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Increased Hydrogen Production by Genetic Engineering of *Escherichia coli*

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**Abstract**

*Escherichia coli* is capable of producing hydrogen under anaerobic growth conditions. Formate is converted to hydrogen in the fermenting cell by the formate hydrogenlyase enzyme system. The specific hydrogen yield from glucose was improved by the modification of transcriptional regulators and metabolic enzymes involved in the dissimulation of pyruvate and formate. The engineered *E. coli* strains ZF1 (ΔfocA; disrupted in a formate transporter gene) and ZF3 (ΔnarL; disrupted in a global transcriptional regulator gene) produced 14.9, and 14.4 μmol of hydrogen/mg of dry cell weight, respectively, compared to 9.8 μmol of hydrogen/mg of dry cell weight generated by wild-type *E. coli* strain W3110. The molar yield of hydrogen for strain ZF3 was 0.96 mol of hydrogen/mol of glucose, compared to 0.54 mol of hydrogen/mol of glucose for the wild-type *E. coli* strain. The expression of the global transcriptional regulator protein FNR at levels above natural abundance had a synergistic effect on increasing the hydrogen yield in the ΔfocA genetic background. The modification of global transcriptional regulators to modulate the expression of multiple operons required for the biosynthesis of formate hydrogenlyase represents a practical approach to improve hydrogen production.

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**Introduction**

The present emphasis on the diversification of global energy sources, while reducing the consumption of fossil fuels, has placed renewed interest on hydrogen as an alternative fuel and its synthesis through microbe-mediated biocorversions. Microbes have evolved unique mechanisms for hydrogen generation, and some of these mechanisms are being explored for biotechnological applications including nitrogenase-mediated and fermentative hydrogen production [1]. Efforts to improve hydrogen production have been focused on pathway redirection, identification and engineering of oxygen-tolerant hydrogenases, improvement in hydrogen molar yields and development of efficient hydrogen separation techniques from bioreactor headspace [1–5].

The production of hydrogen by facultative anaerobic organisms such as *E. coli* is a characteristic of mixed acid fermentation. Under anaerobic conditions, the fermentation products comprise a mixture of ethanol, succinate, lactate, acetate, and formate (Fig. 1). Succinate is produced via the succinate pathway. A key reaction in this pathway is carboxylation of phosphoenolpyruvate (PEP) to the four-carbon compound oxaloacetate by phosphoenolpyruvate carboxylase (PEPC) [6]. PEP is also converted to pyruvate which is subsequently converted to acetyl-CoA and formate by pyruvate formate lyase (PFL), and to lactate by lactate dehydrogenase (LDH). Formate hydrogenlyase (FHL) catalyzes the conversion of formate to carbon dioxide and hydrogen [7,8]. The intracellular level of formate is determined by the rates of biosynthesis and metabolism of formate, and also by formate transporter FocA which is a membrane protein involved in formate transport into and out of the cell [9]. Knockout of the focA gene results in intracellular accumulation of formate [10]. The FHL enzyme complex is comprised of formate dehydrogenase H (FDH_H), and a hydrogen-evolving hydrogenase [hydrogenase 3] [11,12]. The biosynthesis of PFL and FHL are up-regulated by the action of multiple transcriptional regulators including the global transcriptional factors Fnr, ArcAB and integration host factor (IHF) [13–15], and repressed by the dual transcriptional regulator NarL (Table 1) [16]. The transcription of the fhl regulon is regulated by the primary and secondary transcriptional activator FHLA and ModE [17,18]. The expression of fhlA is activated by Fnr in response to the cellular redox state [19,20]. The biosynthesis of FDH_H also requires the expression of selC gene which encodes a tRNA for the incorporation of selenocysteine to FDH_H (Table 1) [8,11,21].

Successful efforts to redirect pyruvate towards hydrogen in *E. coli*, have involved the up-regulation of the FHL complex and the disruption of pathways competing for pyruvate [4,22–24]. It is estimated that over 50 genes distributed across at least 20 distinct genetic loci define fermentative hydrogen metabolism in *E. coli* [8]. In this study, our strategy involves the systematic modification of multiple, discrete, metabolic segments that include global regulatory, transport, and auxiliary components required for FHL biosynthesis, processing and assembly. The modification of key regulatory elements represents a practical strategy for the coordinated engineering of genes and operons that perform distinct biochemical functions related to the production of...
hydrogen. This approach has the potential to achieve the balance of cofactor and precursor supply pathways with the biosynthesis of structural polypeptides without placing an unnecessary metabolic burden on the cells [25]. Here, we describe increases in specific and molar yields of hydrogen achieved by the modification of the focA, ppc, narL, and fnr genes involved in precursor transport and metabolism, and the global regulation of fermentative hydrogen production by E. coli.

Results and Discussion

Strategy for genetic modification

The fermentation pathways initiate by the non-oxidative cleavage of pyruvate to acetyl-CoA and formate by PFL; formate is subsequently metabolized to hydrogen and carbon dioxide by FHL. Our strategy for improving fermentative hydrogen production involved the modification of precursor transport and metabolism, and of regulatory elements, to direct the flow of the two key precursor metabolites pyruvate and formate towards hydrogen, and achieve increased expression of both structural and auxiliary components of the FHL enzyme system. The biochemical reactions and regulatory elements that were modified by the inactivation of specific chromosomal genes, or by plasmid-directed gene overexpression are illustrated schematically in Figure 1; the functions of the corresponding gene products are summarized in Table 1.

The inactivation of the four selected genes in the chromosome of E. coli strain W3110 was hypothesized to increase the cellular concentrations of pyruvate and formate, reduce the re-oxidation of evolved hydrogen, and relieve the repression of genes required for PFL and FHL biosynthesis. The ppc gene encoding phosphoenolpyruvate carboxylase (PEPC) was disrupted to increase cellular pyruvate concentrations available for formate biosynthesis [6,7]. The gene encoding the formate transporter, focA, was inactivated to elevate intra-cellular formate levels for FHL biosynthesis and

![Figure 1. The genetic modification of metabolic pathways and regulatory components for hydrogen production in E. coli.](doi:10.1371/journal.pone.0004432.g001)

| Gene   | Function of gene product                                                                 |
|--------|------------------------------------------------------------------------------------------|
| focA   | Transmembrane protein involved in formate transport.                                     |
| hybC   | Large catalytic subunit of hydrogenase 2; catalyzes dihydrogen oxidation.                |
| narL   | Global transcriptional regulator of anaerobic metabolism; represses the transcription of nik operon genes, fdhF and pflB genes; activates the transcription of formate dehydrogenase N, which couples formate oxidation to nitrate reduction. |
| ppc    | Phosphoenolpyruvate carboxylase (PEPC) catalyzes the carboxylation of phosphoenolpyruvate (PEP) to oxaloacetate (OAA). |
| fnr    | Global transcriptional regulator of anaerobic metabolism; activator of fhlA and pflB genes, and of the hyp, nik and moa operon genes. |
| arcAB  | Global transcriptional regulator of aerobic and anaerobic metabolism; activator of fhlB and pflB genes, and repressor of the hyb and hya operon genes. |
| fhlAB  | Global transcriptional regulator activating the pflB and fhlA genes, and genes of the hyc and hyp operons. |
| modE   | Secondary transcriptional activator of the fhl regulon.                                   |
| selC   | tRNA for selenocysteine incorporation into FDH-F polypeptide of FHL.                     |

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conversion to hydrogen [10]. *E. coli* synthesizes two uptake hydrogenases, hydrogenase 1 and 2, which oxidize hydrogen to protons with the release of electrons. In this study the *hybC* gene, which encodes the large catalytic subunit of hydrogenase 2, was inactivated to reduce the oxidation of hydrogen [26]. The global transcriptional regulator NarL represses transcription of the structural genes of FHL (*fdhF*), PFL (*pflB*), and of the genes of the *nik* operon, which are required for the transport and metabolism of nickel for Hydrogenase cofactor biosynthesis [16]. To eliminate the repression described, the *narL* gene was inactivated.

The global transcriptional regulators Fnr, ArcAB and IhfAB activate multiple genes of anaerobic pyruvate and formate metabolism. Targets for these global regulators include *fhlA* (the specific transcriptional activator of the *fhl* regulon), *pflB*, and the genes of the *moa, nik* and *hyp* operons required for the biosynthesis of the molybdopterin and Ni-Fe cofactors of FHL (Table 1) [15,19,27,28]. The effects on hydrogen yield of multi-copy expression of the global regulators *fnr*, *arcAB* and *ihfAB*, as well as *ModE* (the secondary transcriptional activator of the *fhl* regulon), and the tRNA for selenocysteine incorporation (*selC*), were examined in wild-type W3110 and ΔfocA (ZF1) strains.

### Increased hydrogen yields by gene-inactivated *E. coli* strains

The rate of hydrogen production and of growth by wild-type strain W3110, and four strains containing specific chromosomal gene disruptions were examined (Figures 2A and 2B). The
maximum rate of hydrogen generation occurred between 9 and 16 hours following inoculation of M9 medium under the experimental conditions described. Strains ZF3 (W3110ΔnarL) and ZF4 (W3110Δppc) exhibited the highest rates of hydrogen production, and had accumulated about 20% to 30% more hydrogen in the culture headspace than W3110 in 25 hours (Figure 2A). Strain ZF1 (W3110ΔfocA) exhibited a slightly slower rate of hydrogen generation than W3110 and accumulated 33% less hydrogen by the end of the fermentation experiment. Strains ZF2 (W3110ΔhybC) and W3110 had comparable rates of hydrogen formation to each other. The slower rate of hydrogen generation observed in the later stages of fermentation correlated with an increased accumulation of biomass, possibly resulting from the hydrogen oxidizing activity of uptake hydrogenases 1, 2, and/or 4, generating protons and electrons for cellular ATP synthesis, or by inhibition of hydrogenase 3 by the evolved hydrogen that accumulated in culture headspace [8,26]. The complete consumption of glucose accompanied by reduced glycolytic flux to pyruvate and subsequently to formate could account for the simultaneous formation of glucose accompanied by reduced glycolytic flux to pyruvate accumulated in culture headspace [8,26]. The complete consumption of glucose accompanied by reduced glycolytic flux to pyruvate and subsequently to formate could account for the simultaneous reductions in growth and H2 production rates. Strains W3110, ZF3 (W3110ΔnarL) and ZF4 (W3110Δppc) exhibited similar generation times and reached similar final cell densities. Strain ZF2 (W3110ΔhybC) grew at a slightly slower rate than W3110, while strain ZF1 (W3110ΔfocA) grew at a slower rate than W3110 and reached a 50% lower final culture density (Figure 2B). The lower growth rates of strains ZF1 and ZF2 might result from the increased acidification of cellular cytoplasm from the disruption of formate export, and by the decrease in cellular ATP synthesis by the disruption of an uptake hydrogenase, respectively. The rate of anaerobic growth and of hydrogen production by the strains in TYP medium followed a profile similar to that seen in M9-glucose medium, with the highest rates of hydrogen production occurring between 4 and 7 hours of fermentation. The culture generation times were increased by about 2.5-fold in TYP medium, with A600 values reaching about 6.5 by 10 hours (data not shown).

The specific hydrogen yields were determined for W3110 and the gene-inactivated strains at 17 hours following inoculation of M9-glucose medium (Table 2). All four gene-inactivated strains exhibited 14% to 50% higher specific hydrogen yields compared to W3110. Strains ZF1 (W3110ΔfocA) and ZF3 (W3110ΔnarL) had specific hydrogen yields of approximately 14 μmol/mg dry cell mass, representing an increase of about 46% over the yield by W3110. The increased hydrogen yield by strain ZF1 (W3110ΔfocA) is consistent with the hypothesis that an increase in the level of intracellular formate can either lead to enhanced synthesis of the FHL polypeptides via the transcriptional activator FhA, or elevate the available substrate pool for FHL [29,30]. The negative effect on growth rates by the focA deletion might be alleviated if the intracellular formate can be efficiently metabolized by FHL (further described below). The 50% increase in specific hydrogen yield by strain ZF3 (W3110ΔnarL) showed that the elimination of transcriptional repression of nik operon, and of fhdF and pflB genes by the NarL, results in the increased pyruvate metabolism towards hydrogen [8,31].

The specific hydrogen yield of strain ZF2 (W3110ΔhybC) was 22% higher than W3110 (Table 2). Of the three E. coli uptake hydrogenases, hydrogenase 2 is believed to be the more active one based on in vitro kinetic studies [8]. The double mutant ZF5 (W3110ΔfocAΔhybC) did not produce more hydrogen than the single mutants ZF1 (W3110ΔfocA) and ZF2 (W3110ΔhybC) for reasons that remain unclear. Inactivation of the metabolic enzyme PEPC resulted in approximately 14% increase in specific hydrogen yields. All of the pyruvate is directed towards the PFL reaction the theoretical maximum yield of hydrogen from a hexose sugar is 2 mols of H2/mol glucose [7]. Strain ZF3 (W3110ΔnarL) also displayed the best molar yield of hydrogen (mols of H2/mol of glucose) of approximately 0.96 compared to 0.54 for W3110 (Table 2). The utility of eliminating transcriptional repression of multiple operons in anaerobic metabolism is highlighted by the effects of the narL gene deletion. This deletion confers a 1.5-fold increase in specific hydrogen yield and a 2-fold increase in molar hydrogen yield, while retaining the growth characteristics of the wild-type strain W3110.

To examine the partitioning of glucose in the two high hydrogen yielding strains the distribution of fermentation products of strains ZF1 (W3110ΔfocA) and ZF3 (W3110ΔnarL) were compared to that of W3110 (Table 3). These two strains carried out a balanced fermentation, with over 90% of the added glucose converted to products. No remaining glucose was detected in the medium by HPLC and spectrophotometric assays. Medium from

### Table 2. Hydrogen yields for strain W3110 with or without defined chromosomal gene disruptions.

| Strains          | Hydrogen yield (μmol/mg dry cell mass) | Hydrogen yield from glucose (mol/mol glucose) |
|------------------|---------------------------------------|---------------------------------------------|
| W3110            | 9.80±0.72                             | 0.54±0.04                                   |
| ZF1 (W3310ΔfocA) | 14.85±0.83                            | 0.63±0.04                                   |
| ZF2 (W3310ΔhybC) | 12.14±0.88                            | 0.70±0.05                                   |
| ZF3 (W3310ΔnarL) | 14.39±0.90                            | 0.96±0.07                                   |
| ZF4 (W3310Δppc)  | 11.20±0.89                            | 0.73±0.05                                   |
| ZF5 (W3310ΔfocAΔhybC) | 8.65±0.78                        | Not determined                              |

The hydrogen yields were determined following 17 hours of anaerobic growth on M9 medium containing 415 μmol of glucose.

| Strains          | Hydrogen (μmol) | Formate (μmol) | Lactate (μmol) | Acetate (μmol) | Succinate (μmol) | Ethanol (μmol) |
|------------------|----------------|---------------|----------------|----------------|------------------|---------------|
| W3110            | 91.0±1.34      | 114.24±1.32   | 158.92±1.47    | 106.36±1.17    | 41.48±0.76       | 340.40±2.87   |
| ZF1 (W3110ΔfocA) | 122.84±1.89    | 66.85±0.87    | 216.40±1.88    | 107.96±1.15    | 42.85±0.72       | 285.43±2.25   |
| ZF3 (W3110ΔnarL) | 118.0±1.42     | 112.83±1.26   | 159.66±1.21    | 110.14±1.15    | 43.46±0.81       | 229.0±1.87    |

The concentrations of metabolites in culture media were determined following 34 hours of anaerobic growth on M9 medium containing 830 μmol of glucose.

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strains W3110 and ZF1 (W3110ΔfocA) contained 40% less formate compared to strains W3110 and ZF3 (W3110ΔnarL), consistent with less formate being secreted from the cell due to the disruption of the channel for formate. Lactate formation was increased by about 34% in strain ZF1 (W3110ΔfocA) compared to W3110, perhaps from the efficient induction of ldhA due to lower intracellular pH from formate accumulation, or by the inhibition of PFL activity and/or biosynthesis by formate. The formation of ethanol was lowered by 17% and 33% in strains ZF1 (W3110ΔfocA) and ZF3 (W3110ΔnarL), respectively, compared to that of W3110. These two strains accumulated succinate and acetate to levels similar to those of strain W3110.

### The effect of multi-copy gene expression on hydrogen yields

Recent reports have described improvements in fermentative hydrogen yields in *E. coli* through the modification of the specific transcriptional activator, and repressor proteins of the FHL system, FhlA and HycA, respectively [22]. The three global regulators Fnr, ArcAB and IhfAB modulate the expression of multiple genes involved in the anaerobic metabolism of pyruvate in *E. coli* [9,15,19,27,28]. The impact on fermentative hydrogen yields by the over-production of the Fnr, ArcAB, and IhfAB proteins, and of ModE which is the secondary transcriptional activator of the hyc operon, was examined in strains W3110 and ZF1 (W3110ΔfocA). Strain ZF1 (W3110ΔfocA) was selected because an increased level of cellular formate, combined with the over-expression of formate-metabolizing genes might have a synergistic effect on hydrogen formation. The specific hydrogen yields following induction of the plasmid-bearing strains are shown in Table 4. Strain ZF7, over-expressing of the *fnr* gene in W3110 background, exhibited an approximately 5.5-fold increase in specific hydrogen yield compared to the strain control ZF6 (containing the plasmid without promoter-driven gene expression). Over-expression of the *fnr* gene in the Δ*focA* genetic background (strain ZF13) resulted in the generation of 10 μmol of H2/mg of dry cell mass compared to 6 μmol of H2/mg of dry cell mass obtained with over-expression of the *fhl* gene in the wild-type W3110 background (strain ZF7) in TYP medium. When induced in M9-glucose medium for 8 hours, strains ZF7 and ZF13 accumulated approximately 24 μmol of H2/mg of dry cell mass and 34 μmol of H2/mg of dry cell mass, respectively (data not shown). Over-expression of the global regulators ArcAB and IhfAB, and of the secondary transcriptional activator ModE, had no discernible effects on hydrogen yields in either W3110 or ZF1 strains in TYP or in M9-glucose medium. The synergistic effect on increased hydrogen yield by the over-expression of Fnr in the Δ*focA* genetic background suggests that genetic modifications to simultaneously increase both substrate and enzyme levels for hydrogen production are desirable to prevent the accumulation of formate inside the cell. These results show that specific genetic backgrounds provide additive effects on hydrogen yield when combined with increased gene dosage of particular metabolic enzymes or regulatory components.

While the Fnr protein activates transcription of operons involved in nitrogen and molybdenum metabolism for FHL biosynthesis [32], it has not been shown to affect the transcription of genes involved in selenium metabolism. To determine whether selenium metabolism is limiting to hydrogen production, the *selC* gene, encoding the tRNA for selenocysteine incorporation into the FDHH polypeptide, was over-expressed in strains W3110 and ZF1 (W3110ΔfocA). An approximately 63% increase in hydrogen yield was observed in strain ZF11 which expresses multiple copies of the *selC* gene compared to the negative control, strain ZF6 (Table 4). These results illustrate that hydrogen yields can be increased by improving the efficiency of a pathway for cofactor production for the FHL enzyme system.

One approach to obtaining sustained increases in fermentative hydrogen-yields will be to direct and improve the metabolism of pyruvate and formate towards hydrogen under anaerobic conditions. Genetic modifications that have led to increased hydrogen yields in *E. coli* have thus far focused on the disruption of key metabolic genes of the mixed acid fermentation pathways of *E. coli*, and of specific regulators of the FHL system. This report represents the first example of increased hydrogen production achieved through the modification of two global transcriptional regulators, a cofactor biosynthetic gene, and a transporter for formate. The construction of *E. coli* strains with specific chromosomal gene deletions, have revealed three unique genetic backgrounds that improve fermentative hydrogen production: inactivation of the *focA*, *narL*, and *ppg* genes lead to increased hydrogen yields over that of wild-type *E. coli*. The metabolism of selenium is one of several metabolic pathways required for the processing of metal ions for FHL biosynthesis. The improvement in hydrogen yield observed by the increased expression of the tRNA for selenocysteine suggests that cofactor biosynthesis pathways could be useful targets for modification. The increased hydrogen yields obtained by over-expression of the *fjr* gene, indicates that the anaerobic metabolism of pyruvate and formate can be coordinately up-regulated for improved flux towards hydrogen.

The results presented in this work illustrate the utility of modifying transport, regulatory, and auxiliary pathways related to FHL biosynthesis and function, to achieve increased hydrogen yields. The future application of genome engineering formats in combination with global gene expression analysis to *E. coli* will enable the identification of novel genes and metabolic functions underlying the improved hydrogen producing phenotype [23,33]. Similar approaches might be extended to enteric microbes that contain the FHL enzyme system to improve hydrogen production from alternate carbon sources, and for the simultaneous production of value-added chemical entities derived from the anaerobic metabolism of pyruvate.

### Table 4. Hydrogen yields for strain W3110 and ZF1 (W3110ΔfocA) when expressing plasmid-borne genes.

| Strain | Genotype | Hydrogen yield (μmol/mg dry cell mass) |
|--------|----------|----------------------------------------|
| ZF6    | W3110/pACYC177L | 0.07±0.03 |
| ZF7    | W3110/fnr | 0.63±0.45 |
| ZF8    | W3110/parcAB | 1.11±0.04 |
| ZF9    | W3110/pihfAB | 1.14±0.04 |
| ZF10   | W3110/pmodE | 1.27±0.06 |
| ZF11   | W3110/pseiC | 1.81±0.06 |
| ZF12   | W3110/ΔfocA/pACYC177L | 0.00±0.00 |
| ZF13   | W3110/ΔfocA/pfnr | 10.32±0.58 |
| ZF14   | W3110/ΔfocA/parcAB | 0.19±0.02 |
| ZF15   | W3110/ΔfocA/pihfAB | 0.61±0.03 |
| ZF16   | W3110/ΔfocA/pmodE | 0.63±0.03 |
| ZF17   | W3110/ΔfocA/pseiC | 1.19±0.06 |

The specific hydrogen yields were determined following 4 hours of induction on TYP medium. TYP medium was supplemented with glucose at a final concentration of 0.5% (w/v) for this experiment.

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Materials and Methods

Genetic manipulations

All strains and plasmids used in this study are listed in Table 5, and PCR primers used are shown in Table 6. The disruption of chromosomal genes in E. coli K-12 strain W3110 was performed by the method of Datsenko and Wanner [34]. Plasmid pKD13 was used as the template for amplification of the FRT-flanked KmR gene with primers containing 44 to 50 nucleotide homology extensions. Plasmids pKD46 and pCP20 were used for the replacement of the target gene with the KmR gene, and for subsequent KmR gene removal, respectively. Primer pairs, from genomic DNA of polymerase (Invitrogen) to produce blunt ended PCR products, were amplified with primer pairs: for-FW/for-RV, modE-FW/modE-RV and selC-FW/selC-RV, respectively, digested with PstI and FspI, and ligated into the corresponding sites of pACYC177L. For the construction of parcAB and pihfAB, primer Plac-Sol-RV was used instead of primer Plac-Pst-RV for the amplification of the lacZ promotor, and SolI was used for digestion of the PCR fragment. This modified pACYC177 vector was designated as pACYC177L. The parcAB and pihfAB genes were amplified using primer pairs parcAB-FW/parcAB-RV and pihfAB-FW/pihfAB-RV, respectively. The two forward primers contained a 40-nucleotide linker sequence from genomic DNA of W3110. The PCR products were digested with BglI and ligated to the FspI/BglI site of pACYC177L1. Finally, the parcAB and pihfAB genes were amplified using primer pairs parcAB-FW/parcAB-RV and pihfAB-FW/pihfAB-RV, digested with XhoI, and ligated to SolI/XhoI site of pACYC177L1.

Bacterial strains, media, and growth conditions

All strains of E. coli used in this study are listed in Table 5. E. coli strains were routinely cultured at 37°C in either phosphate-buffered rich medium TYP (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) or minimal medium M9 (10 g/L Na2HPO4, 5 g/L NH4Cl, 10 g/L NaCl) supplemented with antibiotics, if required.

Table 5. E. coli strains and plasmids used in this study.

| Strains and plasmids | Genotype or description | Reference or source |
|----------------------|-------------------------|---------------------|
| Strains              |                         |                     |
| W3110                | F-::NdisrrmA 1 rph-1     | CGSC# 4470          |
| ZF1                  | W3110ΔfocA              | This work           |
| ZF2                  | W3110ΔhybC              | This work           |
| ZF3                  | W3110ΔnarL              | This work           |
| ZF4                  | W3110Δppc               | This work           |
| ZF5                  | W3110ΔfocAΔhybC         | This work           |
| ZF6                  | W3110 containing pACYC177L | This work       |
| ZF7                  | W3110 containing pfir   | This work           |
| ZF8                  | W3110 containing parcAB | This work           |
| ZF9                  | W3110 containing pihfAB | This work           |
| ZF10                 | W3110 containing pmodE  | This work           |
| ZF11                 | W3110 containing selC   | This work           |
| ZF12                 | ZF1 containing pACYC177L| This work           |
| ZF13                 | ZF1 containing pfir     | This work           |
| ZF14                 | ZF1 containing parcAB   | This work           |
| ZF15                 | ZF1 containing pihfAB   | This work           |
| ZF16                 | ZF1 containing pmodE    | This work           |
| ZF17                 | ZF1 containing pselC    | This work           |
| Plasmids             |                         |                     |
| pfir                 | pACYC with LacZ promotor and W3110 fmr gene | This work       |
| parcAB               | pACYC with LacZ promotor and W3110 arcA and arcB genes | This work       |
| pihfAB               | pACYC with LacZ promotor and W3110 ihfA and ihfB genes | This work       |
| pmodE                | pACYC with LacZ promotor and W3110 ihfA and ihfB genes | This work       |
| pselC                | pACYC with LacZ promotor and W3110 selC gene | This work       |

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Fermentations were carried out in 37 ml serum vials (Wheaton) containing 15 ml of TYP or M9 medium. Following the addition of medium, the vials were sealed using silicone stoppers and evacuated with Argon. Following the addition of inoculum culture, prepared under aerobic conditions in TYP, was used to inoculate fermentation medium. Specific hydrogen yields were determined at approximately 5 hours, and 17 hours in TYP and M9 medium, respectively. Fermentations in 10 ml of M9 medium containing 0.5% glucose (w/v) were run for 17 hours to determine the molar yields of hydrogen. The concentrations of all products generated from glucose were determined for strains cultured anaerobically for 54 hours in 15 ml of M9 medium containing 1.0% glucose (w/v) to allow adequate time for the consumption of all of the glucose contained in the medium. All experiments were repeated six times with at least duplicate fermentations contained within each experiment.

The concentrations of all products generated from glucose were determined for strains cultured anaerobically for 54 hours in 15 ml of M9 medium containing 1.0% glucose (w/v) to allow adequate time for the consumption of all of the glucose contained in the medium. All experiments were repeated six times with at least duplicate fermentations contained within each experiment. The ΔA00 for strains cultured anaerobically in M9 medium was measured by removing samples anaerobically with a syringe in intervals for 34 hours.

### Table 6. Primers used in this study.

| Primer     | Sequence                                                                 | Source               |
|------------|--------------------------------------------------------------------------|----------------------|
| focA-H2P4  | 5'-CTTGTAGTATCTGTGACCTAAATAGGAGAGTTAGATTCCGGGGATCGACC-3'                  | This work            |
| focA-H1P1  | 5'-GATCTGATCCTGACCCAT-3'                                                 | This work            |
| focA-FW    | 5'-GCCCAGGAGATATGCTA-3'                                                 | This work            |
| focA-RV    | 5'-GTCCTGAGCTGACCCAT-3'                                                 | This work            |
| hybC-H2P4  | 5'-CTTAAAACACCAACTTACATCCGTAGGACGCAAAAGCAGCATGACATCCGGGATCGACC-3'         | This work            |
| hybC-H1P1  | 5'-CTTAAAACACCAACTTACATCCGTAGGACGCAAAAGCAGCATGACATCCGGGATCGACC-3'         | This work            |
| hybC-FW    | 5'-GTCGCAAGCTGACCCAT-3'                                                 | This work            |
| hybC-RV    | 5'-CAACCGGGAAACCTTCTCTCA-3'                                             | This work            |
| nar-H2P4   | 5'-CACATTATGTTAAGGTATGTTCATTCAAAGCCTGAAGGAAGTTACATTCCGGGATCGACC-3'        | This work            |
| nar-H1P1   | 5'-TTCCGAGATGTCGGAATAGTTGGAATAATTTGAACTTAACAGAGGATGAGCCCTGGTCCCTGAAT-3'  | This work            |
| nar-FW     | 5'-GCCTAAAGCAGGAAATGCTA-3'                                               | This work            |
| nar-RV     | 5'-GACTCGGCCGAGCTCAAACCT-3'                                             | This work            |
| ppc-H2P4   | 5'-CTGTAAGGAGGATTACGGCCATCGAAAGTACAGGGTTCGCTCTGATGAGAGCTGGGACCTCA-3'      | This work            |
| ppc-H1P1   | 5'-ATTTCGATCGGAGGATTACGGCCATCGAAAGTACAGGGTTCGCTCTGATGAGAGCTGGGACCTCA-3'  | This work            |
| ppc-FW     | 5'-CCGATCTTATCCGACTACA-3'                                                | This work            |
| ppc-RV     | 5'-TGCGCTGAGCTGACCCAT-3'                                                | This work            |
| Plac-BamH-FW | 5'-ACATGGATCCTGGGACCTGAAAACTGCAAAC-3'                                     | This work            |
| Plac-Pst-RV | 5'-ACATCTGAGCTGACCCATCGAAACCTTCTC-3'                                     | This work            |
| Plac-Scl-RV | 5'-ACATAGTACTGATCCTTCATTGAAAT-3'                                        | This work            |
| Fnr-FW     | 5'-ACTACTGAGATGTCGGAATGCTGAAAT-3'                                       | This work            |
| Fnr-RV     | 5'-ACTACTGAGATGTCGGAATGCTGAAAT-3'                                       | This work            |
| modE-RV    | 5'-ACTACTGAGATGTCGGAATGCTGAAAT-3'                                       | This work            |
| selC-FW    | 5'-ACTACTGAGATGTCGGAATGCTGAAAT-3'                                       | This work            |
| selC-RV    | 5'-ACTACTGAGATGTCGGAATGCTGAAAT-3'                                       | This work            |
| archB-Xbal-FW | 5'-TCTGATAAGGCGGGAGGATTACATCAGTATGAGAACCTGGGATTCCGGCTCA-3'              | This work            |
| archB-Xbal-RV | 5'-ATCTAGGCGGAAGGCTCTAATTCTATTGGCTTTTGACCC-3'                           | This work            |
| archC-FW   | 5'-ATGCGAACCCTGCTACT-3'                                                 | This work            |
| archC-Xbal-RV | 5'-ACTCTCGATGATCTCCTCAGATCCCGCA-3'                                     | This work            |
| archC-Xbal-RV | 5'-ACTCTCGATGATCTCCTCAGATCCCGCA-3'                                     | This work            |
| ihfB-Xbal-FW | 5'-TCTGATAAGGCGGGAGGATTACATCAGTATGAGAACCTGGGATTCCGGCTCA-3'              | This work            |
| ihfB-Xbal-RV | 5'-ATCTAGGCGGAAGGCTCTAATTCTATTGGCGGC-3'                                 | This work            |
| ihfA-FW    | 5'-ATGCGCATTACAAAGCTCA-3'                                               | This work            |
| ihfA-Xbal-RV | 5'-ACTCTCGATGATCTCCTATTGGCGGA-3'                                       | This work            |

12 g/L K₂HPO₄, 3 g/L KH₂PO₄, pH 7.6) or in M9 salts medium (Sigma-Aldrich) containing trace elements [35,36]. When required, kanamycin was added at a concentration of 30 µg/ml. For studies requiring plasmid-directed gene expression, 1 mM IPTG was added to the cultures 1 hour following inoculation of the fermentation medium, and induction was performed for approximately 4 hours.
Analytical methods

The gas mixture from culture headspace was withdrawn using gastight syringes (Hamilton), and monitored for hydrogen formation. Hydrogen was detected using a Shimadzu GC-17A gas chromatograph (GC) equipped with a thermal conductivity detector (TCD), a 60/60 Molecule Sieve 5A column (Supelco), with argon as the carrier gas. The Clarity software package (DataApex) was used to analyze the chromatograms.

The formation of fermentation products were detected by high performance liquid chromatography (HPLC) using an Aminex HPX-87H ion exchange column (Bio-Rad; 7.8×300 mm) and a VydacProStar 410 liquid chromatography instrument equipped with UV absorbance and refractive index detectors. Culture supernatant was filtered through a 0.45 μm cellulose acetate filters, and 40 μL of sample was injected. The column was eluted isocratically with 5 mM H2SO4 at a flow rate of 0.5 mL/minute. Data was analyzed using the Star Chromatography Workstation software. Ethanol was quantified using the Quantochrome assay kit (BioAssay Systems). Glucose was quantified by both HPLC (described above), and by the glucose oxidase (GO) assay kit (Sigma).

Dry cell mass was determined by filtration of the entire culture through a 0.8 μm nitrocellulose membrane, drying the membrane at 60°C in a vacuum oven for about 14 hours, and determining the weight of the membrane.

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Author Contributions

Conceived and designed the experiments: ZF LY RC. Performed the experiments: ZF RC. Analyzed the data: ZF LY RC. Contributed reagents/materials/analysis tools: ZF RC. Wrote the paper: RC. Revised manuscript drafts: ZF. Revised manuscript: LY.

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Conceived and designed the experiments: ZF LY RC. Performed the experiments: ZF RC. Analyzed the data: ZF LY RC. Contributed reagents/materials/analysis tools: ZF RC. Wrote the paper: RC. Revised manuscript drafts: ZF. Revised manuscript: LY.

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