

**ENHANCED HYDROGEN PRODUCTION IN *ESCHERICHIA COLI* THROUGH
CHEMICAL MUTAGENESIS, GENE DELETION, AND TRANSPOSON
MUTAGENESIS**

A Thesis

by

ANDREA JULIANA GARZON SANABRIA

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

MASTER OF SCIENCE

May 2010

Major Subject: Chemical Engineering

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ABSTRACT

Enhanced Hydrogen Production in *Escherichia coli* Through Chemical Mutagenesis, Gene Deletion, and Transposon Mutagenesis. (May 2010)

Andrea Juliana Garzon Sanabria, B.S., Universidad Industrial de Santander at Bucaramanga

Chair of Advisory Committee: Dr. Thomas K. Wood

We demonstrate that hydrogen production can be increased by random mutagenesis using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and that hydrogen production can be further increased in the chemically-mutagenized strain by targeted gene deletion and overexpression of genes related to formate metabolism. Chemical mutagenesis of *Escherichia coli* BW25113 *hyaB hybC hycE::kan/pBS(Kan)-HycE* to form strain 3/86 resulted in 109 ± 0.5 -fold more hydrogen; 3/86 lacks functional hydrogen uptake hydrogenases 1 and 2, has hydrogen-producing hydrogenase 3 inactivated from the chromosome, and has constitutively active hydrogenase 3 based on expression of the large subunit of hydrogenase 3 from a high copy number plasmid. Deleting *fdoG*, which encodes formate dehydrogenase O, (that diverts formate from hydrogen), from chemical mutagen 3/86 increased hydrogen production 188 ± 0.50 -fold (relative to the unmutagenized strain), and deletion of *hycA*, which encodes the repressor of formate hydrogen lyase (FHL), increased hydrogen production 232 ± 0.50 -fold. Deleting both *fdoG* and *hycA* increased hydrogen production 257 ± 0.50 -fold, and overexpressing *fhIA* along with the *fdoG hycA* mutations increased hydrogen 308 ± 0.52 -fold. Whole-transcriptome analysis of chemical mutagen 3/86 revealed 89 genes were induced and 31 genes were repressed. In an effort to identify chromosomal mutations in chemical mutagen 3/86, we performed comparative genome sequencing and identified two chromosomal loci with mutations in coding

regions of *ftnA* and *yebJ*; however, neither gene was related to the increased hydrogen production as determined by the close vial (short) hydrogen assay.

In addition, transposon mutagenesis, which is one of the most efficient strategies for creating random mutations in the genomic DNA, was performed in two different strains: *E. coli* BW25113 *hyaB hybC hycA fdoG::kan/pCA24N-FhlA* and *E. coli* MG1655 to identify beneficial mutations for hydrogen production. As a result of screening 461 *E. coli* BW25113 *hyaB hybC hycA fdoG::kan/pCA24N-FhlA* transformants and 1000 *E. coli* MG1655 transformants, three interesting mutations have been discovered in *E. coli* BW25113 *hyaB hybC hycA fdoG::kan/pCA24N-FhlA* transformants (*gpsA*, *dipZ*, *glgP*) and 1 beneficial mutation in *E. coli* MG1655 transformants (*malT*). When any of these genes *gpsA*, *dipZ*, or *glgP* is disrupted by Tn5 insertion, hydrogen production decreases 17, 3 and 8-fold, respectively. Additionally, when *malT* gene is disrupted by Tn5 insertion, hydrogen increases 3.4-fold.

DEDICATION

To my mother, father, sister and husband for their unconditional love and support

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I want to profoundly thank my supervisor, Dr. Thomas K. Wood, who guided me during all this time to achieve this academic goal and my committee members, Dr. Arul Jayaraman and Dr. Kung-Hui (Bella) Chu, for being part of this endeavor.

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CHAPTER I

INTRODUCTION

Hydrogen has tremendous potential as an alternative fuel because of the limited availability of fossil fuel resources (13). Energy produced from hydrogen is safe (13) and renewable if produced from renewable feed stocks (20). In addition, hydrogen production by biological methods should reduce energy costs as these processes do not involve extensive heating or electricity as in electrolysis plants (13) since standard conditions are required for biohydrogen synthesis (60). Furthermore, hydrogen produced by microorganisms does not release hazardous compounds such as carbon monoxide which is known to cause damage to the fuel cells electrodes (6, 60); therefore, in the future, biohydrogen could play an important role as a new energy source and replace batteries and fuels that when are burned releases hazardous compounds to the environmental media.

E. coli is a facultative anaerobe that produces hydrogen fermentatively using formate (4, 31, 60, 61) and glucose (28, 59) as substrates. Hydrogen production by *E. coli* can be enhanced by knocking out genes involved in hydrogen uptake or by overexpressing beneficial genes involved in hydrogen synthesis. For example, deletion of *hyaB* and *hybC* that are uptake hydrogenases (Hyd-1 and 2 respectively) resulted in increased hydrogen production (32). In contrast, overexpression of the formate hydrogen lyase (FHL) complex through the activator gene *fhlA* resulted in increased hydrogen production (30, 31, 60, 61).

This thesis follows the style of Applied and Environmental Microbiology.

The engineered *E. coli* *hyaB hybC hycE::kan/pBS(Kan)-HycE* strain (32) was the starting point of the work presented here. Chemical mutagenesis and transposon mutagenesis are two innovative strategies, to engineer *E. coli* bacteria in order to enhance hydrogen production, that have not been used in previous studies. We performed random mutagenesis, targeted gene deletion of interesting genes found in previous studies (31) , and transposon mutagenesis to find novel genes and metabolic pathways related to hydrogen production.

This study is oriented towards making biological hydrogen production from *E. coli* a more efficient energy source; to accomplish this objective, three main goals have been proposed: (i) to enhance hydrogen production by *E. coli* using a combination of genetic manipulation approaches such as chemical mutagenesis, targeted gene deletions and transposon mutagenesis; (ii) to identify novel regulators of hydrogen production by *E. coli* and new metabolic pathways by conducting a whole transcriptome analysis of interesting mutants obtained after chemical mutagenesis; and (iii) to engineer *E. coli* bacteria by overexpressing beneficial genes and deleting deleterious genes to enhance hydrogen production.

CHAPTER II

LITERATURE SEARCH

Escherichia coli produces hydrogen from formate under anaerobic conditions through the reaction $HCOO^- + H_2O \longleftrightarrow H_2 + HCO_3^-$, which is catalyzed by the formate hydrogenlyase (FHL) enzymatic complex (47, 58); this FHL complex is required to achieve formate conversion into hydrogen and carbon dioxide (6, 60).

The FHL enzymatic complex is formed by formate dehydrogenase-H encoded by *fdhF* and hydrogenase 3 encoded by *hycG* (small subunit) and *hycE* (large subunit) (49). Formate dehydrogenase-H is responsible for the formation of $2H^+$, $2e^-$, and CO_2 from formate, while hydrogenase 3 is responsible for the synthesis of hydrogen from $2H^+$ and $2e^-$ (48). FHL has two known regulators, FhlA and HycA (50). FhlA transcription is controlled by P_{hyp} (FhlA-dependent promoter) and by an FNR-dependent placed between *hypA* and its own constitutive promoter (45) (Figure 2-1). Moreover, in order to fully activate the FHL complex, it also requires several proteins such as, the electron transfer proteins encoded by *hycBCDF*, which has been considered to carry electrons between FdhF and Hyd-3 (49), HycI protease (44), HydN (putative electron carrier), and the maturation proteins HycH (48), HypF (34) and HypABCDE (24) (Figure 2-1).

Transcription of the formate regulon starts at -12/-24 promoters through the σ_{54} transcriptional activator FhlA (28). FhlA protein requires formate (47) to bind to the upstream activating sequence (UAS) located 100 bp upstream from the starting site of transcription, where it interacts with the RNA polymerase- σ_{54} complex in order to initiate the transcription of the genes that FhlA regulates (45, 47). Those genes are *fdhF* (7), *hydN-hypF* operon (34, 47), *hyc*

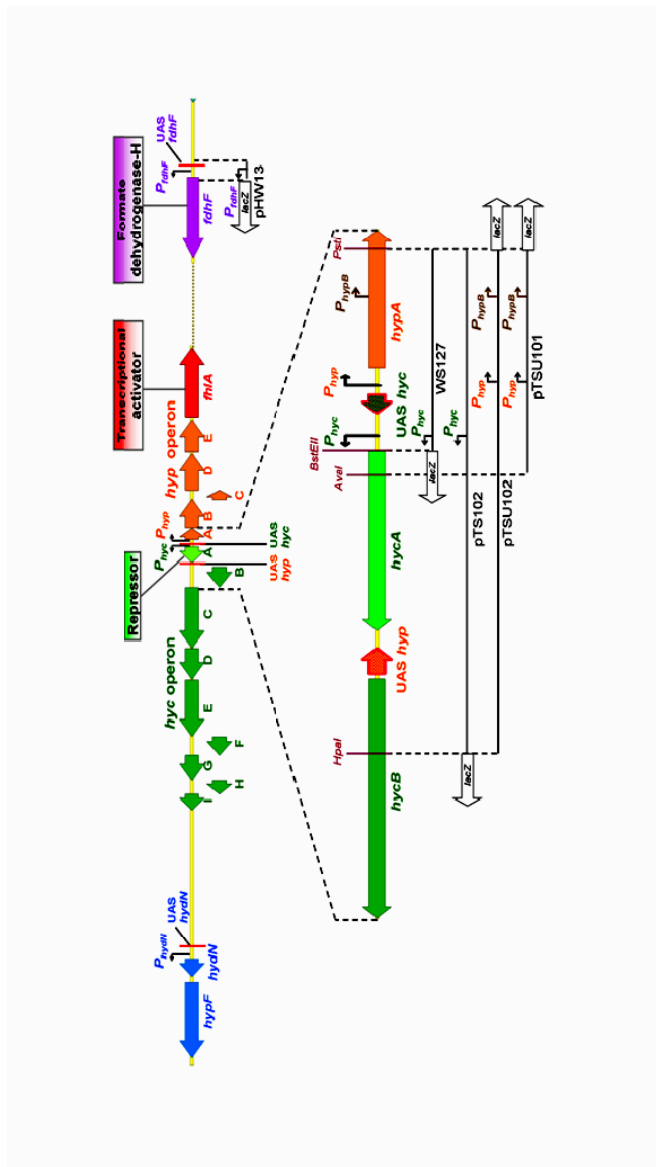


Figure 2-1. Physical map of the transcriptional units activated by FhlA. Codig regions are represented by block arrows, -12/-24 promoters are indicated by black right-angled arrows, the FNR-dependent promoter P_{hypB} is indicated by brown right-angled arrows, and the UAS where FhlA binds (8, 34, 51) 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) (47).

and *hyp* operon (51). FhlA protein binds specifically to the upstream regulatory sequence of the *fdhF* gene (51) and *hydN-hypF* (34, 51) operon, between the *hycA* and *hybB* region, and between the intergenic region of the divergently transcribed *hyc* and *hyp* operon (47, 51) (Figure. 2.1). In addition, the transcription of *fdhF* and the *hyc* operon is also controlled by the accessibility of molybdate (43).

FhlA is a member of the σ_{54} family. It contains three different functional domains (37): the N-terminal domain, the central domain and the C-terminal domain. The N-terminal domain (amino acids 1 to 378) binds formate as a tetramer (47); the central domain (amino acids 379 to 617) is active in ATP hydrolysis. The ATP hydrolysis occurs once formate is bound to the N-domain; this reaction is essential to form the open structure of the complex of RNA polymerase with the DNA, since it activates transcription (23). The C terminal domain (amino acids 918 to 692) (37) forms a helix-turn-helix for DNA binding (37).

Metabolic engineering and genetic manipulation have been powerful tools to enhance hydrogen production in *E. coli* (29, 30, 33, 39, 40, 61). For instance, a significant improvement in H₂ synthesis from *E. coli* by applying a variety of strategies has been accomplished such as *E. coli* BW25113 *hyaB hybC hycA fdoG::kan/pCA24N-FhlA* that is a metabolically-engineered bacterium created in our research lab, which increases hydrogen production from formate 141-fold (31). *E. coli* BW25113 *hyaB hybC hycA fdoG::kan/pCA24N-FhlA* is characterized by the deletion of hydrogenases 1 and 2 (*hyaB* and *hybC* respectively), *hycA*, *fdoG* and overexpression of *fhlA*. Inactivation of hydrogenases 1 and 2 aids to get rid of hydrogen uptake activity (31). Deletion of *hycA* inhibits the repression of formate hydrogen lyase system (FHL) (9, 60). Deletion of *fdoG* is beneficial because FdoG protein diverts formate from hydrogen and overexpression of *fhlA* that activates and regulates genes involved in the FHL regulon (28).

In addition, the large subunit hydrogenase 3 which has uptake and hydrogen synthesis

activities from formate has been studied in order to find beneficial mutation sites to improve hydrogen productivity (32); this work represents the first protein engineering for hydrogen production. Protein engineering of HycE (hydrogenase 3) was conducted through error-prone polymerase chain reaction (epPCR), deoxyribonucleic acid (DNA) shuffling and saturation mutagenesis strategies (32). As a result, from epPCR of *E. coli* BW25113 *hyaB hybC hycE/pBS(Kan)-HycE*, seven enhanced HycE variants were obtained (32). DNA shuffling of the three most active HycE variants obtained from epPCR was conducted and a new strain (*hyaB hybC hycE/pBS(Kan)-shufHycE1-9*) that increased hydrogen production by 23-fold was created (32). Lastly, saturation mutagenesis of *hyaB hybC hycE/pBS(Kan)-shufHycE1-9* was achieved, and the new strain *hyaB hybC hycE/pBS(Kan)-satHycE12366* increases hydrogen synthesis 30-fold (32).

Glycolysis may be used to synthesize hydrogen via microbial fermentation. This glycolysis pathway can be divided into three main steps (7). The first step is the fermentation of glucose, which involves the phosphorylation of glucose to form glucose-6-phosphate (G-6P) by hexokinase; subsequently, G-6P is further converted into fructose-6-phosphate through phosphofructokinase, and to fructose-1, 6-bisphosphate by aldolase enzyme (Figure 2-2). The second stage involves the cleavage of six-carbon fructose to form two three-carbon molecules. In the last stage, ATP generation occurs; two molecules of pyruvate are released. Pyruvate is the precursor of formate which under anaerobic conditions can be converted to hydrogen and carbon dioxide by the FHL (Figure 2-2).

In addition to glucose, fructose can also enter the glycolysis pathway through fructose 1-phosphate, which is further converted into glyceraldehyde and dihydroxyacetone. Glyceraldehyde is converted to glyceraldehydes 3-phosphate (Figure 2-3) (7). Once glyceraldehyde 3-phosphate has been formed, it can continue through the third step of the

glycolysis pathway to form pyruvate synthesis which may be used to form hydrogen (Figure 2-2).

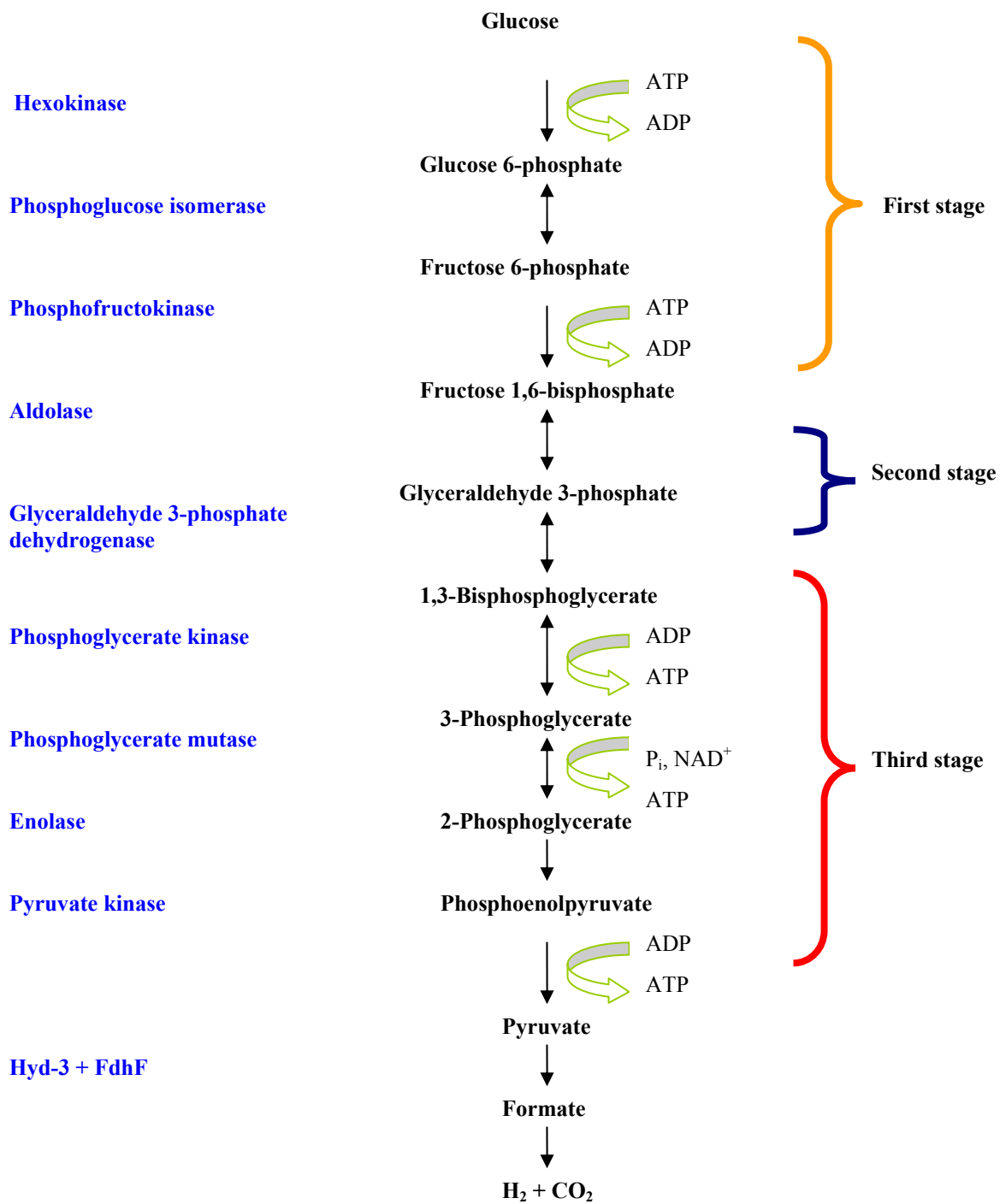


Figure 2-2. Glucose metabolic pathway addressed towards hydrogen and carbon dioxide synthesis.

Taken from (7) with modifications.

CHAPTER III

CHEMICAL MUTAGENESIS AND TARGETED GENE DELETION OF *E.coli*

FOR ENHANCED HYDROGEN PRODUCTION

This chapter is going to be published as “Enhanced Hydrogen Production in *Escherichia coli* Through Chemical Mutagenesis and Gene Deletion”. Dr. Uma Sagaram performed P1 transduction to strain 3/86 to remove deleterious (*fdoG*, and *hycA*) genes, added a plasmid for overexpression of beneficial gene *fhlA*, performed HycE protein sequencing, and performed *E. coli* GeneChip antisense genome array to find the location of mutations in strain 3/86 chemical mutagen. I created the 3/86 chemical mutagen, conducted a whole transcriptome analysis of 3/86 to analyze gene expression, performed mass spectrometry to identify new proteins only in 3/86 (not present in the control strain), performed P1 transduction of *fdoG* gene, and investigated the *gadA* contribution to hydrogen synthesis.

3.1 Overview

Hydrogen has tremendous potential as an alternative fuel, and previously we used protein engineering to evolve hydrogenase 3 from *E. coli* to increase hydrogen production. Here, we demonstrate that hydrogen production can be increased by random mutagenesis using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and that the hydrogen production can be further increased in the chemically mutagenized strain by targeted gene deletion and overexpression of genes related to formate metabolism. Chemical mutagenesis of BW25113 *hyaB hybC hycE::kan/pBS(Kan)-HycE* to form strain 3/86 resulted in 109 ± 0.52 -fold more hydrogen; this strain lacks functional hydrogen uptake hydrogenases 1 and 2, has hydrogen-producing hydrogenase 3 inactivated from the chromosome, and has constitutively active hydrogenase 3 based on expression of the large subunit of hydrogenase 3 from a high copy number plasmid. Deleting *fdoG*, which encodes formate dehydrogenase O, (that diverts formate from hydrogen), from chemical mutagen 3/86 increased hydrogen production 188 ± 0.50 -fold, and deletion of *hycA*, which encodes the repressor of formate hydrogen lyase (FHL), increased hydrogen production 232 ± 0.50 -fold. Deleting both *fdoG* and *hycA* increased hydrogen production 257 ± 0.50 -fold, and overexpressing *fhlA* along with the *fdoG hycA* mutations increased hydrogen 308 ± 0.52 -fold (Figure 3-1). Whole-transcriptome analysis of chemical mutagen 3/86 revealed 89 genes induced and 31 genes repressed. In an effort to identify chromosomal mutations in chemical mutagen 3/86, we performed comparative genome sequencing and identified two chromosomal loci with mutations in coding regions of *ftnA* and *yebJ*; however, neither gene was related to the increased hydrogen production as determined by the close vial (short) hydrogen assay.

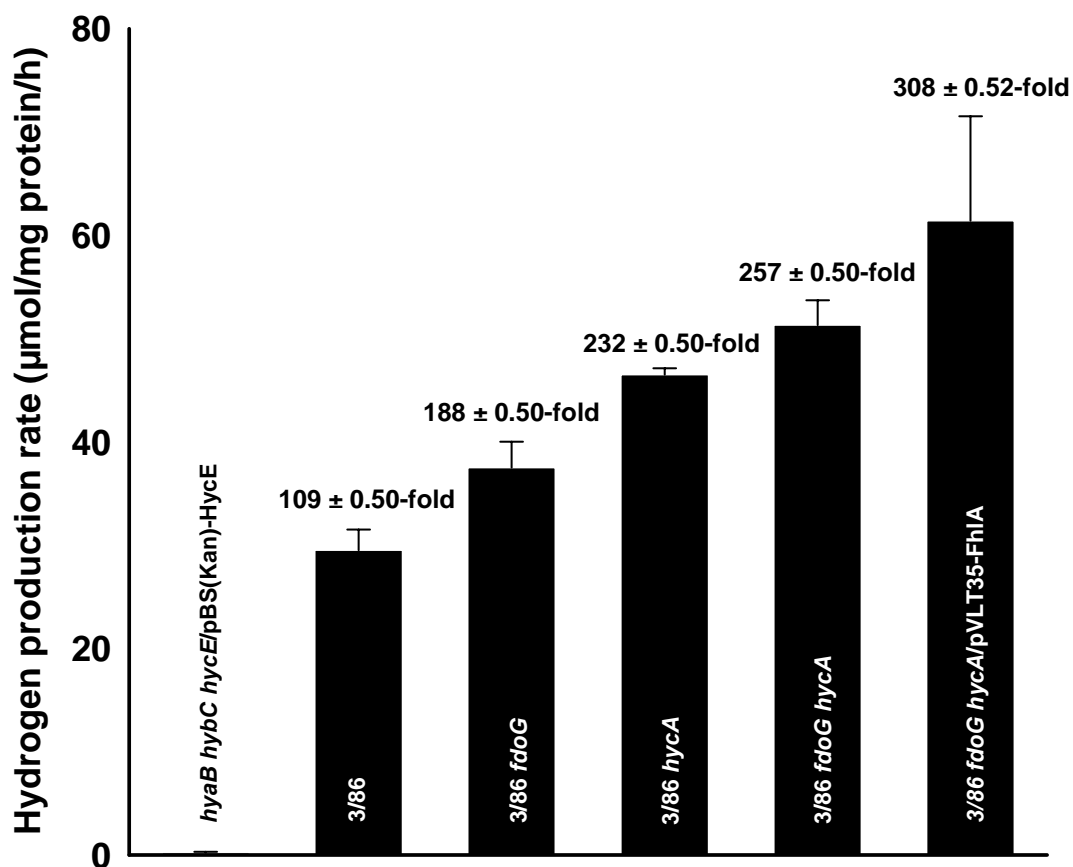


Figure 3-1. Hydrogen production by *hyaB hybC hycE::kan/pBS(Kan)-HycE* after chemical mutagenesis, deletion of *fdoG*, *hycA*, and *fdoG hycA* along with overexpression of *fhlA*. Fold changes are relative to *hyaB hybC hycE::kan/pBS(Kan)-HycE*. 3/86 strain is the chemical mutagen derived from *hyaB hybC hycE::kan/pBS(Kan)-HycE*. The conditions used for hydrogen production were: modified complex formate 100 mM medium, 37 °C and 5 h incubation.

3.2 Introduction

N-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) is an effective mutagen that is widely used to generate random mutations in microorganisms, plants, and animals, including human cells (16). The frequency of mutations (3, 19) and their distribution on the genome vary depending upon the organism (3, 15). In *E. coli*, the mutations occur at the time of DNA replication during the time of exposure to the mutagen (3). The exact mechanism of how NG makes mutations in genomic DNA is not well known (36); however, its mutagenic power could be partially attributed to the diazomethane compound generated during nitrosoguanidine decomposition (11). Diazomethane is a very reactive compound which reacts with a variety of biological molecules such as nucleic acids and their constituents causing structural alterations and DNA methylation (11). Nitrosoguanidine mutagen induces primarily base substitution; nevertheless, small deletions have also been reported (36).

The aim of this study was to enhance hydrogen production in *E. coli* by using a combination of genetic manipulation approaches: chemical mutagenesis and targeted gene deletions of genes involved in hydrogen metabolism. In addition, the transcription profile of chemical mutant 3/86 was studied to identify genetic regulators of hydrogen production in *E. coli*.

3.3 Materials and Methods

Bacterial strains and materials. Bacterial strains used and generated in this study are shown in Table 3.1. 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 (5). *E. coli* cells were streaked from $-80\text{ }^{\circ}\text{C}$ glycerol stocks on Luria-Bertani (LB) agar plates (46) with Kan and/or spectomycin (Sp). The antibiotics concentration was $100\text{ }\mu\text{g/ml}$ unless otherwise specified. After growth on LB agar plates

overnight, a fresh single colony was cultured overnight in 25 ml of LB media with antibiotic at 37 °C and 250 rpm shaking.

Cell growth was measured as turbidity at 600 nm from 0.05 to 0.7 in LB medium and modified complex medium (31) with 100 mM formate under aerobic conditions. Total protein for *E. coli* was 0.22 mg OD⁻¹ ml⁻¹ (31).

Chemical mutagenesis. Chemical mutagenesis of BW25113 *hyaB hybC hycE::kan/pBS(Kan)-HycE* was conducted as explained by Miller (1972) with modifications. An overnight (14-16 h) aerobic cell culture (100 µL) was added to 25 ml of fresh LB media containing Kan and grown under aerobic conditions until a turbidity at 600 nm of 0.4-0.6 was reached. Cell cultures were transferred to 1.5 ml tubes and centrifuged for 5 min at 13,000 rpm. Cells were washed once with 1 ml of 0.1M citrate buffer (citric acid: 21 g/L; NaOH 8.8 g/L; pH 5.5) and resuspended in citrate buffer to obtain a final turbidity of 1.0. MNNG (Sigma) (dissolved in 0.1 M sodium citrate buffer, pH 5.5 that contained 5% dimethyl formamide) was added to 100 µL of cells to a final concentration of 50 µg/ml, and the cells were incubated at 37 °C with shaking at 250 rpm. Cells without the addition of MNNG were used as a negative control. After 30 minutes, cells were centrifuged for 5 min at 13,000 rpm and washed once with 100 µL of 0.1 M phosphate buffer (KH₂PO₄ 13.6 g/L; NaOH 2.32 g/L pH7.0) to remove MNNG. Cells were then resuspended in 100 µL of phosphate buffer and plated using serial dilutions of 10⁻², 10⁻³, 10⁻⁴, and 10⁻⁵ on LB agar plates with Kan; these plates were incubated overnight at 37 °C.

Table 3-1. *E. coli* BW25113 strains and plasmids used in this study. Kan^R, and Sp^R are kanamycin, spectomycin resistance respectively.

Strains and plasmids	Genotype	Source
Strains		
BW25113	<i>lacI rrnB_{T14} ΔlacZ_{WJ16} hsdR514 ΔaraBAD_{AH33} ΔrhaBAD_{LD78}</i>	Yale CGSG Stock Center
<i>hyaB hybC</i> <i>hycE::kan/pBS(Kan)</i> HycE	Defective in large subunit of hydrogenase 1, hydrogenase 2 and hydrogenase 3, Kan ^R plasmid expressing <i>hycE</i>	(31)
3/86	BW25113 <i>hyaB hybC hycE ::kan/pBS(Kan)</i> -HycE with chemical mutagenesis	This study
3/86 <i>fdoG</i>	3/86; defective in formate transporter FdoG	This study
3/86 <i>hycA</i>	3/86; defective in <i>hycA</i> repressor of FHL	This study
3/86 <i>fdoG hycA</i>	3/86; defective in formate dehydrogenase O and <i>hycA</i> repressor of FHL complex.	This study
3/86 <i>fdoG hycA/pVLT-FhlA</i>	3/86; defective in formate dehydrogenase O, <i>hycA</i> repressor of FHL complex, and overexpression of <i>fhlA</i> activator of FHL complex.	This study
<i>ΔgadA</i>	BW25113 defective in <i>gadA</i>	(4)
Plasmids		
pBS(Kan)-HycE	<i>pBS(Kan) pT5-lac::hycE</i> ; expresses <i>hycE</i> derived from <i>Escherichia coli</i>	(31)
pCP20	Ap ^R and Cm ^R plasmid with temperature-sensitive replication and thermal induction of FLP synthesis	(12)

Colony screening. Single colonies obtained after overnight incubation were picked onto LB + Kan 100 µg/ml agar plates and grown overnight at 37 °C, then, these colonies were transferred to modified complex medium with 100 mM formate + 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) agar plates. The plates were incubated in a closed anaerobic chamber (gas packs catalyst anaerobic System BR0038B, Oxoid Limited, Basingstoke, Hampshire, England manufacturer) at 37 °C. After 24 h of incubation, the plates were removed, and hydrogen production was determined using a chemochromic membrane (GVD Corp, Cambridge, MA) (32). The chemochromic membrane is formed by a tungsten oxide (WO₃) thin layer, followed by a catalytic layer of palladium, that in the presence of hydrogen response turning the color of the colony to blue (52). Whatman filter paper was used to avoid direct contact of the colonies with the membrane and then the membrane was placed over the filter paper and pressed hard to remove all the air between the cells and membrane. Bacterial colonies that produce hydrogen released a blue-black coloration upon contact with chemochromic membrane under anaerobic conditions. Mutated strains that produced greater hydrogen were identified by comparing each colony to the original strain (BW25113 *hyaB hybC hycE::kan/pBS(Kan)-HycE* without chemical mutagenesis) from the same plate (Figure 3-2). The putative strains that produced greater hydrogen than BW25113 *hyaB hybC hycE::kan/pBS(Kan)-HycE* were further analyzed using the closed vial hydrogen assays.

Hydrogen determination by the closed vial short hydrogen assay (32). Single colonies were inoculated in 25 ml of LB media containing Kan/Kan + Sp, based on the plasmid used, and cultured overnight at 37 °C and 250 rpm shaking. Overnight cultures (25 ml) and fresh modified complex formate 100 mM media with Kan/Kan + Sp antibiotics were sparged with nitrogen for 5 minutes and sealed. Glass vials (27 ml) were sealed with teflon caps, and sparged with nitrogen for 2 min. Overnight cultures, fresh modified complex formate media and sealed glass vials were

transferred into the anaerobic glove box. Fresh modified complex formate 100 mM (9.5 ml) and overnight culture (0.5 ml) were injected into glass vials inside the anaerobic glove box. These vials were incubated at 37 °C and 250 rpm for hydrogen determinations after 2 and 5 h. The amount of hydrogen generated in the head space of the glass vial was measured using a 50 µL aliquot in a 6890N gas chromatograph as described previously (33). Total protein (whole cells), was calculated using the turbidity at 600 nm at the end of the experiment.

Hydrogen determination by the long closed vial hydrogen assay (31). Single colonies were inoculated in 25 ml of modified complex formate media and cultured overnight at 37 °C and 250 rpm. Overnight cultures (25 ml and 16 h incubation) were mixed with 75 ml complex formate 100 mM (fresh media), then sparged with nitrogen for 5 min and incubated for 6 h at 37 °C and 250 rpm. Glass vials were sealed tightly with teflon caps, and then sparged for 2 min with nitrogen. Anaerobic cultures (100 ml) were transferred into centrifuge bottles inside the glove box and centrifuged for 5 min at 7000 rpm and 4 °C. Cell pellets were resuspended in 20 ml of fresh complex media without formate. Nine ml of resuspended cells and 1 ml of formate (1 M) were injected into glass vials inside the glove box. Vials (10 ml of culture) were incubated for 1 h at 37 °C with shaking and then 50 µL of the head space was analyzed by GC-TCD. Total protein (whole cells) was measured using the turbidity at 600 nm at the end of the experiment.

Targeted gene deletion. Genes of interest were deleted sequentially by P1 transduction and successive selection for Kan resistance that is transferred from the infectious P1 phage along with chromosomal deletion (Figure 3-3). Each Keio mutant (5) has been designed with the ability to delete the Kan resistance marker using thermo regulated FLP recombinase protein from pCP20 (12). Multiple mutations were introduced into a single strain by eliminating Kan resistance gene. All mutant strains generated in this study were confirmed with PCR using gene specific and/or Kan specific primers (data not shown). See Table 3-2.

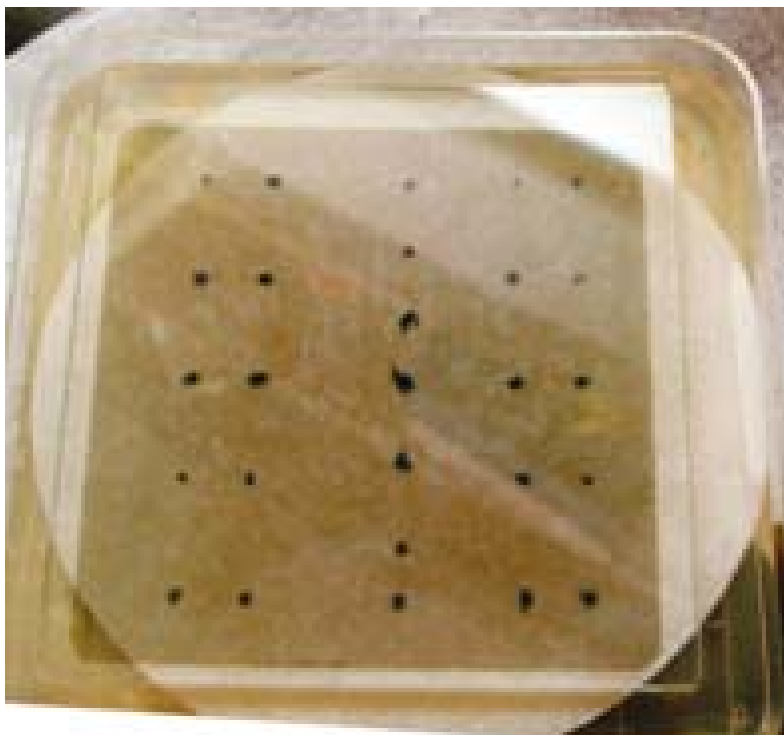


Figure 3-2. Modified complex formate agar screening plates with 100 $\mu\text{g/ml}$ Kan and 0.5 mM IPTG for identifying colonies with enhanced hydrogen production. Agar plate is followed by a whatman filter to avoid direct contact of the colonies with the chemochromic membrane hydrogen detector.

RNA and DNA isolation. To isolate total RNA for the whole-transcriptome studies, BW25113 *hyaB hybC hycE::kan/pBS(Kan)-HycE* (31) and BW25113 *hyaB hybC hycE::kan/pBS(Kan)HycE-3/86* were grown as for the short closed vial hydrogen assay. After 5 hours of incubation, cells were pelleted and stored at -80 °C until RNA isolation. Total RNA was isolated using RNeasy mini kit (Qiagen, CA) as described previously (42) using a bead beater. RNA was reverse transcribed to cDNA through the reaction with poly(A) RNA as is explained previously by (17). cDNA was fragmented to 50-200 bp by digestion with DNase I.

Whole transcriptome analysis. The *E. coli* GeneChip Genome 2.0 Array (P/N: 511302, Affymetrix, Santa Clara, CA) was used to compare the gene expression in BW25113 *hyaB hybC hycE::kan/pBS(Kan)-HycE* and chemical mutagen 3/86. Fragmentation and hybridizations were performed as previously described (17). For transcriptome analysis, if the gene with the greater transcription rate did not have a consistent transcription rate based on the 11 probe pairs (*p*-value less than 0.05 or higher than 0.95), these genes were discarded. A gene was considered differentially expressed when the *p*-value was lower than 0.05 or higher than 0.95 (to assure that the change in gene expression was statistically significant and that false positives arise less than 5 %) and when the expression ratio was lower or higher than standard deviation (± 4.3 -fold), for all genes of the microarray (41). Gene functions were obtained from the Affymetrix–NetAffx Analysis Center (<https://www.affymetrix.com/analysis/netaffx/index.affx>).

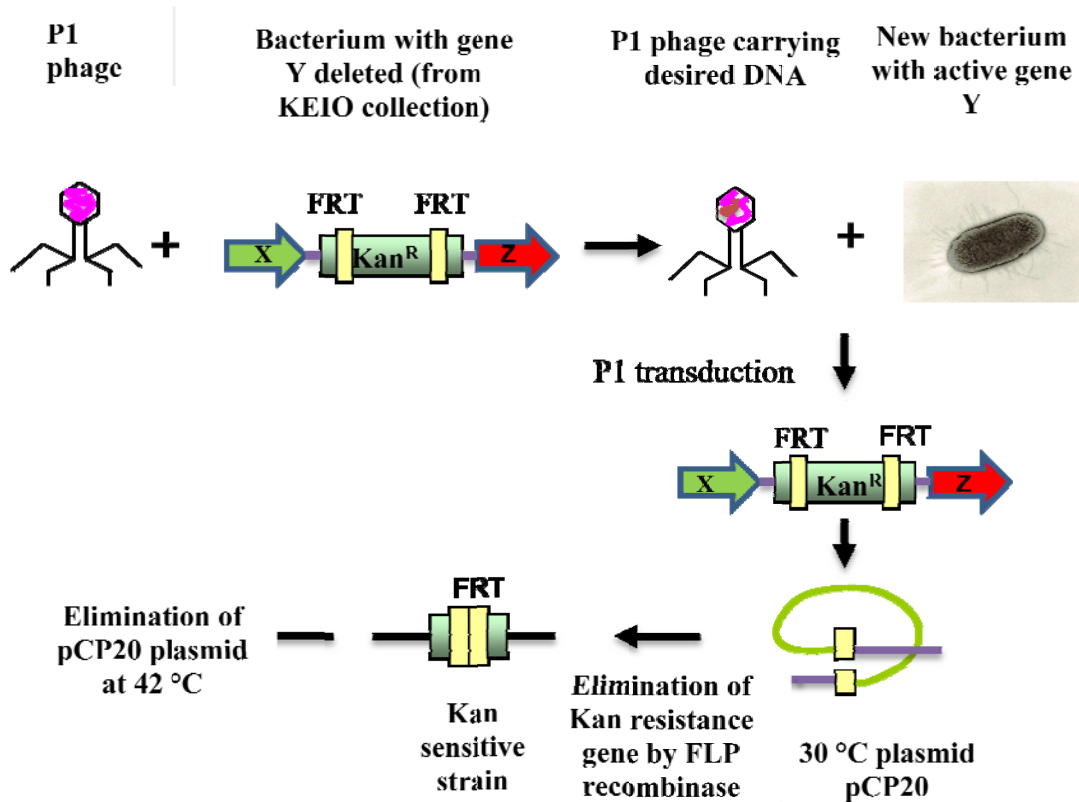


Figure 3-3. Gene deletion through P1 transduction. After construction of P1 phage that transport the DNA surrounding the Kan^R marker of the Keio collection strain; the phage is used to infect the strain we want to engineer by transferring the deletion through homologous recombination. The Kan^R gene is removed from the chromosome of the metabolically-engineered strain by the FLP recombinase of pCP20 at 30 °C. After elimination of the plasmid pCP20 at 42 °C, the new engineered strain is ready to conduct additional mutations. (5)

Table 3-2. Primers used for verifying gene knockout with P1 transduction.

Primer Name	Nucleotide sequence (5' to 3')	Primer Name	Nucleotide sequence (5' to 3')
HypA1	GAGGTGCAGTTTACAACCTTCC	HycB-1	AGTCCGTGTTAAAGGTCAGCTC
fdhD-1	AGACCATTACAGGAAGGAACGAC	fdoH -1	ATACAGGTGGTAACGTCGATGAG
		K1	AGGCTATTCGGCTATGACTG

Chromosome mutation identification. Genomic DNA from *hyaB hybC hycE::kan/pBS(Kan)-HycE* and 3/86 were isolated using the MoBio UltraClean™ Microbial DNA Isolation Kit (Carlsbad, CA) following the manufacturer's instructions. DNA eluted at the final step was dissolved in 500 μ L of TE buffer and treated with 2 μ L of 100 mg/ml RNaseA (Qiagen) at 37 °C for 1 hr to remove RNA contamination. The DNA was again purified once by phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) and chloroform:isoamyl alcohol (24:1, v/v) following the standard protocol (46). The DNA was precipitated, washed with 70 % (v/v) ethanol and resuspended in RNase and DNase free water. DNA concentration and quality was determined using UV-1601 Spectrophotometer (Shimadzu, Columbia, MD) and electrophoresis on 0.9 % agarose gel. The *E. coli* GeneChip antisense genome array (P/N 510052, Affymetrix) which contains probe sets for all 4290 open reading frames (ORF), rRNA, tRNA, and 1350 intergenic regions was used to identify the chromosomal mutations in the 3/86 chemical mutagen. Individual probe data for identifying chromosomal mutations in 3/86 was obtained using dChip software from Harvard University (<http://biosun1.harvard.edu/complab/dchip/>). We used two criteria for considering a probe regions on chromosome for sequencing to identify the mutations (i) the probes that have \geq 4-fold greater signal intensity in 3/86 strain (after subtraction of the background) and (ii) the probes with *hyaB hybC hycE::kan/pBS(Kan)-HycE* signal (background subtracted) of at least 303. As expected, all the probes corresponding to *hyaB* and *hybC* had insignificant signal intensity (due to gene deletions) in arrays of both the strains *hyaB hybC hycE::kan/pBS(Kan)-HycE* and chemical mutagen 3/86.

3.4 Results

Selection of chemical mutagen 3/86 and targeted gene deletion. In this study, we used an *E. coli* strain, BW25113 *hyaB hybC hycE::kan/pBS(Kan)-HycE* that has disrupted *hyaB hybC* and *hycE* genes and a plasmid pBS(Kan)-HycE with HycE under the control of a constitutive *lac*

promoter (32); hence, 0.5 mM IPTG was utilized to express *hycE* which results in hydrogen production. Due to the chromosomal mutation in *hycE* that is not fully complemented by *hycE* on the high-copy plasmid, *hyaB hybC hycE::kan/pBS(Kan)-HycE* has low hydrogen production (32). This feature facilitated the screening for better hydrogen producing strains using a novel chemochromic membrane screen that directly detects hydrogen from individual colonies (32).

Colonies producing more hydrogen were tested three times on agar plates to eliminate false positives. After screening 2130 colonies using the chemochromic membrane method, a strain BW25113 *hyaB hybC hycE::kan/pBS(Kan)-HycE* chemical mutant named 3/86 that was identified to produced significantly higher hydrogen was selected and further analyzed by the closed vial short hydrogen assay, which showed that 3/86 produced 108 ± 0.5 -fold higher hydrogen when compared to BW25113 *hyaB hybC hycE::kan/pBS(Kan)-HycE* (Figure 3-1). Statistical analysis was performed using the *t*-test (54) (assuming unequal variances). It shows that there is a significant difference between these two sets of data (HycE wt strain and 3/86 chemical mutagen) because the Pvalue was 0.0001 for $\alpha = 0.05$ (95 % confidence level).

To study whether hydrogen production could be further increased in a chemically mutagenized strain, we chose to delete/overexpress genes in 3/86 that were previously shown to be involved in hydrogen production (31). Our strategy included deletion of two genes: *fdoG*, encoding formate dehydrogenase-O that serves to consume formate (31) and *hycA*, a repressor of FHL (through a direct or indirect interaction of HycA with FhlA). In addition, we overexpressed *fhlA*, an activator of FHL complex using a compatible plasmid pVLT-FhlA that has *fhlA* under control of the *hyp* FhlA-dependent promoter. The resultant strain 3/86 *fdoG hycA/pVLT-FhlA* was tested for hydrogen production compared to *hyaB hybC hycE/pBS(Kan)-HycE* and 3/86. The short closed vial hydrogen assay results indicated that 3/86 *fdoG hycA/pVLT-FhlA* produced 323 ± 50 $\mu\text{mol/mg}$ protein of hydrogen after 5 h incubation, which is 308 ± 0.52 -fold higher than

hyaB hybC hycE/pBS(Kan)-HycE (Figure 3-1), indicating that the hydrogen production can be further increased in a chemically mutagenized strain by targeted gene deletion. Statistical analysis performed using the *t*-test (54) (assuming unequal variances), shows 95% confident that there is convincing evidence of difference between the following set of data: (i) HycE wt strain and 3/86 *fdoG* (Pvalue = 0.0301); (ii) HycE wt strain and 3/86 *hycA* (Pvalue = 0.0044); (iii) HycE wt strain and 3/86 *fdoG hycA* (Pvalue = 0.0212); 3/86 *fdoG hycA*/pVLT-FhlA (Pvalue = 0.0011); since, the Pvalue for each set of data was $< \alpha = 0.05$, which means 95% confidence level.

Gene expression in chemical mutagen 3/86. In order to determine gene expression in 3/86 strain, we conducted a whole transcriptome analysis and compared gene expression levels between 3/86 and HycE-wild type strain using *E. coli* GeneChip Genome 2.0 Array (Affymetrix, Santa Clara, CA). Overall, 89 genes were induced and 31 genes were repressed, as shown in detail in Tables 3-3 and 3-4, respectively. Some of the genes significantly induced in 3/86 include *slp* (lipoprotein, 36.8 fold increased), *fruBKA* operon (5.0-fold up regulated), and *yhiF* (encoding a putative *luxR* regulator, 8.6 fold increased) see Table 3-3. On the other hand, the *frlABC* operon was down regulated -17, -49 and -24-fold, respectively) (Table 3-4). Most of the genes belonging to the acid resistance system and related with pH response were highly induced such as *gadA* (19.7-fold increased), *gadBC* (22.6-fold increased), *ybaS* (17.1 fold increased), and *ybaT* genes (17.1 fold increased) (Table 3-3). In addition, the *fru* operon containing the *fruB*, *fruK* (encoding fructose-1-P kinase) and *fruA* genes, was induced 5-fold (Table 3-3; while, genes encoding *frl* operon, *frlA* (-17.1 fold), *frlB* (-48.7 fold) and *frlC* (-24.3 fold), were significantly repressed in 3/86 strain. See Table 3-4.

Table 3-3. Induced genes of chemical mutagen 3/86 vs. BW25113 *hyaB hybC hycE::kan/pBS(Kan)-HycE* in modified complex formate 100 mM medium with 100 µg/ml kanamycin for 5 h incubation at 37°C.

Gene Symbol	b #	fold change 3/86 vs. HycE wt strain	Encoded protein
Hydrogenase I			
<i>hyaE</i>	b0976	5.3	Required for the synthesis of active hydrogenase 1 (35).
<i>hyaF</i>	b0977	4.9	Nickel ion binding. The HyaF protein is required for the synthesis of active hydrogenase isoenzyme (35)
pH			
<i>hdeB</i>	b3509	5.7	Periplasmic acid stress chaperone protein
<i>hdeD</i>	b3511	8.6	Inner membrane protein required for the acid resistance phenotype
Acid resistance			
<i>gadA</i>	b3517	19.7	Glutamate decarboxylase activity
<i>gadB</i>	b1493	22.6	Glutamate decarboxylase activity
<i>gadE</i>	b3512	17.1	DNA binding
<i>gadY</i>	b4452	6.1	Small RNA that is highly expressed during entry into stationary phase and upregulated by low pH
<i>ybaS</i>	b0485	17.1	Glutaminase activity
<i>yhjJ</i>	b3527	5.7	Peptidase activity
Tryptophanase activity			
<i>tnaA</i>	b3708	3.7	Pyruvate synthesis
<i>tnaL</i>	b3707	4.9	Required for regulation by attenuation of the <i>tnaCAB</i> operon
Carbon Starvation			
<i>slp</i>	b3506	36.8	Protect cells against effect of toxic metabolites
Sugar Transport			
<i>fruA</i>	b2167	4.9	Sugar porter activity
<i>fruB</i>	b2169	5.3	Sugar porter activity
<i>fruK</i>	b2168	5.7	1-phosphofructokinase activity
<i>gntP</i>	b4321	5.3	Sugar porter activity
Iron			
<i>fhuF</i>	b4367	8.0	Iron ion binding
<i>fepD</i>	b0590	6.1	Transporter activity
<i>tonB</i>	b1252	5.3	Iron ion transporter activity
<i>yheA</i>	b3337	5.3	Iron ion binding
<i>fecR</i>	b4292	4.9	Iron ion binding
<i>feoA</i>	b3408	4.9	Iron ion binding
Electron Transport			
<i>nrdH</i>	b2673	5.7	Electron transporter activity
<i>nrdI</i>	b2674	4.6	Protein function still unknown
<i>trxC</i>	b2582	4.6	Electron transporter activity
DNA Regulation			
<i>yhiF</i>	b3507	8.6	DNA binding
<i>yncE</i>	b1452	7.0	Nucleotide binding
<i>fecI</i>	b4293	6.1	DNA binding
<i>ygaA</i>	b2709	5.7	Nucleotide binding
<i>ykfG</i>	b0247	5.7	Predicted DNA repair protein
Biosynthesis			
<i>aroF</i>	b2601	8.6	3-deoxy-7-phosphoheptulonate synthase
<i>entC</i>	b0593	7.5	Isochorismate syntheses activity
<i>ddg</i>	b2378	6.5	Acyltransferase activity

Table 3-3. Continued

Gene Symbol	b #	fold change 3/86 vs. HycE wt strain	Encoded protein
<i>bioA</i>	b0774	6.1	Adenosylmethionine-8-amino-7-oxonanoate transaminase
<i>tyrA</i>	b2600	5.7	Chorismate mutase activity
<i>entD</i>	b0583	4.6	Magnesium ion binding
Cell Transport			
<i>xasA</i>	b1492	17.1	Amino acid-polyamine transporter activity
<i>ybaT</i>	b0486	13.9	Amino acid-polyamine transporter activity
<i>yhaO</i>	b3110	13.0	Transporter activity
<i>yhiU</i>	b3513	11.3	Protein transporter activity
<i>yhiV</i>	b3514	8.0	Transporter activity
<i>ydhJ</i>	b1644	6.5	Protein transporter activity
<i>exbB</i>	b3006	6.5	Protein transporter activity
Unknown			
<i>yhcS</i>	b1228	27.9	Hypothetical protein
<i>rhcC</i>	b3593	7.5	Encode hydrophilic proteins
<i>yhaM</i>	b3109	9.8	Hypothetical protein
<i>hokD</i>	b1562	9.8	Small toxic polypeptide
<i>yhiD</i>	b3508	9.2	Putative ATP dependent transporter of the MgtC family
<i>yhiM</i>	b3491	8.6	Inner membrane protein
<i>yjcB</i>	b4060	7.5	Predicted inner membrane protein
<i>sixA</i>	b2340	6.1	Phosphohistidine phosphatase activity
<i>yfeK</i>	b2419	6.1	Hypothetical protein
<i>yfgJ</i>	b2510	6.1	Predicted protein
<i>yabl</i>	b0065	6.1	Inner membrane proteins
<i>mokB</i>	b1420	5.7	Hypothetical protein
<i>flu</i>	b2000	5.7	Biofilm formation autotransporter
<i>pspB</i>	b1305	5.7	Transcriptional regulator, toxin-antitoxin pair
<i>ydiE</i>	b1705	5.3	Conserved protein
<i>yeaY</i>	b1806	5.3	Encodes a putative outer membrane lipoprotein
<i>oxyS</i>	b4458	5.3	Plays a regulatory role in the oxidative stress response
<i>yafW</i>	b0246	4.9	Define a protein family of cellular toxins
<i>yafX</i>	b0248	4.6	Hypothetical protein
<i>yebE</i>	b1846	4.6	Inner membrane protein with one predicted transmembrane domain
<i>rseB</i>	b2571	4.6	Negative regulator of sigma E activity
<i>sraD</i>	b4442	4.6	Uncharacterized member of the major facilitator super family (MFS) of transporters
RNA			
<i>rib</i>	b4451	13.9	Appears to function in an indirect role to regulate abundance of the iron-responsive transcriptional regulator Fur at the posttranscriptional level
<i>rib</i>	b4417	8.0	Regulatory RNAs
<i>IS102</i>	b4435	7.5	IS102 encodes a small RNA of approximately 180-190 not in length
<i>rid</i>	b4430	7.5	Rid is a small RNA reported to be approximately 60 not
<i>relic</i>	b4424	4.9	Code for the antisense regulatory RNA part of a toxin-antitoxin pair
<i>miff</i>	b4439	4.6	Antisense regulator of the translation of OmpC poring

Table 3-4. Repressed genes of chemical mutagen 3/86 vs. BW25113 *hyaB hybC hycE::kan/pBS(Kan)-HycE* in modified complex formate (100 mM) medium with 100 µg/ml kanamycin for 5 h incubation at 37°C.

Gene Symbol	b #	fold change 3/86 vs. HycE wt strain	Encoded protein
Formate gene			
<i>fdnG</i>	b1474	-4.9	Formate dehydrogenase activity
Sugar metabolism			
<i>frlA</i>	b3370	-17.1	Putative transporter
<i>yhfN (frlB)</i>	b3371	-48.5	Sugar binding
<i>frlC</i>	b3372	-24.3	Physiological function is to allow the metabolism of the psicoselysine
<i>frlC</i>	b3373	-18.4	Ribonucleoside-diphosphate reductase activity
uid sugar metabolism			
<i>uidA</i>	b1617	-11.3	Hydrolase activity, hydrolyzing O-glycosyl compounds
<i>uidB</i>	b1616	-6.1	Transporter activity
<i>aceK</i>	b4016	-5.7	Nucleotide binding
yjc and yjd sugar transport			
<i>yjcU (alsE)</i>	b4085	-5.7	Ribulose-phosphate 3-epimerase activity
<i>yjcV</i>	b4086	-5.7	Transporter activity
<i>yjcW (alsA)</i>	b4087	-4.9	Nucleotide binding
<i>yjcX</i>	b4088	-9.2	Sugar porter activity
<i>yjdK</i>	b4128	-4.9	Predicted protein
<i>srlA</i>	b2702	-4.9	Sugar porter activity
Other sugar metabolism			
<i>uxaA</i>	b3091	-7.5	Altronate dehydratase activity
<i>uxaC</i>	b3092	-8.0	Glucuronate isomerase activity
Amino acid metabolism			
<i>cadA</i>	b4131	-7.0	Catalytic activity
<i>cadB</i>	b4132	-4.9	Amino acid-polyamine transporter activity
<i>nrdA</i>	b2234	-11.3	Ribonucleoside-diphosphate reductase activity
<i>nrdB</i>	b2235	-6.1	Ribonucleoside-diphosphate reductase activity
<i>nrdD</i>	b4238	-4.6	Catalytic activity
DNA expression regulation			
<i>iclR</i>	b4018	-4.6	DNA binding
<i>rpiR</i>	b4089	-4.6	DNA binding
Misc. enzymes			
<i>yegT</i>	b2098	-9.2	Transporter activity
<i>yegU</i>	b2099	-5.3	Hydrolase activity
<i>glnA</i>	b3870	-4.6	Glutamate-ammonia ligase activity
Transporters			
<i>ompF</i>	b0929	-7.0	Transporter activity
<i>nupG</i>	b2964	-5.7	Transporter activity
Unknown			
<i>ydcX</i>	b1445	-5.7	Predicted inner membrane protein
<i>yeeN</i>	b1983	-4.6	Unknown function

In an effort to identify significant reasons of the hydrogen improvement in 3/86, we wanted to quantify the amount of protein in BW25113 *hyaB hybC hycE::kan/pBS(Kan)*-HycE and BW25113 *hyaB hybC hycE::kan/pBS(Kan)*-HycE 3/86 by performing SDS-PAGE. As an interestingly finding, HycE protein amount in 3/86 was significantly inhibited in regards to the control strain (Figure 3-4). Moreover, five proteins were considerably induced in 3/86 strain and one of them only appeared in the chemical mutagenized 3/86. Based on the mass spectrometry analysis using the MALDI ionization method and based on MASCOT alignments (22), we were able to identify the five different proteins and those are listed in Table 3-5. Fragments that allow protein identification are specified in Table 3.6. The band identified only in 3/86 strain was due to the *tnaA* expression which encodes TnaA protein. Furthermore, *tnaA* was also found induced in the whole transcriptome analysis 3.7-fold; therefore, *tnaA* induction was confirmed by two different methods (whole transcriptome analysis and mass spectrometry).

Identification of chromosomal mutations in chemical mutagen 3/86. To identify chromosomal mutations in chemical mutagen 3/86, we used a modified microarray hybridization-based comparative genome sequencing method that was developed by (1). We hybridized genomic DNAs from *hyaB hybC hycE kan/pBS(Kan)*-HycE and chemical mutagen 3/86 to *E. coli* GeneChip antisense genome array which covers approximately 21% (mostly coding region) of the *E. coli* genome. The oligonucleotide probe sets with differential hybridization intensity ratios were selected and the corresponding genomic regions were sequenced to identify the location of mutations. Based on this method, two mutations were identified in coding regions of *ftnA* and *yebJ*. Single nucleotide replacements led to a nonsense mutation in *ftnA* and an amino acid change from glycine to aspartic acid at position 220 in *yebJ*.

Table 3-5. Identified proteins in *hyaB hybC hycE::kan/pBS(Kan)-HycE-3/86* by using SDS - PAGE, mass spectroscopy, and MASCOT software.

Putative protein	Name of the protein	Score obtained from MASCOT alignments	Protein relationship for hydrogen
TnaA	Tryptophanase	90	Degradation of tryptophan to indole, pyruvate and ammonia (27)
AceA	Isocitrate lyase	68	Enables growth on acetate
TufB	Translation elongation factor EF-Tu.B	60	Essential GTP dependent binding of aa tRNA to the A-site of ribosomes.
AcnB	Aconitate hydratase 2	43	Catalyzes citrate \rightleftharpoons cis-aconitate + H ₂ O for anaerobic growth
OmpC	outer membrane porin C	64	Transport of hydrophilic solute

Table 3-6. Protein fragments identified using mass spectrometry analysis (MALDI ionization method), and MASCOT software.

Matched peptides				
Tna.A	Ace.A	TufB	AcnB	OmpC
MKDYVMENFK.H	K.EWTQPR.W	R.AFDQIDNAPEEK.A	R.CCTGHLPIYK.L	K.GETQVTDQLTGYGQWEYQI QGNSEAENENNSWTR.V
K.HLPEPFR.I	K.LRGSVNPECTLAQLGAAK.M	R.EHILLGR.Q	K.LLSHRSVKR.R	R.VAFAGLKF
R.VIEPVKR.T	R.GSVNPECTLAQLGAAK.M	R.AGENVGILLR.G	K.NPPAGEHEEFL DLLTNR.V	K.FQDVGSFDYGR.N
R.AYREEAIK.S	R.INNTFR.R	K.FESEVYLSK.D	K.GFPLAYVGDV VGTGSSR.K	R.NYGVVYDVTSWTDVLPFEG GDTYGSDFNFMQQR.G
R.SYYALAESVK.N	R.ADQIQWSAGIEPQDPR.Y	R.HITPFFK.G	R.IPLIGR.G	R.GNGFATYR.N
K.KYDIPVVMDSAR.F	K.CGHNMGKVLVPTQEAIQK.L	K.GYRPFYFR.T	R.EALGLPHSDVF R.Q	R.NIDFFGLVDGLNFAVQYQG K.N
K.YDIPVVMDSAR.F	K.VLVPTQEAIQK.L	K.MVVTLIHPIAMDDGLR.F	K.KNFSGR.I	K.NGSVSGEGMTNNGREALR.Q
K.DAMVPMGGLLCKM.K	R.LAADVTGVPTLLVAF.T		K.NIFSGR.I	K.YDANNIVLAAQYQTQYNAT R.V
R.AVEIGSFLLGR.D	R.TSEGFFR.T		K.VEQAFELTDA SAER.S	K.AQNFEAVAQYQDFGLRPSL AYLQSK.G
K.ENAANKGLTFTYEPK.V	R.TSEGFFRTHAGIEQAISR.G		R.VADGATVWST STR.N	K.NLLDDNQFTR.D
K.GLTFTYEPK.V	R.THAGIEQAISR.G		R.YLNFDQLSQ YTEK.A	
	R.RFAQAIHAK.Y			
	K.VQQPEFAAAK.D			

HycE protein expression in 3/86. Although, SDS-PAGE analysis reveals that HycE protein expression in 3/86 is inhibited compared with HycE-wild type (Figure 3-4), protein sequencing reveals no mutation (data not shown). It is tempting to speculate that the decreasing in protein expression is a response of hydrogen production enhancement by 3/86 mutant.

Hydrogen and *gadA*. As an attempt to identify other beneficial genes for hydrogen synthesis, a thorough analysis was performed of one of the genes belonging to the acid resistance system of *E. coli* (*gadA*) which was significantly induced (20-fold) in the microarray results for chemical mutagen 3/86. As a result, from BW25113 deficient in *gadA*, hydrogen production decreases 10 ± 0.91 -fold, 3.2 ± 0.34 -fold, and 1.9 ± 0.14 -fold after 15 min, 30 min, and 1 h incubation respectively by using long closed vial hydrogen determination (Figure 3-5) and consistently 2.0 ± 0.14 -fold by the short closed vial hydrogen assay. Statistical analysis performed using the *t*-test (54) (assuming unequal variances) shows 95% confidence that there is convincing evidence of difference between the hydrogen production measurements of BW25113 vs. BW25113 Δ *gadA* strains after 15 min incubation (Pvalue = 0.0047) and after 30 min Incubation (Pvalue =0.0264). However, it also predicts 95% confidence that there is not convincing evidence of significant difference in the results obtained after 1 hour incubation of BW25113 wild type strain and the mutant BW25113 Δ *gadA* (Pvalue = 0.1002).

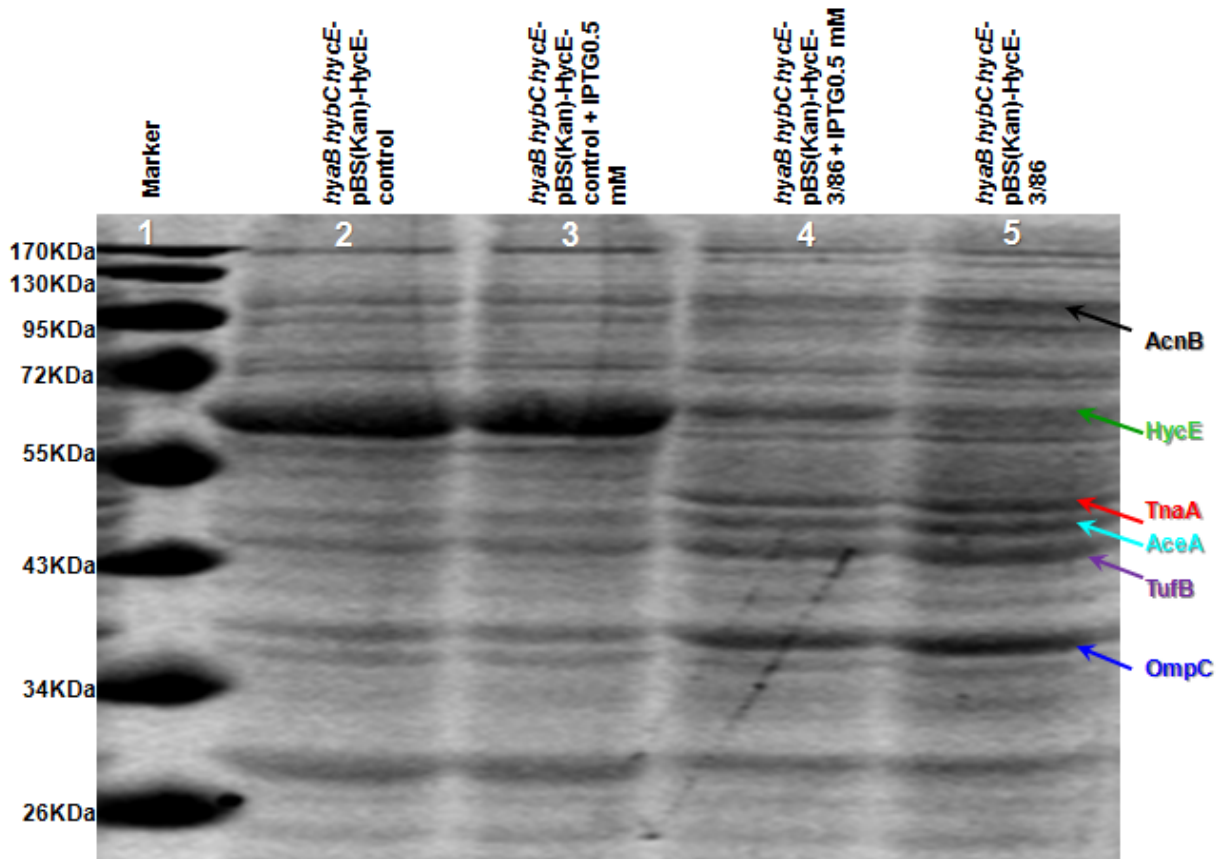


Figure 3-4. SDS-PAGE and protein identifications with mass spectrometry analysis. The first column correspond to EZ run protein marker; the second column correspond to *E. coli* BW25113 *hyaB hybC hycE::kan/pBS(Kan)-HycE-wt*, the third column correspond to *E. coli* BW25113 *hyaB hybC hycE::kan/pBS(Kan)-HycE-wt* with 0.5 mM IPTG, the fourth column correspond to *E. coli* BW25113 *hyaB hybC hycE::kan/pBS(Kan)-HycE-3/86* with 0.5 mM IPTG and the fifth column correspond to *E. coli* BW25113 *hybC hycE::kan/pBS(Kan)-HycE-3/86*. All strains were cultured aerobically in LB medium for 3.5 h consecutives. TnaA, AceA, TufB, AcnB, and OmpC were the proteins identified with mass spectrometry analysis.

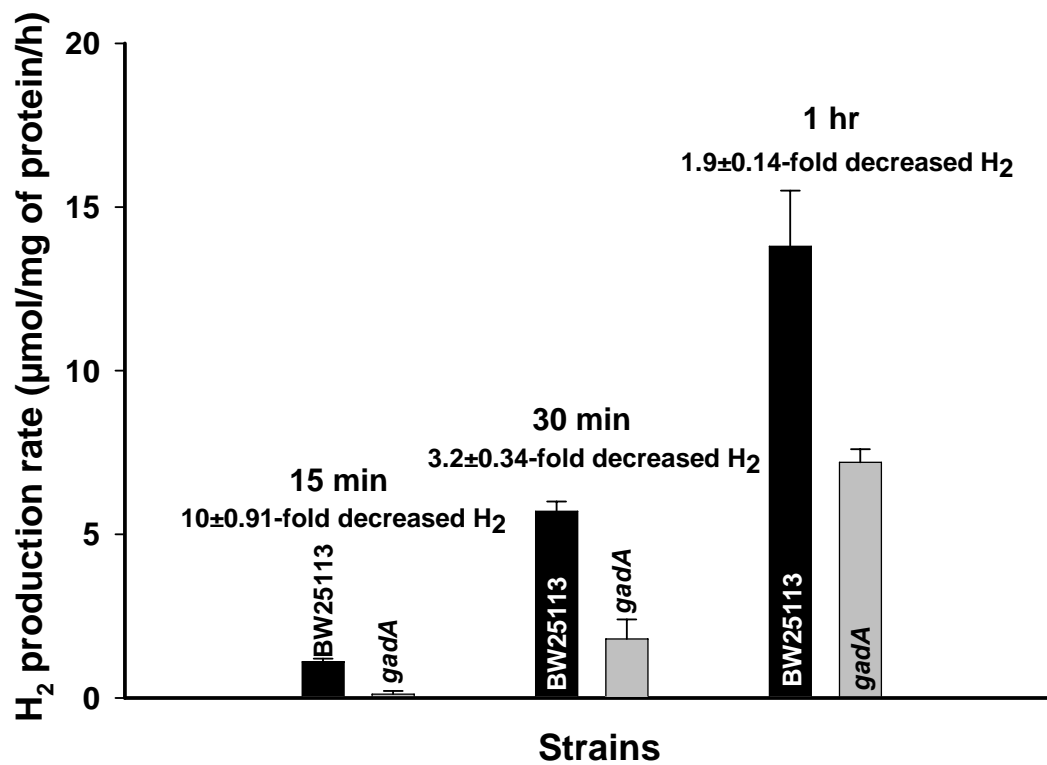


Figure 3-5. H₂ production rate of BW25113 and BW25113 Δ *gadA* after 15 min incubation (first bars set), after 30 min incubation (second bar set) and after 1 h incubation (third bars set).

3.5 Discussion

In this study, we used chemical mutagenesis and gene deletion techniques to enhance hydrogen production from triple mutant BW25113 *hyaB hybC hycE::kan/pBS(Kan)-HycE* as the main host. As a result, it was found a chemical mutagen 3/86 which increase 109-fold hydrogen production than HycE-control, and 308 ± 0.52 -fold after *hycA*, *fdoG* genes deletion and *fhlA* overexpression.

The whole-transcriptome results obtained from chemical mutagen 3/86 vs. the unmutagenized strain indentified several induced and repressed genes related to hydrogen synthesis. Most of the *E. coli* genes that belong to acid resistance were highly induced in the 3/86 mutant such as *ybaS*, *ybaT*, and *gadE*, which is a regulator and activator of *gadA* and *gadBC*; therefore, it is possible that the increased expression of these genes is related to enhanced hydrogen synthesis. In support of this hypothesis that GadA is related to hydrogen production, deletion of *gadA* decreased hydrogen production; hence, *gadA* was probably induced as a way for the cell to reduce hydrogen production in 3/86. Another important result is the reduction of hydrogen production as a function of time for the *gadA* mutation (Figure 3.5). We hypothesize that this effect occurs because hydrogenase 3 is a reversible enzyme (30), which is reflected in hydrogen uptake and synthesis activities. The ratio between the forward reaction (synthesis) and the reverse reaction (uptake) is 30-fold (30). Therefore, as molecular hydrogen is synthesized, some hydrogen might consumed by the uptake activity of Hyd-3.

TnaA protein is other interesting finding in chemical mutagen 3/68. This protein is responsible for the cleavage of tryptophan to indole and pyruvate in *E. coli* (27). Moreover, TnaA protein is only expressed in 3/86 mutant (not in HycE control) as shown in Figure 3-4; hence, there is more pyruvate synthesized by this protein which 3/86 mutant can metabolize and finally converts to molecular hydrogen.

CHAPTER IV

TRANSPOSON MUTAGENESIS OF *E. coli* FOR ENHANCED HYDROGEN PRODUCTION

4.1 Overview

Transposon mutagenesis is one of the most efficient strategies for creating random mutations in the genomic DNA. One of the main advantages of using this kind of mutagenesis is that it is easy to create a complete library of mutants. In this study, transposon mutagenesis was performed in two different strains: *E. coli* BW25113 *hyaB hybC hycA fdoG::kan/pCA24N-FhlA* and *E. coli* MG1655 to identify beneficial mutations for hydrogen production. As a result of screening 461 *E. coli* BW25113 *hyaB hybC hycA fdoG::kan/pCA24N-FhlA* transformants and 1000 *E. coli* MG1655 transformants, three interesting mutations have been discovered in *E. coli* BW25113 *hyaB hybC hycA fdoG::kan/pCA24N-FhlA* transformants (*gpsA*, *dipZ*, *glgP*) and 1 beneficial mutation in *E. coli* MG1655 transformants (*malT*). When any of these genes *gpsA*, *dipZ*, or *glgP* is disrupted by Tn5 insertion, hydrogen production decreases 17, 3 and 8-fold, respectively. In addition, when *malT* gene is disrupted by Tn5 insertion, hydrogen increases 3.4-fold. These results suggest that the total hydrogen amount synthesized by the *E. coli* strains that we are studying is not coming only from formate, but part of it is coming from other sources and reactions that are occurring inside the cells. For instance, (i) from glycerol by the activation of *gpsA*, (ii) through the expression of the electron transport *dipZ*, (iii) the glycogen breakdown by expression of *glgP* and (iv) throughout the inactivation of *malT* gene. Consequently, we present *E. coli* as a versatile bacterium capable to produce hydrogen from a variety of resource.

4.2 Introduction

Transposon mutagenesis is a very common random mutagenesis technique, mostly used because of the easy way of determining which genes have been mutagenized by transposon insertions (53). There are different kinds of transposon; however, not all types of transposon are equally useful for mutagenesis (53). Transposon can be introduced into the cells through plasmids called suicide vectors (Figure 4-1), phages (53), and also by using the EZ::TNTM <DHFR-1> Tnp Transposome Kit, cat # EZI912D manufactured by EPICENTRE Biotechnologies, via electroporation. Tn5 is a transposon formed by two inverted insertion sequences (IS50) (18). The (IS50) region contains the Tnp transposase and at the end of each IS50 sequence there are two 19 bp Tnp recognition sites (59), both of them, IS50 and the 19 bp recognition sites, are required for transposition at specific binding sites (59). The Tnp transposase (476 amino acids) catalyzes transposition by cutting the ends of the transposon, letting the transposon hop into the target DNA as shown in Figure 4-2 (18). The EZ::TNTM <DHFR-1> Tnp Transposome Kit was developed with the transposon and Tnp enzyme separated (there is no gene inside the transposon encoding the Tnp enzyme). The transposon and Tnp enzyme form a complex and only if Mg⁺² is present, the transposition occurs. The use of this kit, allows more stable mutations because the DNA sequence that jumps into the bacterial genomic DNA does not contain the Tnp enzyme; hence it cannot jump more than once.

Tn5 transposon has non-specific DNA binding, as well as a non-specific recombination site (55). Moreover, it is one of the best characterized transposon (21); therefore, Tn5 is ideal for creating random mutants with high frequencies of insertion (53) due to its ability to interact with non-specific DNA (55). Additionally, Tn5 may be used in almost any gram-negative bacterium (53). Transposition involves a hyperactive EZ::TNTM <DHFR-1> Transposome that is

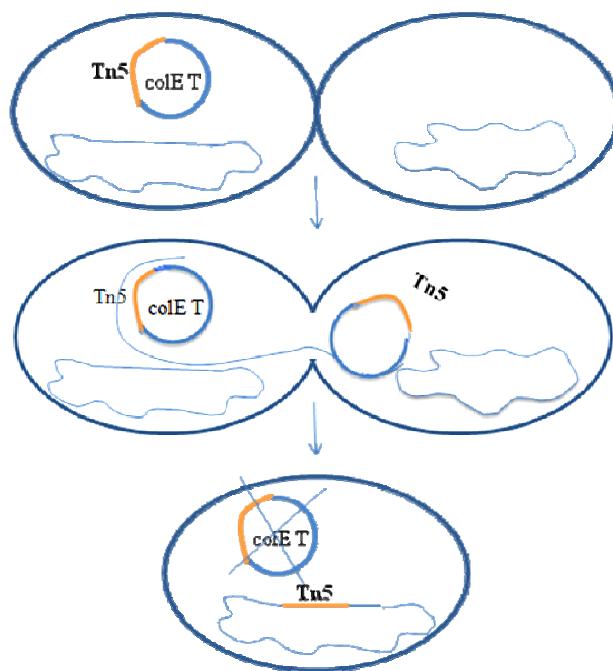


Figure 4-1. Schematic representation of Tn5 transposon insertion into bacterial genomic DNA through a suicide vector derived from the ColE1 plasmid. A ColE1 suicide-derived plasmid, contains the *oriT* sequence of plasmid RP4 and transposon Tn5. Transposon Tn5 integrates into the genomic DNA of each cell and since the derived-suicide plasmid ColE1 cannot replicate by itself, it is lost (53).

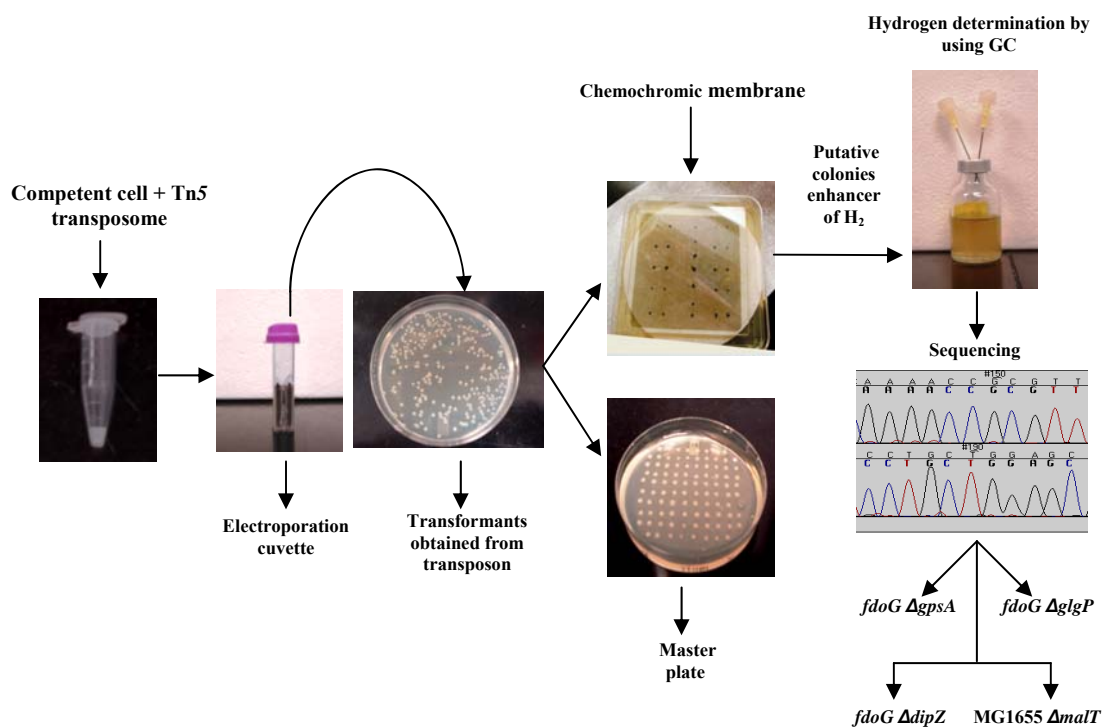


Figure 4-2. Depiction of the whole process to perform transposon mutagenesis and how novel beneficial and deleterious genes to enhance hydrogen production were discovered in BW25113 *hyaB hybC hycA fdoG::kan/pCA24N-FhlA* and MG1655.

a DNA sequence that hops into other DNA molecules catalyzed by a Transposase enzyme, a substrate or desired DNA template to be mutagenized, and depending upon the transposition efficiency of the host, it requires a restriction inhibitor reagent to increase transposition efficiency as indicated by EZ::TNTM <DHFR-1> TransposomeTM kit manufacturers.

The purpose of the work described in this chapter is to discover novel genes related to hydrogen production via transposon inactivation of random genes with the ultimate aim of enhancing hydrogen production in the best strain for hydrogen production, *E. coli* BW25113 *hyaB hybC hycA fdoG::kan/pCA24N-FhlA* (31). One might expect two different classes of interesting mutations: (i) mutations created by genes that when disrupted by transposition enhances H₂ gas synthesis and (ii) mutations created by genes that when disrupted by transposition decrease hydrogen production. Both classes of mutations were considered here during screening.

4.3 Material and Methods

Bacterial growth and materials. Bacterial strains used and created for the development of this study are shown in Table 4.1. *E. coli* strains were initially streaked from -80 °C glycerol stock on Luria-Bertani (LB) agar plates (46) with/without chloramphenicol (Cm 30 µg/ml) and after transposon mutagenesis with Cm + Trimethoprim (Tm) /Tm depending upon plasmid content and then incubated at 37 °C. Fresh single colonies were inoculated in 25 ml of LB media with Cm if necessary and culture overnight at 37 °C and 250 rpm shaking (New Brunswick Scientific, Edison, NJ). The parent strain, *E. coli* K-12 BW25113, was obtained from the Yale University CGSC Stock Center. Cell growth was measured as the turbidity at 600 nm from 0.05 to 0.7 in LB medium and modified complex formate (100 mM) medium (31) under aerobic conditions, and total protein for *E. coli* was 0.22 mg OD⁻¹ ml⁻¹ (31).

Table 4-1. *E. coli* bacterial strains and plasmids used throughout the development of the transposon mutagenesis study.

Strains and plasmids	Genotype	Source
Strains		
BW25113 <i>hyaB hybC hycA fdoG::kan/pCA24N-FhlA</i>	\DeltahyaB , \DeltahybC , \DeltahycA and \DeltafdoG (defective hydrogenases 1 and 2, defective FHL repressor and defective formate dehydrogenase-O) + FhlA	(31)
BW25113 <i>hyaB hybC hycA gpsA fdoG::kan/pCA24N-FhlA (fdoG 4/77)</i>	\DeltahyaB , \DeltahybC , \DeltahycA , \DeltafdoG and \DeltagpsA (defective hydrogenases 1 and 2, defective FHL repressor, defective formate dehydrogenase-O and defective) + FhlA (obtained from transposon mutagenesis)	This study
BW25113 <i>hyaB hybC hycA dipZ fdoG::kan/pCA24N-FhlA (fdoG 4/47)</i>	\DeltahyaB , \DeltahybC , \DeltahycA , \DeltafdoG and \DeltadipZ (defective hydrogenases 1 and 2, defective FHL repressor, defective formate dehydrogenase-O and defective <i>dipZ</i>) + FhlA. (obtained from transposon mutagenesis)	This study
BW25113 <i>hyaB hybC hycA glgP fdoG::kan/pCA24N-FhlA (fdoG 6/1)</i>	\DeltahyaB , \DeltahybC , \DeltahycA , \DeltafdoG and \DeltaglgP (defective hydrogenases 1 and 2, defective FHL repressor, defective formate dehydrogenase-O and defective <i>glgP</i>) + FhlA (obtained from transposon mutagenesis)	This study
MG1655 $\Delta malT$ (MG1655 3/29)	MG1655 $\Delta malT$ (obtained from transposon mutagenesis)	This study
BW25113 <i>hyaB hybC hycA fdoG::kan/pCA24N-GpsA</i>	\DeltahyaB , \DeltahybC , \DeltahycA and \DeltafdoG (defective hydrogenases 1 and 2, defective FHL repressor and defective formate dehydrogenase-O) + GpsA	This study
BW25113 <i>hyaB hybC hycA fdoG::kan/pCA24N-DipZ</i>	\DeltahyaB , \DeltahybC , \DeltahycA and \DeltafdoG (defective hydrogenases 1 and 2, defective FHL repressor and defective formate dehydrogenase-O) + DipZ	This study
BW25113 <i>hyaB hybC hycA fdoG::kan/pCA24N-GlgP</i>	\DeltahyaB , \DeltahybC , \DeltahycA and \DeltafdoG (defective hydrogenases 1 and 2, defective FHL repressor and defective formate dehydrogenase-O) + GlgP	This study
Plasmids		
pCA24N-GpsA		(26)
pCA24N-DipZ		(26)
pCA24N-GlgP		(26)

Competent cells. Single colonies were inoculated in 25 ml of no salt LB media with/without Cm 30 µg/ml depending whether the strain had or not cloned a plasmid and incubated at 37 °C. Overnight cultures (250 µL) were grown in 250 ml of no salt LB media with/without Cm 30 µg/ml at 37 °C under aerobic conditions until turbidity at 600 nm of 0.4-0.5 were reached. Afterward, cultures (250 ml) were cooled on ice immediately after taking them out from the shaker and incubated for the next 45 min. Cultures were transferred into 250 ml pre-cooled centrifuge bottles and centrifuged at 7,000 rpm for 10 min at 4 °C. Cell pellets were re-suspended (with gentle shaking) in 250, 200 and 100 ml consecutively with 10% glycerol and then centrifuged again at 7,000 rpm for 10 min at 4 °C. The cell pellet was re-suspended in the remaining glycerol into the tube after decanting, and 50 µL aliquots of high density cells were stored in pre-cooled in 1.5 ml microcentrifuge tubes at -80 °C for future applications. Cell density was determined at 600 nm (expected ~100).

Transposon mutagenesis. Transposon mutagenesis of BW25113 *hyaB hybC hycA fdoG::kan/pCA24N-FhlA* and MG1655 strains were performed as indicated by the Epicenter EZ::TNTM <DHFR-1> TransposomeTM Kit protocol with modifications. A mix of competent cells, transposome and restriction inhibitor reagent (only for BW25113) to increase transposon insertion efficiency was prepared, followed by a short incubation on ice (15 min). The mix was transferred into an electroporation cuvette and cells were electroporated at 25 °C and 1250 volts. After electroporation, the cells were cultured in 1 ml of SOC medium (46, 56) at 37 °C for 1 h to let them grow and recover. Cultured cells (100 µL) were placed on Muller Hinton (M-H) agar plates (38) containing (Tm) 10 µg/ml + (Cm) 30 µg/ml for *E. coli* BW25113 *hyaB hybC hycA fdoG::kan/pCA24N-FhlA* strain and onto M-H agar plates with Tm 10 µg/ml for *E. coli* MG1655 strain. Plates were incubated at 37 °C overnight. Materials and reagents to conduct transposon mutagenesis and hydrogen assays for this study were prepared the day before to

perform the experiment.

Screening for enhanced hydrogen production. Single colonies obtained from transposon mutagenesis were picked onto M-H agar plates containing Tm 10 µg/ml with/without Cm 30 µg/ml depending upon the strain (for *fdoG* strain the antibiotics used were Tm and Cm and for MG1655 only Tm). Simultaneously, colonies were placed in square modified complex formate 100 mM agar plates with/without Cm 30 µg/ml, conserving the same position that in the M-H agar plates (Figure 4-2). Square plates were incubated in anaerobic chambers (made anaerobic via GAS packs catalyst anaerobic System BR0038B, Oxoid Limited, Basingstoke, Hampshire, England manufacturer) at 37 °C. After seven consecutive hours of anaerobic incubation, hydrogen production was determined through a chemochromic membrane (GVD Corp., Cambridge, MA) (32). A whatman filter paper was used to avoid direct contact of the colonies with the membrane. Chemochromic membrane was placed over the filter paper and pressed hard to remove all the air between the colonies and membrane (Figure 4-2). A blue-black coloration upon contact with the chemochromic membrane was the indicator of the presence of hydrogen. Putative transformants producer (increase or decrease) of hydrogen were chosen for further studies.

Hydrogen determination by the closed vial hydrogen assay (32). Single colonies were inoculated into 25 ml of LB media with/without Cm, based on the plasmid content of the strain, and cultured overnight at 37 °C and 250 rpm shaking. Overnight cultures (25 ml), fresh modified complex medium with formate 100 mM and with/without Cm were sparged with nitrogen for 5 minutes and sealed immediately. Glass vials (27 ml) were sealed tightly with teflon caps, and sparged with nitrogen for 2 min. Overnight cultures, fresh modified complex medium, and sealed glass vials were transferred into the anaerobic glove box. Fresh modified complex formate 100 mM (9.5 ml) and overnight culture (0.5 ml) were injected into glass vials inside the anaerobic

glove box. These vials were immediately incubated at 37 °C at 250 rpm for hydrogen determination after 1 h incubation. The amount of hydrogen generated in the head space of the glass vial was measured using 50 µL aliquots by gas chromatography using a 6890N gas chromatograph as described previously (33). Total protein (whole cells), was calculated using the turbidity at 600 nm at the end of the experiment.

Genomic DNA isolation of interesting transformants. Single colonies of identified transformants were inoculated into 25 ml LB with/without Cm 30 µg/ml and incubated overnight at 37 °C and 250 rpm. Genomic DNA was isolated using the MO BIO UltraClean™ Microbial DNA Isolation Kit (Carlsbad, CA) with modifications, followed by a DNA cleaning procedure with phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) and chloroform:isoamyl alcohol (24:1, v/v) following the standard protocol (46). DNA was precipitated, washed with 70 % (v/v) ethanol and finally incubated at -20 °C for further applications.

Sequencing. The location of the transposon insertion was determined by sequencing the genomic DNA of selected transformants using a Tn5 specific primer DHFR-1 5'-GGCGGAAACATTGGATGCGG -3' homologous to an internal sequence 63 bp before the end of the Tn5 sequence by sequencing out of the transposon and into the surrounding chromosomal DNA. Genomic DNA sequencing requires larger amounts of DNA template; therefore, a polymerase chain reaction (PCR) protocol was used in to obtain sufficient DNA. PCR reactions were subjected to a preheating start at 96 °C for 3 min, followed by incubation at 96 °C for 30 s, then annealing temperature goes down until 57 °C for 15 s to allow primer binding and then temperature goes up until reach 60 °C for 4 min while elongation occurs. This cycle from step 2 to 4 was repeated 60 times and the final product was held at 4 °C for posterior analysis.

Confirmation of mutation. Identified transformants that enhance or decrease hydrogen production were sequenced and then transposon insertions were confirmed by regular PCR by

using two sets of primers: (i) DHFR-1 5'- GGCGGAAACATTGGATGCGG -3' (forward Tn5 primer) and the designed reverse primer of the gene where Tn5 was introduced and (ii) forward and reverse primer designed of the template gene. Each PCR reaction was run at the following conditions: (i) preheating start at 95°C for 10 min, (ii) incubation at 95 °C for 1 min, (iii) primer annealing temperature of 60 °C for 30 s, (iv) elongation time at 72 °C for 2 min, (v) incubation at 72 °C for 7 min. Final product was held at 4 °C. A cycle from step 2 to 4 was conducted 33 times and the final product was kept at -20 °C.

4.4 Results

Hydrogen enhancer transformants. 461 Single transformants of BW25113 *hyaB hybC hycA fdoG::kan/pCA24N-FhlA* and 1000 transformants of MG1655 obtained from transposon mutagenesis were screened using a chemochromic membrane (Figure 4-2). Three BW25113 *hyaB hybC hycA fdoG::kan/pCA24N-FhlA* transformants (*fdoG* 4/77, *fdoG* 4/47, and *fdoG* 6/1) and one MG1655 transformant (MG1655 3/29) were selected containing beneficial or deleterious genes knocked for hydrogen production when compared with control (BW25113 *hyaB hybC hycA fdoG::kan/pCA24N-FhlA* or MG1655). After two confirmatory screens with the chemochromic membranes, transformants that enhanced and decreased H₂ synthesis were analyzed with the closed vial short hydrogen assay. It was determined that *fdoG* 4/77 decreases hydrogen production 17-fold, *fdoG* 4/47 decreases hydrogen 3-fold, *fdoG* 6/1 decreases hydrogen production 8-fold, and MG1655 3/29 increases H₂ 3.4-fold (Figure 4-3 and 4-4).

Novel genes identification and impact on hydrogen production. In an effort to determine why hydrogen production was affected by these mutations, each mutation was identified by DNA sequencing. Sequencing results were analyzed by using the BLAST database, which identified disrupted genes with 99-100 % identity by comparing the sequence results obtained with the DNA sequence of target gene.

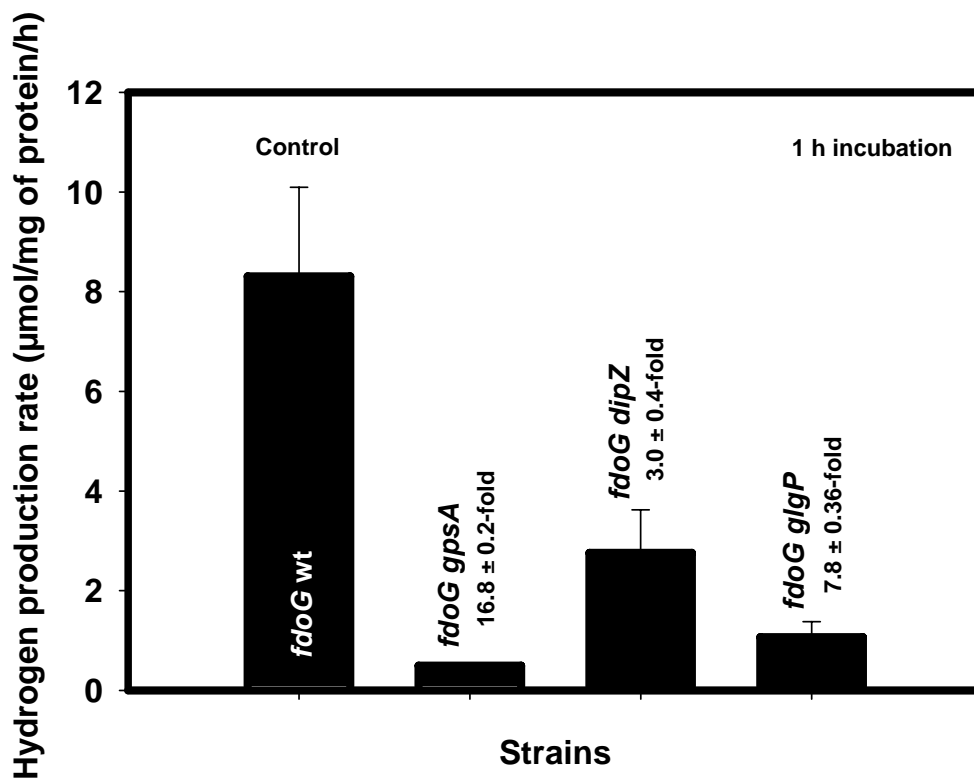


Figure 4-3. Hydrogen production rate of *fdoG* Δ *gpsA*, *fdoG* Δ *dipZ* and *fdoG* Δ *glgP* transformants created through transposon mutagenesis with Tn5. The first bar represents H₂ production of *E. coli* BW25113 *hyaB hybC hycA fdoG::kan/pCA24N-FhlA* wt strain abbreviated as *fdoG wt*; this strain was used as the control to select interesting transformants. Second bar represents the H₂ production by *E. coli* BW25113 *hyaB hybC hycA gpsA fdoG::kan/pCA24N-FhlA* (*fdoG* Δ *gpsA*) strain which decreases H₂ 16.8 fold in comparison with control. The third bar represents H₂ production by *E. coli* BW25113 *hyaB hybC hycA dipZ fdoG::kan/pCA24N FhlA* (*fdoG* Δ *dipZ*) strain which decreases H₂ 3.0-fold, and the fourth bar represents the *E. coli* BW25113 *hyaB hybC hycA glgP fdoG::kan/pCA24N-FhlA* (*fdoG* Δ *glgP*) strain which decreases H₂ 7.8-fold. All these transformants were created due to the transposition of the Tn5 at three different loci.

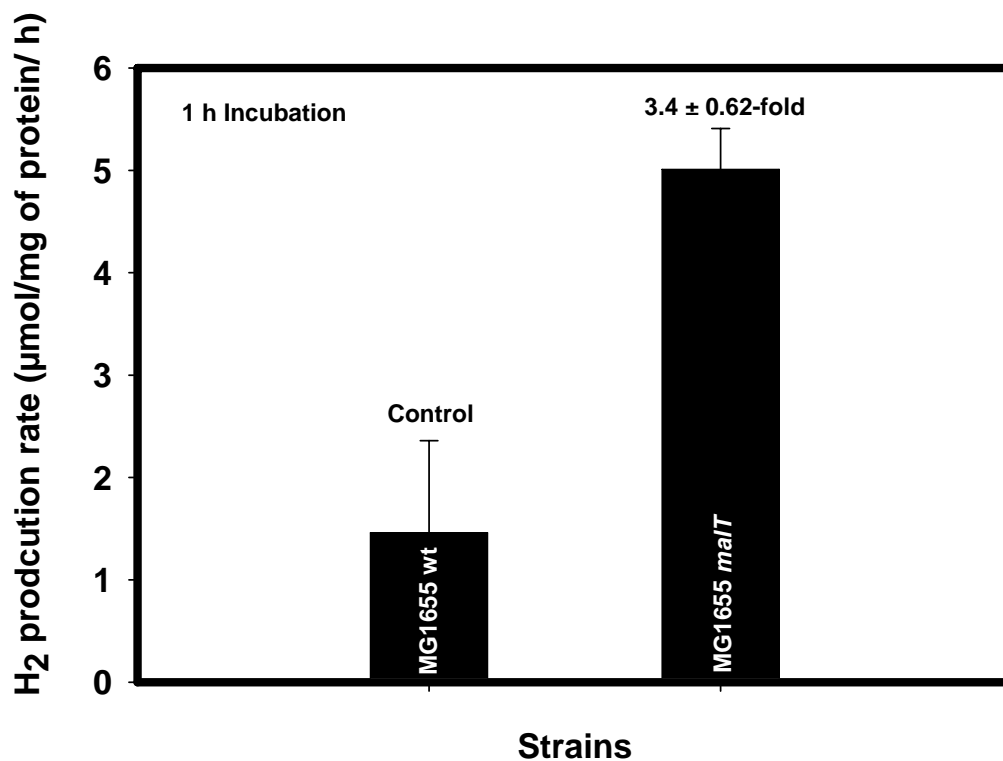


Figure 4-4. Hydrogen production rate of MG1655 wt and MG1655 $\Delta malT$ strain created throughout transposon mutagenesis by using Tn5 transposon. The first bar is the hydrogen production rate of *E. coli* MG1655 wt strain which has been used as the control to select interesting transformants and the second bar is the *E. coli* MG1655 $\Delta malT$ strain which increases H₂ production 3.4-fold due to the *malT* gene knockout.

Hydrogen synthesis was reduced in *fdoG* 4/77 (17 ± 0.2 -fold) due to Tn5 insertion in *gpsA*. *gpsA* has been reported (5) as an essential gene. Since this mutation was achieved in the lab, this gene is important but not essential. This is one of the most important findings of this study.

Furthermore, it was found that the reason hydrogen decreases 3 ± 0.4 -fold in *fdoG* 4/47 was due to the *dipZ* gene knockout and for *fdoG* 6/1 H₂ decreased (8 ± 0.36 -fold) it was due to the *glgP* disruption. Likewise, in MG1655 3/29 transformant the Tn5 insertion occurred into *malt*, which increased hydrogen production 3.4-fold when compared with MG1655 wt strain.

Confirmation of Tn5 insertion by regular polymerase chain reaction (PCR). Although the DNA sequencing results identified that Tn5 was introduced into *gpsA*, *dipZ*, *glgP*, and *malt*, we confirmed the correct size of the expected band with PCR. PCR reactions using two different set of primers for each strain (Table 4-2) established that the mutations created due to the Tn5 transposition identified by sequencing were correct and the disrupted genes were *gpsA*, *dipZ*, *glgP*, and *malt* (data not shown).

Overexpression of novel identified genes influencing hydrogen production. To study whether hydrogen production from *E. coli* bacteria could be further enhanced, genes identified with transposon mutagenesis were overexpressed (*gpsA*, *dipZ*, and *glgP*) using pCA24N vector (26). Eventhough *gpsA* and *glgP* are very important for bacteria to make hydrogen, overexpression of *gpsA* and *glgP* did not significantly improve H₂ production. However, overexpression of *dipZ* increased hydrogen production 1.8 ± 0.43 -fold (Figure 4-5). Statistical analysis using *t*-test (54) (assuming unequal variances) predicts with 95% confidence that there is not convincing evidence of significant difference between the *fdoG*/pCA24N-GpsA, *fdoG*/pCA24N-GlgP, and *fdoG*/pCA24N-DipZ strains in comparison with the *fdoG*/pCA24N-control strain, since their Pvalues are 0.5175, 0.7512, 0.2036 respectively (higher than $\alpha = 0.05$).

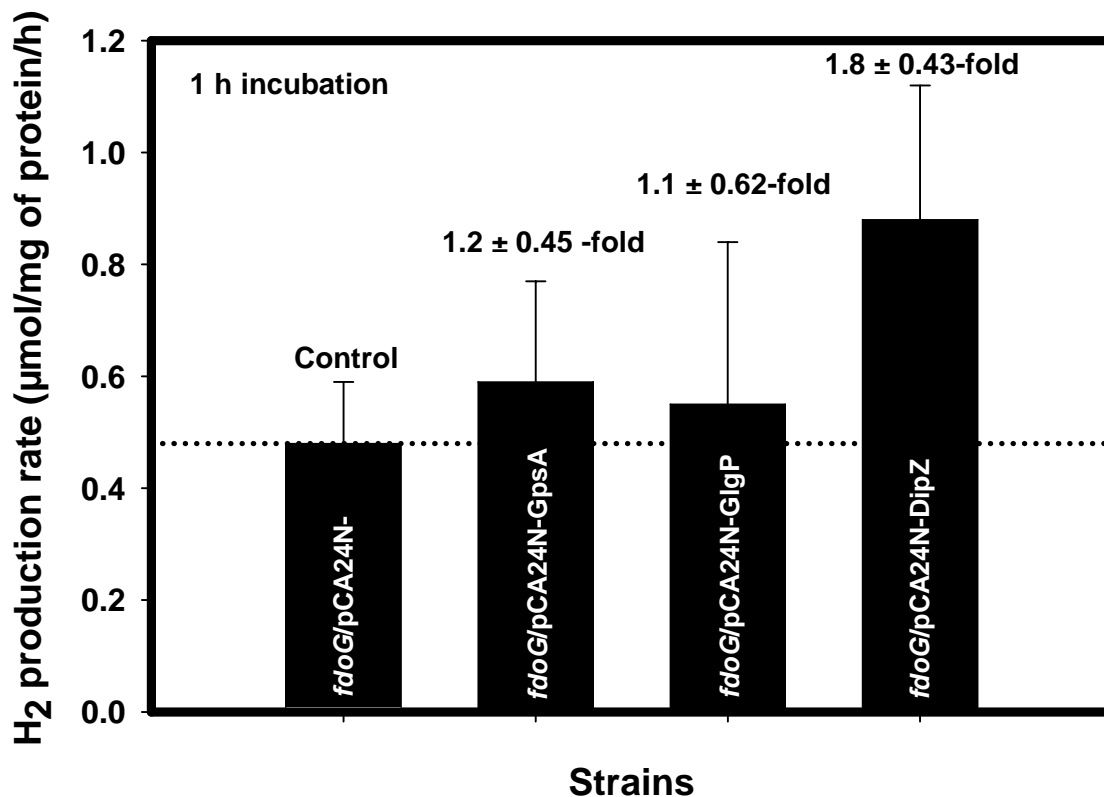


Figure 4-5. Effects on H₂ production rate when overexpressing *gpsA*, *glgP* and *dipZ* genes. The first bar corresponds to the *E. coli* BW25113 *hyaB hybC hycA fdoG::kan/pCA24N*- empty plasmid strain abbreviated *fdoG/pCA24N*- used as control. Second bar corresponds to *E. coli* BW25113 *hyaB hybC hycA fdoG::kan/pCA24N-GpsA* which increases H₂ synthesis 1.2 ± 0.45-fold, the third bar corresponds to *E. coli* BW25113 *hyaB hybC hycA fdoG::kan/pCA24N-GlgP* which remains almost the same (H₂ increase ± 0.62-fold), and the last bar corresponds to the *E. coli* BW25113 *hyaB hybC hycA fdoG::kan/pCA24N - DipZ* which aid bacteria to synthesized 1.8 ± 0.43-fold more H₂ with regards to the control strain.

Table 4-2. Primers used for verifying disrupted genes by Tn5 transposition insertion.

Primer Name	Nucleotide sequence (5' to 3')	Primer Name	Nucleotide sequence (5' to 3')
F-GpsA	CTTACCTGTACCGACAACCAGT	R-GpsA	GTAAAGTCAATGCTGCCTCGC
F- DipZ	GGCTTGTTTACCCTGCAACTCC	R- DipZ	CCTGTAGGCTGGTGATAAAGGC
F-GlgP	GTCCTTCACTGTGGCGGTTTG	R-GlgP	GTAGTCATAGCGGATGCCGTAAC
F-MalT	CACTAATCCAGTGATCCACGAGTC	R-MalT	GACTTATGGGCTGAGTGGGTATT C
DHFR-1	GGCGGAAACATTGGATGCGG		

4.5 Discussion

In the present study, it is clearly shown that transposon mutagenesis is a powerful tool to identify genes related to incompletely-studied pathways for hydrogen production. Through this study, three genes which are necessary to produce fermentative hydrogen from *E. coli* under anaerobic conditions (*gpsA*, *glgP* and *dipZ*) were identified as well as one deleterious gene (*malT*).

gpsA encodes glycerol-3-phosphate dehydrogenase (GpsA), which catalyzes the dehydrogenation of dihydroxyacetone phosphate to glycerol 3-phosphate and then glycerol 3-phosphate is further converted into glycerol (25) as shown in Figure 4.6. We hypothesize that the glycerol that is being formed as a product of this reaction becomes molecular hydrogen; therefore, when *gpsA* gene is disrupted, hydrogen is no longer synthesized from glycerol. Consequently, the hydrogen production rate decreases significantly, which is consistent with the results of hydrogen production obtained experimentally through the closed vial short hydrogen assay shown in Figure 4-3.

Additionally, glycogen phosphorylase (GlgP) breaks down glycogen (2). The glucose-1-phosphate formed is converted into glucose-6-phosphate by the action of phosphoglucomutase and enters into the glycolysis pathway is further converted into pyruvate, molecular hydrogen, and carbon dioxide as indicated in Figure 4-7. Moreover, GlgP also affects glycogen structure by removing glucose from the outer chains in *E. coli* (2); therefore, free glucose molecules created by GlgP are available for glycolysis and finally become hydrogen. A *glgP* mutant lacks glycogen phosphorylase activity (2); hence, there is less glucose available for molecular hydrogen. In agreement with this, the *hyaB hybC hycA glgP fdoG::kan/pCA24N-FhlA (fdoG 4/47)* strain which is able to produce hydrogen from glycerol produces roughly 5.6-fold more hydrogen than

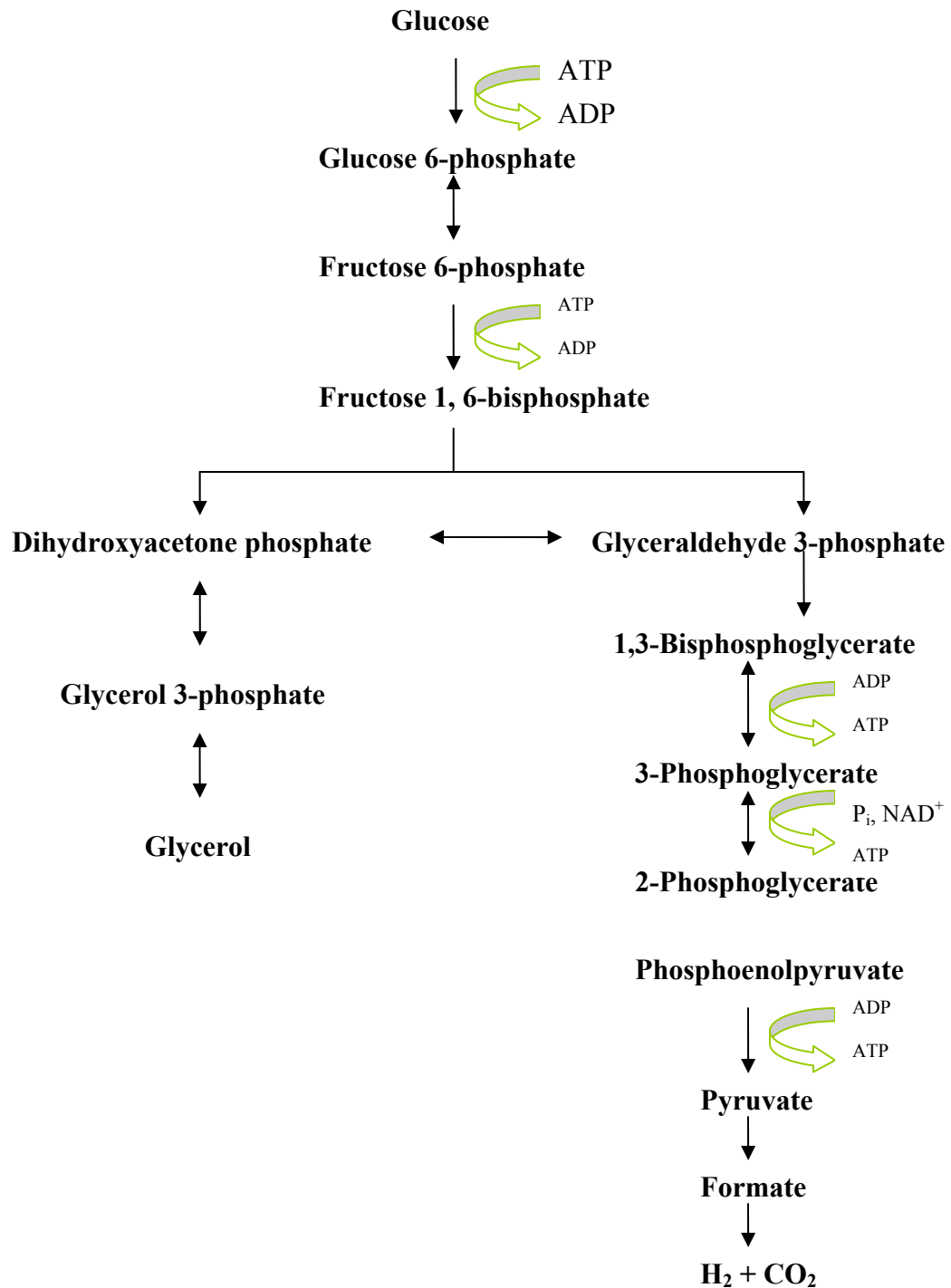


Figure 4-6. Glycerol synthesis pathway via glycerol 3-phosphate dehydrogenase activity.

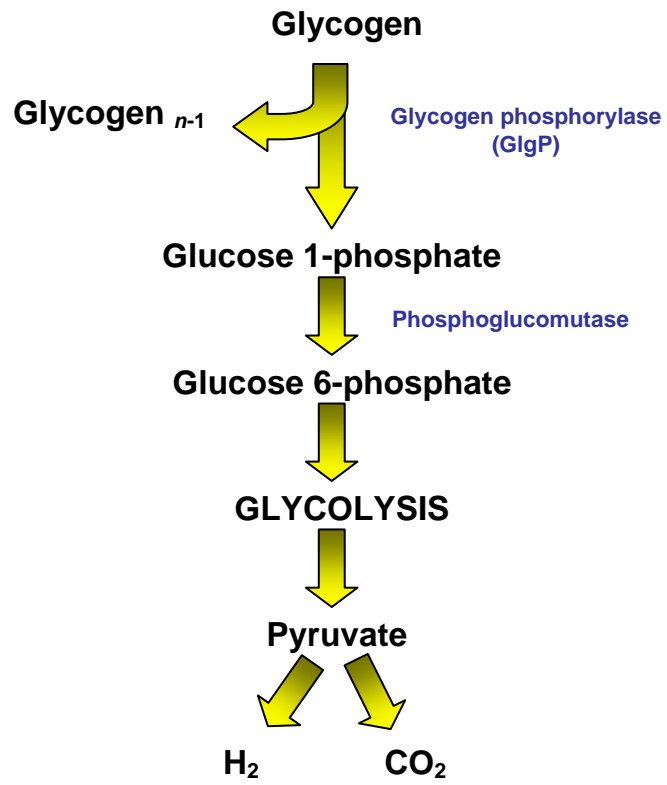


Figure 4-7. Glycogen breakdown using glycogen phosphorylase (GlgP).

hyaB hybC hycA gpsA fdoG::kan/pCA24N-FhlA (fdoG 4/77) which has a *gpsA* gene knockout. Hence, a strain lacking glycogen phosphorylase activity such as *fdoG 4/47*, does not benefit from additional glucose molecules released after glycogen breakdown; however, *fdoG 4/47* can produce hydrogen from glycerol, glucose, and formate; while, *fdoG 4/77* which is defective in *gpsA* gene cannot produce H₂ from glycerol due to the lack of the glycerol 3-phosphate dehydrogenase activity and its only source for hydrogen production would be glucose and formate. This is consistent with the results shown in Figure 4.3, since we can see the difference in hydrogen production rate between *fdoG 4/77*, *fdoG 4/47* and a *fdoG*.

As an unexpected result, *maltT* disruption in MG1655 strain causes a 3.4 ± 0.62 -fold enhancement in hydrogen production. Maltose system has been identified in previous studies to have uptake and metabolism activities of maltodextrins (glucose polymers) (10), which end up in glucose and glucose 1-phosphate (10); therefore, it would be expected that those glucose molecules continue towards glycolysis pathway. Following this, *maltT* expression should be important to obtain more glucose molecules from which molecular hydrogen can be synthesized. Nevertheless, it has been demonstrated in this work that the disruption of *maltT* through Tn5 insertion helps to increase hydrogen production. This unexpected result let us to think that there is something else happening that needs to be investigated in posterior studies.

As well as *gpsA* and *glgP*; *dipZ* also plays an important role as an electron transfer. DipZ is an inner membrane protein whose function is to keep DsbC reduced in the periplasmic space; this reduced form of DsbC protein corrects non-native disulphide bonds (57). DipZ is involved in electron transport, which occurs in the presence of a donor and an electron acceptor to couple the transference of H⁺ across a membrane. Hence, all those H⁺ that can be transported inside the cell can be further taken under the hydrogenases catalytic activity to convert single free hydrogen protons into molecular hydrogen. Consequently, when *dipZ* is disrupted as it was in the

fdoG 4/47 transformant by Tn5 transposition insertion, the free protons found in the periplasmic region cannot be additionally used to form molecular hydrogen inside the cells. This hypothesis agrees with the results found after *dipZ* gene deletion, since once *dipZ* is inactivated hydrogen production decreases 3.0-fold (Figure 4.3) after one hour incubation.

To confirm the contribution of the *gpsA*, *glgP*, and *dipZ* in hydrogen production when overexpressing, closed vial short hydrogen assay was conducted. As a result, it was found that the most significant difference between the non-overexpressing and overexpressing strains tested in this study was due to the *dipZ* overexpression. When *dipZ* gene is overexpressed, hydrogen synthesis increases 1.8 ± 0.43 -fold (Figure 4.5) which confirms a significant contribution of *dipZ* towards hydrogen formation.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

FOR FUTURE WORK

5.1 Conclusions and Recommendations

E. coli strains previously prepared in the lab BW25113 *hyaB hybC hycE::kan/pBS(Kan)-HycE* (32), and BW25113 *hyaB hybC hycA fdoG::kan/pCA24N-FhlA* were (31) the starting point of this study with the final aim of engineering new bacteria with enhanced hydrogen production by applying two different approaches that have not been attempted before for hydrogen production such as, chemical mutagenesis and transposon mutagenesis *E. coli* BW25113 *hyaB hybC hycA fdoG::kan/pCA24N-FhlA* is the best hydrogen producing bacterium to date (31).

Throughout the chemical mutagenesis chapter (Chapter II), *E. coli* BW25113 *hyaB hybC hycE/pBS(Kan)-HycE-3/86* (abbreviated 3/86) was utilized. This strain increased hydrogen 109-fold compared to BW25113 *hyaB hybC hycE/pBS(Kan)-HycE* which expresses a wild-type copy of the large subunit of hydrogenase 3. Chemical mutagen 3/86 strain was analyzed by using a whole transcriptome analysis to identify reasons for the enhancement in hydrogen production, which could elucidate novel regulatory pathways. As it expected, in the whole transcriptome analysis, we found specific genes and operon induced and repressed such as: *fruBKA* (5.0-fold up regulated) and *frlABC* (repressed -17.1, -48.7 and -24.3 respectively). Moreover, transcription of *gadE*, *gadA*, and *gadBC* were highly induced (17.1-fold, 19.7-fold and 22.6-fold, respectively). This finding indicated that *gad* induction is beneficial for hydrogen production. To follow up on this insight, BW25113 Δ *gadA* was tested to see how the *gadA* mutation affects H₂ synthesis by using two closed vial hydrogen assays. It was found that BW25113 Δ *gadA*

decreases H₂ by as much as 10 ± 0.91-fold. This result led us to conclude that *gadA* contributes to biohydrogen synthesis with *E. coli* and for us to speculate that some of the extra H₂ in 3/86 are formed by free protons taken from the media by GadA.

As with *gadA*, further studies with *fruBKA* led to the idea that the fructose metabolic pathway may be used for hydrogen productivity. *fruAB* are responsible for taking up exogenous fructose to release 1-phosphate ester into the cell cytoplasm that would be further converted into fructose-1, 6-bisphosphate by *fruK*, and then easily enters into glycolysis.

In addition, based on previous studies (31), deletion of deleterious genes and overexpression of beneficial genes was tried with the 3/86 chemical mutagen. For example *fdoG*, which encodes formate dehydrogenase O that diverts formate from hydrogen and converts formate to CO₂ (32, 31) and *hycA*, which encodes the repressor of formate hydrogen lyase complex (FHL) (32, 47), were successfully removed from the 3/86 through P1 transduction (12, 14). In addition, overexpressing *fhlA* by compatible plasmid pVLT plasmid via BW25113 *hyaB hybC hycE* 3/86 *fdoG hycA*/pBS(Kan)-HycE3/86/pVLT-FhlA increased hydrogen production rate 308-fold in regards to the starting strain (BW25113 *hyaB hybC hycE*/pBS(Kan)-HycE) control strain. Results obtained from chemical mutagenesis allow us to make three conclusions: (i) it is possible to further increase hydrogen production from a mutagenized *E. coli* bacterium by gene deletion or by overexpressing beneficial genes; (ii) chemical mutagenesis is an efficient method to make random mutations into *E. coli* genome in order to enhance hydrogen production; and (iii) whole transcriptome analysis is an important tool to identify mutations and to elucidate putative regulatory genes that can influence hydrogen synthesis (e.g., *gadA*, *gadBC*, and *fruBKA*).

Transposon mutagenesis has been demonstrated to be an efficient strategy (21) to introduce mutations into bacterial genomic DNA. It offers a significant advantage for identifying

where the mutations occurred compared to random mutagenesis techniques such as chemical mutagenesis. Applying transposon mutagenesis, four important genes were discovered related to hydrogen production: (i) *glgP* and *dipZ* contribute to molecular hydrogen formation and when they are disrupted, H₂ gas synthesis decreases 7.8 ± 0.36 and 3.0 ± 0.4 -fold respectively; (ii) *malT* deletion gene increases H₂ production 3.4 ± 0.62 -fold, and (iii) *gpsA* decreases hydrogen production 16.8-fold. Moreover, when *gpsA* is absent, viability of the bacteria decreases only a little. Even though *gpsA* has been identified as an essential gene (5), in this study, *gpsA* was shown to be not essential, since we were able to knockout *gpsA* from the bacteria genomic DNA through transposon mutagenesis with non-lethal consequences.

As an attempt to quantify how important *gpsA*, *glgP*, and *dipZ* are for hydrogen production, the proteins encoded by these genes were overproduced; the results illustrate DipZ increases hydrogen production by 1.8 ± 0.43 -fold. This result led us to conclude that electron transport proteins like DipZ may play an important role in hydrogen production.

5.2 Future Work

Since, through this research work we have identified that *gpsA*, *dipZ*, *glgP* and *malT*, are strongly related to hydrogen production in *E. coli* bacteria (Chapter III), it is important to continue investigating the best way of expressing them with our two best hydrogen producer bacteria: BW25113 *hyaB hybC hycA fdoG::kan/pCA24N-FhlA* (31) and BW25113 *hyaB hybC hycE 3/86 fdoG hycA/pBS(Kan)-HycE3/86-pVLT-FhlA* (bacterium developed in this work).

In addition, the induction of the *fru* operon and strong repression of the *frlABC* operon in chemical mutant 3/86 bacterium (Chapter II) indicates fructose metabolism should be studied in regard to hydrogen production. Similar to the *fru* operon, the regulator GadE, which activates transcription of *gadA* and other genes belonging to the acid resistance system, is interesting for further study. Since we have evidence that GadA contributes to the H₂ formation perhaps

through *gadE* regulation we can increase even more the amount of H₂ produced from *E. coli*, when engineering a bacterium with *gadA* or *gadE* overexpressed depending upon the results obtained.

REFERENCES

1. **Albert, T. J., D. Dailidienė, G. Dailidienė, J. E. Norton, A. Kalia, T. A. Richmond, M. Molla, J. Singh, R. D. Green, and D. E. Berg.** 2005. Mutation discovery in bacterial genomes: Metronidazole resistance in *Helicobacter pylori*. *Nat. Methods* **2**:951-953.
2. **Alonso-Casajus, N., D. Dauvillee, A. M. Viale, F. J. Munoz, E. Baroja-Fernandez, M. T. Moran-Zorzano, G. Eydallin, S. Ball, and J. Pozueta-Romero.** 2006. Glycogen phosphorylase, the product of the *glgP* Gene, catalyzes glycogen breakdown by removing glucose units from the nonreducing ends in *Escherichia coli*. *J. Bacteriol.* **188**:5266-72.
3. **Avalos, J., J. Casadesus, and C. E. Olmedo.** 1985. *Gibberella fujikuroi* mutants obtained with UV radiation and *N*-Methyl-*N'*-Nitro-*N*-Nitrosoguanidine. *Appl. Environ. Microbiol.* **49**:187-191.
4. **Axley, M. J., D. A. Grahame, and T. C. Stadtman.** 1990. *Escherichia coli* formate-hydrogen lyase. *The J. Biol. Chem.* **265**:18213-18218.
5. **Baba, T., T. Ara, M. Hasegawa, Y. Takai, Y. Okumura, M. Baba, K. A. Datsenko, M. Tomita, B. L. Wanner, and H. Mori.** 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: The Keio collection. *Mol. Syst. Biol.* **2**:2006.0008.
6. **Benemann, J.** 1996. Hydrogen biotechnology: Progress and prospects. *Nat. Biotechnol.* **14**:1101-1103.
7. **Berg, J. M., J. L. Tymoczko, and L. Stryer.** 2001. *Biochemistry*, 5 ed. W. H. Freeman, New York.
8. **Birkmann, A., and A. Böck.** 1989. Characterization of a cis regulatory DNA element necessary for formate induction of the formate dehydrogenase gene (*fdhF*) of *Escherichia coli*. *Mol. Microbiol.* **3**:187-195.

9. **Bohm, R., M. Sauter, and A. Bock.** 1990. Nucleotide sequence and expression of an operon in *Escherichia coli* coding for formate hydrogenylase components. *Mol. Microbiol.* **4**:231-243.
10. **Boos, W., and H. Shuman.** 1998. Maltose/maltodextrin system of *Escherichia coli*: Transport, metabolism, and regulation. *Microbiol. Mol. Biol. Rev.* **62**:204-229.
11. **Cerda-Olmedo, E., and P. C. Hanawalt.** 1968. Diazomethane as the active agent in nitrosoguanidine mutagenesis and lethality. *Molec. Gen. Genetics* **101**:191-202.
12. **Cherepanov, P. P., and W. Wackernagel.** 1995. Gene disruption in *Escherichia coli*: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* **158**:9-14.
13. **Das, D., and T. N. Veziroğlu.** 2001. Hydrogen production by biological processes: A survey of literature. *Int. J. Hydrogen Energy* **26**:13-28.
14. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U.S.A* **97**(12):6640–6645.
15. **Dawes, I. W., and B. L. A. Carter.** 1974. Nitrosoguanidine mutagenesis during nuclear and mitochondrial gene replication *Nature (London)* **250**:709-712.
16. **Gichner, T., and J. Veleminsky.** 1982. Genetic effects of N-methyl-N'-nitro-N-nitrosoguanidine and its homologs **99**:129-242.
17. **González Barrios, A. F., R. Zuo, Y. Hashimoto, L. Yang, W. E. Bentley, and T. K. Wood.** 2006. Autoinducer 2 controls biofilm formation in *Escherichia coli* through a novel motility quorum sensing regulator (MqsR, B3022). *J. Bacteriol.* **188**:305-306.
18. **Goryshin, I. Y., J. A. Miller, Y. V. Kil, V. A. Lanzov, and W. S. Reznikoff.** 1998. Tn5/IS50 target recognition. *Proceedings of the Natl. Acad. Sci. USA* **95**:10716-10721.

19. **Guerola, N., J. L. Ingraham, and E. C. Olmedo.** 1971. Induction of closely linked multiple mutations by nitrosoguanidine. *Nature (London) New Biol.* **230**:122-125.
20. **Hawkes, F. R., I. Hussy, G. Kyazze, R. Dinsdale, and D. L. Hawkes.** 2007. Continuous dark fermentative hydrogen production by mesophilic microflora: Principles and progress. *Int. J. Hydrogen Energy* **32**:172-184.
21. **Herron, P. R., G. Hughes, G. Chandra, S. Fielding, and P. J. Dyson.** 2004. Transposon express, a software application to report the identity of insertions obtained by comprehensive transposon mutagenesis of sequenced genomes: analysis of the reference for *in vitro* Tn5 transposition into GC-rich DNA. *Nucleic Acids Res.* **32**(14): e113.
22. **Hirosawa, M., M. Hoshida, M. Ishikawa, and T. Toya.** 1993. MASCOT: Multiple alignment system for protein sequences based on three-way dynamic programming. *Comput. Appl. Biosci.* **9**:161-167.
23. **Hopper, S., and A. Böck.** 1995. Effector-mediated stimulation of ATPase activity by the σ^{54} -dependent transcriptional activator FHLA from *Escherichia coli*. *J. Bacteriol.* **177**:2798-2803.
24. **Jacobi, A., R. Rossmann, and A. Böck.** 1992. The *hyp* operon gene products are required for the maturation of catalytically active hydrogenase isoenzymes in *Escherichia coli* *Arch. Microbiol.* **158**:444-451.
25. **Keseler, I. M., J. Collado-Vides, S. Gama-Castro, J. Ingraham, S. Paley, I. T. Paulsen, M. Peralta-Gil, and P. D. Karp.** 2005. EcoCyc: A comprehensive data base resource for *Escherichia coli*. *Nucleic Acids Res.* **33**:D334-D337.
26. **Kitagawa, M., T. Ara, M. Arifuzzaman, T. Ioka-Nakamichi, E. Inamoto, H. Toyonaga, and H. Mori.** 2005. Complete set of ORF clones of *Escherichia coli* ASKA library (A

Complete Set of *E. coli* K-12 ORF Archive): Unique resources for biological research. DNA research **12**:291-299.

27. **Lee, J., X.-S. Zhang, M. Hegde, W. E. Bentley, A. Jayaraman, and T. K. Wood.** 2008. Indole cell signaling occurs primarily at low temperatures in *Escherichia coli*. Int. Soc. Microbial Ecology **2**:1007-1023.
28. **Leonhartsberger, S., A. Ehrenreich, and A. Böck.** 2000. Analysis of the domain structure and the DNA binding site of the transcriptional activator FhlA. Eur. J. Biochem. / FEBS **267**:3672-3684.
29. **Maeda, T., V. Sanchez-Torres, and T. K. Wood.** 2007. Enhanced hydrogen production from glucose by metabolically-engineered *Escherichia coli*. Appl. Microbiol. Biotechnol. **77**:879-890.
30. **Maeda, T., V. Sanchez-Torres, and T. K. Wood.** 2007. *Escherichia coli* hydrogenase 3 is a reversible enzyme possessing hydrogen uptake and synthesis activities. Appl. Microbiol. Biotechnol. **76**:1035-1042.
31. **Maeda, T., V. Sanchez-Torres, and T. K. Wood.** 2008. Metabolic engineering to enhance bacterial hydrogen production. Microb. Biotechnol. **1**:30-39.
32. **Maeda, T., V. Sanchez-Torres, and T. K. Wood.** 2008. Protein engineering of hydrogenase 3 to enhance hydrogen production. Appl. Microbiol. Biotechnol. **79**:77-86.
33. **Maeda, T., G. Vardar, W. T. Self, and T. K. Wood.** 2007. Inhibition of hydrogen uptake in *Escherichia coli* by expressing the hydrogenase from the cyanobacterium *Synechocystis* sp. PCC 6803. BMC Biotechnol. **7**:25.
34. **Maier, T., U. Binder, and A. Böck.** 1996. Analysis of the *hydA* locus of *Escherichia coli*: Two genes (*hydN* and *hypF*) involved in formate and hydrogen metabolism. Arch. Microbiol. **165**:333-341.

35. **Menon, N. K., S. J. Robbin, J. S. Wendt, K. T. Shanmugam, and A. E. Przybyca.** 1991. Mutational analysis and characterization of the *Escherichia coli* *hya* operon, which encodes [NiFe] hydrogenase 1. *J. Bacteriol.* **173**:4851-4861.
36. **Miller, J. H.** 1972. *Experiments in molecular genetics.* Cold Spring Harbor, NY. Cold Spring Harbor Laboratory.
37. **Morett, E., and L. Segovia.** 1993. The sigma 54 bacterial enhancer-binding protein family: Mechanism of action and phylogenetic relationship of their functional domains. *J. Bacteriol.* **175**:6067-6074.
38. **Muller, J. H., and J. Hinton.** 1941. A protein-free medium for primary isolation of gonococcus and meningococcus. *Proc. Soc. Exp. Biol. Med.* **48**:3330-3333.
39. **Penfold, D. W., C. F. Forster, and L. E. Macaskie.** 2003. Increased hydrogen production by *Escherichia coli* strain HD701 in comparison with the wild-type parent strain MC4100. *Enzyme Microb. Technol.* **33**:185-189.
40. **Penfold, D. W., F. Sargent, and L. E. Macaskie.** 2006. Inactivation of the *Escherichia coli* K-12 twin-arginine translocation system promotes increased hydrogen production. *FEMS Microbiol. Lett.* **262**:135-137.
41. **Ren, D., L. A. Bedzyk, S. M. Thomas, R. W. Ye, and T. K. Wood.** 2004. Differential gene expression shows natural brominated furanones interfere with the autoinducer-2 bacterial signaling system of *Escherichia coli*. *Biotechnol. Bioeng.* **88**:630-642.
42. **Ren, D., L. A. Bedzyk, S. M. Thomas, R. W. Ye, and T. K. Wood.** 2004. Gene expression in *Escherichia coli* biofilms. *Appl. Microbiol. Biotechnol.* **64**:515-524.
43. **Rosentel, J. K., F. Healy, J. A. Maupin-Furlow, J. H. Lee, and K. T. Shanmugam.** 1995. Molybdate and regulation of *mod* (molybdate transport), *fdhF*, and *hyc* (formate hydrogenlyase) operons in *Escherichia coli*. *J. Bacteriol.* **177**:4857-4864.

44. **Rossmann, R., T. Maier, F. Lottspeich, and A. Böck.** 1995. Characterisation of a protease from *Escherichia coli* involved in hydrogenase maturation. *Eur. J. Biochem.* **227**:545-550.
45. **Rossmann, R., G. Sawers, and A. Böck.** 1991. Mechanism of regulation of the formate-hydrogenlyase pathway by oxygen, nitrate, and pH: Definition of the formate regulon. *Mol. Microbiol.* **5**:2807-2814.
46. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. *Molecular cloning: A laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
47. **Sanchez-Torres, V., T. Maeda, and T. K. Wood.** 2009. Protein engineering of the transcriptional activator FhlA to enhance hydrogen production in *Escherichia coli*. *Appl. Environ. Microbiol.* **75**:5639-5646.
48. **Sauter, M., R. Böhm, and A. Böck.** 1992. Mutational analysis of the operon (*hyc*) determining hydrogenase 3 formation in *Escherichia coli*. *Mol. Microbiol.* **6**:1523-1532.
49. **Sawers, G.** 1994. The hydrogenases and formate dehydrogenases of *Escherichia coli*. *Antonie van Leeuwenhoek* **66**:57-88.
50. **Schlenso, V., and A. Böck.** 1990. Identification and sequence analysis of the gene encoding the transcriptional activator of the formate hydrogenlyase system of *Escherichia coli*. *Mol. Microbiol.* **4**:1319-1327.
51. **Schlenso, V., S. Lutz, and A. Böck.** 1994. Purification and DNA-binding properties of FHLA, the transcriptional activator of the formate hydrogenlyase system from *Escherichia coli*. *J. Biol. Chem.* **269**:19597-19604.
52. **Seibert, M., T. Flynn, D. Benson, E. Tracy, and M. Ghirardi.** 1998. Development of selection and screening procedures for rapid identification of H₂-producing algal mutants with increased O₂ tolerance, p. 227-234. *In* O. R. Zaborsky (ed.), *Biohydrogen*. Plenum Press, New York.

53. **Snyder, L., and W. Champness.** 2003. Molecular genetics of bacteria, 2 ed. ASM Press, Washington, D. C.
54. **Steel, R. G. D., and J. H. Torrie.** 1960. Principles and procedures of statistics. McGraw-Hill, New York.
55. **Steiniger, M., C. D. Adams, J. F. Marko, and W. S. Reznikoff.** 2006. Defining characteristics of Tn5 Transposase non-specific DNA binding. *Nucleic Acids Res.* **34**:2820-2832.
56. **Sun, Q.-Y., L.-W. Ding, L.-L. He, Y.-B. Sun, J.-L. Shao, M. Luo, and Z.-F. Xu.** 2009. Culture of *Escherichia coli* in SOC medium improves the cloning efficiency of toxic protein genes. *Anal. Biochem.* **394**:144-146.
57. **Vertommen, D., M. Depuydt, J. Pan, P. Leverrier, L. Knoops, J. P. Szikora, J. Messens, J. C. Bardwell, and J. F. Collet.** 2008. The disulphide isomerase DsbC cooperates with the oxidase DsbA in a DsbD-independent manner. *Mol. Microbiol.* **67**:336-349.
58. **Woods, D. D.** 1936. Hydrogenlyases. The synthesis of formic acid by bacteria. *Biochem. J.* **30**:515-527.
59. **York, D., K. Welch, I. Y. Gorshin, and W. S. Reznikoff.** 1998. Simple and efficient generation *in vitro* of nested deletions and inversions: Tn5 intramolecular transposition. *Nucleic Acids Res.* **26**:1927-1933.
60. **Yoshida, A., T. Nishimura, H. Kawaguchi, M. Inui, and H. Yukawa.** 2007. Efficient induction of formate hydrogen lyase of aerobically grown *Escherichia coli* in a three-step biohydrogen production process. *Appl. Microbiol. Biotechnol.* **74**:754-760.
61. **Yoshida, A., T. Nishimura, H. Kawaguchi, M. Inui, and H. Yukawa.** 2005. Enhanced hydrogen production from formic acid by formate hydrogen lyase-overexpressing *Escherichia coli* strains. *Appl. Environ. Microbiol.* **71**:6762-6768.

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