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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Approved by:

Chair of Committee,	Thomas K. Wood
Committee Members,	Arul Jayaraman
	Kung Hui (Bella) Chu
Head of Department,	Michael Pishko

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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 *fhlA* 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 Tn*5* insertion, hydrogen production decreases 17, 3 and 8-fold, respectively. Additionally, when *malT* gene is disrupted by Tn*5* insertion, hydrogen increases 3.4-fold.

DEDICATION

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

ACKNOWLEDGMENTS

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.

I would like to extend my thanks to all my coworkers in Dr. Wood's lab who opened their hands when I needed and because together they all contribute to form a great environment for work.

In addition I would like to thank Towanna Arnold, department program coordinator, for her unconditional support and help.

Finally, I would like to thank my husband for being always with me and for his huge patience and love.

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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 overexpresing 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 σ54

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-σ54 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*
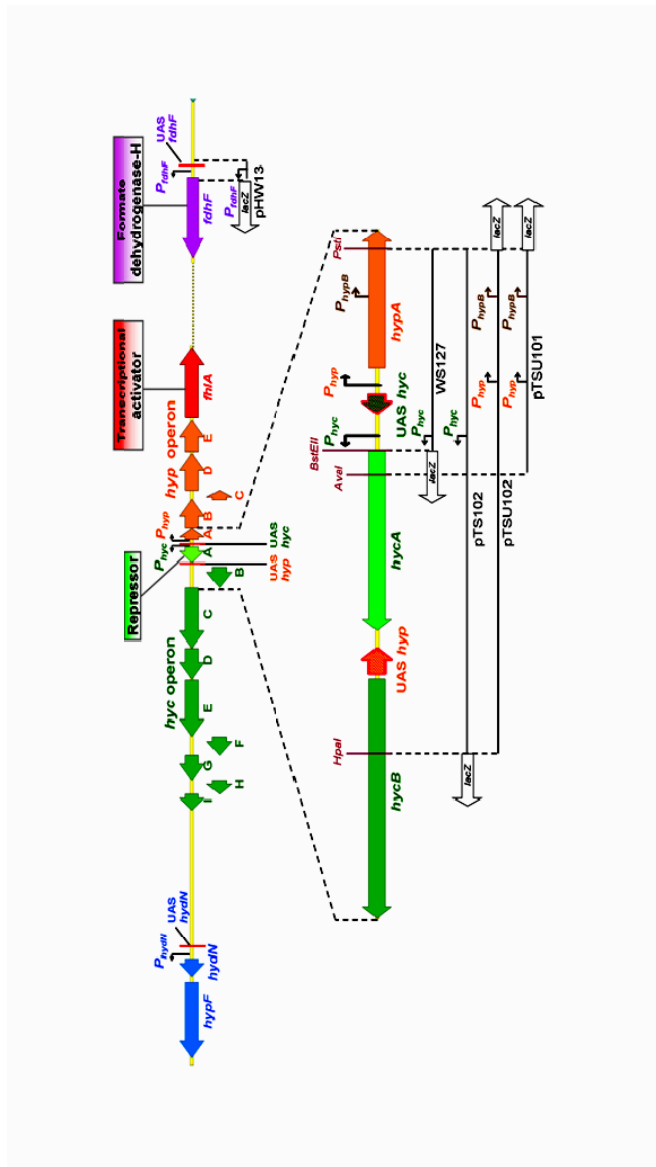
Figure 2-1. Physical map of the transcriptional units activated by FhlA. Codig regions are represented by block arrows, -12/-24 promoters are indicated by black right-angled arrows, the FNR-dependent promoter P_{hypB} is indicated by brown right-angled arrows, and the UAS where FhlA binds (8, 34, 51) 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 σ54 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 H_2 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 glycolisis 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).

Glucose

| | ATP |
| | ADP |

Hexokinase

Glucose 6-phosphate

Phosphoglucose isomerase

Fructose 6-phosphate

First stage

Phosphofructokinase

ATP

ADP

Fructose 1,6-bisphosphate

Aldolase

Glyceraldehyde 3-phosphate

Second stage

Glyceraldehyde 3-phosphate dehydrogenase

1,3-Bisphosphoglycerate

Phosphoglycerate kinase

ADP

ATP

3-Phosphoglycerate

Phosphoglycerate mutase

P_i, NAD^+

ATP

Third stage

Enolase

2-Phosphoglycerate

Pyruvate kinase

Phosphoenolpyruvate

ADP

ATP

Pyruvate

Hyd-3 + FdhF

Formate

H_2 + CO_2

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

Taken from (7) with modifications.

Fructose

Fructokinase　　　↓　ATP

ADP

Fructose 1-phosphate

Fructose 1-phosphate aldolase

Glyceraldehyde + **Dihydroxyacetone phosphate**

Tiose kinase　↓

Glyceraldehyde 3- phosphate

↓　ATP

ADP

Pyruvate

↓

$H_2 + CO_2$
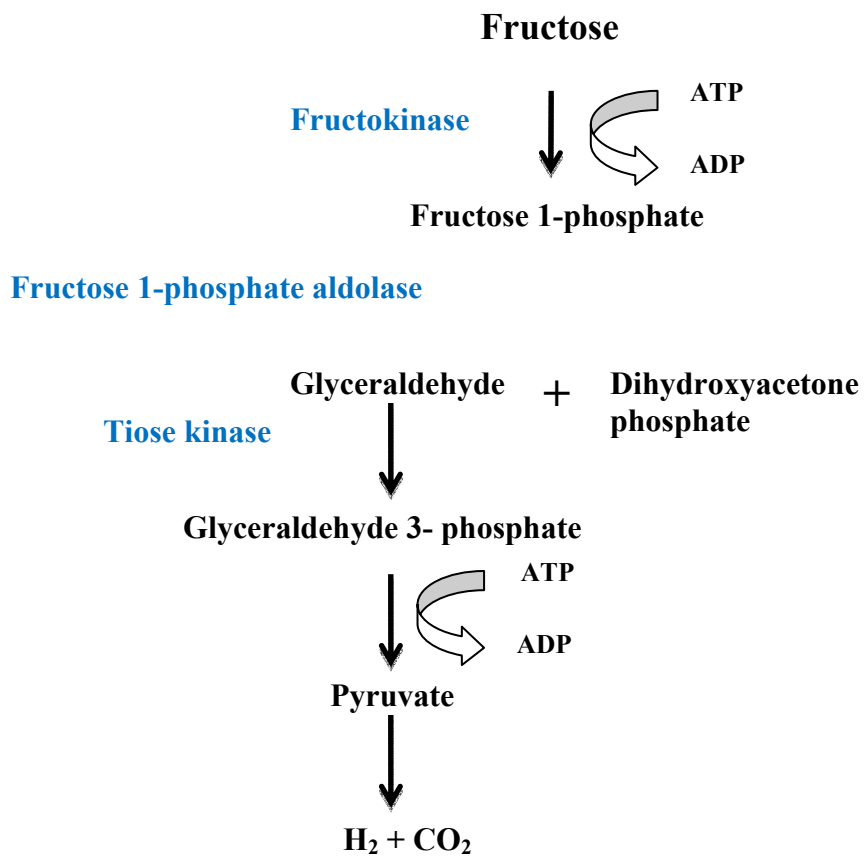
Figure 2-3.　Fructose metabolic pathway addressed towards hydrogen and carbon dioxide

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 -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.
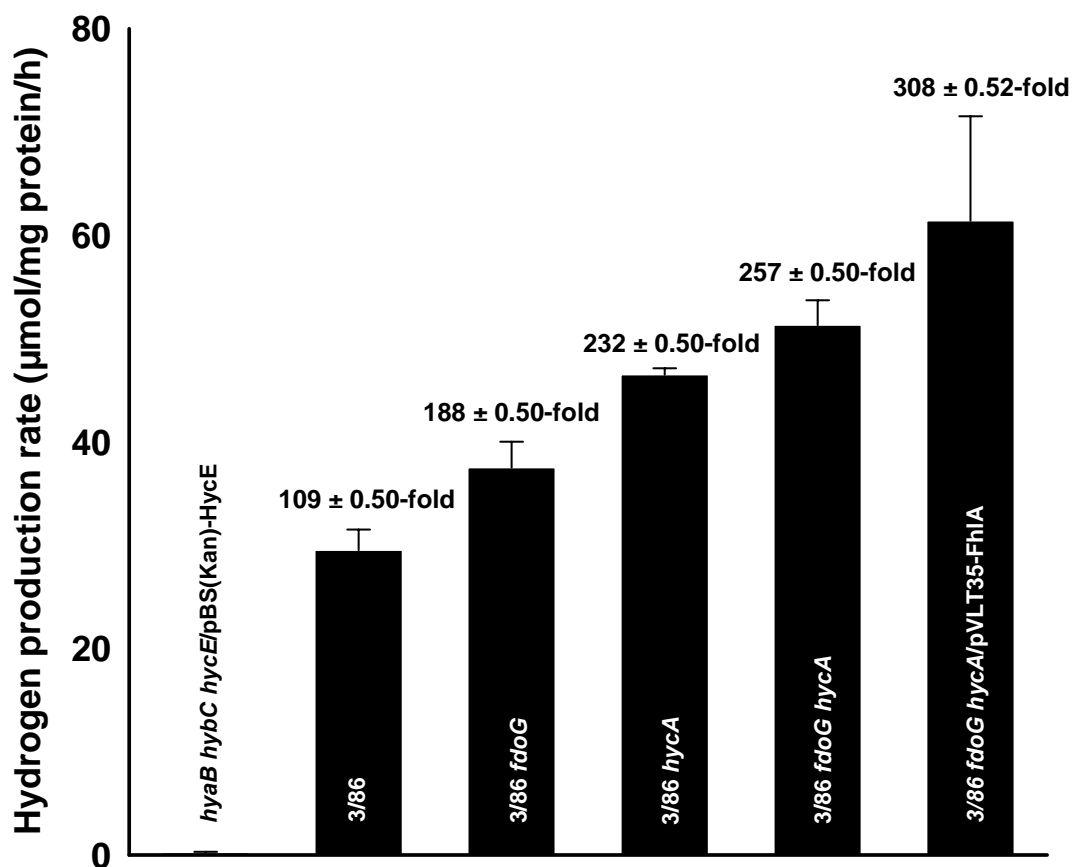
Figure 3-1. Hydrogen production by *hyaB hybC hycE::kan*/pBS(Kan)-HycE after chemical mutagenesis, deletion of *fdoG*, *hycA*, and *fdoG hycA* along with overexpression of *fhlA*. Fold changes are relative to *hyaB hybC hycE::kan*/pBS(Kan)-HycE. 3/86 strain is the chemical mutagen derived from *hyaB hybC hycE::kan*/pBS(Kan)-HycE. The conditions used for hydrogen production were: modified complex formate 100 mM medium, 37 ºC 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 °C glycerol stocks on Luria-Bertani (LB) agar plates (46) with Kan and/or spectomycin (Sp). The antibiotics concentration was 100 µg/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 (KH_2PO_4 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 *s*trains and plasmids used in this study. Kan^R, and Sp^R are kanamycin, spectomycin resistance respectively.

Strains and plasmids	Genotype	Source
Strains		
BW25113	*lacl rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	Yale CGSG Stock Center
*hyaB hybC hycE::kan/*pBS(Kan*)* HycE	Defective in large subunit of hydrogenase 1, hydrogenase 2 and hydrogenase 3, Kan^R plasmid expressing *hycE*	(31)
3/86	BW25113 *hyaB hybC hycE ::kan/*pBS(Kan)-HycE with chemical mutagenesis	This study
3/86 *fdoG*	3/86; defective in formate transporter FdoG	This study
3/86 *hycA*	3/86; defective in *hycA* repressor of FHL	This study
3/86 *fdoG hycA*	3/86; defective in formate dehydrogenase O and *hycA* repressor of FHL complex.	This study
3/86 *fdoG hycA/*pVLT-FhlA	3/86; defective in formate dehydrogenase O, *hycA* repressor of FHL complex, and overexpression of *fhlA* activator of FHL complex.	This study
Δ*gadA*	BW25113 defective in *gadA*	(4)
Plasmids		
pBS(Kan)-HycE	*pBS(Kan) pT5-lac::hycE;* expresses hycE derived from *Escherichia coli*	(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 (WO_3) 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.
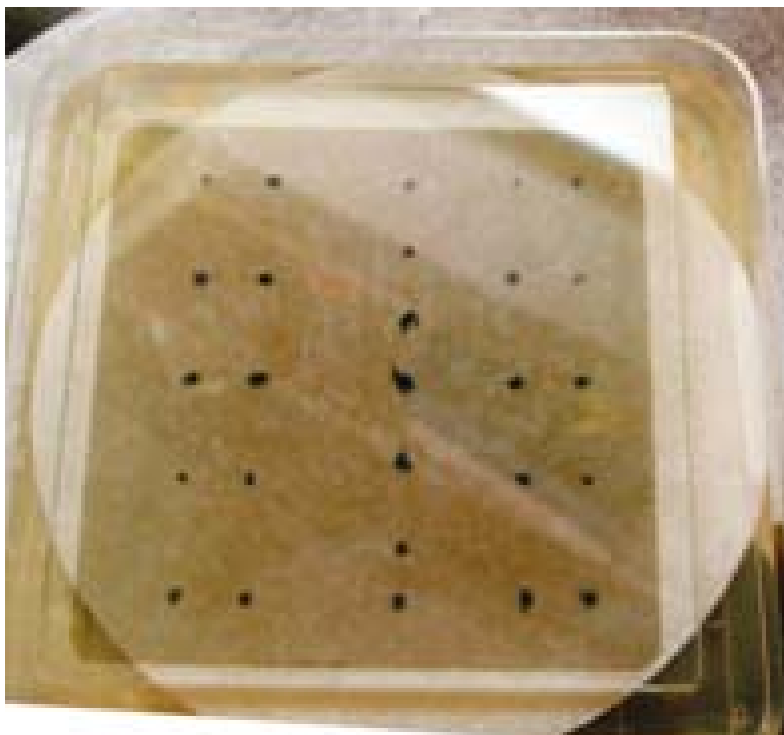
Figure 3-2. Modified complex formate agar screening plates with 100 µg/ml Kan and 0.5 mM IPTG for identifying colonies with enhanced hydrogen production. Agar plate is followed by a whatman filter to avoid direct contact of the colonies with the chemochromic membrane hydrogen detector.

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 ºC 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 ($\pm$ 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).
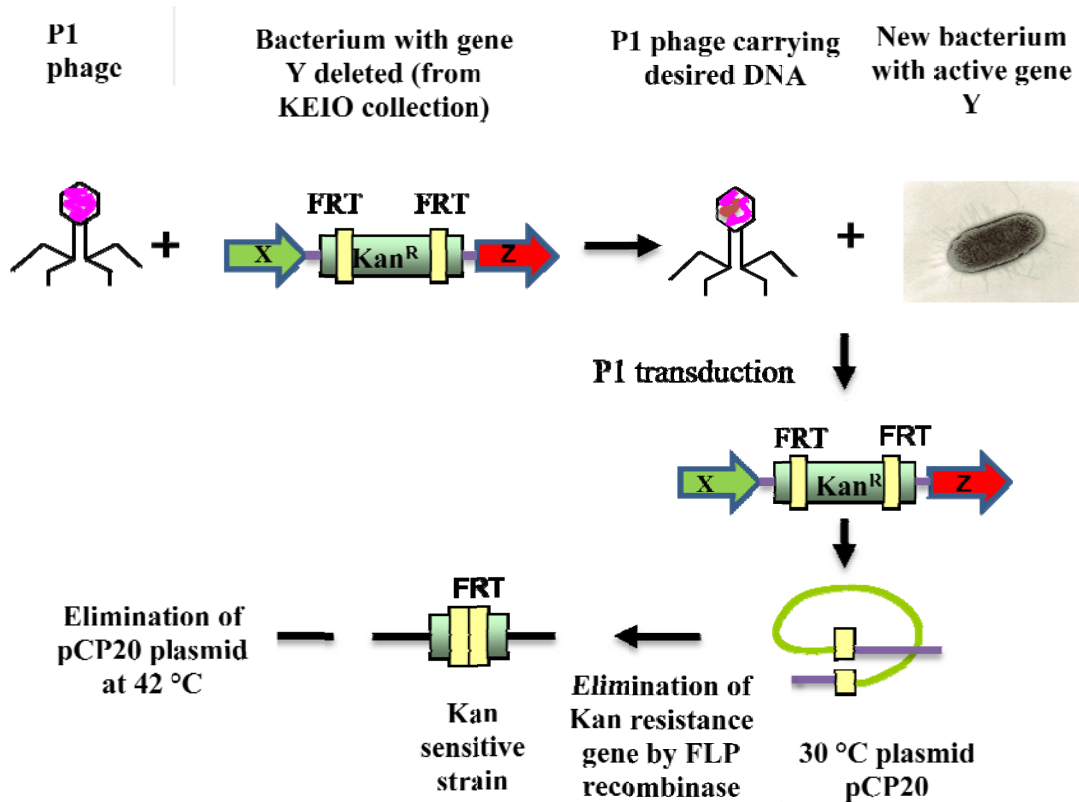
Figure 3-3. Gene deletion through P1 transduction. After construction of P1 phage that transport the DNA surrounding the Kan[R] marker of the Keio collection strain; the phage is used to infect the strain we want to engineer by transferring the deletion through homologous recombination. The Kan[R] gene is removed from the chromosome of the metabolically-engineered strain by the FLP recombinase of pCP20 at 30 ^{o}C. After elimination of the plasmid pCP20 at 42 ^{o}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 µL of TE buffer and treated with 2 µL of 100 mg/ml RNaseA (Qiagen) at 37 $^{\circ}$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 choloroform: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 ≥ 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 µmol/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 s*lp* (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), y*baS* (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			
hyaE	b0976	5.3	Required for the synthesis of active hydrogenase 1 (35).
hyaF	b0977	4.9	Nickel ion binding. The HyaF protein is required for the synthesis of active hydrogenase isoenzyme (35)
pH			
hdeB	b3509	5.7	Periplasmic acid stress chaperone protein
hdeD	b3511	8.6	Inner membrane protein required for the acid resistance phenotype
Acid resistance			
gadA	b3517	19.7	Glutamate decarboxylase activity
gadB	b1493	22.6	Glutamate decarboxylase activity
gadE	b3512	17.1	DNA binding
gadY	b4452	6.1	Small RNA that is highly expressed during entry into stationary phase and upregulated by low pH
ybaS	b0485	17.1	Glutaminase activity
yhjJ	b3527	5.7	Peptidase activity
Triptophanase activity			
tnaA	b3708	3.7	Pyruvate synthesis
tnaL	b3707	4.9	Required for regulation by attenuation of the *tnaCAB* operon
Carbon Starvation			
slp	b3506	36.8	Protect cells against effect of toxic metabolites
Sugar Transport			
fruA	b2167	4.9	Sugar porter activity
fruB	b2169	5.3	Sugar porter activity
fruK	b2168	5.7	1-phosphofructokinase activity
gntP	b4321	5.3	Sugar porter activity
Iron			
fhuF	b4367	8.0	Iron ion binding
fepD	b0590	6.1	Transporter activity
tonB	b1252	5.3	Iron ion transporter activity
yheA	b3337	5.3	Iron ion binding
fecR	b4292	4.9	Iron ion binding
feoA	b3408	4.9	Iron ion binding
Electron Transport			
nrdH	b2673	5.7	Electron transporter activity
nrdI	b2674	4.6	Protein function still unknown
trxC	b2582	4.6	Electron transporter activity
DNA Regulation			
yhiF	b3507	8.6	DNA binding
yncE	b1452	7.0	Nucleotide binding
fecI	b4293	6.1	DNA binding
ygaA	b2709	5.7	Nucleotide binding
ykfG	b0247	5.7	Predicted DNA repair protein
Biosynthesis			
aroF	b2601	8.6	3-deoxy-7-phosphoheptulonate synthase
entC	b0593	7.5	Isochorismate syntheses activity
ddg	b2378	6.5	Acyltransferase activity

Table 3-3. Continued

Gene Symbol	b #	fold change	Encoded protein
		3/86 vs. HycE wt strain	
bioA	b0774	6.1	Adenosylmethionine-8-amino-7-oxononanoate transaminase
tyrA	b2600	5.7	Chorismate mutase activity
entD	b0583	4.6	Magnesium ion binding
Cell Transport			
xasA	b1492	17.1	Amino acid-polyamine transporter activity
ybaT	b0486	13.9	Amino acid-polyamine transporter activity
yhaO	b3110	13.0	Transporter activity
yhiU	b3513	11.3	Protein transporter activity
yhiV	b3514	8.0	Transporter activity
ydhJ	b1644	6.5	Protein transporter activity
exbB	b3006	6.5	Protein transporter activity
Unknown			
ychS	*b1228*	27.9	Hypothetical protein
rhsC	b3593	7.5	Encode hydrophilic proteins
yhaM	b3109	9.8	Hypothetical protein
hokD	b1562	9.8	Small toxic polypeptide
yhiD	b3508	9.2	Putative ATP dependent transporter of the MgtC family
yhiM	b3491	8.6	Inner membrane protein
yjcB	b4060	7.5	Predicted inner membrane protein
sixA	b2340	6.1	Phosphohistidine phosphatase activity
yfeK	b2419	6.1	Hypothetical protein
yfgJ	b2510	6.1	Predicted protein
yabI	b0065	6.1	Inner membrane proteins
mokB	b1420	5.7	Hypothetical protein
flu	b2000	5.7	Biofilm formation autotransporter
pspB	b1305	5.7	Transcriptional regulator, toxin-antitoxin pair
ydiE	b1705	5.3	Conserved protein
yeaY	b1806	5.3	Encodes a putative outer membrane lipoprotein
oxyS	b4458	5.3	Plays a regulatory role in the oxidative stress response
yafW	b0246	4.9	Define a protein family of cellular toxins
yafX	b0248	4.6	Hypothetical protein
yebE	b1846	4.6	Inner membrane protein with one predicted transmembrane domain
rseB	b2571	4.6	Negative regulator of sigma E activity
sraD	b4442	4.6	Uncharacterized member of the major facilitator super family (MFS) of transporters
RNA			
rib	b4451	13.9	Appears to function in an indirect role to regulate abundance of the iron-responsive transcriptional regulator Fur at the posttranscriptional level
rib	b4417	8.0	Regulatory RNAs
IS102	b4435	7.5	IS102 encodes a small RNA of approximately 180-190 not in length
rid	b4430	7.5	Rid is a small RNA reported to be approximately 60 not
relic	b4424	4.9	Code for the antisense regulatory RNA part of a toxin-antitoxin pair
miff	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			
fdnG	b1474	-4.9	Formate dehydrogenase activity
Sugar metabolism			
frlA	b3370	-17.1	Putative transporter
yhfN (frlB)	b3371	-48.5	Sugar binding
frlC	b3372	-24.3	Physiological function is to allow the metabolism of the psicoselysine
frlC	b3373	-18.4	Ribonucleoside-diphosphate reductase activity
uid sugar metabolism			
uidA	b1617	-11.3	Hydrolase activity, hydrolyzing O-glycosyl compounds
uidB	b1616	-6.1	Transporter activity
aceK	b4016	-5.7	Nucleotide binding
yjc and yjd sugar transport			
yjcU (alsE)	b4085	-5.7	Ribulose-phosphate 3-epimerase activity
yjcV	b4086	-5.7	Transporter activity
yjcW (alsA)	b4087	-4.9	Nucleotide binding
yjcX	b4088	-9.2	Sugar porter activity
yjdK	b4128	-4.9	Predicted protein
srlA	b2702	-4.9	Sugar porter activity
Other sugar metabolism			
uxaA	b3091	-7.5	Altronate dehydratase activity
uxaC	b3092	-8.0	Glucuronate isomerase activity
Amino acid metabolism			
cadA	b4131	-7.0	Catalytic activity
cadB	b4132	-4.9	Amino acid-polyamine transporter activity
nrdA	b2234	-11.3	Ribonucleoside-diphosphate reductase activity
nrdB	b2235	-6.1	Ribonucleoside-diphosphate reductase activity
nrdD	b4238	-4.6	Catalytic activity
DNA expression regulation			
iclR	b4018	-4.6	DNA binding
rpiR	b4089	-4.6	DNA binding
Misc. enzymes			
yegT	b2098	-9.2	Transporter activity
yegU	b2099	-5.3	Hydrolase activity
glnA	b3870	-4.6	Glutamate-ammonia ligase activity
Transporters			
ompF	b0929	-7.0	Transporter activity
nupG	b2964	-5.7	Transporter activity
Unknown			
ydcX	b1445	-5.7	Predicted inner membrane protein
yeeN	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 <=> cis-aconitate + H_2O for anaerobic growth
OmpC	outer membrane porin C	64	Transport of hydrophilic solute

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

		Matched peptides		
TnaA	AceA	TufB	AcnB	OmpC
MKDYVMENFK.H	K.EWTQPR.W	R.AFDQIDNAPEEK.A	R.CGTGHLPYK.L	K.GETQVTDQLTGYG-QWEYQI QGNSAENENNSWTR.V
K.HLPEPFR.I	K.LRGSVNPECTLAQLGAAK.M	R.EHILLGR.Q	K.LLSHRSVK.R	R.VAFAGLK.F
R.VIEPVKR.T	R.GSVNPECTLAQLGAAK.M	R.AGENVGVLLR.G	K.NPPAGEEEFLL DLLTNR.V	K.FQDVGSFDYGR.N
R.AYREEAIIK.S	R.INNTFR.R	K.FESEVYILSK.D	K.GFPLAYVGDV VGTGSSR.K	R.NYGVVYDVTSWIDVLPEFG GDTYGSDNFMQQR.G
R.STYALAESVK.N	R.ADQIQWSAGHEPGDPR.Y	R.HTPFFK.G	R.IPLLIGR.G	R.GNGFATYR.N
K.KYDIPVVMDSAR.F	K.CGHMGGKVLVPTQEAIQK.L	K.GYRPQFYFR.T	R.EALGLPHSDVF R.Q	R.NTDFFGLVDGLNFAVQYQG KN
K.YDIPVVMDSAR.F	K.VLVPTQEAIQK.L	K.MVVTLIHPIAMDDGLR.F	K.KNIFSGR.I	K.NGSVSGEIGMTNGREALR.Q
K.DAMVPMGGLLCMK.D	R.LAADVTGVPTLLVAR.T		K.NIFSGR.I	K.YDANNIYLAAQYTQTYNAT R.V
R.AVEIGSFLLGR.D	R.TSEGFFR.T		K.VEQAFELTDA SAER.S	K.AQNFEAVAQYQPDFGLRPSL AYLQSK.G
K.ENAANIKGLTFTYEPK.V	R.TSEGFFR.THAGIEQAISR.G		R.VADGATVVST STR.N	K.INLLDDNQFTR.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).
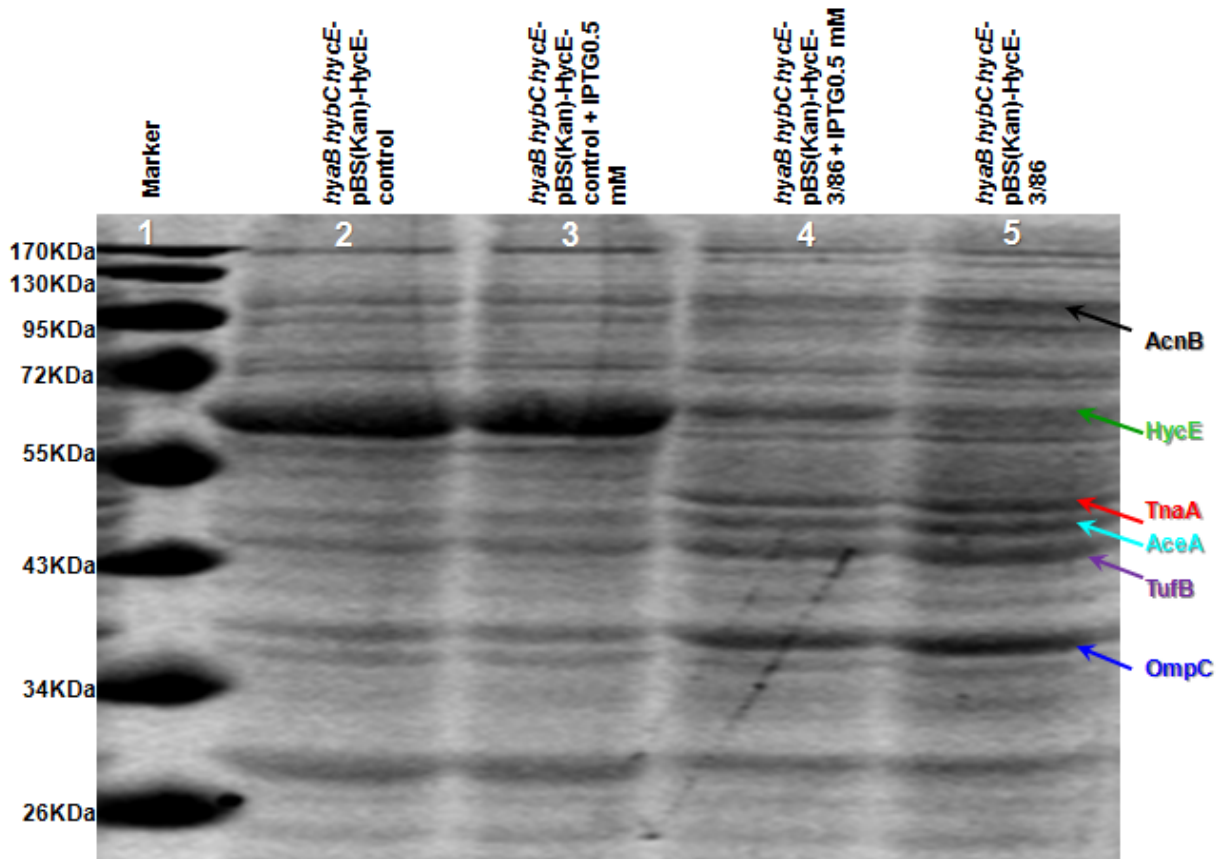
Figure 3-4.   SDS-PAGE and protein identifications with mass spectrometry analysis. The first column correspond to EZ run protein marker; the second column correspond to *E. coli* BW25113 *hyaB hybC hycE::kan*/pBS(Kan)-HycE-wt, the third column correspond to *E. coli* BW25113 *hyaB hybC hycE::kan*/pBS(Kan)-HycE-wt 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.
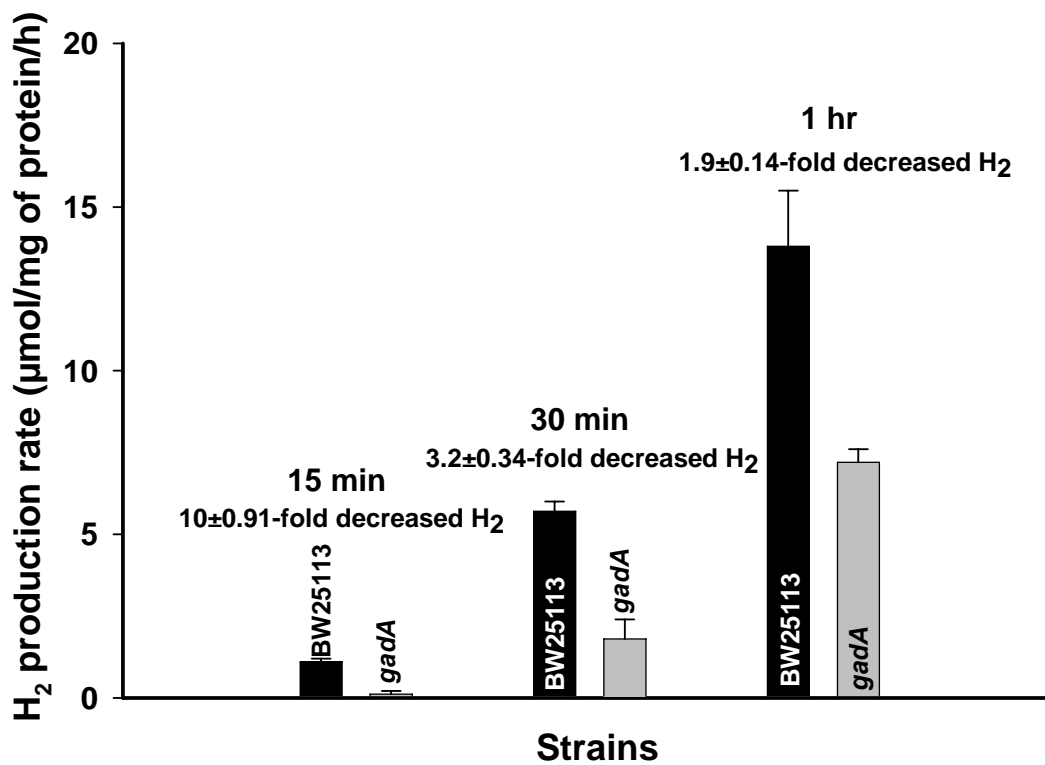
Figure 3-5. H$_2$ production rate of BW25113 and BW25113 $\Delta gadA$ after 15 min incubation (first bars set), after 30 min incubation (second bar set) and after 1 h incubation (third bars set).

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 Tn*5* insertion, hydrogen production decreases 17, 3 and 8-fold, respectively. In addition, when *malT* gene is disrupted by Tn*5* 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::TN™ <DHFR-1> Tnp Transposome Kit, cat # EZI912D manufactured by EPICENTRE Biotechnologies, via electroporation. Tn*5* is a transposon formed by two inverted insertion sequences (IS*50*) (18). The (IS*50*) region contains the Tnp transposase and at the end of each IS*50* sequence there are two 19 bp Tnp recognition sites (59), both of them, IS*50* 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::TN™ <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.

Tn*5* 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, Tn*5* is ideal for creating random mutants with high frequencies of insertion (53) due to the its ability to interact with non-specific DNA (55). Additionally, Tn*5* may be used in almost any gram-negative bacterium (53). Transposition involves a hyperactive EZ::TN™ <DHFR-1> Transposome that is
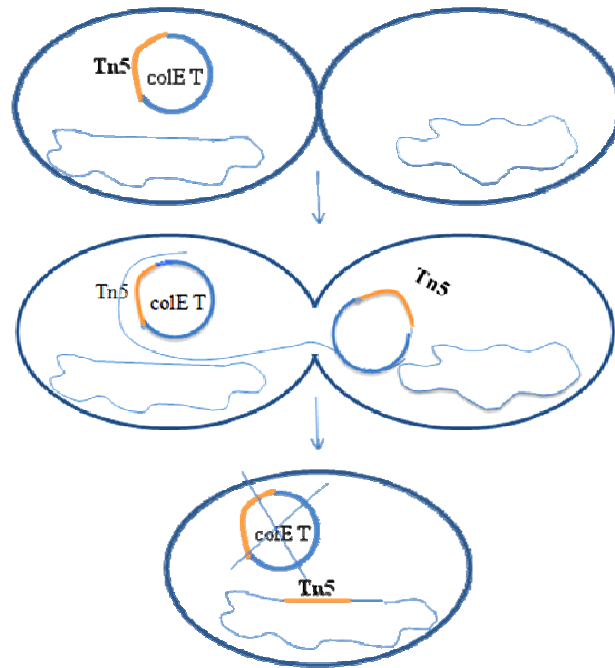
Figure 4-1.   Schematic representation of Tn*5* transposon insertion into bacterial genomic DNA through a suicide vector derived from the ColE1 plasmid. A ColE1 suicide-derived plasmid, contains the *oriT* sequence of plasmid RP4 and transposon Tn*5*. Transposon Tn*5* integrates into the genomic DNA of each cell and since the derived-suicide plasmid ColE1 cannot replicate by itself, it is lost (53).
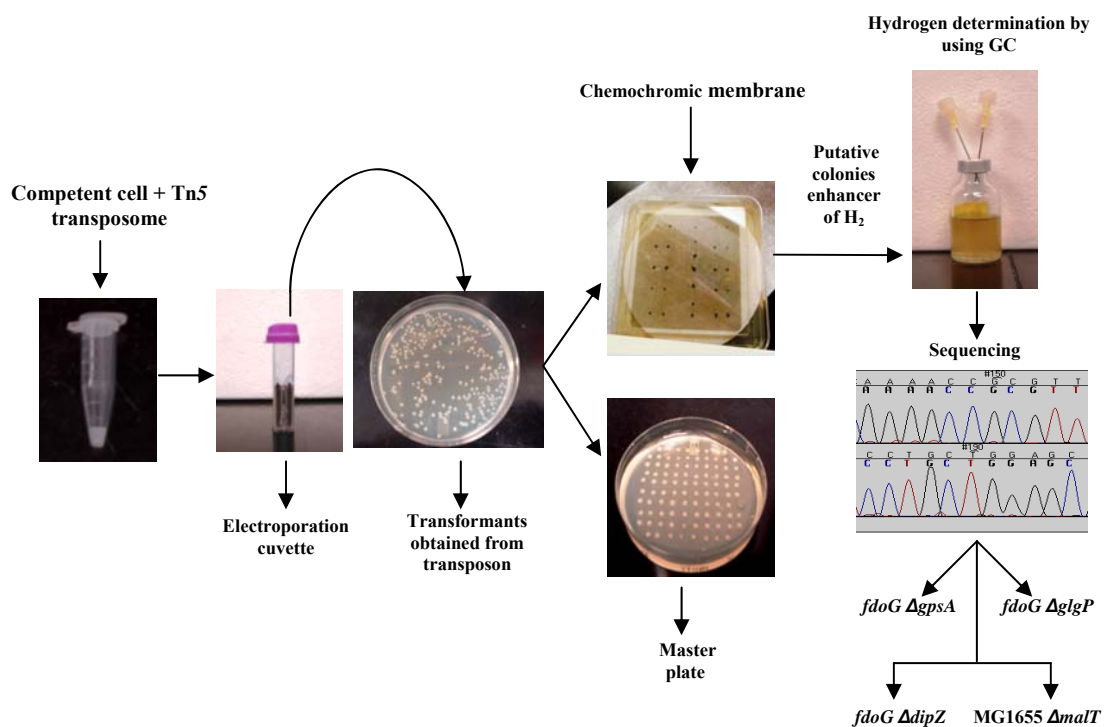
Figure 4-2.   Depiction of the whole process to perform transposon mutagenesis and how novel beneficial and deleterious genes to enhance hydrogen production were discovered in BW25113 *hyaB hybC hycA fdoG::kan*/pCA24N-FhlA and MG1655.

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::TN^{TM} <DHFR-1> Transposome^{TM} 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 H_2 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 *hyaB hybC hycA fdoG::kan*/pCA24N-FhlA	Δ*hyaB*, Δ*hybC*, Δ*hycA* and Δ*fdoG* (defective hydrogenases 1and 2, defective FHL repressor and defective formate dehydrogenase-O) + FhlA	(31)
BW25113 *hyaB hybC hycA gpsA fdoG::kan*/pCA24N-FhlA (*fdoG* 4/77)	Δ*hyaB*, Δ*hybC*, Δ*hycA*, Δ*fdoG* and Δ*gpsA* (defective hydrogenases 1and 2, defective FHL repressor, defective formate dehydrogenase-O and defective) + FhlA (obtained from transposon mutagenesis)	This study
BW25113 *hyaB hybC hycA dipZ fdoG::kan*/pCA24N-FhlA (*fdoG* 4/47)	Δ*hyaB*, Δ*hybC*, Δ*hycA*, Δ*fdoG* and Δ*dipZ* (defective hydrogenases 1and 2, defective FHL repressor, defective formate dehydrogenase-O and defective *dipZ*) + FhlA. (obtained from transposon mutagenesis)	This study
BW25113 *hyaB hybC hycA glgP fdoG::kan*/pCA24N-FhlA (*fdoG* 6/1)	Δ*hyaB*, Δ*hybC*, Δ*hycA*, Δ*fdoG* and Δ*glgP* (defective hydrogenases 1and 2, defective FHL repressor, defective formate dehydrogenase-O and defective *glgP*) + FhlA (obtained from transposon mutagenesis)	This study
MG1655 Δ*malT* (MG1655 3/29)	MG1655 Δ*malT* (obtained from transposon mutagenesis)	This study
BW25113 *hyaB hybC hycA fdoG::kan*/pCA24N-GpsA	Δ*hyaB*, Δ*hybC*, Δ*hycA* and Δ*fdoG* (defective hydrogenases 1and 2, defective FHL repressor and defective formate dehydrogenase-O) + GpsA	This study
BW25113 *hyaB hybC hycA fdoG::kan*/pCA24N-DipZ	Δ*hyaB*, Δ*hybC*, Δ*hycA* and Δ*fdoG* (defective hydrogenases 1and 2, defective FHL repressor and defective formate dehydrogenase-O) + DipZ	This study
BW25113 *hyaB hybC hycA fdoG::kan*/pCA24N-GlgP	Δ*hyaB*, Δ*hybC*, Δ*hycA* and Δ*fdoG* (defective hydrogenases 1and 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::TN™ <DHFR-1> Transposome™ 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 electrophoration cuvette and cells were electroporated at 25 ºC 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 ^{o}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 ^{o}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 ^{o}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 ^{o}C and 250 rpm. Genomic DNA was isolated using the MO BIO UltraClean^{TM} 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 choloroform: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 ^{o}C for further applications.

Sequencing. The location of the transposon insertion was determined by sequencing the genomic DNA of selected transformants using a Tn*5* specific primer DHFR-1 5'- GGCGGAAACATTGGATGCGG -3' homologus to an internal sequence 63 bp before the end of the Tn*5* 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 Tn*5*

primer) and the designed reverse primer of the gene where Tn*5* 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 H_2 synthesis were

analyzed with the closed vial short hydrogen assay. It was determined that *fdoG* 4/77 decreases

hydrogen production 17-fold, *fdoG* 4/47decreases hydrogen 3-fold, *fdoG* 6/1 decreases hydrogen

production 8-fold, and MG1655 3/29 increases H_2 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.
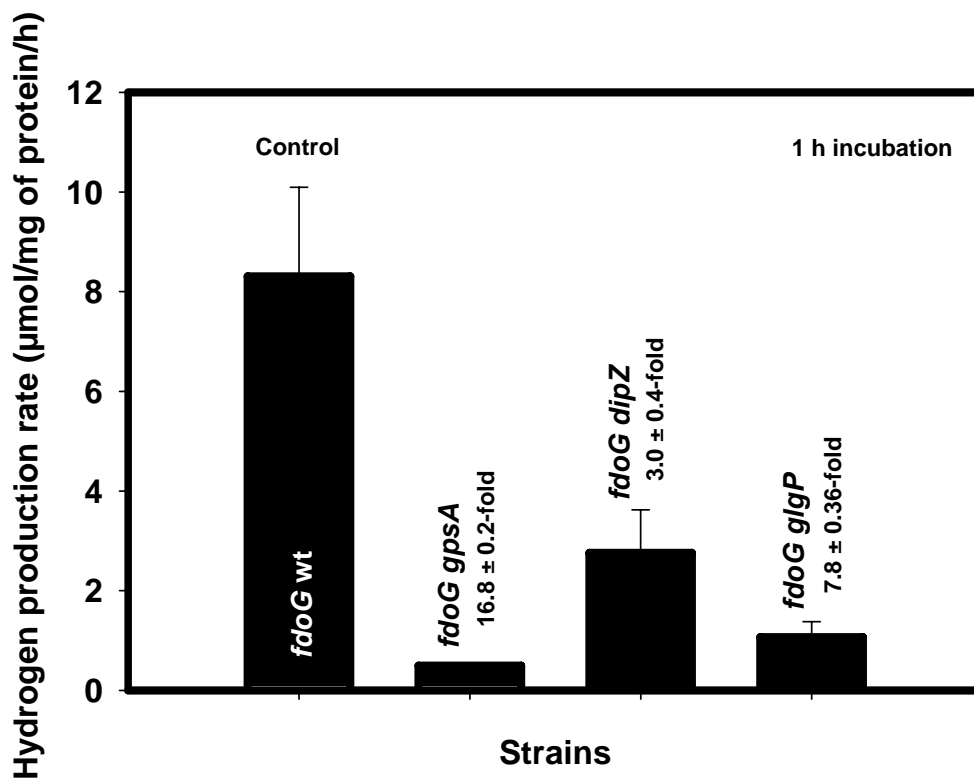
Figure 4-3. Hydrogen production rate of *fdoG* Δ*gpsA, fdoG* Δ*dipZ* and *fdoG* Δ*glgP* transformants created through transposon mutagenesis with Tn*5*. The first bar represents H$_2$ production of *E. coli* BW25113 *hyaB hybC hycA fdoG::kan*/pCA24N-FhlA wt strain abbreviated as *fdoG* wt; this strain was used as the control to select interesting transformants. Second bar represents the H$_2$ production by *E. coli* BW25113*hyaB hybC hycA gpsA fdoG::kan*/pCA24N-FhlA (*fdoG* Δ*gpsA*) strain which decreases H$_2$ 16.8 fold in comparison with control. The third bar represents H$_2$ production by *E. coli* BW25113 *hyaB hybC hycA dipZ fdoG::kan*/pCA24N FhlA (*fdoG* Δ*dipZ*) strain which decreases H$_2$ 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 H$_2$ 7.8-fold. All these transformants were created due to the transposition of the Tn*5* at three different loci.

Figure 4-4. Hydrogen production rate of MG1655 wt and MG1655 Δ*malT* strain created throughout transposon mutagenesis by using Tn*5* tran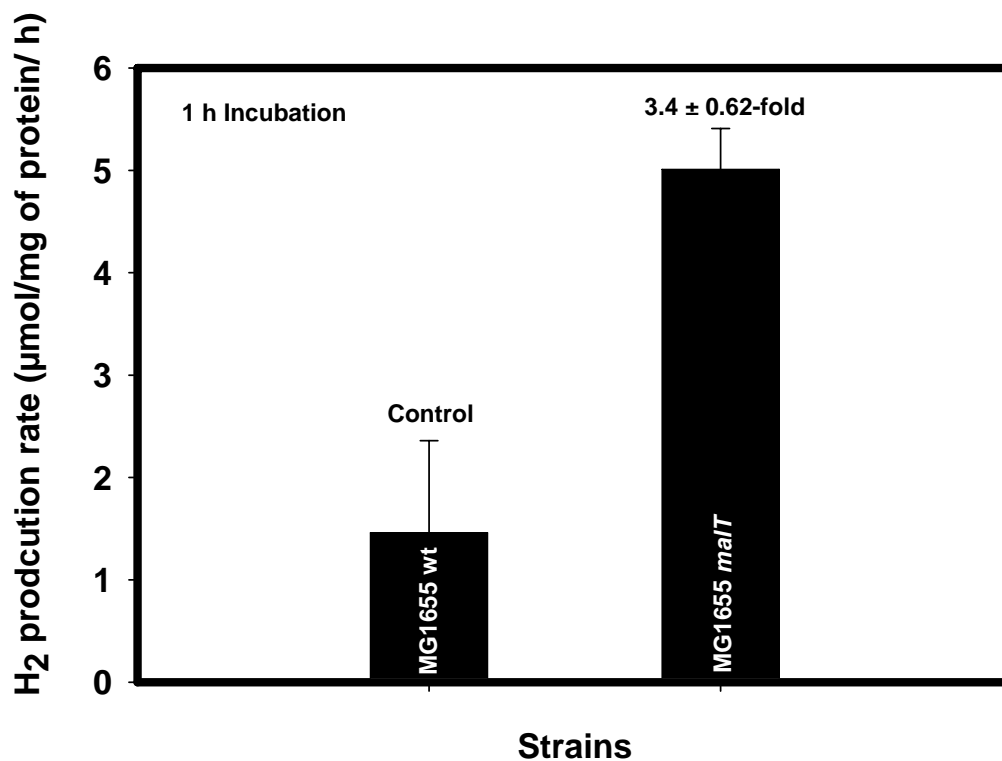sposon. The first bar is the hydrogen production rate of *E. coli* MG1655 wt strain which has been used as the control to select interesting transformants and the second bar is the *E. coli* MG1655 Δ*malT* strain which increases H_2 production 3.4-fold due to the *malT* gene knockout.

Hydrogen synthesis was reduced in *fdoG* 4/77 (17 ± 0.2-fold) due to Tn*5* 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 H_2 decreased (8 ± 0.36-fold) it was due to the *glgP* disruption. Likewise, in MG1655 3/29 transformant the Tn*5* insertion occurred into *malT*, which increased hydrogen production 3.4-fold when compared with MG1655 wt strain.

Confirmation of Tn*5* insertion by regular polymerase chain reaction (PCR). Although the DNA sequencing results identified that Tn*5* 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 Tn*5* 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 H_2 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 α = 0.05).
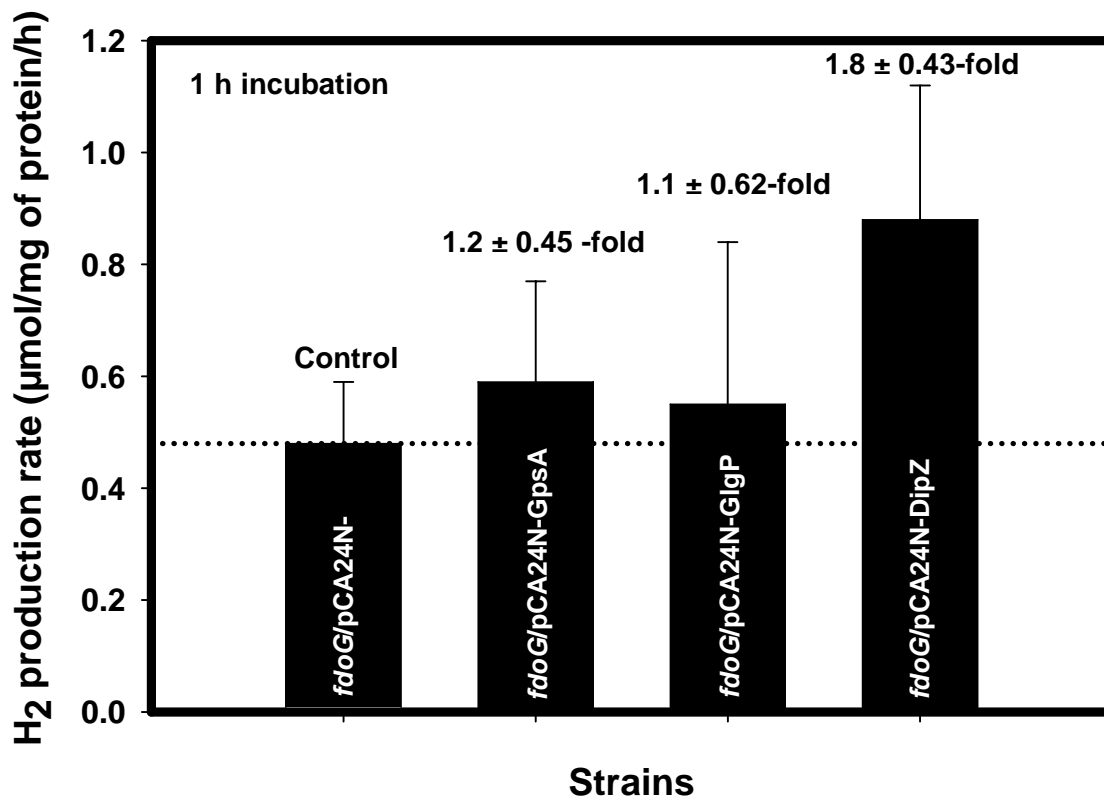
Figure 4-5. Effects on H_2 production rate when overexpressing *gpsA*, *glgP* and *dipZ* genes. The first bar corresponds to the *E. coli* BW25113 *hyaB hybC hycA fdoG::kan*/pCA24N- empty plasmid strain abbreviated *fdoG*/pCA24N- used as control. Second bar corresponds to *E. coli* BW25113 *hyaB hybC hycA fdoG::kan*/pCA24N-GpsA which increases H_2 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 (H_2 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 H_2 with regards to the control strain.

Table 4-2. Primers used for verifying disrupted genes by Tn*5* 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	GACTTATGGGCTGAGTGGGTATTC
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 phophoglucomutase 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

Glucose

ATP

ADP

Glucose 6-phosphate

Fructose 6-phosphate

ATP

ADP

Fructose 1, 6-bisphosphate

Dihydroxyacetone phosphate ⟷ **Glyceraldehyde 3-phosphate**

Glycerol 3-phosphate

1,3-Bisphosphoglycerate

ADP

ATP

Glycerol

3-Phosphoglycerate

P_i, NAD^+

ATP

2-Phosphoglycerate

Phosphoenolpyruvate

ADP

ATP

Pyruvate

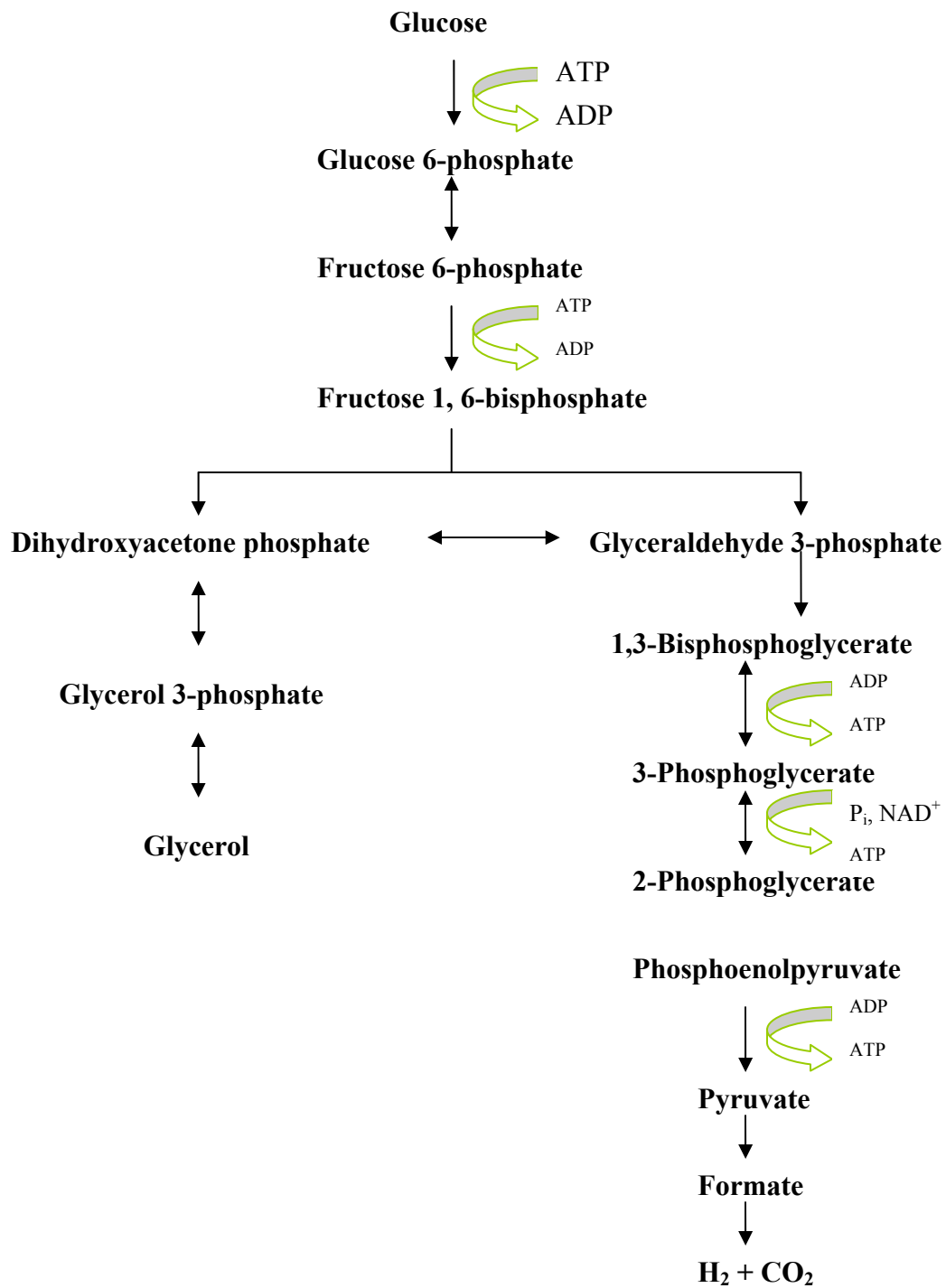**Formate**

$H_2 + CO_2$

Figure 4-6.   Glycerol synthesis pathway via glycerol 3-phosphate dehydrogenase activity.
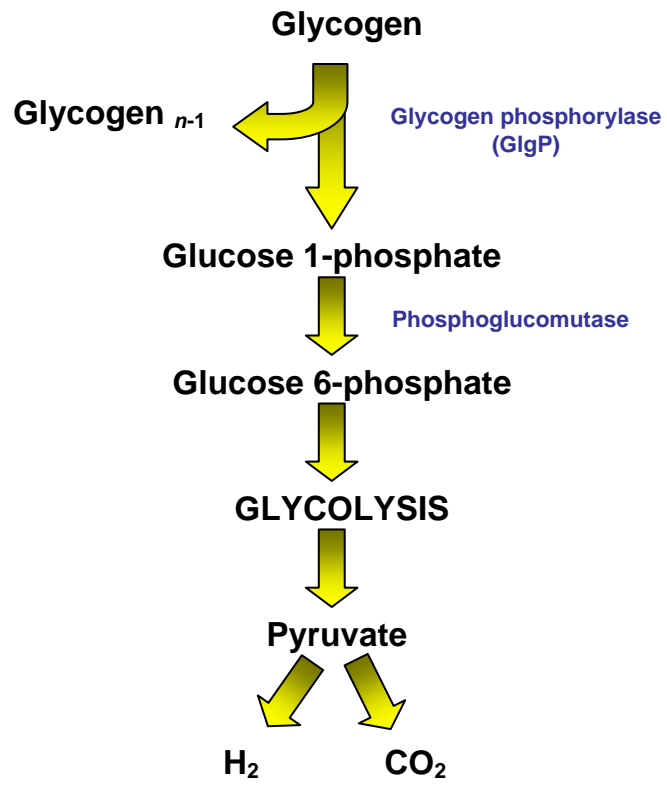
Figure 4-7.   Glycogen breakdown using glycogen phosphorylase (GlgP).

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 H_2 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, *malT* 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, *malT* 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 *malT* through Tn*5* 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 Tn*5* 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* overepression. 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, BW25113Δ*gadA* was tested to see how the *gadA* mutation affects H_2 synthesis by using two closed vial hydrogen assays. It was found that BW25113 Δ*gadA*

decreases H_2 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 H_2 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 CO_2 (32, 31) and *hycA*, which encodes the repressor of formate hydrogen lyase complex (FHL) (32, 47), were successfully removed from the 3/86 though 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 form 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, H_2 gas synthesis decreases 7.8 ± 0.36 and 3.0 ± 0.4-fold respectively; (ii) *malT* deletion gene increases H_2 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 H_2 formation perhaps

through *gadE* regulation we can increase even more the amount of H$_2$ produced from *E. coli,* when engineering a bacterium with *gadA* or *gadE* overexpressed depending upon the results obtained.

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