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Developing a Deletion Construct of the *Halothiobacillus neapolitanus* csoS1C Gene

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Developing a Deletion Construct of the *Halothiobacillus neapolitanus csoS1C* Gene

by

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Abstract

The purpose of this research was to develop a deletion construct for the chemoautotrophic bacterium *Halothiobacillus neapolitanus*, which will be used to generate a mutant lacking a carboxysome shell protein gene. The carboxysome is the location of carbon dioxide fixation. The operon that encodes the carboxysome contains three genes for CsoS1 proteins, the major components of the carboxysome shell. The small CsoS1 proteins self-assemble into hexamers with small central pores. The hexamers arrange into the facets of the icosahedral carboxysome shell. The pores are believed to be involved in selective diffusion of materials necessary for carbon dioxide fixation across the shell.

A deletion construct to replace the *csoS1C* gene with a kanamycin resistance cassette was designed that will allow gene replacement by homologous recombination to determine if the *csoS1C* paralog is necessary to form functional carboxysomes. This deletion construct will allow the function of this paralog to be studied in the resulting mutant. To develop the construct, primers were designed to amplify the kanamycin resistance gene with short ends that are homologous to regions flanking the *csoS1C* gene in the *H. neapolitanus* genome. *E. coli* DY330 was transformed with the amplified resistance cassette and a plasmid containing the *csoS1C* region of genomic DNA for homologous recombination that will yield the deletion construct.

Keywords: carboxysome, carbon dioxide fixation, homologous recombination, paralog, *csoS1C*, kanamycin resistance cassette

Dedication

I would like to dedicate this to my Mom and Daddy for patiently listening to me talk about my research for the better part of my undergraduate career, even when you both didn't know what I was talking about. And thank you both for your comfort and encouragement especially when my colonies didn't grow and my PCRs didn't work—again.

In Memory of Omar Gonzalez (1998-2015)

#flyhighOmar

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I would like to acknowledge all the time, patience, and effort Dr. Sabine Heinhorst has put into my research project and me. Without your stern and caring support, I would not have developed into the scientist I am now. Thank you for being supportive of me especially during times of stress, both academic and personal. And thank you for taking me on as my advisor back when I was just a baby freshman.

I would also like to acknowledge all the help Cassandra Bates has been over the years. Thank you for patiently answering all of my many questions. And thank you especially for laughing off my mistakes; I wouldn't have been able to if you hadn't. Also, thank you to Daniela Del Valle for answering my questions, and listening to me when I was talkative in lab.

Finally, I would like to thank the faculty and staff of the Honors College and the Department of Chemistry and Biochemistry for their support during my undergraduate career.

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Chapter I: Introduction

Bacterial microcompartments (BMCs) are small, polyhedral protein structures found in bacteria.[1,2] These structures are protein shells that encase enzymatic reactions, sequestering the reactants and products from the rest of the cell, increasing enzymatic activity, and/or protecting the cell from potentially harmful by-products.[3] The genes that code for the shell protein components of BMCs are highly conserved among the BMC containing bacteria.[2] There are three types of conserved BMC shell proteins: hexamers, pentamers, and tandem domains.[4] BMCs are interesting to study because they have the capability to self-assemble, which has potential applications in synthetic biology.[5] The BMC of interest to this research is the carboxysome (**Figure 1**).

All cyanobacteria and many chemoautotrophs contain carboxysomes.[3,5] In nature,

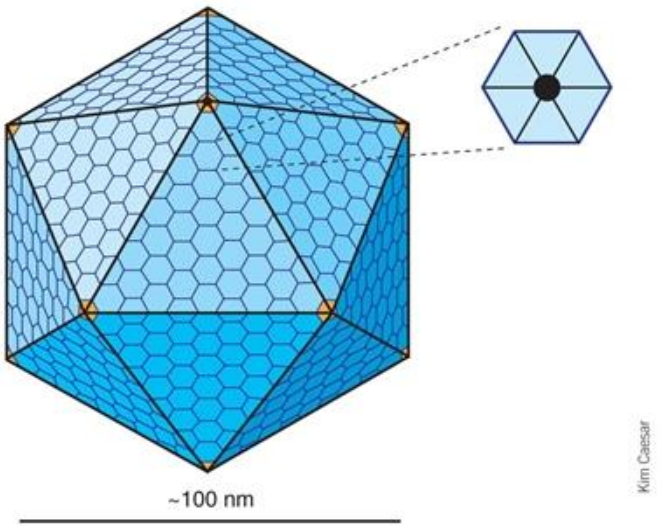

Figure 1. Structure of the Carboxysome. Also showing assembly of hexameric proteins. (Heinhorst, S.; Cannon, G.; *Nat. Struct. Mol. Biol.* **2008.** *15*. 898-898)

there exist two types of carboxysomes, distinguishable by different protein compositions,

the α-carboxysome and the β-carboxysome.[1] The model organism for the study of α-carboxysomes is *Halothiobacillus neapolitanus*, which is a chemoautotroph that utilizes CO_2 and inorganic sulfur compounds as a carbon and energy source, respectively.[3] The predominant enzyme housed in the carboxysome is ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO). RuBisCO is the enzyme that fixes inorganic carbon dioxide in the first step of the Calvin-Bensen-Bassham cycle.[1–3] The proposed higher concentration of CO_2 inside the carboxysome allows the enzyme to function effectively in low CO_2 environments.

The carboxysome shell protein genes are located in the *cso* operon. Within the *cso* operon are three homologous genes—*csoS1C, A, B*—that code for the nearly identical major shell proteins. While it is known that the carboxysome shell is primarily composed of the CsoS1 proteins, it is not yet known if all three paralogs are required for the structure or function of the carboxysome shell.[6] The goal of this research was to develop a deletion construct that will allow for the study of the role of the *csoS1C* gene and CsoS1C protein in the structure and function of the carboxysome.

Chapter II: Literature Review

Bacterial microcompartments (BMCs) are the bacterial equivalents of eukaryotic organelles.[1,2,6] Eukaryotic organelles have specific purposes inside the cell, as do the BMCs. BMCs are polyhedral protein structures with an approximately 100 nm diameter, that contain various enzymatic reactions.[1,2] Containing the reactions benefits the cell by increasing enzyme activity, sequestering dangerous intermediates, and/or shielding the enzyme reaction from inhibitors.[3] The genes that code the three BMC protein components are conserved in roughly a fourth of all bacterial genomes.[2] Additionally, the ability of

BMCs to self-assemble makes them candidates for synthetic biology research.[5] Research into the various components of BMCs may allow for the development of synthetic, self-assembling, molecular structures that could be designed to sequester commercially valuable enzymatic reactions.[7] Alternatively, existing BMCs could be genetically engineered to sequester new and different enzymes of interest.[8]

The carboxysome is the most studied BMC and the only BMC involved in anabolic metabolism.[3,5] This BMC is found in all cyanobacteria and many chemoautotrophs, including the model organism *Halothiobacillus neapolitanus*.[3,5] *Halothiobacillus neapolitanus* is a chemoautotroph that oxidizes inorganic sulfur compounds to obtain energy.[3] *Halothiobacillus neapolitanus* lives in a relatively low CO_2 environment and requires a CO_2-concentrating mechanism (CCM) in which the carboxysome plays a vital role.[1]

The carboxysome encases ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO), which is the CO_2-fixing enzyme of the Calvin-Bensen-Bassham (CBB) cycle.[1–3] RuBisCO catalyzes the attachment of CO_2 to ribulose-1,5-bisphosphate and the following cleavage of the six carbon intermediate into two 3-phosphoglycerate (3-carbon)

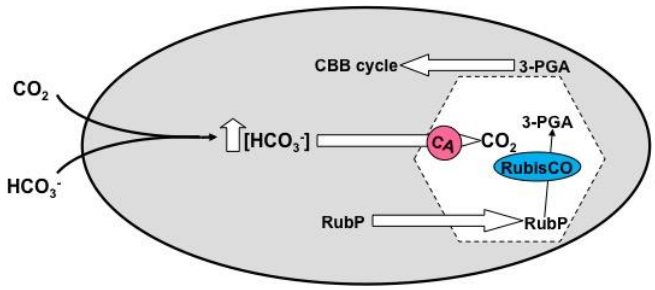

Figure 2. Carbon Fixation Within the Carboxysome. Cytosolic HCO_3^- is converted to CO_2 by carbonic anhydrase inside the carboxysome. RuBisCO then fixes CO_2 onto ribulose-1,5-bisphosphate (RubP), which yields two molecules of 3-phosphoglycerate (3-PGA). Image courtesy of Dr. Sabine Heinhorst.

molecules.[9] As inorganic carbon is transported into the cell, it is equilibrated to mostly bicarbonate, which cannot be used by RuBisCO; however, the charged bicarbonate cannot cross the lipid membrane to exit the cell. The cytosolic concentration of bicarbonate steadily increases and becomes higher than the concentration of bicarbonate inside the carboxysome, creating a concentration gradient across the protein shell (**Figure 2**). The concentration gradient helps to facilitate diffusion of bicarbonate across the carboxysome shell. It is thought that carbonic anhydrase associated with the protein shell accelerates the equilibration between the HCO_3^- and CO_2 as HCO_3^- diffuses across the protein shell into the carboxysome.[1] It is also believed that some feature of the carboxysome shell prevents the CO_2 from diffusing back out of the carboxysome (**Figure 3**).[10,11]

The carboxysome contains the final step in the CCM. The concentrated levels of inorganic carbon (bicarbonate) in the cytosol result in high levels of CO_2 inside the carboxysome (**Figure 2**). The carbonic anhydrase creates a saturated CO_2 environment

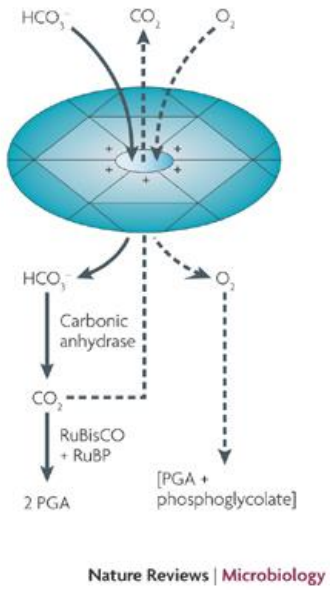

Figure 3. Molecular Transport Through the CsoS1 Hexamer Pores in the Carboxysome Shell. Image source: Yeates, et al. 2008.

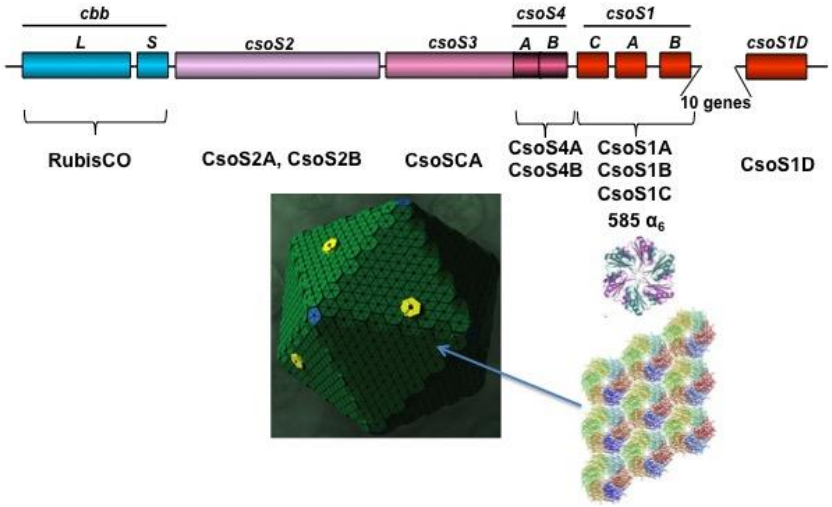

Figure 4. Genes of the *cso* Operon. Also shown are the structures of the carboxysome and of the CsoS1 shell protein hexamers. Image courtesy of Dr. Sabine Heinhorst.

within the carboxysome, allowing the RuBisCO to function with greater efficiency and enhanced catalytic activity.[1] Without the CCM and sequestered RuBisCO the bacterium is unable to grow efficiently in environments with low CO_2 concentrations, such as air.[10]

All bacteria with the potential to develop BMCs have similar hexamer-forming BMC protein components, and genes that tend to be present as multiple paralogs.[4] The major α-carboxysome shell proteins are the CsoS1 proteins. The genes for the CsoS1 proteins are contained in the *cso* operon (**Figure 4**). Within the *cso* operon are three *csoS1* paralogs that code for the predominant shell proteins; however, the *csoS1D* gene is located outside of the operon. The three *csoS1* genes within the operon code for nearly identical proteins. The CsoS1C and CsoS1A proteins are different by only two amino acids and CsoS1B has an additional twelve amino acids on the C-terminus.[5,12,13] The CsoS1 proteins are small monomers that are assembled into hexamers (**Figure 5**). The CsoS1D protein is a pseudo-hexamer that may form a highly selective pore, but still requires more research regarding its function.[14]

The CsoS1 proteins assemble into hexamers with six-fold symmetry. The hexamers have two different sides, one of which is hydrophobic and concave.[15] Hexamer assembly results in the formation of small central pores, which may allow for the selective diffusion of reactants and products of the RuBisCO reaction across the protein shell (**Figures 2 and 5**).[13] The assembly of the hexamers forms the facets of the icosahedral carboxysome shell.

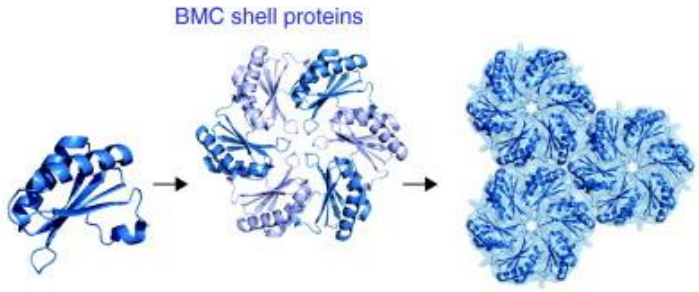

Figure 5. Assembly of CsoS1 Monomers. (Yeates, et al. 2011)

Hydrogen bonding between the hexamers assists the packing of the hexamers into the facets.[15]

It is not yet known if the three CsoS1 proteins are functionally redundant or if they each serve a different purpose in the carboxysome shell. It is hypothesized that each *csoS1* gene codes for a monomer that yields a hexamer with a pore of slightly different permeability.[5,13] For example, it has been proposed that the carboxysome shell prevents the

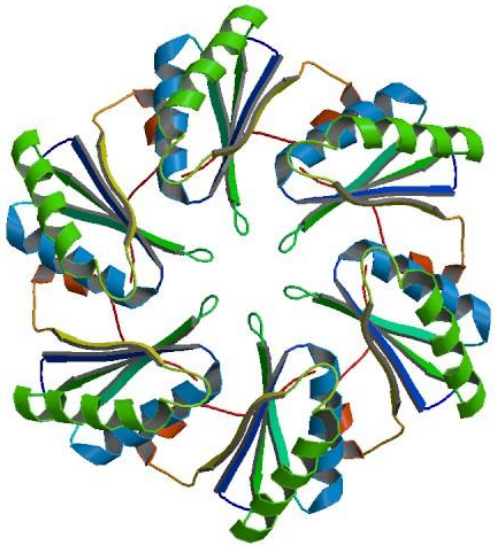

Figure 6. Structure of CsoS1C Hexamer. Six CsoS1C monomers assemble into the hexamer, forming the central pore. Image source: http://www.rcsb.org/pdb/explore/explore.do?structureId=3H8Y

competitive inhibitor O_2 from reaching RuBisCO.[2] It is necessary to study the three homologues separately to determine their biological significance by knocking out their genes and studying the resulting carboxysomes.

The long-term purpose of this research is to study the function of the *csoS1C* gene and consequently the CsoS1C protein (**Figure 6**) in *Halothiobacillus neapolitanus* carboxysomes. By replacing the *csoS1C* gene with a kanamycin resistance cassette, a

mutant can be generated in which the function of the protein encoded by the deleted gene is missing. The ability of *Halothiobacillus neapolitanus* to grow in air is determined by the ability of the carboxysome to function in the CO_2 concentrating mechanism.[13] If the mutant carboxysomes are not fully functional, the bacterium should be unable to effectively concentrate and fix CO_2 at ambient CO_2 concentrations. Such an outcome would suggest that the CsoS1C protein is vital for a functional carboxysome shell. However, if the mutant carboxysomes are unaffected, the CsoS1C protein may not be essential to the formation and/or function of the shell.

Chapter III: Materials and Methods

Materials and Bacterial Strains

Media

Luria-Bertani Broth (LB Broth)
10 g/L NaCl
10 g/L Bacto tryptone
5 g/L Bacto yeast extract

Luria-Bertani Agar (LBA)
15 g/L Agar
10 g/L Bacto tryptone
10 g/L NaCl
5 g/L Bacto yeast extract

S.O.C. Medium (Invitrogen)
20 g/L Bacto tryptone
5 g/L Bacto yeast extract
20 mM Glucose
10 mM NaCl
10 mM $MgCl_2$
10 mM $MgSO_4$
2.5 mM KCl

Dyes

Ethidium Bromide 1% (10 mg/mL)
Fisher BioReagents

6X Gel Loading Dye Blue
New England Biolabs
1X Buffer Components (pH 8.0 at 25℃)
2.5% Ficoll®-400
11 mM EDTA
3.3 mM Tris-HCl
0.017% SDS
0.015% bromophenol blue

Buffers

TBE Buffer (pH 8.0)
108 g/L Tris-Base (pH 7.8)
55 g/L Boric Acid
40 mL 0.5M EDTA (pH 8.0)

10X TE Buffer (pH 8.0)
100 mM Tris-HCl (pH 8.0)
10 mM EDTA (pH 8.0)

Antibiotic Solutions

Aqueous ampicillin (100 µg/mL) and kanamycin (50 µg/mL) were used at 100 mg/mL and 50 mg/mL final concentrations, respectively.

PCR Master Mixes and DNA Ladders

10X ThermoPol Reaction Buffer (pH 8.8 at 25℃)
New England Biolabs
200 mM Tris-HCl
100 mM $(NH_4)_2SO_4$
100 mM KCl
20 mM $MgSO_4$
0.1% Triton® X-100

Deoxynucleotide (dNTP) Solution Mix (pH 7.3)
Thermo Scientific™
Aqueous solution of each dNTP at 10 mM

2X GoTaq® Green Master Mix (pH 8.5)
Promega
GoTaq® DNA Polymerase
400 µM dATP
400 µM dGTP
400 µM dCTP
400 µM dTTP
3 mM $MgCl_2$

1 kb DNA Ladder (pH 8.0 at 25℃)
New England Biolabs
DNA fragments of 500 bp to 10,002 bp size range
10 mM Tris-HCl
1 mM EDTA

100 bp DNA Ladder (pH 8.0 at 25℃)
New England Biolabs
DNA fragments of 100 bp to 1,517 bp size range
10 mM Tris-HCl
1 mM EDTA

Plasmids and E. coli Strains

One Shot®TOP10 chemically competent *E. coli* cells (Invitrogen) for transformation of plasmids

pCR®-Blunt II-TOPO® vector (Invitrogen) for cloning, sequencing and generating glycerol stocks of amplified DNA

pUC18 plasmid DNA (Thermo Scientific) for generation of deletion constructs

E. coli DY330 for electroporation and homologous recombination

Water

Deionized (DI) water from the house line was purified using a Barnstead™ Nanopure Diamond Lab Water Purification System, a Barnstead™ RO System (Thermo Scientific).

Methods

Gel Electrophoresis

To separate and visualize DNA fragments a 0.7% agarose gel was prepared in 1X TBE. Ethidium bromide (40 μg/μL per gel) was added to the still liquid agarose solution to visualize the DNA. The gel was loaded with an appropriate DNA ladder and subjected to 100 V until the bands of loading dye (NEB) migrated the desired distance across the gel. The gel was imaged under UV light (VersaDoc Imaging System, Bio-Rad).

Overnight Bacterial Cultures for Plasmid DNA Purification

A liquid and solid culture of bacteria containing the target plasmid was prepared for plasmid purification using sterile technique. Liquid cultures were prepared in 5 mL Falcon™ Round-Bottom Polystyrene Tubes (Fisher Scientific). Each culture contained 5 mL of LB medium and 5 μL of ampicillin and/or kanamycin solution to select for the desired plasmid. Cells from either glycerol stocks or transformed TOP10 *E. coli* cells were used to inoculate the cultures. The liquid cultures of TOP10 *E. coli* cells were incubated at 37℃ and cultures of *E. coli* DY330 cells were incubated at 30℃ with agitation overnight.

The solid cultures were prepared on LB agar plates containing ampicillin and/or kanamycin. The plates with TOP10 *E. coli* cells were incubated at 37℃ overnight, and the plates with *E. coli* DY330 cells were incubated at 30℃ overnight.

Plasmid Purification Protocol

To isolate the plasmids from the liquid cultures, the cultures were first centrifuged at 9,000 rpm for 10 minutes (JA 25.5 rotor) to pellet the cells; the supernatant was removed from the cell pellet and discarded. Plasmid DNA was then isolated from the pelleted cells using a Fermentas GeneJET Plasmid Miniprep Kit (Thermo Scientific). The pelleted cells were resuspended using 250 μL of the provided Resuspension Solution. The cell suspension was transferred to a 1.5 mL microcentrifuge tube. Next, 250 μL of the included Lysis Solution was added and the cell solution was mixed by inverting the tube. After mixing, 350 μL of the Neutralization Solution was added and the solution quickly mixed by inverting. The solution was centrifuged for 5 minutes at 14,000 rpm. The supernatant

was transferred to the supplied GeneJET™ spin column and centrifuged for 1 minute. The flow-through was discarded. The column was washed with 500 µL of the Wash Solution and centrifuged for 1 minute; this wash step was repeated twice. The empty column was centrifuged an additional minute. The DNA was eluted with 50 µL of deionized (DI) water and incubated at room temperature for 2 minutes before being centrifuged for 2 minutes. The eluate was collected in a fresh microcentrifuge tube.

Determination of DNA Concentration

The concentration of purified plasmid DNA was found using a NanoDrop® ND-1000 Spectrophotometer. The concentration was reported in ng/µL.

Screening for Recombinants/ Colony Lysis PCR

Screening transformants for recombinants included streaking single colonies on LBA plates containing an appropriate antibiotic and colony lysis PCR.

To screen for positive transformants, eight single colonies were selected and streaked on an antibiotic containing LBA plate (**Figure 7**). Transformants of pUC18 constructs required ampicillin, TOPO vector constructs required kanamycin, and pUC18 constructs with Kan^R inserted required both antibiotics on separate plates. The plates were incubated overnight at 37℃ for TOP10 *E. coli* and 30℃ for *E. coli* DY330.

After streaking the LBA plates (**Figure 7**), the inoculating loop was swirled in 25 µL of TE to use for colony lysis PCR. The suspended cells were lysed by boiling at 100℃ for 1 minute. The DNA from the lysed cells was substituted as the DNA template for the

colony PCR (2 µL of lysed cells per PCR reaction).

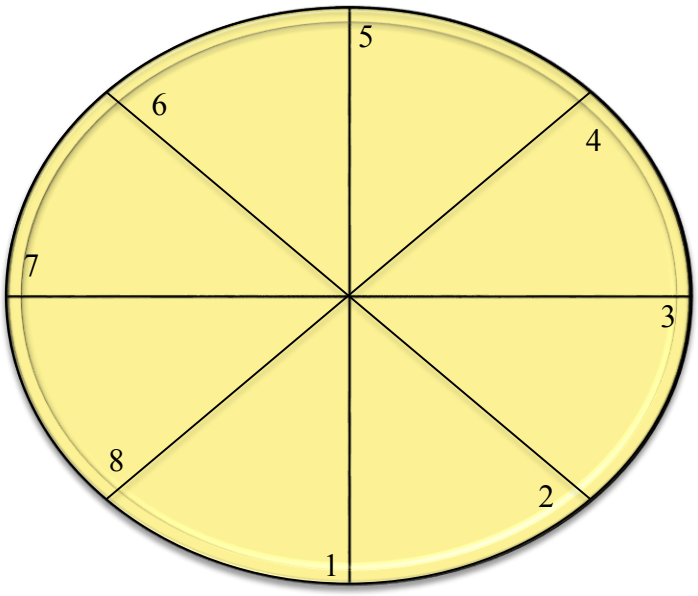

Figure 7. Streaking Pattern for Recombinant Screening.

TOPO-Kan plasmids were screened using the PCR program listed in **Table 1** and the Master Mix in **Table 6**. The primers designed for the Kan^R cassette were used for amplification.

Table 1. PCR parameters for Colony PCR of TOPO-Kan Transformants (program name: edkan1)

Temperature	Time	Cycles
95℃	3 minutes	1
95℃	30 seconds	30
50℃	30 seconds	
72℃	1 minute	
72℃	10 minute	1
12℃	∞	Hold

Recombinant pUC18 constructs were screened using the GoTaq® Green Master Mix (**Table 5**) and CBStandard PCR program listed in **Table 2**. The M13-20F and M13-26R (Eurofins) primers were used for these amplifications.

Table 2. Reaction Parameters of CBStandard PCR Program

Temperature	Time	Cycles
95℃	3 minutes	1
95℃	30 seconds	30
55℃	30 seconds	
72℃	1 minute	
72℃	5 minutes	1
12℃	∞	Hold

Restriction Digest

Restriction enzymes were used to linearize circular DNA into fragments of identifiable size. These fragments were used to generate new constructs and to test for the

presence of specific fragments in generated constructs. The restriction digest reaction (**Table 3**) contained a specific restriction enzyme(s) that cut the DNA at the desired location. A buffer (NEB) was chosen according to the enzyme used. The reaction was incubated at an appropriate temperature for 1 hour. The restriction products were separated and visualized by gel electrophoresis.

Table 3. General Reaction for Restriction Digestions

Reagent	Amount
NEB 10X Buffer	2.5 μL
DNA Template	1 μg (500 ng)
Restriction Enzyme	1 μL
DI water	Up to 24 μL
Final Volume	**24 μL**

DNA Recovery by Gel Extraction

Once the DNA fragments were separated on the agarose gel, the desired fragment was excised from the gel. The fragment was extracted from the gel using the QIAEX II® Gel Extraction Kit from Qiagen. The mass of gel excised was determined, and Buffer QXI (included in kit) was added using 3 volumes of buffer for fragments 100 bp-4 kb. The QIAEX II reagent (included in kit) was resuspended by vortexing for 30 s, and 10 μL were added to the gel. The reaction was incubated at 50℃ for 10 min, vortexing every 2 min to help dissolve the gel and allow the DNA to bind to the QIAEX II reagent. Next the reaction was centrifuged at 13,000 rpm for 30-60 s and the supernatant discarded. The pellet was washed with 500 μL of Buffer QXI and resuspended by vortexing. The solution was centrifuged again for 30-60 s and the supernatant discarded. The pellet was then washed twice with 500 μL Buffer PE by resuspending the pellet, centrifuging for 30-60 s, and

discarding the supernatant. The pellet was then allowed to air dry until white. The pellet was resuspended in 20 µL of water and allowed to incubate at room temperature for 5 min then centrifuged for 30-60 s. The supernatant containing the isolated DNA was removed and saved. The concentration of DNA extracted was determined by UV absorbance.

Preparation of the Kan^R Cassette

Primer Design

In order to amplify the Kan^R cassette and replace the *csoS1C* gene, primers (**Figure 14**) were designed with sequence homology to both the sequence surrounding the *Halothiobacillus neapolitanus csoS1C* gene and the Kan^R cassette. Working stock primers

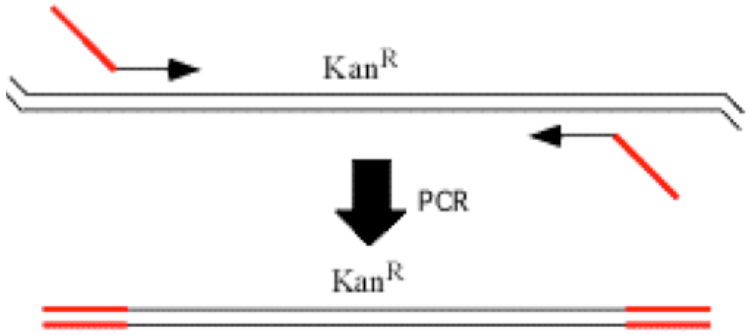

Figure 8. Amplification of Kan^R Cassette. The black lines represent DNA containing the kanamycin resistance gene. The black arrows are the portion of the designed primers that anneal to the kanamycin resistance gene, and the red lines are the base pairs that anneal to the regions flanking *csoS1C* in the *Halothiobacillus neapolitanus* genome. Source: http://www.sci.sdsu.edu/~smaloy/MicrobialGenetics/topics/in-vitro-genetics/red-swap.html

were created by diluting 5 µL of stock primer with 45 µL DI H_2O.

Amplification of Kan^R cassette via Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) was used to amplify the Kan^R cassette with ends homologous to sequences up- and downstream of *csoS1C* using the previously designed primers. Amplified Kan^R PCR products were used for transformation of *E. coli* DY330 cells, and for insertion into the pCR®-Blunt II-TOPO® vector for sequencing and glycerol stocks. PCR reactions were performed using a Bio-Rad MyCycler™ thermal cycler. The program used for amplification of the Kan^R cassette was 95℃ for 3 min; 5 cycles of 95℃ for 30 s, 49℃ for 30 s, and 72℃ for 1 min; 25 cycles of 95℃ for 30 s, 62.9 ℃ for 30 s, and 72℃ for 1 min; 72℃ for 5 min, and a hold temperature of 12℃ (**Table 2**). Five microliters of the PCR products were used for visualization via gel electrophoresis.

Table 4. Reaction Parameters for Amplification of Kan^R (program name: edkansh)

Temperature	Time	Cycles
95℃	3 minutes	1
95℃	30 seconds	5
49℃	30 seconds	
72℃	1 minute	
95℃	30 seconds	25
62.9℃	30 seconds	
72℃	1 minutes	
72℃	5 minutes	1
12℃	∞	Hold

The Kan^R cassette prepared for transformation of *E. coli* DY330 used the GoTaq® Green Master Mix provided by Promega. The reaction mixture is listed in **Table 5**.

Table 5. GoTaq® PCR Reaction Master Mix

Reagent	Volume	Concentration
GoTaq® Green Master Mix, 2X	12.5 µL	1X
Forward Primer	1 µL	0.5 µM
Reverse Primer	1 µL	0.5 µM
Water	9.5 µL	N/A
Kan^R Template	1 µL	50 ng
Total	**25 µL per reaction**	

The *E. coli* DY330 cells were transformed using amplified Kan^R from the PCR reaction listed in **Table 2**, and with purified PCR products as a control. The products of an identical PCR reaction were purified using the GeneJET™ PCR Purification Kit and included protocol from Fermentas.

The Kan^R cassette amplified for insertion into the pCR®-Blunt II-TOPO® vector for sequencing and glycerol stocks used the "Routine Deep Vent PCR" Master Mix outlined by NEB and listed in **Table 6**.

Table 6. Master Mix Reagents for Deep Vent PCR

Reagents	Volume	Concentration
ThermoPol Reaction Buffer (10X)	2.5 µL	0.5X
Deoxynucleotide (dNTP) Solution Mix (10 mM)	2.0 µL	200 µM
Forward Primer	1.0 µL	0.5 µM
Reverse Primer	1.0 µL	0.5 µM
Deep Vent (Taq) Polymerase	0.5 µL	1 U
Water	17 µL	N/A
Total (per reaction)	**24 µL**	

The reaction parameters are listed in **Table 4**.

TOPO® Cloning Reaction/Transforming One Shot® Competent Cells

The Kan^R PCR product was inserted into the pCR®-Blunt II-TOPO® vector (**Figure 9**) for sequencing and generation of glycerol stocks (700 µL liquid culture and 300 µL 80% glycerol) for long term storage. The Kan^R PCR product was cloned using the Zero Blunt® TOPO® PCR Cloning Kit supplied by Invitrogen™.

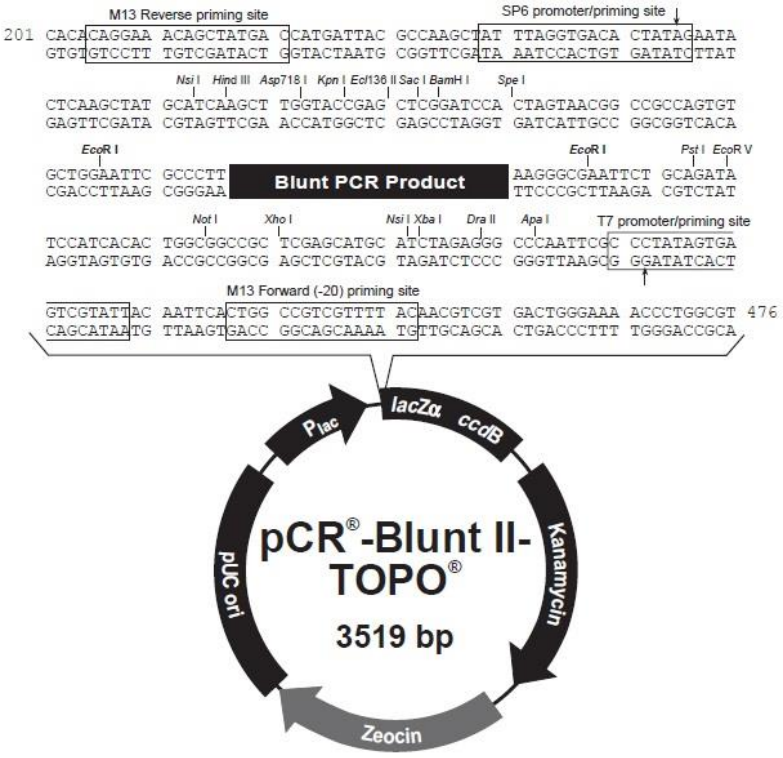

Figure 9. pCR®-Blunt II-TOPO® Vector Map. From http://tools.thermofisher.com/content/sfs/vectors/pcrbluntiitopo_map.pdf

Next, One Shot®TOP10 chemically competent *E. coli* cells (Invitrogen) were transformed with the plasmid DNA. A vial of cells were thawed on ice before adding 2 µL of the TOPO® cloning reaction. The cells were incubated on ice for 30 minutes. The cells were then heat-shocked for exactly 30 seconds at 42℃ without shaking, and immediately transferred back to ice. Next, 250 µL of room temperature S.O.C medium were added. The cells were agitated (200 rpm) at 37℃ horizontally for 1 hour. While the reaction was incubating, two LB plates containing kanamycin were warmed to room temperature. After incubation, 50 µL of the reaction were plated on one plate, and 200 µL on the other. These plates were incubated at 37℃ overnight. Positive recombinants were selected and screened using colony lysis PCR (**Table 1**).

Sequencing

Positive transformants were selected for plasmid purification and subsequent sequencing. The purified recombinant TOPO plasmids were sent to Eurofins Genomics for sequencing—the company outlined the reaction specifications (150 ng DNA in water for a total of 15 µL). The sequencing results were used to determine if the Kan^R PCR product contained the *Halothiobacillus neapolitanus* flanking sequence.

Preparation of pUC18-HnPE2.0 Vector Plasmid

The pUC18-HnPE2.0 construct was previously created in the Shively lab. The glycerol stocks were used to inoculate overnight cultures that were used for plasmid purification.

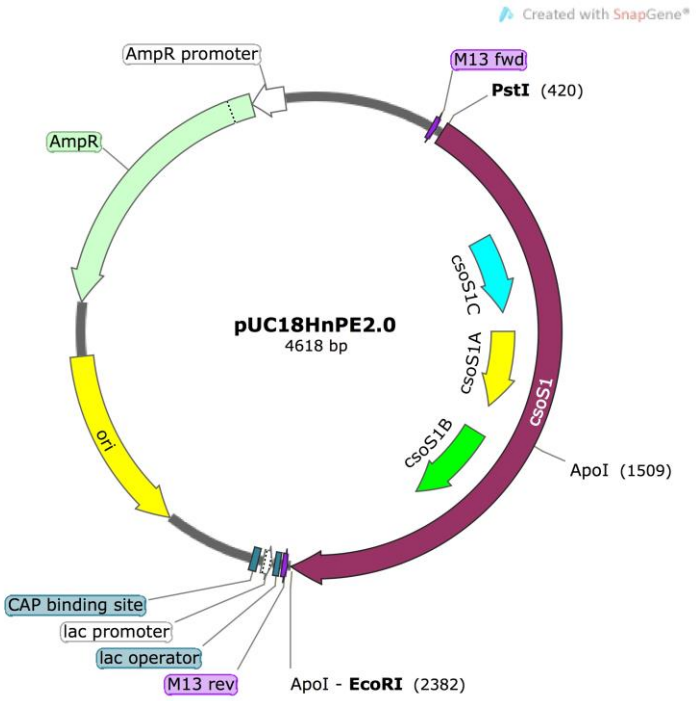

Figure 10. Plasmid Map of pUC18-HnPE2.0. Fragment PstI-EcoRI is 1,962 bp and fragment EcoRI-PstI is 2,656 bp.

Generation of pUC18-HnPE2.0csoS1C Plasmid

An additional plasmid was generated that contains 372 bp upstream of *csoS1C* and 431 bp downstream of the gene. The plasmid was generated by excising an 873 bp fragment from the pUC18-HnPE2.0 plasmid by digestion with ApoI, and re-ligating the plasmid. ApoI only cuts within the *H. neapolitanus* region of the pUC18-HnPE2.0 plasmid (**Figure 10**).

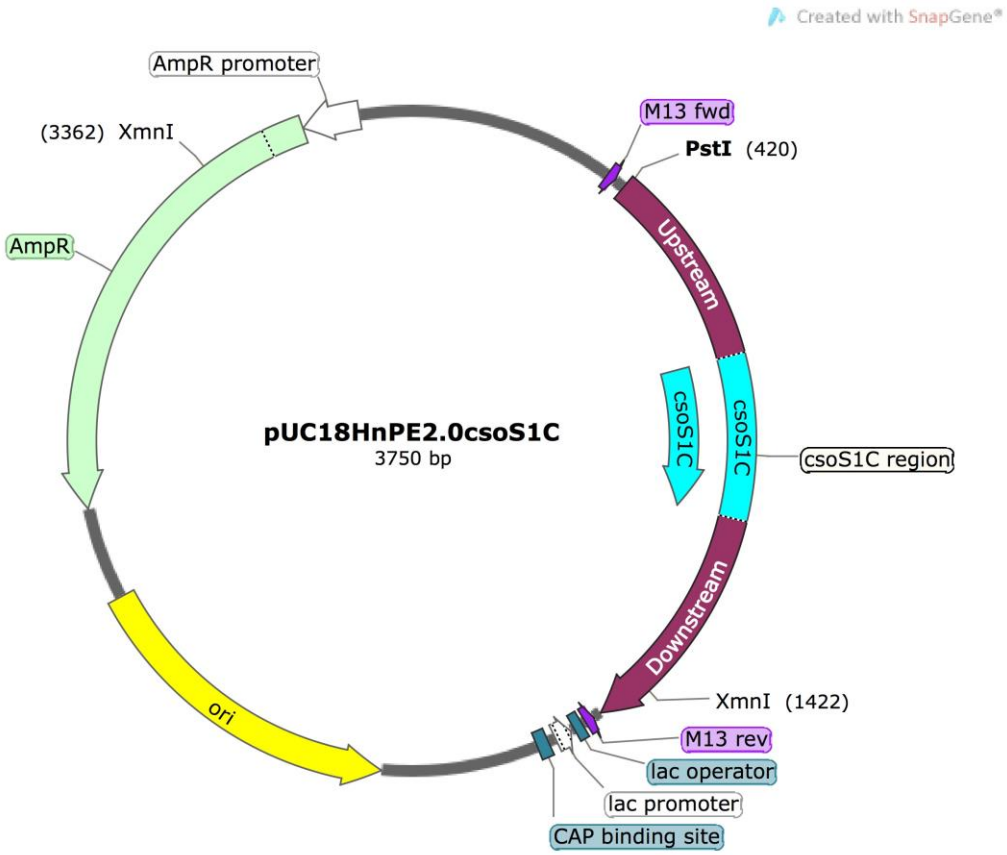

Figure 11. Vector Map of pUC18HnPE2.0csoS1C. Fragment PstI-XmnI is 1,002 bp. Fragment XmnI-XmnI is 1,940 bp, and fragment XmnI-PstI is 808 bp.

Digestion of pUC18-HnPE2.0 with ApoI

The restriction digest is listed in **Table 7**. The reaction was incubated at 50℃ for 1 hour and stopped by the addition of 5 µL of gel loading dye. The 3750 bp fragment was excised from the agarose gel using the QIAEX II® Gel Extraction Kit; however, an additional centrifugation step was added to each spin cycle to completely remove the supernatant. Additionally, two 10 µL elution steps were used to increase the yield in the eluate.

Table 7. Restriction Digest Reaction for ApoI

Reagent	**Amount**
pUC18HnPE2.0 #1 339.3 ED 11.5.15	2.95 µL (1000.9 ng)
NEB Buffer 3.1 (or 3.0)	2.5 µL
ApoI	1.0 µL
Water to 20 µL	13.55 µL

Ligation of ApoI Digested Plasmid

The plasmid digested with ApoI was ligated back to itself using the T4 DNA Ligase provided by NEB. The reaction used followed the general protocol suggested for use with the T4 DNA Ligase (**Table 8**). The reaction was mixed gently by pipetting followed by a brief centrifugation. The reaction was incubated overnight at 16℃ and the ligase inactivated by freezing.

Table 8. Ligation Reaction using T4 DNA Ligase from NEB

Reagent	Amount
10XT4 DNA Ligase Buffer	2 μL
Plasmid DNA	50 ng
Water	To 20 μL
T4 DNA Ligase	1 μL

Transformation of TOP10 cells

The same protocol listed under *TOPO® Cloning Reaction/Transforming One Shot® Competent Cells* was used to transform a tube of TOP10 *E. coli* cells with 2 μL of chilled ligation product. The transformed cells were plated on LBA plates containing ampicillin. Eight colonies from the transformation step were screened (**Figure 7**). Four positive transformants were selected for glycerol stocks and plasmid purification. The pUC18-HnPE2.0csoS1C plasmid with the highest concentration was selected to continue to DY330.

Transformation of DY330

Preparation of competent DY330 cells

Overnight cultures of DY330 were prepared and incubated at 30°C. The liquid culture was used to inoculate two 50 mL subcultures in baffled flasks. The subcultures were incubated at 30°C (225 rpm) until the OD_{600} reached 0.5. One of the 50 mL subcultures was divided into two 25 mL subcultures. The 50 mL and one 25 mL subculture was incubated at 42°C and the other 25 mL subculture was incubated at 30℃ creating the uninduced control. All cultures were then transferred to an ice/water slurry and cooled while shaking for 15 minutes. The cells were transferred to Falcon tubes and harvested by

centrifuging (JA 25.5 rotor) at 4℃ for 10 minutes 4,000 rpm. The supernatant was discarded and the cells were resuspended in 1 mL of ice-cold DI water. The cell suspension was transferred to microcentrifuge tubes and centrifuged at 14,000 rpm and 4℃ for 20 seconds. The supernatant was discarded and the pellet washed with 1 mL ice-cold DI water. The centrifugation and wash step was repeated a total of three times. The final pellet was resuspended in 200 μL ice-cold water and kept on ice until electroporation.

Electroporation of DY330 cells

Equimolar amounts of the linear donor DNA (Kan^R amplification product) and plasmid acceptor DNA (pUC18-HnPE2.0 or pUC18-HnPE2.0csoS1C at approximately 0.14 pmol) were added to a pre-cooled electroporation cuvette along with 100 μL of competent DY330 cells. The cells were electroporated at 2.0 kV, 25 μF with the pulse controller set to 200 Ohms. Immediately after electroporation, 1 mL of LB was added to the cuvette and the reaction was transferred to a Falcon tube. The electroporated cells were incubated on ice for 5 minutes, then shaken at 30°C for 1.5 h. The cells were plated in 200 μL aliquots on LBA plates containing kanamycin and ampicillin and incubated at 30°C overnight. A total of six different reactions were conducted using both pUC18-HnPE2.0csoS1C and pUC18-HnPE2.0 and both purified and unpurified Kan^R PCR products and two control reactions. One control reaction used cells from the uninduced culture. The other control introduced the pUC18-HnPE2.0 plasmid to DY330 to insure that the electroporation step did not kill the cells. This reaction was plated on a LBA plate containing only ampicillin.

Table 9. Reactions used for the Electroporation of DY330

Reaction Number	Vector Plasmid	Linear Donor
Reaction 1	pUC18-HnPE2.0csoS1C	Purified Kan^R PCR Product
Reaction 2	pUC1-HnPE2.0csoS1C	Unpurified Kan^R PCR Product
Reaction 3	pUC18-HnPE2.0	Purified Kan^R PCR Product
Reaction 4	pUC18-HnPE2.0	Unpurified Kan^R PCR Product
Reaction 5	pUC18-HnPE2.0	Unpurified Kan^R PCR Product
Reaction 6	pUC18-HnPE2.0	N/A

Colonies from the overnight plates of electroporated cells were selected for colony PCR using GoTaq® Green Master Mix (**Table 5**) and standard M13 primers. The PCR program was CBStandard (**Table 2**) using 23 μL of master mix and 2 μL of template (lysate). The screened colonies were also streaked on LBA plates containing kanamycin (**Figure 7**). The colony PCR products were visualized using gel electrophoresis.

Isolation of Deletion Construct

Colonies from Reaction 2 and Reaction 4 were selected and grown on kanamycin plates at 30℃ overnight. Colonies from these cultures were prepared for colony PCR as described in the preceding section. To obtain single plasmid colonies, 4 colonies from Reaction 2 and Reaction 4 were selected on LBA plates containing kanamycin.

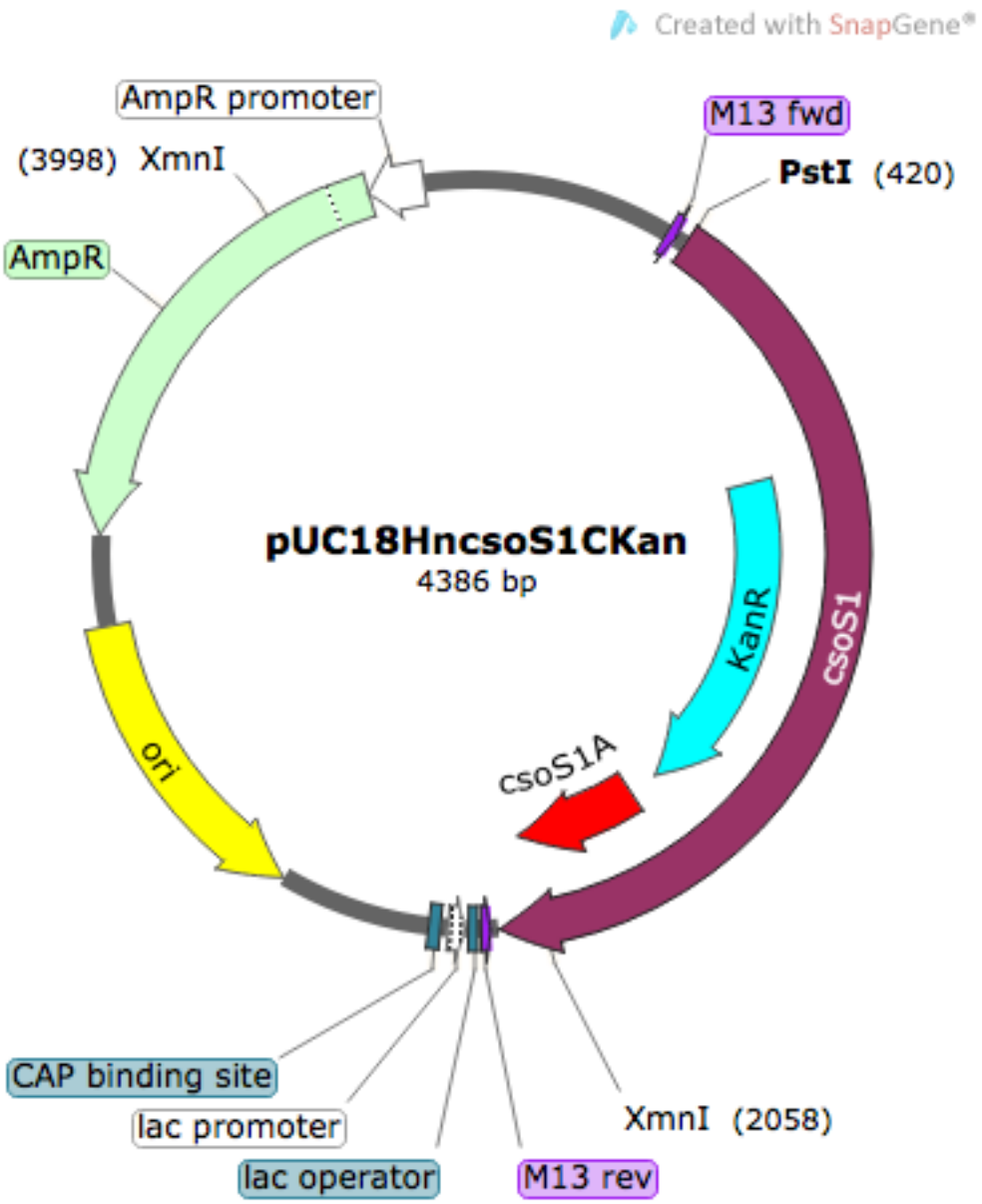

Figure 12. Predicted Deletion Construct of pUC18HnPE2.0csoS1C Recombination. Fragment PstI-XmnI is 1,638 bp; fragment XmnI-XmnI is 1,940 bp, and fragment XmnI-PstI is 808 bp.

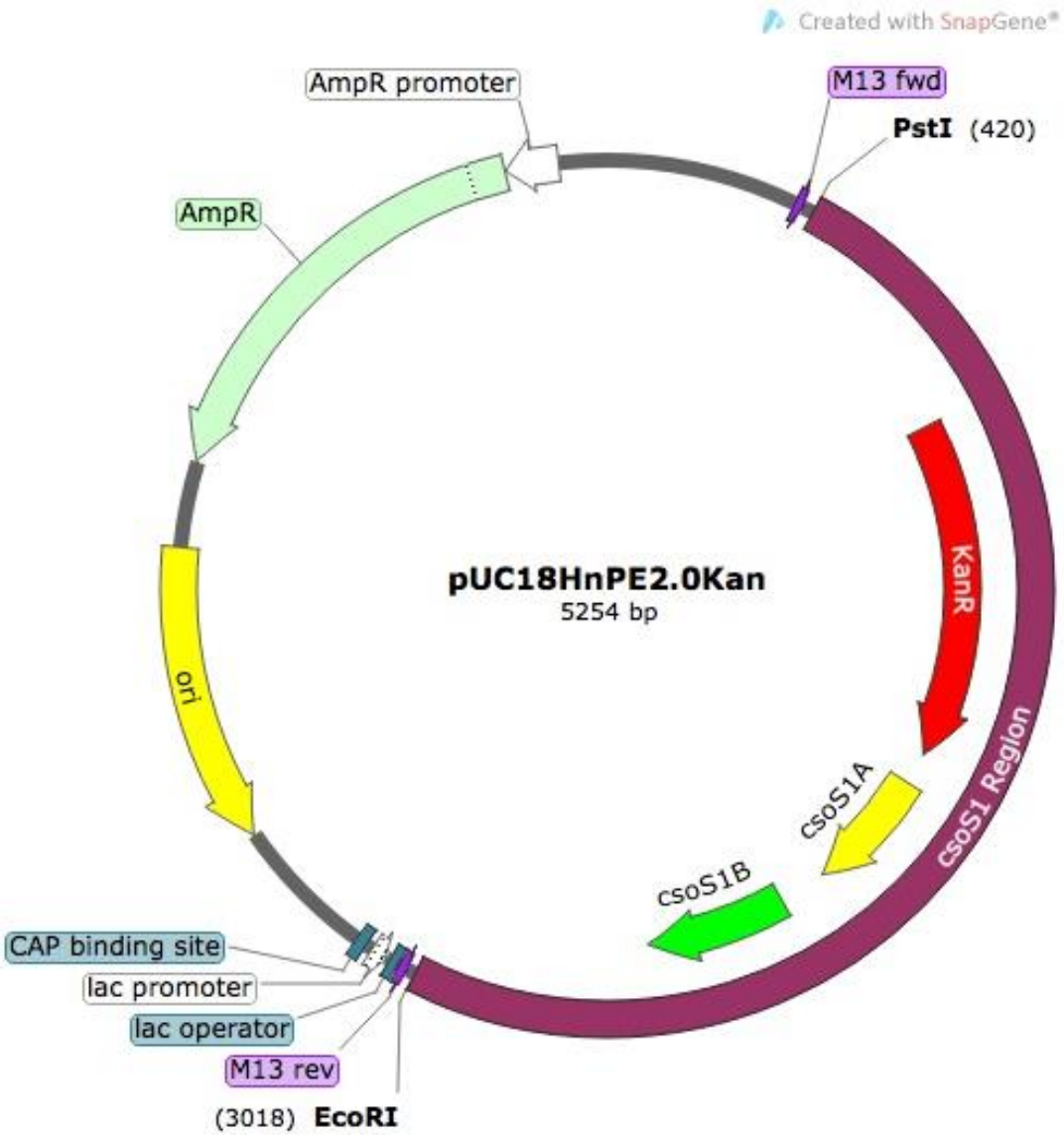

Figure 13. Predicted Deletion Construct of pUC18HnPE2.0 Recombination. Fragment PstI-EcoRI is 2,598 bp and fragment EcoRI-PstI is 2,656 bp.

Colonies from these cultures were again selected and prepared for colony PCR (**Table 2**). Liquid LB cultures containing kanamycin and ampicillin were inoculated with 2 colonies from each reaction. The cultures were incubated overnight at 30℃ with agitation. The plasmids from these cultures were isolated (see *Plasmid Purification Protocol*).

To further study the results of the recombination, the isolated plasmids were digested to identify characteristic fragments. Each restriction digestion contained 500 ng of plasmid DNA. The plasmids from Reaction 2 were digested with PstI and XmnI (NEB), in a reaction containing NEBuffer 2.1. The plasmids from Reaction 4 were digested with PstI and EcoRI (NEB) in a reaction containing NEBuffer 3.1.

The plasmid from Reaction 2 clone 7 was diluted to 210 pg/μL and used to transform TOP10 *E. coli* cells. The transformants were plated on a LBA plate containing kanamycin, one containing ampicillin, and one without an antibiotic for control. Cultures, both liquid LB and a LBA plate with kanamycin, were inoculated with 6 transformant colonies and incubated overnight at 37℃. Glycerol stocks were prepared from the liquid cultures. The plasmids were isolated (see *Plasmid Purification Protocol*), and subsequently digested as described previously. Two plasmids were sequenced.

Chapter IV: Results

Preparation of the Kan^R Cassette

The primers designed to amplify the Kan^R cassette with ends homologous to the flanking regions of *csoS1C* were designed. The preferred length of the region of homology is 45 nucleotides up- and downstream of the *csoS1C* gene, to allow for efficient homologous recombination in *E. coli* DY330. The primers should not self-anneal or anneal

to each other; additionally, both primers needed to denature around the same temperature for amplification. The Oligo Analyzer program on the Integrated DNA Technologies (IDT) site was used to analyze potential primer designs (http://www.idtdna.com/site). The resulting primers used for the amplification of the kanamycin resistance cassette are shown in **Figure 14**. The forward primer added 47 bp of *H. neapolitanus* DNA, and the reverse primer added 44 bp to the end of the Kan^R cassette (**Figure 15**).

Forward Primer (62 bp) T_m=74.5℃
5'-cgctagatgagttgattttgaatgagtctttattgaggagagaagaaCCGGAATTGCCAGCTG-3'
Reverse Primer (65 bp), T_m=78.7℃
5'-aaagaaccggaacaagcctgcgccggttcgtctttcccaatcctCAGAAGAACTCGTCAAGAAGG-3'

Figure 14. Primer Design for Amplification of Kan^R Cassette with Regions of Homology Flanking the *H. neapolitanus csoS1C* gene. Lowercase sequence designates sequence from *Halothiobacillus neapolitanus*. Uppercase sequence designates sequence that anneals to the kanamycin resistance gene.

In order to confirm the sequence and generate glycerol stocks of the Kan^R cassette, the amplified PCR product was cloned into the pCR®-Blunt II-TOPO vector.

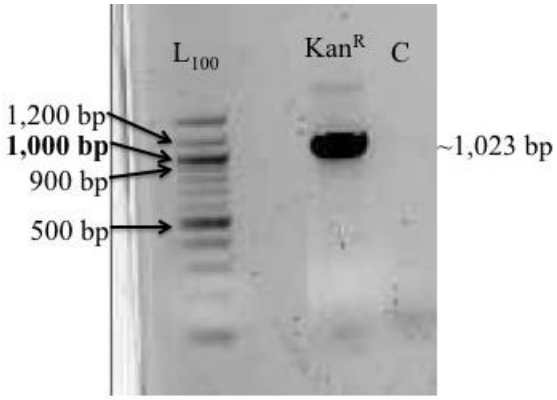

Figure 15. Amplification of Kan^R Cassette. Lane Kan^R contains product cloned into pCR®-Blunt II-TOPO Vector. Water was used as negative control (no DNA template; lane C). Lane L_{100} contains the 100 bp ladder.

The recombinant construct was transformed into One Shot® TOP10 competent *E. coli* cells for selection of positive recombinants. Plasmids from positive clones (**Figure 16**) were isolated and used for sequence confirmation and for the generation of glycerol stocks. The sequencing confirmed the

presence of the Kan^R cassette with ends homologous to *H. neapolitanus* DNA. The PCR products obtained were confirmed to be the Kan^R cassette, and the PCR primers and

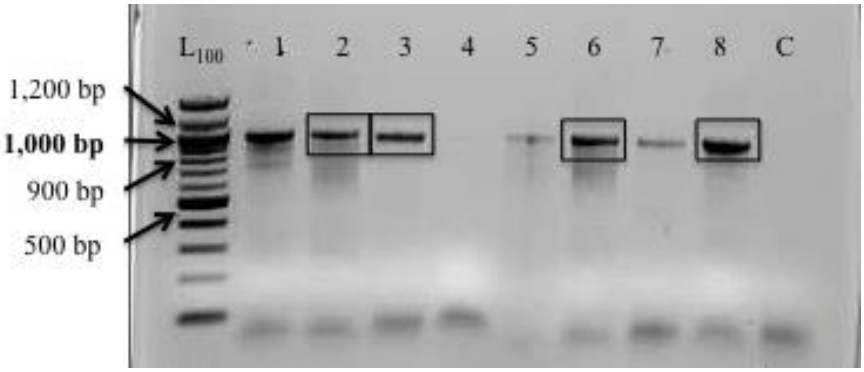

Figure 16. Colony Lysis PCR of TOPO-Kan Clones. The expected fragment was 1,023 bp. The negative control used water instead of template DNA. L_{100} is 100 bp DNA ladder, 1-8 are the screened clones, C is the control.

protocols were used subsequently.

The linear Kan^R cassette used for the electroporation of DY330 was obtained by amplification by PCR using the GoTaq® master mix and a TOPO-Kan cassette as the DNA template. The two sets of PCR products were obtained. One set was purified to remove residual PCR reagents and loading dye and the other was not. The purified PCR product had a concentration of approximately 24 ng/μL, and the concentration of the unpurified

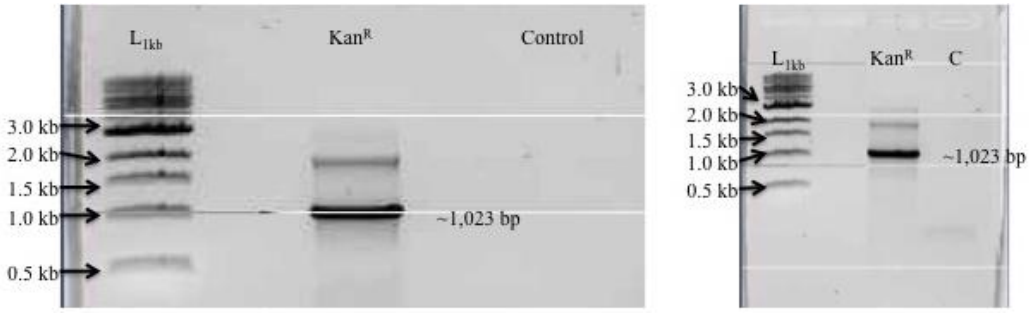

Figure 17. Kan^R Cassettes Used as Donor DNA for Homologous Recombination. Left: PCR product used unpurified. Right: PCR product before purification. L_{1kb} is 1 kb DNA ladder, Kan^R is PCR product, Control or C is the water control.

PCR product was estimated to be 25 ng/µL by comparison to the concentration of the NEB ladder fragments.

Preparation of pUC18-HnPE2.0 Vector Plasmid

The pUC18-HnPE2.0 plasmid was isolated from glycerol stocks of TOP10 cells containing the plasmid. The purified plasmid used for consecutive steps had a final concentration of approximately 340 ng/µL.

Generation of pUC18-HnPE2.0csoS1C Plasmid

To develop the pUC18-HnPE2.0csoS1C plasmid, the pUC18-HnPE2.0 plasmid was digested with the restriction enzyme ApoI. The digestion was expected to yield two fragments, one 873 bp and the other 3,750 bp. The 873 bp fragment corresponds to the region of DNA to be removed from the pUC18-HnPE2.0 insert, and the 3,750 bp

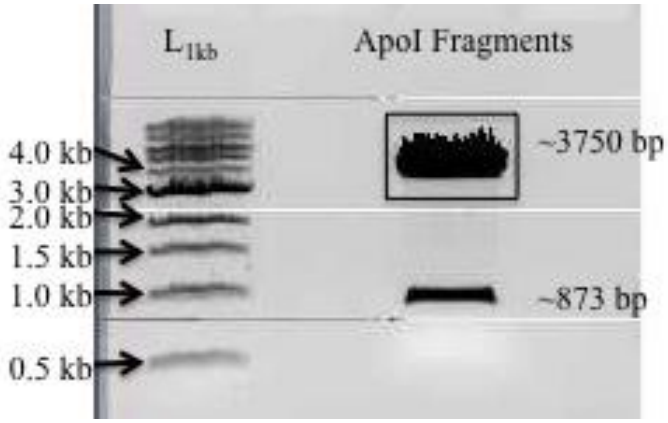

Figure 18. Digestion of pUC18HnPE2.0 with ApoI. The desired fragment indicated at 3,750 bp was excised from the gel. L_{1kb} contains the 1 kb DNA ladder, ApoI Fragments are the resulting fragments from digestion.

corresponds to the remaining fragment. Both fragments were visualized in the expected region on the gel (**Figure 18**). The desired fragment at 3,750 bp was excised from the gel. The resulting concentration of the fragment was 26 ng/µL. The excised fragment underwent self-ligation to yield the new plasmid construct. The ligation products were used

to transform TOP10 *E. coli* cells. Eight colonies were randomly selected for screening with colony lysis PCR. The amplified fragment was expected to be approximately 1,099 bp, which was seen on the gel (**Figure 19**). Four of the positive colonies were used to generate

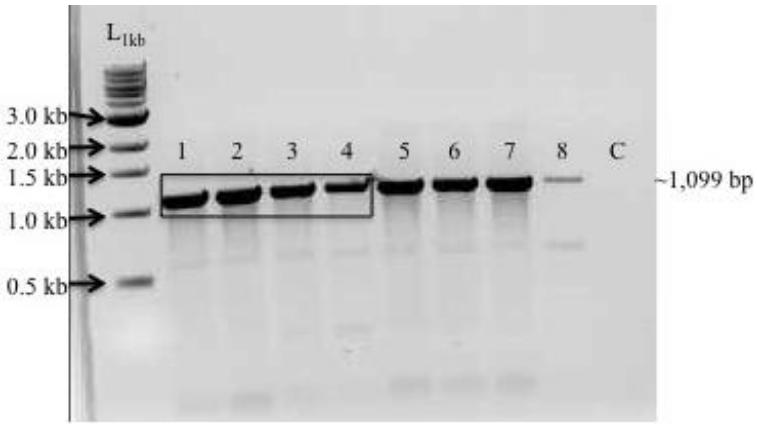

Figure 19. Colony PCR of pUC18HnPE2.0csoS1C. Clones selected for the generation of glycerol stocks are indicated with a square, the clone used as acceptor DNA was clone 3. The negative control used water instead of template DNA. L_{1kb} is 1kb DNA ladder, 1-3 are screened colonies, C is water control.

glycerol stocks. The pUC18-HnPE2.0csoS1C used for the electroporation of DY330 had a final concentration of 361 ng/μL.

Transformation of DY330

The *E. coli* DY330 cells were co-transformed with an acceptor plasmid and the linear Kan^R cassettes generated through PCR amplification (see **Table 9** for reactions). All transformants were able to grow on LBA plates containing ampicillin and kanamycin. However, colony lysis PCR of the first generation clones revealed double fragments, indicative of mixed plasmids, and fragments of incorrect size (**Figure 20**). Recombinants of the pUC18HnPE2.0 plasmid were expected to yield a fragment of approximately 2,700 bp and recombinants of pUC18HnPE2.0csoS1C were expected to yield a fragment of approximately 1,800 bp after colony lysis PCR amplification. However, amplification of

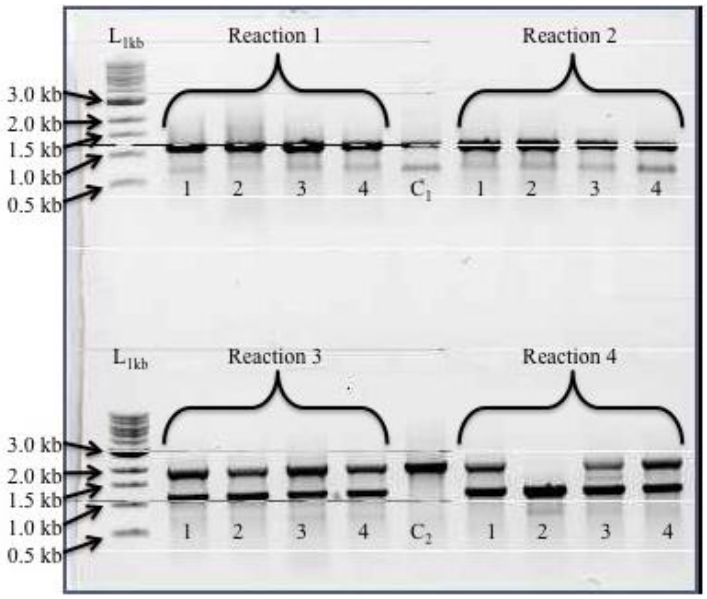

Figure 20. Colony PCR of First Generation DY330 Clones. The control labeled C_1 amplified pUC18HnPE2.0csoS1C and the C_2 control amplified pUC18HnPE2.0. L_{1kb} is 1kb DNA ladder, 1-4 are screened colonies, C are the controls.

Reactions 1 and 2 resulted in fragments approximately 1,000 bp and 600 bp, and Reactions 3 and 4 showed fragments approximately 1,200 bp and 1,900 bp long.

In an attempt to separate the plasmids, colonies from Reaction 4 (pUC18-HnPE2.0 = acceptor plasmid) and colonies from Reaction 2 (pUC18-HnPE2.0csoS1C = acceptor plasmid) were further isolated by selection on kanamycin plates. These second generation clones were able to grow in the presence of kanamycin; however, the colony lysis PCR again resulted in double bands (**Figure 21**). Amplification of Reaction 2 resulted in a fragment approximately 1,000 bp long and amplification of Reaction 4 resulted in fragments approximately 1,200 bp and 1,900 bp.

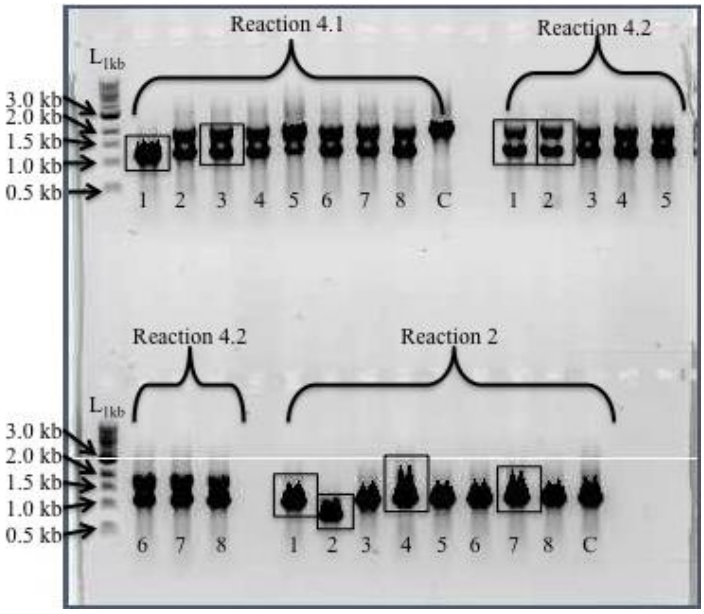

Figure 21. Second Generation Colony PCR Results. Clones from Reaction 4.1 and 4.2 were selected from different LBA plates. Reaction 4 control was pUC18HnPE2.0, and Reaction 2 control was pUC18HnPE2.0csoS1C. Colonies selected to continue are indicated by a square. L_{1kb} is 1kb DNA ladder, 1-8 are screened colonies, C is control.

Four colonies from each reaction were selected and grown on kanamycin plates in an attempt to isolate the recombinant plasmid. **Figure 22** shows the results of the colony lysis PCR. Amplification of the third generation clones still resulted in double fragments,

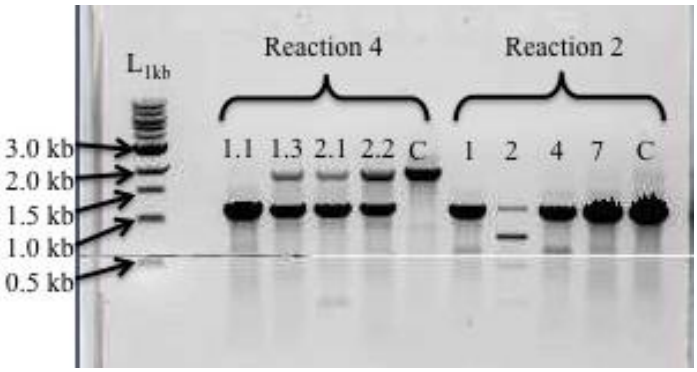

Figure 22. Colony PCR on Third Generation Clones. The control for Reaction 4 was pUC18HnPE2.0, and the control for Reaction 2 was pUC18HnPE2.0csoS1C. L_{1kb} is 1kb DNA ladder, 1.1 is clone 1 from Reaction 4 plate 1, 1.3 was clone 3 from Reaction 4 plate 1, 2.1 is clone 1 from Reaction 4 plate 2, 2.2 is clone 2 from Reaction 4 plate 2. 1 is clone 1 from Reaction 2, 2 is clone 2 from Reaction 2, 4 is clone 4 from Reaction 2, 7 is clone 7 from Reaction 2. C indicates controls.

and fragments that were of similar sizes to the original plasmids (**Figure 22**). Reaction 4 had two bands of approximately 1,200 bp and 1,900 bp. Reaction 2 exhibited fragments of approximately 1,000 bp and 800 bp.

Identification of Recombination Events

Since the colony PCR results were inconclusive, a different method was used to determine the results of the recombination. Plasmids were isolated from Reaction 2 numbers 1 and 7, Reaction 4 number 1.1, and Reaction 4 number 2.2 (**Figure 22**). Plasmids from Reaction 2 were digested with PstI and XmnI, and plasmids from Reaction 4 were

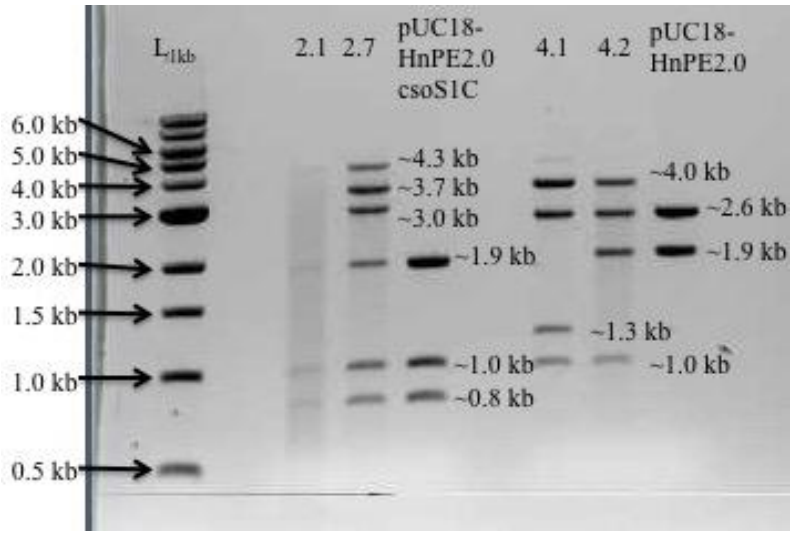

Figure 23. Restriction Digest of Plasmids from Reactions 2 and 4. Digestion of plasmid 2.1 contained an error, 2.7 shows a pattern indicative of mixed original and recombinant plasmids. Digestion of plasmids 4.1 and 4.2 did not result in expected fragments. L_{1kb} is 1kb DNA ladder. pUC18-HnPE2.0csoS1C and pUC18-HnPE2.0 were used as controls.

digested with PstI and EcoRI. The resulting fragment pattern (**Figure 23**) was used to identify which plasmids were present in the cells (**Figure 23**).

Different fragments were expected from the digestion of the recombinant plasmid and the original plasmid (**Figures 13 and 14**). Digestion of pUC18HnPE2.0csos1C plasmid with PstI and XmnI produced three fragments approximately 1.9 kb, 1.0 kb, and 0.8 kb, which can be seen in **Figure 23**. Digestion of pUC18HnPE2.0 with PstI and EcoRI results in two fragments approximately 2.6 kb and 1.9 kb (**Figure 23**). The recombinant pUC18HnPE2.0csoS1C should show fragments approximately 1.6 kb, 1.9 kb, and 0.8 kb. The fragments from the digested pUC18HnPE2.0 recombinant should be approximately 2.7 kb and 2.6 kb. As shown in **Figure 23**, the plasmids isolated from the *E. coli* DY330 cells appeared to be a mixture of original and recombinant plasmids. To isolate the recombinant plasmid, TOP10 *E. coli* cells were transformed with 210 pg of the purified plasmid DNA isolated from one of the transformants (Reaction 2 number 7, **Figure 22**). From the transformants that were able to grow in the presence of kanamycin, plasmid DNA was isolated from selected colonies and digested with PstI and XmnI (**Figure 24**). It appears that separation of the original and recombinant plasmids was obtained. As seen in **Figure 24**, lanes 2 and 5 contained a fragment of approximately 4.3 kb, which corresponds to the length of the linearized recombinant plasmid. In lanes 1, 3, and 6 the 4.3 kb fragment is not present; however, the fragments characteristic of the original acceptor plasmid DNA are present. Since the two fragment patterns are not present in the same lane, and thus the same bacterial colony, the original and recombinant plasmids have been separated. Possible linearization of the recombinant occurred as well. The presence of a single fragment at approximately 4.3 kb instead of the predicted three fragments suggests that the recombinant

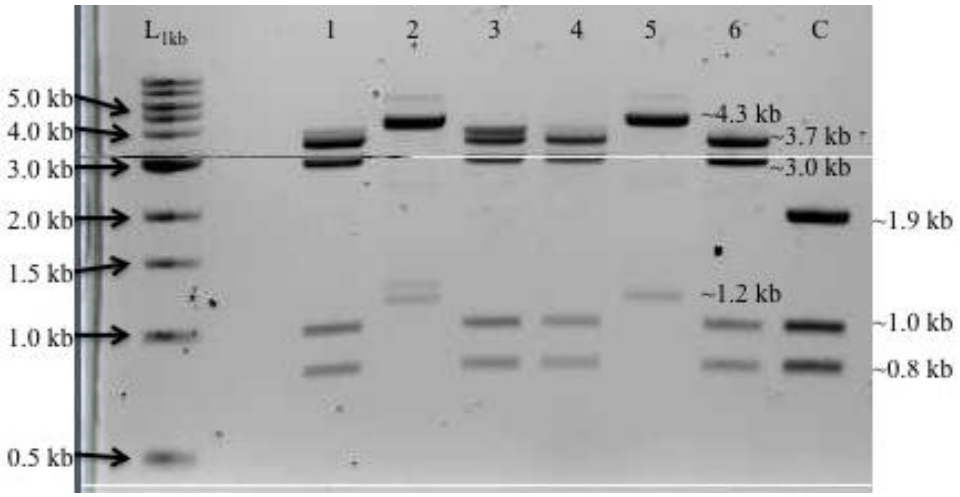

Figure 24. Restriction Digest of Diluted Plasmids. Plasmids digested were isolated from TOP10 *E. coli* transformed with diluted 2.7 plasmids. Clone #5 and #6 were used for sequencing. L_{1kb} is 1kb DNA ladder. #1-6 are screened colonies, C is digested pUC18-HnPE2.0csoS1C.

plasmid was simply linearized rather than completely digested. To examine the recombination site, plasmid DNA from two colonies was sequenced.

The plasmid DNA from clones 5 and 6 (**Figure 24**) was sequenced. The sequencing results from the M13 forward primer of plasmid 5 did not show any homology to the expected recombinant sequence. The sequencing results using the M13 reverse primer for plasmid 5 exhibited alignment with the kanamycin resistance gene. The forward sequencing reaction of plasmid 6 indicated homology with the expected recombinant sequence upstream of the predicted insertion site of the kanamycin cassette and downstream of the cassette, but no homology with the predicted cassette site. A BLAST alignment using the original *csoS1* region as the query revealed that *csoS1C* had not been replaced by the kanamycin cassette. However, the reverse reaction showed alignment with the kanamycin cassette. The sequencing results of both plasmids suggest that the

kanamycin cassette was inserted in the acceptor plasmid, but did not replace the *csoS1C* gene.

Chapter V: Discussion and Conclusion

The Kan^R cassette was generated to replace the *csoS1C* gene in the *cso* operon (**Figure 4**). The Kan^R cassette was amplified with approximately 45 base pairs of *H. neapolitanus* DNA on each end to allow for efficient sequence recognition by the lambda recombinase that is overexpressed in *E. coli* DY330. A shorter sequence would have resulted in less efficient recombination.[16] The amplification of the Kan^R cassette was successful as indicated by the sequencing results. A purified PCR product and an unpurified PCR product were used as linear donor DNA for the transformation of *E. coli* DY330. Transformations with both PCR products resulted in colonies able to grow on the LBA plates containing kanamycin and ampicillin after electroporation. The transformants from purified PCR reactions resulted in more, smaller colonies after plating, while the transformants from unpurified PCR reactions grew fewer, larger colonies. A higher colony count suggests that the purified PCR products had a higher rate of transformation and recombination. The size difference in the colonies may simply be the result of resource competition. More colonies on a plate mean less nutrients for each colony, thus restricting the size of the colony. Since both reactions resulted in colonies, both were screened for recombinants.

The pUC18-HnPE2.0csoS1C plasmid used as the acceptor DNA was designed to include approximately 500 base pairs of DNA up- and downstream of the *csoS1C* gene to increase the success rate of recombination. The number of *csoS1* paralogs (2 paralogs) in this plasmid is lower than in the original pUC18-HnPE2.0 plasmid (3 paralogs). The

pUC18-HnPE2.0 plasmid contains *csoS1C, A, B,* and *csoS4A/B* while the new plasmid lacks the *csoS1B* gene that may interfere with correct insertion of the kanamycin cassette into *csoS1C*. The purpose of the pUC18-HnPE2.0csoS1C plasmid was to obtain a circular acceptor containing the *csoS1C* gene with approximately a 500 bp buffer region on each end. The resulting fragment contains 372 bp upstream of the gene and 431 bp downstream of the gene. Since intermolecular ligation is more favorable than intramolecular ligation, a restriction enzyme was selected that cut the original plasmid in two locations only in the *csoS1* region of the plasmid. The restriction enzyme ApoI was the only enzyme that cut only twice and in the desired location on the insert. The digest and subsequent gel extraction removed 873 bp from the *csoS1* region (**Figure 18**). The new plasmid was used along with the original plasmid as the circular receptor DNA for homologous recombination.

Both plasmids were used for electroporation of DY330, and both plasmids resulted in colonies. Colonies from both plasmids and both PCR products were screened for recombinants. Successful homologous recombination should have resulted in a single band at approximately 1,800 bp fragment after colony PCR from the pUC18-HnPE2.0csoS1C plasmid and a 2,670 bp fragment from the pUC18-HnPE2.0 plasmid. Additionally, if homologous recombination had not occurred the bacteria should not have been able to grow on LBA plates containing kanamycin.

The colony lysis PCR showed double bands, and bands similar in size to the original plasmids. Although the expected fragments were not seen, the bacteria were able to grow on kanamycin plates indicating a recombination event had taken place. The double fragments suggest that both original acceptor plasmid DNA and recombinant plasmid DNA

were present in the cells. To determine if the kanamycin cassette had been incorporated into the genome of DY330 instead of the acceptor plasmids, the plasmids were purified and used to transform TOP10 *E. coli*. The transformants were able to grow on kanamycin plates, which confirms that the recombination event did occur in the acceptor plasmids.

A restriction digest of the plasmids was conducted to examine the location of the recombination event(s). The resulting fragmenting patterns of the plasmids in **Figure 23** (Reaction 4) were inconclusive. The resulting fragments either matched the original plasmid, or were smaller than any predicted fragment from either original or the expected recombined plasmids. The remaining fragments in **Figure 23** (Reaction 2) were as expected for a mixture of both original and recombinant plasmids. Fragments corresponding to linearized original plasmid (3.7 kb) and recombinant plasmid (4.3 kb) were seen, as well as fragments expected from the digestion of the original pUC18HnPE2.0csoS1C plasmid. In a mixture of both plasmids, a double band was expected approximately at 1.9 kb. Both plasmids were expected to show a fragment at 1.9 kb, and the recombinant was expected to have a fragment at 1.6 kb. The only fragment that was not expected from the digestion of Reaction 2 was the fragment approximately 3.0 kb. It is possible that this fragment was the result of partial digestion of a plasmid.

In order to isolate the recombinant plasmid, the purified plasmids were diluted before being used to transform TOP10 *E. coli* cells to increase the likely hood of each cell taking up only a single plasmid. Cells transformed with the recombinant plasmid were able to grow on LBA plates containing kanamycin. Plasmids were isolated from six of the positive colonies, and digested with PstI and XmnI. Of the six, two digestions did not show fragments corresponding to the original plasmid; the other four digestions did not show a

band corresponding to linearized recombinant plasmid DNA. All six reactions lacked a fragment at 1.9 kb. Both enzymes were functional since control digestion of the original acceptor plasmid DNA yielded the expected digestion pattern. Plasmid DNA from transformants resulting in each digestion pattern (**Figure 24**, clones 5 and 6) was sequenced to determine the outcome(s) of the recombination.

The sequencing results obtained from the extension of the M13 reverse primer of the linearized single band plasmid (**Figure 24**, clone 5), showed sequence similarity to the kanamycin resistance gene, indicating that the recombination event did occur in or near the *csoS1* insert in pUC18. The sequencing results from the extension of the M13 forward primer did not show homology to the predicted recombinant construct, suggesting that the kanamycin construct did not replace the *csoS1C* gene, rather inserted near the end of the *csoS1* insert. The sequencing of the second plasmid (**Figure 24**, clone 6) obtained from the extension of the M13 forward primer revealed homology to the original acceptor plasmid. The sequence obtained using the M13 reverse primer was homologous to the kanamycin cassette. Again, the kanamycin cassette did not replace the *csoS1C* gene, but inserted toward the end of the *csoS1* insert or on the pUC18 plasmid itself. The presence of the kanamycin cassette in the extension of the M13 reverse primer, suggests that the cassette was closer to the M13 reverse primer site on the plasmid (**Figure 12**).

The expected deletion construct was not generated; however, a recombination event did occur. The linearization of recombinant plasmid DNA (**Figure 24**) resulted in a 4.3 kb fragment consistent with the predicted size of the recombinant. However, sequencing determined that the kanamycin cassette did not replace the *csoS1C* gene but inserted further downstream. It is possible that some epigenetic characteristic that prevented the desired

recombination developed during the generation of the pUC18HnPE2.0 plasmid. Further research into the recombination event is necessary to determine why the kanamycin cassette did not replace the *csoS1C* gene.

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