# Development of a Novel Shuttle Vector for *Tetragenococcus halophilus*

By

Carl Bowman Womack

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of
Master of Science in Biology

Middle Tennessee State University
April 2024

Thesis Committee:
Dr. Brian Robertson, Chair
Dr. Rebecca Seipelt-Thiemann
Dr. April Weissmiller

## Acknowledgements

I would like to thank Dr. Robertson for guiding me through my academic career. As an undergrad he was willing to bring me into the lab and begin teaching me the fundamentals. His involvement in this formative stage of my studies led me to desire to pursue a master's degree at MTSU. I would also like to thank him for becoming my thesis advisor and guiding me through the process of investigating, testing, and writing my thesis project. I am thankful he was willing to provide his time, resources, and knowledge and help me see through my time as a graduate student.

I would also like to thank Dr. Seipelt-Thiemann for my time as a graduate teaching assistant in the genetics lab. She taught me all of the skills needed to successfully teach a class and guide students in developing their skillsets. I was fortunate enough to also learn from her in multiple classes and am thankful for all of the guidance she provided that helped me. Her willingness to help me meet class goals and complete class projects was instrumental to my success in graduate school.

Dr. Weissmiller was also kind enough to accept me into her lab as a graduate researcher. Thanks to her I was able to secure my financial needs and have learned a wide variety of skills while also having the opportunity to be involved in a number of papers that have been published. Her guidance has shaped me into an even better person than I was before. I thank all three of them tremendously for their willingness to help guide me and be involved on my committee.

## Abstract

*Tetragenococcus halophilus* is used by companies in a variety of fermented foods while also being shown to be capable of being a potent probiotic. *T. halophilus* fermentative batches are at risk of being compromised by viral pathogens, which leads to product loss in the industry. To address the need for a molecular toolset that was capable of being maintained in *T. halophilus* that expresses desired genes, a plasmid (pCBW2) was designed and built in *E. coli.* With *T. halophilus* having no prior optimized way of transformation, multiple methods of transformation were tested in an attempt to introduce pCBW2 to *T. halophilus*. The plasmid pCBW2 was built using PCR and restriction enzymes to insert a combination of preexisting reporter elements paired with hypothetical promoters from *T. halophilus* genomic DNA. After construction, chemical transformation, electroporation, and biolistic transformation methods were attempted under a variety of conditions to introduce the constructed plasmid to *T. halophilus*. While no attempts of the transformation of *T. halophilus* were successful, conventional chemical transformation of *E. coli* was successful. *E. coli* were able to utilize promoters endogenous to *T. halophilus*, suggesting pCBW2 is a functional plasmid capable of expressing provided genes of interest.

## TABLE OF CONTENTS

## List of figures

## List of Tables

# Introduction

A variety of microorganisms are crucial to the fermentation industry in the production of various food products. The ability of these organisms to carry out carbohydrate fermentation and produce lactic acid has led to the creation and preservation of a variety of food sources such as soy sauce and miso (Uchida, 1982). This natural process is the means by which these organisms are able to process sugar monomers such as xylose, glucose, and galactose as their substrates. Companies are able to use the natural ability of these organisms to process complex plant and animal biomass into edible or more palatable items, resulting in commercially available products such as yogurt, miso, soy sauce, beer, and bread (Mathur et al., 2020). The flavor profile of fermented products tends to be improved by fermentative bacteria through the production of short chain acids (Link et al., 2021). The economic viability of these products often relies on specific mixtures of microbial species and the loss of any particular one can lead to a failure in the production of the desired fermented product (Fukushima, 1981). This can lead to financial loss for corporations as well as strain the food supply due to the loss of food and resource.

Not only is economic loss a consideration, but many fermented foods, sometimes called probiotics, are associated with having a positive effect on the wellbeing and microbiome of people. The consumption of fermented foods leads to an increase in the number and diversity of microbial species in the gastrointestinal tract, an increase in beneficial microbial species, and a decrease in pathogenic microbial species (Islam et al.

2022). Beneficial bacterial species tend to limit growth of pathogenic bacteria through the excretion of inhibitory molecules and through outcompeting them (Kumazawa et al., 2018). In some situations, an increase in a single species of beneficial bacteria in the intestinal tract promotes an increase in both the diversity and number of other species of beneficial bacteria. This enrichment in the number and diversity of species leads to a larger assortment of inhibitory molecules being produced with antioxidant potential (Islam et al., 2022).

One probiotic bacterium that is widely used in the fermentation of plant and meat products such as miso, soy sauce, silage, and salted anchovies is *Tetragenococcus halophilus. T. halophilus* is a halophilic, gram-positive, facultative anaerobic lactic acid bacterium that was originally classified as *Pediococcus halophilus* until a comparison of 16S rRNA with other lactic acid bacteria lead to a reclassification The halophilic nature of *T. halophilus* allows it to grow optimally at concentrations of 5-10% sodium chloride with some strains possessing the ability to grow in up to 26% sodium chloride media (Collins et al., 1990). This ability to survive in high salinity environments has led to this species being used in the fermentation industry when processing high salinity foodstuffs such as soy sauce. (Gürtler et al., 1998). *T. halophilus* contributes to the fermentation process by breaking down sugars and by the production of organic acids. *T. halophilus* also contributes to the flavor profile of fermented by preventing growth of many bacterial strains that produce biogenic amines that are considered unpalatable (Link et al., 2021).

One issue with utilizing *T. Halophilus* in commercial fermentation is that it is susceptible to bacteriophages that can prevent the fermentation of lactic acid, disrupting

the fermentation process and leading to quality issues or complete batch losses. This can impact product quality leading to major financial losses. Bacteriophages use the wide variety of polysaccharides and peptidoglycan in the lactic acid bacterial cell wall to enter these cells (Wakinaka et al., 2023). Unfortunately, these same structures also form a complex barrier that has resulted in a lack of molecular tools that can be easily used in certain gram-positive species (Rogers et al., 1980). With *T. halophilus* being a target for these bacteriophages and being a highly valued organism in the production process of multiple foodstuffs and fermentation products, the threat of batch loss increasing financial costs make developing molecular tools for *T. halophilus* an important goal.

In food fermentation, bacteria can be engineered through recombinant DNA technology to produce strains that are resistant to viral pathogens, are able to produce products quicker, provide greater shelf stability and flavor profile, and with traits that consumers find pleasing. This same method can also be used to engineer strains that lack toxic products or decrease the quality of fermented products (Geisen & Holzapfel, 1996). The long-term goal of this ongoing project is to engineer a strain of T. halophilus that is resistant to bacteriophages that damage this industry's potential.

Three of the most important aspects off developing molecular tools to engineer a viral resistant *T. halophilus,* or any strain are 1) working out conditions for DNA transfer, 2) identifying sequences that enable expression of genes in the new host, and 3) identification and validation of reporter genes and selectable markers. Transformation of genetic material into cells, transformation, has been accomplished in different species using a number of techniques including chemical, electrical, and biolistic (Sirajuddin &

Sundram, 2020). Gram positive bacteria have a complex barrier of exterior peptidoglycan that prevents the physical passage of exogenous DNA across the cell membrane and internally to the cell. This nature of the bacterial cell wall differs for gram positive and gram-negative species and must be considered in any transformation protocol (Hahn, et al., 2021).

Chemical transformation is a process by which cells are made competent through chemical exposure followed by heat shock to induce cells to allow permeability. A classical method to make chemocompetent cells is by using $CaCl_2$ to break down the lipids in the cell membrane. This process disrupts the membrane and allows exogenous DNA to enter the cell internally (Nemeth et. al, 2021). A period of heat shock followed by a recovery phase usually results in transformation. While used predominately for gram-negative bacteria, there are examples of conventional chemical transformation working in some gram-positive species. It is perhaps the least used method for gram-positive bacteria due to issues regarding cell wall complexity (Sirajuddin & Sundram, 2020)., the successful proliferation of transformed cells. This method is convenient as it is fairly cost effective and does not require complex equipment. However, the process of making chemocompetent cells can be tedious, and it is not as efficient at producing transformed cells as transformation that uses electricity, known as electroporation (Liu et al., 2018).

Electroporation uses a high voltage discharge that causes the cell membrane to become permeable (Jin et al., 2012). This allows exogenous molecules such as a plasmid to pass through the membrane and be taken internally to the cell (Wang et al., 2020). This

method of transformation works well in gram-negative species with permeable membranes, but not so well in gram positive species that have a thick peptidoglycan layer (Cho et al., 2020). One method to remedy this problem is to perform a chemical pre-treatment to weaken the peptidoglycan layer followed by electroporation (Jin et al., 2012). The pulse length and the strength of the voltage discharge are the two main variables of electroporation that should be considered and tested (Dower et al., 1988).

A third strategy for transforming bacteria is biolistic transformation. This process uses high velocity microparticles that are bombarded onto cells that can cross through the cell membrane and deliver a molecular payload (Sanford, 1990). Biolistic transformation overcomes many of the hurdles encountered in transforming gram-positive species, specifically their thick peptidoglycan layer. Gold particles are useful in biolistic transformation due to their biocompatibility, ease of interaction with molecules, and non-toxicity in cells (Kumari et al., 2017). In this method, no pre-treatment is necessary as any actively growing cells can be used. Disadvantages to this approach are its inefficiency compared to electroporation and chemical transformation, the need for specialized equipment, and the oftentimes need for optimization in specific species (Sanford, 1990). Another disadvantage to this approach is the invasiveness of the bombardment, which results in damage to healthy cells. This approach requires the appropriate amount of microparticle velocity; too little and the particle does not enter the cell, too much and the particle passes through and out of the cell (Kumari et al., 2017). This method of transformation is often used in the transformation of plant tissues and yeast and is not as prevalent in bacterial transformations (Bonnefoy et al, 2023).

Promoters are regions upstream of genes that are used to drive the expression of genes. Often transcription factors aid in this process by driving transcription through the recruitment of polymerase (Pátek et al., 2012). While some promoters are species specific, others can be utilized by multiple species. Promoters can be used to engineer bacteria to produce proteins of interest to companies or in the lab (Myers et al., 2021).

Finally the last molecular development tool of interest in this study is the identification and validation of reporter genes and selectable markers for *T. halophilus*. When developing plasmids that will be introduced to new species, a method to observe successful transformation and gene expression may be included as a way to reveal information about the plasmid or host microbe. Many common ways to achieve this is through the inclusion of antibiotic resistance genes that allow transformed species to survive in media containing the chosen antibiotic (Liu et al., 2022), and bioluminescent genes that confer the ability to produce proteins that produce measurable light that can be measured. These characteristics can be used to determine strength and regulation of promoters as well as screen for successful transformation of bacterial colonies (Robertson & Johnson, 2011).

One reporter gene is *CBG99* found in the insect *Pyrophorus plagiophthalamus*. *CBG99* encodes a bioluminescent protein click beetle luciferase, which emits a green light that can be observed and quantified (Mezzanotte et al., 2011). When luciferase comes into contact with a luciferin substrate containing ATP, an oxidative reaction causes it to emits a light at 537 nm. Placing this gene downstream of a constitutive

promoter in a plasmid will enable screening of transformed colonies in the presence of luciferin as the would emit an observable signal (Robertson & Johnson, 2011).

Another useful set of genes that can be included in a plasmid are selectable marker genes that confer antibiotic resistance. Many bacteria have innate antibiotic immunity, but some are sensitive to antibiotics (Liu et al., 2022). By including a gene with a plasmid that confers antibiotic resistance that is driven by a constitutive promoter, successfully transformed bacteria will be able to survive in media containing antibiotics. The usefulness of this approach allows an easy method to discern successful transformants without expensive equipment (Liu et al., 2022).

The objective of this research was to design a novel plasmid and transform *T. halophilus* with said plasmid that would express genes in the species *T. halophilus* and have reporter and selectable markers. To achieve this goal, a variety of native promoters paired with reporter and selectable genes were assembled into a plasmid named pCBW2. This plasmid was used with a variety of transformation conditions including electroporation, chemical, and biolistic transformation to determine an optimized method to transform E. coli (a gram-negative bacterium) and *T. halophilus (*a gram-positive bacterium).

Based on successful transformation of other gram-positive bacteria (Wang et al., 2020) pCBW2 will express reporter genes paired with native promoters of *T. halophilus* after being successfully transformed through electroporation, chemical transformation, or biolistic transformation.

# Materials and Methods

## 2.1 Microbial Strains and Growth Conditions

Tryptic soy (Tsoy) broth and solid media (containing 1.5% agar) at an optimal temperature of 30°C were used to grow *T. halophilus*. *E. coli* was grown in Luria broth (LB) at 37°C. *T. halophilus* was grown in a variety of NaCl concentrations and it was determined 5-10% NaCl concentration was suitable for growth conditions. For strain maintenance, a concentration of 7% NaCl was most frequently used in the broth. *T. halophilus* strain maintenance involved inoculating 5 ml of Tsoy broth with 100 µl of freshly suspended cells from a prior batch every 4-5 days. When needed, ampicillin at a concentration of 100 µM or chloramphenicol at a concentration of 10 µg/ml was added to the plates to select for successfully transformed colonies. Strain ATCC 33315 was used for *T. halophilus* experiments while the Top10 strain (Thermofisher) was used for *E. coli.*

## 2.2 PCR

Phusion High-Fidelity DNA Polymerase (Thermo Fisher) was used to perform PCR products used in plasmid construction and GoTaq Polymerase (Promega) was used for PCR screening of colonies. Reactions consisted of 50 µl mixtures with a final concentration of 1X Buffer, 200 µM dNTP's, 0.5 µM forward and 0.5 µM reverse primers, a provided DNA sample, 0.02 U/µl Polymerase, and $H_2O$ to volume. In the case of Taq Polymerase reactions, 1X Buffer, 4 mM $MgCl_2$, 0.2 mM each dNTP, 1 µM

forward and 1 μM reverse primers, a provided DNA sample, 1.25 U Polymerase, and $H_2O$ to volume. An extension time of 15-30 seconds per kilobase was used.

**TABLE 1: Primer Sequences**

| Primer Number | Name | Sequence | Annealing Temp |
|---|---|---|---|
| 1 | KanR(Nde)5 | actact**CATATG**AGCCATATTCAACGG | 61 |
| 2 | KanRterm(Afl)3 | actact**CTTAAG**GTTTACAATTTCAGGTGG | 60 |
| 3 | AmpR(Nde)5 | actact**CATATG**AGTATTCAACATTTCCG | 61 |
| 4 | AmpRterm(Afl)3 | actact**CTTAAG**AGTGGAACGAAAACTCACG | 60 |
| 5 | PcypX(Nhe)5 | aagatc**GCTAGC**AGAATGGCAGAAACTCGTC | 61 |
| 6 | PcypX(Nde)3 | actact**CATATG**CAATATTCCTCCTTAGTAATAATATCC | 61 |
| 7 | 287ORlus(SacI)5 | actact**GAGCTC**TTAATCTCGAACAGCGGTG | 64 |
| 8 | 287REPBds(SacI)3 | actact**GAGCTC**GACATCACCTGCAGCCTG | 64 |
| 9 | PPolA(Xho)5 | actact**CTCGAG**TGGCTGAAATCATTTTAGTGG | 64 |
| 10 | PPol1(BsrGI)3 | actact**TGTACA**CCCCTCACTCAAATTCTCTTTAC | 64 |
| 11 | CBG99(BsrGI)5 | actact**TGTACA**GCAAATGGTGAAGCGTGAGAAAAATG | 66 |
| 12 | CBG99(Sal)3 | actact**GTCGAC**CTAACCGCCGGCCTTCTC | 67 |
| 13 | TDegV(Sal)5 | actact**GTCGAC**AAAACAAGCCTCTCCAAATTC | 64 |
| 14 | TDegV(Bgl)3 | actact**AGATCT**TAGAATGGTTGCGTCAATATG | 64 |
| 15 | HSPscrn5 | GGAGTGCTAATTAAAGGGTG | 61 |
| 16 | HSPscrn3 | GACAATTCACGCAGATCAGCCG | 73 |
| 17 | xylIlscrn5 | GGCATATTGCTCATGACGAGG | 71 |
| 18 | xylIlscrn3 | GGTGCACAAAACTCTACAGC | 62 |
| 19 | greAscrn5 | GACCAACAAACGTGTTCGTCC | 71 |
| 20 | greAscrn3 | CTGTTACTTATGAGGACCTTGAG | 61 |
| 21 | Galscrn5 | GCTTGCCTACATATACAAATATGCC | 67 |
| 22 | Galscrn3 | CATCCGGCACAAGGGACTTAC | 70 |
| 23 | CatScrn5 | AAAAACAATTGCAAAAGCAG | 62 |
| 24 | CatScrn3 | AATCAGTCCATAAGTTCAAAACC | 62 |

Lowercase letters show nucleotides added to increase restriction enzyme binding activity. Sections in bold denote restriction enzyme sites added to the ends of designed primers. Underlined portions represent the portion of the primer that anneals to the target template. Annealing temperatures were in Celsius.

Restriction digestions were set up in 20 µl reactions consisting of around 1000 ng of mini prep purified DNA, 2 µl of 10x buffer, 0.5 µl of restriction enzyme (New England Biolabs), and the remaining volume filled to 20 µl with molecular-grade water. Digestions were allowed to incubate at minimum one hour and at maximum overnight at 37°C.

Ligation reactions consisted of 20 µl reactions of 10x T4 DNA ligase buffer, 50 ng of vector, 3x nanogram quantities of insert to vector, T4 DNA ligase enzyme (New England Biolabs), at 200 U per reaction and $H_2O$ to volume. Ligation reactions were allowed to incubate overnight at 4°C before being used in transformation.

For *E. coli* transformations, a 100 µl sample of chemically competent cells were aliquoted into a pre-chilled tube alongside 2-5 µl of ligated plasmid and incubated on ice for 30 minutes. Cells were then heat shocked at 42°C for 45 seconds and placed back on ice for 2 minutes to recover. Cells were then allowed to grow in 400 µl of LB broth for one hour and then 50-200 µl of cells were plated on kanamycin plates at a concentration of 50 µM.

Agarose gel electrophoresis was performed using 1% gels made by heating 40 ml of 1X TAE combined with 0.4 g of agarose. To visualize DNA, 2 µl of SybrSafe (Thermo Scientific) was added to the gel and mixed. Samples were loaded into wells after submerging the gels in 1X TAE and were separated through 70-100 V. Gels were visualized in a ChemiDoc (Bio-Rad), and fragment size was determined by comparison to a 1 kb ladder (N3232 New England Biolabs).

## 2.3 Construction of pCBW2 plasmid

The pCBW2 plasmid was constructed using the following workflow. Sequences to be added to the plasmid (inserts) were PCR amplified then purified with a GeneJet PCR Purification Kit (Thermo Scientific). Appropriate sticky ends were created on the PCR product and target vector by digesting the PCR product and target vector with one or a pair of restriction enzymes. *Tetragenococcus halophilus* strain ATCC 33315 was used as the DNA template for the amplification of the promoters, *repAB* origin of replication, and the *degV* terminator. The *KanR* gene and origin of replication PBR322 was amplified from the donor plasmid pCambia2300, while the *AmpR* gene was PCR amplified from pAllet (Reichard et al., 2023). Lastly, the bioluminescent reporter was PCR amplified from pCBG99-basic (Promega). The digestion products were fractionated on a 1% agarose gel and appropriate bands were excised from the gel and purified using a GeneJet Gel Extraction Kit (Thermo Scientific). Insert and vector were ligated, and the resulting product transformed into *E. coli* (Top10) using chemical transformation and selected on LB plates containing Ampicillin. Several colonies were screened for the presence of the plasmid's modification using PCR, and one or more of the successfully screened colonies were cultured for plasmid miniprep by GeneJet Miniprep Kit (Thermo Scientific). The purified plasmid was confirmed by restriction analysis using the restriction enzymes matching each primer and then the process repeated for the next modification. The specifics for each modification are described below.

**Table 2: Plasmids Used as Templates**

| Plasmid Name | Source | Relevant Features |
|---|---|---|
| CBG99-basic | Promega | Contains *CBG* reporter element |
| pCambia2300 | Hajdukiewicz et al., 1994 | Contains *KanR* reporter element |
| pUCL287 | ATCC | Contains *repAB* origin of replication |
| pAllet | Reichard et al., 2023 | Contains multiple cloning sites and used as the backbone in pCBW2 construction |
| pCBW2 | This Work | Contains reporter elements driven by native promoters |

The *KanR* segment was PCR amplified using primers 1 and 2 (Table 1) from the pCambia2300 template and digested using restriction enzymes *NdeI* and *AflII*. The *AmpR* segment was amplified from pAllet using primer 3 and 4 (Table 1) and digested with restriction enzymes *AflII* and *NdeI*. The *cypX* promoter was PCR amplified using primers 5 and 6 from *T. halophilus* and digested using the restriction enzymes *NheI* and *NdeI*. *RepAB* was PCR amplified using primers 7 and 8 from *T. halophilus* native plasmid pUCL287 and digested with the *SacI* restriction enzyme. The *polA* promoter was PCR amplified using primers 9 and 10 from *T. halophilus* and digested with *XhoI* and *BsrGI*. The bioluminescent reporter *CBG99* was PCR amplified from pCBG-99-basic using primers 11 and 12 and digested with *BsrGI* and *SalI*. The *degV* terminator was amplified using primers 13 and 14 from *T. halophilus* and digested with *SalI* and *BglII*. After the vector and PCR inserts were digested, ligation was performed overnight, and the resulting ligation product was transformed into *E. coli* using chemical transformation.

## 2.4 Chemical Transformation

Conventional chemical transformations were assessed in *T. halophilus.* Cells were grown and made chemically competent using a modified RbCl method commonly used for *E. coli* (openwetware.com). Briefly, this involved taking *T. halophilus* cells grown for 1-2 days and inoculating a flask with 20 ml of tryptic soy media (Tsoy) with 0.8 ml of fresh cells. Cells were grown to an $OD_{550}$ of 0.45. The cells were then placed into conical tubes and chilled on ice for 15 minutes then centrifuged at 2.5k RPM 4°C for 5 minutes. Cells were then resuspended in 1.6 ml of transformation buffer #1 (1 L volume of 1.2% RbCl, 1% of $MnCl_2$, 3% 1 M potassium acetate, 0.15 % $CaCl_2$, pH adjusted to 5.8, and 15% glycerol) and allowed to chill on ice for 15 minutes. *E. coli* or *T. halophilus* were then pelleted and resuspended in 400 μL of transformation buffer #2 (2% 0.5 M MOPS at 6.8 pH, 0.12% RbCl, 1.1% $CaCL_2$, and 15% glycerol). A 100 μl sample of competent cells were then placed into a pre-chilled tube with 2-5 μl of pCBW2 purified via mini-prep and incubated on ice for 30 minutes. Cells then underwent heat shock at 42°C for 45 seconds and placed back on ice for 2 minutes to recover. Cells were then allowed to grow in 400 μl of tryptic soy media (Tsoy) overnight and then plated on ampicillin plates at a concentration of 100 μM.

## 2.5 Biolistic Transformation

Biolistic transformation tests were performed under the conditions as follows. To prepare the delivery system, 0.6 μg gold particles (Bio-Rad) were coated with pCBW2 and loaded onto macrocarrier disks (Bio-Rad) that would be used in the biolistic delivery system. To achieve this, 30 mg of gold particle beads were weighed out and placed into a 1.5 ml tube with 1 ml of 70% ethanol. The tube was then shaken vigorously with a vortex for 3-5 minutes then allowed to soak in 70% ethanol for 15 minutes. The 1.5 ml tube was then centrifuged at 13,000 rpm for 5 minutes to pellet and the supernatant was removed. After the supernatant was removed, 1 ml of dd$H_2O$ was added and the tube was vortexed for 1 minute then allowed to settle 1 minute before being briefly spun to a pellet before the supernatant was removed 3 separate times. Then 500 μl of sterile 50% glycerol was added to bring the particle concentration to 60 mg/ml and then stored at 4°C for at most two weeks. At time of use, the beads were resuspended through vigorous vortexing for at least 5 minutes. Fifty microliters of the microcarriers were added to a new 1.5 ml tube. While continuously vortexing and in precise order, 5 μl of DNA at a concentration of 1 μg/ml was added, following 50 μl of sterile 2.5 M $CaCl_2$, then 20 μl of 0.1 M spermidine. The mixture was vortexed for 2-3 minutes then allowed to settle for 1 minute. The tube was then spun for a brief 2-3 seconds and supernatant was removed. After removal, 140 μl of 70% ethanol was added then removed, followed by 140 μl of 100% ethanol. Supernatant was removed again and 48 μl of 100% ethanol was added. To keep final mixture suspended during application, the tube was lightly tapped and vortexed for 2-3 seconds.

To load the macrocarrier disk with DNA coated microcarrier gold particles, the macrocarrier disk was submerged in 100% ethanol using forceps and placed into a petri dish with drierite to allow efficient drying and then loaded into the macrocarrier holder. Stopping screens (Bio-Rad) and 1100 psi rupture disks (Bio-Rad) were dipped in 70% ethanol and allowed to dry, with the rupture disks only being dipped for 2-3 seconds. Macrocarriers were coated with a range of bead mix preparations from 1 µl to 25 µl and loaded into the biolistic delivery system (Bio-Rad). Plates containing *T. halophilus were* grown in a lawn for 1-3 days to use in the biolistic delivery system. Plates were then loaded into the biolistic delivery system at the target distance of 6 cm and the prepared gold particles were dispersed at a vacuum of 29 Hg and allowed a recovery period at 30°C for a minimum of 3 hours to a maximum of overnight. An antibiotic overlay was then added consisting of sterile Tsoy and 1.5% agar. The overlay mix contained of 100 µM ampicillin. Plates were then allowed to grow for another 2-3 days in a 30°C incubator to allow successful transformants to grow. Colonies were unable to be determined through this method, so modifications were made. On subsequent tests, cells were plated on Tsoy plates without antibiotic and following bombardment were transported from the plate using a sterile velveteen cloth stamp and pressed into a new plate containing ampicillin at a concentration of 100 µM or chloramphenicol at a concentration of 10 µg/ml.

## 2.6 Electroporation

For electroporation, *T. halophilus* were grown in Tsoy media containing 0.25 M sucrose to a $OD_{660}$ of 0.2. Next Penicillin G was added at a concentration of 0.8 μg/ml. Cells were then allowed to grow to an $OD_{660}$ of 0.6-0.8. Cells were then treated with 600 U/ml of Lysozyme for 10-20 minutes. Cells were resuspended in an ice-cold solution of 0.5 M sucrose, 1 mM $K_2HPO_4$, 1 mM $MgCl_2$, at pH 7.4 then placed into 0.2 mm electroporation cuvettes with mini-prep purified DNA and electroporated with a voltage between 2000-3000, a capacitance of 25 uF, and a resistance between 200-400. Cells were then allowed to recover in 1 mL Tsoy media containing 2% sucrose for a minimum of 1 hour at 30°C and a maximum of 5 hours then plated on chloramphenicol plates at a concentration of 10 μg/ml.

## 2.7 Bioluminescent Imaging

*E. coli* containing pCBW2 were grown overnight at 37°C on LB plates containing 100 μM beetle luciferin (Promega) and either 100 μM ampicillin or 10 μg/ml chloramphenicol. Bioluminescence was recorded using a ChemiDoc MP (Bio-Rad) with 4 × 4 binning for an exposure time of 5 seconds.

# Results

## 3.1 Plasmid Construction

The goal of this project was to build an easily modifiable shuttle-expression vector that could be maintained in *E. coli* but shuttled to *T. halophilus* and express genes of interest in that target bacteria. This plasmid was designed with the major plasmid components for maintenance in the hosts (origin of replication) expression of reporter genes and selectable markers. The bioluminescent reporter *CBG* was chosen to provide a green bioluminescent signal. Two separate origins of replication were included, one for *E. coli* and one for *T. halophilus* so that the plasmids can be maintained in both species. Antibiotic resistance genes to confer resistance to kanamycin, ampicillin, and chloramphenicol were selected based on pre-existing susceptibility of *E. coli* and *T. halophilus* to those antibiotics. The *KanR* and *CamR* genes were already regulated using endogenous promoters for expression, while *CBG* and *AmpR* and would need to be paired with promoters specific to *T. halophilus*.

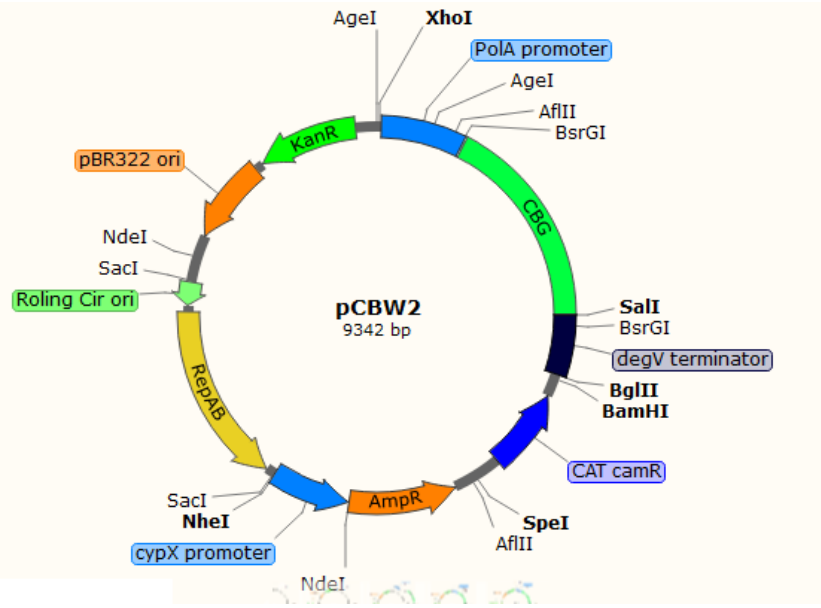


**Figure 1**. pCBW2 map. Plasmid contains a chloramphenicol, ampicillin, and kanamycin resistance marker, the bioluminescent reporter element *CBG*, the *RepAB* ori from pUCL287, the *degV* terminator, and the *T. halophilus* native promoters *polA* and *cypX*. Plasmid was built using pAllet as a template.

To achieve this goal, *T. halophilus* promoters were first identified. Partial genome sequences of ATCC 33315 (accession number GCA_003841405.1) were located through the NCBI database and were downloaded. No prior accessible annotations were discovered for *T. halophilus.* Putative promoters were identified by annotating *T. halophilus* genomic DNA using the DFast program to annotate possible genes. The DFast program (an online tool) requires the use of a FASTA format sequence loaded into the program that will generate an output annotating hypothetical genes. After the genome was characterized, genes expected to have constitutive expression to allow the novel

plasmid to continuously express desired products. Based on expression data from other bacteria like *E. coli*, The *polA, cypX, lig,* and *dnaQ* genes were identified as potential candidates having constitutive promoters. The start codon of each gene was identified and the portion of the sequence before the gene that would theoretically contain the promoter was targeted. A 500 base pair section of DNA upstream of the gene that contained each promoter was then amplified using genomic DNA as a template using primers with additional restriction sites designed into the ends (Table 1, primers 5,6,9,10, and 15-18). PCR products were verified through agarose gel electrophoresis. Next the putative promoters of *T. halophilus* were engineered upstream of genes and tested for expression in *E. coli*.

Out of the initial promoters selected, the *polA* and *cypX* genes were chosen as the promoters used in the construction of pCBW2. The *lig* and *dnaQ* promoters were also attempted to be added but were unsuccessfully ligated. To test the functionality of the regions of DNA suspected to contain these promoters, an ampicillin resistance gene (*AmpR*) and a bioluminescent gene (*CBG*) were selected as expression subjects to test both promoters simultaneously. Previous work in the Robertson Lab had shown *T. halophilus* to be susceptible to ampicillin but resistant to kanamycin, so ampicillin resistance was selected as our antibiotic reporter.

To create the shuttle-expression vector pCBW2 with the ampicillin resistance gene under control of the *T. halophilus* promoters, the plasmid pAllet was used as the plasmid backbone, but it contained an ampicillin resistant cassette under the control of an *E. coli*-sensitive promoter that is normally used in *E. coli* for plasmid selection. It was

uncertain whether *T. halophilus* could express the ampicillin resistance gene if under an *E. coli* promoter, so steps were taken to create an ampicillin resistance gene under the control of the *T. halophilus cypX* promoter. To prevent the addition of duplicate ampicillin resistance genes in pCBW2, the native ampicillin resistant gene was swapped for an *E. coli* regulated kanamycin resistant gene using the restriction enzymes *AgeI* and *SacI.* The native ampicillin resistance gene and plasmid's origin of replication were removed, and a PCR product containing the *KanR* gene and origin of replication PBR322 from the template plasmid pCambia2300 was added. This PCR product was generated using primers 1 and 2 (Table 1). After The addition of the kanamycin resistance and ligation, the plasmid modification was confirmed through PCR (Figure 2).

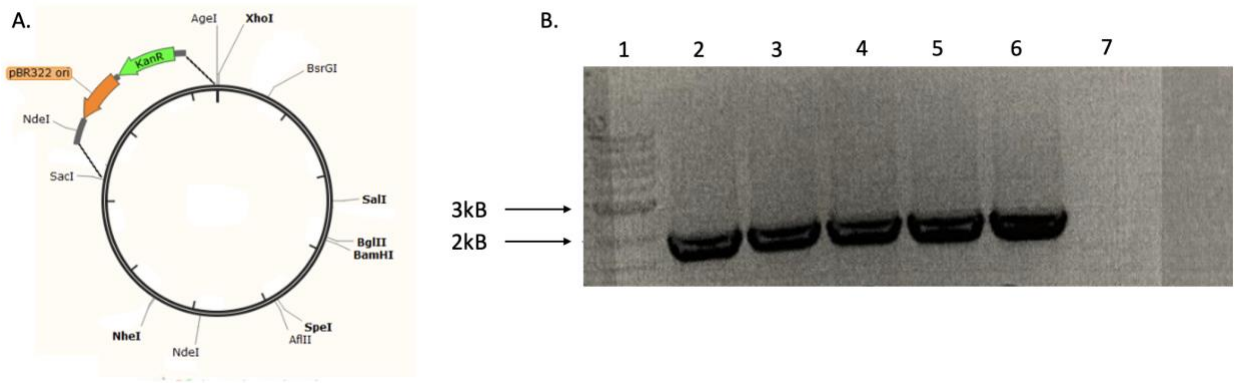


**Figure 2.** Confirmation of insertion of Kanamycin resistant marker. **(A)** Plasmid map showing the insertion of *KanR* and PBR322 ori. **(B)** Kanamycin resistant cassette was PCR amplified from donor source pCambia2300 and digested with the restriction enzymes *AgeI* and SacI then ligated into plasmid. After chemical transformation in *E. coli*, colonies were selected and screened through PCR. Lane 1 is the ladder, Lanes 2-6 are selected colonies, and lane 7 is a negative control. Expected PCR size was ~2kB.

Next the *AmpR* gene was added back to the plasmid using the enzymes *AflII* and *NdeI*. First the *AmpR* gene was amplified using high fidelity PCR with primers 3 and 4 (Table 1) using pAllet template. This addition was verified through PCR screening for the *AmpR* gene as well as a restriction digest confirmation using the *AflII* and *NdeI* restriction enzymes (Fig. 3).

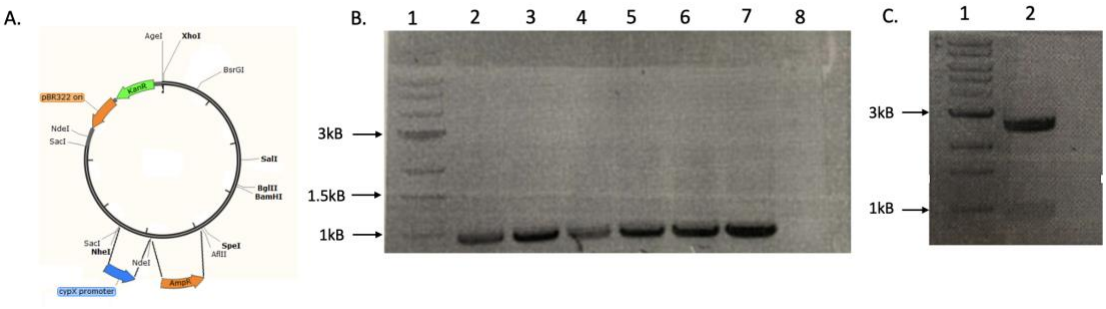


**Figure 3.** Confirmation of insertion of Ampicillin resistant marker. **(A)** plasmid map showing the addition of *AmpR*. **(B)** *AmpR* was PCR amplified and digested with the restriction enzymes *AflII* and *NdeI* then ligated into plasmid. After chemical transformation in E. coli, colonies were selected and screened through PCR. Lane 1 is the ladder, lanes 2-6 are selected colonies, and lane 7 is a positive control, lane 8 is a negative control. **(C)** Restriction digestion confirmation showing a 1kB band for *AmpR* and a 2.9kB band for the vector plasmid.

So that *T. halophilus* could express the *AmpR* gene, the *cypX* promoter was added immediately upstream of the *AmpR* sequence. This was done by PCR amplifying the *cypX* promoter from *T. halophilus* genomic DNA using primers 5 and 6 (Table 1) and

ligating this *NheI-NdeI* cut PCR product into constructed vector cut with the same enzymes. This modification was confirmed by PCR and restriction analysis (Fig. 4).

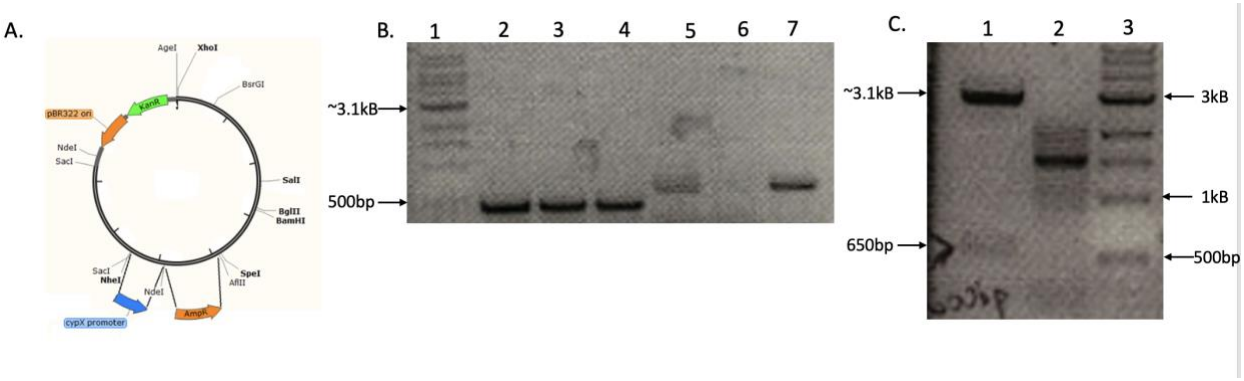


**Figure 4.** Confirmation of insertion of *cypX* promoter. **(A)** Plasmid map showing the addition of *cypX*. **(B)** *The cypX* promoter was PCR amplified from *T. halophilus* and digested with the restriction enzymes NheI and NdeI then ligated into plasmid. After chemical transformation in *E. coli*, colonies were selected and screened through PCR. Left image Row 1 is the ladder, rows 2-4 are selected colonies involving a different promoter, row 5-7 are colonies selected for *cypX* . **(C)** Confirmation through restriction digestion. Lane 1 was digested with *NheI* and *NdeI*, lane 2 was an unrelated digestion, while lane 3 is the ladder. Promoter *cypX* size is 650bp while total plasmid size was 3.7kB.

*T. halophilus* strain ATCC 33315 was determined to possess a native plasmid known as pUCL287 and was acquired to use in our experiments. Native pUCL287 has an origin of replication known as *RepAB* that operates through rolling circle replication. This fragment was amplified using high fidelity PCR using pUCL287 using primers 7 and 8 (Table 1). The *RepAB* PCR product underwent restriction digestion with SacI and was then ligated into the shuttle vector. Insertion was confirmed through PCR (Fig. 5).

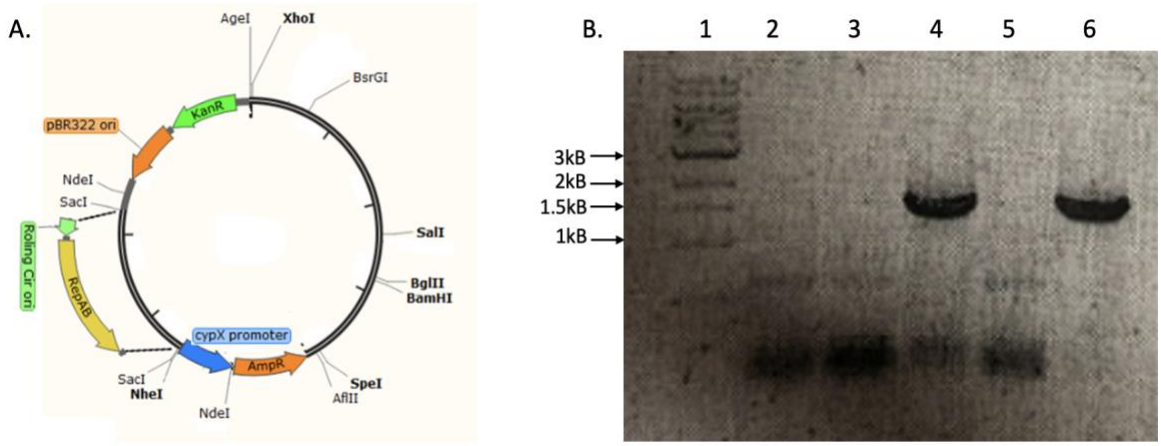


**Figure 5.** Confirmation of insertion of *RepAB* origin of replication. (A) Plasmid map showing *RepAB* insertion. **(B)** *RepAB* was PCR amplified from the native plasmid pUCL287 found in *T. halophilus* and digested with the restriction enzyme *SacI* then ligated into vector plasmid. After chemical transformation in *E. coli*, colonies were selected and screened through PCR. Lane 1 is the ladder, lanes 2-5 are selected colonies, and lane 6 is a positive control. *RepAB* size is ~1.7kB.

The next sequence of additions to the shuttle vector were designed around the *polA* promoter driving the expression of the bioluminescent reporter *CBG*. Where the ampicillin resistance cassette and the kanamycin resistance cassette both had naturally occurring terminators, the new addition of *CBG* did not. To remedy this a terminator from *T. halophilus* was used from the *degV* gene. *TdegV* contains a restriction site for BsrGI, which would be problematic if *CBG* was added before *TdegV* so the *CBG* reporter was added before the addition of *TdegV*. Promoter *polA* was amplified using high fidelity PCR from *T. halophilus* using primers 9 and 10 (Table 1) and digested with *XhoI* and *BsrGI*. After ligation and transformation, colonies were plated onto kanamycin plates and screened for the addition (Fig. 6).

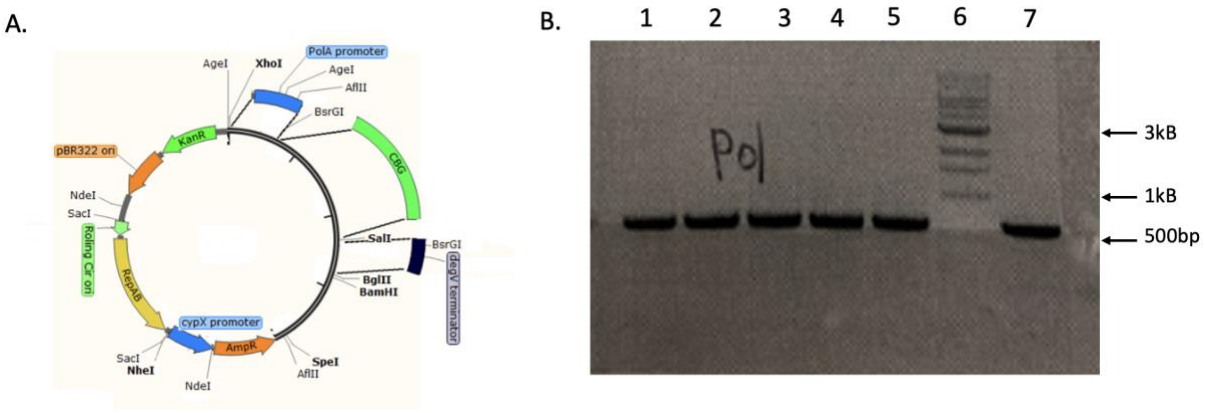


**Figure 6.** Confirmation of insertion of *T. halophilus* native *PolA* promoter. **(A)**Plasmid map showing the insertion of the *PolA* promoter. **(B)** *PolA* promoter was PCR amplified from *T. halophilus* and digested with the restriction enzymes *XhoI* and *BsrGI* then ligated into plasmid. After chemical transformation in *E. coli*, colonies were selected and screened through PCR. Rows 1-5 are selected colonies, row 6 is the ladder, and row 7 is a positive control. *PolA* size is ~650bp.

Following the addition of *polA*, the bioluminescent reporter *CBG* was added to the shuttle vector. The *CBG* gene was amplified using high fidelity PCR using pCBG99-basic as template using primers 11 and 12 (Table 1). The product was digested with *BsrGI* and *SalI* then ligated into the shuttle vector. After transformation, colonies were plated on kanamycin plates and screened using the same primers to target *CBG*.

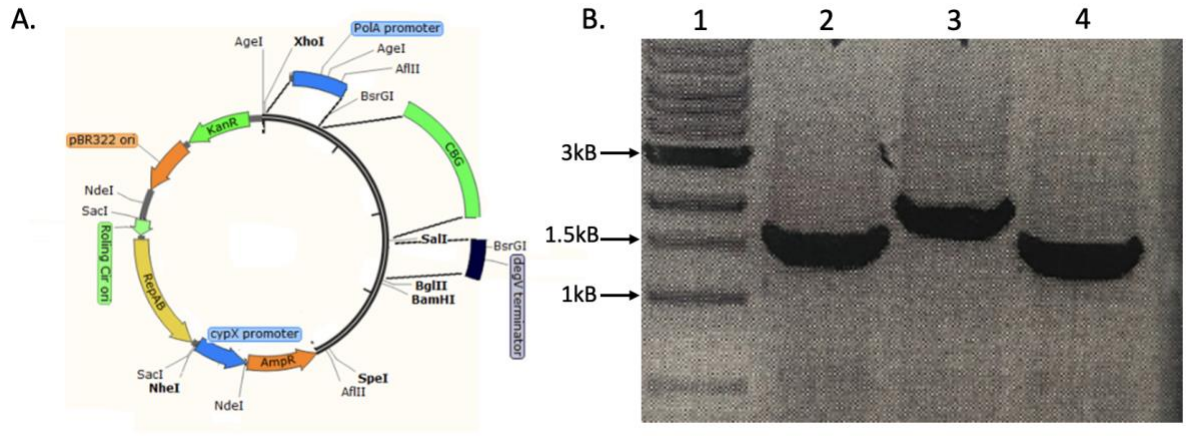


**Figure 7. (A)** Plasmid map showing the insertion of the *CBG* reporter. **(B)** Confirmation of insertion of *CBG*. *CBG* was PCR amplified using high fidelity PCR and digested with the restriction enzymes *BsrGI* and *SalI* then ligated into plasmid. After chemical transformation in *E. coli*, colonies were selected and screened through PCR. Lane 1 is the ladder, lanes 2-4 are selected colonies. *CBG* size is 1.6kB and colony from lane 2 was selected for future work.

Finally, to complete the bioluminescent portion of the shuttle vector, *Tdegv* was added. With *CBG* not having its own terminator, one was selected that would theoretically work in *T. halophilus*. TdegV was chosen by looking for a sequence that followed one coding sequence that was upstream from an adjacent gene that was transcribed in the opposite direction. To increase the likelihood that any potential sequence contained a terminator, we sought candidate sequences between stop codes of adjunct genes that were transcribed in opposite directions. That way there was likely a terminator between them, rather than the coding sequence being part of an operon that didn't have a terminator after the stop codon. Primers were designed to target the *degV* terminator from the genomic DNA of *T. halophilus* which was amplified using high

fidelity PCR and primers 13 and 14 (Table 1). The PCR product was digested using *BglII* and *SalI* then ligated into the plasmid. Transformed colonies were screened using PCR and a following restriction digestion analysis was performed (Fig. 8).

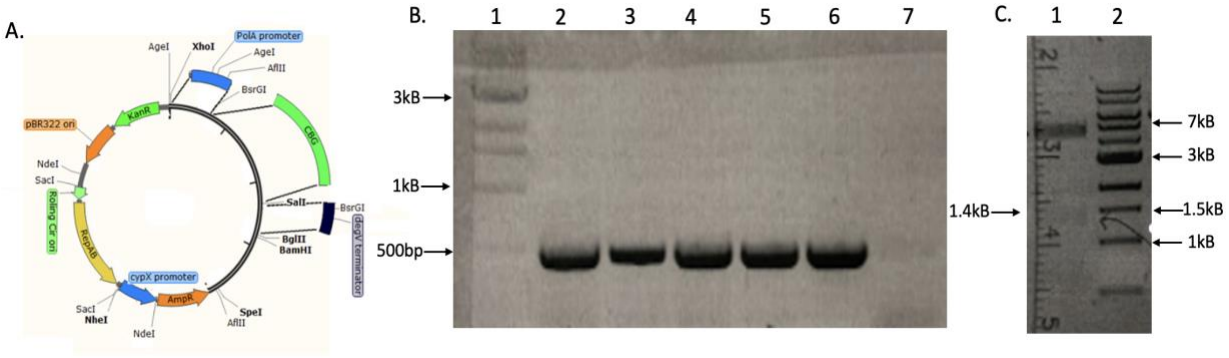


**Figure 8.** Confirmation of insertion of *degV* terminator. (A) Plasmid map of the insertion of *TdegV. (B) TdegV* was PCR amplified from *T. halophilus* and digested with the restriction enzymes *SalI* and *BglII* then ligated into plasmid. After chemical transformation in *E. coli*, colonies were selected and screened through PCR. Lane 1 is the ladder, lanes 2-6 are selected colonies, lane 7 is a negative control. **(C)** Confirmation through restriction digestion. Restriction enzymes *MluI* and *BglII* were selected to allow visualization of the terminator band with an expected size of around 1.4-1.5kB. Plasmid total size was 6.4kB.

To further show the transformation of our plasmid in the event *T. halophilus* was unable to produce stable ampicillin resistance, a chloramphenicol cassette was added to the plasmid. Previous studies showed that this addition was found to work in species similar to *T. halophilus* that were sensitive to chloramphenicol and would further validate successful transformation. The chloramphenicol resistance marker was synthesized by Genscript using the pIP501 sequence (accession number X65462.1) acquired from

Genebank with built in restriction sites for *SpeI* and *BamHI*. After using the appropriate enzymes and ligating the plasmid, the addition was confirmed using PCR with primers 23 and 24 (Table 1).

## 3.2 Transformations

After the plasmid was fully constructed and confirmed through PCR, the task of determining how to transform *T. halophilus* began. The novel plasmid was easily transformed into *E. coli* through standard chemical transformation. Ampicillin resistant *E. coli* showed that *E. coli* utilized the ampicillin resistance gene driven by the *T. halophilus cypX* promoter. Similarly, transformed *E. coli* grew on chloramphenicol plates as well (Fig. 10). Transformed *E. coli* also expressed *CBG* when exposed to luciferin substrate (Fig. 10) while also having a perceivable signal when assessed in the luminometer. These data suggest that the promoters were in fact functional and constitutively expressed. With *E. coli* being able to express genes in the plasmid, it would suggest *T. halophilus* would also be capable of utilizing the novel plasmid. Despite success in *E. coli*, we found no successful transformations of *T. halophilus* using this method (Fig. 9). The peptidoglycan layer that normally causes gram-positive bacteria to resist conventional transformation methods could possibly be preventing our plasmid from being taken in, so different methods of transformation were next attempted: Electroporation and biolistic transformation.

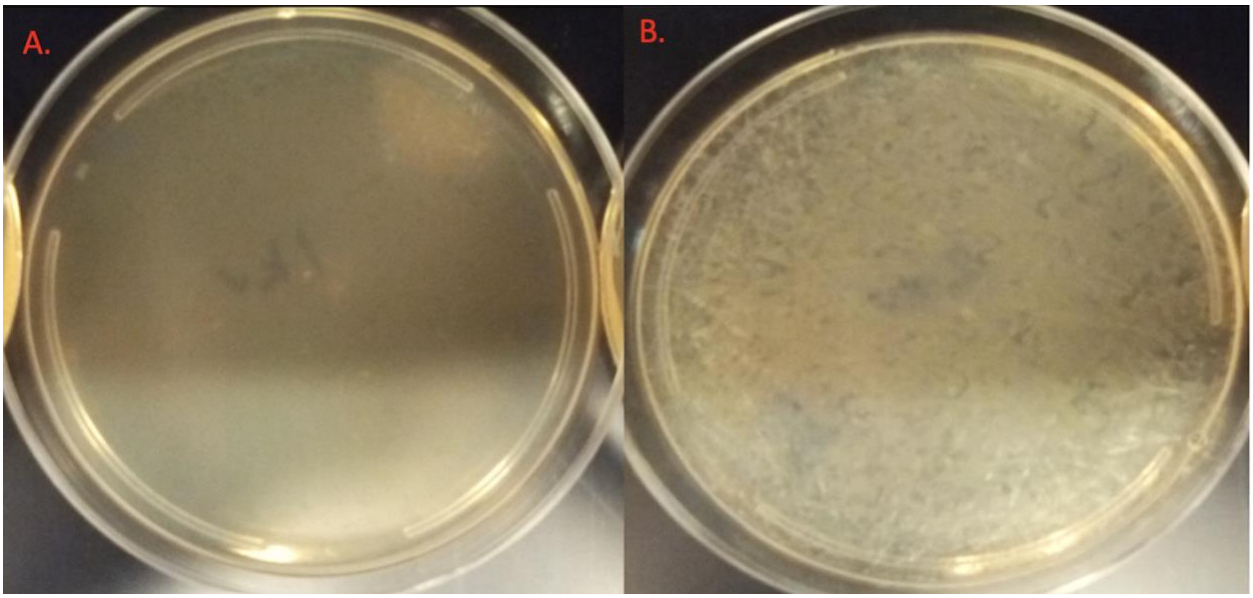


**Figure 9.** Representation of *T. halophilus* Transformation Results. **(A)**Tryptic Soy Agar plate with Ampicillin. **(B)** Tryptic Soy Agar containing no antibiotics. Cells were made electrocompetent and subjected to 3000V in the electroporator followed by an overnight recovery period before plating. Plates were allowed to grow for 3 days before being imaged.

Electroporation was attempted under a variety of conditions (Table 3). Initially *T. halophilus* were made electrocompetent and an electroporation protocol used in *E. coli* was attempted that resulted in no success. Due to the intricacies of the cell membrane, modifications were made to the protocol for making electrocompetent *T. halophilus*. Previous work in other species showed a dual use of Penicillin G and lysozyme was effective in weakening the cell membrane enough to allow successful electroporation (Jin et al., 2012). Despite these modifications, no electroporation trials resulted in successful transformations in *T. halophilus* with a representation of consistent results seen across all trials (Fig. 9). *E. coli* were able to be successfully transformed through electroporation

and were confirmed through survival on chloramphenicol and ampicillin plates while also bioluminesceing (Fig. 10).

**TABLE 3: Electroporation Conditions Attempted and Results**

| Antibiotic | Voltage | Total Attempts | Results |
|---|---|---|---|
| Ampicillin | 2000 | 4 | Negative |
| Ampicillin | 2500 | 9 | Negative |
| Ampicillin | 2750 | 6 | Negative |
| Ampicillin | 3000 | 15 | Negative |
| Chloramphenicol | 2500 | 2 | Negative |
| Chloramphenicol | 2750 | 2 | Negative |
| Chloramphenicol | 3000 | 8 | Negative |

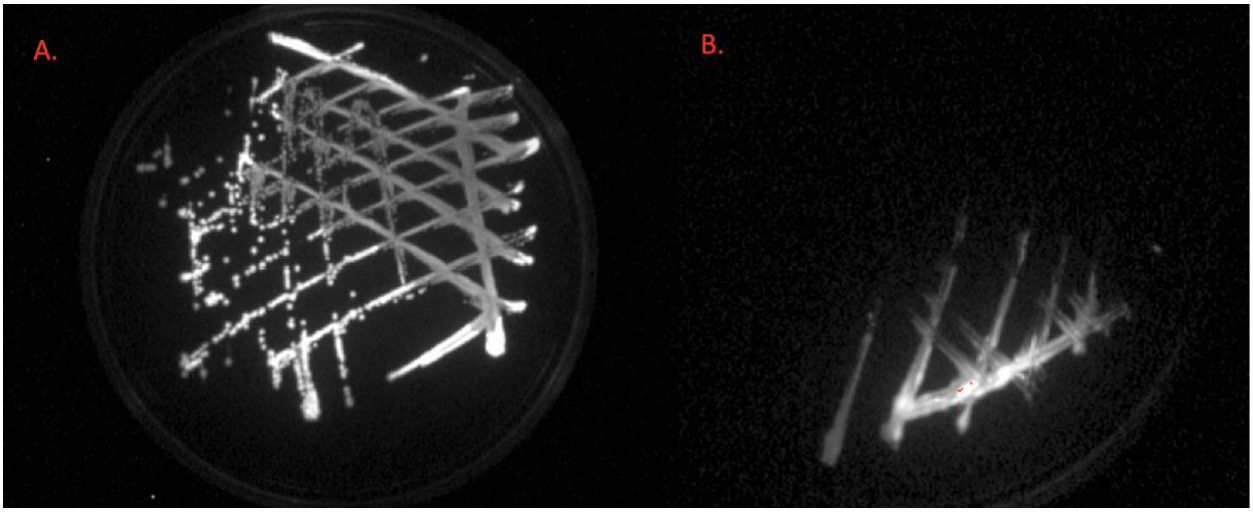


**Figure 10.** *E. coli* transformed with pCBW2. **(A)** LB agar with ampicillin containing luciferin substrate. **(B)** LB Agar with chloramphenicol containing luciferin substrate. *E. coli* transformed with pCBW2 was visualized with a 4X4 binning and exposure time of 5 seconds.

Biolistic transformation was also attempted under a variety of conditions (Table 4). All transformation attempts were unsuccessful, with only a similar morphology contaminant being transformed in one trial that was later shown to not be *T. halophilus* through the use of PCR using primers that were designed to target various genomic regions of *T. halophilus* (Table 1 primers 15-22). Both ampicillin and chloramphenicol plates were used with a visual representation of results seen in figure 9 above.

**TABLE 4: Biolistic Transformation Conditions Attempted and Results**

| **Antibiotic** | **Microcarrier Volume** | **Total Attempts** | **Results** |
|---|---|---|---|
| Ampicillin | 1 μl | 5 | Negative |
| Ampicillin | 2.5 μl | 5 | Negative |
| Ampicillin | 5 μl | 35 | Negative |
| Ampicillin | 7.5 μl | 5 | Negative |
| Ampicillin | 10 μl | 10 | Negative |
| Ampicillin | 15 μl | 2 | Negative |
| Ampicillin | 20 μl | 4 | Negative |
| Ampicillin | 25 μl | 1 | Negative |
| Chloramphenicol | 1 μl | 1 | Negative |
| Chloramphenicol | 2.5 μl | 1 | Negative |
| Chloramphenicol | 5 μl | 3 | Negative |
| Chloramphenicol | 7.5 μl | 1 | Negative |
| Chloramphenicol | 10 μl | 3 | Negative |
| Chloramphenicol | 15 μl | 2 | Negative |
| Chloramphenicol | 20 μl | 2 | Negative |

Another likely issue that was suspected was potential mutations to the *RepAB* origin of replication as it was propagated through *E. coli*. To disprove the possibility of this occurring and causing the origin of replication from being unusable in *T. Halophilus*, pCBW2 was sequenced using Sanger sequencing by MClab to determine if any changes had occurred. After successful sequencing, it was confirmed that there had not been any mutations in this region and that it should still function as intended in *T. halophilus*.

# Discussion

Although there were no transformants of *T. halophilus* using chemical, electroporation, or biolistic transformation, it was shown that the expression vector and *T. halophilus* promoters are capable of being utilized by *E. coli.* Many of the elements that went into the plasmid were theoretical, such as the promoters and *degV* terminator. The hypothetical promoters were shown to be functional as seen by the expression of *CBG* driven by the *polA* promoter in *E. Coli* showing bioluminescence in transformants as well as the ampicillin resistance gene driven by the *cypX* promoter allowing transformed *E. coli* to survive ampicillin plates. The *degV* terminator confirmed as a working terminator through the successful expression of *CBG* and bioluminescence. *RepAB* was also shown to not compete with the origin or replication in *E. coli*. Finally, the synthesized chloramphenicol gene was utilized as well, suggesting it would likely be utilized by *T. halophilus.*

Having successfully transformed *E. coli* with pCBW2 and showing that the promoters can drive transcription of the reporters, it can be assumed that if pCBW2 was able to be transformed into *T. halophilus*, we would see similar if not better results. It is possible there are barriers to transformation of *T. halophilus* both known and unknown. The peptidoglycan layer may be too thick alongside other cell wall barriers, preventing the plasmid from being taken in. It was theorized that treating the cells with a Penicillin G-lysozyme mix as described in the materials and method section would be sufficient to allow the barrier to weaken enough to allow cells to take in the plasmid. It was also

hypothesized that biolistic transformation would remedy this same problem and allow the plasmid to bypass the cell wall and be successfully incorporated into the cell. Both avenues resulted in unsuccessful trials suggesting there may be unknown variables at play.

Another possibility is the bacterial defense system. Bacteria often possess inherent restriction enzymes that provide defenses from the introduction of foreign genetic material, such as from a virus. This could cause issues with the introduction of pCBW2 in that if the restriction enzymes are able to target the plasmid, the plasmid could be destroyed (Allers et al, 2010). An investigation into what restriction enzymes *T. halophilus* generates could be pursued to determine if this is a barrier to entry. If it was determined that *T. halophilus* possesses genes to create restriction enzymes, a knockout strain could be engineered to no longer express those enzymes. This would allow stable use of pCBW2 in *T. halophilus* and provide an optimized strain for transformation.

While controls showed that *T. halophilus* was capable of surviving electroporation and biolistic transformation, it could be that those cells successfully affected by the electrical current or pierced by the gold particles were unable to survive, while those in the population that were unaffected by the transformation method survived. With these avenues of transformation having been tested with *T. halophilus*, other avenues can now be pursued.

Conjugation is another plausible method that could be conducted in future studies. Unfortunately, conjugation has its own set of problems. A suitable donor species would need to be identified, the plasmid would need to include components that allow the

plasmid to be maintained in the donor species, and the donor species would need to be capable of growing in compatible media with *T. halophilus* while being able to be selected against post transformation (Alderliesten et al., 2020). If this avenue resulted in success, then a useable modifiable plasmid would be available to any companies or researchers who are working with *T. halophilus*. Other closely related species may also be able to utilize pCBW2 and any who pursue that path could attempt to use pCBW2 in transformation attempts. After determining a method of transformation, pCBW2 could be easily modified to express a number of genes of interest. With CRISPR, pCBW2 could be provided the sgRNA and Cas9 elements to target viral pathogens that are a threat to *T. halophilus*. This would provide researchers with a modifiable system in which to engineer strains that possess a variety of viral defenses. With *T. halophilus* being used in food fermentation, strains could also be developed that express proteins associated with desired flavor in the food products. With many companies seeking to improve the quality of their product, this could prove a useful tool in that endeavor.

# References


Alderliesten JB, Duxbury SJ, Zwart MP, de Visser JA, Stegeman A, Fischer EA. 2020. Effect of donor-recipient relatedness on the plasmid conjugation frequency: A meta-analysis. BMC Microbiology. 20(1). doi:10.1186/s12866-020-01825-4

Allers T, Barak S, Liddell S, Wardell K, Mevarech M. 2010. Improved strains and plasmid vectors for conditional overexpression of his-tagged proteins in *haloferax volcanii*. Applied and Environmental Microbiology.;76(6):1759–1769. doi:10.1128/aem.02670-09

Bonnefoy N, Remacle C. 2023. Biolistic Transformation of Chlamydomonas reinhardtii and Saccharomyces cerevisiae Mitochondria. Methods Mol Biol. 2615:345-364. doi: 10.1007/978-1-0716-2922-2_24.

Cho SW, Yim J, Seo SW. 2020. Engineering tools for the development of recombinant lactic acid bacteria. Biotechnology Journal.;15(6). doi:10.1002/biot.201900344

Collins MD, Williams AM, S. Wallbanks. 1990. The phylogeny of Aerococcus and Pediococcus as determined by 16S rRNA sequence analysis: description of Tetragenococcus gen. nov. Fems Microbiology Letters. 70(3):255–262. https://doi.org/10.1111/j.1574-6968.1990.tb14006.x.

Dower WJ, Miller JF, Ragsdale CW. 1988. High efficiency transformation of e.coli by high voltage electroporation. Nucleic Acids Research. 16(13):6127–6145. doi:10.1093/nar/16.13.6127

Fukushima D. 1981. Soy proteins for foods centering around soy sauce and tofu. Journal of the American Oil Chemists' Society. 58(3Part2):346–354. https://doi.org/10.1007/bf02582376.

Geisen R, Holzapfel WH. 1996. Genetically modified starter and protective cultures. International Journal of Food Microbiology. 30(3):315–324. doi:10.1016/0168-1605(96)00959-2

Gürtler M, Gänzle MG, Wolf G, Hammes WP. 1998. Physiological Diversity Among Strains of Tetragenococcus halophilus. Systematic and Applied Microbiology. 21(1):107–112. https://doi.org/10.1016/s0723-2020(98)80013-1.

Hahn J, DeSantis M, Dubnau D. 2021. Mechanisms of transforming DNA uptake to the periplasm of bacillus subtilis. mBio.;12(3). doi:10.1128/mbio.01061-21

Hajdukiewicz P, Svab Z, Maliga P. 1994. The small, versatile ppzp family of agrobacterium binary vectors for plant transformation. Plant Molecular Biology.;25(6):989–994. doi:10.1007/bf00014672

Hanahan D. 1983. Studies on transformation of escherichia coli with plasmids. Journal of Molecular Biology. 1983;166(4):557–580. doi:10.1016/s0022-2836(83)80284-8

Islam SM, Ryu H-M, Sohn S. 2022. Tetragenococcus halophilus alleviates intestinal inflammation in mice by altering gut microbiota and regulating dendritic cell activation via CD83. Cells. 11(12):1903. doi:10.3390/cells11121903

Jin Q, Eom H-J, Jung JY, Moon JS, Kim JH, Han NS. 2012. Optimization of electrotransformation conditions for Leuconostoc mesenteroides subsp. mesenteroides ATCC8293. Letters in Applied Microbiology. 55(4):314–321. doi:10.1111/j.1472-765x.2012.03300.x

Kumari M, Pandey S, Mishra A, Nautiyal CS. 2017. Finding a facile way for the bacterial DNA transformation by biosynthesized gold nanoparticles. FEMS Microbiology Letters. 364(12). doi:10.1093/femsle/fnx081

Kumazawa T, Nishimura A, Asai N, Adachi T. 2018. Isolation of immune-regulatory tetragenococcus halophilus from miso. PLOS ONE. 13(12). doi:10.1371/journal.pone.0208821

Link T, Vogel RF, Ehrmann MA. 2021. The diversity among the species Tetragenococcus Halophilus including new isolates from a lupine seed fermentation. BMC Microbiology. 21(1). doi:10.1186/s12866-021-02381-1

Liu J, Chang W, Pan L, Liu X, Su L, Zhang W, Li Q, Zheng Y. 2018. An improved method of preparing high efficiency transformation escherichia coli with both plasmids and larger DNA fragments. Indian Journal of Microbiology. 58(4):448–456. doi:10.1007/s12088-018-0743-z

Liu X, Li J, Li Y, Li J, Sun H, Zheng J, Zhang J, Tan H. 2022. A visualization reporter system for characterizing antibiotic biosynthetic gene clusters expression with high-sensitivity. Communications Biology. 5(1). doi:10.1038/s42003-022-03832-9

Mathur H, Beresford TP, Cotter PD. 2020. Health Benefits of Lactic Acid Bacteria (LAB) Fermentates. Nutrients. 12(6):1679. https://doi.org/10.3390/nu12061679.

Mezzanotte L, Que I, Kaijzel E, Branchini B, Roda A, Löwik C. 2011. Sensitive dual color in vivo bioluminescence imaging using a new red codon optimized firefly luciferase and a green click beetle luciferase. PLoS ONE. 6(4). doi:10.1371/journal.pone.0019277

Myers KS, Noguera DR, Donohue TJ. 2021. Promoter architecture differences among alphaproteobacteria and other bacterial taxa. mSystems.;6(4). doi:10.1128/msystems.00526-21

Németh T, D. Nosanchuk J, Vagvolgyi C, Gacser A. 2021. Enhancing the chemical transformation of candida parapsilosis. Virulence.;12(1):937–950. doi:10.1080/21505594.2021.1893008

Pátek M, Nešvera J. 2012. Promoters and plasmid vectors of Corynebacterium glutamicum. J. Biotechnology Jul 2:51–88. doi:10.1007/978-3-642-29857-8_2

Reichard WD, Smith SE, Robertson JB. 2023. Blincar: A reusable bioluminescent and cas9-based genetic toolset for repeatedly modifying wild-type scheffersomyces stipitis. mSphere. 8(4). doi:10.1128/msphere.00224-23

Robertson JB, Johnson CH. 2011. Luminescence as a continuous real-time reporter of promoter activity in yeast undergoing respiratory oscillations or cell division rhythms. Methods in Molecular Biology. 63–79. doi:10.1007/978-1-61779-086-7_4

Rogers HJ, Perkins HR, Ward JB. 1980. Microbial cell walls and membranes. London: Chapman & Hall.

Sanford JC. 1990. Biolistic Plant Transformation. Physiologia Plantarum. 79(1):206–209. doi:10.1034/j.1399-3054.1990.790131.x

Sirajuddin SA, Sundram S. 2020. Evaluation of two transformation protocols and screening of positive plasmid introduction into bacillus cereus EB2, a gram-positive bacterium using qualitative analyses. Brazilian Journal of Microbiology. 51(3):919–929. doi:10.1007/s42770-020-00241-0

Uchida K. 1982. Multiplicity in soy pediococci carbohydrate fermentation and its application for analysis of their flora. Journal of General and Applied Microbiology. 28(3):215–223. https://doi.org/10.2323/jgam.28.215.

Wakinake T, Matsutani M, Watanabe J, Yoshinobu M, Masafumi T, Akihiro O. 2023. Identification of Capsular Polysaccharide Synthesis Loci Determining Bacteriophage Susceptibility in Tetragenococcus halophilus. American Society for Microbiology: Microbiology Spectrum. https://doi.org/10.1128/spectrum.00385-23

Wang C, Cui Y, Qu X. 2020. Optimization of electrotransformation (ETF) conditions in lactic acid bacteria (LAB). Journal of Microbiological Methods. 174:105944. doi:https://doi.org/10.1016/j.mimet.2020.105944.