Evaluation of the Role of *gsp*, *ygiC,* and *yjfC* Genes in Glutathione Metabolism in *Escherichia coli* by Gene Disruption

by

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Abstract

Glutathione metabolism differs amongst humans and bacteria and has the potential to be a good target for antibacterial agents. Within *Escherichia coli*, glutathione can form an amide bond with spermidine to produce the conjugate glutathionylspermidine. This reaction is catalyzed by the enzyme glutathionylspermidine synthetase/amidase. Three genes, *gsp*, *ygiC* and *yjfC* have previously been identified and proposed to be associated with glutathionylspermidine metabolism; however, the specific function of *ygiC* and *yjfC* has yet to be determined. The objective of this research is to eliminate these genes from the *E. coli* genome and evaluate the responsiveness of the knockout strains to various stress conditions. Corresponding genes were replaced by antibiotic resistance genes through homologous recombination. Once the gene disruptions had been achieved, the antibiotic resistance gene was eliminated utilizing a helper plasmid pCP20. The techniques used to create gene disruptions, or knockouts, included polymerase chain reaction and electroporation. Seven strains have been created: single knockout (*gsp, ygiC*, or *yjfC*), double knockout (*gsp/ygiC, gsp/yjfC*, or *ygiC/yjfC*), and triple knockout (*gsp/ygiC/yjfC*). The sensitivity of the knockout strains were tested by stressors including antibiotics, transition metals, and hydrogen peroxide.

Acknowledgements

I would like to thank Dr. Nina Stourman for being my advisor. She has directed my research with the upmost interest and patience. She has shown me how to conduct my research knowledgeably, professionally, and logically. Her hard work and dedication has inspired me to develop and learn the work ethic needed to complete this work in a timely manner. I would also like to thank my committee members Dr. Jonathan Caguiat and Dr. Micheal Serra who besides accepting to be on my committee have allowed me to use equipment throughout my research. Without them my research would not be possible.

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Table of Abbreviations

E. coli	*Escherichia coli*
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
H_2O_2	Hydrogen Peroxide
O_2^-	Superoxide
OH·	Hydroxyl Radical
O_3	Ozone
GSH	Glutathione
Glu	Glutamic Acid
Gly	Glycine
Cys	Cysteine
His	Histidine
GSp	Glutathionylspermidine
kb	Kilobase
kDa	Kilodaltons
ATP	Adenosine Triphosphate
ADP	Adenosine Diphosphate
$O.D._{600}$	Optical Density at 600 nm
cm	Centimeters
nm	Nanometers
ng	Nanograms
µL	Microliters
mL	Mililiters
^0C	Degrees Celcius
sec	Seconds
min	Minutes
Am	Ampicillin
Cm	Chloramphenicol
Kan	Kanamycin
Tet	Tetracycline
TAE	Buffer containing Tris base, acetic acid and EDTA
PCR	Polmerase Chain Reaction
SOC	Super Optimal Broth with Catabolite Repression
SOB	Super Optimal Broth
LB	Luria-Bertani medium
bp	Base Pairs
V	Volts

Chapter 1: Introduction

Escherichia coli

Escherichia coli (E. coli) are important bacteria for both human life and research. *E. coli* is a gram negative facultative anaerobe that can grow either aerobically or anaerobically. The bacteria can also grow over a very wide range of pH. These qualities make *E. coli* easy to grow under different conditions and favorable to study.[1]

In humans, *E. coli* is found in the intestinal tracts. In the tracts, *E. coli* uses amino acids and peptides that are acquired from food. If the bacteria becomes overpopulated, it will intake more of these nutrients than the host causing serious health problems.[1] The bacterium causes several diseases including urinary tract infections, new born meningitis, and septicemia.[2] One concern within the medical field is that drug resistance has become transferable from bacteria to bacteria.[3] Because of this phenomenon, drug resistant strains of some bacteria, including *E. coli*, have been found in nonselective environments. This introduces new problems for health professionals and requires new medicines to combat pathogenic strains of bacteria.[3]

Oxidative Stress

Oxidative stress within a cell is the result of excess oxidants. There are a number of different oxidants found in biological systems. Some of the more common are hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl radicals ($OH\cdot$), and ozone (O_3). Oxidative stressors are extremely toxic to cells and are responsible for several diseases including rheumatoid arthritis, inflammatory bowel disorder, and atherosclerosis among others.[4] Beyond causing specific diseases, oxidative stressors cause damage to

deoxyribonucleic acid (DNA), ribonucleic acid (RNA), proteins, and lipids. The damage can ultimately lead to cell death. Of the oxidative stressors, the hydroxyl radical is one of the most reactive species.[4]

The reactivity of a certain oxidative species can be predicted by comparing the standard electrode potential of the species.[4] The higher the standard electrode potential of a species, the more reactive that species will be. The hydroxyl radical (standard electrode potential approximately 2.3 V)[4] has a much higher potential than most other species making it more reactive than others. The hydroxyl radical is so reactive it will oxidize nearly any molecule that it is in contact with, with the exception of ozone. The O_2^- species will typically react with olefins, more specifically with conjugated systems. Hydrogen peroxide is not a radical but can be converted to the hydroxyl ion and the hydroxyl radical.[4]

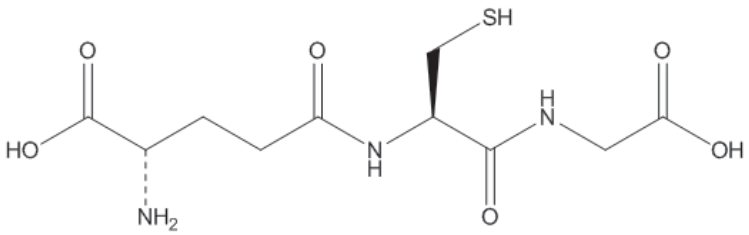
Oxidative stressors are naturally created within cells as byproducts of reactions between many different biological compounds. They can also be acquired via other intracellular or extracellular sources. Each oxidant can be generated by autoxidation, the spontaneous formation of oxidants without the use of a catalyst. The superoxide anion can be generated via paraquat, plumbagin, ubiquinone, menadione, tert-butyl hydroperoxide, and cumene hydroperoxide.[4] Several of these molecules, referred to as redox-cycling agents can go through a cycling process that will continue producing the superoxide anion. There are several enzymes that will generate radicals in *E. coli*. These enzymes include NADH dehydrogenase, succinate dehydrogenase, D-lactate

dehydrogenase, and glutathione reductase.[4] Several of these enzymes catalyze reactions of the citric acid cycle or glycolysis.

Avoiding production of oxidative species is nearly impossible. Therefore, cells need to be able to convert the oxidants to other harmless compounds. Under aerobic conditions, *E. coli* has two specific superoxide dismutases. One is Mn-containing, while the other is Fe-containing. Each of these generates H_2O_2 from O_2^-, which is further degraded to H_2O and O_2 by catalase.[4] Although oxidative stress is harmful, organisms have metabolic pathways to relieve them from stress. One of the most prominent pathways to relieve the stress is through the use of glutathione.[4]

Glutathione

Glutathione (GSH) is a tripeptide of Glu-Cys-Gly (Figure 1-1). It is a major thiol compound with a low molecular mass found in nearly all biological cells. GSH is not toxic to the cell and can be used by cells to maintain a thiol/disulfide redox potential by acting as a buffer.[5] In this case, glutathione acts as a nucleophile by attracting electrophilic centers of many compounds.[5] Glutathione has the ability to reduce hydrogen peroxide via a redox reaction, where glutathione acts as the reducing agent.[6] In the reaction, oxygen can act as a hydrogen acceptor and use the hydrogen atom bonded to sulfur in GSH to generate water. Once two GSH molecules have been oxidized, the two deprotonated GSH molecules can form a disulfide bond. Oxidized glutathione can then get reduced back to reduced GSH by the enzyme glutathione reductase.[6]



Glutathione (GSH)

Figure 1-1. Structure of the tripeptide glutathione

In *E. coli*, glutathione is a major metabolite. The concentration of GSH changes directly due to osmotic pressure.[7] Glutathione regulates the potassium release channels within the cytosol of the bacteria. Because glutathione is responsible for this, it aids in *E. coli's* ability to grow in anaerobic conditions. Glutathione is also responsible for detoxification of the superoxide oxidant within the bacteria. The detoxification is thought to be possible via a redox reaction producing oxidized glutathione (Figure 1-2).[7]

$$R^{\bullet} + GSH \leftrightarrow RH + GS^{\bullet}$$

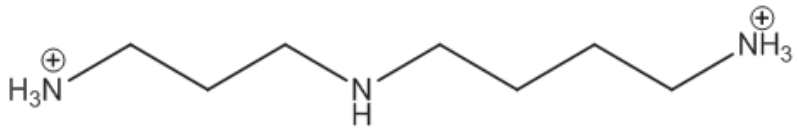
$$GS^{\bullet} + GS^{-} \leftrightarrow GSSG^{\bullet -}$$

Figure 1-2. Reaction scheme of reduced glutathione to oxidized glutathione. (Adapted from ref 8)

Spermidine

Spermidine, a polyamine found in most mammalian cells and in *E. coli,* is polycationic (Figure 1-3) and can react with several biological molecules including proteins, phospholipids, and nucleic acids.[9,10] Polyamines are important compounds for the growth and health of an organism. Spermidine functions within cells as a charge

neutralizer and can form a complex with the phosphodiester backbone of DNA. Polyamines can be generated via an addition of aminopropyl groups. Spermidine, a molecule with two terminal amino groups and one secondary amine group, can undergo further addition with an aminopropyl group to generate spermine.[9]



Spermidine

Figure 1-3. Structure of the polyamine spermidine.

Synthesis of Glutathionylspermidine from Glutathione and Spermidine

Glutathionylspermidine (GSp) is a compound composed of a glutathione molecule and a spermidine molecule.[11] Some organisms, such as *E. coli* and other gram negative bacteria are dependent on GSP conjugates. This is supported by three observations. First, they lack catalases and peroxidase hemoproteins that defend other organisms such as *Trypanosoma* and *Leishmania* from oxidants. Second, a large amount of their glutathione is used to produce the glutathionylspermidine conjugate. Third, the normal glutathione peroxidase-glutathione reductase enzyme couple is replaced with a trypanothione metabolic pathway in *E. coli*.[11]

In organisms such as *Trypanosoma* and *Leishmania*, the production of glutathionylspermidine is one of the last steps in the process generating trypanothione.

These organisms are dependent on the metabolism of trypanothione instead of that of glutathione.[12] *E. coli however*, are not dependent on the generation of trypanothione for defense but might utilize the synthesis of glutathionylspermidine. In this reaction, the C-carboxyl group of the glycine residue of glutathione forms an amide bond with the N^1-amino group of spermidine. The reaction is catalyzed by the enzyme glutathionylspermidine synthetase/amidase (Figure 1-2).[13]

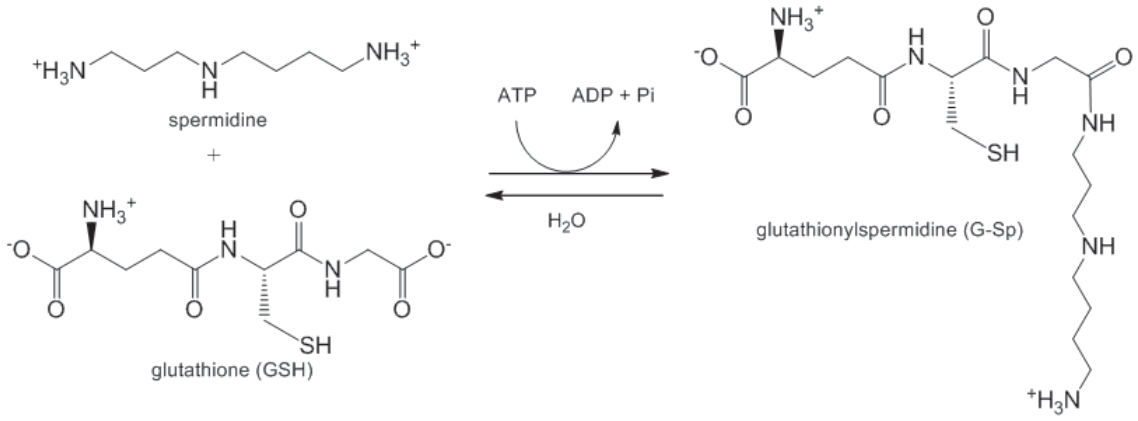


Figure 1-2. Reaction catalyzed by bifunctional enzyme glutathionylspermidine synthetase/amidase. (Reproduced from ref 11)

Glutathionylspermidine synthetase/amidase

Glutathionylspermidine synthetase/amidase is a homodimer of 70 kDa monomers that are composed of 619 amino acid residues.[14] The enzyme consists of two domains: the C-terminal domain and the N- terminal domain. The C-terminal domain is responsible for catalyzing the forward, synthetase, reaction producing glutathionylspermidine. The C-terminal domain is 45 kDa in size and is composed of residues 206-619 of the protein.

The N-terminal domain catalyzes the hydrolysis of glutathionylspermidine to glutathione and spermidine. The N- terminal domain is 25 kDa in size and is composed of residues 1-195.[14,15] The residues connecting the two domains form the linker region and account for residues 196-205 (Figure 1-3).[10]

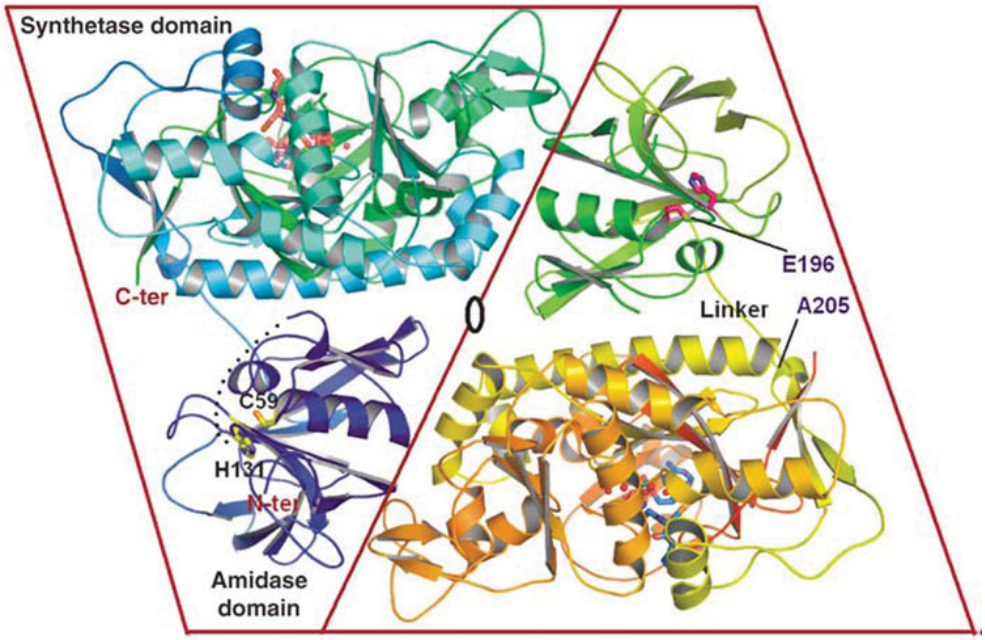


Figure 1-3. The structure of the glutathionylspermidine synthetase/amidase homodimer. The regions of the enzyme are labeled accordingly. (Adapted from ref 10).

The C-terminal domain of the enzyme belongs to the superfamily known as the ATP-grasp superfamily. This domain is composed of 9 alpha helices and 20 beta sheets. Although there is no homology between the two, the structure of the C-terminal domain is very similar to that of human glutathione synthetase and most likely functions in a similar fashion. The domain includes a lid domain that closes around substrates or

inhibitors upon binding (Figure 1-4). This domain also encompasses a hydrophobic pocket that contains the ATP-binding site.[10]

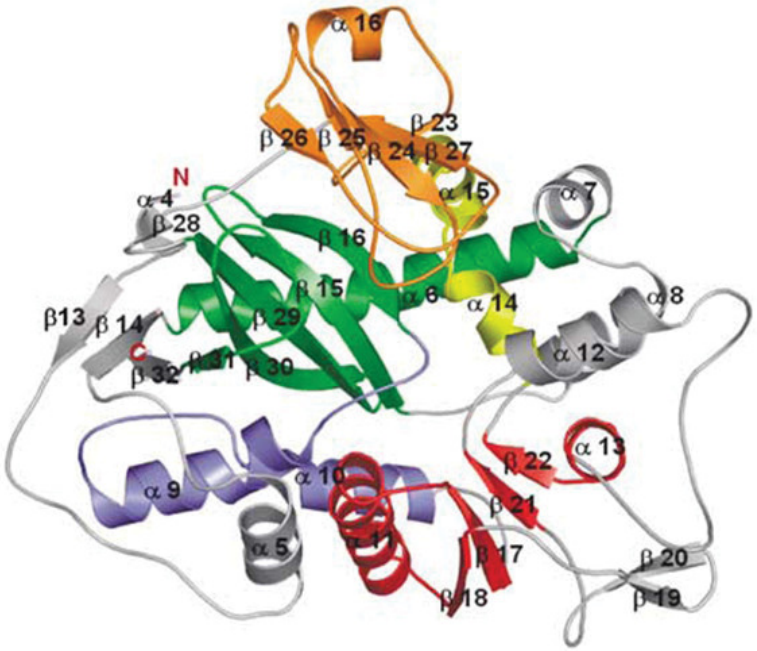


Figure 1-4. Structure of the C-terminal synthetase domain of the enzyme. The orange region shows the lid region that folds over upon binding to substrate or inhibitors. (Reproduced from ref 10).

The N-terminal domain of the enzyme belongs to the superfamily known as the cysteine, histidine dependent amidohydrolases/peptidases superfamily. The N-terminal domain contains alpha helices that are surrounded by 12 antiparallel beta sheets oriented in an open-sandwich configuration (Figure 1-5). The residues Cys59 and His131 are the catalytically active residues that are highly conserved in the enzyme throughout organisms.[10]

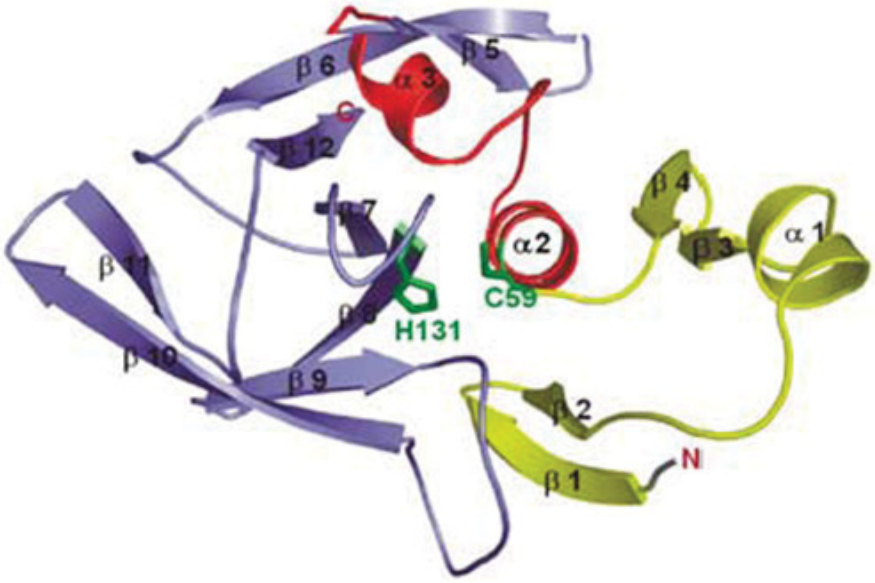


Figure 1-5. Structure of the N-terminal amidase domain of the enzyme. Conserved catalytic residues H131 and C59 are depicted in green and labeled accordingly. (reproduced from ref 10).

gsp Homologs in E. coli

Glutathionylspermidine synthetase/amidase is encoded by the *gsp* gene.[11] From the *E. coli* genome, it is predicted that there are two other genes that could produce proteins with similar function as a GSp synthetase. These genes are *ygiC* and *yjfC*. The GSP and YgiC proteins have significant homology where catalytic residues from the C-terminal domain in GSP remain conserved in YgiC.[11] Due to these similarities, the *ygiC* gene is proposed to code for a protein that acts as a glutathionylspermidine synthetase. YgiC and YjfC proteins have about 50% homology between them and it is proposed that *yjfC* codes for a protein that is similar to that coded for by *ygiC* and functions as GSp synthetase.[16]

Recombinase System

E. coli BW25113-pKD46 cells are specifically designed to contain the 6.3 kb pKD46 plasmid that expresses the Lambda Red recombinase operon and is ampicillin resistant (Figure 1-6).[17] The recombinase operon is induced by L-arabinose. AraC is a regulatory protein that inhibits the P_{araB}. Binding of L-arabinose to the AraC protein initiates transcription from ParaB ultimately leading to expression of the recombinase genes.[18]

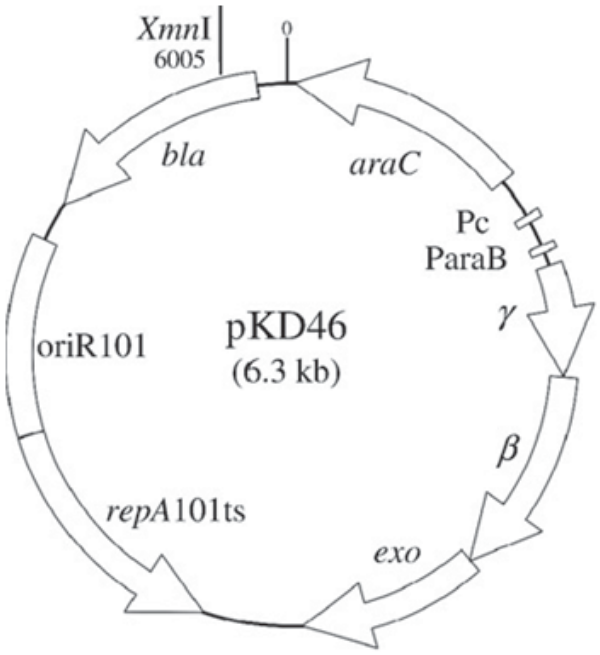


Figure 1-6. Plasmid map of pKD46. This figure shows the genes γ, β, and *exo* that are responsible for the recombinase activity that is induced with arabinose. (Reproduced from ref 17).

The operon contains three genes designated γ, β, and *exo* which are responsible for producing the respective proteins Gam, Beta, and Exo (Figure 1-8).[19] The Gam

protein is responsible for inhibiting the intracellular exonuclease RecBCD which naturally degrades linear DNA inside *E. coli* cells. The Beta protein acts as an annealing protein which recombines an inserted fragment of DNA into the genome sequence. The annealing protein can recombine sequences of approximately 50 bases. The Exo protein acts as a 5' → 3' exonuclease that hydrolyzes the phosphodiester bonds of the targeted genomic sequence.[20] In tandem, by means of homologous recombination, these proteins can eliminate a targeted gene and replace it with a specific fragment of DNA, produced using PCR.[19]

First, DNA fragments that contain a homologous region targeted for disruption are transformed into the cells. Results are best if the homologous sequence is between 30 and 50 base pairs in length.[20] The fragments also need to have an antibiotic resistant cassette flanked by an *frt* region. This allows for selection after tansformation. Two template plasmids have been created that can produce a DNA fragment useful in gene elimination. The two plasmids are pKD3 and pKD13 that are chloramphenicol resistant and kanamycin resistant respectively.[20] With these plasmids as templates, the PCR reaction produces DNA fragments that are approximately 1.1 kb and 1.4 kb in length respectively (Figure 1-9).[20]

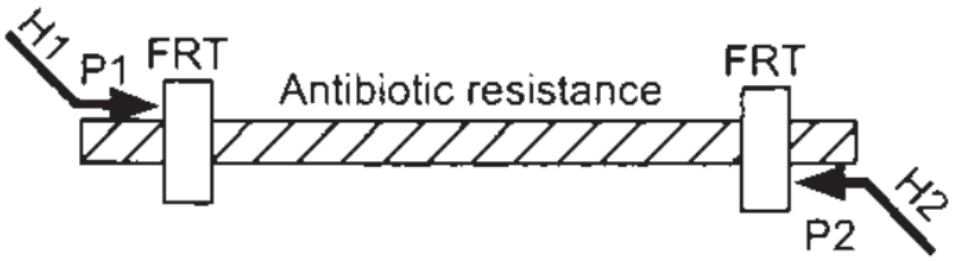


Figure 1-9. Replicate DNA fragment to be transformed into cells for disruptions where H1 and H2 and homologous ends with the gene to be disrupted, P1 and P2 are priming sites for antibiotic resistant cassettes, and FRT is the frt regions flanking the antibiotic resistant cassette. (Reproduced from ref 20).

The recombinase activity allows for homologous recombination between a generated PCR fragment and a region of genomic DNA of the targeted cells.[21] The *frt* region is used as an flp recombinase marker that is present on the helper plasmid pCP20.[17] After transformation of the cells by electroporation, the PCR fragments can be recombined into the genome through the recombinase activity of pKD46.[20] Selection for successful transformants can be accomplished through growth on medium supplemented with an antibiotic that corresponds to the antibiotic cassette of the PCR product (Figure 1-1-10).[20]

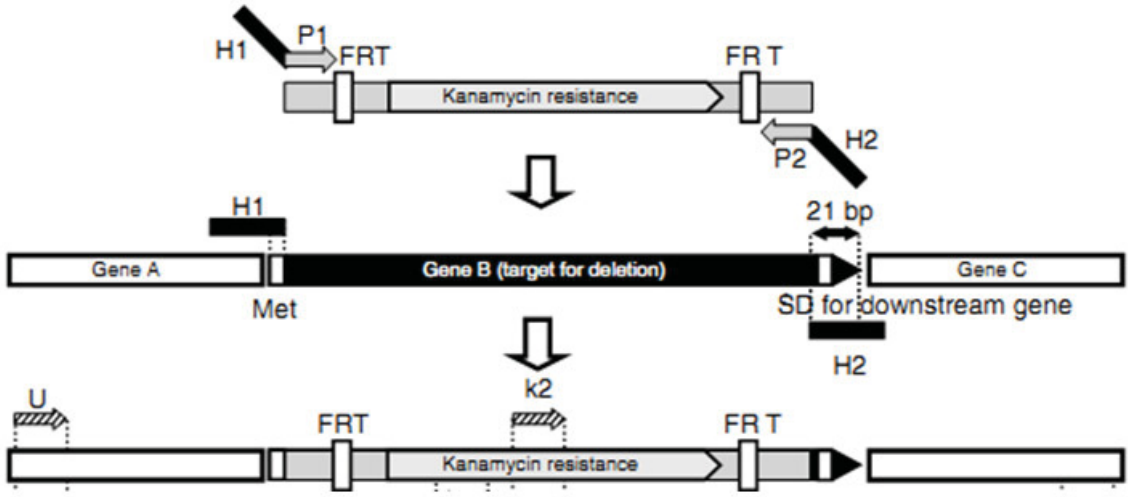


Figure 1-10. Depiction of the recombination of the PCR fragment into the genome in place of the targeted gene. The top DNA fragment represents the PCR fragment, the second DNA fragment represents the gene of interest in the genome, and the bottom DNA fragment represents the PCR fragment replacing the gene of interest. (Adapted from ref 22).

After recombination, the cells will be resistant to an antibiotic, either chloramphenicol or kanamycin. This antibiotic resistance presents an obstacle in the creation of multiple gene knockouts. Therefore, that antibiotic resistance must be eliminated before transformation.[22] Failure to eliminate the antibiotic resistant gene would result in the lack of ability to select for successful transformants. The antibiotic resistance can be removed via thermal induction with the helper plasmid pCP20.[16]

pCP20 is a helper plasmid that contains an *flp* gene that codes for flp recombinase (figure 1-11).[17] The flp recombinase acts on the *frt* sites that are flanking the antibiotic resistant cassette. The 9.4 kb pCP20 plasmid contains resistance to both chloramphenicol and ampicillin. The plasmid is temperature sensitive and upon thermal induction can be used to eliminate both the genomic antibiotic resistance along with the pKD46 plasmid.[20]

In order for the thermal induction to work, cells must be grown in a temperature range between 37 °C and 43 °C.[20]

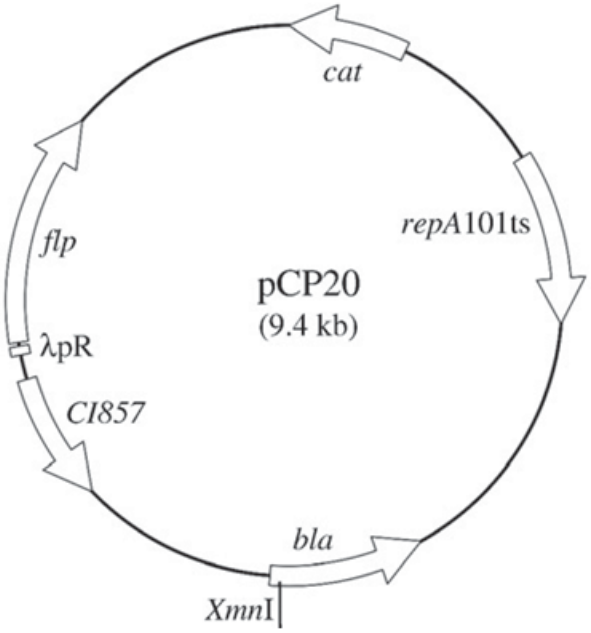


Figure 1-11. Plasmid map of pCP20, the helper plasmid that contains the *flp* gene. The *flp* gene codes for the protein that targets the *frt* region of the transformed cells. This is a temperature sensitive plasmid that is used to eliminate antibiotic resistance along with the pKD46 plasmid. (Reproduced from ref 13).

Upon thermal induction, the flp recombinase protein acts on the *frt* regions that were designed as part of the transposon. The flp recombinase acts in such a way that the two flanking regions of the gene form a loop-like structure and recombine with one another (Figure 1-12). As a result, the antibiotic resistant cassette is excised from the genome. The flp recombinase also acts on the helper plasmid pKD46 and all antibiotic resistance can be eliminated.

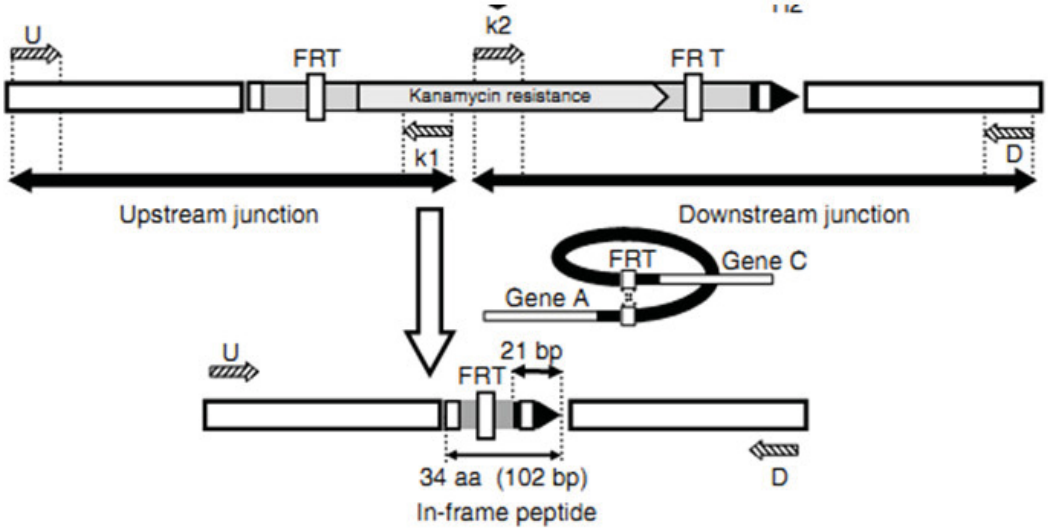


Figure 1-12. Depiction of the final product after the antibiotic resistance has been eliminated by the pCP20 helper plasmid. (Adapted from ref 22).

Goal of Research

The goal of this research is to provide insight for the function of the genes *gsp*, *ygiC,* and *yjfC* in *E. coli* by gene disruptions. The intention is to create a cascade of gene disruptions that include three single knockouts of the genes, three double knockouts of the genes, and one triple knockout. The knockouts are made utilizing the Lambda Red Recombinase system. Once gene disruptions are created, mutant cells will be studied to determine how they react to different stress conditions. High performance liquid chromatography (HPLC) will be used to determine the small thiol content within the cells.

Chapter 2: Materials and Methods

Materials

Strains and Plasmids of Bacteria

The strain of *E. coli* used to create gene disruptions were derivatives of BW25113 cells (designated as wild type cells). pKD46 in BW25113, pKD13 in XL1Blue, pCP20 in XL1Blue, pKD3 in XL1Blue, BW25113 (*Δgsp*), and BW25113 (*ΔygiC*), and BW25113 (*Δgsp/ygiC*) were all gifts from Dr. Armstrong at Vanderbilt University (Nashville, Tennessee).

Media and Antibiotics

SOB Broth, LB Broth, and Agar to make media were purchased from Amresco (Solon, Ohio). The antibiotics ampicillin (Am), chloramphenicol (Cm), kanamycin (Kan), and tetracycline (Tet) were all purchased from Amresco (Solon, Ohio). D-Glucose was purchase from Mallinckrodt (Maywood, New Jersey). L-Arabinose was given as a gift from Dr. Armstrong from Vanderbilt University in (Nashville, Tennessee).

PCR

Go Taq Green Master Mix for PCR was purchased from Promega (Madison, Wisconsin). QIAprep spin miniprep kit (50) and QIAquick gel extraction kit (50) were purchased from Qiagen (Valencia, California). An Eppendorf Centrifuge 5415C Tabletop centrifuge was used in conjunction with the Qiagen kits. All PCR primers were ordered

through Invitrogen (Carlsbad, California). The PCR machine used was a TECHNE TC-312. 10X TAE buffer was purchased from Amresco (Solon, Ohio).

Electroporation

Two different types of electroporation cuvettes were used. The first were purchased from Bio-Rad (Hercules, California) and had a 0.2 cm gap. The other cuvettes were BTX Cuvettes with a 0.1 cm gap. The electroporator used was a Bio-Rad (Hercules, California) Gene Pulser II.

Transition metals

1) 1M Na_2SeO_3, 2) 50 mM $AuCl_3$ 3) 50 mM $CdCl_2$, 4) 1M K_2CrO_4, 5) 1M $NaAsO_2$, 6) 100 mM $CuSO_4$, 7) 10 mM $HgCl_2$, 8) 50 mM $Pb(NO_3)_2$, 9)1M $ZnCl_2$ in 3.6 mM HCl, and 10) 50 mM $AgNO_3$ were used to test inhibition of growth for mutant strains and given as a gift from Dr. Jonathan Caguiat, Youngstown State University (Youngstown, Ohio). 30% hydrogen peroxide was purchased from Amresco (Solon, Ohio).

Analysis

A Hewlett Packard Diode Array 8452A Spectrophotometer was used. The Agarose, 1 kb DNA Ladder, EZ-Vision Three DNA Loading Buffer were purchased from Amresco (Solon, Ohio).

HPLC

The HPLC used for analysis used a Waters 1525 binary HPLC Pump with a Waters 2487 Daul λ Absorbance Detector. The column used was an Atlantis T3 5µm 4.6

x 250 µm C18 reverse phase column from Waters in Milford Massachusetts. Formic acid was purchased from Amresco and acetonitrile was purchased from Fischer Scientific. 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) was purchased from Amresco.

Methods

Generating PCR Fragment for Knockouts

PCR fragments (~1.1 kb) were generated to create gene disruptions. The plasmids pKD3 (146 ng/µL) and pKD13 (33 ng/µL) were used as templates to create antibiotic resistant cassettes for chloramphenicol and kanamycin respectively. The template plasmids were purified using a QIAprep spin miniprep kit following the directions suggested by the manufacturer. The concentration of purified plasmids was calculated using an absorbance at 260 nm and Beer's law. Primers used to create the *yjfC*/chloramphenicol fragments included the forward primer FL1: ACAACGTTCCTGT GCGACGGGATCTGGACCAGATCGCCGC*GTGTAGGCTGGGCTGCTTC* and the reverse primer RL1: ATGTAATGCGGAACGAAACGTGAGGTGTCTTTGGTGATC*A CATATGAATATCCTCCTTG*. Primers used to create the *ygiC*/kanamycin fragments included forward primer FL2: GAAAGAGTCAGTATTACCGAGCGCCCGGACTGGC GTGAGGT*GTGTAGGCTGGAGCTGCTTC* and the reverse primer RL2: AACCCAAG CTTATTCAACAAAAATATGTGGATAAAACCG*ATTCCGGGGATCCGTCGACC*.

The ends homologous to the plasmids pKD3 and pKD13 are shown in italics. The reactions were prepared as 50 µL samples. The reactions contained 25 µL Go Taq Green Master Mix, 1 µL forward primer (10 µM initial concentration), 1 µL reverse primer (10

µM initial concentration), 3.5 µL template (about 120 ng), and 19.5 µL nuclease free water. The cycling parameters were an initial denaturation temperature of 95°C for 2 minutes followed by 30 cycles of a denaturation temperature of 95°C for 45 seconds, an annealing temperature of 60°C for 45 seconds, an extension temperature of 72°C for 90 seconds, and a final hold of 72°C for 10 minutes. The reaction mixtures were loaded on a 1% agarose gel using EZ-vision Three DNA loading buffer. The gel was run at 90 V for about 45 minutes. The fragments were gel purified using the QIAquick gel extraction kit following the directions suggested by the manufacturer. The concentration of PCR fragments to create the *yjfC* knockout (60 ng/µL) and *ygiC* knockout (61 ng/µL) were determined using an absorbance at 260 nm. The purified fragments were stored at -20 °C in elution buffer.

Preparing Electrocompetent Cells

Cells of interest were grown in 5 mL SOC media at 30°C in a shaking incubator overnight. Antibiotics were used to supplement the overnight culture if cells were antibiotic resistant. The culture was diluted 100 fold with SOB media supplemented with 1 mM L-arabinose and grown to an $O.D._{600}$ ~ 0.6 at 30°C. 15 mL aliquots were centrifuged at ~1000 x g for 20 minutes. The cells were washed twice with ice cold sterile water and twice with ice cold 10% (v/v) glycerol. The amount of water started at 15 mL and with each successive wash decreased in volume by half. After each wash, cells were centrifuged at ~1000 x g for 15 minutes and the supernatant was discarded.

After the final wash, cells were resuspended in 150 µL 10% (v/v) glycerol solution, flash frozen with dry ice, and stored at -80°C.

Creating Gene Disruptions

Electrocompetent cells were transformed with the PCR fragments by electroporation. 100 µL electrocompetent cells along with 1.5 µL (about 90 ng) PCR fragment were placed in the electroporation cuvettes with a 0.1 cm gap. The settings for the electroporator were at 2.5 kV, 200 ohms, and 25 µF. After electroporation, the cells were suspended in 900 µL SOC media and incubated at 37°C for about 1 hour. 400 µL of the culture were plated on selective LB agar and grown overnight at 30°C. If no colonies grew overnight, the remaining cell culture that was kept at room temperature was plated on LB agar selective medium and grown overnight at 30°C. Gene disruptions were confirmed by PCR. The primers used for this purpose produce an approximately 350 bp fragment that is located in the middle of the gene being disrupted. Primers used to confirm the *yjfC* gene knockout included forward primer FS1: GCGCGGTAAAAGATGAAGAG and the reverse primer RS1: GTGTCCTGACAGCA GCAAAA. Primers used to confirm the *ygiC* gene knockout included forward primer FS2: GCCTGAAAGTGGTGGAAAAA and the reverse primer RS2: GTGAGATGCAG CAACTGGAA. The reactions were prepared as 26 µL samples. The reactions contained 11 µL nuclease free water with resuspended colonies along with 13 µL Go Taq Green Master Mix, 1 µL forward primer (10 µM initial concentration), 1 µL reverse primer (10 µM initial concentration). The cycling parameters for the PCR reactions had an initial denaturation temperature of 95°C for 2 minutes followed by 30 cycles with a

denaturation temperature of 95°C for 45 seconds, an annealing temperature of 60°C for 45 seconds, an extension temperature of 72°C for 30 seconds, and a final hold of 72°C for 10 minutes. The reaction mixtures were loaded on a 1% agarose gel using EZ-vision Three DNA loading buffer. The gel was run at 90 V for about 45 minutes. The absence of a 350 base band confirmed a successful gene disruption in the genome.

Elimination of Antibiotic Resistant Genes

Cells that had successful gene disruptions were made electrocompetent by the method described earlier. Using cuvettes with a 0.2 cm gap, 100 µL electrocompetent cells were transformed with the 1.5 µL of the helper plasmid pCP20 (20 ng/µL) and grown at 30°C on LB agar selective medium. Cell colonies were grown once nonselectively at either 37°C or 43°C overnight on LB agar medium to promote thermal induction. Individual colonies were then plated on LB agar nonselective and LB agar selective medium and grown at 30°C to determine the successful elimination of antibiotic resistance.

Creating Multiple Gene Knockouts

Electrocompetent cells with either a single knockout or double knockout were transformed with pKD46 by electroporation. 100 µL of electrocompetent cells and 1.5 µL (45 ng/µL) of pKD46 were electroporated in cuvettes with a 0.1 cm gap. After electroporation, cells were suspended in 900 µL SOC media and incubated at 30°C in a

shaking incubator for about 1 hour. 400 µL of the SOC culture was plated on LB agar medium, selective for ampicillin-resistant transformants, and grown overnight 30°C. If no colonies grew, the remainder of the SOC culture that was kept at room temperature overnight was plated on LB agar selective medium and grown overnight at 30°C. Transformed cells were made electrocompetent by the method described above.

Electrocompetent cells were transformed with the PCR fragments by electroporation. 100 µL of electrocompetent cells along with 1.5 µL of PCR fragment were transformed by electroporation in cuvettes with a 0.1 cm gap. After electroporation the cells were suspended in 900 µL SOC media and incubated at 37 °C for about 1 hour. 400 µL of the SOC culture was plated on selective LB agar plate and grown overnight at 30°C. If no colonies grew overnight, the remaining cell culture that was kept at room temperature overnight was plated on LB agar selective medium and grown overnight at 30 °C. Gene disruptions were confirmed by PCR. The primer sequence for the confirmation produces an approximately 500 bp fragment. Cloning primers are homologous to either end of the gene and the corresponding short primers are homologous to a 20 bp fragment located in the middle of the gene being disrupted. Primers used to confirm the *yjfC* gene knockout included forward primer FC1: AAAAAAAACATATGCTGAGACACAACGTTCCTGTG, and the reverse primer RS1: GTGTCCTGACAGCAGCAAAA, the forward primer FS1: GCGCGGTAAAAGATGA AGAG, and the reverse primer RC1: AAGAATTCTTATCCAGCAATGTAATGCGGT TCG. Primers used to confirm the *ygiC* gene knockout included the forward primer FC2:

AAAAGGATCCATGGAAAGAGTCAGTATTACCGAGCG, the reverse primer RS2: GTGAGATGCAGCAACTGGAA, the forward primer FS2: GCCTGAAAGTGGTGGA AAAA, and the reverse primer RC2: AACCCAAGCTTATTCAACAAAAATATGTGG ATAAAACCG. The reactions were prepared as 26 µL samples. The reactions contained 11 µL nuclease free water with resuspended colonies along with 13 µL Go Taq Green Master Mix, 1 µL forward primer (10 µM initial concentration), 1 µL reverse primer (10 µM initial concentration). The cycling parameters for the PCR reactions had an initial denaturation temperature of 94°C for 2 minutes followed by 30 cycles of a denaturation temperature of 94°C for 30 seconds, an annealing temperature of 66°C for 30 seconds, an extention temperature of 72°C for 1.5 minutes, and a final hold of 72°C for 10 minutes. The reaction mixtures were loaded on a 1% agarose gel using EZ-vision Three DNA loading buffer. The gel was run at 90 V for about 45 minutes. The absence of a band confirmed a successful gene disruption in the genome.

Testing for Antibiotic Sensitivity

Cultures of wild type and antibiotic susceptible mutants were grown to the stationary phase or to an $O.D._{600}$ ~ 0.7 at 37°C. 200 µL were plated on LB agar medium. 0.6 cm discs cut out of filter paper were placed on the plates and 5 µL of antibiotic solution was pipetted onto the center of the discs. The plates were incubated overnight at 37 °C. Sensitivity was determined by measuring the diameter of growth inhibition around the disc.

The concentrations of antibiotics tested were: 100 mg/mL ampicillin, 30 mg/mL chloramphenicol, 50 mg/mL kanamycin, and 12 mg/mL tetracycline.

Testing for Transition Metal Sensitivity

Cultures of wild type and antibiotic susceptible mutants were grown to the stationary phase or to an $O.D._{600}$ ~ 0.8 at 37 °C. 200 µL were plated on LB agar medium. 0.6 cm discs cut out of filter paper were placed on the plates and 5 µL of transition metal solution was pipetted onto the center of the discs. The plates were incubated overnight at 37 °C. Sensitivity was determined by measuring the diameter of growth inhibition around the disc.

The transition metals used were numbered 1-10 and correspond from plate to number were: 1) 1M Na_2SeO_3, 2) 50 mM $AuCl_3$ 3) 50 mM $CdCl_2$, 4) 1M K_2CrO_4, 5) 1M Na_2AsO_3, 6) 100 mM $CuSO_4$, 7) 10 mM $HgCl_2$, 8) 50 mM $Pb(NO_3)_2$, 9)1M $ZnCl_2$ in 3.6 mM HCl, and 10) 50 mM $AgNO_3$.

Testing the Sensitivity to Hydrogen Peroxide by Serial Dilutions

5 mL of cells grown in LB media at 37°C overnight were treated with 18 mM hydrogen peroxide and incubated for 1 hour at 37°C. 10 µL of the culture was added to 990 µL LB media. The cells were diluted 4 times with each successive dilution being 100 x the previous dilution. 5 µL of each dilution was plated on LB agar and minimal media (0.4% glucose) agar and incubated overnight at 37°C.

Analysis of Thiol Content by HPLC

Wild type, *Δgsp, ΔygiC*, and *ΔyjfC* were grown in minimal media (0.4% glucose) anaerobically overnight at 37 °C. Cells were pelleted by centrifugation at 14,000 rpm for 5 minutes in a tabletop centrifuge. The pellet was resuspended in 1.5 mL minimal media (0.4% glucose). The $O.D._{600}$ was adjusted to the same value to ensure approximately the same amount of cells used for analysis. The pellet was resuspended in 100 µL of lysozyme (2 mg/ml) and incubated at room temperature for 5 minutes. 25 µL of 5 mM DTNB was added and cells were incubated at room temperature for 5 minutes. The solution was then incubated at 95°C for 3 minutes. The mixture was centrifuged at 14,000 rpm in a tabletop centrifuge for 10 minutes. The supernatant was collected and diluted in a 1:1 ratio with 200 mM Tris buffer pH 8.0 and analyzed by HPLC. A 50 µL loading volume was used. The flow rate was set at 0.8 ml/min throughout the entire cycle. Buffer A was 0.9% formic acid and Buffer B was 100% acetonitrile. The buffer gradient was 95% A for the first minute then decreased to 70% A over 9 minutes. Buffer A was decreased to 20% over 7 minutes and held at 20% for an additional 5 minutes. Buffer A was increased to 95% over 1 minute and held there for 10 minutes. The total cycle was 33 minutes. The products were visualized at 330 nm.

Chapter 3: Results

Design of Primers for Gene Disruptions

The fragments of DNA that are designed to replace the existing genes were created via PCR. The primers used to amplify the DNA were 60 bases in length. 40 bases were homologous to the genomic DNA that is located at either end of the targeted gene. The other 20 bases were homologous to the sequence immediately preceding the *frt* region of the antibiotic resistant cassette on the plasmids used as a template for PCR. The plasmids pKD3 or pKD13 are chloramphenicol resistant and kanamycin resistant respectively. The reaction produces a DNA fragment that is approximately 1.1 kb in length when the amplified fragment contains a chloramphenicol resistant cassette (Figure 3-1) and a 1.4 kb fragment when the amplified fragment contains a kanamycin resistant cassette. For this study, *gsp* and *yjfC* genes were replaced by the chloramphenicol resistant cassette while *ygiC* was replaced by the kanamycin resistant cassette.

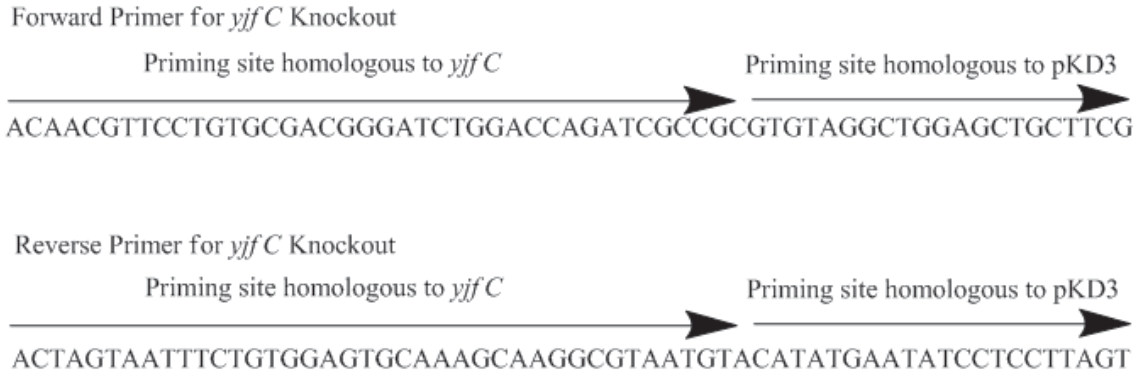


Figure 3-1. PCR primer design used to create the *yjfC* gene knockout. The *ygiC* and *gsp* knockout primers are very similar with homologous regions to the gene to the pKD3 plasmid.

Gene Knockouts

Six sets of cells were created that contained disruptions in their respective genes: *gsp, ygiC, yjfC, gsp/ygiC, gsp/yjfC, ygiC/yjfC, and gsp/ygiC/yjfC.* The cells were transformed by electroporation and elimination was confirmed by PCR followed by an agarose gel. *Δgsp*, Δ*ygiC, and Δgsp/ygiC* strains were provided by Dr. Armstrong from Vanderbilt University.

gsp, ygiC, gsp/ygiC Knockouts

The *Δgsp, ΔygiC,* and *Δgsp/ygiC* knockout strains still contained chloramphenicol resistance. The antibiotic resistance was eliminated with the use of the helper plasmid pCP20. After transformation and thermal induction, the three strains grew on nonselective medium while they did not grow on chloramphenicol, ampicillin or kanamycin selective medium indicating loss of antibiotic resistance (Figure 3-2).

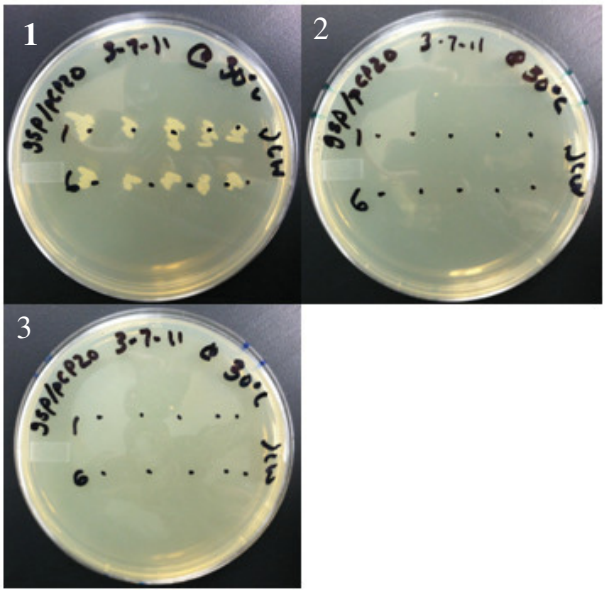


Figure 3-2. LB agar plates used to confirm the elimination of antibiotic resistance in Δgsp cells. Plate 1 shows nonselective medium Plate 2 shows medium selective for ampicillin and Plate 3 shows medium selective for chloramphenicol. Results were the same for *ΔygiC* and *Δgsp/ygiC* strains.

yjfC Knockout

Wild type cells were transformed with a DNA fragment that contained a chloramphenicol resistance cassette. After the transformation, PCR reactions were carried out to confirm successful elimination of the gene. To test whether the *yjfC* primers work under the PCR parameters set, control reactions were set up using the wild type cells and the *yjfC* primers. A 1% agarose gel was used to visualize the results and bands of approximately 350 bp were seen (Figure 3-3) indicating the primers did work under these conditions. Lane 1 shows a DNA ladder. Lanes 2 and 3 show the results of the PCR product.

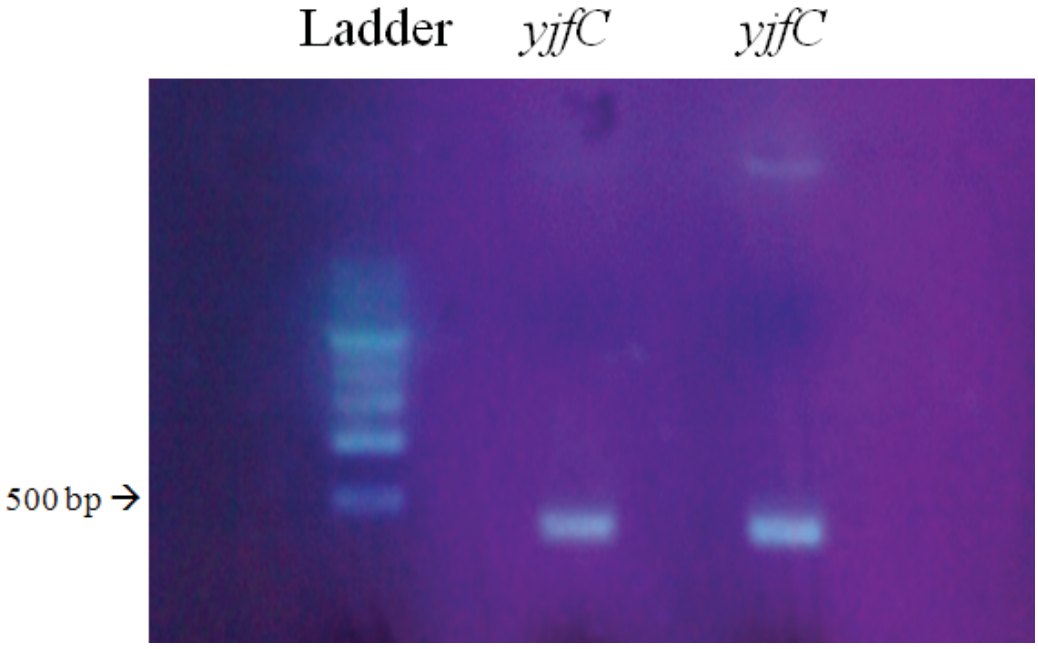


Figure 3-3. PCR product obtained from WT DNA using *yjfC* primers, FS1 and RS1, as control.

The primers used for PCR allow for amplification of about 350 bp fragment of *yjfC* genes, therefore, the presence of a band on the gel indicates the presence of the gene while the absence of a band indicates successful elimination of the gene. After PCR, the DNA was visualized on a 1% agarose gel (Figure 3-4). Lanes 2, 4, 6, and 8 are reactions with *yjfC* primers and do not contain bands. This indicates the successful elimination of the *yjfC* gene. Lanes 3, 5, and 7 are control reactions with *ygiC* primers. Presence of the bands confirms availability of bacterial DNA for the PCR reactions.

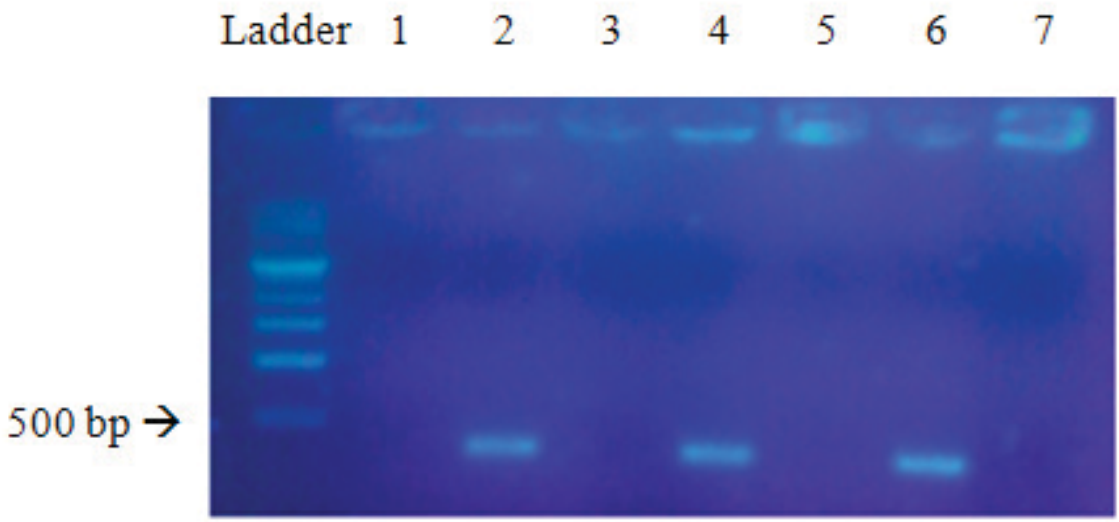


Figure 3-4. PCR with *yjfC* primers, FS1 and RS1, (lanes 1, 3, 5, 7) and *ygiC* primers, FS2 and RS2, as a control (lanes 2, 4, 6) after knockout of *yjfC* gene from WT cells.

Next, the antibiotic resistance was eliminated. The resistance was eliminated with the use of the helper plasmid pCP20 as described earlier. Cells grew nonselectively, but did not grow selectively confirming the elimination of antibiotic resistance in the cells (Figure 3-5).

Figure 3-5. LB agar plates used to confirm the elimination of antibiotic resistance. Plate 1 shows nonselective medium and Plate 2 shows medium selective for chloramphenicol.

gsp/yjfC Knockout

The antibiotic susceptible *Δgsp* cells were transformed with pKD46. The cells were then made electrocompetent and transformed with the PCR product for the *yjfC* knockout. A 1% agarose gel confirmed elimination of the *yjfC* gene from the genome (Figure 3-6). Lane 1 shows the DNA ladder. Lanes 2 and 3 are controls using wild type cells with *yjfC* primers, and Lanes 4-7 show transformed colonies with reactions using *yjfC* primers.

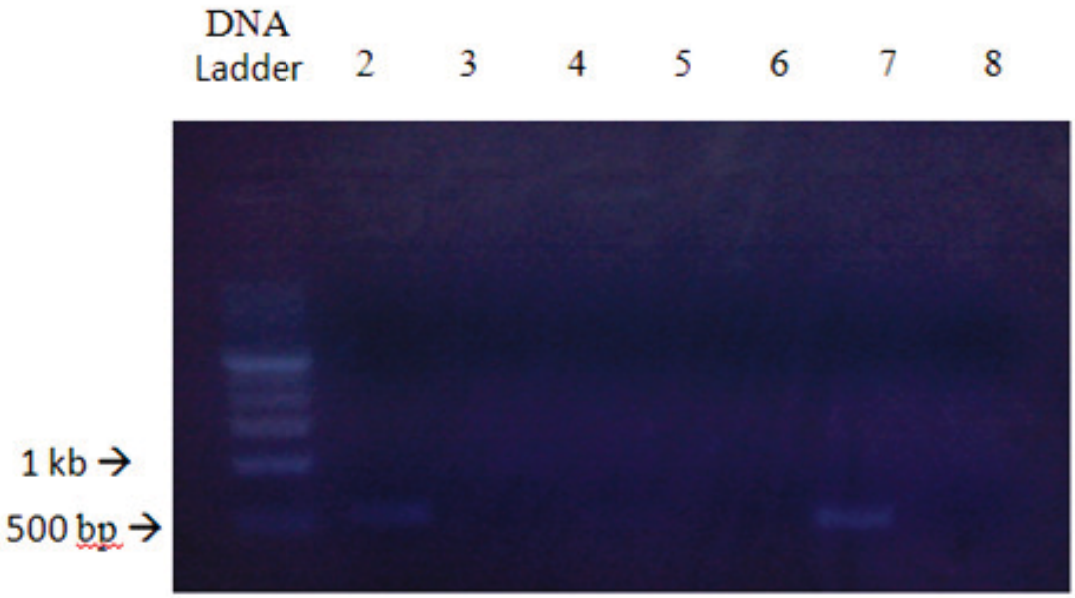


Figure 3-6. PCR with *yjfC* primers, FC1/RS1and FS1/RC1 in an alternating fashion, after knockout of *yjfC* gene from *Δgsp* cells. Lanes 2 and 3 serve as control lanes.

A band is seen in the control lane 2 showing that the primer combination will produce a DNA fragment under the PCR parameters. Lanes 4 and 6 are reactions that used this same primer combination and can be used to determine a successful mutant. The absence of a band in lane 4 signifies the disruption of the gene while the band in lane 6 signifies the gene has not been disrupted.

No band is seen in the control lane 3 showing that the primer combination will not work for the PCR parameters. Therefore, the absence of a band in lanes 5 and 7 cannot be used to confirm a mutant.

After mutant cells were obtained, they were transformed with pCP20. After thermal induction, cells were grown on selective medium. Cells grew on LB/agar

medium but did not grow on plates that contained ampicillin or chloramphenicol indicating the loss of antibiotic resistance (Figure 3-7).

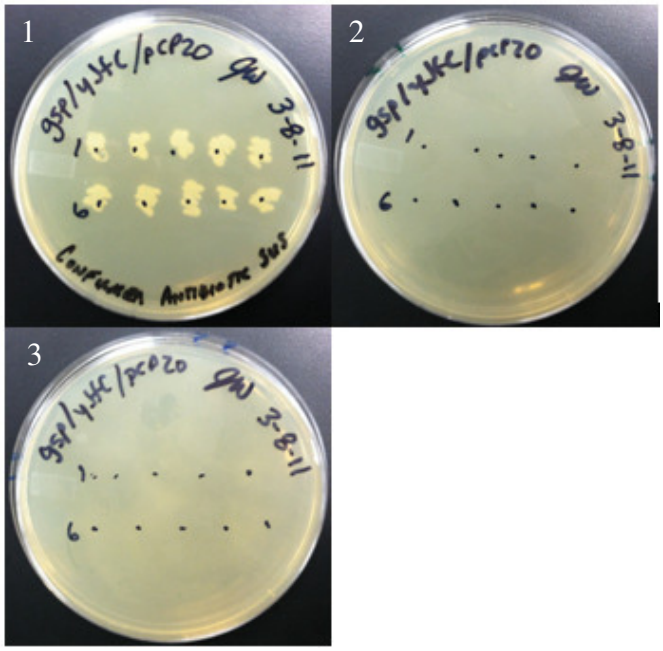


Figure 3-7. LB agar plates used to confirm the elimination of antibiotic resistance. Plate 1 shows nonselective medium, Plate 2 shows medium selective for ampicillin, and Plate 3 shows medium selective for chloramphenicol.

ygiC/yjfC Knockout

The cells with the *ygiC* knockout were transformed with pKD46 then transformed with the PCR product for the *yjfC* knockout and the successful elimination of the gene was confirmed by PCR with the specific primers (Figure 3-8).

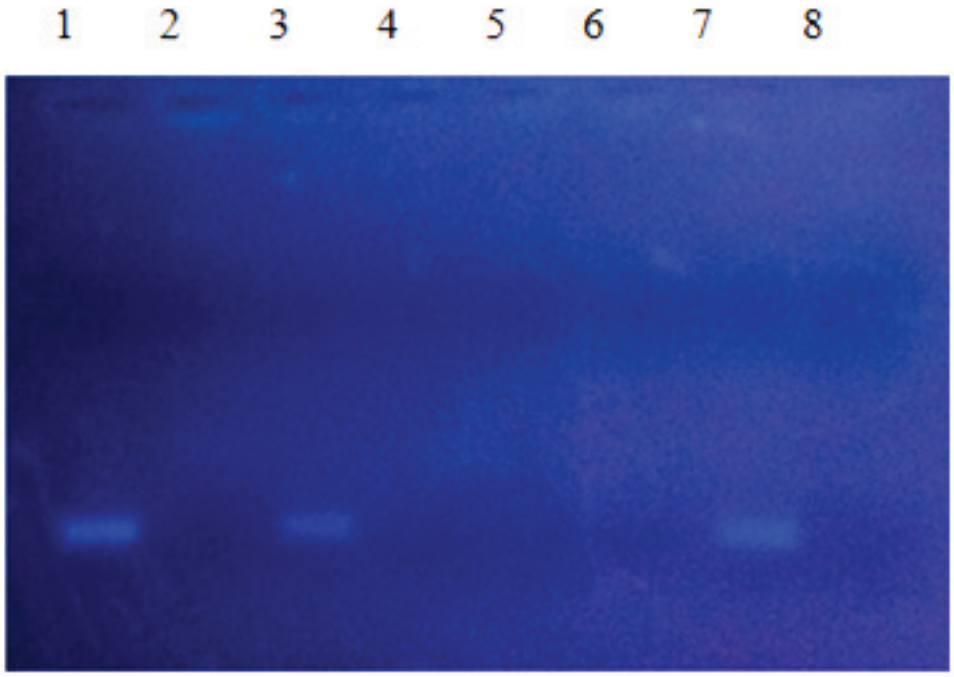


Figure 3-8. PCR with *yjfC* primers, FC1/RS1and FS1/RC1 in an alternating fashion, after knockout of *yjfC* gene from *ΔygiC* cells. Lanes 1 and 2 serve as control lanes.

After mutant cells were obtained, they were transformed with pCP20. After thermal induction, cells were grown on selective medium. Cells grew on LB/agar medium but did not grow on plates that contained ampicillin, chloramphenicol, or kanamycin indicating the loss of antibiotic resistance (Figure 3-9).

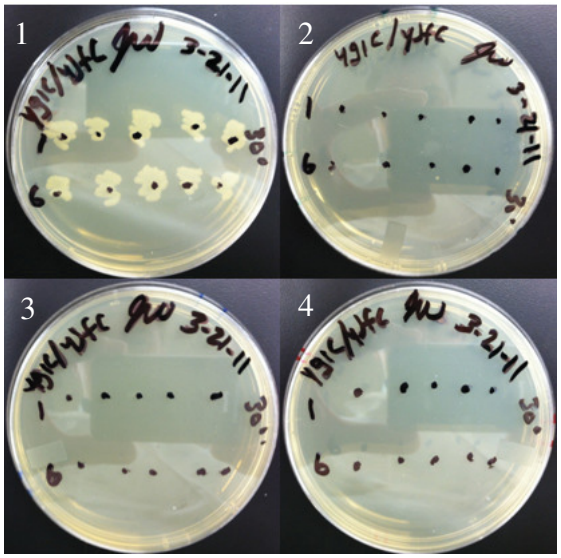


Figure 3-9. LB agar plates used to confirm the elimination of antibiotic resistance. Plate 1 shows nonselective medium, plate 2 shows medium selective for ampicillin, Plate 4 shows medium selective for chloramphenicol, and Plate 4 shows medium selective for kanamycin.

gsp/ygiC/yjfC Triple Knockout

The cells with the *gsp/yjfC* double knockout were transformed with pKD46 then transformed with the PCR product for the *ygiC* knockout. A 1% agarose gel confirmed elimination of the *ygiC* gene from the genome (Figure 3-10).

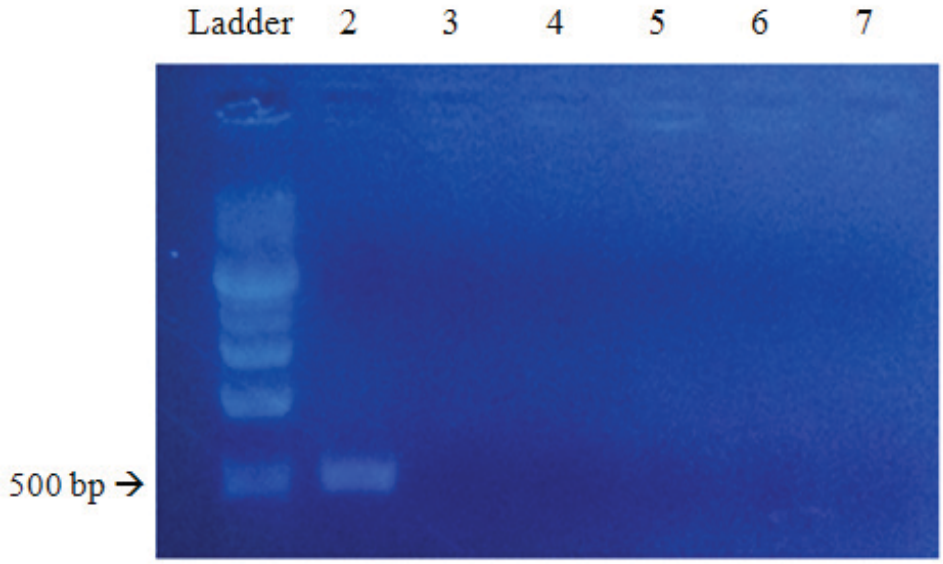


Figure 3-10. PCR with *ygiC* primers, FC2/RS2and FS2/RC2 in an alternating fashion, after knockout of *ygiC* gene from *Δgsp/yjfC* cells. Lanes 2 and 3 serve as control lanes using wild type cells as a template.

After mutant cells were obtained, they were transformed with pCP20. After thermal induction, cells were grown on selective medium. Cells grew on LB/agar medium but did not grow on plates that contained ampicillin, or kanamycin. Cells grew very minimally on chloramphenicol plates. Spots 3, 5, 6, and 7 show the most growth. Spot 2 showed no growth at all indicating the loss of all antibiotic resistance (Figure 3-11).

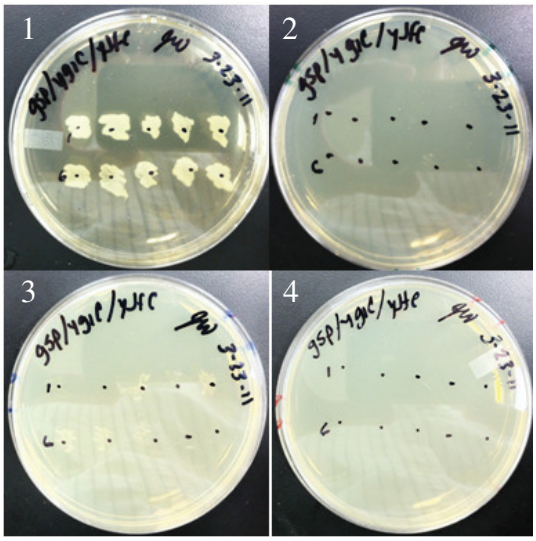


Figure 3-11. LB agar plates used to confirm the elimination of antibiotic resistance. Plate 1 shows nonselective medium, plate 2 shows medium selective for ampicillin, Plate 4 shows medium selective for chloramphenicol, and Plate 4 shows medium selective for kanamycin.

Testing for Antibiotic Sensitivity

The *E. coli* strains (wild type and seven knockouts) were exposed to 4 different antibiotics including ampicillin, chloramphenicol, kanamycin, and tetracycline using the disc method of testing (Figure 3-12). Cultures were grown on LB media overnight at 37 ^{o}C then plated on LB agar.

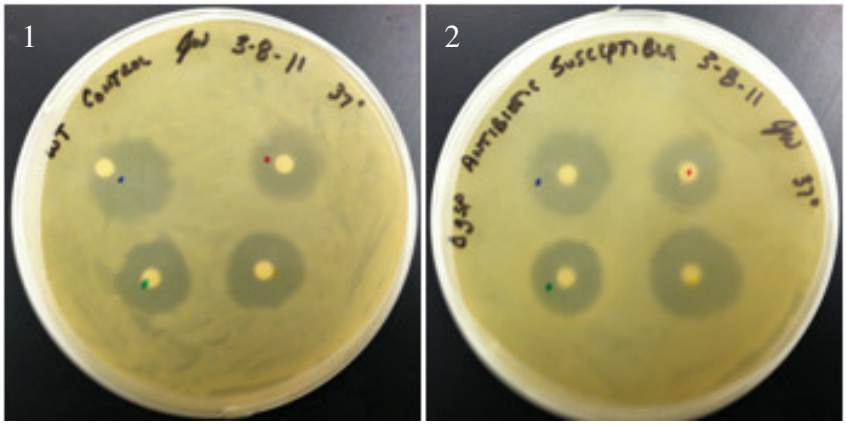


Figure 3-12. Plate 1 shows wild type cells and the Plate 2 shows *Δgsp* cells both grown on LB agar medium. The antibiotics are from top left in a clockwise fashion, chloramphenicol, kanamycin, ampicillin, and tetracycline.

Only the double knockout, *ΔygiC/yjfC*, showed significant difference in sensitivity to ampicillin where the diameter of growth inhibition increased from 2.4 cm to 3 cm indicating the cells are more sensitive to the antibiotic than the wild type. The double knockout *Δgsp/yjfC* and the triple knockout *Δgsp/ygiC/yjfC* showed a decrease in growth inhibition when exposed to chloramphenicol indicating an increase of resistance to the antibiotic. All strains with the exception of *Δgsp* showed an increase in sensitivity to kanamycin while *Δgsp* showed an increase in resistance to the antibiotic. All strains of cells showed an increase in sensitivity to tetracycline. The double knockout *Δgsp/yjfC* and triple knockout showed the least increase in sensitivity with a diameter changing from 2.3 cm in the wild type to 2.5 cm in the two strains (Table 3-1).

Table 3-1. Growth inhibition of antibiotics for all strains created grown in LB medium at 37°C overnight.

	Wild Type (cm)	*Δgsp* (cm)	*ΔygiC* (cm)	*ΔyjfC* (cm)	*Δgsp/ygiC* (cm)	*Δgsp/yjfC* (cm)	*ΔygiC/yjfC* (cm)	*Δgsp/ygiC/yjfC* (cm)
100 mg/mL Ampicillin	2.4	2.4	2.5	2.6	2.4	2.4	3.0	2.5
30 mg/mL Chloramphenicol	2.7	2.7	2.8	2.6	2.8	2.4	2.6	2.4
50 mg/mL Kanamycin	2.3	2.1	2.6	2.6	2.5	2.6	2.6	2.5
12 mg/mL Tetracycline	2.3	3.1	2.9	2.8	3.0	2.5	2.9	2.5

The strains were also tested after being grown to an $O.D._{600}$ ~ 0.7. This was performed to determine differences in growth between cells grown to the log phase and cells grown to the stationary phase. No significant differences were seen amongst cells exposed to ampicillin. *Δgsp* and *Δgsp/ygiC/yjfC* showed little to no change in sensitivity to chloramphenicol. *ΔygiC*, *ΔyjfC*, *Δgsp/ygiC*, and *ΔygiC/yjfC* all showed an increase in sensitivity to chloramphenicol. *Δgsp/yjfC* showed significant increase in resistance to chloramphenicol. All strains with the exception of *ΔyjfC* showed no significant changes to kanamycin. *ΔyjfC* showed a slight increase in resistance to the antibiotic. *Δgsp/yjfC* showed an increase in resistance to tetracycline. The rest of the strains showed an increase in sensitivity to tetracycline with *ΔygiC/yjfC* being the most sensitive (Table 3-2).

Table 3-2. Growth inhibition of antibiotics for all strains created grown in LB medium to specified O.D.

	Wild Type O.D.$_{600}$=0.70 (cm)	*Δgsp* O.D.$_{600}$=0.69 (cm)	*ΔygiC* O.D.$_{600}$=0.70 (cm)	*ΔyjfC* O.D.$_{600}$=0.75 (cm)	*Δgsp/ygiC* O.D.$_{600}$=0.66 (cm)	*Δgsp/yjfC* O.D.$_{600}$=0.65 (cm)	*ΔygiC/yjfC* O.D.$_{600}$=0.66 (cm)	*Δgsp/ygiC/yjfC* O.D.$_{600}$=0.71 (cm)
100 mg/mL Ampicillin	2.6	2.6	2.6	2.5	2.7	2.6	2.7	2.5
30 mg/mL Chloramphenicol	2.5	2.6	3.0	2.7	2.8	2.0	2.9	2.6
50 mg/mL Kanamycin	2.5	2.5	2.4	2.2	2.6	2.6	2.7	2.6
12 mg/mL Tetracycline	2.5	2.9	3.0	3.0	2.6	2.3	3.4	2.8

Testing for Transition Metal Sensitivity

Overnight cultures were grown and plated on LB agar plates. The seven knockout strains were tested for sensitivity to 10 different transition metals by the disc method (Figure 3-15). Wild type cells were used as a control group. The wild type and all knockout strains showed no sensitivity to Na_2SeO_3, $CdCl_2$, $CuSO_4$, or $Pb(NO_3)_2$. Most knockout strains except *ΔygiC* and the triple knockout showed an increase in sensitivity to Na_2AsO_3 compared to the wild type. On the contrary, while *ΔygiC* showed no change in sensitivity, the triple knockout showed an increase in resistance to Na_2AsO_3. No significant differences in growth inhibition were observed amongst other metal/strain pairs compared to the wild type control (Table3-13).

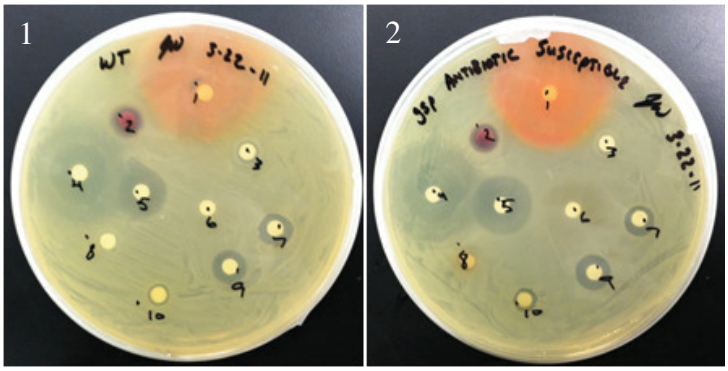


Figure 3-13. Plate 1 shows Wild type cells while Plate 2 shows *Δgsp* cells being tested for sensitivity to 10 transition metals. Other plates were made in a similar fashion. The metals tested were 1) 1M Na_2SeO_3, 2) 50 mM $AuCl_3$ 3) 50 mM $CdCl_2$, 4) 1M K_2CrO_4, 5) 1M Na_2AsO_3, 6) 100 mM $CuSO_4$, 7) 10 mM $HgCl_2$, 8) 50 mM $Pb(NO_3)_2$, 9)1M $ZnCl_2$ in 3.6 mM HCl, and 10) 50 mM $AgNO_3$.

Table 3-3. Growth inhibition for all strains created grown in LB media overnight being tested for sensitivity to transition metals.

	Wild Type (cm)	*Δgsp* (cm)	*Δygi C*	*ΔyjfC* (cm)	*Δgsp/ygi C* (cm)	*Δgsp/yjf C* (cm)	*ΔygiC/yjf C* (cm)	*Δgsp/ygiC/yjf C* (cm)
1) 1M Na_2SeO_3	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S
2) 50 mM $AuCl_3$	1.2	1.2	1.2	1.2	1.1	1.1	1.1	1.1
3) 50 mM $CdCl_2$	0.8	N/S	N/S	N/S	N/S	N/S	N/S	N/S
4) 1M K_2CrO_4	2.3	2.3	2.2	2.2	2.1	2.1	2.0	2.3
5) 1M Na_2AsO_3	2.1	2.4	2.2	2.5	2.7	2.5	2.6	1.5
6) 100 mM $CuSO_4$	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S
7) 10 mM $HgCl_2$	1.4	1.3	1.3	1.2	1.3	1.3	1.4	1.5
8) 50 mM $Pb(NO_3)_2$	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S
9)1M $ZnCl_2$ in 3.6 mM HCl	1.4	1.4	1.3	1.3	1.5	1.4	1.3	1.4
10) 50 mM $AgNO_3$	0.9	1.0	1.0	1.0	1.0	1.0	1.0	1.1

*N/S indicates a field in which no growth inhibition was observed.

When cultures were grown to an $O.D._{600}$ ~ 0.80, Na_2SeO_3, $CuSO_4$, and $Pb(NO_3)_2$ showed no cell growth inhibition in any of the cell strains. $CdCl_2$ showed some minor inhibition in the wild type cells while the rest of the strains showed complete resistance to the solution. All double knockout and the triple knockout strains showed an increase in

resistance to Na_2AsO_3. Amongst the rest of the strains, no significant differences were observed between the knockout strains and the wild type cells amongst the other transition metal solutions (Table 3-4).

Table 3-4. Growth inhibition for all strains created grown in LB media to specified O.D. being tested for sensitivity to transition metals.

	Wild Type O.D.$_{600}$=0.78 (cm)	*Δgsp* O.D.$_{600}$=0.83 (cm)	*ΔygiC* O.D.$_{600}$=0.83 (cm)	*ΔyjfC* O.D.$_{600}$=0.83 (cm)	*Δgsp/ygiC O.D.$_{600}$=0.82 (cm)*	*Δgsp/yjfC* O.D.$_{600}$=0.84 (cm)	*ΔygiC/yjfC* O.D.$_{600}$=0.84 (cm)	*Δgsp/ygiC/yjfC* O.D.$_{600}$=0.83 (cm)
1) 1M Na_2SeO_3	N/S	N/S	N/S	N/S	N/S	N/S	0.8	N/S
2) 50 mM $AuCl_3$	1.3	1.2	1.1	1.2	1.2	1.1	1.2	1.1
3) 50 mM $CdCl_2$	0.9	N/S	N/S	N/S	N/S	N/S	N/S	N/S
4) 1M K_2CrO_4	2.1	2.2	2.2	2.2	2.3	2.5	2.2	2.1
5) 1M Na_2AsO_3	2.3	2.3	2.3	2.2	1.9	1.9	1.9	1.9
6) 100 mM $CuSO_4$	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S
7) 10 mM $HgCl_2$	1.5	1.4	1.4	1.4	1.3	1.4	1.3	1.5
8) 50 mM $Pb(NO_3)_2$	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S
9)1M $ZnCl_2$ in 3.6 mM HCl	1.5	1.5	1.5	1.5	1.4	1.4	1.5	1.4
10) 50 mM $AgNO_3$	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

*N/S indicates a field in which no growth inhibition was observed.

Analysis of the Sensitivity to Hydrogen Peroxide by Method of Serial Dilution

Cultures, both before and after exposure to hydrogen peroxide, were diluted and plated to evaluate the effects of hydrogen peroxide on growth of cells. Each strain was diluted 100 x 5 consecutive times. Each sample was plated on both LB agar and MM9 (0.4% glucose) agar (Figure 3-14).

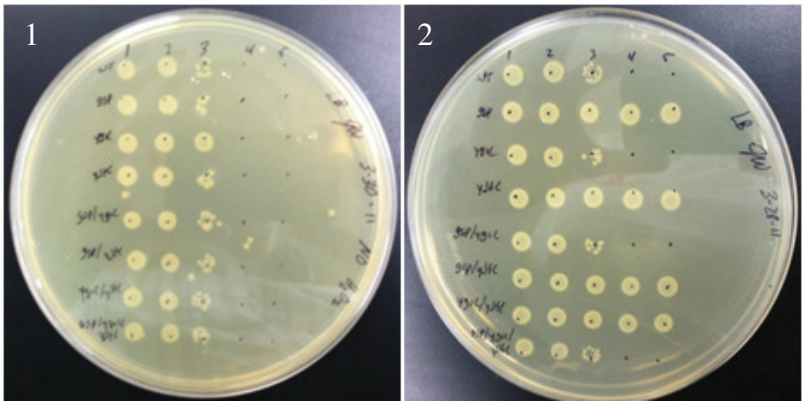


Figure 3-14. Plate 1 shows serial dilutions of an overnight culture before exposure to H_2O_2. Plate 2 shows serial dilutions of an overnight culture after exposure to 18mM H_2O_2 for 1 hour.

In the culture grown without exposure to hydrogen peroxide, all strains of the bacteria grew well in dilutions 1 and 2, grew good in dilution 3, and no growth was observed in dilutions 4 and 5 (Table 3-5). After exposure to hydrogen peroxide, the mutant strains *Δgsp*, *ΔyjfC, Δgsp/yjfC,* and *ΔygiC/yjfC* grew well throughout all dilutions. Wild type cells along with *ΔygiC, Δgsp/ygiC*, and *Δgsp/ygiC/yjfC* grew well in dilutions 1 and 2, good in dilution 3 and not at all in dilutions 4 and 5 which do not differ from cells grown without exposure to hydrogen peroxide (Table 3-6).

Table 3-5. Growth of *E. coli* cells after serial dilutions on LB agar before exposure to hydrogen peroxide.

Strain	*Growth for dilution 1	Growth for dilution 2	Growth for dilution 3	Growth for dilution 4	Growth for dilution 5
WT	++	++	+	-	-
Δgsp	++	++	+	-	-
ΔygiC	++	++	+	-	-
ΔyjfC	++	++	+	-	-
Δgsp/ygiC	++	++	+	-	-
Δgsp/yjfC	++	++	+	-	-
ΔygiC/yjfC	++	++	+	-	-
Δgsp/ygiC/yjfC	++	++	+	-	-

* ++ Vigorous growth
+ Good growth
- No growth

Table 3-6. Growth of *E. coli* cells treated with hydrogen peroxide after serial dilutions on LB agar.

Strain	*Growth for dilution 1	Growth for dilution 2	Growth for dilution 3	Growth for dilution 4	Growth for dilution 5
WT	++	++	+	-	-
Δgsp	++	++	++	++	++
ΔygiC	++	++	+	-	-
ΔyjfC	++	++	++	++	++
Δgsp/ygiC	++	++	+	-	-
Δgsp/yjfC	++	++	++	++	++
ΔygiC/yjfC	++	++	++	++	++
Δgsp/ygiC/yjfC	++	++	+	-	-

* ++ Vigorous growth
+ Good growth
- No growth

Growth on minimal media agar showed similar results to that on LB agar with the exception the wild type cells did not grow well on minimal media (Table 3-7). After exposure to hydrogen peroxide, wild type cells in dilutions 1-3 showed minimal growth and dilutions 4 and 5 showed no growth. The other strains had similar results to the cells grown on LB agar where the mutant strains *Δgsp, ΔyjfC, Δgsp/yjfC*, and *ΔygiC/yjfC* grew well throughout all dilutions. *ΔygiC, Δgsp/ygiC*, and *Δgsp/ygiC/yjfC* grew well in dilutions 1 and 2, good in dilution 3, and not at all in dilutions 4 and 5 (Table 3-8).

Table 3-7. Growth of *E. coli* cells after serial dilutions on minimal media agar before exposure to hydrogen peroxide.

Strain	*Growth for dilution 1	Growth for dilution 2	Growth for dilution 3	Growth for dilution 4	Growth for dilution 5
WT	+	+	+	-	-
Δgsp	++	++	+	-	-
ΔygiC	++	++	+	-	-
ΔyjfC	++	++	+	-	-
Δgsp/ygiC	++	++	+	-	-
Δgsp/yjfC	++	++	+	-	-
ΔygiC/yjfC	++	++	+	-	-
Δgsp/ygiC/yjfC	++	++	+	-	-

* ++ Vigorous growth
+ Good growth
- No growth

Table 3-8. Growth of *E. coli* cells treated with hydrogen peroxide after serial dilutions on minimal media agar.

Strain	*Growth for dilution 1	Growth for dilution 2	Growth for dilution 3	Growth for dilution 4	Growth for dilution 5
WT	+	+	+	-	-
Δgsp	++	++	++	++	++
ΔygiC	++	++	+	-	-
ΔyjfC	++	++	++	++	++
Δgsp/ygiC	++	++	+	-	-
Δgsp/yjfC	++	++	++	++	++
ΔygiC/yjfC	++	++	++	++	++
Δgsp/ygiC/yjfC	++	++	+	-	-

* ++ Vigorous growth
+ Good growth
- No growth

Analysis of Thiol Content in E. coli Cells by HPLC

After samples had been prepared and run through the HPLC, three peaks appeared in the chromatogram. The first peak eluted at ~13.5 minutes and is indicative of GSH. The second peak eluted at ~18 minutes and is indicative of TNB and the third peak eluted at ~20.5 minutes and is indicative of DTNB (Figure 3-15).

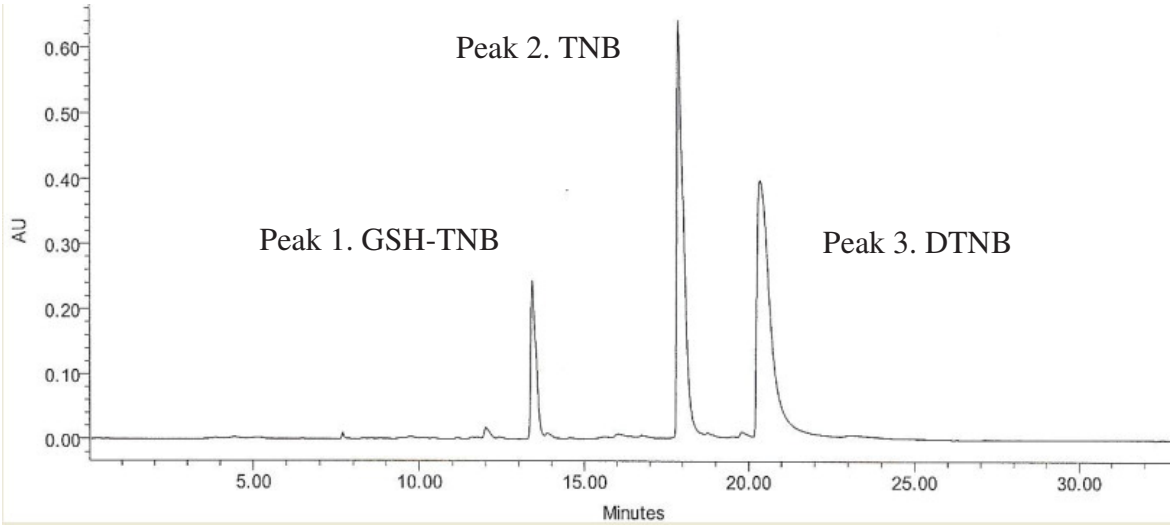


Figure 3-15. Chromatogram of thiol content *of Δgsp* cells.

Chapter 4: Discussion

In order to study the function of the genes *gsp, ygiC*, and *yjfC*, and their possible role in glutathione metabolism, gene disruptions were created. The knockout strains were exposed to antibiotics, transition metals, and hydrogen peroxide to determine the effect of mutations to the sensitivity of the stressors. Analysis of GSH and GSp content in the strains was performed using HPLC.

A cassette of seven mutant strains was created via electroporation and homologous recombination. The absence of bands on the agarose gels (Figures 3-4, 3-5, 3-8, 3-10, and 3-12) indicated that four sets of gene disruptions including Δ*yjfC,* Δ*gsp/yjfC,* Δ*ygiC/yjfC,* and Δ*gsp/ygiC/yjfC* had been successfully created. Δ*gsp*, Δ*ygiC*, and Δ*gsp/ygiC* were provided.

Once the seven mutant strains were obtained, they were made antibiotic susceptible via the helper plasmid pCP20. After thermal induction, presence of growth on LB agar plates with no antibiotics and the absence of growth on LB agar plates supplemented with antibiotics (Figures 3-2, 3-3, 3-6, 3-7, 3-9, 3-11, 3-13) signified that antibiotic resistance had been lost throughout all strains.

Antibiotic Sensitivity

It has been speculated by previous research[23] that GSH concentration positively correlates to the antibiotic resistance of *E. coli* by changing the oxidative protection that GSH offers. An increase in exogenous GSH showed an increase in resistance to cells

beyond just those antibiotics that use oxidative damage as a mechanism of inhibition. This suggests that GSH plays a role in cellular functions beyond oxidative stress relief.[23]

According to the results (Table 3-1 and 3-2), sensitivity to ampicillin was not altered in any of the mutants. However, mutants showed an increase in sensitivity to kanamycin in the stationary phase of growth (Table 3-1). Our data as well as the literature[23] suggests that GSH promotes resistance to kanamycin, the three genes *gsp, ygiC*, and *yjfC* may promote an increase in GSH levels in the log phase of growth and decrease of GSH levels in the stationary phase.

Chloramphenicol acts on the 50S ribosomal subunit during translation, inhibiting protein synthesis.[23] In the cells grown to an $O.D._{600}$ ~ 0.7 mutant strains lacking the *ygiC* gene became more sensitive to chloramphenicol in all cases except in the triple knockout strain (Table 3-2). The mutant *Δgsp/yjfC*, lacking both *gsp* and *yjfC* genes, showed an increase in resistance to this antibiotic. This suggests that the YgiC may function in protecting the 50S subunit from exogenous inhibitors or affects the transport of the antibiotic into the cell.

Mutant strains grown both to the stationary phase and to an $O.D._{600}$ ~ 0.7 showed more sensitivity to tetracycline (Table 3-1 and 3-2). Tetracycline acts by binding to the 30S ribosomal subunit and ultimately inhibits elongation of the amino acid chain during translation.[24] The increase in sensitivity to tetracycline suggests that proteins produced from the three genes may play a role in protecting the 30S subunit from exogenous inhibitors such as tetracycline.

Transition Metal Sensitivity.

It has been found that increased GSH levels promote resistance to arsenite, mercury, and cadmium.[25] The mechanism of resistance remains unknown. The mutant strains showed no change in the resistance compared to the wild type cells with the exception of arsenite (Tables 3-3 and 3-4). In the stationary phase, all mutants were more sensitive to arsenite. When grown to an $O.D._{600}$ ~ 0.8 all double knockouts and the triple knockout mutant showed an increase in resistance. Being consistent with the literature, if GSH levels positively correlate with resistance to arsenite, the results indicate that levels of glutathione may be increased in the log phase of growth, promoting resistance, while they may be decreased in the stationary phase, promoting sensitivity. These results are congruent with the results found in the kanamycin test.

Effects of Hydrogen Peroxide

The three genes *gsp, ygiC*, and *yjfC* have been identified in *E. coli* as participating in the metabolism of glutathione. In most organisms, glutathione acts as a relief to oxidative stress. Hydrogen peroxide is an easily accessible oxidative stressor and is of interest in determining the function of the three genes.

Δgsp, ΔyjfC, Δgsp/yjfC, and *ΔygiC/yjfC* showed an increase in growth after being treated with hydrogen peroxide and plated in serial dilutions on both LB agar and MM9 (0.4% glucose) plates (Table 3-5, 3-6, 3-7, and 3-8). The results were relatively unexpected due to hydrogen peroxide forming free radicals that usually kill cells. It is suggested that hydrogen peroxide can rapidly oxidizes Fe(II) to Fe(III).[26] If this is in fact

the case, an increase in GSH concentration should allow for the cells to overcome the oxidative stress provided by H_2O_2 and survive.

It had also been found that the mutant strains grow better on MM9 (0.4% glucose) media as opposed to the wild type that shows little to no growth at all. If *gsp, ygiC*, or *yjfC* genes are prominent in the cell and utilize major sources of nutrients, the absence would allow the cell to utilize the nutrients for other purposes such as metabolism to make energy and allow the cell to grow better in these conditions instead of using them for biosynthesis.

Analysis of Thiol Content in the E. coli Cells by HPLC

HPLC was performed to determine if glutathione and glutathionylspermidine concentrations could be quantified within the cell. Three peaks were observed, one for GSH, one for TNB, and one for DTNB. Therefore under these parameters, only GSH levels can be analyzed. The results do show a higher concentration of GSH in *Δgsp* cells as compared to those of both *ΔygiC* and *ΔyjfC* strains grown in MM9 (0.4% glucose) anaerobically.

Future Work

Now that the cassette of gene disruptions have been created, studying the function of these genes can be accomplished in more depth. Preliminary experiments indicate that GSH levels may be higher in the log phase of growth compared to that of the stationary phase of growth. HPLC has been shown to have potential in determining the GSH

concentrations within the cells. Future work in the project would include quantification of the GSH and GSp content in the mutant strains growing under different stress conditions and to different phases of growth. Further studies on the effects of hydrogen peroxide and the reason for increased growth after exposure needs to be completed.

Chapter 5: Conclusion

The three genes *gsp, ygiC*, and *yjfC* have been identified as genes that participate in glutathione metabolism in *E. coli*. Seven mutant strains were created via homologous recombination using the λ Red Recombinase mechanism. PCR was used to confirm successful elimination of the genes. The mutants were made antibiotic susceptible with use of the helper plasmid pCP20. Growth on nonselective and selective medium confirmed the loss of antibiotic resistance within the cells. The mutants were tested for sensitivity to antibiotics, transition metals, and hydrogen peroxide. It was found that there was an increase in sensitivity to tetracycline and chloramphenicol indicating the gene products may act in protecting the 50S and 30S ribosomal subunit or act in the transport of molecules within the cell. The cells show no change in resistance to kanamycin in the log phase of growth while they became more sensitive to it in the stationary phase suggesting an decrease of GSH concentration in the stationary phase. Arsenic was the only metalloid that effected growth. These results are congruent with the data for the kanamycin test that indicated an increase in GSH concentration in the log phase while there is a decrease in GSH concentration in the stationary phase. Hydrogen peroxide was found to increase growth in four of the seven mutant strains. Further analysis is required to better understand this observation. Further studies are needed to quantify the concentration of GSH in the cell under different growth conditions.

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