC1157, pVSC14, and pVSC363 indicated that the higher hydrogen production

rates were not due to changes in the amount of FhlA (data not shown).

To confirm that the hydrogen produced by the strains studied came from the formate added to the medium (rather than from other medium components), we compared hydrogen production by JW2701-1(pVSC133) and JW2701-1(pASKA2701) with and without formate. Hydrogen production in the absence of formate was 0.5 ± 0.1 μmol H_2 mg protein^{-1} h^{-1} for JW2701-1(pVSC133) and 0.290 ± 0.005 μmol H_2 mg protein^{-1} h^{-1} for JW2701-1(pASKA2701). The hydrogen production of JW2701-1(pVSC133) in modified complex medium with 20 mM formate was 14 ± 5-fold higher than the hydrogen production without formate; for JW2701-1(pASKA2701), hydrogen production with 20 mM formate was 3 ± 1-fold higher than without formate. Since the amount of hydrogen produced in the medium without formate was very small relative to the amount produced with 20 mM formate, we concluded that the hydrogen produced by the strains studied came predominantly from the formate added to the medium.

3.4.3 Transcription of fdhF, the hyc operon, and the hyp operon

Since FhlA is the transcriptional activator of the genes of the FHL complex, the impacts of formate (20 mM) on the transcription of *fdhF* (which encodes formate dehydrogenase H), the *hyc* operon (which encodes the structural proteins of the FHL complex), and the *hyp* operon (which encodes maturation proteins) (Fig. 3.1) were evaluated by the β-galactosidase assay. With *fhlA133* expressed from pVSC133, P_{hyc} transcription in strain WS127 (including the *hyc* UAS and P_{hyc}) was increased 2.3-fold with 20 mM formate and 8-fold in the absence of formate (Fig. 3.2A). Using plasmid pTS102, which contains the *hyc* UAS, *hycA*, and the *hyp* UAS, P_{hyc} transcription in the strain harboring *fhlA133* was 1.7-fold higher than in the strain with *fhlA* with and without formate (Fig. 3.2A). Using pTSU101 (including the *hyc* UAS and P_{hyp}), pTSU102

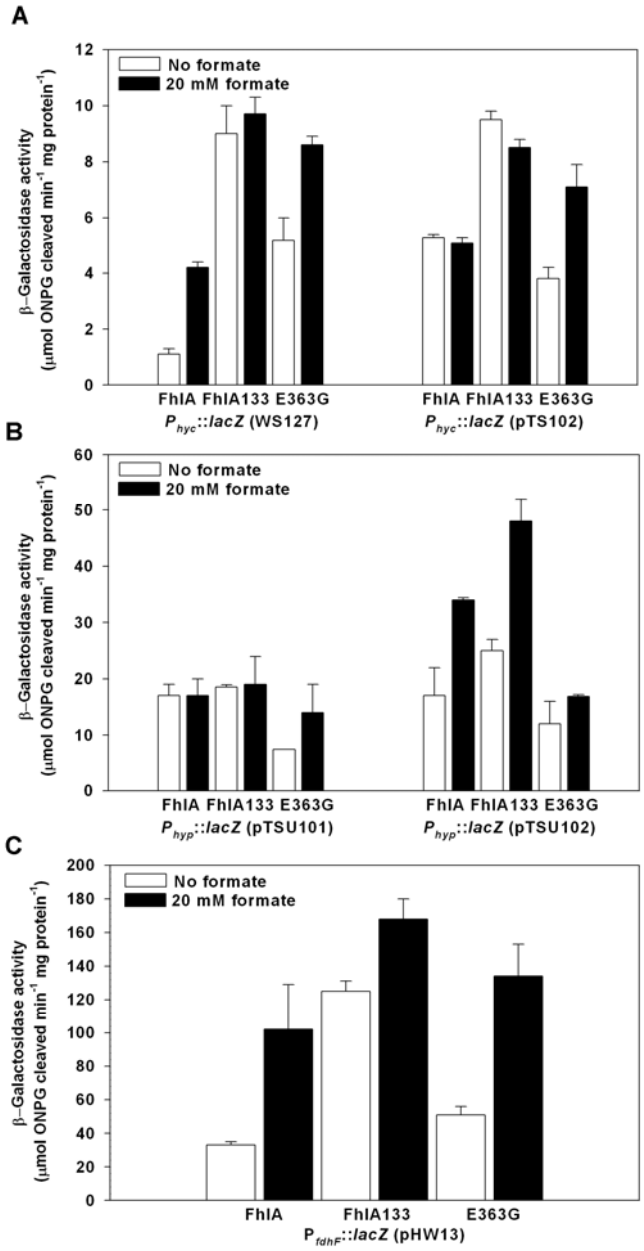

Fig. 3.2. Transcriptional activation of *hyc*, *hyp*, and *fdhF* by FhlA variants. Cells were cultured anaerobically in modified complex medium without formate or with 20 mM formate for 40 min. The results are the averages of two independent cultures. (A) Transcription of P_{hyc}::*lacZ* using *E. coli* WS127 (the *fhlA* alleles were expressed via pCA24N) and *E. coli* JW2701-1 (Δ*fhlA*) harboring plasmid pTS102 (the *fhlA* alleles were expressed via pVLT35). (B) Transcription of P_{hyp}::*lacZ* using plasmids pTSU101 and pTSU102 with JW2701-1 (the *fhlA* alleles were expressed via pVLT35). (C) Transcription of P_{fdhF}::*lacZ* using plasmid pHW13 with JW2701-1 (the *fhlA* alleles were expressed via pVLT35). ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

transcription rates of P_{hyp} in the strains with *fhlA133* and *fhlA* were very similar (Fig. 3.2B); for (including the *hyp* UAS), P_{hyp} transcription increased 1.5-fold in the strain with *fhlA133* relative to the strain with *fhlA* (Fig. 3.2B). Increased transcription due to the mutations in *fhlA133* was also observed for P_{fdhF}; transcription of this promoter was 1.7-fold higher with 20 mM formate and 3.8-fold higher without formate (Fig. 3.2C). In addition, P_{hyc} and P_{fdhF} transcriptions were less affected by formate addition in the strains with *fhlA133* than in the strains with *fhlA* (Fig. 3.2A and C). Hence, the 9-fold higher hydrogen production attained by JW2701-1(pVSC133) appears to be due to an increase in transcription of all three promoters of *hyc*, *hyp*, and *fdhF*.

In the presence of 20 mM formate, the replacement E363G in FhlA increased P_{hyc} transcription; WS127(pVSC363) had 2.1-fold higher P_{hyc} transcription than WS127(pASKA2701), and using the pTS102 reporter, the strain with *fhlA363* had 1.4-fold higher P_{hyc} transcription than the wild-type strain. A higher P_{hyc} transcription rate was also observed in the absence of formate for WS127(pVSC363) (fivefold) (Fig. 3.2A). The transcription of P_{fdhF} was slightly higher in the strain with *fhlA363* than in the strain with *fhlA* (1.5-fold without formate and 1.3-fold with 20 mM formate) (Fig. 3.2C), while the transcription of P_{hyp} decreased (Fig. 3.2B). Therefore, the E363G replacement confers a higher transcription rate for *hyc* and *fdhF* that appears to lead to increased hydrogen production.

3.4.4 Whole-transcriptome analysis

To investigate why the mutant JW2701-1(pVSC133) produced more hydrogen than the wild-type strain, we performed a whole-transcriptome analysis. The amino acid changes in FhlA133 induced all four of the transcriptional units regulated by FhlA: the *hyc* operon (1.6-fold), the *hyp* operon (1.5-fold), *fdhF* (1.7-fold), and *hydN-hypF* (1.7-fold) (Table 3.4). These results corroborate those found using the promoter reporters (1.7-fold for the *hyc* operon, 1.5-

Table 3.4. Differential expression of genes activated by FhlA in the JW2701-1(pVSC133) vs. JW2701-1(pASKA2701) whole-transcriptome analysis. Cells were incubated anaerobically at 37°C for 0.5 h in modified-complex medium supplemented with 20 mM formate.

Gene	b-number	Wild-type signal	Mutant signal	Fold change	Change p-value	Function (139)
fhlA	b2731	2080.3	2531.7	1.2	0.10566	Transcriptional activator of the FHL complex
Hydrogenase 3 (*hyc* operon)						
hycA	b2725	2452.8	4154.8	1.6	0.00002	Regulatory gene for *hyc* and *hyp* operons; counteracts activation by FhlA
hycB	b2724	801.2	1431.9	1.7	0.00002	Formate hydrogenlyase complex iron-sulfur protein
hycC	b2723	1774	2883.2	1.6	0.00002	Formate hydrogenlyase complex inner membrane protein
hycD	b2722	2165.2	3537.9	1.6	0.00002	Formate hydrogenlyase complex inner membrane protein
hycE	b2721	1915.1	2907.9	1.5	0.00002	Large subunit of hydrogenase 3
hycF	b2720	2421.6	3618.5	1.5	0.00002	Formate hydrogenlyase complex iron-sulfur protein
hycG	b2719	2480.3	3877.1	1.6	0.00002	Hydrogenase 3 small subunit
hycH	b2718	1970.1	3357.4	1.6	0.00002	Required for converting HycE precursor to mature Hyd-3 large subunit
hycI	b2717	1955.4	3061.3	1.6	0.00002	Maturation endoprotease for Hydrogenase 3 large subunit HycE
Maturation proteins (*hyp* operon)						
hypA	b2726	1772.7	2730.3	1.5	0.00002	Hydrogenase 3 accessory protein required for activity
hypB	b2727	1930.5	2993	1.5	0.00002	Required for metallocenter assembly in Hydrogenases 1,2,3; Ni donor for HycE
hypC	b2728	2108.1	3263.2	1.6	0.00002	Hydrogenase 3 chaperone-type protein; required for metallocenter assembly
hypD	b2729	1452.5	2069.6	1.5	0.00002	Hydrogenases 1,2,3 accessory protein; required for metallocenter assembly
hypE	b2730	2122.6	3020.4	1.4	0.00002	Hydrogenases 1,2,3 accessory protein; required for CN ligand synthesis.
Formate dehydrogenase H						
fdhF	b4079	1660.4	2368.8	1.7	0.00002	Formate dehydrogenase H, selenopeptide
hydN-hypF operons						
hydN	b2713	1720.2	3039.4	1.7	0.00002	Iron-sulfur protein required for Hyd-3 activity
hypF	b2712	1072.2	1814.7	1.6	0.00002	Hydrogenases 1,2,3 accessory protein; required for CN ligand synthesis.

fold for the *hyp* operon, and 1.6-fold for *fdhF*) and demonstrate that the *hydN-hypF* operon is also induced by this protein variant.

Surprisingly, the genes of the FHL complex were not the most induced genes. Instead, the highest induction was observed for genes activated by OxyR under conditions of oxidative stress (193): *grxA* (2.8-fold), *ahpF* (2.8-fold), and *ahpC* (2.5-fold) Table 3.5). Other stress-related genes were also induced, such as the *psp* operon, which is transcribed by σ^{54}-RNA polymerase (183).

3.4.5 qRT-PCR

qRT-PCR was used to verify the expression of the most induced genes (*grxA* and *ahpF*) and of some genes activated by FhlA (*hycE*, *hypD*, *fdhF*, and *hydN*). The differential changes in expression were comparable to those in the whole-transcriptome analysis: *grxA*, 3.0-fold versus 2.8-fold; *ahpF*, 3.9-fold versus 2.8-fold; *hycE*, 1.2-fold versus 1.5-fold; *hypD*, 1.4-fold versus 1.5-fold; *fdhF*, 1.2-fold versus 1.7-fold; and *hydN*, 1.5-fold versus 1.7-fold.

3.4.6 grxA, ahpF, and oxyS mutations and hydrogen production

To explore whether the oxidative-stress genes induced in the whole-transcriptome analysis were related to hydrogen production, we analyzed the effects of deleting *grxA*, *ahpF*, and *oxyS* on hydrogen production by BW25113. The rate of hydrogen production by BW25113 was slightly reduced upon deletion of *grxA* (1.6 ± 0.2-fold) and *ahpF* (1.4 ± 0.4-fold); however, the *oxyS* deletion increased hydrogen production by 1.7 ± 0.4-fold.

3.4.7 Hydrogen uptake assay

Hydrogen uptake was assayed directly to determine if the increase in hydrogen

Table 3.5. Relevant stress-related genes induced in the JW2701-1(pVSC133) vs. JW2701-1(pASKA2701) whole-transcriptome analysis. Cells were incubated anaerobically at 37°C for 0.5 h in modified-complex medium supplemented with 20 mM formate.

Gene	b-number	Wild-type signal	Mutant signal	Fold change	Change p-value	Function (139)
OxyR activated, induced by hydrogen-peroxide (193)						
grxA	b0849	1091.8	2998.6	2.8	0.00002	Glutaredoxin 1
ahpF	b0606	922.9	2870.2	2.8	0.00002	Alkyl hydroperoxide reductase, subunit F; NAD(P)H:peroxiredoxin oxidoreductase
ahpC	b0605	1402.6	3278.7	2.5	0.00002	Alkyl hydroperoxide reductase, subunit C; reduced by the AhpF subunit
dpS	b0812	1526.7	3062.7	2.1	0.00002	DNA-binding protein; starvation-induced resistance to H_2O_2; Fe-binding and storage protein
oxyS	b4458	2093.8	3709.8	1.9	0.00002	OxyS sRNA helps to protect cells against oxidative damage
trxC	b2582	914.4	1647	1.7	0.00002	Thioredoxin 2, zinc-binding
sufA	b1684	906.8	1352.3	1.6	0.00002	Scaffold protein for assembly of iron-sulfur clusters; facilitates delivery to target proteins
fur	b0683	1453	2495.8	1.5	0.00002	Ferric iron uptake global transcriptional repressor; activated by Fe^{+2}; zinc metalloprotein
stress-related						
pspA	b1304	1462.4	2574.4	1.7	0.00002	Negative regulator of *psp* operon; binds PspB and PspC; enhances protein export through Tat pathway
pspB	b1305	1670.8	2665.6	1.6	0.00002	Regulator, with PspC, activates expression of *psp* operon; binds PspA
pspC	b1306	1024.7	1628	1.5	0.00002	Positive regulatory gene, cooperatively with PspB; facilitates binding of PspA to PspB
pspD	b1307	970	1442.5	1.5	0.00002	Expressed protein in *psp* operon; peripheral inner membrane protein
pspE	b1308		1414.4	1.4	0.00002	Thiosulfate sulfurtransferase
hns	b1237	1353.1	2048.5	1.5	0.00002	DNA-binding global regulator H-NS; diverse mutant phenotypes affecting transcription, transposition, inversion, cryptic-gene expression; involved in chromosome organization
stpA	b2669	744.9	1193	1.6	0.00002	RNA chaperone and DNA-binding protein

Table 3.5. (continued)

Gene	b-number	Wild-type signal	Mutant signal	Fold change	Change p-value	Function (139)
bhsA	b1112	1238.2	1954.5	1.5	0.00005	Biofilm, cell surface and signaling defects
marA	b1531	2104.2	3138	1.5	0.00002	Transcription activator of multiple antibiotic resistance
ibpA	b3687	2125.8	3492.7	1.5	0.00002	Chaperone, heat-inducible protein of HSP20 family
ibpB	b3686	1519.7	2216.4	1.5	0.00003	Chaperone, heat-inducible protein of HSP20 family
clpB	b2592	1479.5	2291.1	1.5	0.00002	ATP-dependent protease and chaperone; protein disaggregation chaperone
dnaK	b0014	1751.8	2539	1.5	0.00002	Hsp70 molecular chaperone, heat-inducible

production by JW2701-1(pVSC133) was due to a decrease in Hyd-3-mediated hydrogen uptake. MW1002 was used because it lacks *fhlA* and the large subunits of uptake hydrogenases Hyd-1 and Hyd-2; thus, only uptake by Hyd-3, a reversible enzyme capable of hydrogen uptake (92) that is activated by FhlA, is possible. There was no significant difference in the hydrogen uptake activity of MW1002(pVSC133) (0.53 nmol min^{-1} mg $protein^{-1}$) relative to that of MW1002(pASKA2701) (0.55 nmol min^{-1} mg $protein^{-1}$); therefore, the increase in hydrogen production by JW2701-1(pVSC133) was not due to a change in hydrogen uptake.

3.4.8 Hydrogen production with overexpression of hycA

To ascertain if the mutations in *fhlA133* and *fhlA363* alter the HycA-mediated repression of genes encoding FHL (147), we evaluated the effect of *hycA* overexpression on hydrogen production. pASKA2695 (expressing *hycA*) was electroporated into JW2701-1(pVSV2701) and the derived strains harboring *fhlA133* and *fhlA363*. As expected, overexpression of *hycA* reduced hydrogen production; however, the strain with *fhlA363* was repressed less by HycA (1.4 ± 0.7-fold) than the strain with *fhlA* (3.7 ± 0.9-fold) and the strain with *fhlA133* (5 ± 1-fold). These results indicate that E363 of FhlA may be involved in the HycA-mediated inhibition of transcription of the genes encoding FHL.

3.5 Discussion

Random mutagenesis of *fhlA* had been conducted previously by Korsa and Böck (79) to find FhlA variants that activate P_{hyc} transcription independently of formate; however, mutagenesis was not conducted to increase hydrogen production, as these variants were used to study the kinetics of ATP hydrolysis in the presence and absence of formate. They found that FhlA with amino acid replacements E358K and E363K activated *hyc* transcription with reduced

dependence on formate, whereas E183K conferred a constitutive phenotype (79). Similarly, Self and Shanmugam (156) found several FhlA variants that activated *hyc* transcription without molybdate. Here, using direct screening for hydrogen production, we identified four mutants with increased hydrogen production obtained through epPCR and saturation mutagenesis (Table 3.3) of *fhlA* and discovered the importance of position L14 of FhlA.

The N-terminal domains of some σ^{54} regulators (e.g., DmpR (159) and XylR (121)) inhibit transcription activation in the absence of their corresponding effectors. FhlA-C, an N-terminally truncated FhlA protein lacking the first 378 amino acids, is active independently of formate and is not affected by the repressor HycA (84). Similarly, FhlA165, which has a deletion from amino acids 5 to 374, activates *hyc* transcription independently of formate, but unlike FhlA-C, its activity was reduced by HycA (155). FhlA-N, a C-terminally and central-domain-truncated protein lacking the last 314 amino acids, repressed transcriptional activation of the *hyc* operon by FhlA in the presence and absence of formate (84). Hence, the N domain of FhlA inhibits FhlA transcriptional activation and is influenced by formate and HycA (155).

Even though the entire *fhlA* gene was mutated here, all six amino acid replacements in FhlA133 were in the N domain. This suggests that these replacements may decrease the repressive effect of the N domain. Saturation mutagenesis at each codon affected by the mutations in *fhlA133* led only to the discovery of replacement L14G, which stimulates hydrogen production 4 fold in a medium supplemented with 20 mM formate. L14 is in the region between amino acids 7 and 37 of FhlA, and Self et al.(155) showed that a truncation of this region abolishes transcriptional activation of the *hyc* operon; therefore, this region is important for hydrogen production. Since FhlA is a transcriptional activator of four loci (96, 151, 152), the increase in hydrogen production due to the mutations in *fhlA* should be related to changes in the

transcription of the units activated by FhlA; therefore, we studied the transcriptional activation of the *fdhF* gene and the *hyc* and *hyp* operons by strains harboring *fhlA*, *fhlA133*, and *fhlA363*.

The results of the β-galactosidase transcription assay for strains with *fhlA* (Fig. 3.2) agree with the data reported previously in which formate induced an increase in P_{fdhF} transcription (181)and an increase in P_{hyp} when the *hyc* UAS and *hyp* UAS were present (using pTSU102) (152). Also, as reported by Schlensog et al. (152), P_{hyc} transcription was not induced by formate using the pTS102 reporter plasmid (Fig. 3.2A) and P_{hyp} transcription was not induced by formate using pTSU101. From these transcription reporter results, the replacements in FhlA133 led to increased transcription of all three of the promoters studied (P_{hyc}, P_{hyp}, and P_{fdhF}) with and without formate (Fig. 3.2). Moreover, transcription from P_{hyc} and P_{fdhF} in strains with FhlA133 was less dependent on formate. This is reflected in the hydrogen production rate in the absence of formate [JW2701-1(pVSC133) had 1.7 ± 0.3-fold higher hydrogen production than JW2701-1(pASKA2701)]. Since the intracellular level of formate determines the transcription rate of the FHL genes by FhlA(138), FhlA133 may be able to activate transcription with a smaller internal concentration of formate than FhlA.

Strain WS127, which was used to measure P_{hyc} transcription, has a deletion of all of the genes of the formate regulon except *fdhF* (156). Thus, we studied P_{hyc} transcription in the absence of the repressor HycA and with only the *hyc* UAS and the *fdhF* UAS present, since the other UAS were deleted. Using this strain, replacements in FhlA133 led to an 8-fold increase in P_{hyc} transcription in the absence of formate and a 2.3-fold increase in P_{hyc} transcription in the presence of 20 mM formate.

Along with indicating that all four of the known FhlA-controlled operons are induced in JW2701-1(pVSC133) versus JW2701-1(pASKA2701), the whole-transcriptome analysis indicated that the replacements in FhlA133 also induced eight genes related to oxidative stress

(Table 3.5). A role for oxidative-stress proteins during anaerobic fermentations is surprising. However, removal of OxyS inhibition of FhlA translation by deleting *oxyS* from BW25113 was expected to provide a small beneficial effect on hydrogen production, and a nearly 2-fold increase was measured. OxyS RNA forms a stable antisense-target complex with *fhlA* mRNA by binding to a sequence overlapping the ribosome binding site and to a sequence located in the *fhlA* coding region; mutations at either site decrease the stability of the complex (6). For the JW2701-1(pASKA2701) derivatives, deletion of *oxyS* should have less impact on hydrogen production, since the ribosome binding sequence from plasmid pASKA2701 differs from the native sequence where OxyS binds to *fhlA* mRNA. In addition, for *fhlA133* and *fhlA14*, the replacement at position L14 is located in one of the OxyS binding regions (6). Another 12 stress-related genes were also induced (Table 3.5), which suggests that the increased hydrogen production affects cell physiology and that increases in hydrogen production may be facilitated by increasing the production of proteins that alleviate stress.

Among these stress-related genes is the *psp* operon; transcription of this operon, as well as that of the operons activated by FhlA, depends on σ^{54}. Among the other 16 σ^{54}-dependent promoters (129), the promoter with the highest similarity to the *psp* promoter is P_{hyc} (66.7% identity). Therefore, FhlA133 may increase transcription of the *psp* operon because of its similarity to the promoters controlling the expression of the genes of the FHL complex.

Mutagenesis in the *fhlA* region coding for the N domain of FhlA produced variant FhlA1157 with replacements M6T, S35T, L113P, S146C, and E363K. In the absence of formate, FhlAE363K has kinetic parameters (K_m and V_{max}) for ATP hydrolysis similar to those of FhlA with bound formate; therefore, mutation E363K renders FhlA less sensitive to formate (79). Here, saturation mutagenesis at position E363 produced the replacement E363G, which increased hydrogen production 6 fold. E363G, like E363K, increased transcription of P_{hyc} with formate;

E363G also slightly increased P_{fdhF} transcription (50% without formate and 30% with 20 mM formate).

Transcription of P_{hyp} in the presence of *fhlA363* decreased for the two systems studied, pTSU101 (*hyc* UAS) and pTSU102 (*hyc* and *hyp* UAS). Transcription of *hypBCDE* in the presence of only the *hyc* UAS (pTSU101) is due to the FNR-dependent promoter located within *hypA* (88) and is not due to the FhlA-dependent promoter P_{hyp} (Fig. 3.2B); this promoter did not show significant induction with formate (152). However, using pTSU101, the E363G mutation led to a 2-fold induction of P_{hyp} transcription by formate (Fig. 3.2B). Hence, the mechanism for increasing hydrogen production of strains harboring *fhlA133* is different than that of strains harboring *fhlA363*. Strains with *fhlA133* have increased transcription of all of the genes of the FHL complex and have P_{fdhF} and P_{hyc} transcription that is less sensitive to formate regulation, whereas strains harboring *fhlA363* have increased P_{hyc} and P_{fdhF} transcription and less P_{hyp} transcription and have hydrogen production that is less affected by the repressor HycA.

CHAPTER IV

GLOBAL REGULATOR H-NS AND LIPOPROTEIN NLPI INFLUENCE PRODUCTION OF EXTRACELLULAR DNA IN *ESCHERICHIA COLI* *

4.1 Overview

Extracellular DNA (eDNA) is a structural component of the polymeric matrix of biofilms from different species. Different mechanisms for DNA release have been proposed including lysis of cells, lysis of DNA-containing vesicles, and DNA secretion. Here, a genome-wide screen of 3985 non-lethal mutations was performed to identify genes whose deletion alters eDNA release in *Escherichia coli*. Deleting *nlpI*, *yfeC*, and *rna* increased eDNA from planktonic cultures while deleting *hns* and *rfaD* decreased eDNA production. The lipoprotein NlpI negatively affects eDNA since the overexpression of *nlpI* decreases eDNA 16 fold while deleting *nlpI* increases eDNA 3 fold. The global regulator H-NS is required for eDNA production since DNA was not detected for the *hns* mutant and production of H-NS restored eDNA production to wild-type levels. Therefore our results suggest that secretion may play a role in eDNA release in *E. coli* since the effect of the *hns* deletion on cell lysis (slight decrease) and membrane vesicles (3-fold increase) does not account for the reduction in eDNA.

4.2 Introduction

Bacteria accumulate at interfaces forming biofilms, communities of cells embedded in a self-produced polymeric matrix. The matrix constitutes about 90% of the mass of the biofilm

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*Reprinted with permission from "Global regulator H-NS and lipoprotein NlpI influence production of extracellular DNA in *Escherichia coli*" by Viviana Sanchez-Torres, Toshinari Maeda, and Thomas K. Wood, 2010, Biochemical and Biophysical Research Communications, Copyright 2010, Elsevier, doi:10.1016/j.bbrc.2010.09.026. V. Sanchez-Torres and T. Maeda performed the genome-wide screening, and V. Sanchez-Torres was responsible for the qPCR experiments.

and mainly consists of extracellular polysaccharides, proteins, lipids, and nucleic acids (45). The extracellular DNA (eDNA) component of the biofilm matrix has been found in many Gram positive (55, 132, 171, 178) and Gram negative bacteria (57, 81, 184) and serves many roles in different bacteria; eDNA is required for initial attachment to a surface (55, 126, 132), has a structural role connecting the cells in the biofilm (1, 20, 81, 132), works as a nutrient source (41, 114), contributes to cation gradients, induces antibiotic resistance, and promotes its own release via cell lysis by destabilizing membranes through cation chelation (106). eDNA also facilitates horizontal gene transfer and DNA uptake (165).

eDNA also works as an interconnecting material for planktonic cells for *Pseudomonas aeruginosa* where microscopic observation and DNase I treatment indicate that planktonic cells are connected by eDNA-forming clumps (1). Another example is the marine photosynthetic bacterium *Rhodovulum sulfidophilum* which forms aggregated communities of cells called flocs (168) joined by extracellular DNA and RNA (110).

The origin of eDNA is not clear since some reports indicate that eDNA is similar to genomic DNA (gDNA) (1, 126, 168) but other studies revealed, by comparing eDNA and gDNA through random amplification, that they are different (20, 110). The most common mechanism of eDNA release is cell lysis (1, 115, 126, 165). However, it has been proposed that membrane vesicles (MVs) released from the outer membrane also participate in eDNA production (184) since when MVs are opened, eDNA and enzymes that promote lysis are liberated (68). Some bacteria produce eDNA by direct secretion from intact cells such as *Neisseria gonorrhoeae* that produces eDNA via type IV secretion system (54).

eDNA release has been related to quorum-sensing in *Streptococcus pneumoniae* via the competence-stimulating peptide (CSP) (165) and in *P. aeruginosa* via acylhomoserine lactones (AHLs) and PQS signaling (1). We also have reported that eDNA levels are inversely related to

c-di-GMP in *P. aeruginosa* (174) as regulated by tyrosine phosphorylation regulator TpbA (173).

Here, we sought to identify the genes controlling the release of eDNA in *E. coli* (190) in order to understand better the nature of its release from this strain; to date the mechanism of eDNA release in this best-studied strain has not been addressed. We screened the entire Keio collection of 3985 *E. coli* K-12 BW25113 single gene knock-out mutants for eDNA using a fluorescence dye to stain the DNA present in the supernatant of cultures grown quiescently in minimal media in microtiter plates. The mutations altering eDNA are related to general cellular processes such as DNA replication, transcription, translation, nutrient transport and metabolism, and cell envelope. Specifically, the *nlpI*, *yfeC*, and *rna* mutants increased eDNA production and the *hns* and *rfaD* mutants decreased eDNA production. The role of cell lysis and MVs on eDNA with the *nlpI* and *hns* mutants was also investigated; these results suggest DNA is secreted by a process controlled by H-NS.

4.3 Materials and methods

4.3.1 Bacterial strains, media, and growth conditions

The *E. coli* strains and plasmids used in this study are listed in Table 4.1. We used the 3985 *E. coli* K-12 BW25113 single gene knock-out mutants from the Keio collection (9) for the eDNA screening and the ASKA library (76) for overexpression of specific genes. Cultures were made in Luria-Bertani (LB) (142). Kanamycin (50 μg/mL) was used for pre-culturing the knock-out mutants, carbenicillin (100 μg/mL) was used for pLP170, and chloramphenicol (30 μg/mL) was used for selecting plasmid pCA24N and its derivatives. All experiments were conducted at 37^{o}C.

Table 4.1. *E. coli* strains and plasmids used in this study.

Strains and plasmids	Genotype/relevant characteristics[a]	Source
Strains		
BW25113	F$^-$ *Δ(araD-araB)567 ΔlacZ4787*(*::*rrnB-3) λ$^-$ *rph-1 Δ(rhaD-rhaB)568 hsdR514*; parental strain for the Keio collection.	Yale Coli Genetic Stock Center
BW25113 *hns*	BW25113 *Δhns746::kan* Km^R	(9)
BW25113 *nlpI*	BW25113 *ΔnlpI775::kan* Km^R	(9)
BW25113 *rfaD*	BW25113 *ΔrfaD731::kan* Km^R	(9)
BW25113 *rna*	BW25113 *Δrna749::kan* Km^R	(9)
BW25113 *yfeC*	BW25113 *ΔyfeC732::kan* Km^R	(9)
BW25113 *hha*	BW25113 *Δhha745::kan* Km^R	(9)
BW25113 *hha hns*	BW25113 *Δhha845 Δhns746::kan* Km^R	(60)
Plasmids		
pCA24N	*lacI*^{q}, Cm^R	(76)
pCA24N-*hns*	pCA24N $P_{T5\text{-}lac}$::*hns* Cm^R	(76)
pCA24N-*nlpI*	pCA24N $P_{T5\text{-}lac}$::*nlpI* Cm^R	(76)
pLP170	promoterless *lacZ* fusion vector Cb^R	(125)

[a] Km^R, Cm^R, Cb^R are kanamycin, chloramphenicol, and carbenicillin resistance, respectively.

4.3.2 eDNA screening

The mutants from the Keio collection were transferred from glycerol stocks, using a 96 pin replicator (Boekel Scientific, Feasterville, PA), to 96-well polystyrene plates (Corning, Lowell, MA) containing 300 µL of AB medium (29) supplemented with 0.2 % glucose and 0.4% casamino acids and were incubated for 24 h without shaking. AB medium (1) was used for the screening since LB medium interfered with the fluorescence dye used for detecting eDNA. Cell density was measured at 620 nm with a Sunrise microplate reader (Tecan, Salzburg, Austria), and the 96-well plates were centrifuged at 4150 rpm for 10 min using an AccuSpin 3R centrifuge (Fisher Scientific Co, Pittsburgh, PA). The amount of DNA in 100 µL of supernatant was determined with Quant-iT PicoGreen dsDNA kit (Molecular Probes, Eugene, OR) using a Spectra Max Germini EM fluorescence microplate reader (Molecular Devices, Sunnyvale, CA) with an excitation wavelength of 480 nm and emission wavelength of 520 nm. The amount of DNA was normalized by the cell density, and the mutants that significantly altered eDNA were screened again against the wild-type BW25113 using at least three independent colonies of each strain.

4.3.3 Quantitative polymerase chain reaction (qPCR)

eDNA was purified as described previously (174) from cells cultured for 24 h in LB with shaking at 250 rpm starting from an initial turbidity at 600 nm of 0.05. The culture (1 mL) was centrifuged at 13 krpm for 10 min, and the supernatant was used for eDNA purification using phenol:chloroform:isoamyl alcohol (25:24:1) extraction and sodium acetate and isopropanol precipitation. To normalize the eDNA by the total amount of DNA in the cells and in the supernatant, 1 mL of culture was sonicated for 45 s at 10 W (60 Sonic Dismembrator, Fisher Scientific Co, Pittsburgh, PA) and centrifuged at 13 krpm for 10 min; the supernatant was

used for total DNA purification. The purified eDNA and total DNA from at least two independent cultures of each strain was quantified by qPCR using the StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA) and the SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR Kit (Invitrogen, Carlsbad, CA) with primers for the reference gene *purA* (purA-f 5'-GGGCCTGCTTATGAAGATAAAGT-3' and purA-r 5'-CAACCACCA TAGAAGTCAGGT-3').

4.3.4 Cell lysis assay

BW25113 and the *hns* and *nlpI* mutants expressing *lacZ* from pLP170 were cultured into 25 mL of LB medium starting from a cell density of 0.05 at 600 nm for 24 h, 250 rpm. The β-galactosidase activity of the culture supernatants was normalized by the total β-galactosidase activity of the sonicated cultures and used to evaluate cell lysis as described previously (89).

4.3.5 Membrane vesicles

MVs were purified as described previously (180), with some modifications. BW25113, *nlpI*, and *hns* cultures in LB with an initial turbidity at 600 nm of 0.03 were grown for 14 h then centrifuged at 6000 g for 10 min at 4°C. The supernatants were filtered through a 0.22 μm vacuum filter (Millipore Co., Billerica, MA) and concentrated by ultrafiltration using a 100 kDa cut-off Diaflo membrane (Amicon Co., Lexington, MA) in a stirred ultrafiltration cell (model 8200, Amicon Co., Lexington, MA). The concentrated supernatants were ultracentrifuged at 30 krpm for 1 h at 4°C in a SW41 Ti rotor (154,100 g) using Beckman L8-M ultracentrifuge (Beckman Coulter Inc., Brea, CA); the supernatants were decanted and the precipitated membrane vesicles were resuspended with 50 mM HEPES pH 6.8 buffer. The amount of MVs was determined using Bio-Rad protein assay kit (Bio-Rad, Richmond, CA).

4.4 Results

4.4.1 Screening of the genes involved in eDNA production

To identify genes involved in *E. coli* eDNA production, we screened 3985 nonessential gene knock-out mutants of the Keio Collection (9). Since the mutants were grown quiescently in microtiter plates, the eDNA detected in the screening was produced from both biofilm and planktonic cells. The screening was performed based on the fluorescence of Quant-iT PicoGreen reagent upon binding to double stranded DNA (dsDNA); the sensitivity with the conditions used for the assay was 0.004 ng dsDNA/μL. After two rounds of screening, four mutants that increase eDNA and 31 mutants that decrease eDNA more than 2.5 fold were identified (Table 4.2). These genes encode proteins mainly located in the cytoplasm that are related to different cellular processes including the synthesis of components of the cell envelope such as lipopolysaccharide (LPS).

Fifteen mutants indentified in the initial screen which had the biggest impact on eDNA within various functional groups were further verified via qPCR with eDNA samples purified from planktonic cells cultured in LB: *rna, hns, pnp, groL, cyaA, aspC*, *moaE*, *menD*, *pstA*, *rfaD*, *rfaG*, *ybgF*, *nlpI*, *yfeC*, and *yieL*. Of these 15, the *nlpI*, *yfeC*, and *rna* mutants increased eDNA, and the *rfaD* mutant decreased eDNA as expected based on the initial screen (Fig. 4.1A). There was no amplification via qPCR for the eDNA samples of the *hns* mutant (even after a 50-fold concentration); hence, the *hns* deletion abolishes the formation of eDNA.

Hha is a global regulator (49) with nonspecific DNA binding (109) that alters the production of multiple proteins (11) and which forms a complex with H-NS that binds DNA (109). To evaluate if the H-NS regulation of eDNA occurs through its interaction with Hha, we evaluated via qPCR the eDNA produced by the *hha* mutant and *hha hns* double mutant. The *hha*

Table 4.2. ***E. coli* BW25113 genes whose mutations altered eDNA as detected by Quant-iT PicoGreen.** The BW25113 value corresponds to the average of 31 independent colonies and for each mutant at least 3 colonies were assayed. For all listed mutants, differences in eDNA compared to the wild-type are significant based on a Student's T test ($P < 0.05$). Locations are from (101) and function are from (73, 139).

Strain	OD_{620}	ng DNA μL^{-1} OD_{620}^{-1}	Location[a]	Fold	Function
BW25113	1.1 ± 0.2	0.53 ± 0.07	-	1	-
Replication, recombination and repair					
priA	0.62 ± 0.03	0.17 ± 0.01	C	-2.9	PriA participates in DNA replication
Transcription and translation					
rna	0.9 ± 0.2	2.2 ± 0.6	P	4.4	RNase I, cleaves phosphodiester bonds in RNA
hns	0.67 ± 0.05	0.17 ± 0.02	C	-2.9	DNA-binding global regulator H-NS
pnp	1.1 ± 0.1	0.16 ± 0.01	C	-3.1	PNPase, involved in general mRNA degradation
Posttranslational modification, protein turnover, chaperones					
groL	1.2 ± 0.3	0.15 ± 0.01	C	-3.3	Chaperone Hsp60
sspA	1.1 ± 0.2	0.16 ± 0.01	C	-3.1	Protein essential for cell survival under acid-stress
Metabolism					
cyaA	0.99 ± 0.07	0.10 ± 0.01	C	-5.0	Adenylate cyclase CyaA catalyzes the synthesis of cyclic AMP
aspC	0.76 ± 0.02	0.11 ± 0.01	C	-4.5	Aspartate aminotransferase
gmhB	0.85 ± 0.08	0.13 ± 0.02	C	-3.8	D,D-heptose 1,7-bisphosphate phosphatase
btuB	1.0 ± 0.3	0.15 ± 0.06	OM	-3.3	Receptor for transport of vitamin B12, E colicins, and phages BF23 and C1
moaA	0.7 ± 0.04	0.15 ± 0.03	C	-3.3	Protein that participates in the synthesis of molybdopterin guanine dinucleotide
moaC	0.59 ± 0.04	0.19 ± 0.03	C	-2.6	Protein that participates in the MPT biosynthesis
moaE	0.8 ± 0.3	0.13 ± 0.03	C	-3.8	MPT synthase
mog	0.82 ± 0.07	0.21 ± 0.01	C	-2.8	Protein that participates in the MPT biosynthesis
menD	1.16 ± 0.05	0.14 ± 0.02	C	-3.6	Protein that participates in menaquinone biosynthesis
menE	1.14 ± 0.08	0.11 ± 0.01	C	-4.5	Protein that participates in menaquinone biosynthesis
nudB	1.1 ± 0.2	0.20 ± 0.04	C	-2.5	Protein that participates in the early steps in folate synthesis
Inorganic ion transport					
pstA	1.7 ± 0.07	0.12 ± 0.01	IM	-4.2	Part of the ATP-dependent phosphate uptake system PstABCS
pstS	1.0 ± 0.3	0.13 ± 0.04	P	-3.8	Part of the ATP-dependent phosphate uptake system PstABCS

Table 4.2. (continued)

Strain	OD_{620}	ng DNA μL^{-1} OD_{620}^{-1}	Location[a]	Fold	Function
phoU	1.1 ± 0.3	0.15 ± 0.04	C	-3.3	Negative regulator of pho regulon (phosphate transport system)
modC	0.6 ± 0.2	0.18 ± 0.01	C	-2.8	ATP-binding component of the molybdate ABC transporter
Cell envelope					
lpcA	0.65 ± 0.08	0.19 ± 0.02	C	-2.6	Catalyzes the first step in the synthesis of core lipopolysaccharide (LPS)
rfaD	0.63 ± 0.02	0.16 ± 0.03	C	-3.1	Involved in the synthesis of the precursor of core LPS
rfaE	0.61 ± 0.05	0.16 ± 0.01	C	-3.1	Involved in the synthesis of the precursor of core LPS
rfaF	0.75 ± 0.07	0.20 ± 0.02	C	-2.5	LPS heptosyltransferase II
rfaG	0.7 ± 0.2	0.19 ± 0.04	C	-2.6	Glucosyltransferase I involved in LPS core biosynthesis
nlpD	1.0 ± 0.1	0.17 ± 0.02	OM	-2.9	Protein related to cell division
tolC	0.6 ± 0.2	2.0 ± 0.4	OM	4.0	Porin component of several multi-drug efflux systems
ybgF	0.86 ± 0.03	0.18 ± 0.02	P	-2.8	Part of the Tol-Pal contributing to maintain cell envelope integrity
yfgA	0.7 ± 0.1	0.18 ± 0.04	IM	-2.8	Protein responsible for maintaining the rod shape of the *E. coli* cell
Function unknown					
nlpI	0.7 ± 0.2	2.1 ± 0.1	OM	4.2	Lipoprotein related to osmotic sensitivity, filamentation, and virulence
yfeC	0.93 ± 0.03	1.4 ± 0.3	C	2.8	Predicted DNA-binding transcriptional regulator
yieL	0.70 ± 0.02	0.15 ± 0.01	P	-3.3	Predicted xylanase
yhbP	0.63 ± 0.03	0.18 ± 0.01	C	-2.8	Function unknown.
yjiP	1.10 ± 0.07	0.2 ± 0.1	C	-2.6	Predicted transposase involved in biofilm formation

[a] C, cytoplasm; IM, inner membrane; P, periplasm; OM, outer membrane

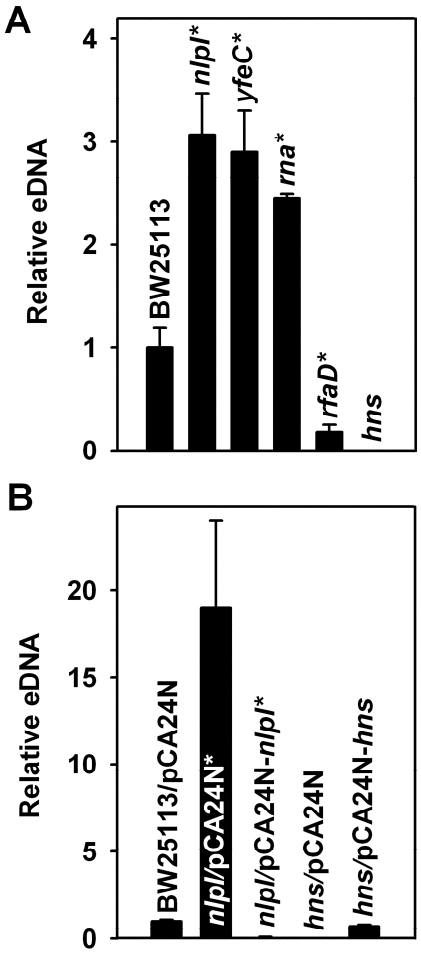

Fig. 4.1. **eDNA quantified by qPCR.** The values are the average of at least 2 independent cultures assayed in duplicate, the error bars correspond to the standard deviation, and an asterisk indicates P-values < 0.05 using Student's T test. (A) Knock-outs mutants that altered eDNA production. Cells were grown in LB for 24 h, 250 rpm at 37°C. (B) Complementation of *hns* and *nlpI* eDNA. Cells were grown in LB for 24 h, 250 rpm at 37°C with 21.5 h of induction with 0.1 mM IPTG.

mutant produces the same amount of eDNA as wild-type BW25113, and the eDNA of the *hha hns* mutant was not detected; therefore, H-NS regulation of eDNA is not related to Hha.

The *aspC*, *ybgF*, *moaE*, *menD*, *pstA*, *cyaA, pnp* and *yieL* mutants did not have statistically significant differences in their eDNA compared to the wild-type strain as assayed by qPCR. The *rfaG* (2.4 fold) and *groL* (2.9 fold) mutations increased eDNA via qPCR but decreased eDNA based on the initial screening with Quant-iT PicoGreen. These discrepancies may be due to differences in the growth conditions since the initial screen was performed with cells grown quiescently in AB minimal media supplemented with glucose and casamino acids in microtiter plates, but the cells for the qPCR screen were grown in LB media in flasks with shaking.

4.4.2 Complementation of nlpI and hns eDNA

The mutants with the highest impact on eDNA were *nlpI* and *hns.* To confirm that NlpI and H-NS regulate eDNA production, plasmids pCA24N-*hns* and pCA24N-*nlpI* were used to overexpress *hns* and *nlpI* (Fig. 4.1B). As expected, eDNA was not produced by BW25113 *hns*/pCA24N, and overexpressing *hns* in BW25113 *hns*/pCA24N-*hns* restored eDNA to 70% of the wild-type BW25113/pCA24N. Similarly, as expected, the *nlpI* mutation in BW25113 *nlpI*/pCA24N increased eDNA 19 fold while overexpressing *nlpI* in BW25113 *nlpI*/pCA24N-*nlpI* decreased eDNA 16 fold (Fig. 4.1B). Hence, our results indicate that H-NS enhances eDNA production and that NlpI negatively controls eDNA in *E. coli*.

4.4.3 Cell lysis assay

Since β-galactosidase is a cytoplasmic enzyme, its activity in culture supernatants has been used previously to determine if eDNA production occurs via lysis of a subpopulation of the

culture (1, 115, 132, 165). Therefore, plasmid pLP170 harboring *lacZ* was electroporated into BW25113 containing the *hns* or *nlpI* mutations to evaluate the β-galactosidase in the culture supernatants normalized by the β-galactosidase activity of cell lysates; *lacZ* is inactivated in wild-type BW25113. The *nlpI* mutant increased cell lysis 6.4 ± 0.9 fold which is similar to its increase in eDNA (3.1 ± 0.4 fold). However, cell lysis does not explain the decrease in eDNA in the *hns* mutant since the deletion of *hns* abolished *E. coli* eDNA production but cell lysis decreased by 1.8 ± 0.6 fold. These results suggest that cell lysis contributes to eDNA release in *E. coli;* however, another mechanism may also be present.

4.4.4 Membrane vesicles

To investigate whether MVs were altered by the *nlpI* and *hns* mutations, we purified MVs from supernatants of BW25113 and the *nlpI* and *hns* mutants cultures made in LB medium. The *nlpI* mutant has 107-fold more vesicles (cf., 3-fold more eDNA) and the *hns* mutant has 3-fold more vesicles (cf., no eDNA) than the wild-type BW25113. These results for MVs agree with the values reported previously for the *nlpI* (97) and *hns* (63) mutants. Therefore, eDNA production via MVs is not the main mechanism of eDNA production in *E. coli* since the changes in MVs do not match the changes in eDNA for these two mutants.

4.5 Discussion

Our results show that *E. coli* releases eDNA during static growth, where there are planktonic and sessile cells. Furthermore, we identified 35 proteins with a greater than 2.5-fold difference in eDNA production and characterized the *nlpI* and *hns* mutations more fully. Mutations in *yfeC*, *rna*, and *nlpI* increased eDNA. YfeC is an uncharacterized protein that has a helix-turn-helix domain (42); hence, it probably is a negative transcriptional regulator of genes

encoding proteins related to eDNA. *rna* encodes RNase I; hence, the increase in eDNA by the *rna* deletion may be related to the reduced degradation of DNA that occurs in the *rna* mutant (189). Since RNase I is a periplasmic protein, the increase in eDNA by the *rna* deletion suggests that DNA is present in the periplasm which agrees with eDNA release via secretion.

The largest increase in eDNA was obtained with the *nlpI* mutant (Fig. 4.1A). NlpI is an outer membrane lipoprotein that probably participates in cell division (113) and is related to bacterial virulence in pathogenic *E. coli* strains by promoting adhesion to intestinal epithelial cells (14) and human brain microvascular endothelial cells (170). The *nlpI* mutant shows elongation at 42^{o}C at low osmolarity (113) and produces more than 100 fold more membrane vesicles (97). Cells overexpressing *nlpI* have a prolate ellipsoidal shape and have some cells joined by partial constrictions which suggest that cell division is altered due to defects in chain elongation and the formation of the septal ring (113). Hence, deletion of *nlpI* probably leads to more eDNA that may decorate the exterior of the cell and render it less able to bind epithelial and endothelial cells.

Mutations in *hns* and *rfaD* decreased eDNA. RfaD is an enzyme that participates in the synthesis of a precursor of LPS. The *rfaD* mutant forms mini-cells which indicate cell division defects, has a mucoid phenotype, has resistance to λ phage, and cannot growth at temperatures higher than 42^{o}C or in media containing bile salts (72).

For the *hns* mutant, eDNA production was abolished since eDNA was not detected by qPCR. H-NS is an abundant protein (approximately 20,000 copies per cell) (38) that binds to DNA and condenses the nucleoid (34). H-NS functions as a transcriptional global regulator controlling genes encoding proteins related to the cell envelope and adaptation to environmental conditions (59) including 69% of temperature regulated genes (185). The *hns* mutant forms 3-fold more membrane vesicles (63) and has altered chromosome partitioning and replication (69).

Since the reduction in cell lysis by the *hns* mutant is not comparable to the reduction in eDNA production, *E. coli* should have another mechanism other than lysis for eDNA production. Similarly, the production of eDNA via membrane vesicles may not be the main mechanism of eDNA production in *E. coli* since the *hns* deletion increases vesiculation but decreases eDNA. Hence, it is possible that *E. coli* produces eDNA via direct secretion from living cells. Therefore, although speculative, our data suggest that H-NS regulates eDNA secretion in *E. coli* in a manner that is not dependent on Hha.

Given that *E. coli* is a Gram negative bacterium, to be secreted, DNA should go through the inner membrane, the cell wall, and the outer membrane. This transport of DNA may also occur through the points where the inner and outer membranes are joined to each other through the cell wall (47). The inner and outer membrane are involved in DNA replication, and the outer membrane fractions contain newly replicated DNA. During cell division, on each side of the septum, two rings are formed where the inner and outer membranes are fussed. Since both *nlpI* and *hns* mutants have altered cell division and have the biggest effect on eDNA release, secretion in *E. coli* may be related to DNA replication and cell division. An eDNA secretion mechanism related to DNA replication occurs in the Gram positive *Bacillus subtilis*. During spore germination, *B. subtillis* releases eDNA following replication, and the rate of DNA synthesis is similar to the rate of DNA release (24).

CHAPTER V

GGDEF PROTEINS YEAI, YEDQ, AND YFIN REDUCE EARLY BIOFILM FORMATION AND SWIMMING MOTILITY IN *ESCHERICHIA COLI*

5.1 Overview

The second messenger 3'-5'-cyclic diguanylic acid (c-di-GMP) promotes biofilm formation by regulating different phenotypes such as exopolysaccharide production, aggregation, motility, and extracellular DNA (eDNA). c-di-GMP is synthesized by diguanylate cyclases characterized by the GGDEF domain and degraded by phosphodiesterases characterized by EAL or HD-GYP domains. *Escherichia coli* K-12 has 12 proteins with a GGDEF domain (AdrA, YdaM, YcdT, YddV, YdeH, YeaP, YeaI, YeaJ, YedQ, YfiN, YliF, and YneF), 10 proteins with an EAL domain (Rtn, YcgF, YcgG, YdiV, YhjH, YjcC, YlaB, YliE, YoaD and YahA), and 7 proteins with both GGDEF and EAL domains (YciR, Dos, YegE, YfeA, YfgF, YhdA, and YhjK).

Here, we evaluated the effect of the 12 *E. coli* GGDEF-only proteins on biofilm formation and motility. Deletions of the genes encoding the GGDEF proteins YeaI, YedQ, YfiN, YeaJ, and YneF increased swimming motility as expected for strains with reduced c-di-GMP, and alanine substitution in the EGEVF motif of YeaI abolished its impact on swimming motility. In addition, eDNA was increased as expected with the *yeaI* (10 fold), *yedQ* (1.8 fold) and *yfiN* (3.2 fold) deletions.

As a result of the significantly enhanced motility, but contrary to current models of decreased biofilm formation with decreased diguanylate cyclase activity, early biofilm formation increased dramatically for the *yeaI* (30 fold), *yedQ* (12 fold), and *yfiN* (18 fold) deletions. Our results indicate that YeaI, YedQ, and YfiN are active diguanylate cyclases that reduce motility,

eDNA, and early biofilm formation and contrary to the current paradigm, the results indicate that c-di-GMP levels should be reduced, not increased, for initial biofilm formation so c-di-GMP levels must be regulated in a temporal fashion in biofilms.

5.2 Introduction

Bacterial cells can adopt two different lifestyles, the planktonic mode characterized by single motile cells, or the biofilm mode where bacterial cells form sedentary multicellular communities attached themselves or to a surface (78). The second messenger 3'-5'-cyclic diguanylic acid (c-di-GMP) acts as a switch promoting the transition from the planktonic to the biofilm lifestyle (58). The c-di-GMP paradigm is that high intracellular c-di-GMP levels promote the synthesis of exopolysaccharides which are components of the biofilm matrix, promote the formation of adhesive fimbriae, and inhibit motility (123).

c-di-GMP is synthesized from guanosine-5'-triphosphate by diguanylate cyclases which are characterized by the GGDEF domain (58). Diguanylate cyclases have a conservative GG[D/E]EF motif in their active site (A-site), and some of them also have an inhibitory site for c-di-GMP binding (I-site) (162). Degradation of c-di-GMP is catalyzed by phosphodiesterases, characterized by EAL or HD-GYP domains (150). Most bacteria have multiple diguanylate cyclases and phosphodiesterases. *Escherichia coli* K-12 has 12 proteins with a GGDEF domain, 10 proteins with an EAL domain, and 7 proteins with both GGDEF and EAL domains in a single polypeptide (182).

Among the 12 GGDEF-only proteins in *E. coli*, the diguanylate cyclase activity of AdrA (5), YdaM (182), YddV (98), YcdT, YdeH (67), and YeaP (141) has been confirmed either in vitro, using purified protein, or in vivo by measuring the effect of deleting or overexpressing the corresponding genes in the c-di-GMP intracellular levels. Some of these *E. coli* GGDEF-only

proteins alter biofilm related phenotypes since YdaM (182), YeaP (162), and YddV (169) promote curli formation, AdrA (5) and YedQ (33) activate cellulose production, and YdeH activates production of the polysaccharide adhesin poly-b-1,6-N-acetyl-glucosamine (22). Also, YdeH, YcdT (67), YeaJ, YedQ (122), YddV, and YfiN (21) negatively regulate motility. The phenotypes controlled by the other GGDEF-only proteins (YeaI, YliF, and YneF) have not been characterized.

Mature *E. coli* biofilm formation increased by overexpressing the genes encoding the GGDEF-only diguanylate cyclases AdrA (5), and YddV (98). Hence, high c-di-GMP concentrations enhance late biofilm formation while reducing motility. However, mutants with decreased motility have reduced initial biofilm formation (50); hence, we reasoned that decreasing diguanylate cyclase activity should increase motility and lead to increased early biofilm formation, even though diguanylate cyclase activity is known to increase biofilm formation. To address this paradox and to investigate the role of heretofore unstudied *E. coli* diguanylate cyclases on biofilm formation, we compared biofilm formation of the wild-type BW25113 and its isogenic mutants defective in each of the 12 genes encoding GGDEF-only proteins and found knock-outs of the genes encoding the GGDEF proteins YeaI, YedQ, and YfiN enhanced early biofilm formation dramatically.

Our results suggest that a reduction in the c-di-GMP levels caused by inactivating *yeaI*, *yedQ*, and *yfiN* enhances swimming motility which contributes to enhanced initial attachment to the polystyrene surface thus promoting early biofilm formation. Therefore, the current paradigm for c-di-GMP should be refined to indicate its inverse relationship to initial biofilm formation and to indicate that the timing of c-di-GMP production is likely more sophisticated than just elevated in biofilms.

5.3 Materials and methods

5.3.1 Bacterial strains, media, and growth conditions

The *E. coli* strains and plasmids used in this study are listed in Table 5.1. Single deletion mutants of the parental strain *E. coli* K-12 BW25113 were obtained from the Keio collection (9). The double deletion strains BW25113 *yeaI yedQ* and BW25113 *yeaI yfiN* were constructed via P1 transduction as described previously (93). The deletions of *yeaI*, *yedQ*, and *yfiN* were verified via polymerase chain reaction (PCR) using primers listed in Table 5.2 as described previously (144). Primers flhDC-F2 and flhDC-R (Table 5.2) were used to verify by PCR that the wild-type strain and mutants used here *adrA, ydaM*, *ycdT*, *yddV*, *ydeH*, *yeaP*, *yeaI*, *yeaJ*, *yedQ*, *yfiN*, *yliF*, *yneF,* and *ycgR*, do not contain IS insertions in the regulatory sequence of *flhDC* that may increase motility (13).

Experiments were conducted at 37^{o}C in either in Luria-Bertani (LB) (142) or M9 minimal medium supplemented with 0.4% casamino acids (M9C). Kanamycin (50 μg/mL) was used for pre-culturing the knock-out mutants and chloramphenicol (30 μg/mL) was used for selecting plasmid pCA24N (76) and its derivatives. The specific growth rates of BW25113 and the *yeaI, yedQ,* and *yfiN* knock-out mutants were measured in LB using two independent cultures for each strain with the turbidity measured at 600 nm from 0.05 to 0.7.

Table 5.1. Strains and plasmids used.

Strains and plasmids	Genotype/relevant characteristics[a]	Source
Strains		
BW25113	F⁻ *Δ(araD-araB)567 ΔlacZ4787*(::rrnB-3) λ⁻ *rhp-1 Δ(rhaD-rhaB)568 hsdR514*; parental strain for the Keio collection.	Yale Coli Genetic Stock Center
BW25113 *adrA* (*yaiC*)	BW25113 *ΔyaiC750::kan* Km^R	(9)
BW25113 *ydaM*	BW25113 *ΔydaM778::kan* Km^R	(9)
BW25113 *ycdT*	BW25113 *ΔycdT771::kan* Km^R	(9)
BW25113 *yddV*	BW25113 *ΔyddV783::kan* Km^R	(9)
BW25113 *ydeH*	BW25113 *ΔydeH756::kan* Km^R	(9)
BW25113 *yeaP*	BW25113 *ΔyeaP790::kan* Km^R	(9)
BW25113 *yeaI*	BW25113 *ΔyeaI782::kan* Km^R	(9)
BW25113 *yeaJ*	BW25113 *ΔyeaJ783::kan* Km^R	(9)
BW25113 *yedQ*	BW25113 *ΔyedQ730::kan* Km^R	(9)
BW25113 *yfiN*	BW25113 *ΔyfiN767::kan* Km^R	(9)
BW25113 *yliF*	BW25113 *ΔyliF734::kan* Km^R	(9)
BW25113 *yneF*	BW25113 *ΔyneF743::kan* Km^R	(9)
BW25113 *yeaI yedQ*	BW25113 Δ*yeaI882 ΔyedQ730::kan* Km^R	This study
BW25113 *yeaI yfiN*	BW25113 Δ*yeaI882 ΔyfiN767::kan* Km^R	This study
Plasmids		
pCA24N	$lacI^q$, Cm^R	(76)
pCA24N-*yeaI*	pCA24N $P_{T5\text{-}lac}$::*yeaI* Cm^R	
pCA24N-*yeaIE407A*	pCA24N $P_{T5\text{-}lac}$::*yeaI407* Cm^R; encodes YeaI with E407A	This study

[a] Km^R and Cm^R are kanamycin and chloramphenicol resistance, respectively.

Table 5.2 Primers used for site-directed mutagenesis, qPCR, DNA sequencing, and verification of the relevant deletions in the strains used.

Primer	Sequence[a]
Site-directed mutagenesis at position E407 of YeaI	
yeaIE407-F	5'-ATTTTAGCGCGACTGGAGGGTGCGGTGTTTGGCTTGCTATTTACC-3'
yeaIE407-R	5'-GTAAATAGCAAGCCAAACACCGCACCCTCCAGTCGCGCTAAAAT-3'
qPCR	
purA-f	5'-GGGCCTGCTTATGAAGATAAAGT-3'
purA-r	5'-TCAACCACCATAGAAGTCAGGAT-3'
DNA sequencing of pCA24N-*yeaIE407A*	
hha rear	5'-GAACAAATCCAGATGGAGTTCTGAGGTCATT-3'
Verification of strains	
yeaI front	5'-GTGGCGAGAATATGAGCATCTG-3'
yeaI rev	5'-CTGGATCAGTGTACTGCCGTTA-3'
yedQ front	5'-GAGTGTCGTTGGTATGACGGTTAC-3'
yedQ rev	5'-GTTCCCAGCTAACATAGCGACT-3'
yfiN front	5'-AGTACCGCCCTACAAGAGAATG-3'
yfiN rev	5'-CAGAATACAACCGGTCAGTACG-3'
kanrev	5'-ATCACGGGTAGCCAACGCTATGTC-3'
flhDC-F2	5'-CCTGTTTCATTTTTGCTTGCTAGC-3'
flhDC-R	5'-GGAATGTTGCGCCTCACCG-3'

[a] Underlined text indicate the site-directed mutation for the codon corresponding to E407 (5'-GAG to 5'-GCG for E407A).

5.3.2 Crystal violet biofilm assay

Biofilm formation was assayed in 96-well polystyrene plates (Corning, Lowell, MA) as described previously (46). Wells were inoculated with overnight cultures at an initial turbidity at 600 nm of 0.05 in LB and incubated for 7 h and 24 h quiescently. Biofilm formation was also assayed in M9C (142) for 7 h. For each strain, at least 2 independent cultures were assayed in 12 replicate wells.

5.3.3 Site-directed mutagenesis

The codon encoding the second glutamic acid of the EGEVF motif of YeaI GAG was mutated to the alanine codon GCG to yield a EGAVF motif. Site-directed mutagenesis was performed using pCA24N-*yeaI* as template with complementary primers containing the target mutation (Table 5.2) as described previously (164).

5.3.4 Swimming motility assay

Single colonies were inoculated onto motility plates (1% tryptone, 0.25% NaCl, and 0.3% agar) (163) using a toothpick. For cells with pCA24N-based plasmids, the motility plates were supplemented with 30 μg/mL chloramphenicol and 0.1 mM IPTG. The motility halos were measured after 12 h incubation and at least two independent cultures for each strain were used.

5.3.5 eDNA assay

eDNA was assayed as described previously using quantitative PCR (qPCR) (174). Briefly, LB cultures with an initial turbidity at 600 nm of 0.05 were incubated for 24 h. Supernatants (1 mL) were centrifuged for 10 min at 13 krpm to find eDNA, and the total amount of DNA in the culture (outside and inside the cells) was determined using 1 mL of culture that

was sonicated for 45 s at 10 W (60 Sonic Dismembrator, Fisher Scientific Co, Pittsburgh, PA) and centrifuged at 13 krpm for 10 min. eDNA and total DNA were purified using phenol:chloroform:isoamyl alcohol (25:24:1) extraction and sodium acetate and isopropanol precipitation. eDNA and total DNA were quantified by qPCR using the StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA) and the SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR Kit (Invitrogen, Carlsbad, CA) with primers for the reference gene *purA* (Table 5.2). At least two independent cultures were used.

5.3.6 Quantification of c-di-GMP

-di-GMP was assayed as described previously using HPLC (Ueda and Wood 2009). Cells from overnight cultures were inoculated into 350 mL of LB medium and cultured for 16 h with shaking (250 rpm). Nucleotides were extracted using 65% ethanol and c-di-GMP was quantified using HPLC (Waters 515 with photodiode array detector, Milford, MA). Commercial c-di-GMP (BIOLOG Life Science Institute, Bremen, Germany) was used as the standard. The c-di-GMP peak was verified by spiking each sample with the commercial c-di-GMP and the in vitro degradation of c-di-GMP by purified phosphodiesterase YahA was used as control.

5.4 Results

5.4.1 Deletions of yeaI, yedQ, and yfiN increase early biofilm formation

To investigate whether reductions in diguanylate cyclase activity (which serve to decrease c-di-GMP concentrations) increase initial biofilm formation due to increased cell motility, we assayed biofilm formation after 7 h upon deleting 12 genes encoding GGDEF proteins in *E. coli*. In LB medium, there were not significant differences in biofilm formation

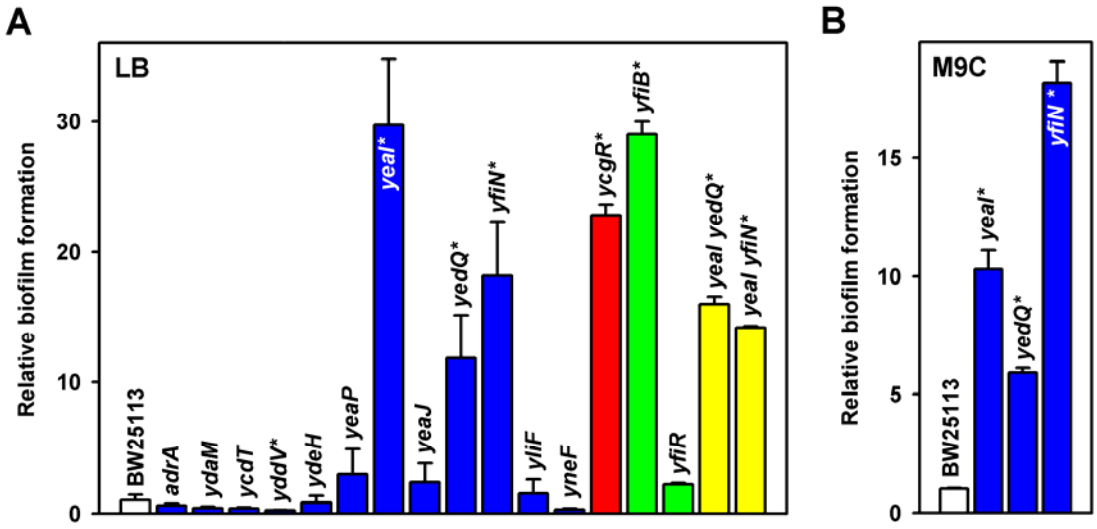

Fig. 5.1. Relative normalized biofilm formation in polystyrene microtiter plates after 7 h at 37°C. (A) Biofilm in LB (B) Biofilm in M9C. Wild-type BW25113 is shown in white; single mutants defective in genes encoding GGDEF-only proteins are in blue, the *ycgR* mutant, which is defective in the gene encoding the c-di-GMP regulated flagellar velocity braking protein, is red, the *yfiB* and *yfiR* mutants which lack genes in the same operon as *yfiN* are in green, and the double deletion mutants *yeaI yedQ* and *yeaI yfiN* are yellow. Biofilm formation (turbidity at 540 nm) was normalized by the amount of planktonic growth (turbidity at 620 nm) and is shown relative to the BW25113 normalized biofilm value. Each data point is the average of at least twelve replicate wells from two independent cultures. The error bars correspond to the standard deviation, and an asterisk indicates P-values < 0.05 using a Student's T test.

increased biofilm formation dramatically: *yeaI* (30 fold), *yedQ* (12 fold), and *yfiN* (18 fold) (Fig. 5.1A). These same three mutations also increased biofilm formation in minimal medium (Fig. 5.1B). After 7 h of incubation, biofilm formation increased 10 fold for the *yeaI* mutant, 6 fold for *yedQ* mutant, and 18 fold for the *yfiN* mutant relative to the wild-type (Fig 5.1B). Hence, deleting *yeaI, yedQ,* and *yfiN* increase biofilm formation dramatically in both rich and minimal medium. Note there was no change in the specific growth rate in rich medium so these changes in biofilm formation are not related to changes in growth.

Since the double deletion mutants *yeaI yedQ* and *yeaI yfiN* did not increase further biofilm formation (Fig. 5.1A), YeaI appears to regulate the same process controlled by YedQ and YfiN. In addition, after 24 h of incubation, none of the mutants significantly altered biofilm formation (data not shown). These results indicate that the deletions of *yeaI, yedQ*, and *yfiN* mainly alter the initial stages of biofilm formation which are influenced by motility (124).

YfiN was first characterized by us in *Pseudomonas aeruginosa* where we showed it was related to rugose colony formation due to its diguanylate cyclase activity that is controlled by a tyrosine phosphatase (173). Since *yfiN* is part of the operon *yfiRNB* in *E. coli*, the biofilm formation of the *yfiB* and *yfiR* mutants was also assayed to evaluate the effect of YfiB and YfiR on the activity of YfiN. While the *yfiR* mutant has biofilm formation similar to the wild-type, the *yfiB* mutant increases biofilm formation similarly to the *yfiN* mutant (Fig. 5.1A). These results indicate that YfiB is a positive regulator of YfiN activity and that the activity of YfiN is not significantly altered by deleting *yfiR*.

To confirm that initial biofilm formation is enhanced when motility is not inhibited by c-di-GMP, we investigated biofilm formation with a *ycgR* deletion. YcgR with bound c-di-GMP inhibits swimming motility by reducing the flagella motor speed through its interaction with MotA (21), FliG, and FliM (118). We found the *ycgR* mutant, like the *yeaI, yedQ*, and *yfiN*

mutants, increases biofilm formation (23 fold) after 7 h (Fig. 5.1A) but not after 24 h (data not shown). These results suggest that the *yeaI, yedQ*, and *yfiN* deletions decrease the levels of c-di-GMP which promotes motility via YcgR inactivation thus increasing early biofilm formation.

5.4.2 Deletions of yeaI, yedQ, and yfiN increase swimming motility and the EGEVF motif of YeaI is necessary to reduce swimming motility

Low levels of c-di-GMP promote swimming and swarming motility (135); hence, we investigated swimming with the *yeaI, yedQ,* and *yfiN* mutants since inactivation of these genes should decrease c-di-GMP levels by inactivating diguanylate cyclase activity. An large increase in swimming motility was observed for the single deletions in *yeaI* (4 fold), *yedQ* (6 fold), and *yfiN* (10 fold) (Fig. 5.2). We also evaluated swimming motility for the remaining 9 mutants encoding GGDEF-only proteins and found that *yeaJ* (2 fold), and *yneF* (4 fold) also have increased motility, suggesting that the GGDEF proteins encoded by these genes are diguanylate cyclases that control swimming motility.

The increase in swimming motility phenotype caused by the *yeaI* deletion was complemented by plasmid pCA24N-*yeaI* (encoding YeaI with EGEVF) (Fig. 5.2). Since YeaI has a EGEVF motif instead of the conserved GG[D/E]EF motif characteristic of active diguanylate cyclases, a single amino acid change of the second glutamic acid of EGEVF (corresponding to the catalytic residue of GGDEF (27) to alanine was introduced via site-directed mutagenesis to show that YeaI is an active diguanylate cyclase. Since motility was not complemented by pCA24N-*yeaIE407A* (encoding YeaI with EGAVF) (Fig. 5.2), the EGEVP domain of YeaI is necessary to reduce swimming motility which provides additional evidence that YeaI increases c-di-GMP as a diguanylate cyclase.

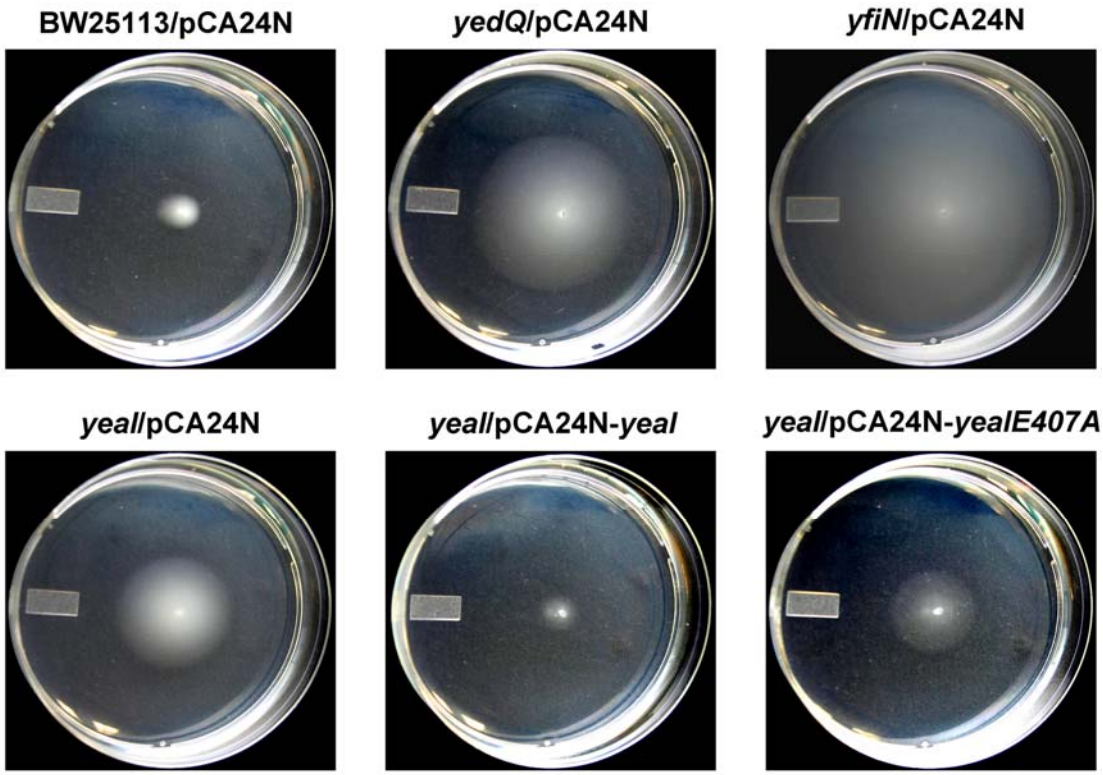

Fig 5.2. Swimming motility for strains with mutations in the genes encoding diguanylate cyclases YeaI (EGEVF), YedQ (GGEEF), and YfiN (GGDEF) and complementation studies for the *yeaI* mutant using pCA24N-*yeaI* producing YeaI and pCA24N-*yeaIE407A* that produces YeaI E407A (EGAVF). Swimming motility was assayed after 12 h at 37°C using motility plates supplemented with 30 μg/mL Cm and 0.1 mM IPTG to induce diguanylate cyclase production from the pCA24N-based plasmids.

5.4.3 Deletions of yeaI, yedQ, and yfiN increase eDNA

eDNA is an important component of the bacterial biofilm matrix (184). c-di-GMP is inversely proportional to eDNA in *P. aeruginosa* cultures (174); therefore, we investigated if the deletions of *yeaI*, *yedQ*, and *yfiN* alter eDNA in *E. coli* with the expectation that deleting these genes would reduce c-di-GMP and thereby increase eDNA. For planktonic cells cultured for 24 h in LB medium, deletion of *yeaI* increased eDNA 10 ± 3 fold, deletion of *yedQ* increased eDNA slightly (1.8 ± 0.3 fold), and deletion of *yfiN* increased eDNA 3.2 ± 0.1 fold. These results suggest that the mutations in *yeaI, yedQ,* and *yfiN* decrease c-di-GMP which results in increased eDNA.

5.4.4 Deletions of yeaI, yedQ, and yfiN do not alter the total concentration of c-di-GMP

We evaluated the c-di-GMP concentrations from cell extracts of the BW25113 and the *yeaI*, *yedQ*, and *yfiN* mutants from planktonic cultures in LB medium. There were not significant differences in the total concentration of c-di-GMP inside the cells of BW25113 and the *yeaI*, *yedQ*, and *yfiN* mutants. These results suggest that the effect of YeaI, YedQ, and YfiN on the c-di-GMP levels may occur locally, close to the inner membrane, since YeaI, YedQ, and YfiN are integral inner membrane proteins (Misra et al. 2005).

5.5 Discussion

Biofilm formation occurs following a developmental sequence (111). First, bacteria move in the liquid culture and reach a surface where cells are reversibly attached, and some cells have a strong adhesion to the surface and become sessile (53). These cells replicate and aggregate in a self-produced polymeric matrix thus forming a mature biofilm (53). Finally cells disperse from the biofilm and return to a motile state (71). For all of these steps, c-di-GMP plays

a role (66).

In many bacteria including *E. coli*, high concentrations of c-di-GMP promote biofilm formation (39). For example, overexpression of the genes encoding the diguanylate cyclases AdrA (5) and YddV (98) increase *E. coli* mature biofilm formation. c-di-GMP also inversely regulates motility (98), and motility is important for initial attachment to a surface; non-flagellated cells or cells with paralyzed flagella have reduced initial biofilm formation (124), and *E. coli* strains with high motility make more biofilm than strains with poor motility (186).

Motility also affects biofilm architecture since biofilms of strains with high motility make vertical structures while strains with poor motility form flat biofilms (186). Hence, opposite to the current understanding that c-di-GMP promotes biofilm formation, we hypothesized that deletion of the genes encoding diguanylate cyclases should decrease c-di-GMP levels thus increasing motility and early biofilm formation. Although we found a significant reduction in early biofilm formation for *yddV* (4 fold), deletions of *yeaI*, *yedQ*, and *yfiN* increased dramatically biofilm formation after 7 h of incubation in LB at 37^{o}C (Fig. 5.1) while increasing motility (Fig. 5.2). These results suggest that the *yeaI*, *yedQ*, and *yfiN* mutations affect the initial steps of biofilm formation by decreasing c-di-GMP which results in higher motility. Hence, c-di-GMP levels should be low for initial biofilm formation. Furthermore, predicted inner membrane proteins (101) YeaI, YedQ, and YfiN function as active diguanylate cyclases producing c-di-GMP.

A previous report indicated that single deletions of genes encoding *E. coli* GGDEF proteins do not have a significant effect on motility (21). In contrast, we found a dramatic increase in motility after 11 h of incubation at 37^{o}C for the knock-out mutants of genes encoding the GGDEF proteins YeaI, YedQ, YfiN (Fig. 5.2), YeaJ, and YneF. These increases in motility combined with an increase in initial biofilm formation were corroborated by an increase in

biofilm formation by the *ycgR* strain which lacks the YcgR motility brake that is activated by c-di-GMP (Fig. 5.1).

eDNA is required for initial attachment to a surface and has a structural role connecting the cells in the biofilms (132). Previously, we reported that in *P. aeruginosa* eDNA is inversely regulated by c-di-GMP (174). To determine if the same eDNA regulation occurs in *E. coli*, we evaluated eDNA for the *yeaI*, *yedQ*, and *yfiN* mutants and found that eDNA increases as expected for low c-di-GMP levels. Recently, we reported that deletion of *hns* (encoding the global regulator H-NS) abolished eDNA production in *E. coli* (143). The *hns* mutant is also defective in swimming motility (77). Since deletion of *ycgR* (encodes a motility brake) and overexpression of *yhjH* (encodes a phosphodiesterase) restore the motility defect of the *hns* mutant (77), the *hns* deletion may increase c-di-GMP levels thus inhibiting motility via YcgR. Hence, our results for *yeaI*, *yedQ*, *yfiN*, and *hns* mutants suggest that c-di-GMP negatively regulate eDNA production in *E. coli*.

Our results suggest that the network of diguanylate cyclases and phosphodiesterases in *E. coli* tune the c-di-GMP concentrations according to the developmental sequence of biofilm formation. Initially, c-di-GMP concentrations should be low to promote early biofilm through increased swimming motility and increased eDNA as reported here for *yeaI*, *yedQ*, and *yfiN*, then c-di-GMP should be increased to promote biofilm maturation by inducing exopolysacharide production, formation of adhesive fimbriae, and sessility (123). Finally, c-di-GMP should decrease to produce biofilm dispersal through higher motility as we reported recently (90).

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The *Escherichia coli* hydrogen production rate was increased 9-fold through random mutagenesis of *fhlA*. Variant FhlA133 (Q11H, L14V, Y177F, K245R, M288K, and I342F) enhances hydrogen production by increasing transcription of the four transcriptional units related to the structure and function of the FHL complex, which are activated by FhlA. FhlA133 also requires less formate than wild-type FhlA to activate the transcription of P_{hyc} and P_{fdhF}.

E. coli hydrogen production rate was increased 6-fold by mutation E363G in FhlA, obtained through saturation mutagenesis. The mechanism for this increase is that FhlA E363G is less affected by the repressor HycA and increases P_{hyc} transcription. We identified for the first time that position L14 is important for FhlA transcriptional activation and hydrogen production. The amino acid replacement L14G of FhlA obtained through saturation mutagenesis of *fhlA* leads to a 4-fold increment of hydrogen production.

A whole transcriptome-analysis performed to evaluate the impact of the amino acid changes of FhlA133 in gene expression revealed that oxidative genes regulated by OxyR are expressed under anaerobic conditions and are related to hydrogen production. *E. coli* hydrogen production was increased 1.7 fold by deleting *oxyS* and slightly reduced upon deletion of *grxA* (1.6 fold) and *ahpF* (1.4 fold).

With the recognition that *E. coli* biofilm reactors will probably used to form hydrogen, an effort was undertaken to understand biofilm formation with this strain. Therefore, the complete *E. coli* genome was screened to identify proteins that affect extracellular DNA (eDNA) since this polymer is an important constituent of bioflms. The *nlpI*, *yfeC*, and *rna* mutants

increased eDNA production and the *hns* and *rfaD* mutants decreased eDNA production for both planktonic cells grown in flasks in rich medium and for static cultures in microtiter plates in glucose minimal media.

The *nlpI* and *hns* deletions were complemented for eDNA. Deletion of *nlpI* increases eDNA 3 fold while overexpression of *nlpI* decreases eDNA 16 fold. Global regulator H-NS is required for eDNA with *E. coli* since deletion of *hns* abolished eDNA production while overexpression of *hns* restored eDNA to 70% of the wild-type levels. Since cell lysis and membrane vesicles do not explain the effect of *hns* on eDNA, probably the mechanism of eDNA production in *E. coli* is related to direct secretion.

Deletions of the genes encoding the GGDEF proteins YeaI, YedQ, and YfiN increased swimming motility and eDNA as expected for low leves of 3'-5'-cyclic diguanylic acid (c-di-GMP). An alanine substitution in the EGEVF motif of YeaI abolished its impact on swimming motility indicating that EGEVF is necessary for YeaI activity. Therefore, our results suggest that YeaI, YedQ, and YfiN are active diguanylate cyclases synthesizing c-di-GMP.

Early biofilm formation increased dramatically for the *yeaI* (30 fold), *yedQ* (12 fold), and *yfiN* (18 fold) deletions as a result of the significantly enhanced motility due to low levels of c-di-GMP. Supporting these results we obtained a comparable increase in early biofilm formation by deleting *ycgR* (encoding a motility brake activated by c-di-GMP (118)). Hence, contrary to the current paradigm, our results indicate that c-di-GMP levels should be reduced for initial biofilm formation.

6.2 Recommendations

Since formate is required for the activity of FhlA (62) and is the direct substrate for *E. coli* hydrogen production (149), an increased knowledge of the mechanisms controlling the

intracellular concentration of formate will facilitate the design of new strategies to enhance hydrogen production. Through a whole-transcriptome analysis of BW25113 cells grown under anaerobic conditions in the presence and absence of formate, we would be able to identify the genes involved in formate transport, other transcriptional unis activated by FhlA, and the metabolic pathways that consume formate.

We found that the deletion of *nlpI* increases eDNA 3-fold and the deletion of *hns* abolished eDNA production for planktonic cultures in LB (143). However, we did not explore the impact of eDNA on *E. coli* biofilm formation. By comparing biofilm formation in flow cells chambers by BW25113, BW25113 *nlpI*, and BW25113 *hns*, expressing GFP we would observe biofilms via confocal laser scanning microscopy and determine if eDNA affects biofilm architechture, biomass, or surface coverage. eDNA may be also observed by staining the biofilms with impermeant fluorescent dyes that bind DNA such as Toto-3 (Molecular Probes, Eugene, OR).

By evaluating biofilm formation of single deletion mutants of genes encoding the 12 GGDEF proteins in *E.coli* we identified 3 mutants that significantly increased early biofilm formation. A similar approach may be followed to study the impact on biofilm formation of the other *E. coli* proteins with domains related to c-di-GMP turnover: the 7 mutants of genes encoding proteins with both GGGDEF and EAL domains, and the 10 mutants of genes encoding EAL proteins.

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APPENDIX A

ESCHERICHIA COLI HYDROGENASE 3 IS A REVERSIBLE ENZYME POSSESSING HYDROGEN UPTAKE AND SYNTHESIS ACTIVITIES*

Abstract

In the past it has been difficult to discriminate between hydrogen synthesis and uptake for the three active hydrogenases in *Escherichia coli* (hydrogenase 1, 2, and 3); however, by combining isogenic deletion mutations from the Keio collection, we were able to see the role of hydrogenase 3. In a cell which lacks hydrogen uptake via hydrogenase 1 (*hyaB*) and via hydrogenase 2 (*hybC*), inactivation of hydrogenase 3 (*hycE*) decreased hydrogen uptake. Similarly, inactivation of the formate hydrogen lyase complex which produces hydrogen from formate (*fhlA*) in the *hyaB hybC* background also decreased hydrogen uptake; hence, hydrogenase 3 has significant hydrogen uptake activity. Moreover, hydrogen uptake could be restored in the *hyaB hybC hycE* and *hyaB hybC fhlA* mutants by expressing *hycE* and *fhlA*, respectively, from a plasmid. The hydrogen uptake results were corroborated using two independent methods (both filter plate assays and a gas chromatography-based hydrogen uptake assay). A 30-fold increase in the forward reaction, hydrogen formation by hydrogenase 3, was also detected for the strain containing active hydrogenase 3 activity but no hydrogenase 1 or 2 activity relative to the strain lacking all 3 hydrogenases. These results indicate clearly that hydrogenase 3 is a reversible hydrogenase.

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*Reprinted with permission from "*Escherichia coli* hydrogenase 3 is a reversible enzyme possessing hydrogen uptake and synthesis activities" by Toshinari Maeda, Viviana Sanchez-Torres, and Thomas K. Wood, 2007, Applied Microbiology and Biotechnology 76:1035-104, Copyright 2007, Springer-Verlag, doi:10.1007/s00253-007-1086-6. The original publication is available at www.springerlink.com. T. Maeda constructed the strains, performed hydrogen uptake assays and hydrogen production assays for all of the strains except BW25113 *hyaB hybC fhlA*/pCA24N and BW25113 *hyaB hybC fhlA*/pCA24N-FhlA (hydrogen production for these strains was evaluated by V. Sanchez-Torres).

Introduction

Molecular hydrogen is a 100% renewable fuel that burns cleanly and efficiently and which generates no toxic by-products (Hansel and Lindblad 1998). Use of biological methods for hydrogen production promises significant energy reduction costs compared to non-biological methods (Das and Veziroğlu 2001). Biological methods depend on hydrogenases which catalyze the reaction $2H^+ + 2e^- \leftrightarrow H_2$ (g). Hydrogenases are found in archaea, anaerobic bacteria, and some eukaryotes, such as unicellular green algae, anaerobic ciliates, and anaerobic fungi (Horner et al. 2002). Biohydrogen may be produced through either photosynthetic or fermentative processes by these hydrogenases; in general, fermentative hydrogen production is more efficient than photosynthetic ones (Yoshida et al. 2005).

Hydrogenases are largely classified into three classes based on the metals at their active sites: [FeFe]-hydrogenases from *Clostridium pasteurianum* (Peters et al. 1998) and *Desulfovibrio desufuricans* (Nicolet et al. 1999), [NiFe]-hydrogenases from *D. gigas* (Volbeda et al. 1995), and [Fe]-hydrogenases from *Methanobacterium thermoautotrophicum* (Zirngibl et al. 1992). [FeFe]- and [NiFe]-hydrogenases have a similar overall structure as the active site is located within a large subunit and electrons are delivered to this center via iron-sulfur (Fe-S) centers located in the small subunit (Forzi and Sawers 2007). [Fe]-hydrogenases lack Fe-S clusters and are found only in a small group of methanogenic archaea (Zirngibl et al. 1992).

E. coli cells have 4 hydrogenases (hydrogenases 1, 2, 3, and 4). Hydrogenase 1 and 2 have hydrogen uptake activity only (Ballantine and Boxer 1986; King and Przybyla 1999), hydrogenase 4 appears to be inactive (Self et al. 2004), and hydrogenase 3 is reported to have the only hydrogen production activity (Bagramyan et al. 2002; Mnatsakanyan et al. 2004). To our knowledge, there are no reports indicating *E. coli* hydrogenase 3 is a hydrogen uptake enzyme,

but instead it has been reported to be a synthesis enzyme (Böck and Sawers 1996). Hydrogenase 1 (encoded by *hyaABCDEF* (Richard et al. 1999)), hydrogenase 2 (encoded by *hybOABCDEFG* (Richard et al. 1999)), and hydrogenase 3 (encoded by *hycABCDEFGHI* (Bagramyan and Trchounian 2003)) are [NiFe]-hydrogenases that contain two cyanide molecules and a carbon monoxide molecule at the active site (Blokesch et al. 2002); these hydrogenases rely on the auxiliary proteins such as HypABCDEF (metalochaperones for NiFe insertion) and SlyD (nickel insertion) for maturation as well as may possibly rely on the chaperones GroEL/GroES (Zhang et al. 2005). In *E. coli*, hydrogen is produced by the formate hydrogenlyase system (FHL) complex that consists of two separable enzymatic activities: a formate dehydrogenase-H for producing $2H^+$, $2e^-$, and CO_2 from formate and a hydrogenase 3 for synthesizing hydrogen from $2H^+$ and $2e^-$ (Sawers et al. 1985); the source of the two electrons for H_2 is formate (Bagramyan and Trchounian 2003). The FHL system may be used for the regulation of internal pH in cells (Böck and Sawers 1996). *E. coli* hydrogenases are sensitive to oxygen (Glick et al. 1980) like other hydrogenases derived from *D. desufuricans* (Vincent et al. 2005) so their activities are assayed under anaerobic conditions.

It has been reported that the [NiFe]-hydrogenase from *D. gigas* (De Lacey et al. 2000) and *Synechocystis* sp. PCC 6803 (Gutekunst et al. 2005) are enzymes possessing both hydrogen production and uptake activity; i.e., they function physiologically in either direction: hydrogen production from NADH oxidation or NAD^+ reduction with hydrogen as the electron donor. To date it has been difficult to determine if hydrogenase 3 is a reversible hydrogenase because it was not easy to discriminate between the 3 different hydrogenase activities. Here we report that hydrogenase 3 is a bidirectional enzyme having significant hydrogen uptake and synthesis activity.

Materials and methods

Bacterial strains, growth, and total protein

Strains are shown in Table A.1. *E. coli* cells were initially streaked from -80°C glycerol stocks on Luria-Bertani (LB) agar plates (Sambrook et al. 1989) containing 100 μg/mL kanamycin and 30 μg/mL chloramphenicol where appropriate and incubated at 37°C. After growth on LB agar plates, these strains were cultured at 37°C with shaking at 250 rpm (New Brunswick Scientific Co., Edison, NJ) from a fresh single colony in complex medium (Rachman et al. 1997) or complex-fructose medium in which fructose (20 g, J.T. Baker Chemical, Phillipsburg, NJ) was substituted for glucose (20 g, Fisher Scientific), and 100 μg/mL kanamycin or 30 μg/mL chloramphenicol were added where appropriate. Plasmids pBS(Kan) (Canada et al. 2002), pBS(Kan)HycE (below), pCA24N (Kitagawa et al. 2005), and pCA24N-FhlA (Kitagawa et al. 2005) were electroporated into the mutants (Table A.1). Cell growth was measured using turbidity at 600 nm, and total protein concentrations for *E. coli* were 0.22 mg/OD/mL (Protein assay kit, Sigma Diagnostics, St. Louis, MO).

Eliminating kanamycin resistance and P1 transduction

Plasmid pCP20 (Cherepanov and Wackernagel 1995) was used as described previously (Datsenko and Wanner 2000) to eliminate the kanamycin resistance gene (kan^R) from the isogenic BW25113 mutants (Keio strains) (Baba et al. 2006). This enabled strains to be generated with multiple mutations via sequential P1 transductions and allowed for the use of the kanamycin-resistant plasmid pBS(Kan)HycE (Table A.1). P1 transduction (Silhavy et al. 1984) and pCP20 were used to create *E. coli* MW1000 (*hyaB hybC Δkan*) from BW25113 *hybC Δkan* by transferring *hyaB* kan^R via P1 transduction and using pCP20 to eliminate the kanamycin

resistance marker. Similarly, MW1001 (*hyaB hybC hycE Δkan*) and MW1002 (*hyaB hybC fhlA Δkan*) were created from BW25113 *hyaB hybC Δkan* by transferring *hycE* kan^R or *fhlA* kan^R and by eliminating the kanamycin resistance marker.

Construction of pBS(Kan)HycE

E. coli K-12 genomic DNA was obtained as described previously (Zhu et al. 1993). The 1811 bp chromosomal DNA fragment encoding *hycE* was amplified using *Pfu* polymerase and primers HycEKpnI Front [5'-CTCCTTGCTGGGTACCTGATTAAAGAGAGTTTGAG CATGTC-3'] and HycEEcoRI Rear [5'-GGATAAGACGAATTCGCCGTGCCGGTTTTGATG AC-3'] with 30 cycles and 52°C annealing. The PCR product was cloned into the multiple cloning site in pBS(Kan) (Canada et al. 2002) after double digestion with *Kpn*I and *Eco*RI to create pBS(Kan)HycE. Plasmid DNA was isolated using a Mini Kit (Qiagen, Inc., Chatsworth, CA). The correct plasmid was verified by digesting the plasmid with the restriction enzymes *Eco*RV, *Dra*III, *Nru*I, *Fsp*I, and *Xho*I.

Hydrogen production assay

For all experiments using pBS(Kan)HycE, complex-fructose medium was used to avoid catabolite repression by glucose of the *lac* promoter for expressing *hycE*. Overnight aerobic cultures (25 mL) and fresh media were sparged for 5 min with nitrogen to remove oxygen. Sealed crimp-top vials (27 mL) were sparged for 5 min with nitrogen, and 1 mL of the cell suspension and 9 mL of fresh medium (as needed including 1 mM IPTG) was added to the bottles which were incubated at 37°C with shaking for 2 to 20 h. The amount of hydrogen generated in the 17 mL head space was measured using a 50 μL aliquot by gas chromatography (GC) as described previously (Maeda et al. 2007).

Hydrogen uptake assay and SDS-PAGE

Three independent hydrogen uptake assays (oxidized methylviologen-based uptake assay, uptake plate assay, and GC-based hydrogen uptake assay) were performed as previously described (Maeda et al. 2007). Expression of complemented proteins from samples was analyzed with standard Laemmli discontinuous SDS-PAGE (12%) (Sambrook et al. 1989).

Results

Construction of E. coli strains defective in hydrogenases 1, 2, and 3 and FHL

The goals of this work were to assay hydrogen uptake and synthesis activity of *E. coli* hydrogenase 3 in a background devoid of competing hydrogenase 1 and 2 activity so that the role of hydrogenase 3 in hydrogen production may be more clearly defined. To achieve this aim, we used the *E. coli* K-12 BW25113 isogenic single deletion mutants (Keio collection), which were obtained from the Genome Analysis Project in Japan (Baba et al. 2006), since these strains allowed us to introduce multiple mutations into *E. coli* cells through P1 transduction and multiple rounds of selection on kanamycin plates. Each Keio deletion mutant is designed with the ability to eliminate the kanamycin-resistance selection marker by expressing the flippase (FLP) recombinase protein from pCP20 since each kanamycin resistance gene is flanked by a FLP recognition target that is excised by FLP recombinase. Hence we created a double mutant defective in hydrogenases 1 and 2 (*hyaB hybC*), a triple mutant defective in hydrogenases 1, 2, and 3 (*hyaB hybC hycE*), and a triple mutant defective in hydrogenases 1 and 2 and the FHL system (*hyaB hybC fhlA*) (Table A.1). For eliminating hydrogenase 1, 2, and 3 activities, the large subunit of each hydrogenase (*hyaB*, *hybC*, and *hycE*) was deleted because the active site of catalysis is located within each large subunit in [NiFe]-hydrogenases. Also, the *fhlA* mutation abolishes hydrogenase 3 activity related to FHL (Sankar et al. 1988).

Hydrogen uptake activity in E. coli strains defective in hydrogenases 1, 2, 3, and 4

To assay the hydrogen uptake activity in various *E. coli* cells, the oxidized methylviologen-based hydrogen uptake assay was performed (Table A.2). As expected, elimination of hydrogenase 1 and 2, the two known *E. coli* hydrogenases with uptake activity, led to a 3.2 ± 0.4-fold decrease in hydrogen uptake, and the *hyfG* mutation (inactivates vestigial hydrogenase 4) did not decrease hydrogen uptake (Table A.2).

Importantly, the hydrogen uptake activity in the hydrogenase 1 and 2 double mutant (*hyaB hybC*) was reduced 2.7 ± 0.4-fold by addition of the *hycE* mutation (*hyaB hybC hycE*) and was reduced 6.0 ± 0.5-fold by addition of the *fhlA* mutation (*hyaB hybC fhlA*); hence, hydrogenase 3 has significant hydrogen uptake activity. Also, corroborating this result, BW25113 *hycE*/pBS(Kan) showed 3.8 ± 0.1-fold lower hydrogen uptake activity than wild type BW25113/pBS(Kan), and the uptake activity in HD705/pBS(Kan), which is defective hydrogenase 3, was 4.4 ± 0.4-fold lower than that in wild type MC4100/pBS(Kan) (Table A.2).

Hydrogen synthesis by hydrogenase 3

As shown in Table A.3, both mutations for hydrogenase 3 (*hycE* and *fhlA*) nearly abolished hydrogen production activity in the wild-type background in complex medium after 2 h. Furthermore, these two mutations also abolished hydrogen production activity in the *hyaB hybC* background (strain defective in hydrogenase 1 and hydrogenase 2 activity). As expected, inactivating the vestigial hydrogenase 4 via the *hyfG* mutation did not change hydrogen production. These results indicate clearly that the activity of hydrogenase 3 is essential for producing hydrogen in *E. coli*.

Complementation of the hycE and fhlA mutations for H_2 synthesis

To further demonstrate *E. coli* hydrogenase 3 has hydrogen uptake activity, pBS(Kan)HycE was constructed to complement the defective *hycE* allele. Also, to complement the defective FHL activator (*fhlA*), pCA24N-FhlA was used. These plasmids were introduced into the single *hycE* or *fhlA* mutant and the triple mutants *hyaB hybC hycE* and *hyaB hybC fhlA*, and hydrogen production in BW25113 *hycE*/pBS(Kan)HycE, *hyaB hybC hycE*/pBS(Kan)HycE, *fhlA*/pCA24N-FhlA, and *hyaB hybC fhlA*/pCA24N-FhlA was assayed. The deficiency in hydrogen production in a *hycE* background was complemented by introducing pBS(Kan)HycE since hydrogen production in both the *hycE* and *hyaB hybC hycE* strains with pBS(Kan)HycE was increased 9.4 ± 0.5-fold and 14.4 ± 0.3-fold compared to those with the empty vector pBS(Kan) in complex-fructose after 20 h (Table A.3). Note that the *plac* promoter is leaky in this vector. Similarly, BW25113 *fhlA* or *hyaB hybC fhlA* with pCA24N-FhlA showed 45.6 ± 0.3- or 45.6 ± 0.1-fold higher hydrogen production activities than those with empty vector pCA24N in complex medium after 3 h (Table A.3). These results indicate that these two plasmids are certainly active in *E. coli*, and confirm that hydrogenase 3 is responsible for hydrogen synthesis. Also, the expression of both HycE (65.0 kDa) and FhlA (78.5 kDa) from the complementation plasmids was confirmed by SDS-PAGE (data not shown).

Complementation of the hycE and fhlA mutations for H_2 uptake activity

To ascertain if the two active plasmids can restore the hydrogen uptake activity that was abolished in the two triple mutants *hyaB hybC hycE* or *hyaB hybC fhlA*, hydrogen uptake activity was assayed (Table A.2). These results show that BW25113 *hyaB hybC hycE*/pBS(Kan)HycE had 6.1 ± 0.2-fold more hydrogen uptake activity compared to BW25113 *hyaB hybC hycE* with empty vector pBS(Kan) and comparable hydrogen uptake activity relative to BW25113 *hyaB*

hybC/pBS(Kan), which has active native hydrogenase 3.

Similarly, hydrogen uptake activity in a *hyaB hybC fhlA* background was restored as indicated by the 3.7 ± 0.9-fold increase in activity by expressing active FhlA (BW25113 *hyaB hybC fhlA*/pCA24N-FhlA vs. BW25113 *hyaB hybC fhlA*/pCA24N). These hydrogen uptake results were also corroborated using a plate assay for reversible hydrogenase activity which showed blue color (showing hydrogen uptake) upon expressing HycE and FhlA in the two triple mutant strains (*hyaB hybC hycE* or *hyaB hybC fhlA*); the mutants without the complementation plasmids remained colorless in this assay. Additionally, a GC-based hydrogen uptake assay indicated that H_2 uptake activity in BW25113 *hyaB hybC fhlA*/pCA24N-FhlA is 10 ± 1-fold more than BW25113 *hyaB hybC fhlA*/pCA24N over 0 to 3 h. Taken together, these results show that the HycE and FhlA proteins are responsible for hydrogen uptake, i.e., that hydrogenase 3 has uptake activity. In addition, these three consistent results clearly indicate that methylviologen can assay hydrogenase activity including hydrogenase 1 and hydrogenase 2 without interference from other non-hydrogenase activities of other present enzymes in *E. coli*.

Discussion

We show here that *E. coli* hydrogenase 3 is a reversible hydrogenase possessing two clear activities: hydrogen production and hydrogen uptake activity. In *E. coli*, there are 4 [NiFe] hydrogenases (hydrogenase 1, 2, 3, and 4), and hydrogenase 1 and hydrogenase 2 are irreversible hydrogenases equipped with only hydrogen uptake activity since negligible hydrogen production occurred in the *hycE* or *fhlA* background (defective hydrogenase 3) although active hydrogenases 1 and 2 were present (Table A.3). These results agree with previous studies (Menon et al. 1994) who found hydrogenase 2 had uptake activity. Complementing *hycE* via pBS(Kan)HycE in BW25113 *hyaB hybC hycE* restored roughly half of the hydrogen uptake activity in BW25113

hyaB hybC/pBS(Kan) (Table A.2); this agrees with the hydrogen production results in that complementing *hycE* via pBS(Kan)HycE in BW25113 *hycE* restored about 1/3 of hydrogen production of BW25113/pBS(Kan) (Table A.3). Hence, these results showed expression of *hycE* from chromosomal DNA is more effective than that from plasmid pBS(Kan) for both hydrogen production and hydrogen uptake. In contrast, overexpression of *fhlA* via pCA24N-FhlA in BW25113 *fhlA* led to small (24%) increase of hydrogen production compared to BW25113/pCA24N (Table A.3). This indicates that overproducing *fhlA* may trigger increased hydrogen production (note that FhlA is an activator for FHL system (Sankar et al. 1988)); however, overexpressing FhlA (BW25113 *hyaB hybC fhlA*/pCA24N-FhlA) only restored 1/2 of hydrogen uptake relative to BW25113 *hyaB hybC*/pBS(Kan) (Table A.2). Although the reason is unclear, it may be due to other functions of FhlA because FhlA is a complex regulator protein with at least three functions: controlling the *hyp* operon for protein maturation, controlling the *hyc* operon for hydrogenase 3, and controlling *fdhF* for formate dehydrogenase-H (Schlensog et al. 1994). Note also that the hydrogen uptake and hydrogen production values are roughly comparable; for example, hydrogen production by the wild-type strain (Table A.3 with units converted) is 200 nmol/min/mg protein in complex medium vs. hydrogen uptake by the same strain in the same medium is 76 nmol/min/mg protein.

Our bioinformatics analysis indicates there are 6 conserved metal-binding motifs (L0-L5 motifs) (Burgdorf et al. 2002) surrounding the [NiFe] site in the large subunits of *E. coli* hydrogenase 1 and hydrogenase 2 which is similar to the catalytic subunit HoxH in *Ralstonia eutropha*, *D. gigas*, and *Synecocystis* sp. PCC 6803. However, even though hydrogenase 3 is a [NiFe] hydrogenase (Drapal and Böck 1998), the large subunit of hydrogenase 3 (and also hydrogenase 4) is different since there is no L0 motif, and the order of the remaining 5 motifs are different than those of hydrogenase 1 and 2. This difference in the [NiFe]-active site in the large

subunit of hydrogenase apparently allows hydrogen production for hydrogenases 3.

Previous studies have implied that *E. coli* hydrogenase 3 mainly is responsible for hydrogen formation based on results of low hydrogen productivity in *E. coli* cells with a single mutation for *hycE* (hydrogenase 3 large subunit) (Bagramyan et al. 2002; Mnatsakanyan et al. 2004) or *fhlA* (Sankar et al. 1988); however, these results are not completely satisfying in that they were conducted in a hydrogenase 1 and hydrogenase 2 background. Here we provide clear experimental evidence that (i) hydrogenase 1 and hydrogenase 2 have probably no hydrogen synthesis activity (results obtained without hydrogenase 3, Table A.3), (ii) that hydrogenase 3 is the primary hydrogenase for producing hydrogen in *E. coli* (results obtained without hydrogenase 1 and 2, Table A.3), (iii) that hydrogenase 3 has significant hydrogen uptake activity (results obtained without hydrogenase 1 and 2 and by using two mutations to eliminate hydrogenase 3, Table A.2), and (iv) that hydrogenase 4 is inactive (results obtained without hydrogenase 4, Table A.2 and A.3).

Hydrogen in *E. coli* is produced by the collaborative activity between formate dehydrogenase-H, which converts formate into H^+, carbon dioxide, and electrons, and between hydrogenase 3, which converts H^+ and electrons into hydrogen (forward reaction) (Bagramyan and Trchounian 2003). Although we show here that hydrogenase 3 has significant hydrogen uptake activity, it is unlikely that formate is synthesized from carbon dioxide and hydrogen by the reverse reaction of hydrogenase 3 but instead it is more likely that the reverse activity of hydrogenase 3 produces H^+ from hydrogen with the concomitant reduction of a species other than carbon dioxide.

Hydrogenase 3 is essential for producing hydrogen in *E. coli* since a single mutant in *hycE* and *fhlA* was not able to produce hydrogen; these results are consistent with previous studies (Bagramyan et al. 2002). Also, hydrogen production in a double mutant (*hyaB hybC*;

defective in hydrogenase 1 and hydrogenase 2) increased up to 32% compared to that in the wild-type strain (Table A.3); this result agrees well with our previous study that engineered *E. coli* cells expressing the cyanobacterial hydrogenase HoxEFUYH derived from *Synechocystis* sp. PCC 6803 enhanced hydrogen production by inhibiting the hydrogen uptake activity by hydrogenase 1 and hydrogenase 2 (Maeda et al. 2007). Therefore, if there is no uptake from hydrogenases 1 and 2, more hydrogen may be produced, and this *hyaB hybC* mutant has importance for fermentative hydrogen generation.

It is still not well understood why hydrogenase 4, which is a homolog of reversible hydrogenase 3, does not function in *E. coli* although our biofilm microarray studies show transcription of this operon is differentially regulated (Domka et al. 2007). Here we show via an isogenic *hyfG* mutation (defective in hydrogenase 4) that hydrogenase 4 has no detectable hydrogenase activity (both synthesis and uptake) under the experimental conditions used (Table A.2 and A.3). In addition, it is not clear why hydrogenases 1 and 2 have hydrogen uptake but not synthesis activity and why there are two of these enzymes. Hence, hydrogen production in *E. coli* is a complex process that is only beginning to be unraveled.

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Table A.1. **Strains and plasmids used.** Km^R, Cm^R, and Ap^R are kanamycin, chloramphenicol and ampicillin resistance, respectively.

Strains and plasmids	Genotype	Source
Strains		
E. coli MC4100	F- *araD139* Δ*lacU169 rpsL thi fla*	(Casadaban 1976)
E. coli HD705	MC4100 Δ*hycE*; defective in large subunit of the hydrogenase 3 subunit	(Sauter et al. 1992)
E. coli BW25113	$lacI^q$ $rrnB_{T14}$ $\Delta lacZ_{WJ16}$ *hsdR514* $\Delta araBAD_{AH33}$ $\Delta rhaBAD_{LD78}$	Yale CGSG Stock Center
E. coli BW25113 *ΔfhlA*	*E. coli* JW2701 Km^R; defective in FHL activator	(Baba et al. 2006)
E. coli BW25113 *ΔhyfG*	*E. coli* JW2472 Km^R; defective in large subunit of hydrogenase 4	(Baba et al. 2006)
E. coli BW25113 *ΔhyaB Δkan*	*E. coli* JW0955 (Baba et al. 2006) *Δkan*; defective in large subunit of hydrogenase 1	this study
E. coli BW25113 *ΔhybC Δkan*	*E. coli* JW2962 (Baba et al. 2006) *Δkan*; defective in probable large subunit of hydrogenase 2	this study
E. coli BW25113 *ΔhycE Δkan*	*E. coli* JW2691 (Baba et al. 2006) *Δkan*; defective in large subunit of hydrogenase 3	this study
E. coli MW1000	BW25113 *ΔhyaB ΔhybC Δkan*; defective in large subunit of hydrogenase 1 and 2	this study
E. coli MW1001	MW1000 *ΔhycE Δkan*; defective in large subunit of hydrogenases 1, 2, and 3	this study
E. coli MW1002	MW1000 *ΔfhlA* Km^R; defective in large subunit of hydrogenases 1 and 2, and in FHL activator	this study
Plasmids		
pBS(Kan)	Cloning vector; Km^R	(Canada et al. 2002)
pBS(Kan)HycE	pBS(Kan) *plac*::*hycE*; expresses HycE derived from *Escherichia coli*	this study
pCA24N	Empty vector; Cm^R	(Kitagawa et al. 2005)
pCA24N-FhlA	pCA24N *pT5-lac*::*fhlA*; expresses FhlA derived from *Escherichia coli*	(Kitagawa et al. 2005)
pCP20	Ap^R and Cm^R plasmid with temperature-sensitive replication and thermal induction of flippase (FLP) recombinase	(Cherepanov and Wackernagel 1995)

Table A.2. **Hydrogen uptake activity with various *E. coli* BW25113 strains in complex and complex-fructose medium as determined by the oxidized methylviologen-based hydrogen uptake assay after 5 min.** Standard deviations shown from one representative experiment with 2 replicates.

Strain	Description	Hydrogen uptake	
		nmol/min/ mg protein	relative
E. coli BW25113	wild type[a]	76 ± 4	3.2 ± 0.4
E. coli BW25113 *hyfG*	*ΔhyfG* (defective hydrogenase 4)[a]	70 ± 5	2.9 ± 0.4
E. coli BW25113 *hyaB hybC*	*ΔhyaB* and *ΔhybC* (defective hydrogenase 1 and 2)[a]	24 ± 9	1
E. coli BW25113 *hyaB hybC hycE*	*ΔhyaB*, *ΔhybC*, and *ΔhycE* (defective hydrogenases 1, 2, and 3)[a]	9 ± 1	0.4 ± 0.4
E. coli BW25113 *hyaB hybC fhlA*	*ΔhyaB*, *ΔhybC*, and *ΔfhlA* (defective hydrogenase 1 and 2, and defective FHL activator)[a]	4 ± 1	0.2 ± 0.5
E. coli BW25113/pBS(Kan)	wild type[b]	94 ± 12	4.5 ± 0.1
E. coli BW25113 *hycE*/pBS(Kan)	*ΔhycE* (defective hydrogenase 3)[b]	25 ± 1	1.2 ± 0.1
E. coli BW25113 *hyaB hybC*/pBS(Kan)	*ΔhyaB* and *ΔhybC* (defective hydrogenase 1 and 2)[b]	21 ± 1	1
E. coli BW25113 *hyaB hybC hycE*/pBS(Kan)	*ΔhyaB*, *ΔhybC*, and *ΔhycE* (defective hydrogenases 1, 2, and 3)[c]	1.8 ± 0.4	0.1 ± 0.2
E. coli BW25113 *hyaB hybC hycE*/pBS(Kan)HycE	*ΔhyaB*, *ΔhybC*, and *ΔhycE* (defective hydrogenases 1, 2, and 3) + HycE[c]	11 ± 1	0.5 ± 0.1
E. coli BW25113 *hyaB hybC fhlA*/pCA24N	*ΔhyaB*, *ΔhybC*, and *ΔfhlA* (defective hydrogenase 1 and 2, and defective FHL activator)[c]	3 ± 2	0.1 ± 0.7
E. coli BW25113 *hyaB hybC fhlA*/pCA24N-FhlA	*ΔhyaB*, *ΔhybC*, and *ΔfhlA* (defective hydrogenase 1 and 2, and defective FHL activator) + FhlA[c]	10 ± 3	0.5 ± 0.3
E. coli MC4100/pBS(Kan)	wild type[b]	35 ± 15	1
E. coli HD705/pBS(Kan)	defective hydrogenase 3[b]	8 ± 1	0.2 ± 0.4

[a] Complex medium without IPTG
[b] Complex-fructose medium with 1 mM IPTG
[c] Complex-fructose medium without IPTG

Table A.3. **Hydrogen production in various *E. coli* BW25113 strains in complex-fructose or complex medium after 2 to 20 h.** Standard deviations shown from one representative experiment with 2 replicates.

Strain	Description	Hydrogen production	
		μmol/mg protein	Relative
E. coli BW25113	Wild type[a]	24 ± 1	27.6 ± 0.1
E. coli BW25113 *hycE*	*ΔhycE* (defective hydrogenase 3)[a]	0.87 ± 0.06	1
E. coli BW25113 *fhlA*	*ΔfhlA* (defective FHL activator)[a]	0.99 ± 0.05	1.1 ± 0.1
E. coli BW25113 *hyfG*	*ΔhyfG* (defective hydrogenase 4)[a]	23 ± 1	26.4 ± 0.1
E. coli BW25113 *hyaB hybC*	*ΔhyaB* and *ΔhybC* (defective hydrogenase 1 and 2)[a]	32 ± 3	36.8 ± 0.1
E. coli BW25113 *hyaB hybC hycE*	*ΔhyaB*, *ΔhybC*, and *ΔhycE* (defective hydrogenases 1, 2, and 3)[a]	0.91 ± 0.01	1.0 ± 0.1
E. coli BW25113 *hyaB hybC fhlA*	*ΔhyaB*, *ΔhybC*, and *ΔfhlA* (defective hydrogenase 1 and 2, and defectivw FHL activator)[a]	0.79 ± 0.00	0.9 ± 0.1
E. coli BW25113/pBS(Kan)	Wild type[b]	45 ± 7	28.1 ± 0.3
E. coli BW25113 *hycE*/pBS(Kan)	*ΔhycE* (defective hydrogenase 3)[b]	1.6 ± 0.4	1
E. coli BW25113 *hycE*/pBS(Kan)HycE	*ΔhycE* (defective hydrogenase 3) + HycE[b]	15 ± 6	9.4 ± 0.5
E. coli BW25113 *hyaB hybC hycE*/pBS(Kan)	*ΔhyaB*, *ΔhybC*, and *ΔhycE* (defective hydrogenases 1, 2, and 3)[b]	1.9 ± 0.1	1.2 ± 0.3
E. coli BW25113 *hyaB hybC hycE*/pBS(Kan)HycE	*ΔhyaB*, *ΔhybC*, and *ΔhycE* (defective hydrogenases 1, 2, and 3) + HycE[b]	23 ± 3	14.4 ± 0.3
E. coli BW25113/pCA24N	Wild type[c]	33 ± 0	36.7 ± 0.1
E. coli BW25113 *fhlA*/pCA24N	*ΔfhlA* (defective FHL activator)[c]	0.9 ± 0.1	1
E. coli BW25113 *fhlA*/pCA24N-FhlA	*ΔfhlA* (defective FHL activator) + FhlA[c]	41 ± 10	45.6 ± 0.3
E. coli BW25113 *hyaB hybC fhlA*/pCA24N	*ΔhyaB*, *ΔhybC*, and *ΔfhlA* (defective hydrogenase 1 and 2, and defective FHL activator)[c]	0.9 ± 0.1	1
E. coli BW25113 *hyaB hybC fhlA*/pCA24N-FhlA	*ΔhyaB*, *ΔhybC*, and *ΔfhlA* (defective hydrogenase 1 and 2, and defective FHL activator) + FhlA[c]	41 ± 1	45.6 ± 0.1

[a] Complex medium without IPTG after 2 h
[b] Complex-fructose medium without IPTG after 20 h
[c] Complex medium without IPTG after 3 h

APPENDIX B

METABOLIC ENGINEERING TO ENHANCE BACTERIAL HYDROGEN PRODUCTION*

Summary

Hydrogen fuel is renewable, efficient, and clean, and fermentative bacteria hold great promise for its generation. Here we use the isogenic *E. coli* K-12 KEIO library to rapidly construct multiple, precise deletions in the *E. coli* genome to direct the metabolic flux toward hydrogen production. *E. 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 fdoG*/pCA24N-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 3-fold higher hydrogen production from glucose with this strain.

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*Reprinted with permission from "Metabolic engineering to enhance bacterial hydrogen production" by Toshinari Maeda, Viviana Sanchez-Torres, and Thomas K. Wood, 2008, Microbial Biotechnology 1:30-39, Copyright 2008, Blackwell Publishing, doi: 10.1111/j.1751-7915.2007.00003.x. The definitive version is available at www.blackwell-synergy.com. T. Maeda constructed the strains and performed hydrogen production assays. V. Sanchez-Torres determined the specific growth rate of the metabolically-engineered strains.

Introduction

Hydrogen is a promising fuel as it has a higher energy content than oil (142 MJ/kg for H_2 vs. 42 MJ/kg 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 Veziroğlu, 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 Veziroğlu, 2001). Biological methods depend on hydrogenases which catalyze the reaction $2H^+ + 2e^- \leftrightarrow H_2$ (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 since 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* (Bagramyan and Trchounian, 2003; Sauter et al., 1992)) and formate dehydrogenase-H (encoded by *fdhF* (Axley et al., 1990)) which are the key enzymes of the formate hydrogen lyase system (FHL); these enzymes catalyze the reaction $HCOO^- + H_2O \leftrightarrow H_2 + HCO_3^-$ (Woods, 1936) (Fig. B.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. B.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 *fnr* mutation leads to 3-fold 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 two days), the resulting deletion mutations are more stable for eliminating target genes compared to point mutations or frame-shift 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 metabolic 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 LB medium (Table B.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 B.2); for example, the specific growth rates of BW25113 *hyaB hybC hycA*/pCA24N-FhlA and BW25113 *hyaB hybC hycA focB*/pCA24N-FhlA were reduced 2-fold compared to 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 since these experiments were conducted at turbidities of 1.3 to 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 since 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 since 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 B.1). Since 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 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 to the wild type (Table B.1).

Formate-related deletions

E. coli has three pathways for eliminating formate produced by fermentation: (i) export of formate by the putative formate transporters FocA (Suppmann and Sawers, 1994) and its homolog 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 CO_2; 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, a *focA*, *focB*, *narG*, *fdnG*, and *fdoG* mutation, were introduced to the triple mutant (*hyaB hybC hycA*) to make 5 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* since the *fnr* mutation activates expression of FhlA by 3-fold (Self and Shanmugam, 2000); unexpectedly, the *fnr* mutation in the *hyaB hybC hycA* background decreased hydrogen production (Table B.1).

To further test the combination of the *focA*, *focB*, *narG*, *fdnG*, and *fdoG* mutations, 7 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 2 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.

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 B.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 vs. BW25113/pCA24N-FhlA, BW25113 *hyaB hybC hycA*/pCA24N-FhlA vs. BW25113 *hyaB hybC hycA*/pCA24N, and BW25113 *hyaB hybC hycA fdoG*/pCA24N-FhlA vs. 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

Since 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. B.2 and the results are shown in Table B.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*/pCA24N-FhlA, BW25113 *hyaB hybC hycA*/pCA24N-FhlA, and BW25113 *hyaB hybC hycA focB*/pCA24N-FhlA was 9-, 71-, 80-, and 76-fold higher than that in the wild type strain (Table B.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 B.2).

The hydrogen yield in BW25113 *hyaB hybC hycA fdoG*/pCA24N-FhlA was 1.15 ± 0.01 mol hydrogen/mol formate compared to 0.64 ± 0.01 mol-hydrogen/mol-formate for BW25113/pCA24N. This indicates that the metabolically-engineered *E. coli* cells with 5 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

Since 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. B.2). Compared to 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 vs. 12 ± 1 μmol/mg-protein/h). Also, the hydrogen yield from glucose was increased by 50% compared to that in the original strain (0.47 ± 0.06 for BW25113/pCA24N vs. 0.70 ± 0.02 mol-H_2/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 than the wild-type strain at a rate of 113 μmol/mg/h 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 from 47 to 70%.

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 2-fold higher hydrogen production compared to 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 μmol/mg/h) (Oh et al., 2003), *Rhodopseudomonas palustris* JA1 (60 μmol/mg/h) (Archana et al., 2003), *Rhodopseudomonas palustris* P4 (41 μmol/mg/h) (Jung et al., 1999), and *Klebsiella oxytoca* HP1 (30 μmol/mg/h) (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 B.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 homolog of *focA* (Andrews et al., 1997)) was more effective although it only enhanced hydrogen production slightly (Table B.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 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 B.1). Therefore, these results indicate that consumption of formate that does not lead to hydrogen (formate hydrogenase-H is required for hydrogenase 3, Fig. B.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 3-fold higher FhlA expression (Self and Shanmugam, 2000), decreased hydrogen production (Table B.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 modeling 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 B.3. *E. coli* cells were initially streaked from -80°C glycerol stocks on Luria-Bertani (LB) agar plates (Sambrook et al., 1989) containing 100 μg/mL kanamycin (for those with chromosomal kanamycin resistance markers) and 30 μg/mL 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 rpm (New Brunswick Scientific Co., Edison, NJ) in LB medium (Sambrook et al., 1989), modified complex-glucose medium (Rachman et al., 1997) to which 0.4 mg/L $(NH_4)_6Mo_7O_{24}$ was added, or modified complex-formate medium in which formate (100 mM, Fisher Scientific, Fair Lawn, NJ) was substituted for glucose along and 0.4 mg/L $(NH_4)_6Mo_7O_{24}$, was added; 100 μg/mL kanamycin or 30 μg/mL 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-FhlA were electroporated into hydrogen-overproducing *E. coli* strains (Table B.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/mL (Protein assay kit, Sigma Diagnostics, St. Louis, MO).

Multiple chromosomal mutations

P1 transduction (Silhavy et al., 1984) was performed in succession to knockout specific genes by selecting for the kanamycin-resistance gene that is transferred along with each

chromosomal deletion that are 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) since 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^{o}C for 6h. 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^{o}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 1M formate were added to the bottles which were incubated at 37^{o}C with shaking for 1 h. The amount of hydrogen generated in the head space 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. B.2). The vials were incubated at 37^{o}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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Table B.1. Effect of metabolic mutations on the aerobic specific growth rate in LB medium and on hydrogen production from formate by *E. coli* BW25113 in the closed system.

Strain	Growth rate		Hydrogen production[1]	
	1/hr	Relative	μmol/mg protein	Relative
BW25113	1.6 ± 0.1	1	5 ± 2	1
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 fdnG*	1.6 ± 0.1	1	14 ± 1	2.8
BW25113 *hyaB hybC hycA fdoG*	1.4 ± 0.1	0.9	53 ± 2	10.6
BW25113 *hyaB hybC hycA fdnG 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 focA 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*	1.6 ± 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 *hyaB hybC 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

Table B.1. (continued)

Strain	Growth rate	Hydrogen production[1]	Strain	Growth rate
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 hycE*/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 *hyaB hybC hycA*/pCA24N-FhlA	1.39 ± 0.02	1	55 ± 5	18.3
BW25113 *hyaB hybC hycA focA*/pCA24N-FhlA	1.4 ± 0.1	1	58 ± 12	19.3
BW25113 *hyaB hybC hycA focB*/pCA24N-FhlA	1.4 ± 0.2	1	59 ± 3	19.7
BW25113 *hyaB hybC hycA narG*/pCA24N-FhlA	1.38 ± 0.06	0.9	56 ± 11	18.7
BW25113 *hyaB hybC hycA focB narG*/pCA24N-FhlA	ND[2]	-	48 ± 1	16.0
BW25113 *hyaB hybC hycA focA focB narG*/pCA24N-FhlA	ND	-	35 ± 12	11.7
BW25113 *hyaB hybC hycA fdoG*/pCA24N	1.5 ± 0.2	1	66 ± 1	22.0
BW25113 *hyaB hybC hycA fdoG*/pCA24N-FhlA	1.47 ± 0.03	1	79 ± 7	26.3

[1] 1 h in complex-formate medium
[2] Not determined

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

Strain	Description	Growth rate		H_2 production rate[1]	
		1/h	relative	µmol/mg protein/h	relative
BW25113/pCA24N	wild type	0.95 ± 0.01	1	0.8 ± 0.3	1
BW25113/pCA24N-FhlA	wild type + FhlA	ND[2]	-	7 ± 4	9
BW25113 *hyaB hybC*/pCA24N-FhlA	*ΔhyaB* and *ΔhybC* (defective hydrogenases 1 and 2) + FhlA	ND	-	57 ± 10	71
BW25113 *hyaB hybC hycE*/pCA24N	*ΔhyaB, ΔhybC*, and *ΔhycE* (defective hydrogenases 1, 2, and 3)	ND	-	0.3 ± 0.03	0.4
BW25113 *hyaB hybC hycA*/pCA24N-FhlA	*ΔhyaB, ΔhybC*, and *ΔhycA* (defective hydrogenases 1 and 2, and defective FHL repressor) + FhlA	0.42 ± 0.07	0.44	64 ± 3	80
BW25113 *hyaB hybC hycA focB*/pCA24N-FhlA	*ΔhyaB, ΔhybC*, *ΔhycA*, and *ΔfocB* (defective hydrogenases 1 and 2, defective FHL repressor, and defective putative formate transporter) + FhlA	0.47 ± 0.07	0.49	61 ± 16	76
BW25113 *hyaB hybC hycA fdoG*/pCA24N-FhlA	*ΔhyaB, ΔhybC*, *ΔhycA*, and *ΔfdoG* (defective hydrogenases 1 and 2, defective FHL repressor, and defective formate dehydrogenase-O) + FhlA	0.23 ± 0.07	0.24	113 ± 12	141

[1] Hydrogen production rate was calculated from 30 min-incubation in complex-formate medium
[2] Not determined

Table B.3. Stains and plasmids used. Km^R, Cm^R, and Ap^R are kanamycin, chloramphenicol and ampicillin resistance, respectively.

Strains and plasmids	Genotype	Source
Strains		
E. coli BW25113	*lacI*^{q} *rrnB*$_{T14}$ *ΔlacZ*$_{WJ16}$ *hsdR514 ΔaraBAD*$_{AH33}$ *ΔrhaBAD*$_{LD78}$	Yale CGSG Stock Center
E. coli BW25113 *ΔhyaB*	BW25113 *hyaB* Km^R; defective in large subunit of hydrogenase 1	(Baba et al., 2006)
E. coli BW25113 *ΔhybC*	BW25113 *hybC* Km^R; defective in large subunit of hydrogenase 2	(Baba et al., 2006)
E. coli BW25113 *ΔhycA*	BW25113 *hycA* Km^R; defective in repressor of FHL	(Baba et al., 2006)
E. coli BW25113 *ΔhycE*	BW25113 *hycE* Km^R; defective in large subunit of hydrogenase 3	(Baba et al., 2006)
E. coli BW25113 *ΔfocA*	BW25113 *focA* Km^R; defective in formate transporter	(Baba et al., 2006)
E. coli BW25113 *ΔfocB*	BW25113 *focB* Km^R; defective in putative formate transporter	(Baba et al., 2006)
E. coli BW25113 *ΔnarG*	BW25113 *narG* Km^R; defective in α-subunit of nitrate reductase A	(Baba et al., 2006)
E. coli BW25113 *Δfnr*	BW25113 *fnr* Km^R; defective in FNR transcriptional dual regulator	(Baba et al., 2006)
E. coli BW25113 *ΔfdnG*	BW25113 *fdnG* Km^R; defective in α-subunit of formate dehydrogenase-N	(Baba et al., 2006)
E. coli BW25113 *ΔfdoG*	BW25113 *fdoG* Km^R; defective in α-subunit of formate dehydrogenase-O	(Baba et al., 2006)
E. coli BW25113 *ΔhyaB ΔhybC*	BW25113 *hyaB hybC Δkan*; defective in large subunit of hydrogenase 1and hydrogenase 2	this study
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA*	BW25113 *hyaB hybC hycA Δkan*; defective in large subunit of hydrogenase 1and hydrogenase 2, and defective in repressor of FHL	this study
E. coli BW25113 *ΔhyaB ΔhybC ΔhycE*	BW25113 *hyaB hybC hycE Δkan*; defective in large subunit of hydrogenase 1, hydrogenase 2, and hydrogenase 3	this study
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔfocA*	BW25113 *hyaB hybC hycA focA Δkan*; defective in large subunit of hydrogenase 1and hydrogenase 2, defective in repressor of FHL, and defective in formate transporter	this study
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔfocB*	BW25113 *hyaB hybC hycA focB Δkan*; defective in large subunit of hydrogenase 1and hydrogenase 2, defective in repressor of FHL, and defective in putative formate transporter	this study
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔnarG*	BW25113 *hyaB hybC hycA narG Δkan*; defective in large subunit of hydrogenase 1and hydrogenase 2, defective in repressor of FHL, and defective in α-subunit of nitrate reductase A	this study
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA Δfnr*	BW25113 *hyaB hybC hycA fnr* Km^R; defective in large subunit of hydrogenase 1and hydrogenase 2, defective in repressor of FHL, and defective in FNR transcriptional dual regulator	this study

Table B.3. (continued)

Strains and plasmids	Genotype	Source
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔfdnG*	BW25113 *hyaB hybC hycA fdnG* Km^R; defective in large subunit of hydrogenase 1and hydrogenase 2, defective in repressor of FHL, and defective in α-subunit of formate dehydrogenase-N	this study
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔfdoG*	BW25113 *hyaB hybC hycA fdoG Δkan*; defective in large subunit of hydrogenase 1and hydrogenase 2, defective in repressor of FHL, and defective in α-subunit of formate dehydrogenase-O	this study
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔfdoG ΔfocA*	BW25113 *hyaB hybC hycA fdoG focA Δkan*; defective in large subunit of hydrogenase 1and hydrogenase 2, defective in repressor of FHL, defective in α-subunit of formate dehydrogenase-O, and defective in formate transporter	this study
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔfdnG ΔfdoG*	BW25113 *hyaB hybC hycA fdoG fdnG* Km^R; defective in large subunit of hydrogenase 1and hydrogenase 2, defective in repressor of FHL, and defective in α-subunit of formate dehydrogenase-N and formate dehydrogenase-O	this study
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔfocA ΔfocB*	BW25113 *hyaB hybC hycA focA focB* Km^R; defective in large subunit of hydrogenase 1and hydrogenase 2, defective in repressor of FHL, and defective in formate transporter and putative formate transporter	this study
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔfocA ΔnarG*	BW25113 *hyaB hybC hycA focA narG* Km^R; defective in large subunit of hydrogenase 1and hydrogenase 2, defective in repressor of FHL, defective in formate transporter, and defective in α-subunit of nitrate reductase A	this study
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔfocB ΔnarG*	BW25113 *hyaB hybC hycA focB narG Δkan*; defective in large subunit of hydrogenase 1and hydrogenase 2, defective in repressor of FHL, defective in putative formate transporter, and defective in α-subunit of nitrate reductase A	this study
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔfocA ΔfocB ΔnarG*	BW25113 *hyaB hybC hycA focA focB narG* Km^R; defective in large subunit of hydrogenase 1and 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
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔfocB ΔfdnG*	BW25113 *hyaB hybC hycA focB fdnG* Km^R; defective in large subunit of hydrogenase 1and hydrogenase 2, defective in repressor of FHL, defective in putative formate transporter, and defective in α-subunit of formate dehydrogenase-N	this study
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔfocB ΔfdoG*	BW25113 *hyaB hybC hycA focB fdoG Δkan*; defective in large subunit of hydrogenase 1and hydrogenase 2, defective in repressor of FHL, defective in putative formate transporter, and defective in α-subunit of formate dehydrogenase-O	this study

Table B.3. (continued)

Strains and plasmids	Genotype	Source
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔfocB ΔfdnG ΔfdoG*	BW25113 *hyaB hybC hycA focB fdnG fdoG* Km^R; defective in large subunit of hydrogenase 1and 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 et al., 2005)
pCA24N-FhlA	pCA24N *pT5-lac*::*fhlA*; expresses FhlA derived from *Escherichia coli*	(Kitagawa et al., 2005)
pCP20	Ap^R and Cm^R plasmid with temperature-sensitive replication and thermal induction of FLP synthesis	(Cherepanov and Wackernagel, 1995)

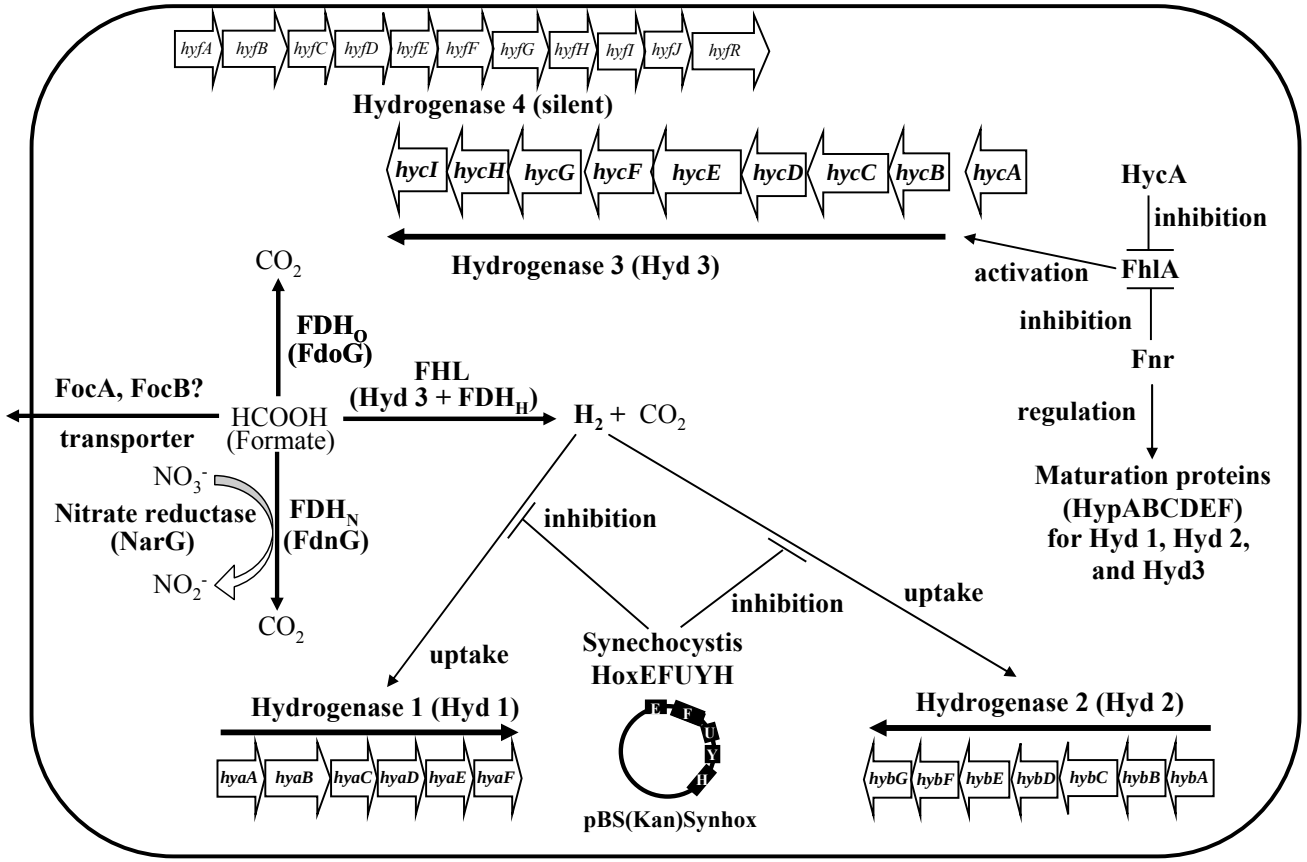

Fig. B.1. Schematic of fermentative hydrogen production in *E. coli.* Hydrogen is produced from formate by the formate hydrogen lyase (FHL) system (hydrogenase 3 and formate dehydrogenase-H (FDH_H)), which is activated by FhlA (that is regulated by Fnr) and repressed by HycA. Evolved hydrogen is consumed 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.

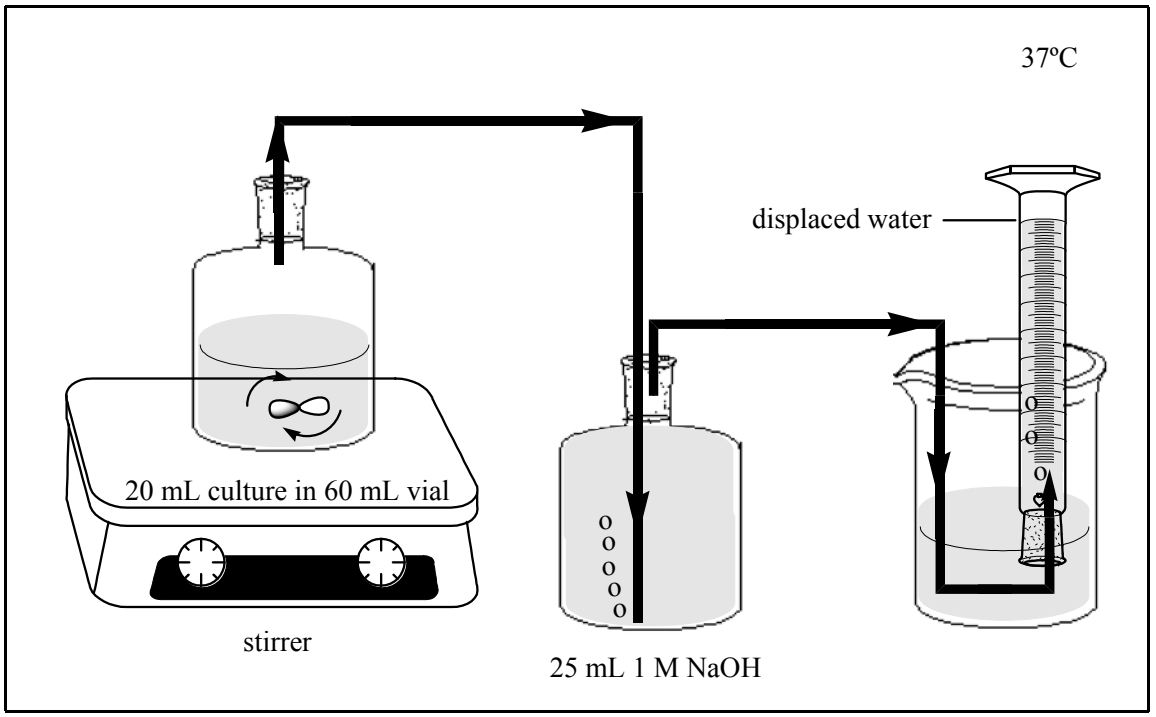

Fig. B.2. **Low-pressure, anaerobic hydrogen production in a simple batch reactor incubated at 37°C.** That allows the produced hydrogen to escape from the reactor vessel. The volume of hydrogen gas was measured after 30 min, and complex-formate medium was used.

APPENDIX C

ENHANCED HYDROGEN PRODUCTION FROM GLUCOSE BY METABOLICALLY-ENGINEERED *ESCHERICHIA COLI**

Abstract

To utilize fermentative bacteria for producing the alternative fuel hydrogen, we performed successive rounds of P1 transduction from the KEIO *Escherichia coli* K-12 library to introduce multiple, stable mutations into a single bacterium to direct the metabolic flux toward hydrogen production. *E. coli* cells convert glucose to various organic acids (such as succinate, pyruvate, lactate, formate, and acetate) to synthesize energy and synthesize hydrogen from formate by the formate hydrogen lyase (FHL) system that consists of hydrogenase 3 and formate dehydrogenase-H. We altered the regulation of FHL by inactivating the repressor encoded by *hycA* and by overexpressing the activator encoded by *fhlA*, removed hydrogen uptake activity by deleting *hyaB* (hydrogenase 1) and *hybC* (hydrogenase 2), re-directed glucose metabolism to formate by using the *fdnG*, *fdoG*, *narG, focA*, *focB*, *poxB*, and *aceE* mutations, and inactivated the succinate and lactate synthesis pathways by deleting *frdC* and *ldhA*, respectively. The best of the metabolically engineered strains, BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE*, increased hydrogen production 4.6-fold from glucose and increased the hydrogen yield 2-fold from 0.65 to 1.3 mol H_2/mol glucose (maximum 2 mol H_2/mol glucose).

*Reprinted with the permission from "Enhanced hydrogen production from glucose by metabolically-engineered *Escherichia coli*" by Toshinari Maeda, Viviana Sanchez-Torres, and Thomas K. Wood, 2007, Applied Microbiology and Biotechnology 77:879-890, Copyright 2007, Springer-Verlag, doi: 10.1007/s00253-007-1217-0. The original publication is available at www.springerlink.com. T. Maeda constructed the strains and performed hydrogen production assays. V. Sanchez-Torres determined the specific growth rate of the metabolically-engineered strains and evaluated hydrogen production from glucose for 12 strains.

Introduction

Hydrogen 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 the internal combustion engine (Dunn 2002). It is estimated that the global energy system will shift from fossil fuels to hydrogen and methane (Dunn 2002). 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 chemical production (Das and Veziroğlu 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 Veziroğlu 2001). Biological methods depend on hydrogenases which catalyze the reaction $2H^+ + 2e^- \leftrightarrow H_2$ (g) (Evans and Pickett 2003). Hydrogen may be produced through either photosynthetic or fermentative processes; but, fermentative hydrogen production is more efficient than photosynthetic production (Yoshida et al. 2005).

Escherichia coli is used here for hydrogen production since it is easy to manipulate genetically, and it is the best-characterized bacterium (Blattner et al. 1997). For example, the glucose glycolytic pathway to phosphoenolpyruvate, pyruvate, acetate, ethanol, and formate via bacterial fermentation is well established (Bagramyan and Trchounian 2003), and P1 phage transduction allows one to easily introduce mutations into *E. coli* cells. Previously, we (Maeda et al. 2007b) used the isogenic *E. coli* K-12 KEIO collection of the Genome Analysis Project in Japan (Baba et al. 2006), which contains all non-lethal deletion mutations (3985 genes), to introduce as many as six mutations in a single *E. coli* strain for directing cell metabolism from formate to hydrogen without diminishing cell growth. The simple technique consisted of removing the kanamycin antibiotic resistance marker (kan^R) after each round of P1 transduction

by using the flanking flippase (FLP) recognition target sequences with FLP recombinase (Datsenko and Wanner 2000).

E. coli produces hydrogen from formate by the formate hydrogen lyase system (FHL) that consists of hydrogenase 3 (encoded by *hycABCDEFGHI* (Bagramyan and Trchounian 2003)) and formate dehydrogenase-H (encoded by *fdhF* (Axley et al. 1990)); these enzymes catalyze the reaction $HCOO^- + H_2O \leftrightarrow H_2 + HCO_3^-$ (Woods 1936) (Fig. C.1) and are probably used to help regulate internal pH (Böck and Sawers 1996). FHL activity is repressed by the *hycA* gene product (Bagramyan and Trchounian 2003) and activated by the *fhlA* gene product (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). The evolved hydrogen from the FHL is consumed by *E. coli* hydrogenase 1 (*hyaB* encodes the large subunit (Forzi and Sawers 2007)) and hydrogenase 2 (*hybC* encodes the large subunit (Forzi and Sawers 2007)) (Fig. C.1). In *E. coli*, 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). Hence, by deleting *hyaB*, *hybC*, *fdnG*, *fdoG*, *focAB*, and *narG*, hydrogen production should be enhanced, and we have found that a quintuple mutant (BW25113 *hyaB hybC hycA fdoG*/pCA24N-FhlA) increases hydrogen production from formate by over two orders of magnitude (Maeda et al. 2007b). In addition, pyruvate dehydrogenase (encoded by *aceE*) and pyruvate oxidase (encoded by *poxB*) consume pyruvate produced from glucose (Abdel-Hamid et al. 2001; Angelides et al. 1979) (Fig. C.1), so inactivating these genes may be

useful for enhancing hydrogen production by preventing pyruvate consumption. Also, the succinate-producing pathway (phosphoenolpyruvate to succinate) and lactate-producing pathway (pyruvate to lactate) may be inactivated to direct glucose metabolism toward hydrogen (Fig. C.1); therefore, deletion of fumarate reductase (*frdC*) and lactate dehydrogenase (*ldhA*) increases hydrogen production from glucose (Yoshida et al. 2006).

Since it may be more practical to produce hydrogen from glucose (Kraemer and Bagley 2007) rather than to add or overproduce formate, here we create one septuple mutant (BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE*) that produces 4.6-fold more hydrogen than the wild-type strain and that enhances the yield of hydrogen 2-fold as a result of manipulating the pathway mutations *hyaB*, *hybC*, *hycA*, *fhlA, focA*, *focB*, *narG*, *fdoG*, *fdnG*, *frdC*, *ldhA*, *poxB*, and *aceE*. This is the first report of strains harboring these seven mutations for converting glucose to hydrogen (previously we reported on an *E. coli* strain harboring the *hyaB hybC hycA, fdoG,* and *fhlA* mutations for converting formate to hydrogen (Maeda et al. 2007b)), and the first investigation of the importance of the *poxB* and *aceE* mutations for hydrogen production.

Materials and methods

Bacterial strains, growth rates, and total protein

Strains are shown in Table C.1. *E. coli* cells were initially streaked from -80°C glycerol stocks on Luria-Bertani (LB) agar plates (Sambrook et al. 1989) containing 100 μg/mL kanamycin (for those with chromosomal kanamycin resistance markers) and 30 μg/mL 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 rpm (New Brunswick Scientific Co., Edison, NJ) in LB medium (Sambrook et al. 1989) or in

modified complex-glucose medium (Rachman et al. 1997) in which 0.4 mg/L $(NH_4)_6Mo_7O_{24}$, was added; 100 μg/mL kanamycin or 30 μg/mL chloramphenicol were also added where appropriate. Wild-type *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 based on pCA24N (Kitagawa et al. 2005) were electroporated into hydrogen-overproducing *E. coli* strains (Table C.1). Aerobic cell growth was measured using turbidity at 600 nm from 0.05 to 0.7, and total protein for *E. coli* was 0.22 mg/OD/mL (Protein assay kit, Sigma Diagnostics, St. Louis, MO).

Multiple chromosomal mutations

Repeated rounds of P1 transduction (Silhavy et al. 1984) were performed to knockout specific genes by selecting for the kanamycin-resistance gene that is transferred along with each chromosomal deletion that are 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) since 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 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 modified complex-glucose medium (111 mM glucose) 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, 20 mL of modified complex medium without glucose was added, and then the cells were suspended to a turbidity of 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 glucose were added to the bottles which were incubated at 37°C with shaking for 30 min to 17 h. The amount of hydrogen generated in the head space 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. 2007c).

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 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 (Maeda et al. 2007b). The vials were incubated at 37°C with stirring for 15 min, and hydrogen was assayed with a GC. As a negative control, cell suspensions (20 mL) without glucose were also used. Glucose concentrations in complex-glucose media were measured using the HK assay (Sigma). For yield calculations, the vials were incubated for 16 h.

Results

Our strategy for metabolic engineering *E. coli* for enhanced hydrogen production from

glucose via formate was six-fold (Fig. C.1) and based on our initial success of using some of these mutations for increasing the yield of hydrogen from formate using strain BW25113 *hyaB hybC hycA fdoG*/pCA24N-FhlA (Maeda et al. 2007b); note all the original mutations had to be re-evaluated since they were originally assayed for their effect on producing hydrogen starting from formate, and two new mutations were evaluated here (*poxB* and *aceE*). We (i) prevented hydrogen consumption by inactivating hydrogenase 1 (HyaB, large subunit) and hydrogenase 2 (HybC, large subunit), (ii) inactivated the FHL repressor HycA, (iii) overexpressed the FHL activator FhlA (FhlA binds directly to the intergenic region between the *hyc* and *hyp* operons and between the *hycA* and *hycB* genes (Schlensog et al. 1994), (iv) eliminated the formate exporters FocA and its homolog FocB (Andrews et al. 1997; Suppmann and Sawers 1994), (v) prevented formate consumption by formate dehydrogenase-N (FdnG, α-subunit) coupled with nitrate reductase A (NarG, α-subunit) (Rossmann et al. 1991) and dehydrogenase-O (FdoG, α-subunit) (Rossmann et al. 1991), and (vi) altered glucose metabolism to efficiently synthesize formate from glucose by preventing lactate and succinate formation as well as pyruvate consumption. *E. coli* cells metabolize glucose into formate via phosphoenolpyruvate and pyruvate by the glycolytic system (Bagramyan and Trchounian 2003); phosphoenolpyruvate may also be converted into succinate by fumarate reductase (FrdC) (Iverson et al. 1999), pyruvate may be converted into lactate by lactate dehydrogenase (LdhA) (Sode et al. 1999), and pyruvate may be consumed by pyruvate dehydrogenase (AceE) (Angelides et al. 1979) and pyruvate oxidase (PoxB) (Abdel-Hamid et al. 2001) (Fig. C.1). Therefore, deleting *frdC*, *ldhA*, *aceE*, and *poxB* should enhance hydrogen production by increasing formate production.

Another goal was to introduce mutations that did not make the cell less viable so specific growth rates were quantified after each mutation was added. Cell viability was not significantly affected for all strains (46 strains) except the two septuple mutants with the *aceE* mutations

(*hyaB hybC hycA fdoG frdC ldhA aceE* and *hyaB hybC hycA fdnG frdC ldhA aceE*) that had a 3.6-fold reduced aerobic specific growth rate compared to the wild type strain in LB medium (Table C.2). In addition, the specific growth rate of BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE* was 2.7 times lower than that of wild-type cells in complex-glucose medium (1.6 ± 0.1 for BW25113 vs. 0.59 ± 0.02 1/h for BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE*); however, there was no difference in the amount of cellular protein after overnight growth for the low partial pressure/closed hydrogen assay experiments between the wild type strain and the *hyaB hybC hycA fdoG frdC ldhA aceE* strain (data not shown). These results are primarily in contrast to other approaches in which cell viability has been reduced (Penfold et al. 2006).

To decrease hydrogen uptake activity in *E. coli*, the genes encoding the large subunits of hydrogenase 1 (*hyaB*) and hydrogenase 2 (*hybC*) were deleted since 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) which led to a 1.4-fold increase in hydrogen production compared to the wild-type strain in complex-glucose medium after 30 min (Table C.2); however, there was only a slight change in hydrogen production rates for each single mutation (*hyaB* or *hybC*). Also, adding the *hycA* mutation to the *hyaB hybC* double mutant did not show a significant increase in hydrogen production from glucose (Table C.2) although BW25113 *hyaB hybC hycA* produced 1.5-fold more hydrogen than *E. coli* from formate compared to cells defective in both hydrogenase 1 (*hyaB*) and hydrogenase 2 (*hybC*) (Maeda et al. 2007b).

Formate, which is the substrate for producing hydrogen in *E. coli,* is depleted by two non-hydrogen-producing pathways: (i) excretion by the putative formate transporters FocA (Suppmann and Sawers 1994) and its homolog FocB (Andrews et al. 1997), and (ii) degradation by formate dehydrogenase-N (coupled with nitrate reductase A) and degradation by formate

dehydrogenase-O, which convert formate to ATP (Wang and Gunsalus 2003). 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, 5 quadruple mutants (*hyaB hybC hycA focA*, *hyaB hybC hycA focB*, *hyaB hybC hycA narG*, *hyaB hybC hycA fdnG*, and *hyaB hybC hycA fdoG*) were constructed by introducing a *focA*, *focB*, *narG*, *fdnG*, and *fdoG* mutation to the triple mutant (*hyaB hybC hycA*), and then hydrogen production was assayed. The addition of the *focB* and *narG* mutations to the *hyaB hybC hycA* mutant increased hydrogen production 1.6-fold compared to the wild type strain in complex-glucose medium after 30 min (Table C.2). Also, hydrogen production in BW25113 *hyaB hybC hycA fdoG* was increased 1.7-fold compared to that in the wild-type cells (Table C.2). The *focA* and *fdnG* mutation were not effective for producing more hydrogen in the *hyaB hybC hycA* background.

To further test the combination of the *focA*, *focB*, *narG*, *fdnG*, and *fdoG* mutations, 7 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 2 sextuple strains (*hyaB hybC hycA focA focB narG* and *hyaB hybC hycA focB fdnG fdoG*) were constructed and hydrogen production was assayed. Three quintuple mutants (*hyaB hybC hycA fdnG fdoG*, *hyaB hybC hycA fdoG focA*, and *hyaB hybC hycA focA focB*) produced 1.5-1.7 times more hydrogen than the wild type strain; hydrogen production in 2 quintuple mutants BW25113 *hyaB hybC hycA focA narG* and *hyaB hybC hycA focB narG* was the same level with that in BW25113 *hyaB hybC hycA*. On the other hand, 2 quintuple mutants (*hyaB hybC hycA focB fdnG* and *hyaB hybC hycA focB fdoG*) and 2 sextuple strains (*hyaB hybC hycA focA focB narG* and *hyaB hybC hycA focB fdnG fdoG*) had lower hydrogen production activity than the wild-type cells.

To test the effect of deleting the succinate-producing pathway (*frdC*) and the lactate-

producing pathway (*ldhA*), 2 quadruple mutants (*hyaB hybC hycA ldhA* and *hyaB hybC hycA frdC*), 7 quintuple mutants (*hyaB hybC hycA ldhA frdC*, *hyaB hybC hycA fdoG ldhA*, *hyaB hybC hycA fdoG frdC*, *hyaB hybC hycA focB ldhA*, *hyaB hybC hycA focB frdC*, *hyaB hybC hycA narG ldhA*, and *hyaB hybC hycA narG frdC*), three sextuple mutants (*hyaB hybC hycA fdnG fdoG ldhA*, *hyaB hybC hycA fdoG ldhA frdC*, and *hyaB hybC hycA fdnG ldhA frdC*), and 1 septuple mutant (*hyaB hybC hycA fdoG fdnG ldhA frdC*) were constructed and the hydrogen was assayed. One quintuple (BW25113 *hyaB hybc hycA frdC ldhA*) and two sextuple mutants (BW25113 *hyaB hybC hycA fdoG ldhA frdC* and *hyaB hybC hycA fdnG ldhA frdC*) produced 2-fold more higher hydrogen than the wild type strain after 30 min in complex-glucose medium (Table C.2). Also, hydrogen production in all strains harboring the *ldhA* mutation was increased by 20-50% compared to that in the wild-type cells after 17 h in complex-glucose medium (Table C.2). One septuple mutant (*hyaB hybC hycA fdoG fdnG ldhA frdC*) showed lower hydrogen production than two sextuple mutants (*hyaB hybC hycA fdoG ldhA frdC* and *hyaB hybC hycA fdnG ldhA frdC*) that have high hydrogen production potential (Table C.2).

Previously, we found that expressing the FhlA protein (FHL activator) led to a 9-fold increase in hydrogen production in medium containing formate (BW25113/pCA24N-FhlA vs. BW25113) (Maeda et al. 2007b). Hence, to boost hydrogen productivity further, plasmid pCA24N-FhlA was added to the metabolically engineered strains, and hydrogen production was assayed (Table C.2). Unexpectedly, the expression of *fhlA* did not lead to a significant increase of hydrogen production from the modified complex-glucose medium in BW25113, BW25113 *hyaB hybC hycA*, BW25113 *hyaB hybC hycA fdoG*, and BW25113 *hyaB hybC hycA fdoG ldhA frdC* in the closed hydrogen assay. Also, overexpressing FhlA by adding IPTG (0.01 to 1 mM) led to a significant decrease in hydrogen production; hydrogen production with 1 mM IPTG was 3-fold less than that without IPTG (data not shown).

To investigate whether pyruvate consumption by the PoxB and AceE pathways (Fig. C.1) is significant for hydrogen production, 4 septuple mutants (BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE*, BW25113 *hyaB hybC hycA fdoG frdC ldhA poxB*, BW25113 *hyaB hybC hycA fdnG frdC ldhA aceE*, and BW25113 *hyaB hybC hycA fdnG frdC ldhA poxB*) were constructed and then hydrogen production was assayed. Two septuple strains with the *aceE* mutation (BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE* and BW25113 *hyaB hybC hycA fdnG frdC ldhA aceE*) had a slight increase of hydrogen production (8 to 12%) compared to BW25113 *hyaB hybC hycA fdoG frdC ldhA* or BW25113 *hyaB hybC hycA fdnG frdC ldhA*; hydrogen production in these two strains was 2.2 times higher than that in wild type strain (Table C.2).

With BW25113 *hyaB hybC hycA fdoG frdC aceE* we also tested whether the enhanced hydrogen production was from the added glucose. As expected, this strain produced hydrogen only slightly from complex medium that lacked glucose (4.4% of that from complex-glucose medium). This indicates that hydrogen from complex-glucose is derived from glucose.

Since the accumulation of hydrogen in the headspace in the closed system reduces hydrogen production (Kraemer and Bagley 2007), hydrogen production for the nine best strains was measured using an anaerobic system that maintained low hydrogen headspace pressure, and the results are shown in Table C.3. BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE* produced 4.6-fold more hydrogen than the wild type strain, whereas BW25113 *hyaB hybC hycA fdnG frdC ldhA*, BW25113 *hyaB hybC hycA fdnG frdC ldhA aceE*, and BW25113 *hyaB hybC hycA fdoG frdC ldhA* had 4.1- to 4.3-fold higher hydrogen production. Similarly, BW25113 *hyaB hybC hycA frdC*, BW25113 *hyaB hybC hycA ldhA*, and BW25113 *hyaB hybC hycA frdC ldhA* synthesized 2.9- to 3.3-fold more hydrogen relative to the wild type strain (Table C.3). As a negative control, BW25113 *hyaB hybC hycE*, which lacks an active hydrogenase 3, showed

negligible hydrogen production (8.8-fold less) than that of the wild-type cells for both the low partial pressure (Table C.3) as well as the closed hydrogen assays (Table C.2). Also, BW25113 *hyaB hybC hycA fdoG ldhA frdC* with pCA24N or pCA24N-FhlA produced up to 4.8-fold higher hydrogen than BW25113/pCA24N, although expression of FhlA protein did not lead to a significant increase of hydrogen production.

Along with hydrogen production, hydrogen yields are important. For BW25113 *hyaB hybC hycA frdC ldhA*, BW25113 *hyaB hybC hycA fdnG frdC ldhA*, BW25113 *hyaB hybC hycA fdnG frdC ldhA aceE*, BW25113 *hyaB hybC hycA fdnG frdC ldhA poxB*, BW25113 *hyaB hybC hycA fdoG frdC ldhA*, and BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE*, the hydrogen yield increased by 2-fold compared to that in BW25113 wild-type cells. In addition, BW25113 *hyaB hybC hycA fdoG frdC ldhA* with pCA24N and pCA24N-FhlA had 2.5-fold higher hydrogen yields than BW25113/pCA24N. Also, the yield of BW25113 *hyaB hybC hycA frdC* and BW25113 *hyaB hybC hycA ldhA* increased 1.4 to 1.8-fold compared to BW25113 *hyaB hybC hycA* (Table C.3), indicating that these two mutations (*frdC* and *ldhA*) are effective for enhancing hydrogen yields from glucose. Deleting *aceE* in BW25113 *hyaB hybC hycA fdoG frdC ldhA* (i.e., BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE*) had a slight increase (up to 7%) in hydrogen yield compared to that in BW25113 *hyaB hybC hycA fdoG frdC ldhA* (Table C.3). Assaying glucose in complex-glucose medium demonstrated clearly that the septuple strain consumed over 97% of glucose after 16 h.

Discussion

In this work, we show that a fermentative *E. coli* strain with seven mutations, BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE* produces 4.6-fold more hydrogen than the wild-type strain (~32 μmol/h/mg protein vs. 7 μmol/h/mg protein) as a result of inactivating hydrogen

consumption by hydrogenase 1 (*hyaB*) and hydrogenase 2 (*hybC*), activation of FHL by deleting the FHL repressor (*hycA*), inactivation of formate dehydrogenase-O (*fdoG*) to prevent formate consumption, inactivation of the succinate-synthesis (*frdC*) and lactate-synthesis (*ldhA*) pathways, and inactivation of pyruvate dehydrogenase (*aceE*) to prevent pyruvate consumption. Also, the hydrogen yield with BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE* by the strain increased two fold (~1.32 vs. 0.7 mol H_2/mol glucose).

We used the *E. coli* KEIO collection here to introduce as many as seven mutations into a single strain. Thus, the use of this library is a breakthrough in that it has been difficult to make strains with multiple mutations using other methods that depend on different selection makers for each gene inactivated (Lee et al. 2005; Yoshida et al. 2006). This method is general and simple (repetition of resistance-gene elimination and P1 transduction), and may be used to engineer *E. coli* for many applications where multiple chromosomal genes must be eliminated.

Previously, three groups have enhanced hydrogen production in *E. coli*. Inactivation of the FHL repressor (HycA) and overexpression of the FHL activator (FhlA) led to a 2.8-fold increase of hydrogen production from formate (Yoshida et al. 2005). Deleting the twin-arginine translocation system for transporting proteins into the periplasm resulted in 2-fold higher hydrogen production from glucose by indirectly inactivating hydrogenase 1, hydrogenase 2, formate dehydrogenase-N, and formate dehydrogenase-O; however, this mutation led to a significant decrease in cell viability (Penfold et al. 2006). Also, deletions of lactate dehydrogenase (*ldhA*) and fumarate reductase (*frdBC*) resulted in only a 1.4-fold increase in hydrogen production compared to the wild-type strain from glucose (Yoshida et al. 2006). In comparison, our metabolically-engineered *E. coli* cells have as much as 4.6-fold greater hydrogen production and the method remains robust since it is still possible to introduce further mutations to enhance hydrogen production. Due to the ease of its genetic manipulation, *E. coli*

may also be a better model than other hydrogen-producing strains such as *Citrobacter* sp. Y19 (Oh et al. 2003), *Rhodopseudomonas palustris* JA1 (Archana et al. 2003), *Rhodopseudomonas palustris* P4 (Jung et al. 1999), and *Klebsiella oxytoca* HP1 (Minnan et al. 2005) that have high maximum hydrogen activity (up to 65 μmol/mg/h).

The deletion of succinate-producing pathway (*frdC*) and lactate-producing pathway (*ldhA*) in the *hyaB hybC hycA* background led to a 3-fold higher increase of hydrogen production rate and a 2-fold higher hydrogen yield compared to the wild-type strain (Table C.3); these results are consistent with the results described previously (Sode et al. 1999; Yoshida et al. 2006). Since the two quadruple mutants (BW25113 *hyaB hybC hycA frdC* and BW25113 *hyaB hybC hycA ldhA*) increased hydrogen production 1.5- and 1.4-fold, respectively, vs. BW25113 *hyaB hybC hycA* (Table C.3) and resulted in a 1.1- or 1.5-fold higher hydrogen yield relative to BW25113 *hyaB hybC hycA*, both the *frdC* and *ldhA* mutations are important for both hydrogen production from glucose and yield with the *ldhA* mutation more effective than the *frdC* mutation for increasing the hydrogen yield (Table C.3).

It has been reported that the deficiency of formate dehydrogenase-N leads to an accumulation of intracellular formate and activation of the FHL pathway (Suppmann and Sawers 1994); hence, mutating *fdnG* should be effective for enhancing hydrogen production. As expected, the deletion of *fdnG* was significant as seen by comparing hydrogen production between BW25113 *hyaB hybC hycA frdC ldhA* vs. BW25113 *hyaB hybC hycA fdnG frdC ldhA* (Table C.3); the additional *fdnG* deletion led to a 45% increase in the hydrogen production rate. Similarly, deleting *fdoG* also increased hydrogen production by about 45% (BW25113 *hyaB hybC hycA frdC ldhA* vs. BW25113 *hyaB hybC hycA fdoG frdC ldhA*, Table C.3); however, the effect was not as large as the effect for growth on formate where there was a 2.2-fold increase in hydrogen production (BW25113 *hyaB hybC hycA* vs. BW25113 *hyaB hybC hycA fdoG* in a

closed system assay) (Maeda et al. 2007b). On the other hand, deleing both formate dehydrogenase-N and formate dehydrogenase-O led to a significant decrease in the hydrogen production rate (Table C.2 and Table C.3), although these mutations did not influence hydrogen yield. These results show that either active formate dehydrogenase-N or formate dehydrogenase-O is essential for producing hydrogen from glucose whereas increasing hydrogen production from formate requires inactivation of formate dehydrogenase-O (Maeda et al. 2007b).

Since our metabolically-engineered *E. coli* strains had a 1.3 mol H_2/mol glucose of hydrogen yield instead of the theoretical hydrogen yield for facultative anaerobes of 2 mol H_2/mol glucose (Yoshida et al. 2006), the *E. coli* cells metabolize glucose by pathways other than those remaining to make formate. For example, *E. coli* cells have three lactate dehydrogenases (*ldhA*, *dld*, and *lldD*) and two of them are membrane-bound flavoproteins linked with the respiratory chain (Mat-Jan et al. 1989); hence, these other two lactate dehyrogenases may prevent the cell from producing even more hydrogen.

The deletion of pyruvate oxidase (*poxB*) in the BW25113 *hyaB hybC hycA fdoG frdC ldhA* and BW25113 *hyaB hybC hycA fdnG frdC ldhA* backgrounds was not effective for enhancing hydrogen production and hydrogen yields (Table C.2 and Table C.3). The reason may be that PoxB is more important under aerobic conditions (Abdel-Hamid et al. 2001). Note that *E. coli* cells require anaerobic conditions to synthesize hydrogen since *E. coli* hydrogenases are sensitive to oxygen (Glick et al. 1980); therefore, PoxB product may not be important for enhanced hydrogen production. On the other hand, the inactivation of pyruvate dehydrogenase (AceE) was effective for enhancing both hydrogen production and hydrogen yield, although the effect is slight (BW25113 *hyaB hybC hycA fdoG frdC ldhA* vs. BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE*, Table C.3); this may be due to increased metabolic flux to formate during

glucose metabolism.

E. coli is robust since many technologies are available for its manipulation; for example, classical chemical mutagenesis followed by genome breeding (Patnaik et al. 2002) may provide other important genes for enhanced hydrogen production since there are indubitably unanticipated interactions in the metabolic pathways and their regulators. Microarray analysis (Maeda et al. 2007c) would then enable the molecular basis of the beneficial mutations to be discerned. Such approaches may hold promise for constructing even better strains for enhanced hydrogen production in glucose metabolism.

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Table C.1. **Strains and plasmids used.** Km^R, Cm^R and Ap^R are kanamycin, chloramphenicol and ampicillin resistance, respectively.

Strains and plasmids	Genotype	Source
Strains		
E. coli BW25113	*lacI*^{q} *rrnB*$_{T14}$ *ΔlacZ*$_{WJ16}$ *hsdR514 ΔaraBAD*$_{AH33}$ *ΔrhaBAD*$_{LD78}$	Yale CGSG Stock Center
E. coli BW25113 *ΔhyaB*	BW25113 *hyaB* Km^R; defective in large subunit of hydrogenase 1	(Baba et al. 2006)
E. coli BW25113 *ΔhybC*	BW25113 *hybC* Km^R; defective in large subunit of hydrogenase 2	(Baba et al. 2006)
E. coli BW25113 *ΔhyaB ΔhybC*	BW25113 *hyaB hybC Δkan*; defective in large subunit of hydrogenase 1 and hydrogenase 2	this study
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA*	BW25113 *hyaB hybC hycA Δkan*; defective in large subunit of hydrogenase 1 and hydrogenase 2, and defective in repressor of FHL	this study
E. coli BW25113 *ΔhyaB ΔhybC ΔhycE*	BW25113 *hyaB hybC hycE Δkan*; defective in large subunit of hydrogenase 1, hydrogenase 2, and hydrogenase 3	this study
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔfocA*	BW25113 *hyaB hybC hycA focA Δkan*; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, and defective in formate transporter	this study
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔfocB*	BW25113 *hyaB hybC hycA focB Δkan*; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, and defective in putative formate transporter	this study
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔnarG*	BW25113 *hyaB hybC hycA narG Δkan*; 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
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA Δfnr*	BW25113 *hyaB hybC hycA fnr* 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
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔfdnG*	BW25113 *hyaB hybC hycA fdnG* 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
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔfdoG*	BW25113 *hyaB hybC hycA fdoG Δkan*; 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
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔldhA*	BW25113 *hyaB hybC hycA ldhA Δkan*; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, and defective in lactate dehydrogenase	this study
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔfrdC*	BW25113 *hyaB hybC hycA frdC Δkan*; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, and defective in fumarate reductase	this study

Table C.1. (continued)

Strains and plasmids	Genotype	Source
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔfrdC ΔldhA*	BW25113 *hyaB hybC hycA frdC ldhA* Km^R; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in fumarate reductase, and defective in lactate dehydrogenase	this study
E. coli BW25113 ΔhyaB ΔhybC ΔhycA ΔfdnG ΔfdoG	BW25113 hyaB hybC hycA fdnG fdoG Δkan; 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
E. coli BW25113 ΔhyaB ΔhybC ΔhycA ΔfdnG ΔfdoG ΔldhA	BW25113 hyaB hybC hycA fdnG fdoG ldhA KmR; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in α-subunit of formate dehydrogenase-N and formate dehydrogenase-O, and defective in lactate dehydrogenase	this study
E. coli BW25113 ΔhyaB ΔhybC ΔhycA ΔfdoG ΔfocA	BW25113 hyaB hybC hycA fdoG focA KmR; 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
E. coli BW25113 ΔhyaB ΔhybC ΔhycA ΔfdoG ΔldhA	BW25113 hyaB hybC hycA fdoG ldhA KmR; 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 lactate dehydrogenase	this study
E. coli BW25113 ΔhyaB ΔhybC ΔhycA ΔfdoG ΔfrdC	BW25113 hyaB hybC hycA fdoG frdC Δkan; 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 fumarate reductase	this study
E. coli BW25113 ΔhyaB ΔhybC ΔhycA ΔfdoG ΔldhA ΔfrdC	BW25113 hyaB hybC hycA fdoG ldhA frdC KmR; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in α-subunit of formate dehydrogenase-O, defective in lactate dehydrogenase, and defective in fumarate reductase	this study
E. coli BW25113 ΔhyaB ΔhybC ΔhycA ΔfdoG ΔldhA ΔfrdC ΔaceE	BW25113 hyaB hybC hycA fdoG ldhA frdC aceE KmR; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in α-subunit of formate dehydrogenase-O, defective in lactate dehydrogenase, defective in fumarate reductase, and defective in pyruvate dehydrogenase	this study
E. coli BW25113 ΔhyaB ΔhybC ΔhycA ΔfdoG ΔldhA ΔfrdC ΔpoxB	BW25113 hyaB hybC hycA fdoG ldhA frdC poxB KmR; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in α-subunit of formate dehydrogenase-O, defective in lactate dehydrogenase, defective in fumarate reductase, and defective in pyruvate oxidase	this study
E. coli BW25113 ΔhyaB ΔhybC ΔhycA ΔfdnG ΔldhA ΔfrdC	BW25113 hyaB hybC hycA fdnG ldhA frdC KmR; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in α-subunit of formate dehydrogenase-N, defective in lactate dehydrogenase, and defective in fumarate reductase	this study

Table C.1. (continued)

Strains and plasmids	Genotype	Source
E. coli BW25113 ΔhyaB ΔhybC ΔhycA ΔfdnG ΔldhA ΔfrdC ΔaceE	BW25113 hyaB hybC hycA fdnG ldhA frdC aceE KmR; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in α-subunit of formate dehydrogenase-N, defective in lactate dehydrogenase, defective in fumarate reductase, and defective in pyruvate dehydrogenase	this study
E. coli BW25113 ΔhyaB ΔhybC ΔhycA ΔfdnG ΔldhA ΔfrdC ΔpoxB	BW25113 hyaB hybC hycA fdnG ldhA frdC poxB KmR; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in α-subunit of formate dehydrogenase-N, defective in lactate dehydrogenase, defective in fumarate reductase, and defective in pyruvate oxidase	this study
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔfdoG ΔfdnG ΔldhA ΔfrdC*	BW25113 *hyaB hybC hycA fdoG fdnG ldhA frdC* Km^R; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in *α*-subunit of formate dehydrogenase-O and formate dehydrogenase-N, defective in lactate dehydrogenase, and defective in fumarate reductase	this study
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔfocA ΔfocB*	BW25113 *hyaB hybC hycA focA focB* 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
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔfocA ΔnarG*	BW25113 *hyaB hybC hycA focA narG Δkan*; 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
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔfocB ΔnarG*	BW25113 *hyaB hybC hycA focB narG Δkan*; 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
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔfocA ΔfocB ΔnarG*	BW25113 *hyaB hybC hycA focA focB narG* 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
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔfocB ΔfdnG*	BW25113 *hyaB hybC hycA focB fdnG* 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
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔfocB ΔfdoG*	BW25113 *hyaB hybC hycA focB fdoG Δkan*; 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
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔfocB ΔfdnG ΔfdoG*	BW25113 *hyaB hybC hycA focB fdnG fdoG* 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

Table C.1. (continued)

Strains and plasmids	Genotype	Source
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔfocB ΔldhA*	BW25113 *hyaB hybC hycA focB ldhA Δkan*; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in putative formate transporter, and defective in lactate dehydrogenase	this study
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔfocB ΔfrdC*	BW25113 *hyaB hybC hycA focB frdC* 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 fumarate reductase	this study
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔnarG ΔldhA*	BW25113 *hyaB hybC hycA narG ldhA Δkan*; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in α-subunit of nitrate reductase A, and defective in lactate dehydrogenase	this study
E. coli BW25113 *ΔhyaB ΔhybC ΔhycA ΔnarG ΔfrdC*	BW25113 *hyaB hybC hycA narG frdC* Km^R; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in α-subunit of nitrate reductase A, and defective in fumarate reductase	this study
Plasmids		
pCA24N	Empty vector; Cm^R	(Kitagawa et al. 2005)
pCA24N-FhlA	pCA24N *pT5-lac*::*fhlA*; expresses FhlA derived from *Escherichia coli*	(Kitagawa et al. 2005)
pCP20	Ap^R and Cm^R plasmid with temperature-sensitive replication and thermal induction of FLP synthesis	(Cherepanov and Wackernagel 1995)

Table C.2. Effect of metabolic mutations on hydrogen production from glucose by *E. coli* BW25113 in the closed system.

Strain	Growth rate		H_2 production[1]		H_2 production[2]	
	1/hr	Relative	μmol/mg protein	Relative	μmol/mg protein	Relative
BW25113	1.6 ± 0.1	1	15 ± 4	1	35 ± 1	1
BW25113 *hyaB*	1.42 ± 0.01	0.9	15.6 ± 0.5	1.1	35 ± 1	1
BW25113 *hybC*	1.6 ± 0.1	1	17.5 ± 0.04	1.2	37 ± 1	1.1
BW25113 *hyaB hybC*	1.6 ± 0.1	1	21 ± 1	1.4	38.3 ± 0.6	1.1
BW25113 *hyaB hybC hycA*	1.4 ± 0.2	0.9	21 ± 1	1.4	36 ± 2	1
BW25113 *hyaB hybC hycE*	1.5 ± 0.1	0.9	0.57 ± 0.02	0.04	1.74 ± 0.01	0.05
BW25113 *hyaB hybC hycA focA*	1.6 ± 0.1	1	20 ± 2	1.4	35 ± 1	1
BW25113 *hyaB hybC hycA focB*	1.58 ± 0.01	1	24 ± 1	1.6	32 ± 5	0.9
BW25113 *hyaB hybC hycA narG*	1.46 ± 0.00	0.9	24 ± 4	1.6	34 ± 4	1
BW25113 *hyaB hybC hycA fnr*	1.6 ± 0.2	1	0.6 ± 0.2	0.04	1.7 ± 0.1	0.05
BW25113 *hyaB hybC hycA fdnG*	1.6 ± 0.1	1	20.7 ± 0.8	1.4	34.2 ± 0.2	1
BW25113 *hyaB hybC hycA fdoG*	1.44 ± 0.05	0.9	25 ± 3	1.7	35 ± 1	1
BW25113 *hyaB hybC hycA ldhA*	1.58 ± 0.06	1	22 ± 1	1.5	43 ± 1	1.2
BW25113 *hyaB hybC hycA frdC*	1.6 ± 0.1	1	20.5 ± 0.8	1.4	35.4 ± 0.5	1
BW25113 *hyaB hybC hycA frdC ldhA*	1.51± 0.03	0.9	30 ± 1	2.0	43 ± 3	1.2
BW25113 *hyaB hybC hycA fdnG fdoG*	1.46 ± 0.07	0.9	25.3 ± 0.5	1.7	36.3 ± 0.8	1
BW25113 *hyaB hybC hycA fdnG fdoG ldhA*	1.53 ± 0.05	1	27.4 ± 0.7	1.8	45.4 ± 0.9	1.3
BW25113 *hyaB hybC hycA fdoG focA*	1.5 ± 0.2	0.9	23.0 ± 0.2	1.6	34.6 ± 0.2	1
BW25113 *hyaB hybC hycA fdoG ldhA*	1.5 ± 0.1	0.9	26.8 ± 0.7	1.7	52 ± 12	1.5
BW25113 *hyaB hybC hycA fdoG frdC*	1.55 ± 0.07	1	26 ± 1	1.7	41 ± 11	1.2
BW25113 *hyaB hybC hycA fdoG ldhA frdC*	1.5 ± 0.2	0.9	29.1 ± 0.9	1.9	44.64 ± 0.01	1.3
BW25113 *hyaB hybC hycA fdoG ldhA frdC aceE*	0.44 ± 0.03	0.3	32.5 ± 0.8	2.2	45.2 ± 0.3	1.3
BW25113 *hyaB hybC hycA fdoG ldhA frdC poxB*	1.40 ± 0.09	0.9	30 ± 1	2.0	43 ± 1	1.2

Table C.2. (continued)

Strain	Growth rate		H_2 production[1]		H_2 production[2]	
	1/hr	Relative	μmol/mg protein		1/hr	Relative
BW25113 *hyaB hybC hycA fdnG ldhA frdC*	1.49 ± 0.02	0.9	30 ± 1	2.0	42.9 ± 0.4	1.2
BW25113 *hyaB hybC hycA fdnG ldhA frdC aceE*	0.46 ± 0.01	0.3	32.4 ± 0.2	2.2	44.6 ± 0.7	1.3
BW25113 *hyaB hybC hycA fdnG ldhA frdC poxB*	1.2 ± 0.1	0.8	28.9 ± 0.5	1.9	41± 0.5	1.2
BW25113 *hyaB hybC hycA fdoG fdnG ldhA frdC*	1.48 ± 0.05	0.9	15.7 ± 0.1	1.0	45 ± 2	1.3
BW25113 *hyaB hybC hycA focA focB*	1.3 ± 0.2	0.8	22.3 ± 0.5	1.5	36.0 ± 0.6	1
BW25113 *hyaB hybC hycA focA narG*	1.3 ± 0.1	0.8	20.7 ± 0.2	1.4	33.0 ± 0.1	0.9
BW25113 *hyaB hybC hycA focB narG*	1.4 ± 0.1	0.9	19 ± 2	1.3	34.1 ± 0.1	1
BW25113 *hyaB hybC hycA focA focB narG*	1.4 ± 0.3	0.9	9.7 ± 0.8	0.7	26.3 ± 0.6	0.8
BW25113 *hyaB hybC hycA focB fdnG*	1.6 ± 0.1	1	1.2 ± 0.1	0.08	3.6 ± 0.1	0.1
BW25113 *hyaB hybC hycA focB fdoG*	1.5 ± 0.1	0.9	1.2 ± 0.1	0.08	3.6 ± 0.2	0.1
BW25113 *hyaB hybC hycA focB fdnG fdoG*	1.54 ± 0.09	1	1.2 ± 0.1	0.08	3.7 ± 0.2	0.1
BW25113 *hyaB hybC hycA focB ldhA*	1.44 ± 0.05	0.9	0.86 ± 0.03	0.06	4.0 ± 0.2	0.1
BW25113 *hyaB hybC hycA focB frdC*	1.47 ± 0.04	0.9	0.87 ± 0.00	0.06	3.9 ± 0.4	0.1
BW25113 *hyaB hybC hycA narG ldhA*	1.54 ± 0.01	1	23 ± 2	1.5	47 ± 3	1.3
BW25113 *hyaB hybC hycA narG frdC*	1.5 ± 0.1	0.9	22.0 ± 0.3	1.5	29.7 ± 0.4	0.8
BW25113/pCA24N	1.46 ± 0.03	1	12.4 ± 0.3	1	30 ± 2	1
BW25113/pCA24N-FhlA	1.47 ± 0.03	1	13.2 ± 0.0	1.1	31 ± 1	1
BW25113 *hyaB hybC hycA*/pCA24N	1.39 ± 0.08	1	18 ± 2	1.5	35 ± 4	1.2
BW25113 *hyaB hybC hycA*/pCA24N-FhlA	1.39 ± 0.02	1	18 ± 2	1.5	31 ± 6	1
BW25113 *hyaB hybC hycA focA*/pCA24N-FhlA	1.4 ± 0.1	1	16 ± 1	1.3	33.4 ± 0.2	1.1
BW25113 *hyaB hybC hycA focB*/pCA24N-FhlA	1.4 ± 0.2	1	16.8 ± 0.2	1.4	33.61 ± 0.03	1.1
BW25113 *hyaB hybC hycA narG*/pCA24N-FhlA	1.38 ± 0.06	0.9	16.6 ± 0.3	1.3	33.8 ± 0.4	1.1
BW25113 *hyaB hybC hycA focB narG*/pCA24N-FhlA	ND[3]	-	15 ± 4	1.2	27 ± 7	0.9

Table C.2. (continued)

Strain	Growth rate		H_2 production[1]		H_2 production[2]	
	1/hr	Relative	μmol/mg protein		1/hr	Relative
BW25113 *hyaB hybC hycA focA focB narG*/pCA24N-FhlA	ND	-	4 ± 3	0.3	8 ± 5	0.3
BW25113 *hyaB hybC hycA fdoG*/pCA24N	1.5 ± 0.2	1	19.9 ± 0.3	1.6	28 ± 3	0.9
BW25113 *hyaB hybC hycA fdoG*/pCA24N-FhlA	1.47 ± 0.03	1	22.0 ± 0.9	1.8	29 ± 1	1
BW25113 *hyaB hybC hycA fdoG ldhA frdC*/pCA24N	1.47 ± 0.09	1	21.6 ± 0.8	1.7	42.7 ± 0.1	1.4
BW25113 *hyaB hybC hycA fdoG ldhA frdC*/pCA24N-FhlA	1.52 ± 0.01	1	20.6 ± 0.5	1.7	44 ± 1	1.5

[1] 30 min in complex-glucose medium
[2] 17 h in complex-glucose medium
[3] Not determined

Table C.3. Hydrogen production from glucose by metabolically-engineered *E. coli* strains using the low partial pressure assay and hydrogen yields.

Strain	Description	Hydrogen production rate[1]		Hydrogen yield	
		μmol/mg protein/h	relative	mol/mol[2]	Relative
BW25113	wild type	7 ± 1	1	0.65 ± 0.08	1
BW25113 *hyaB hybC hycE*	*ΔhyaB, ΔhybC*, and *ΔhycE* (defective hydrogenases 1, 2, and 3)	0.8 ± 0.1	0.1	0.02 ± 0.00	0.03
BW25113 *hyaB hybC hycA*	*ΔhyaB, ΔhybC*, and *ΔhycA* (defective hydrogenase 1 and 2, and defective FHL repressor)	16 ± 4	2.3	0.78 ± 0.06	1.2
BW25113 *hyaB hybC hycA frdC*	*ΔhyaB, ΔhybC*, *ΔhycA*, and *ΔfrdC* (defective hydrogenase 1 and 2, defective FHL repressor, and defective fumarate reductase membrane protein)	23.7 ± 0.8	3.4	0.89 ± 0.01	1.4
BW25113 *hyaB hybC hycA ldhA*	*ΔhyaB, ΔhybC*, *ΔhycA*, and *ΔldhA* (defective hydrogenase 1 and 2, defective FHL repressor, and defective D-lactate dehydrogenase)	22.9 ± 0.7	3.3	1.15 ± 0.03	1.8
BW25113 *hyaB hybC hycA fdoG*	*ΔhyaB, ΔhybC*, *ΔhycA*, and *ΔfdoG* (defective hydrogenase 1 and 2, defective FHL repressor, and defective formate dehydrogenase-O)	16 ± 7	2.3	0.81 ± 0.06	1.2
BW25113 *hyaB hybC hycA frdC ldhA*	*ΔhyaB, ΔhybC*, *ΔhycA*, *ΔfrdC*, and *ΔldhA* (defective hydrogenase 1 and 2, defective FHL repressor, defective fumarate reductase membrane protein, and defective D-lactate dehydrogenase)	20 ± 5	2.9	1.34 ± 0.04	2.1
BW25113 *hyaB hybC hycA fdnG frdC ldhA*	*ΔhyaB, ΔhybC*, *ΔhycA*, *ΔfdnG*, *ΔfrdC*, and *ΔldhA* (defective hydrogenase 1 and 2, defective FHL repressor, defective formate dehydrogenase-N, defective fumarate reductase membrane protein, and defective D-lactate dehydrogenase)	29 ± 3	4.1	1.35 ± 0.06	2.1
BW25113 *hyaB hybC hycA fdnG frdC ldhA aceE*	*ΔhyaB, ΔhybC*, *ΔhycA*, *ΔfdnG*, *ΔfrdC*, *ΔldhA,* and *ΔaceE* (defective hydrogenase 1 and 2, defective FHL repressor, defective formate dehydrogenase-N, defective fumarate reductase membrane protein, defective D-lactate dehydrogenase, and defective pyruvate dehydrogenase)	30 ± 2	4.3	1.28 ± 0.01	2.0

Table C.3. (continued)

Strain	Description	Hydrogen production rate[1]		Hydrogen yield	
		μmol/mg protein/h	relative	mol/mol[2]	Relative
BW25113 *hyaB hybC hycA fdnG frdC ldhA poxB*	*ΔhyaB, ΔhybC*, *ΔhycA*, *ΔfdnG*, *ΔfrdC*, *ΔldhA,* and *ΔpoxB* (defective hydrogenase 1 and 2, defective FHL repressor, defective formate dehydrogenase-N, defective fumarate reductase membrane protein, defective D-lactate dehydrogenase, and defective pyruvate oxidase)	25.2 ± 0.7	3.6	1.33 ± 0.02	2.0
BW25113 *hyaB hybC hycA fdoG frdC ldhA*	*ΔhyaB, ΔhybC*, *ΔhycA*, *ΔfdoG*, *ΔfrdC*, and *ΔldhA* (defective hydrogenase 1 and 2, defective FHL repressor, defective formate dehydrogenase-O, defective fumarate reductase membrane protein, and defective D-lactate dehydrogenase)	29 ± 3	4.1	1.23 ± 0.05	1.9
BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE*	*ΔhyaB, ΔhybC*, *ΔhycA*, *ΔfdoG*, *ΔfrdC*, *ΔldhA,* and *ΔaceE* (defective hydrogenase 1 and 2, defective FHL repressor, defective formate dehydrogenase-O, defective fumarate reductase membrane protein, defective D-lactate dehydrogenase, and defective pyruvate dehydrogenase)	32 ± 6	4.6	1.32 ± 0.04	2.0
BW25113 *hyaB hybC hycA fdoG frdC ldhA poxB*	*ΔhyaB, ΔhybC*, *ΔhycA*, *ΔfdoG*, *ΔfrdC*, *ΔldhA,* and *ΔpoxB* (defective hydrogenase 1 and 2, defective FHL repressor, defective formate dehydrogenase-O, defective fumarate reductase membrane protein, defective D-lactate dehydrogenase, and defective pyruvate oxidase)	22 ± 4	3.1	1.20 ± 0.04	1.8
BW25113 *hyaB hybC hycA fdoG fdnG frdC ldhA*	*ΔhyaB, ΔhybC*, *ΔhycA*, *ΔfdoG*, *ΔfdnG*, *ΔfrdC*, and *ΔldhA* (defective hydrogenase 1 and 2, defective FHL repressor, defective formate dehydrogenase-O and formate dehydrogenase-N, defective fumarate reductase membrane protein, and defective D-lactate dehydrogenase)	8 ± 1	1.1	1.15 ± 0.05	1.8
BW25113/pCA24N	wild type	5 ± 2	1	0.49 ± 0.02	1
BW25113/pCA24N-FhlA	wild type + FhlA	4.1 ± 0.6	0.8	0.57 ± 0.06	1.2
BW25113 *hyaB hybC hycA*/pCA24N	*ΔhyaB, ΔhybC*, and *ΔhycA* (defective hydrogenase 1 and 2, and defective FHL repressor)	13 ± 3	2.6	0.74 ± 0.04	1.5
BW25113 *hyaB hybC hycA*/pCA24N-FhlA	*ΔhyaB, ΔhybC*, and *ΔhycA* (defective hydrogenase 1 and 2, and defective FHL repressor) + FhlA	13 ± 1	2.6	0.77 ± 0.03	1.6

Table C.3. (continued)

Strain	Description	Hydrogen production rate[1]		Hydrogen yield	
		μmol/mg protein/h	relative	μmol/mg protein/h	relative
BW25113 *hyaB hybC hycA fdoG frdC ldhA*/pCA24N	*ΔhyaB, ΔhybC*, *ΔhycA*, *ΔfdoG*, *ΔfrdC*, and *ΔldhA* (defective hydrogenase 1 and 2, defective FHL repressor, defective formate dehydrogenase-O, defective fumarate reductase membrane protein, and defective D-lactate dehydrogenase)	24.1 ± 0.4	4.8	1.13 ± 0.04	2.4
BW25113 *hyaB hybC hycA fdoG frdC ldhA*/pCA24N-FhlA	*ΔhyaB, ΔhybC*, *ΔhycA*, *ΔfdoG*, *ΔfrdC*, and *ΔldhA* (defective hydrogenase 1 and 2, defective FHL repressor, defective formate dehydrogenase-O fumarate reductase membrane protein, and defective D-lactate dehydrogenase) + FhlA	22 ± 0.7	4.4	1.2 ± 0.1	2.5

[1] An initial hydrogen production rate was calculated from 15 min-incubation in complex-glucose medium
[2] Hydrogen yields were calculated as mol-H_2/mol-glucose from the results after 16 h

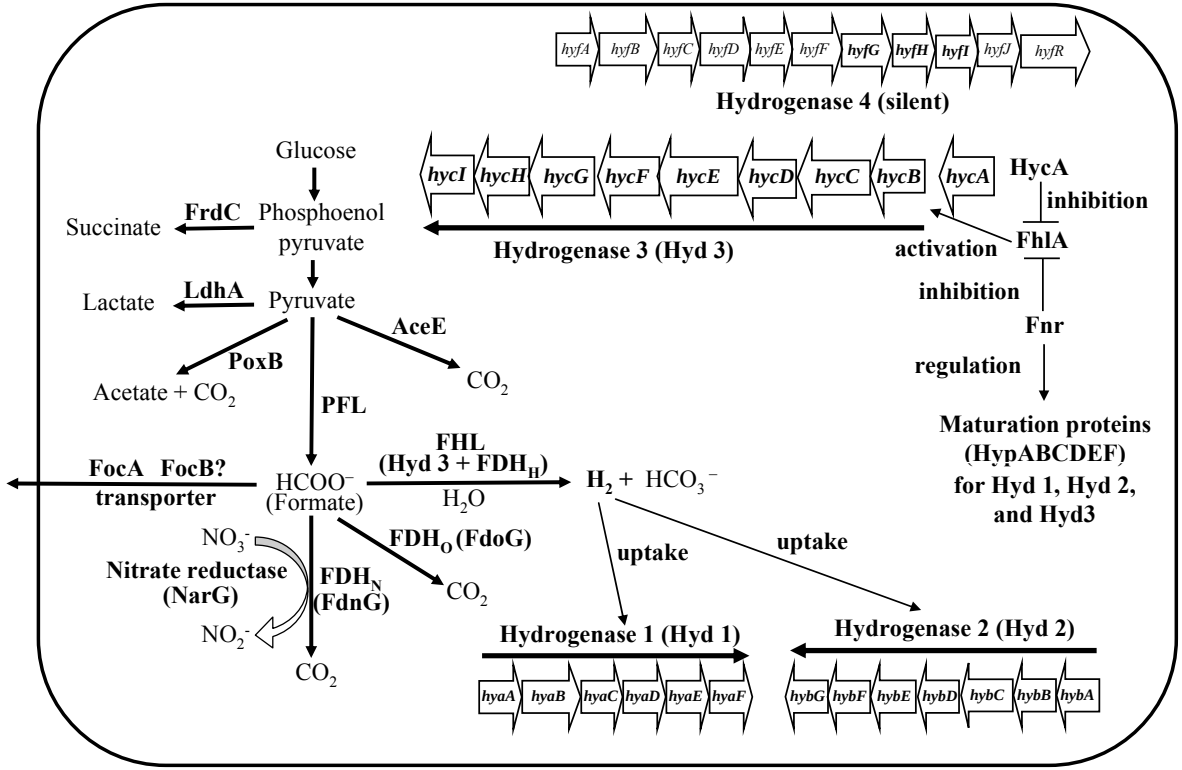

Fig. C.1. Schematic of fermentative hydrogen production in *E. coli.* Cells metabolize glucose into phosphoenolpyruvate, pyruvate, and formate. Phosphoenolpyruvate is converted to succinate by fumarate reductase (FrdC), and pyruvate is converted to either lactate by lactate dehydrogenase (LdhA), to carbon dioxide (CO_2) and acetate by pyruvate oxidase (PoxB), to carbon dioxide by pyruvate dehydrogenase (AceE), or to formate by pyruvate formate lyase (PFL). Hydrogen is produced from formate by the formate hydrogen lyase (FHL) system consisting of hydrogenase 3 (Hyd 3) and formate dehydrogenase-H (FDH_H); the FHL 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 (Hyd 1) and hydrogenase 2 (Hyd 2). Formate is exported by FocA and/or FocB and is metabolized by formate dehydrogenase-N (FDH_N; FdnG) which is linked with nitrate reductase A (NarG) and formate dehydrogenase-O (FDH_O; FdoG). HypABCDEF are maturation proteins for hydrogenases 1, 2, and 3.

APPENDIX D

PROTEIN ENGINEERING OF HYDROGENASE 3 TO ENHANCE HYDROGEN PRODUCTION*

Abstract

The large subunit (HycE, 569 amino acids) of *Escherichia coli* hydrogenase 3 produces hydrogen from formate via its Ni-Fe binding site. Here we engineered HycE for enhanced hydrogen production by an error-prone polymerase chain reaction (epPCR) using a host that lacked hydrogenase activity via the *hyaB hybC hycE* mutations. Seven enhanced HycE variants were obtained with a novel chemochromic membrane screen that directly detected hydrogen from individual colonies. The best epPCR variant contained eight mutations (S2T, Y50F, I171T, A291V, T366S, V433L, M444I, and L523Q) and had 17-fold higher hydrogen-producing activity than wild-type HycE. In addition, this variant had 8-fold higher hydrogen yield from formate compared to wild-type HycE. DNA shuffling using the three most-active HycE variants created a variant that has 23-fold higher hydrogen production and 9-fold higher yield on formate due to a 74-amino acid carboxy-terminus truncation. Saturation mutagenesis at T366 of HycE also led to increased hydrogen production via a truncation at this position; hence, 204 amino acids at the carboxy terminus may be deleted to increase hydrogen production by 30-fold. This is the first random protein engineering of a hydrogenase.

*Reprinted with the permission from "Protein engineering of hydrogenase 3 to enhance hydrogen production" by Toshinari Maeda, Viviana Sanchez-Torres, and Thomas K. Wood, 2007, Applied Microbiology and Biotechnology 79:77-86, Copyright 2008, Springer-Verlag, doi: 10.1007/s00253-008-1416-3. The original publication is available at www.springerlink.com. T. Maeda performed the mutagenesis of *hycE*, the screening, and hydrogen assays. V. Sanchez-Torres worked on screening, sequencing, and evalutating hydrogen production with the saturation mutagenesis library.

Introduction

Fermentative hydrogen production has much potential as a renewable energy source (Das and Veziroglu 2001), and *Escherichia coli* is amenable to genetic manipulation (Blattner et al. 1997). *E. coli* produces hydrogen from formate by the formate hydrogen lyase (FHL) complex that consists of formate dehydrogenase-H (encoded by *fdhF* (Axley et al. 1990)) for forming $2H^+$, $2e^-$, and CO_2 from formate and hydrogenase 3 (encoded by *hycABCDEFGHI* (Bagramyan and Trchounian 2003; Sauter et al. 1992)) for synthesizing hydrogen from $2H^+$ and $2e^-$ (Sawers et al. 1985).

Hydrogenase 3 is the large subunit (537 amino acids after a 32 aa truncation) of a [NiFe]-type hydrogenase (Drapal and Böck 1998) whose Ni-Fe active site contains one CO and two CN ligands (Blokesch et al. 2002). The CN and CO ligands may be synthesized from carbamoylphosphate via the postulated reaction: $2L_nFe + 2H_2NCOOPO_3^- \rightarrow 2L_nFeCONH2 \rightarrow L_nFeCO + L_nFeCN$) (Paschos et al. 2001); however, CO synthesis is unknown (Forzi et al. 2007). For [NiFe]-hydrogenase from *Ralstonia eutropha*, carbamoylphosphate serves as the source of CN^-, but not of the intrinsic CO (Lenz et al. 2007).

Seven accessory proteins are required for maturing HycE (Drapal and Böck 1998) including the HycI protease that catalyzes a 32 amino acid C-terminal proteolytic cleavage of the HycE apoenzyme (Rossmann et al. 1994) and HypA, HypB, HypC, HypD, HypE, and HypF for assembling the Ni-Fe metallocenter (Drapal and Böck 1998). HypA and HypB play a role in the nickel insertion followed by proteolytic removal of a C-terminal extension (Blokesch et al. 2002). HypB has GTP activity (Maier et al. 1993); GTP hydrolysis is required for Ni insertion (Maier et al. 1995). HypC is a chaperone (Drapal and Böck 1998), HypD is a Fe-S protein (Blokesch et al. 2004a), and seven conserved cysteine residues in HypD are essential for hydrogenase maturation (Blokesch and Böck 2006); the HypC-HypD complex is involved in Fe

insertion (Blokesch et al. 2002). Also, HypC interacts with the HycE apoenzyme to avoid misfolding or to maintain a suitable conformation for metal incorporation (Drapal and Böck 1998). The cysteine residue in the motif MC(L/I/V)(G/A)(L/I/V)P at the amino terminus in HypC is necessary for the interaction with the HycE apoenzyme residues Cys241, Cys244, Cys531, and Cys534 for Fe binding, Ni binding, and cysteine bridging (Magalon and Böck 2000). HypE has ATPase activity (Blokesch et al. 2002), and HypF has carbamoyl transferase activity (Blokesch et al. 2004b), and these proteins contribute to the synthesis of the CN ligands (Blokesch et al. 2004b). Thus, maturation for hydrogenase 3 is complex (Blokesch et al. 2002).

In formate dehydrogenase-H, the cofactors are selenocysteine, molybdenum, two molybdopterin guanine dinucleotides, and a Fe_4S_4 cluster at the active site (Boyington et al. 1997). The *fdhF* mRNA hairpin structure promotes selenocysteine incorporation through binding to SelB which is a selenocysteinyl-tRNA-specific translation factor (Hüttenhofer et al. 1996). There is little known about how hydrogenase interacts with formate dehydrogenase-H, but it is postulated that formate dehydrogenase-H binds to HycB, a [4Fe-4S] ferredoxin-type peptide (Bagramyan and Trchounian 2003). Hence, random mutagenesis should help determine how HycE functions.

Metabolic engineering has been used successfully to enhance hydrogen production from *E. coli*. For example, we have created a quintuple mutant by inactivating hydrogenase 1 via *hyaB* (to prevent hydrogen consumption), inactivating hydrogenase 2 via *hybC* (to prevent hydrogen consumption), inactivating the FHL repressor via *hycA*, inactivating formate dehydrogenase-O via *fdoG* (to prevent formate consumption), and overexpression of the FHL activator via $fhlA^+$; these five mutations enhanced hydrogen production from formate 141-fold and achieved the theoretical hydrogen yield (1 mol H_2/mol formate) (Maeda et al. 2008). In addition, we created a septuple mutant (*hyaB hybC hycA fdoG frdC ldhA aceE*) that increased

hydrogen production 5-fold from glucose and improved the hydrogen yield 2-fold from 0.65 to 1.3 mol H_2/mol glucose (Maeda et al. 2007b). Previously, a 3-fold increase in hydrogen production from formate was also obtained by inactivating the FHL repressor (HycA) and by overexpressing the FHL activator (FhlA) (Yoshida et al. 2005). Inactivation of the twin-arginine translocation system for transporting proteins into the periplasm led to a 2-fold increase of hydrogen production from glucose although this mutation led to a significant decrease in cell viability (Penfold et al. 2006). Also, deleting lactate dehydrogenase (*ldhA*) for converting pyruvate to lactate and fumarate reductase (*frdBC*) for converting phosphopyruvate to succinate resulted in a 1.4-fold increase in hydrogen production (Yoshida et al. 2006).

In contrast, protein engineering studies for hydrogenases to enhance bacterial hydrogen production have not been developed extensively as there have been no high-throughput methods to readily measure hydrogenase activity (either directly or indirectly) whereas many beneficial proteins such as epoxide hydrolase (van Loo et al. 2004), amine oxidase (Carr et al. 2003), alkane hydroxylase (Glieder et al. 2002), toluene para-monooxygenase (Fishman et al. 2005), toluene-*o*-xylene monooxygenase (Vardar and Wood 2005), 2,4-dinitrotoluene dioxygenase (Leungsakul et al. 2005), and acetyltransferase(Castle et al. 2004) have been engineered for enhanced catalytic function through DNA shuffling, epPCR, and saturation mutagenesis followed by novel high-throughput screening methods that enable the evaluation of many samples (100 to 1000 colonies/screen). There is only one report concerning the evolution of a hydrogenase, one derived from *Clostridia* sp. via DNA shuffling (Nagy et al. 2007); however, no screening method was used and little improvement occurred.

A novel chemochromic membrane to easily detect hydrogen produced by single colonies by colorimetric response by binding of hydrogen to a thin-film WO_3 sensor (Seibert et al. 1998) is now available and should speed research in this area. Additionally, a facile method to measure

hydrogenase activity based on formate consumption has been reported recently (Maeda and Wood 2008). Herein we report on evolving HycE for hydrogen production using epPCR, DNA shuffling, and saturation mutagenesis.

Material and methods

Bacterial strains, growth, and total protein

Parent strain *E. coli* K-12 BW25113 was obtained from the Yale University CGSC Stock Center, and its isogenic deletion *hycE* was obtained from the Genome Analysis Project in Japan (Keio collection) (Baba et al. 2006); *E. coli* BW25113 *hyaB hybC hycE ΔkanR* was constructed as described previously (Maeda et al. 2007c) and used as the host for screening the *hycE* variants since it lacks hydrogen production, hydrogen consumption, and kanamycin resistance. All *E. coli* strains were initially streaked from −80°C glycerol stocks on Luria-Bertani (LB) agar plates (Sambrook et al. 1989) containing 100 μg/mL kanamycin (Kan) (for maintaining pBS(Kan)-based plasmids) and incubated at 37°C. Overnight cultures were made from fresh, single colonies (37°C, 250 rpm) in LB medium-Kan. The total protein concentrations were 0.22 mg/OD/mL (Fishman et al. 2005).

epPCR and DNA shuffling

Plasmid pBS(Kan)HycE was constructed as described previously (Maeda et al. 2007a) and has *hycE* under control of a constitutive *lac* promoter. To introduce random mutations into the whole *hycE* locus, epPCR was performed using pBS(Kan)HycE as the template with two primers (epHycE-forward: 5'-ACAGCTATGACCATGATTACGCC-3' and epHycE-reverse: 5'-AAGGCGATTAAGTTGGGTAA CGC-3') as described previously (Cadwell and Joyce

1992). The ep-PCR products were cloned into the multiple cloning site (*Kpn*I and *Eco*RI) in pBS(Kan) after double digestion with *Kpn*I and *Eco*RI, and the ligation mixture was electroporated into BW25113 *hyaB hybC hycE*.

DNA shuffling was conducted as described previously (Canada et al. 2002). To isolate template DNA for DNA shuffling, the ep-HycE alleles harboring increased HycE activity were PCR-amplified using *Taq* polymerase with two primers (shufHycE-forward: 5'-TGCAGCTGGCACGACAGGTTTCC-3' and shufHycE-reverse: 5'-CAGGCTGCGCAACTGT TGGGAAGG-3'). Fragments (20 to 100 bp) for DNA shuffling were created by digesting the cleaned PCR product with DNase I and purified by using a Centri-Sep spin column (Princeton Separations, Adelphia, N.J.). The fragments were reassembled by PCR without primers. The 2.0-kb HycE allele was recovered by PCR with nested front primer (epHycE-forward: 5'-ACAGCTATGACCATGATTACGCC-3') and nested rear primer (epHycE-reverse: 5'-AAGGC GATTAAGTTGGGTAACGC-3'). The shuffled PCR products were then cloned into pBS(Kan) and these plasmids were electroporated into BW25113 *hyaB hybC hycE*.

Saturation mutagenesis

Saturation mutagenesis was performed at codon T366 of HycE using a QuikChange® XL Site-Directed Mutagenesis Kit (Stratagene; La Jolla, CA) and DNA primers designed to vary 32 codons to allow for substitution of all 20 amino acids as described previously (Leungsakul et al. 2006). The 5'-ACT codon for HycE T366 was varied using primers with the variable NNS codon where N is A, G, C, or T and S is G or C: HycE366front 5'-GGTGGATGTGCTGCTGAGC**NNS**CCGAACATGGAACAGC-3' and HycE366Rear 5'-CGACAGTGCGCTGTTCCATGTTCGG**SNN**GCTCAGC AGC-3'. The constructed plasmids were electroporated into BW25113 *hyaB hybC hycE,* and 360 of the generated colonies were

screened with the chemochromic membranes to find variants showing high hydrogen-producing activity.

Screening

Chemochromic membranes (GVD Corp., Cambridge, MA) (Seibert et al. 1998) were used to identify HycE variants with beneficial mutations; i.e., colonies were chosen on the basis of enhanced hydrogen production. The colonies from the epPCR, DNA shuffling, and saturation mutagenesis libraries were transferred to square agar plates (100 × 100 × 15 mm) containing modified complex-formate medium (100 mM formate) (Maeda et al. 2008), and the plates were incubated anaerobically at 37^{o}C for 14 h using a Gas-Pak anaerobic system. In the presence of oxygen, Whatman filter paper was placed firmly on top of the colonies on each plate, and the glass plates coated with the chemochromic membrane were placed on top of the Whatman paper. Colonies showing deep blue were chosen as candidates. The negative control, BW25113 *hyaB hybC hycE*, did not produce hydrogen (Maeda et al. 2007a) and remained colorless on the membrane. At least three replicates were checked for each candidate before proceeding to a gas chromatography (GC)-based hydrogen assay.

Closed hydrogen assay

Overnight aerobic LB-Kan cultures (25 mL) and fresh modified complex-formate medium were sparged for 5 min with nitrogen to remove oxygen. Sealed crimp-top vials (27 mL) were sparged for 5 min with nitrogen, and 0.5 mL of the cell suspension and 9.5 mL of fresh medium (formate concentration 100 mM) was added to the bottles which were incubated at 37^{o}C with shaking for 2 to 20 h. The amount of hydrogen generated in the head space of the recombinant system was measured using a 50 µL aliquot by GC using a 6890N gas

chromatograph as described previously (Maeda et al. 2007c).

Low partial pressure hydrogen assay

Overnight, aerobic LB cultures (25 mL, turbidity at 600 nm of 3.5) were used to inoculate 75 mL of the modified complex-formate medium in 250 mL shake flasks, and the 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 were centrifuged (7350 × *g*) for 5 min at 4°C. The supernatant was decanted in the glove box, 30 mL of fresh modified complex medium without formate was added anaerobically, and then the cells were resuspended. Sealed crimp-top vials (60 mL) were sparged for 5 min with nitrogen, and 18 mL of the cell suspension and 2 mL of 1 M formate (final concentration 100 mM) were added to the vials which were then incubated at 37°C anaerobically with shaking as described previously (Maeda et al. 2008). The hydrogen gas generated passed 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 was collected in an inverted graduated cylinder which was used to measure the volume of the gas (Maeda et al. 2008). Hence, low partial pressure was maintained in the head space of the vials. The vials were incubated at 37°C with stirring for 2.5 h, and hydrogen was assayed with the GC as described above. For yield calculations, the vials were incubated for 16 h.

SDS-PAGE, DNA sequencing, and modeling

Expression of recombinant proteins was analyzed with standard Laemmli discontinuous SDS-PAGE (12%) (Sambrook et al. 1989). A dideoxy chain termination technique (Sanger et al. 1977) with the ABI™ Prism BigDye Terminator Cycle Sequencing Ready Kit (PerkinElmer,

Wellesley, MA) was used to determine the *hycE* nucleotide sequences. HycE was modeled using MOE software (Chemical Computing group, Montreal, Canada) and was based on the large subunit of hydrogenase derived from *Desulfovibrio gigas* (Volbeda et al. 1995).

Results

Hydrogenase 3 expression host

E. coli cells possess four hydrogenases (hydrogenase 1 encoded by *hyaABCDEF*(Richard et al. 1999), hydrogenase 2 encoded by *hybOABCDEFG* (Richard et al. 1999), hydrogenase 3 encoded by *hycABCDEFGHI* (Bagramyan and Trchounian 2003; Sauter et al. 1992), and hydrogenase 4 encoded by *hyfABCDEFGHIR* (Andrews et al. 1997)). Hydrogenases 1 and 2 have hydrogen uptake activity only (Ballantine and Boxer 1986; King and Przybyla 1999), hydrogenase 4 appears to be inactive and remains cryptic (Self et al. 2004), and hydrogenase 3 has not only hydrogen synthesis activity but also hydrogen uptake activity (Maeda et al. 2007a). In this study, BW25113 *hyaB hybC hycE* was used as the host for cloning since it produces minimal hydrogen due to inactivating hydrogen consumption by hydrogenases 1, 2, and 3 and synthesis by hydrogenase 3 (Maeda et al. 2007a). Our goal was to engineer HycE for hydrogen synthesis using error-prone PCR and DNA shuffling and to identify beneficial mutations as there have been no structure/function studies for this enzyme beyond studies concerning the four cysteines in its active site for binding nickel and iron (Magalon and Böck 2000). Plasmid pBS(Kan)HycE, which can complement the chromosomal *hycE* mutation (Maeda et al. 2007a), was used for protein engineering of HycE.

epPCR

To introduce random mutations into *hycE,* epPCR was performed. From sequencing 10 colonies, the maximum error rate was 0.53%. 4540 colonies with ep-HycE alleles were screened using the chemochromic sensor method which resulted in the identification of seven variants with beneficial mutations (Table D.1) that showed high hydrogen-producing activity compared to BW25113 *hyaB hybC hycE* with wild-type HycE. To confirm the phenotype was due to plasmid-based HycE, the plasmids were isolated and re-electroporated into the original host BW25113 *hyaB hybC hycE* host. It was also confirmed via gel electrophoresis that each plasmid from the seven HycE variants was correct by digesting with *Kpn*I and *Eco*RI, and SDS-PAGE showed no change in expression of mutated HycE among the variants (data not shown), indicating that the difference of hydrogen-producing activity in HycE variants is due to the HycE mutations.

Closed vial hydrogen assay for the epHycE variants

Hydrogen production with the seven plasmids was assayed using a closed hydrogen assay. The HycE variants epHycE17, epHycE23-2, and epHycE39 had 7- to 8-fold higher hydrogen production than BW25113 *hyaB hybC hycE* expressing wild-type HycE from pBS(Kan)HycE in the closed hydrogen assay (Table D.1). Also, HycE67 and epHycE70 produced over 11- to 13-fold more hydrogen, and epHycE21 and epHycE95 produced 16- to 17-fold more hydrogen. These increases in hydrogen production were due to one to eight amino acid changes (Table D.1 and Fig. D.1). epHycE39 had a truncation of 16 aa, and epHycE70 also had a truncation of 78 aa as well as a mutation in the ribosome-binding site.

Low-partial pressure hydrogen assay in epHycE variants

To confirm the results from the closed vial assay, a low-partial pressure assay was performed on the seven HycE variants. By maintaining low partial pressures of hydrogen, feedback inhibition is avoided (Maeda et al. 2008). The results agreed well with hydrogen production values slightly higher in the low partial pressure assay (the relatives rates demonstrated similar trends in both assay systems) (Table D.1). During long incubations (16 h), these epHycE variants also had significantly-improved hydrogen yields from formate with 3- to 8-fold enhancements relative to BW25113 *hyaB hybC hycE* expressing wild-type HycE. This indicates that the beneficial mutations make the strains more capable of producing hydrogen for long periods.

DNA shuffling

To identify additional HycE variants that produce more hydrogen than the best error-prone PCR variant epHycE95, DNA shuffling was conducted with two pools consisting of either three best variants (epHycE21, epHycE67, and epHycE95) or four best variants (epHycE21, epHycE67, epHycE70, and epHycE95). 8160 colonies with shuffled HycE alleles were screened using the chemochromic sensor screening system, and out of nine possible variants studied via the closed system assay (with the GC-based hydrogen assay), one variant with significantly greater hydrogen production was identified, shufHycE1-9. The shufHycE1-9 plasmid was digested by *Kpn*I and *Eco*RI to verify correct construction, and no change in expression of the shufHycE1-9 HycE variant was observed with SDS-PAGE (Fig. D.2). After re-electroporating the plasmid, shufHycE1-9 was found to have 23-fold higher hydrogen-synthesis compared to wild-type HycE in both the closed and low partial pressure hydrogen assays (Table D.1). In addition, the yield was increased 9.3-fold. These increases in hydrogen production were the

result of truncating 74 aa from C-terminus of the mature protein; since 32 aa are removed from the C-terminus upon HycE maturation, this mutation results in a deletion of 106 aa compared to the apoenzyme) (Fig. D.1). Surprisingly, variant shufHycE1-9 did not have any mutations from epHycE21, epHycE67, and epHycE95; however, the results of DNA sequencing of two random colonies from the shuffling library showed that DNA shuffling worked well since one random clone had a silent mutation derived from epHycE21 and the V394D mutation derived from epHycE67, and the other random clone had a silent mutation from epHycE95, mutation F409L from epHycE21, and one new mutation (data not shown). This indicates that in the 8000 variant protein space sampled, the 74 aa truncation was superior to a recombination of the beneficial mutations from epHycE21, epHycE67, and epHycE95. The benefit of the carboxy-terminus truncation of the shuffling mutant was corroborated by the epPCR variant epHycE70 that had a similar mutation (Fig. D.1). SDS-PAGE also showed that both the shufHycE1-9 (Fig. D.2) and epHycE70 variants had truncated HycE alleles.

Saturation mutagenesis

Since two of the epPCR HycE variants (epHycE67 and epHycE95) had the same amino acid change, T366S (Table D.1), we investigated the importance of this position of HycE for enhanced hydrogen production by substituting all possible aa at this position via saturation mutagenesis. After screening 360 colonies to ensure with a probability of 99.999% that all possible codons were utilized (Rui et al. 2004), three variants (satHycE12T366, satHycE18T366, and satHycE19T366) were identified that had elevated hydrogen-producing activity compared to the epHycE95 epPCR variant using the chemochromic membranes. DNA sequencing revealed that all three of these mutants had the TAG stop codon at T366 (note that with NNS mutagenesis only the TAG stop codon is possible). After re-electroporating the plasmid into the original host

BW25113 *hyaB hybC hycE* to confirm the phenotype was due to plasmid-based HycE, hydrogen production in two of the saturation mutagenesis mutants, satHycE12T366 and satHycE19T366, was assayed using the closed hydrogen assay; these variants produced 18 to 32% more hydrogen than the best DNA shuffling mutant, shufHycE1-9 (Table D.1); hence, the 204 aa truncation was consistently found and consistently led to enhanced hydrogen production. The 204 aa HycE truncation of the saturation mutagenesis variant was verified using SDS-PAGE (Fig. D.2).

Protein Modeling of HycE

To evaluate where the beneficial mutations lie, MOE software was used to create a homology model based on the large subunit of the *D. gigas* [Ni-Fe]-hydrogenase (Volbeda et al. 1995); these proteins share 14% identity. Seven of the eight mutations in epHycE95 are shown in Fig. D.3.

Discussion

In this study, we constructed nine HycE variants (Table D.1) that can produce up to 30-fold higher hydrogen than BW25113 *hyaB hybC hycE*/pBS(Kan)HycE through ep-PCR, DNA shuffling, and saturation mutagenesis. Notably, the hydrogen yield was increased by an order of magnitude to become nearly equal to the theoretical maximum of 1 mol H_2/mol formate (Woods 1936). These results may be improved by eliminating formate dehydrogenase-N and formate dehydrogenase-O (Maeda et al. 2008).

Unlike many engineered proteins that are a single polypeptide, the large subunit of hydrogenase 3, HycE, is part of the membrane-bound FHL complex (FdhH-HycB-HycC-HycD-HycF-HycG-HycE) and binds HycG, the small subunit of hydrogenase 3 (Bagramyan and Trchounian 2003). Hence, the improvements in hydrogen production may be due to enhanced

catalytic reactions by the large subunit, enhanced maturation, or improved FHL complex formation. For example, epHycE70 and shufHycE1-9 should produce hydrogen without requiring HycI because these HycE alleles have unexpected termination codons at position 492 and 464, respectively (note that HycI cleaves wild-type HycE at position 537 (Rossmann et al. 1994)). Also, via saturation mutagenesis, we also found a variant satHycE12T366 with a truncation at codon T366 of HycE which produces more hydrogen than the DNA shuffling variant shufHycE1-9. All three of these truncations cause the loss of two important cysteine residues at C_{531} and C_{534} that are used by the wild-type HycE for Ni and Fe binding as part of a $DPCX_2CX_2(H/R)$ motif; previously, the replacement of these cysteine residue with alanine residues led to a significant decrease in hydrogenase activity (Magalon and Böck 2000). Our surprising truncation results (Fig. D.1) found through three independent protein engineering methods (DNA shuffling, epPCR, and saturation mutagenesis) suggest that perhaps some new interaction with HycG is required to incorporate Ni and Fe after the beneficial truncations; this hydrogenase small subunit has seven cysteines in its 255 aa and may be a suitable target for additional mutagenesis. Also, HycE may have two distinct functions: the N-terminal region may be most significant for producing hydrogen and the C-terminal region may repress hydrogen production; hence, deleting the C-terminus by truncation triggers enhanced hydrogen production. Other possibilities for the increased hydrogen production include alterations in membrane insertion of the proteins and changes in allosteric regulation.

From the eight HycE variants (seven epPCR variants and one shuffling variant), there are no mutations from aa positions 51 to 111 and 113 to 170; hence, these regions appear important for hydrogenase activity. Also, epHycE67 and epHycE95 have amino acid changes at the same positions (2 and 366) indicating that the amino acid changes S2P/S2T and T366S may be important for improved activity (note at position 266 a hydroxyl appears to be required since

serine was substituted for threonine). Therefore, these two positions may be significant for enhanced hydrogen production and may be good targets for saturation mutagenesis.

E. coli hydrogenase 3 and *D. gigas* hydrogenase are [Ni-Fe]-type hydrogenases (Drapal and Böck 1998; Volbeda et al. 1995); however, our bioinformatics analysis (Vardar-Schara et al. 2008) indicates that HycE of hydrogenase 3 does not have all six [Ni-Fe]-binding motifs of the *D. gigas* hydrogenase and the four present motifs are not in the same order in the primary sequence (Burgdorf et al. 2002), whereas *E. coli* hydrogenase 1 and hydrogenase 2 (hydrogen uptake activity) (Maeda et al. 2007a) contain all six motifs in the same order as the *D. gigas* hydrogenase (Vardar-Schara et al. 2008). Also, the large subunit of hydrogenase 1 and 2 have 40 and 42% identity, respectively, compared to that of *D. gigas* whereas HycE has only 14% identity. This indicates that *E. coli* hydrogenase 3 differs significantly from that of *D. gigas*.

Given the low identity, the homology modeling for HycE is just a starting point for identifying the relevance of the beneficial mutations found here. With this caveat, the modeling suggests the mutations in the HycE variants are positioned primarily on the surface opposite the small subunit of hydrogenase such as A291V, T366S, and V433L shown for the epHycE95 variant (Fig. D.3). This position was also observed for the beneficial mutations in the epHycE17, epHycE21, epHycE23-2, epHycE39, and epHycE67 variants. In contrast, D202V of the epHycE70 variant and Y50F/I171T/M441I of the epHycE95 variant were adjacent to the small subunit of the hydrogenase; these mutations may strengthen the interaction between the large subunit and small subunit and thereby enhance the flow of electrons generated from formate by formate dehydrogenase-H. Note that the electrons created by formate dehydrogenase-H flow to HycB to HycF to HycG to HycE (Bagramyan and Trchounian 2003). Since metabolic engineering (rational pathway engineering approach) has been used to enhance hydrogen production with *E. coli* (Bisaillon et al. 2006; Maeda et al. 2007b, 2008; Penfold et al.

2006; Yoshida et al. 2005, 2006), the HycE variants described here using a random approach may be combined with these systems to increase hydrogen production further. Also, since all of the metabolic pathways in *E. coli* are not fully elucidated, other random technologies might be performed including classical chemical mutagenesis followed by genome breeding (Patnaik et al. 2002) and DNA microarray analysis (Maeda et al. 2007c) to discern where the random chromosomal mutations lie. Hence, using *E. coli* as a reference system for producing hydrogen has many advantages.

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Table D.1. Hydrogen production and yield in *E. coli* BW25113 *hyaB hybC hycE* expressing wild-type HycE or re-electroporated HycE variants via pBS(Kan)HycE using the closed and low-partial pressure assays (n = 2). Asterisk (*) indicates nonsense mutations that result in truncation.

HycE variants	HycE aa changes	H_2 production rate[1]		H_2 production rate[2]		H_2 yield[3]	
		μmol/mg protein/h	Relative	μmol/mg protein/h	Relative	mol H_2/mol formate	Relative
wild-type	wild-type HycE	0.3 ± 0.4	1	0.6 ± 0.5	1	0.09 ± 0.03	1
epHycE17	F297L, L327Q, E382K, L415M, A504T, D542N	2.1 ± 0.5	7	2.4 ± 0.3	4	0.40 ± 0.04	4
epHycE21	Q32R, V112L, G245C, F409L	4.6 ± 0.2	15	4.68 ± 0.06	8	0.558 ± 0.004	6
epHycE23-2	D210N, I271F, K545R	2.3 ± 0.6	8	2.3 ± 0.6	4	0.33 ± 0.04	4
epHycE39	I333F, K554*	2 ± 1	7	1.43 ± 0.03	2.4	0.23 ± 0.01	3
epHycE67	S2P, E4G, M314V, T366S, V394D, S397C	4 ± 2	13	4 ± 1	7	0.44 ± 0.03	5
epHycE70	D202V, K492*	3.2 ± 0.1	11	5 ± 2	8	0.60 ± 0.08	7
epHycE95	S2T, Y50F, I171T, A291V, T366S, V433L, M444I, L523Q	5.0 ± 0.6	17	10.3 ± 0.5	17	0.7 ± 0.1	8
shufHycE1-9	Y464*	6.8 ± 0.5	23	13.6 ± 0.5	23	0.84 ± 0.02	9
satHycE12T366	T366*	9 ± 4	30	-	-	-	-
satHycE19T366	T366*	8 ± 1	27	-	-	-	-

[1] Hydrogen production rate by the closed assay system; the rate was calculated from hydrogen production after 5 h in complex-formate medium.
[2] Hydrogen production rate by the low partial pressure assay system; the rate was calculated from hydrogen production after 2.5 h in complex-formate medium.
[3] Hydrogen yield was calculated as mol-H_2/mol-formate from hydrogen production after 16 h in complex-formate medium by the low partial pressure assay system.

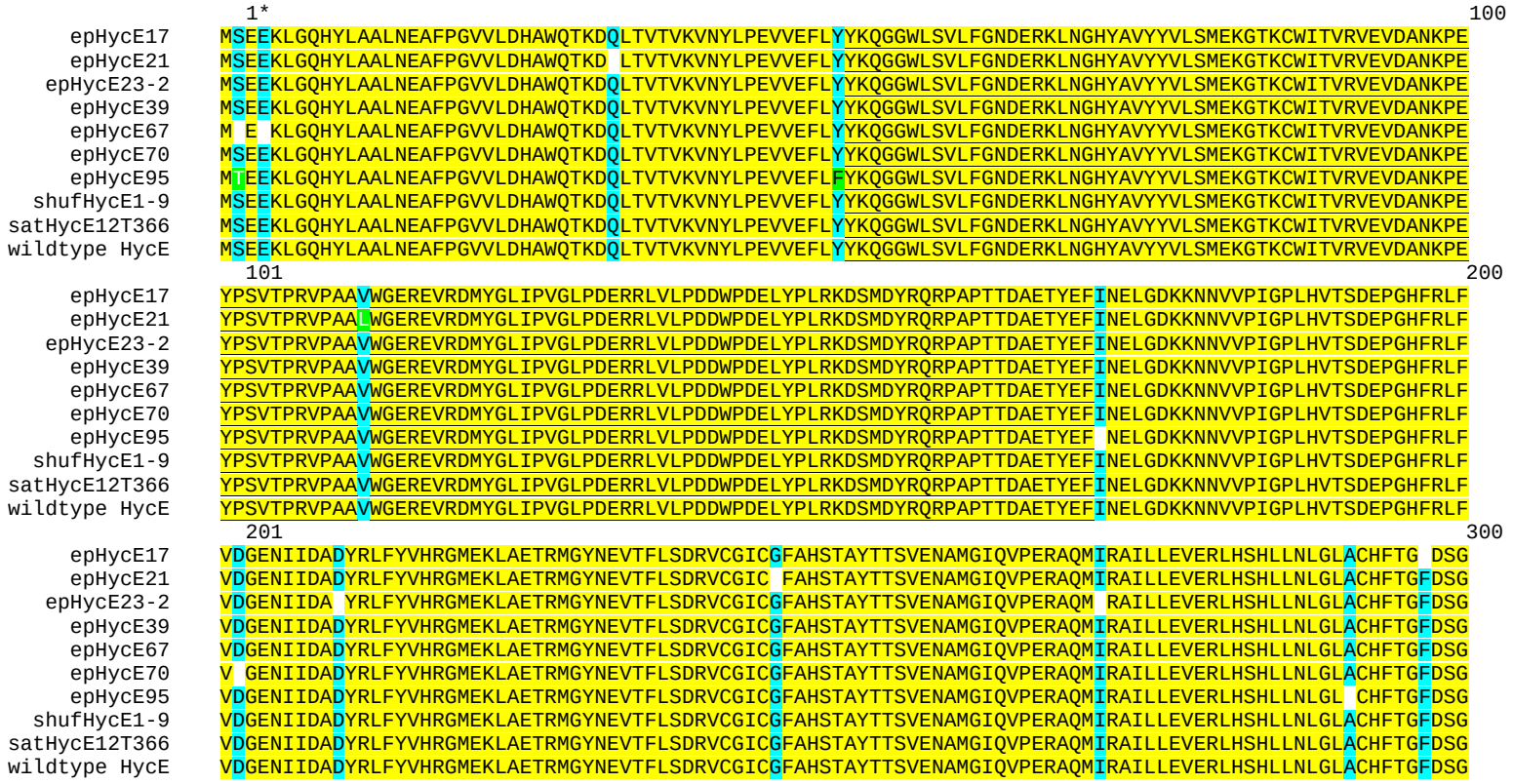

Fig. D.1. Protein sequences of the hydrogenase 3 error-prone PCR, DNA shuffling, and saturation mutagenesis variants. Amino acid changes are indicated by black highlight, and conserved Ni-Fe hydrogenase cysteines are indicated by no shading (Cys_{241}, Cys_{244}, Cys_{531}, and Cys_{534}). Underline indicates aa positions 51 to 111 and 113 to 170 in which there are no mutations, and two asterisks indicate aa positions 2 and 366 in which amino acids were changed in both epHycE67 and epHycE95.

Fig. D.1. (continued)

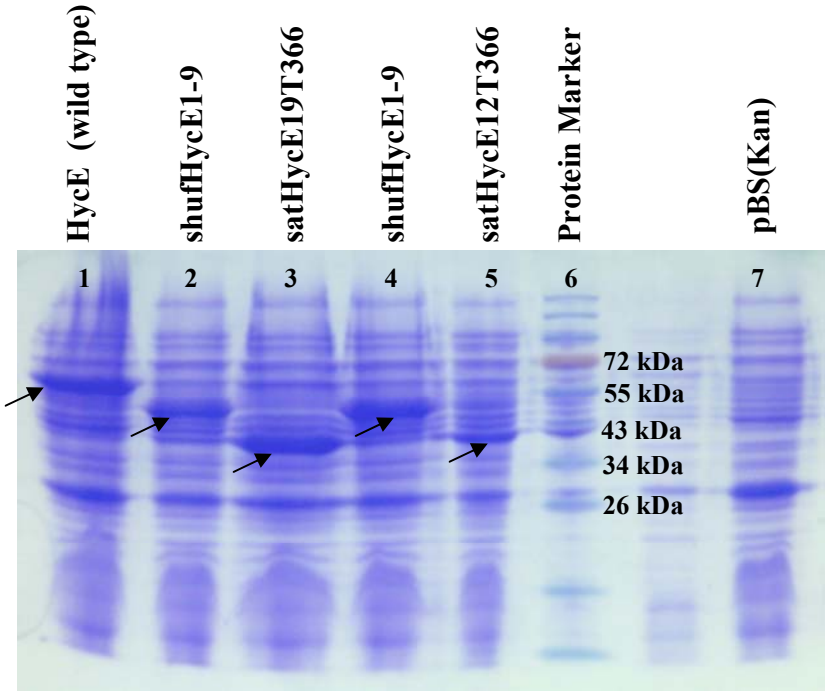

Fig. D.2. Expression of the truncated HycE variants shufHycE1-9, satHycE19T366, and satHycE12T366 along with wild-type HycE in *E. coli hyaB hybC hycE*/pBS(Kan)HycE as visualized by SDS-PAGE. Arrows indicate bands corresponding to HycE and HycE variants. *E. coli hyaB hybC hycE*/pBS(Kan) (empty vector) was used as a negative control (no expression of HycE).

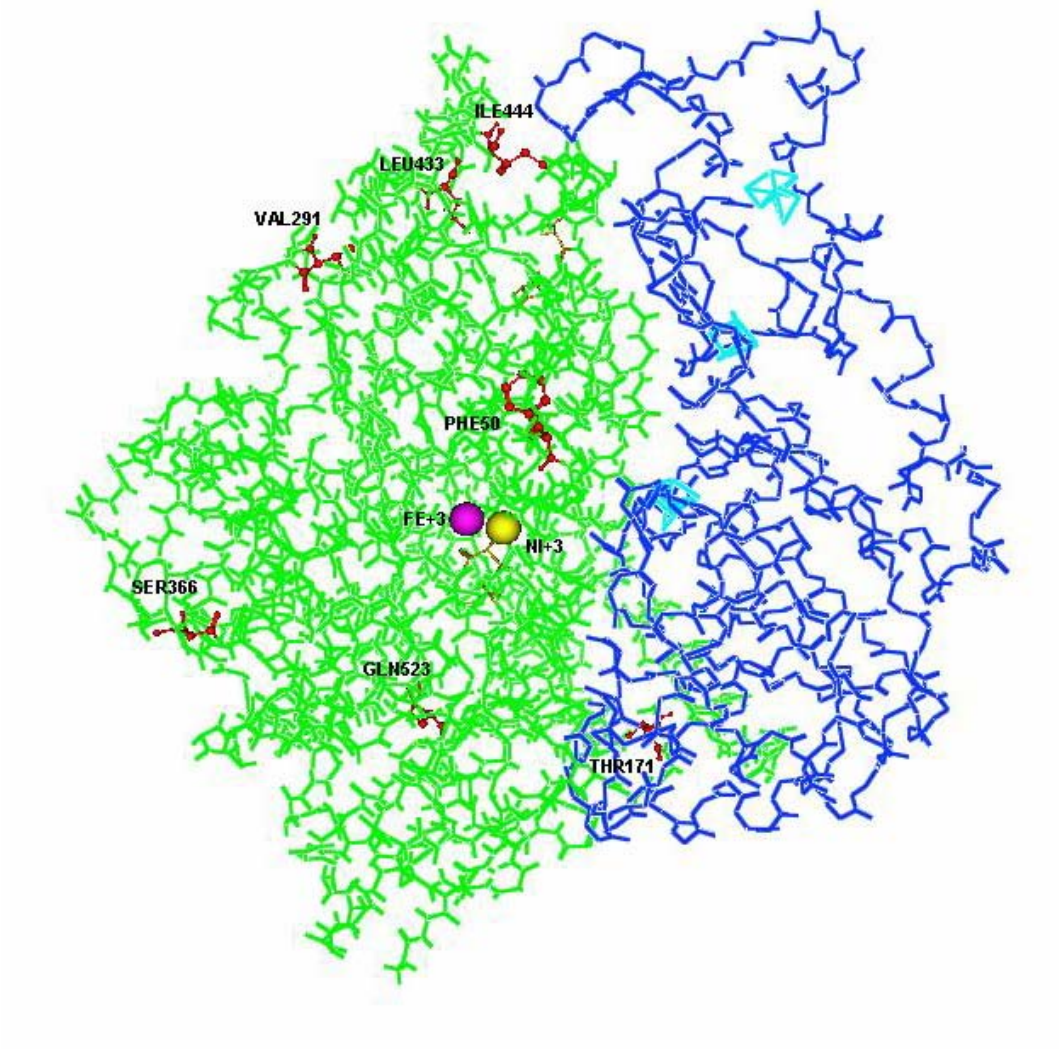

Fig. D.3. Protein modeling of the large subunit of hydrogenase 3, HycE (yellow) along with the small subunit HycG (blue). Mutations in epHycE95 are displayed in red (PHE50 for Y50F, THR171 for I171T, VAL291 for A291V, SER366 for T366S, LEU433 for V433L, ILE444 for M444I, and GLN523 for L523Q; S2T not shown). Metal cofactors Ni^{+} (yellow), Fe^{+3} (pink), and Fe-S clusters (blue) are indicated.

VITA

Name:	Viviana Sanchez Torres
Address:	Texas A&M University, Department of Chemical Engineering, 3122 TAMU, College Station, TX 77843-3122
Email:	viviana.sanchez@neo.tamu.edu
Education:	B.S., Chemical Engineering, Universidad Industrial de Santander, 2005
	Ph.D., Chemical Engineering, Texas A&M University, 2010