Knockout of the *lacZ* Gene in *Enterobacter* sp. YSU

by

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Submitted in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the

Biological Sciences

Program

YOUNGSTOWN STATE UNIVERSITY

August 2018

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ABSTRACT

The lactose operon is responsible for the import and metabolism of lactose. β-galactosidase is a lactose operon enzyme that hydrolyzes lactose into glucose and galactose. *Enterobacter* sp. YSU, which was isolated from a heavy metal contaminated site in Oak Ridge, Tennessee, contains a lactose operon. Many studies used β-galactosidase as a reporter gene to study the expression levels of other genes. This was accomplished by replacing the gene of interest with the gene for β-galactosidase, *lacZ*. Thus, the gene of interest controls the expression of *lacZ*. Then, LacZ activity is detected using a color indicator such as 5-bromo-4-chloro-3-indolyl- β-D-galactopyranoside (X-gal) or o-nitrophenyl-β-D-galactopyranoside (ONPG) which turn blue or yellow, respectively, when hydrolyzed by β-galactosidase. The overall goal is to use *lacZ* as a reporter gene to study metal resistance genes in *Enterobacter* sp. YSU. Before *lacZ* can be used as a reporter, it must be removed from the genome of *Enterobacter* sp. YSU to eliminate background activity. A large section of the *lac* operon was cloned into a suicide plasmid and sequenced. Then, PCR was used to amplify the whole plasmid lacking the *lacZ* gene. The DNA was ligated to produce a new recombinant plasmid lacking *lacZ* or it was ligated with a kanR/sacB DNA fragment to produce a new recombinant plasmid with *lacZ* replaced by the kanR/sacB genes. KanR is a selectable marker for kanamycin resistance and *sacB* is a counter-selectable marker. Cells containing the *sacB* gene die when spread on agar plates containing 5% sucrose. Deletion of the lacZ gene in *Enterobacter* sp. YSU will be a two-step process. First, the recombinant with the kanR/sacB replacement will be electroporated into *Enterobacter* sp. YSU. Homologous recombination into the chromosome of *Enterobacter* sp. YSU will allow it to grow on

kanamycin plates. Second, the *lacZ* deletion plasmid will be electroporated into the new strain. Homologous recombination will remove the *sacB* gene allowing for growth on plates containing 5% sucrose and result in the complete removal of the *lacZ* gene. Bioinformatics analysis of the *Enterobacter* sp. YSU *lacZ* gene and LacZ protein showed that it is highly similar to the *lacZ* gene and LacZ protein from *E. coli*.

ACKNOWLEDGEMENTS

I would like to acknowledge Dr. Jonathan Caguiat for taking me on for this project and for being my primary thesis advisor. Thank you for always supporting me with the many questions I had and for allowing me to work and learn in his lab. I am confident that the knowledge and skills I have learned in your lab will prove very useful in the work force. Thank you for everything you have done.

I would like to thank my other committee members, Dr. David Asch and Dr. Xiangjia Min, for being supportive and always being ready to answer any questions I had. Both of you have taught me very valuable lessons in and out of the classroom. I would also like to thank Dr. Gary Walker for helping me since the first day I started my Master's Degree.

A very generous thank you to not only my colleagues, but also the many friends I have made along the way: Jamielynn Doyle, Sarah Eisnaugle, and Tim Daugherty to name a few. I am especially thankful to my boyfriend, Michael Juhasz, for loving and supporting me 100% since the day I met him, not just with my studies, but with everyday life. I want to especially thank my parents, Connie and Richard Ford, and my sister and her husband, Kristen and Anthony Tiefenbach, for supporting me in wanting to go back for my degree and being there for me with anything I ever needed since day one. Just the love and support you have all shown me is amazing.

Thank you to the Department of Biological Sciences for providing funds to support my research and everyone at YSU has been so wonderful to me since the first day I stepped foot on campus at Youngstown State University.

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CHAPTER I: INTRODUCTION

1.1 Y-12 Plant and *Enterobacter* sp. YSU

In the early 1940s, the Y-12 plant in Oak Ridge, Tennessee processed uranium to make nuclear bombs. Later during the Cold War, the plant solely processed lithium for the creation of hydrogen bombs. This technique required substantial amounts of mercury, which was released into the surrounding air, soil, and water. Substantial amounts of this mercury ended up in East Fork Poplar Creek. In addition, four close-by S3 ponds were used to store waste products of these processes. During storage, mass amounts of waste products leeched into the surrounding earth. Eventually, these ponds were dredged, drained, capped, covered with asphalt and converted into a large parking lot [4, 19, 29, 34]. The multi-metal resistant strains, *Stenotrophomonas maltophilia* Oak Ridge strain 02 (*S. maltophilia* 02) and *Enterobacter* sp. YSU were isolated from this site [20].

Enterobacter sp. YSU is resistant to salts of mercury, zinc, cadmium, copper, gold, silver, arsenic and selenium. This strain also has the ability to use lactose as a sole carbon source using β-galactosidase (*LacZ*), a gene that *S. maltophilia* 02 does not have [20]. The overall goal is to clone and sequence the *Enterobacter* sp. YSU β-galactosidase gene (*lacZ*) and use it as a reporter gene to study metal-resistances in *S. maltophilia* 02 and *Enterobacter* sp. YSU.

1.2 *Escherichia coli* lactose operon

The *Escherichia coli* (*E. coli*) lactose operon consists of the genes, *lacI*, *lacZ*, *lacY* and *lacA* [48], which encode certain enzymes that break down and metabolize lactose, hence the name “lac” (Figure 1). The *lacI* gene encodes the *lac* repressor, LacI,

which inhibits the transcription of the other *lac* operon structural genes when lactose is absent. The catabolite activator protein (CAP) binding site is the site where the catabolite activator protein/cyclic adenosine monophosphate complex binds to DNA and activates the expression of the *lac* structural genes. The gene for CAP is not located in the operon. The *lac* promoter is the RNA polymerase binding site where transcription begins. There are three operator sites that are binding sites for LacI. The LacI protein can either bind to sites 1 and 3 or sites 1 and 2. Operator site 1 is located just downstream of the promoter. Operator site 2 is located downstream of site 1, in the *lacZ* gene. Operator site 3 is located just upstream of the promoter. The *lacZ* gene codes for the enzyme, *β*-galactosidase, which hydrolyzes lactose, a disaccharide sugar, to form two simpler sugars, these sugars being glucose and galactose (Figure 2). The *lacY* gene contains the lactose permease enzyme (LacY) which imports lactose into the cell. The *lacA* gene encodes the enzyme, galactoside transacetylase (LacA), whose function is unknown, but recent studies show it may be involved in removing toxins [45].

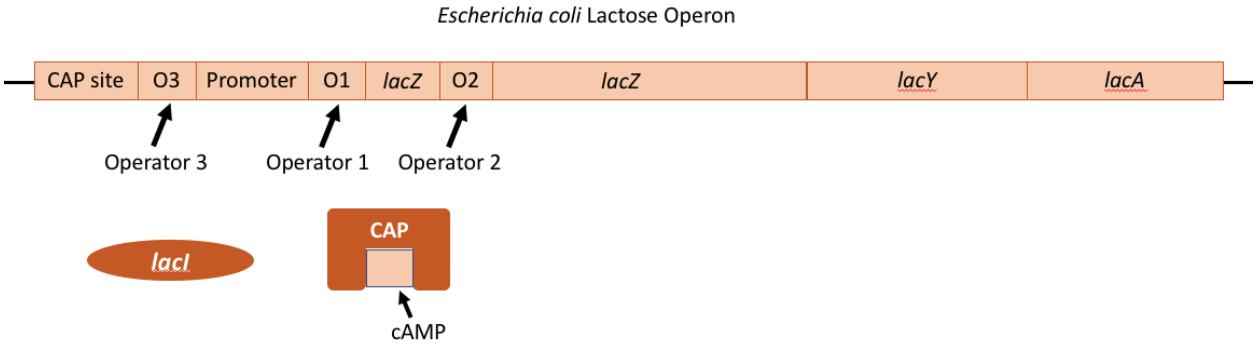

Figure 1: Schematic of the *E. coli* lactose operon.

The *lac* repressor (LacI) negatively regulates the expression of the β-galactosidase gene. When lactose is absent, LacI prevents RNA polymerase from transcribing *lacZ* by

binding to operator sites 1 and 3 or 1 and 2. When lactose is present, β-galactosidase hydrolyzes lactose into glucose and galactose, but a small portion of lactose undergoes a side reaction to produce allolactose (Figure 2). β-galactosidase is always regulated and is expressed only when needed during low levels of glucose. It is known as an inducible enzyme, meaning its level rises in the presence of an inducer, allolactose. This inducer binds to the repressor, causing a conformational change, making the repressor unable to bind to any of the three operator sites. The absence of LacI binding allows RNA polymerase to transcribe *lacZ*, which is translated to form LacZ.

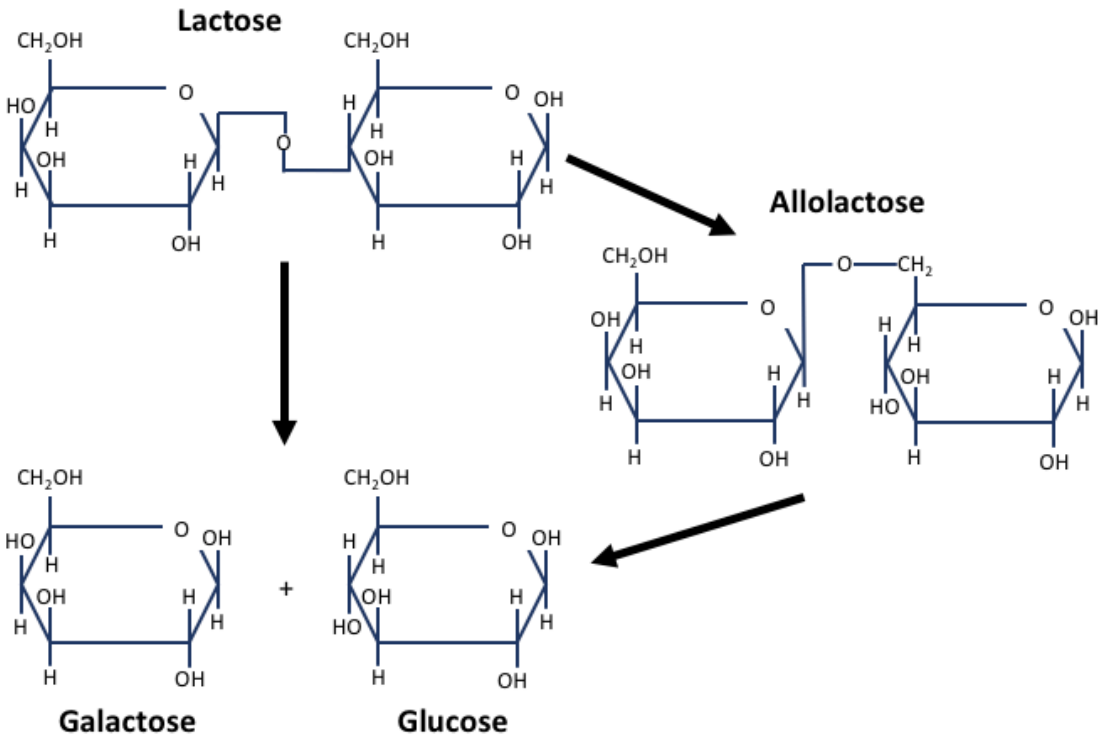

Figure 2: A recreation of the reaction catalyzed by LacZ. [49]

The catabolite activator protein (CAP) positively regulates the expression of β-galactosidase. When glucose levels are high, the levels of cyclic adenosine

monophosphate (cAMP) (Figure 3), are low and CAP cannot bind to the CAP binding site to activate the expression of β-galactosidase. When glucose levels are low, the presence of high phosphoenolpyruvate levels activates adenyl cyclase, which converts adenosine triphosphate (ATP) to cAMP. CAP binding of cAMP changes the conformation of CAP so that it can bind to the CAP binding site and activate the expression of β-galactosidase.

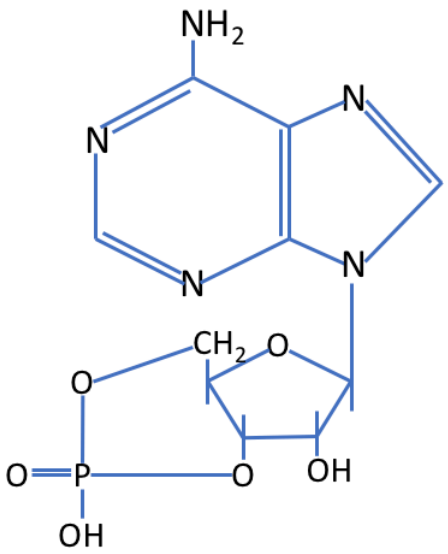

Figure 3: Anionic structure of cyclic adenosine monophosphate

1.3 Operon Control

The entire *lac* operon is regulated under control of the promoter and the operators. The expression of the β-galactosidase gene is related to the presence or absence of glucose and lactose. When lactose is present and glucose is absent, allolactose binds to the repressor, Lac I, rendering it inactive and making it unable to bind to the operator and

repress β-galactosidase expression (Figure 4A). Then, the CAP/cAMP complex binds to the CAP site, allowing transcription to occur. When both lactose and glucose are present (Figure 4B) the LacI/allolactose complex cannot bind to the operator and CAP cannot bind to the CAP binding site. β-galactosidase expression is low because CAP cannot activate transcription. When neither lactose nor glucose are present (Figure 4C), LacI binds to the operator and the CAP/cAMP complex binds to the CAP binding site, but β-galactosidase expression is low because LacI represses transcription. When lactose is absent and glucose is present, LacI binds to the operator and CAP cannot bind to the CAP binding site (Figure 4D). β-galactosidase expression is low because LacI repressed transcription and CAP cannot activate transcription. The best situation for the highest rate of transcription is when lactose is available, and glucose is not. Any time that the *lac* repressor is bound to the operator, preventing transcription from taking place, negative control of the operon is exhibited. Whenever CAP is bound, it helps activate transcription in the most effective way possible, therefore exhibiting positive control of the operon. [30]

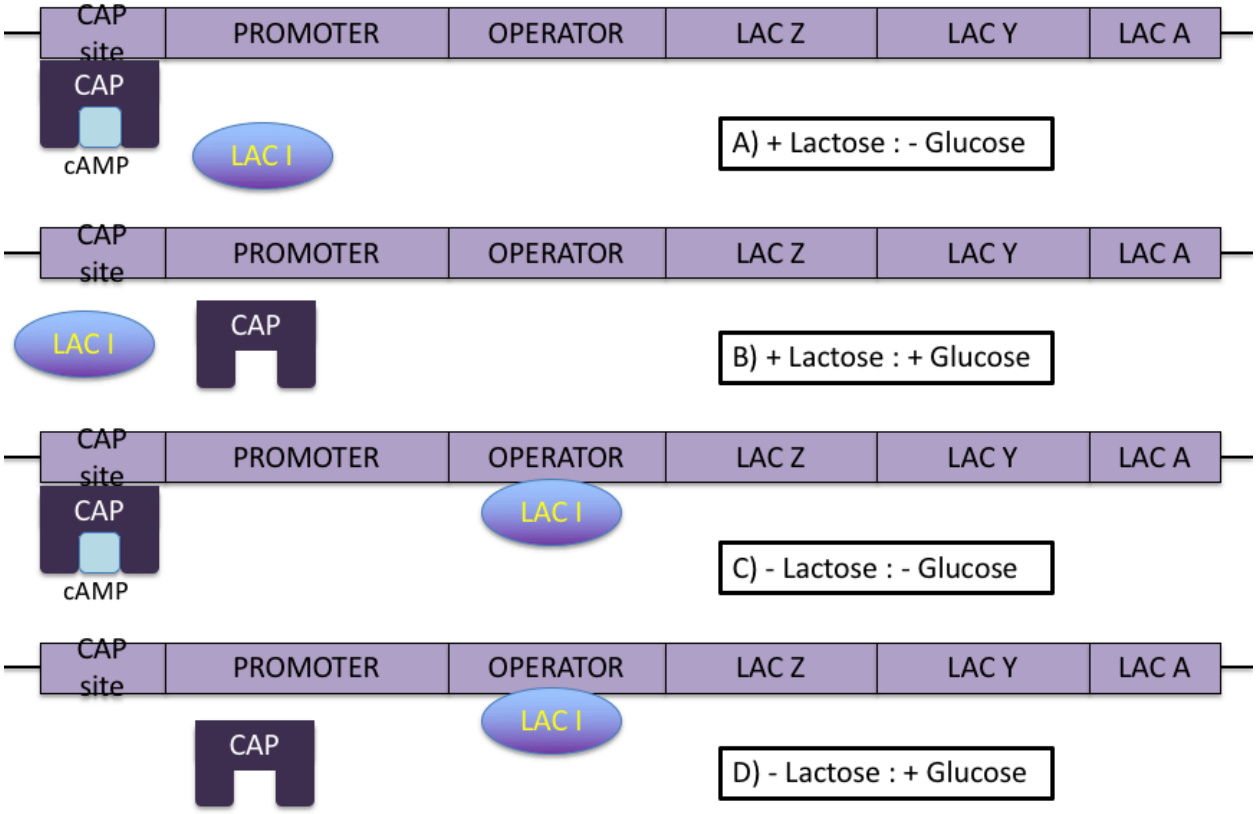

Figure 4: The four outcomes that take place in the *Escherichia coli* lac operon.

1.4 β-galactosidase

β-galactosidase activity can be detected using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), o-nitrophenyl-*β*-D-galactopyranoside (ONPG) and MacConkey agar [1, 30]. When a *lacZ*$^{+}$ bacterial strain is spread on agar plates containing isopropyl β-D-1-thiogalactopyranoside (IPTG) and X-gal, IPTG acts similarly to allolactose and induces the expression of *lacZ*, and β-galactosidase hydrolyzes X-gal to produce blue colonies. Colonies that are *lacZ*$^{-}$ appear white. MacConkey agar plates contain peptone, lactose, bile salts, sodium chloride, crystal violet, neutral red, agar and distilled water [1]. When β-galactosidase hydrolyzes lactose and it is fermented, the decrease in pH causes neutral red to switch from colorless to red. Thus, *lac*$^{+}$ bacteria

form pink colonies. ONPG is used to detect *lacZ* expression in liquid cultures. After induction with IPTG, the bacteria are harvested by centrifugation and lysed using chloroform. Then, LacZ in the bacterial lysates hydrolyzes ONPG to produce a yellow color which can be detected at 600 nm using a spectrophotometer.

1.5 pMOD5 Plasmid

The plasmid that is going to be used in this experiment is the pMOD5 plasmid, obtained from Illumina, Inc (Madison, WI) (Figure 5). This plasmid is known as a suicide plasmid because it cannot replicate in the *Enterobacter* sp. YSU strain. This plasmid contains the R6Kγ replication origin which can only replicate in bacterial strains that contain the *pir* gene. This will force the plasmid to recombine with the strain to survive.

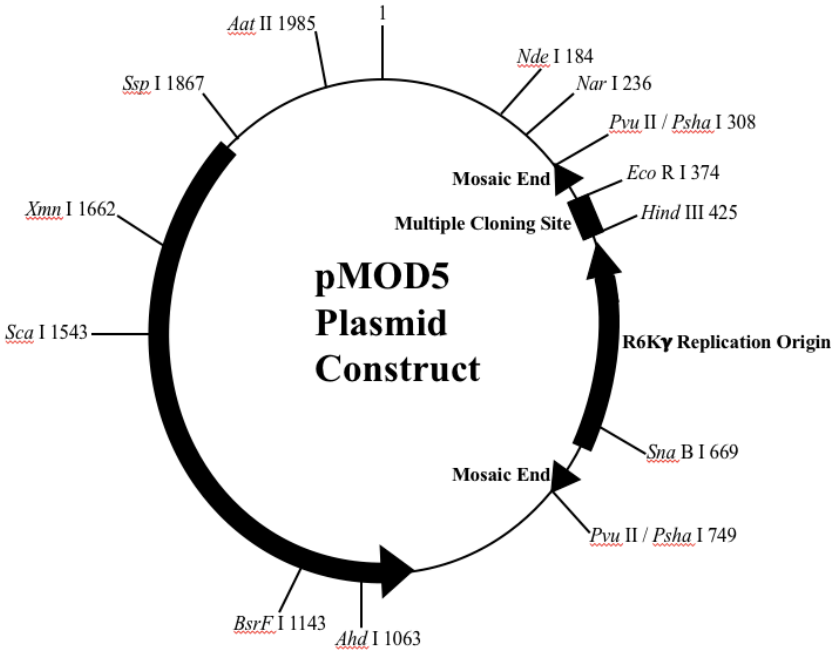

Figure 5: pMOD5 suicide plasmid, as mapped by Illumina, Inc (Madison, WI). Outer markers show restriction enzymes and the R6Kγ replication origin.

1.6 *lacZ* Gene as a Reporter

The *lacZ* gene can also be used as a reporter. Previous studies on *T. cruzi* successfully engineered trypomastigote parasites to express the β-galactosidase gene. The levels of expression were then followed using chlorophenol red β -D-galactopyranoside assays [6]. The *lacZ* gene has also been used as a reporter to study mercury resistance gene expression from Tn*21* in *E. coli* [36].

1.7 Bioinformatics Analysis

Bioinformatics involves the process of using software programs and sites to evaluate and interpret information and data related to biology. There are many different ways of performing bioinformatics analyses. These can range from using programs such as Clustal X for multiple to alignments, to Basic Local Alignment Search Tool (BLAST) [2, 38] to find homologous and orthologous sequences. Bioinformatics analyses have been used to look at protein complexes in *Saccharomyces cerevisiae* [12] and to develop different *E. coli*-specific virulence determinants [25]. The most useful tools used involve running a BLAST on a sequence of interest to find how many other species may have similar sequences to the one being studied. This helps in determining what experiments have been done on other strains that may prove useful during research. Multiple sequence alignments are useful in comparing more than one sequence to recognize similar residues and amino acids. Creating phylogenetic trees and bootstrapping those trees are useful in identifying lineage and possible ancestral domains. The phylogenetic tree shows the evolutionary relationship between species, and it most commonly used to create neighbor-joining trees. After this is completed, a bootstrap analysis is done to assess the

accuracy of that estimate. This usually involves the statistical analysis of at least 100 replications, if not 1000, to show that the phylogenetic tree analysis was accurate in placing the strains in the clade closest to their sister phylogenies.

CHAPTER II: SPECIFIC AIMS

The overall goal is to use the β-galactosidase gene as a reporter gene to study metal-resistance gene expression in *Enterobacter* sp. YSU. To prevent background activity, the *lacZ* gene from *Enterobacter* sp. YSU must first be deleted. This plan will be accomplished by cloning a large segment of the *lac* operon into the vector, pMOD5. Next, the *lacZ* gene will be eliminated from this plasmid using a CRISPR or PCR technique and replaced with a kanamycin resistance gene. Then, the new kanamycin resistance plasmid will be transformed into the *Enterobacter* sp. YSU, and the transformed cells will be spread on agar plates containing kanamycin. Since this strain lacks the *pir* gene and the plasmid cannot replicate in this strain, it must recombine through a double crossover event with the *lacZ* gene to acquire kanamycin resistance and grow on the kanamycin plates [21]. Mutants will be detected by the appearance of white colonies on plates containing kanamycin, IPTG and X-gal.

A bioinformatics analysis of the *lac* operon will be done to compare against *Escherichia coli*, *Enterobacter* sp. YSU and other lacZ sequences. A multiple sequence alignment will be completed, and the number of mismatches will be calculated to determine how similar the sequences are. Bootstrapping is a statistical analysis that will also be done to prove accuracy of the alignments.

CHAPTER III: MATERIALS AND METHODS

3.1 Overview

Enterobacter sp. YSU was isolated from East Fork Poplar Creek (Holmes Reference). *E. coli* strain ECD100D *pir116* was obtained from Illumina, Inc. (Madison, WI). Its genotype is *F* - *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*dlac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ- *rps*L *nup*G.

The plasmid, pMOD5 was purchased from Illumina, Inc. (Madison, WI).

3.2 Growth Medium

Lennox Lysogeny broth (LB) was obtained from Molecular Biologicals International, Inc. (Irvine, CA) and consisted of 10 g/L tryptone, 5 g/L yeast extract and 5 g/L sodium chloride in deionized water. The medium was autoclaved for 20 minutes at 121°C. When required, LB medium was supplemented with 50 μg/ml kanamycin (Amresco, Solon, OH), 100 μg/ml ampicillin (Amresco, Solon, OH), 1 μM IPTG (Amresco, Solon, OH) and 40 μg/ml X-gal (Amresco, Solon, OH).

3.3 Agarose Gel Electrophoresis

A 0.8% agarose gel was prepared by adding 1.04 g of agar (Fisher Scientific, FairLawn, NJ) to 130 ml of Tris Borate EDTA buffer containing tris, Borate and EDTA (Amresco, Solon, OH). The mixture was then heated in the microwave at 1-minute

intervals for 5 minutes until the agar was completely dissolved in the EDTA buffer. GelGreen (Embi Tec, San Diego, CA) was then added to the warm mixture and stirred using a stirring plate until the red color was uniform throughout. Trays were prepared by pouring the mixture into each tray and adding combs to make wells. Once solidified, the combs were removed, and the agarose gels were stored until use. When needed, the gel was placed into a gel box and covered with 1X TBE buffer. 6X loading dye (Amresco, Solon, OH) was added to the DNA sample on parafilm and loaded into each well of the gel. The box was covered with the lid, connected to the power supply, and the 100V current was then passed through the gel. After approximately 40 minutes, the power supply was shut off, the gel was removed and subsequently analyzed.

3.4 Preparation of Electrocompetent Cells

100 ml of cells were grown at 30ºC, for about two hours, to an optical density (600nm) between 0.6 and 0.8. The cells were then harvested by centrifugation at 4ºC for 7 minutes at 7,000 x g. After centrifugation, the supernatant was poured off, and the pellet was resuspended in 20 ml of ice cold water. Additional ice-cold water was added to a final volume of 100 ml. The centrifugation, combined with washing and resuspending, was repeated twice. After the last wash, the supernatant was poured off and the pellet was resuspended in 250µl of ice cold water, which was equal volume to the pellet.

3.5 Transformation Methods

There are two ways in which the transformation was carried out in this experiment. The first was done by electroporation, and the second was by the calcium chloride ($CaCl_2$) method. The electroporation method creates pores through which the DNA enters. The pores then reseal themselves with the DNA inside. The $CaCl_2$ method uses a heat shock method to alter the membrane so the DNA enters the cell.

3.5.1 Electroporation Method

Electroporation competent cells were prepared as described above. A mixture containing 1 µl of DNA and 40 µl of the electrocompetent cells was then added to an electroporation cuvette with a 2 mm gap (BioExpress, Kaysville, UT). The DNA was then shocked into the cells at 25 µF, 200 ohms and 2.5 kV using a Gene Pulser® II Electroporation System (Hercules, CA). Immediately after electroporation, 960 µl of SOC was added to the cuvette to prevent the cells from dying. The cells were then shaken at 30ºC for 1 hour and 30 minutes. Upon completion of shaking and incubation, 100 µl of cells was plated on LB-agar-Xgal-IPTG plates containing the appropriate antibiotic and incubated overnight at 30ºC.

3.5.2 Calcium Chloride Method

The cells were grown in a 100 ml culture to an optical density of 1.0, placed on ice and pelleted by centrifugation at 7,000X g and 4ºC for 7 minutes. After pouring off the supernatant, the cells were resuspended in 20 ml of ice cold 0.15 M sodium chloride and pelleted at 7,000X g and 4ºC for 7 minutes. The supernatant was removed, and the cells were resuspended in 1 ml of a solution containing 15% glycerol, 10 mM Tris-HCl,

pH 8.0, 100 mM $CaCl_2$, and 10 mM $MgCl_2$. The cells were incubated on ice overnight in the refrigerator and frozen at -80ºC. Before each transformation, the cells were thawed on ice, and 100 µl of thawed cells were mixed with 1 µl of DNA. This transformation mixture was incubated on ice for 30 minutes and heat shocked for 50 seconds at 42°C. The mixture was placed back on ice, mixed with 900 µl of LB medium and incubated with shaking at 37°C. Finally, 100 µl of cells were spread on agar plates containing the appropriate supplements.

3.6 Oligonucleotides

Table 1: List of oligonucleotides used, along with their corresponding sequence.

PRIMERS	SEQUENCE
LACI-Y_1 LACI-Y_2	5'-TCA AAA GAT CAC GCC CCT TG -3' 5'-TCA CCG GTA GCA AAG AAC GA-3'
LACZ15 F LACZ16 F	5'-CAT TAC CAG GCC GAA GCA-3' 5'-CAC CGC TGG ATA ACG ACA T-3'
LACY2 R LACY3 R	5'-GCC AAC ACA GCC AAA CAT C-3' 5'-ACC ACC AAC AAT CGA TCC TAC-3'
KANF14-H3 SacB R	5'-ACC AAG CTT GCA TTC ACA GGG TGT CTC A-3' 5'-TTA TTT GTT AAC TGT TAA TTG TCC TTG -3'
LACZ_Del_1 LACZ_Del_2	5'-GGC TAC ATG ACA TCA ACC ATA TC-3' 5'-CGT GAA TTG TTA TCC GCT CAC-3'

3.7 Q5 PCR Reactions

Q5 PCR reactions contained 1.25 µl of 10 µM forward primer, 1.25 µl of 10 µM reverse primer, 9 µl of nuclease free water, 1 µl of DNA template and 12.5 µl of proprietary Q5 High Fidelity 2X Mix (New England Biolabs, Beverly, MA). The following thermal cycling program was run on the Q5 samples: one cycle of 98ºC for 30 seconds, 35 cycles of 98ºC for ten seconds, 54.4ºC for 15 seconds and 72ºC for 50 seconds and one cycle of 72ºC for 2 minutes. Samples were then held at 10ºC.

3.8 QIAquick PCR Purification Kit Obtained from QIAGEN (Hilden, Germany)

Five volumes of Buffer PB was added to one volume of the PCR sample. 10 µl of 3M sodium acetate, pH 5.0, was then added to each tube, and the total sample was transferred to a QIAquick 2 ml spin column with a collection tube. The samples were centrifuged for 60 seconds, and the flow-through was subsequently discarded. 750 µl of Buffer PE was then added to the column, and the samples were again centrifuged for 60 seconds. The flow-through was discarded, and the samples were re-centrifuged for an additional 60 seconds to remove all residual ethanol. The QIAquick column was then placed in a clean 1.5 ml microcentrifuge tube and 30 µl of Buffer EB was added. After incubating at room temperature for one minute, the samples were centrifuged for 60 seconds. The QIAquick column was discarded, and the eluted DNA was stores at -20ºC in the 1.5 ml microcentrifuge tube.

3.9 Strataclone PCR Cloning

The Strataclone PCR Cloning Kit was obtained from Agilent Technologies (Santa Clara, CA). 3 µl of Strataclone blunt cloning buffer was added to a 1.5 ml microcentrifuge tube, along with 2 µl of PCR product and 1 µl of Strataclone vector mix. The sample was incubated at room temperature for 5 minutes, then subsequently placed on ice. 1 µl of the ligation reaction mix was added to competent cells on ice. After incubating on ice for 20 minutes, the mixture was heat shocked at 42°C for 45 seconds, then immediately placed back on ice for 2 minutes. 250 µl of, prewarmed to 42°C, SOC was added, and the sample was shaken for one hour at 37°C. 100 µl of sample was spread on LB-Amp-X-gal plates. The plates were then incubated overnight at 37°C.

3.10 Plasmid Preparations

Plasmid DNA was purified using a Promega Wizard Plus SV Minipreps DNA Purification System obtained from Promega (Madison, WI)

Overnight, 5 ml cultures were centrifuged at 7,000X g and 4ºC for 5 minutes. The pelleted cells were resuspended in 250 µl of Cell Resuspension Solution by pipetting up and down. The cell solution mixed with 250 µl of Cell Lysis Solution, mixed by inversion and incubated for five minutes at room temperature until the suspension is cleared. 10 µl of Alkaline Protease Solution was added, and the tubes were then subsequently inverted multiple times to mix. This solution was also incubated at room temperature for five minutes. After adding 350 µl of Neutralization Solution and mixing by inversion, the lysate was centrifuged at room temperature at 14000 rpm for 10 minutes. The cleared lysate was transferred to a 2 ml spin column with a collection tube.

The supernatant was then centrifuged at 14000 rpm for one minute. The flow-through was discarded, and 750 µl of Column Wash Solution was added to the column. The sample was then centrifuged for one minute, and the flow-through was discarded. The wash procedure was repeated using 250 µl of Column Wash Solution. The sample was then centrifuged for an additional 2 minutes, again at room temperature, and the column was transferred to a clean 1.5ml microcentrifuge tube. 100 µl of Nuclease-Free Water was added to elute the DNA, and the sample was centrifuged at the same speed for 60 seconds at room temperature. The column was then discarded, and the microcentrifuge tube with the purified DNA sample was stored at -20°C.

3.11 Ligations

3.11.1 Ligation of digested pMOD5

The digested sample of pMOD5 was incubated at 80°C for 20 minutes. After the incubation period was over, 1 µl ligase buffer, 2 µl DNA ligase, 5 µl *lac* insert and 2 µl of the digested pMOD5 plasmid was combined in a 0.2ml microcentrifuge tube and incubated at 4°C overnight.

3.11.2 Ligation of pMOD-LacZ and Kan-Sac PCR Product

10 µl pMOD-LacZ was added to a 0.2ml tube with 2 µl DNA ligase buffer, 7 µl nuclease-free water, and 1 µl Polynucleotide Kinase. The sample was then incubated for 30 minutes at 37°C and subsequently incubated for 20 minutes at 65°C.

10 µl of purified, PCR Kan-Sac fragment was placed in a separate 0.2ml tube with 2 µl DNA ligase buffer, 7 µl nuclease-free water, and 1 µl Polynucleotide Kinase. The sample was then incubated at 37°C for 30 minutes and subsequently incubated at

65°C for 20 minutes. This polynucleotide kinase reaction provided a treated pMOD-LacZ plasmid and a Kan-Sac PCR fragment for the following ligation. Polynucleotide kinase adds a 5' phosphoryl group onto the DNA for subsequent ligation. In one tube 1 µl DNA ligase buffer was mixed with 7 µl of the treated pMOD-LacZ plasmid and 2 µl T4 DNA ligase. In a second tube, 1 µl ligase buffer was combined with 3 µl untreated pMOD-lacZ, 4 µl of the Kan-Sac PCR fragment that was just treated during the polynucleotide kinase reaction, and 2 µl T4 DNA ligase. The samples were then incubated at 16°C for 2 hours.

After the 2-hour incubation period, 100 µl *pir* competent cells were combined with 2 µl of each ligation. The samples were then cooled for 30 minutes and heat shocked at 42C for 50 seconds. After the heat shock, they were immediately placed back on ice and 900 µl SOC was added. The samples were then shaken at 37C for one hour, and 100 µl of each tube was spread onto plates. The tube that contained the treated pMOD-LacZ plasmid was plated onto a LB-Amp-Xgal-IPTG plate. The tube that contained the untreated pMOD-LacZ and the treated Kan-Sac PCR fragment was plated onto a LB-Amp-Kan-Xgal-IPTG plate.

3.12 Restriction Endonuclease Digestions

For single restriction endonuclease digestions, 12 µl of DNA was placed in a 0.2 ml microcentrifuge tube with 2 µl Cutsmart buffer (New England Biolabs Inc., Beverly, MA), 5 µl nuclease-free water and 1 µl of restriction endonuclease. All samples were digested for 2 hours at 37°C, except for those digested with *Sma* I. The digestion was incubated at 25°C.

For double restriction endonuclease digestions, 12 µl of DNA was mixed with 2 µl of 10X Cutsmart buffer, 6 µl nuclease-free water and 1 µl each restriction endonuclease. The mixture was incubated at 37°C for 2 hours.

3.13 QIAquick Gel Extraction Kit Obtained from QIAGEN (Hilden, Germany)

This protocol was carried out to excise and purify DNA fragments from gels. All centrifugation steps were done at 14,000 rpm. The DNA fragment was excised with a clean razor blade and placed into a pre-weighed microcentrifuge tube. The 1.5 ml microcentrifuge tube was then weighed again to obtain the weight of the fragment alone. Three volumes of Buffer QG was added per one volume of gel. The fragment sample was then placed in a hot water bath set to 50°C. The sample was vortexed every three minutes to help the gel dissolve. After the gel was completely dissolved, 10 µl of 3 M sodium acetate, pH 5.0, was added. One gel volume of isopropanol was then added to the tube. The entire sample was then transferred into a 2ml collection tube with a spin column. The sample was then centrifuged for 60 seconds at room temperature, and the flow-through was subsequently discarded. 500 µl of Buffer QG was then added, and the solution was centrifuged for an additional minute, with the flow-through being discarded. 750 µl of Buffer PE was added to the column, the solution was centrifuged for one minute and the flow-through was discarded. The tube was then placed back into the centrifuge and spun for an additional 60 seconds to get rid of any residual buffer. The column was then placed into a 1.5 ml microcentrifuge tube, and 30 µl Buffer EB was added. The sample was allowed to stand for one minute, and then centrifuged for one minute. The purified DNA sample was analyzed by agarose gel electrophoresis.

3.14 DNA Sequencing

The GenomeLab Dye Terminator Cycle Sequencing with Quick Start Kit was purchased from AB SCIEX (Framingham, MA). The concentration of each plasmid for sequencing was measured using a Nanodrop 2000 to determine the volume required for 50 fmol DNA. A 10 µl solution of 50 fmol of plasmid and water was heated at 96ºC for 1 minute and cooled to 25ºC. Then, 8 µl of DTCS Sequencing Mix and 2 µl of 1.6 µM primer was added to the DNA. The thermal cycling program was 30 cycles of 96 ºC for 20 seconds, 50 ºC for 20 seconds and 60 ºC for 4 minutes. Samples were then held at 4ºC. Sequencing cleanup was then performed. 5 µl of a stop solution containing 2 µl of 3 M sodium acetate, pH 5.2, 2 µl of 100 µM EDTA and 1 µl glycogen was added to the sequencing sample, followed by 60 µl of ice-cold 95% ethanol. The sample was centrifuged at 14,000X g for 15 minutes and the supernatant was discarded. To wash the pellet, 200 µl of 70% ethanol was added, the tube containing the pellet was gently inverted and the sample was centrifuged at 14,000X g for 2 minutes at 4ºC. This washing process was then completed once more, and the samples were dried in a Centrivap for 10 minutes. Upon completion of drying, 40 µl Sample Loading Solution (SLS) was added, and the samples were separated on a Beckman Coulter CEQ™ 2000 XL DNA Analysis System.

3.15 CRISPR Obtained from Integrated DNA Technologies (Skokie, IL)

CRISPR was used to cut the strands of DNA at more specific sites. No ideal restriction enzymes were where the *lacZ* gene was to be cut. Two different crRNAs were used. The first, CD.Cas9.YPRP2489.AA had a sequence of 5'-GUG UCA AAA AUA

AUA-3'. The second, CD.Cas9.PVKZ2075.AA had a sequence of 5'-UGU GAG UCA GCU CAC-3'. The double-stranded DNA template was created using a 10:1 molar ratio of Cas9 RNP:DNA. The substrate was then resuspended in nuclease-free water. The crRNA:tracrRNA duplex was then created. The RNA oligos were resuspended in IDTE buffer to 100 μm. 1 μl of 100 μm of the Alt-R CRISPR-Cas9 crRNA was placed in a 0.65ml microcentrifuge tube with 1 μl Alt-R CRISPR-Cas9 tracrRNA and 8 μl nuclease-free duplex buffer and heated to 95°C for 5 minutes, and then cooled to room temperature. 1.6 μl of the Alt-R S.p. Cas9 Nuclease 3 NLS was then added to the Alt-R crRNA:tracrRNA complex along with 88.4 μl of phosphate-buffered saline and incubated for 10 minutes at room temperature. The digestion reaction was then created in a 0.2 ml microcentrifuge tube. 2 μl of the 10x Cas9 Nuclease Reaction Buffer was placed with 1 μl each of the two Cas9 RNPs (Cas9.YPRP2489.AA and Cas9.PVKZ2075.AA). After these were each added, 10 μl of the 100 nM DNA substrate was added with 6 μl of nuclease-free water. The entire 20 μl solution was incubated at 37°C for 60 minutes. After this incubation period was over, 1 μl of Proteinase K was added to the mixture and incubated for 10 minutes at 56°C and subsequently analyzed on an agarose gel.

3.16 Bioinformatics Analysis

The free software package, GenomeCompiler (http://www.genomecompiler.com/) (Los Altos, CA), was used to compare all sequences obtained, design primers for PCR and draw maps of the *lac* operon.

DNA sequences were assembled using the computer program Contig Editor from the free software package, GeneStudio (www.genestudio.com). Tentative identification

of *lac* operon genes was determined using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome#). [2,38]. The phylogenetic tree was drawn using Clustal X (www.clustal.org/clustal2/) and MEGA [41] software. Bootstrapping was then completed using the same MEGA software. After bootstrapping, Genedoc [33] software was used to view the full alignment on a single page.

CHAPTER IV: RESULTS

4.1 Previous Work and Cloning

Most of the *lac* operon from *Enterobacter* sp. YSU was sequenced previously. A group of students from Gene Manipulation, BIOL5827, used PCR and Southern blotting to identify a 7 kb *Eco*R I fragment that contained the operon. Several different students working for research credit, cloned the 7 kb fragment into pMOD5 and sequenced it. The fragment contained part of a gene for a zinc-dependent oxidoreductase, the complete gene for the *lac* repressor and most of the gene for β-galactosidase. Even though a short segment of the C-terminal end of this protein was missing, it still produced blue colonies when it was transformed into a *lac*$^-$ strain of *E. coli* which was spread on X-gal, IPTG plates. The complete *lacI* and *lacZ* genes were PCR amplified using the primers LacI-Y_1 and LacI-Y_2 and cloned into pMOD5 (Figure 6). DNA sequencing provided the sequence for the unsequenced C-terminal end of LacZ.

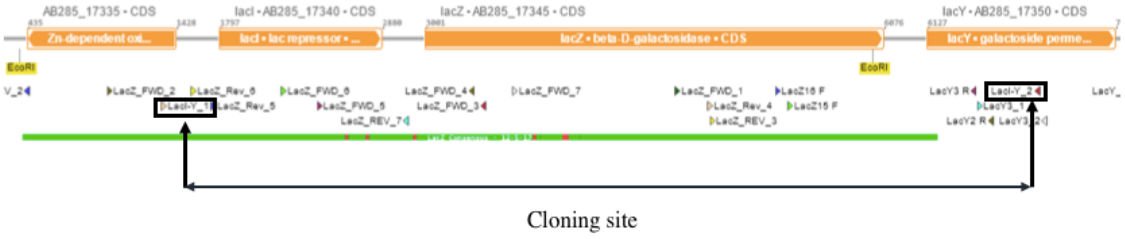

Figure 6: *Lac* operon region that was cloned into pMOD5. The green line represents the previously cloned and sequenced fragment. The black line with arrows represents the PCR section that was completed, amplified, and then cloned using the LacI-Y primers.

4.2 Alignment Comparison

A BLAST analysis was done on the *Enterobacter* sp. YSU lactose operon. The best strain to use as a reference sequence was *Escherichia coli*. Specific sites of each strain were aligned to determine sequence similarity.

The CAP binding site of *E. coli* was compared to *Enterobacter* sp. YSU (Figure 7). Both sequences showed high similarity, although there was one mismatch between a thymine and cytosine residue in the middle of the motif.

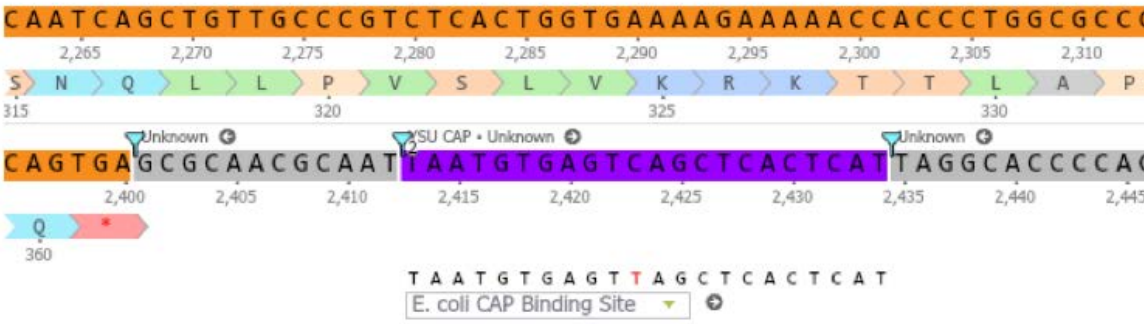

Figure 7: Sequence alignment of *Enterobacter* sp. YSU CAP site vs *Escherichia coli* CAP site.

Next, one of the operator sites, as well as the promoter site, was subjected to sequence alignment to understand the orthology of both sequences (Figure 8). The operator sequence showed one mismatch between an adenine and thymine. The promoter site denoted four mismatches. Three were in the spacer region where RNA polymerase does not bind. The last mismatch was in 3' end of the -10 signal of the *Enterobacter* sp. YSU chain between an adenine and a guanine. There was only one difference in the 5' end of the operator region. The comparison of these key elements of the *lac* operon prove

that the DNA binding sites for CAP, RNA polymerase and LacI are highly conserved between *Escherichia coli* and *Enterobacter* sp. YSU.

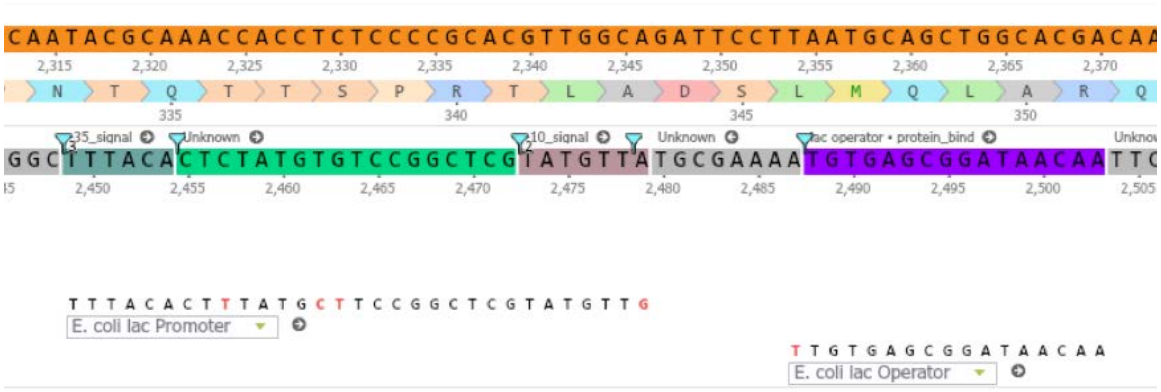

Figure 8: Operator and promoter sequences of both *Enterobacter* sp. YSU and *Escherichia coli*.

4.3 Cloning into pMOD5

The *lac* operon region was cloned into pMOD5, using *lacI-Y* primers. The fragment was blunt-end cloned into a *Sma* I site in pMOD5 (Figure 9). Four transformants were saved for further analysis.

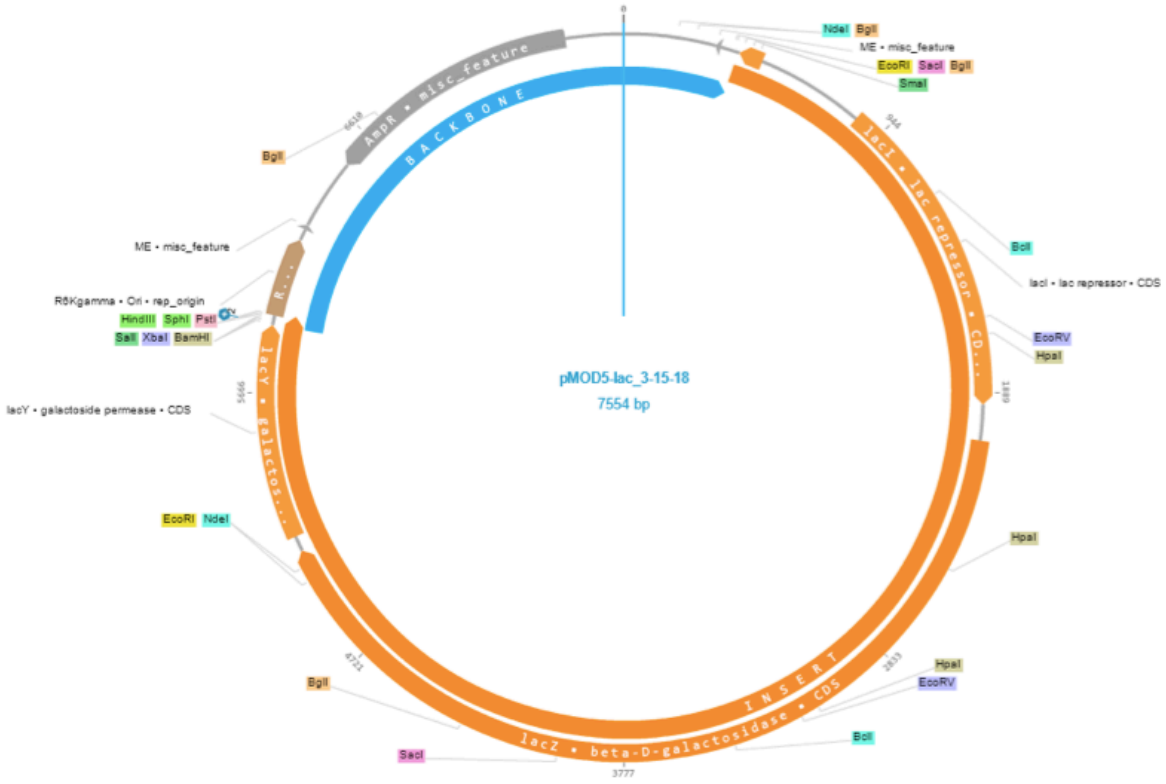

Figure 9: LacZ cloned into the pMOD5 suicide plasmid. Every restriction enzyme is colored differently. ME is a mosaic end. LacI is the lac repressor. LacY is the galactoside permease. The *R6Kγ* is the replication origin and is not compatible with *Enterobacter* sp. YSU.

4.4 CRISPR

When looking into cutting the plasmid at specific sites, no restriction enzymes seemed to be a good fit. CRISPR was used as an alternate means of cutting at the specific sites of the DNA (Figure 10).

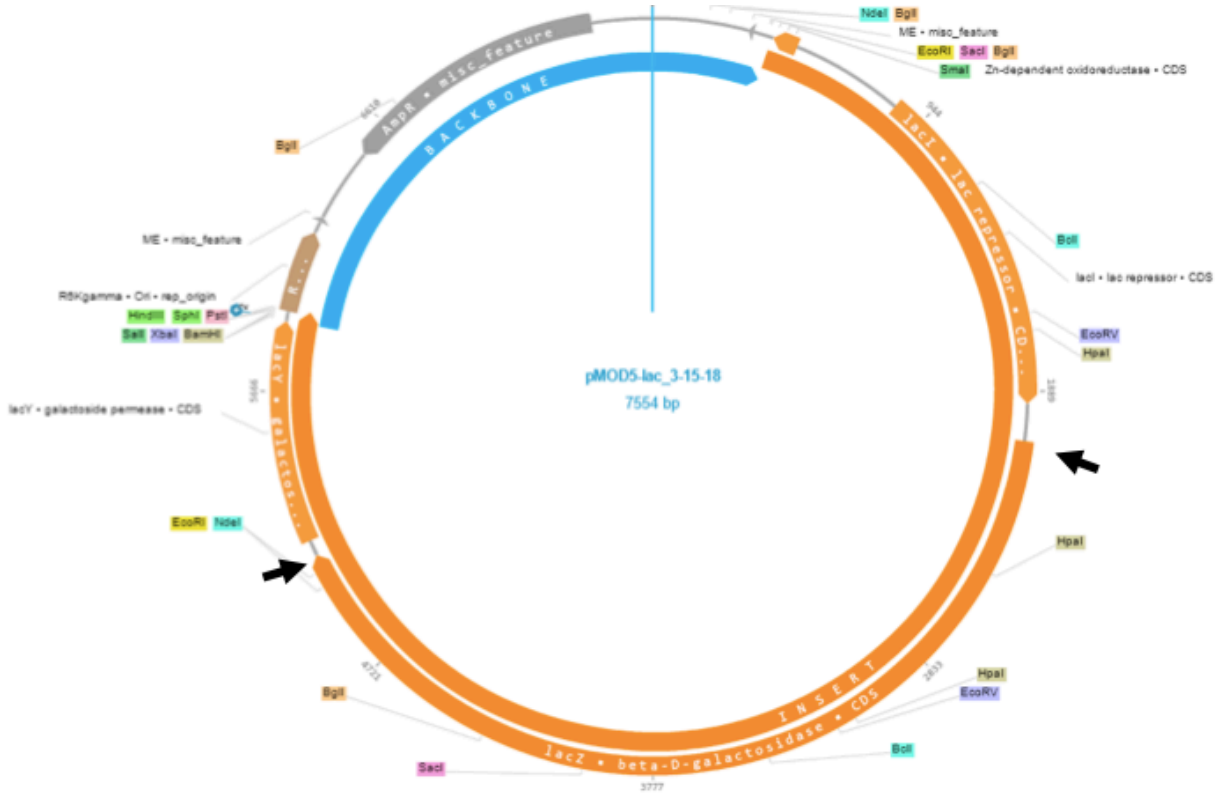

Figure 10: PMOD/LacZ plasmid. The black arrows indicate the CRISPR guide RNA sites.

After the above CRISPR guide RNA (gRNA) sites were obtained and ordered, CRISPR was completed using the four pMOD5-lacZ plasmids that were isolated previously. The double digestions appeared to be partially successful because a 1.8 kb fragment and a 4.5 kb fragment were observed in lanes 2-5. The gRNAs may have not cut at the intended target to excise the *lacZ* gene because the expected size was 3 kb, not 1.8 kb. Also, the digestions were not complete because there are additional larger bands in each lane.

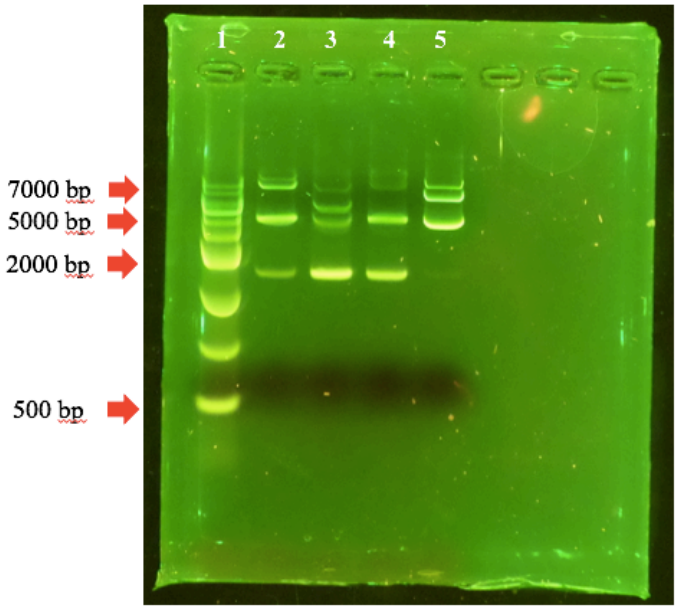

Figure 11: CRISPR results on gel electrophoresis. First time running CRISPR. Lane 1 is a 1 kb ladder. Lanes 2-5 are all lacZ gene substrate.

4.5 Kan-Sac Insertion

The digested CRISPR reactions were cleaned up using a QiaQuick PCR Cleanup kit and ligated with the Kan-Sac PCR fragments. Unfortunately, no transformants were obtained on kanamycin plates when the mixture was transformed into *E. coli* strain, ECD100D *pir116*. Instead, the primers, were used to amplify all of the pMOD5-lacZ plasmid, except the *lacZ* gene. The 4.5 kb PCR product was cleaned up using the QiaQuick PCR Cleanup kit and ligated with the Kan-Sac PCR fragment that was treated with polynucleotide kinase. Transformation of ECD100D *pir116* with the ligation mixture yielded four colonies on LB-Kan plates. Figure 12 is an overview of how the fragment could have inserted itself. The purified recombinant plasmids were digested with the restriction endonuclease, *Hin*d III.

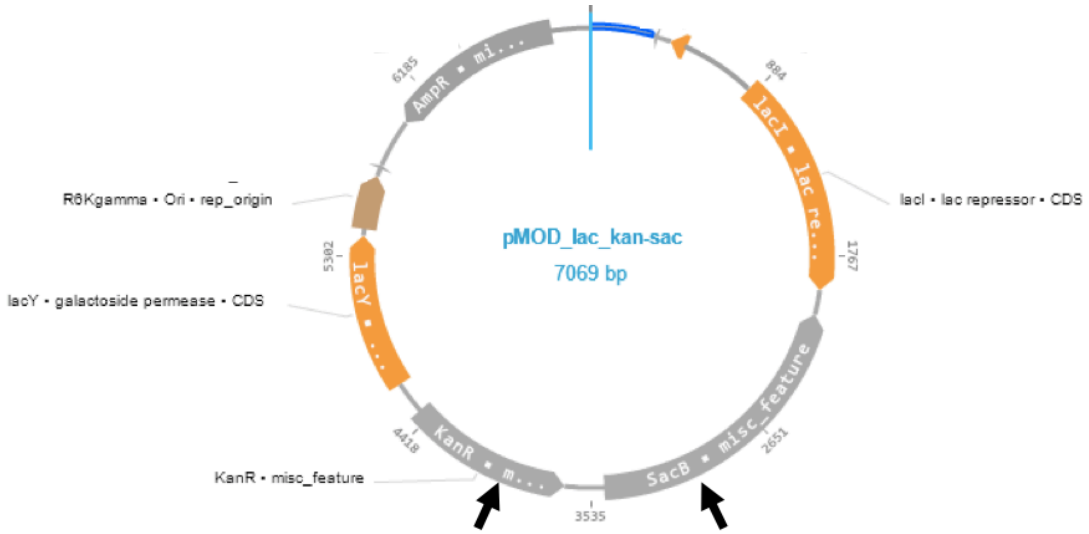

Figure 12: Overview of the pMOD5/Kan-Sac plasmid. Counter-clockwise: AmpR is the ampicillin resistance gene. R6kγ is the replication origin. LacY is the galactoside permease. KanR and SacB replace the lacZ gene. LacI is the repressor. Arrows indicate the two fragments that replaced lacZ.

A *Hin*d III digestion gel simulation from Genome Compiler (Figure 13A) is compared to the gel of the *Hin*d III digested recombinant plasmids (Figure 13B). The gel profiles in lanes 5 and 10 of figure 13B suggest that these recombinants have inserts in the orientation shown in figure 12 because the *Hind* III digestions produces fragments of approximately 963, 2388, and 3727, as shown in figure 13A. The insert in the recombinant plasmid in lane 11 of figure 13B probably has inserted in the opposite direction because the fragment sizes are slightly different from the ones in figure 13A.

To create a *lacZ* deletion without an insert, the cleaned up, 4.5 kb, pMOD-lacZ PCR product was also treated with polynucleotide kinase, ligated and transformed into ECD100D *pir116*. Plasmids from two white transformants were digested with *Hin*d III and separated on an agarose gel. Lanes 12 and 13 in Figure 13B show pMOD5-lacZ

missing the *lacZ* fragment. It does not contain a Kan-Sac insert because there is only one 4.5 kb fragment.

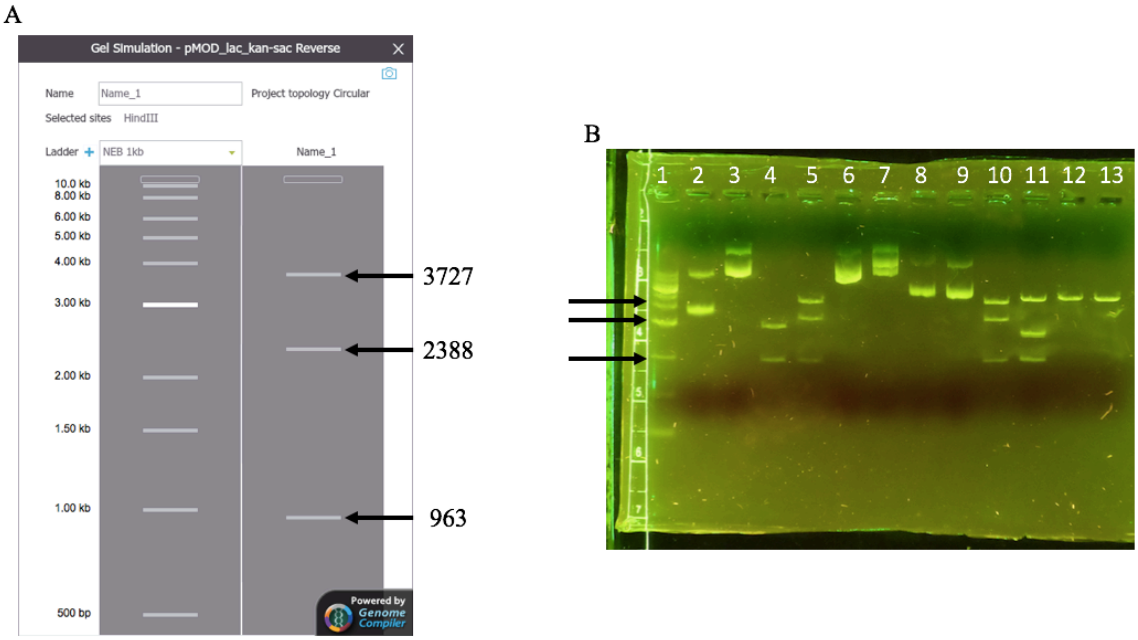

Figure 13: A) A gel simulation of the Kan-Sac fragment digested with *Hind* III. Simulation was completed through Genome Compiler. B) Gel electrophoresis results based on the Kan-Sac digestion. Lane 1 is the 1kb ladder. Lanes 2, 3, 6, 7, 8, and 9 are all undigested fragments. Lanes 4, 5, 10, 11, 12 and 13 are all digested with *Hind* III. Lanes 5,10 and 11 were chosen for electroporation in future studies. Black arrows show the closest results to the gel simulation, with a two out of three fragment match for each sample in the chosen lanes.

4.6 Bioinformatics Analysis

Seven different LacZ sequences were aligned with each other using ExPASy, which is a system to find sequences and run a BLAST on them to find other similar sequences. Six of those sequences had at least a 77% positive match to the seventh sequence, which was linked to a chain of β-galactosidase through the *Escherichia coli K-12* strain. The *Enterobacter* sp. YSU strain had the highest sequence similarity to the

4ckd sequence with 1002 out of 1024 positive matches, denoting a 98% match. The second highest was the *Escherichia coli* strain that had been used as a reference to *Enterobacter* with 993 out of 1024 positive pairs; a 97% match. *Citrobacter freundii* MF466 and *Salmonella* sp. CDC 158-87 both were fairly similar, with 724/823 and 707/820 positive matches; 88% and 86% respectively. *Pseudo Escherichia vulneris* ATCC 29943, a strain known to be similar to the family *Enterobacteraceae*, came in with 301 out of 382 positive matches, denoting a 79% similarity. The least similar of the lacZ sequences obtained was *Serratia* sp. MF416 with only 634/824 positive matches, a 77% similarity.

4.6.1 Multiple Sequence Alignment

Six other *lacZ* amino acid residue sequences were identified by BLAST from a PDB database that contains sequences of proteins whose structures have been resolved through X-ray crystallography and other biochemical methods. Figure 14 shows an alignment of these proteins using MEGA software [41]. The alignment of *Enterobacter* sp. YSU with *E. coli* shows high similarity and suggests that they are closely related. The other four sequences cover only part of the LacZ protein. The first 205 amino acids are not present. The sequence of the *Pseudoescherichia* covers only up to position 590 of this 1024 amino acid residue protein. The other three strains have high similarity to

Enterobacter sp. YSU and *E. coli* on the C-terminal end of the protein.

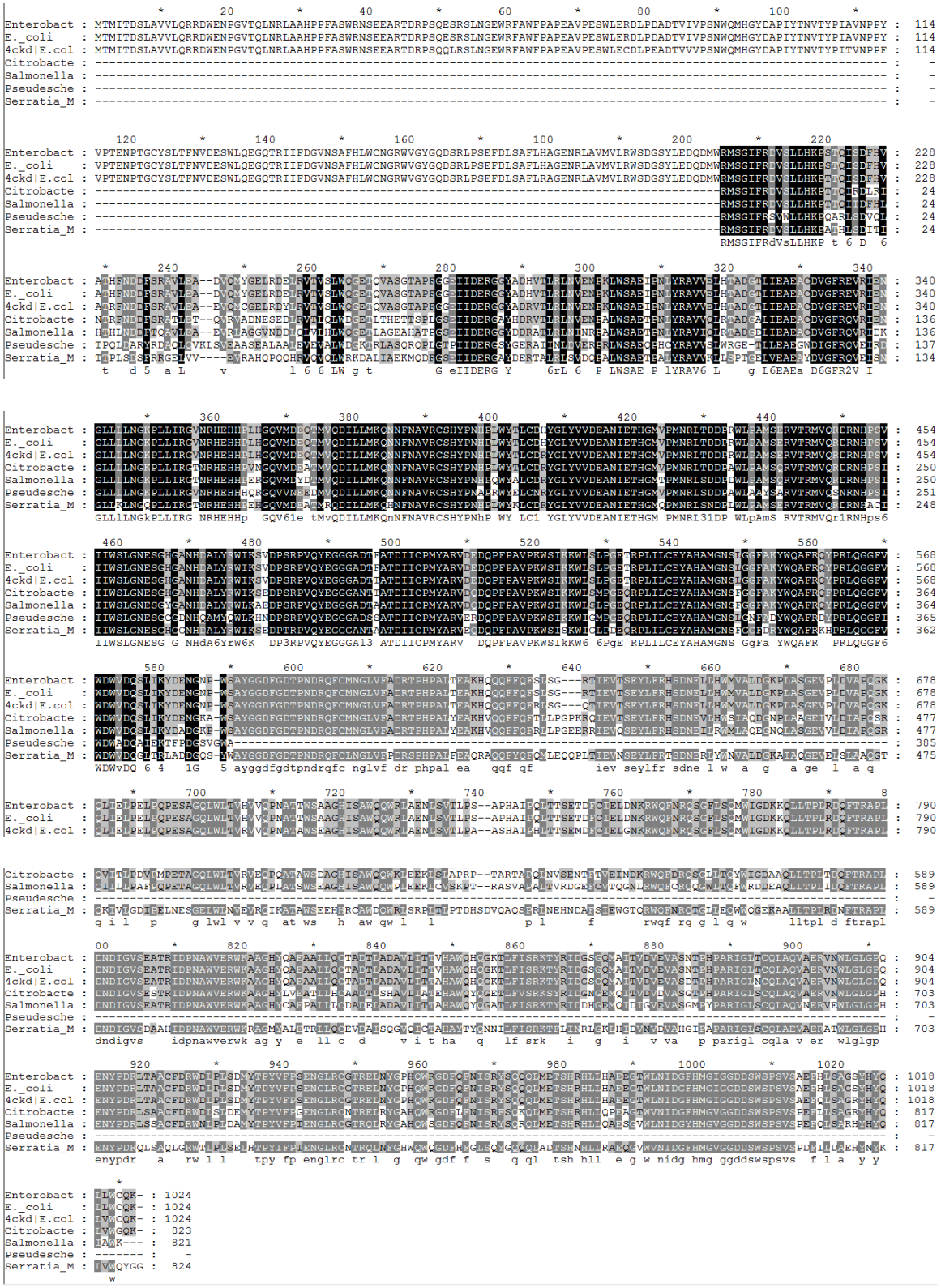

Figure 14: Full LacZ protein alignment

4.6.2 Phylogenetic Tree and Bootstrapping

Upon completion of running the multiple sequence alignment in MEGA, a phylogenetic neighbor-joining tree was produced using the same software on both the protein and DNA sequences (Figures 18 and 19, respectively). The phylogenetic tree showed high similarity between *E. coli* for both the *lacZ* DNA sequence and the LacZ protein sequences.

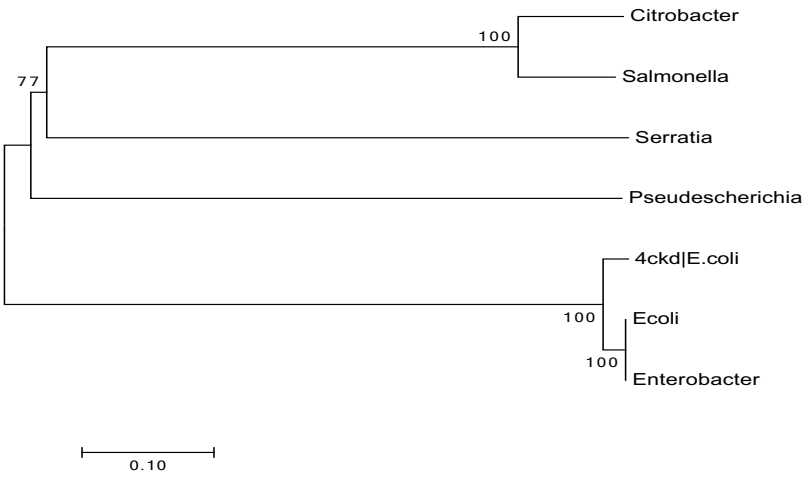

Figure 15: Phylogenetic tree of the LacZ protein sequences analyzed.

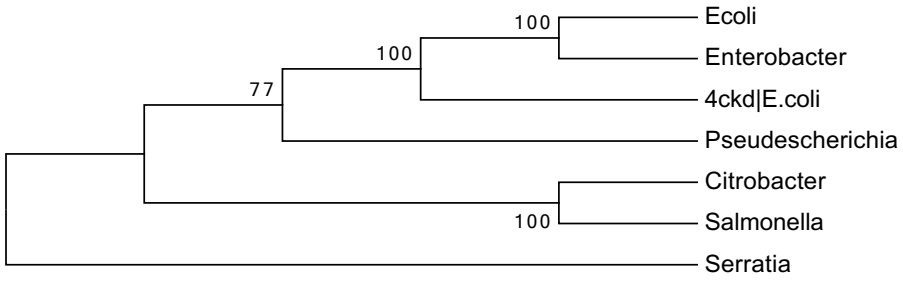

Figure 16: Phylogenetic tree of the LacZ DNA sequences analyzed.

A bootstrap on each phylogenetic tree was then completed using the MEGA software using 500 replications to ensure confidence in the alignments (Figures 20 and 21, respectively). Bootstraps of over 70% are said to be highly accurate. [16]

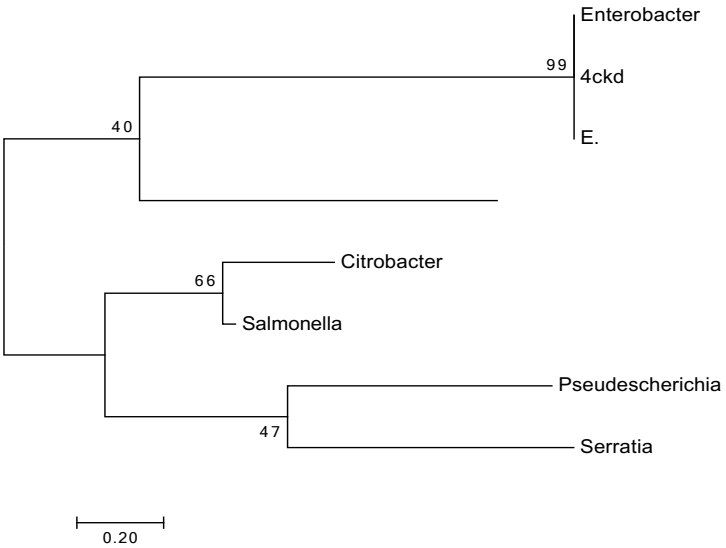

Figure 17: Bootstrap of protein sequences. Values over 70% are considered highly accurate.

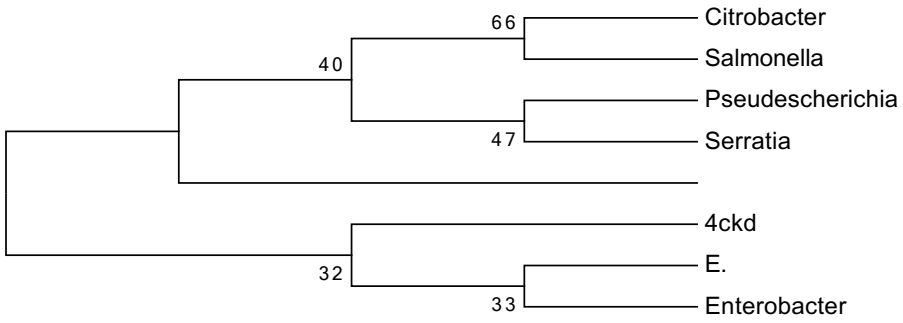

Figure 18: Bootstrap of DNA sequences.

CHAPTER V: DISCUSSION

5.1 Hypothesis Overview

The goal of this work was to delete the *lacZ* gene in *Enterobacter* sp. YSU. A segment of the *lac* operon from this strain was cloned into the suicide plasmid pMOD5. Then, two different deletion plasmids were constructed from this recombinant plasmid. One plasmid lacked the complete *lacZ* gene and the other replaced the *lacZ* gene with a kan/sac fragment. The simplest strategy was to electroporate the deletion plasmid into *Enterobacter* sp. YSU and look for white colonies amongst all the blue colonies on X-gal/IPTG plates. Unfortunately, all the white colonies that were observed gave false results. Streaking them out onto fresh IPTG/X-gal plates showed that they produced blue colonies are were still *lacZ* positive.

The next strategy will use the plasmid containing the *lacZ* gene interrupted by the kan/sac genes. *Enterobacter* sp. YSU will be transformed with this plasmid and spread on plates containing kanamycin, IPTG and X-gal. Since the pMOD5 replication origin is not compatible with this bacterial strain, the only way that it can become resistant to kanamycin is by homologous recombination with the lac operon of *Enterobacter* sp. YSU. During this process the *lacZ* gene will be replaced by the kan/sac fragment. This new strain will be *lacZ*$^-$, kanamycin resistant and sensitive to sucrose. To remove the kan/sac fragment, the *lacZ* deletion plasmid will be electroporated into new the kanamycin resistant strain, and the transformation will be plated on a 5% sucrose plate. Again, since the pMOD5 replication origin is not compatible *Enterobacter* sp. YSU, cells that undergo homologous recombination will lose the *sacB* gene and be able to grow on

the sucrose plate. After the successful knockout has taken place, the β-galactosidase gene will be able to be used to measure expression levels of metal resistance genes in *Enterobacter* sp. YSU.

5.2 Electroporation Strategy for Homologous Recombination

The plasmid containing the *lacZ* gene interrupted by the kan/sac genes was electroporated into *Enterobacter* sp. YSU but no colonies appeared on the kanamycin plates. The crossover did not occur. After electroporation, incubating the cells for a longer period of time might allow for the crossover to occur before plating the cells. Another study that has recently looked into the same methods has been employed using *Pseudomonas aeruginosa* [21].

5.3 Using CRISPR for Cloning

CRISPR is more formally known as Clustered Regularly Interspaced Short Palindromic Repeats. CRISPR-Cas9 is very useful when researchers are looking to cut a part of a sequence that may not have any type of restriction enzymes available. It is more specific than other ways of cutting sequences. The way it works is that the guide RNA is a piece that is used to find a specific target sequence of DNA. The Cas9 enzyme, or CRISPR-Associated Protein 9, cuts the DNA at that specific location, allowing researchers to add, delete or alter the sequence. The digestion was successful, and it was possible to ligate the digested plasmid to construct the *lacZ* deletion pMOD5 plasmid, but the ligation of the kan/sac fragment into the digestion was not successful. Thus, the PCR

strategy which amplified the whole pMOD5-lac plasmid except for the *lacZ* gene was used instead.

5.4 Alignment of Enterobacter sp. YSU to *Escherichia coli*

Comparing the *Enterobacter* sp. YSU strain to a consensus sequence of *Escherichia coli* confirmed our assumption that *E. coli* would make an excellent reference strain when working with *Enterobacter* sp. YSU. One other experiment that may be of use would be to compare the mismatches of the analysis to identify if those mismatches affect the promoter, operator, and CAP regions in a negative or positive way for *Enterobacter* sp. YSU. Comparing the entire sequences in a pairwise alignment would be ideal to look for mutations and compare every mismatch and the spacers between the promoter, operator and CAP regions.

5.5 Bioinformatics Analysis

Using programs such as BLAST, ClustalX, ExPASy, MEGA and Jalview all proved very useful in obtaining specific sequences, doing multiple sequence alignments, and building phylogenetic trees. Genedoc was the most useful in being able to analyze an entire sequence on a single page.

The most conserved areas of the multiple sequence alignments involved a leucine and an alanine residue. These residues are known for being involved in and stabilizing α -helical structures. The 4ckd domain of the *Escherichia coli* K-12 strain had 1024 residues and was responsible for the glycoside hydrolase family 2. This family is responsible for conserving the glutamic acid in *E. coli*. Glycoside hydrolase family 2 is

the family that contained the enzyme β-galactosidase, though there are more than 45 families of glycosyl hydrolases. The topology of β-galactosidase involves a crater-like structure and a TIM barrel fold. This type of protein fold that contains a mix of α-helices and β-strands, which confirms why the two most highly conserved residues were involved in these types of helices. These kinds of folds can catalyze a very vast array of reactions, some involving nucleotide and amino acid metabolism, all depending on what kind of protein is being investigated.

CHAPTER VI: REFERENCES

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