Solving for Tomorrow: A glimpse into Drought Tolerance in Soybean-Bacteria Symbiosis

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Abstract

Bradyrhizobium japonicum creates a symbiotic relationship with soybeans in nature. *B. Japonicum* is a species of nitrogen-fixing bacteria that soybeans protect in order to help them grow in low-nitrogen conditions. The goal of this project was to genetically modify *E. Coli* plasmids to express salt tolerant genes to be inserted into *B. Japonicum* to make soybeans drought resistant. When these transformed cells infect a soybean plant, it will increase root growth, and increase tolerance to low-water conditions. To demonstrate this, two genes were inserted into an *E. Coli* plasmid to be able to be expressed and later to demonstrate increased salt tolerance. We will then clone these plasmids into *B. Japonicum* and test the effectiveness in soybeans.

Introduction

As concerns with global warming begin to rise, solutions to its harmful effects are constantly being proposed to counteract the warming climate. One of the most essential necessities to modern human life, affected by climate change, is agriculture. Drought conditions across the globe are causing a drastic decrease in crop yields, leading to food shortages in certain parts of the world. As the global population increases, increased crop yield and cultivation area expansion will be needed. To meet the needs of the growing global population it is imperative to find ways to utilize marginal soils, due to high salt content, for crop cultivation.

Legumes such as soybeans are of high interest because of their positive association with nitrogen-fixing bacteria which reduces the need for commercial farmers to use nitrogen rich fertilizer in their soil. However, soybeans are not well suited for drought-stricken areas or marginal soils. The primary complication for these types of soils in the regions with drought conditions is the impact of low water activity. Decreased water concentration due to drought or high salt concentrations result in similar effects on the plant. The high salt concentrations cause the plants' cells to lose water and shrivel up due to osmosis, becoming useless and killing the plants. Drought also affects the bacteria that associate with plant roots, especially nitrogen-fixing symbionts of legumes. The osmotic effect of drought and high salt concentrations are similar; therefore, a single approach can be used to address both problems. Genes found in naturally salt tolerant organisms that may increase salt tolerance of *B. Japonicum* and drought tolerance in soybean plants are glycine sarcosine methyltransferase (GSMT) and sarcosine dimethylglycine methyltransferase (SDMT).

Betaine is a compatible solute that is crucial as an osmoprotectant in plants, animals, bacteria, and archaea. It was found in the organism *M. portucalensis*, SDMT responsible for betaine synthesis, is an indication of halophilic methanoarchaea having more than one betaine synthesis system to ensure survival in hypersaline conditions.[7] The catalytic efficiency of SDMT is higher than GSDMT, which indicates that the GSMT/SDMT system may be responsible for more immediate osmotic stresses.

Naturally salt tolerant bacteria can be found in various environments. It is dependent on the level of salt present in the environment that these bacteria will employ different strategies to combat osmotic stresses. The most common approach is to synthesize compatible solutes such as glycine-betaine. Introducing compatible solutes into the cytoplasm of the cells counteracts high salt concentration outside the cell without interfering with the normal function of cellular enzymes which may be inhibited by salts. Dr. Mei-Chin Lai and colleagues are the

National Chung-Hsing University in Taiwan cloned the betaine bio synthetic genes from a marine bacterium, *Methanohalophilus portucalensis* FDF1T, and introduced them into *E. Coli* and into model plant Arabidopsis thaliana. Her and her colleagues demonstrated increased levels of salt tolerance caused by these genes. Therefore, it is feasible that introducing the same cloned genes into *B. Japonicum* will increase the salt tolerance of the organism. *B. Japonicum* is naturally sensitive to salt and generally will not exhibit growth at salt concentrations as low as 0.5%. Consequently, increasing salt tolerance should have a significant impact on the utility of this organism in more extreme salt conditions.

This project has potential to let agriculture be introduced to drier, saltier regions and allow more crops to be grown and food to be produced due to the increased survival rate in drought and high salt conditions. The goal is to develop effective salt tolerant strains of *B. Japonicum* that would protect crops from dry conditions. The success of this project would bring many economic and agricultural benefits to not only Missouri, but other regions of the world.

Materials and Methods

The first step that needed to be done was the cloning of the genes. Polymerase chain reaction (PCR) was used to amplify the genes and introduce restriction endonuclease cleavage sites that corresponded to the synthetic biology standard assembly. Bullseye Taq Polymerase master mix and the primers, combinations of foreword GSMT (GSMT-F), reverse GSMT (GSMT-R), and reverse SDMT (SDMT-R), designed to amplify the GSMT and SDMT genes from plasmids were used to introduce the genes into *E. Coli.*

The PCR reactions were performed by adding 4 microliters of milliQ water, 2 microliters of pUHE21 plasmid DNA, 2 microliters of GSMT-F, 2 microliters of GSMT-R, and 10 microliters of master mix. The same solution was put into another PCR tube with SDMT-R instead of GSMT-R.

Once the structural genes were individually cloned, the promoters and ribosomal binding sites (RBS) from *B. Japonicum* were cloned to ensure expression ability in the organism using PCR reactions. After cloning, a gel electrophoresis was run to check the sizes of the genes were correct. The gel for the electrophoresis was made by mixing 0.8 grams of agarose with 50 mL of TAE buffer in a 250 mL flask and microwaved for two minutes in a beaker with 50mL of water to prevent solidifying. Once everything was dissolved, another 50mL of TAE buffer was poured into the flask and 3 microliters of ethidium bromide was pipetted into the liquid. The liquid was then poured into a mold and set to solidify for 40 minutes. 10 microliters of a lambda size ladder were used to check DNA sizes, 10 microliters of each PCR reaction were used in each corresponding lane.

The GSMT and SDMT clones were then combined with the promoter and RBS. The *E. Coli* strain that contains the genes was grown by inoculating a culture in 5mL LB broth and 5 microliters of ampicillin at 37˚C overnight in an incubated shaker. After an adequate amount of growth, plasmid purification was done with the MidSci Mini High-Speed Plasmid prep kit. A digest was run afterwards using 2 microliters of specified buffer, 5 microliters of purified plasmid, 12.5 microliters sterile water, and 0.5 microliters of EcoRI restriction enzyme. The solution as incubated in a 37˚C water bath for one hour. After one hour, a gel electrophoresis was run to ensure the promoter and RBS was taken up by the DNA.

The DNA was extracted from the gel and placed in a 1.5 mL micro-centrifuge tube. 200 microliters of buffer NTI were pipetted into the tube and incubated in a 50˚C water

bath until the solution becomes homologous. A filter column was placed in a collection tube and 210 microliters of the sample was loaded into the column. The tube was centrifuged for 30 seconds at 11,000xg and the flow through was discarded. 700 microliters of wash buffer were added and centrifuged for 20 seconds and the flow through was discarded again. The sample was dried, and 50 microliters of elution buffer was added and let sit for one minute and centrifuged for one minute.

Three tubes of competent cells were acquired, and one tube was inoculated with plasmid DNA, one with plasmid and PCR product, and another with a different plasmid used as a control. After 15 minutes in a 42˚C water bath, the cells were plated onto LAX plates and incubated at 37˚C overnight. 8 colonies were isolated from the plasmid/PCR plate and DNA from the G template were used to make 9 total PCR products.10 microliters of PCR was used for a gel run and the best two PCR products were chosen to be colonies 3 and 5.

Many attempts were made to utilize colonies 3 and 5 for gene insertion and expression. MpGSMT and MpSDMT were replaced with TRCSDMT and TRCGSMT in order to make more effective PCR reactions and fresh primer dilutions were made. Both attempts to resolve the issue did not work and working with the DNA of colonies 3 and 5 was abandoned and working with the TRCGSMT/TRCSDMT genes seemed ineffective. It was decided that new dilutions of the original MpGSMT/MpSDMT genes were to be made and used to rerun future experiments. Since the experiments with the original 9 colonies resulted in insufficient results, a transformation was done to new cells for fresh pUHE21 DNA. An ice box was retrieved and filled with ice for the transformation. 2 tubes of competent cells were placed in the ice to keep the tubes chilled. 2 micro-centrifuge tubes were labeled 3, 5, and the competent cell tubes were labeled pUHE21 and pB121. Once thawed, 50 microliters were transferred to the empty tubes making 50 microliters in each of the four tubes. 2 microliters of corresponding DNA were pipetted into each tube and kept on ice for 15 minutes. The tubes were then heated in a 42˚C water bath for 1 minute and then put back on ice for 30 seconds. 200 microliters of S.O.C. was pipetted into each tube and the tubes were then placed in a 37˚C water bath for 45 minutes. 100 microliters of cells were streaked on each corresponding antibiotic plate and were incubated at 37˚C overnight. Colonies were isolated from each plate and were streaked onto new kanamycin plates and incubated at 37˚C overnight. New dilutions were made for MpGSMT-R, MpGSMT-F, MpSDMT-F, MpSDMT-R because the only GSMT/SDMT PCR reaction that worked were from the Mp primers. Plasmid DNA was then purified from colonies 3, 5, pUHE21, and pB121. A digest was performed using 5 microliters plasmid DNA, 2 microliters of buffer, 1 microliter EcoR1 enzyme, and 12 microliter sterile water. The solution was then incubated in a37˚C water bath for one hour.

The GSMT, GSMT/SDMT, and SDMT reactions worked well after the digest was rerun. Colonies 3 and 5 seemed to be inconclusive which is an indication of the original gel not being read correctly. Due to this oversight, new broth cultures were made of colonies 1-8 with 5 mL LB broth, 5 microliters ampicillin, and 50 microliters of the original colony broth cultures and were incubated at 37˚C overnight. After further consideration, it was decided to find a new approach to modifying GSMT and SDMT. Four competent cell transformations were done with a new plasmid, pUC19, GSMT, SDMT, GSMT/SDMT. After transforming the cells, 100 microliters were plated onto ampicillin plates and incubated at 37˚C for two days. Colonies were isolated from each plate and inoculated into 5 mL LB broth and 5 microliters ampicillin and incubated at 37˚C for another two days. After enough growth was observed, plasmid DNA was purified from arch tube. 4 GSMT/SDMT colonies, 3 SDMT colonies, and 2 GSMT colonies

were checked with a digest to see if the genes were properly inserted into the plasmid. One colony isolated from each original plate was then selected for further testing.

Using DNA from pTRC99A, GSMT/SDMT-3, SDMT-1, and GSMT-2 DNA, a digest was run using 12 microliters sterile water, 5 microliters of DNA, 2 microliters EcoR1 buffer, 0.5 microliter EcoR1 enzyme and 0.5 microliter Pst1 enzyme. It was observed that SDMT-1 had two bands on the gel that was run after the digest, this is an indication that a restriction site may be impeding the desired results because SDMT should not have two bands on the gel with the enzymes that it was digested with.

Due to a shortage of pUC19 DNA with no active culture, a cell transformation was performed with pUC19, and four colonies were isolated from the streaked plate. These four colonies were then grown in 5 mL LB broth and 5 microliter ampicillin. The plasmid DNA was then purified, and two digests was done using all colonies of pUC19, GSMT/SDMT, GSMT, SDMT, making a total of 13 solutions for each digest. One digest used the enzyme Xba1 with 2.1 buffer to check the restriction sites on the plasmids. The other digest that was performed used EcoRV enzyme and 2.1 buffer to check to orientation in which the PCR product was oriented in the plasmid.

Results

The sequences of GSMT and SDMT are shown in Figure 1. Both genes produce glycine-betaine in *M. portucalensis* and both genes were taken from *M. portucalensis* to be placed into *E. coli* to be transformed into *B. japonicum.*

Figure 1:

MpGSMT	MpSDMT
atgaaccaat acggaaaaca ggattttgga gataatccaa tagaagtaag ggaatcggac cattatgagg aagagtatgt tcttggattt gttgataaat gggacgaact catcgattgg gaaagccgtg ccgagagcga aggggatact atcataaaca tcctgaaaga aaggggtgtt aagaaagtac tcgatgtggc aacaggcacc ggttttaatt cggttcgttt acttcaggca ggttttgatg tcgtaagtgc agatggcagt gcagaaatgc ttgtcaaggc ttttgataat gcccgtgatc atgggtattt gatgaggacc gttcaggctg attggaggtg gatgaataag gacatccatg acaaattcga tgctattgta tgtctgggta attcatttac acatcttttc gatgaaggcg accgcagaaa agctctggct gagttctatg cgctgctgaa acatgatggt gtattgcttc ttgaccagcg taactatgat gccatactgg atgatggtta ttccagcaag catgcccatt attactgtgg ggatactgta tcagtctatc cggaacatgt tgatgagggg	atgtctgaaa accaaaaaac cgcagtagat aaagcacagg aatattacaa tagcgatgat gccgacaatt tttactttac catctggggc ggcgaggata ttcatgtcgg tctctataat tcagaagatg aaccaatatt tgacgccagt agacgtacga ttgaaaggat ggcatcaaaa ataagtaatc tggataagga tagtaagatt ctggatatcg gagctggtta tggtggagct gccaggtatc tggccaaaaa atatgggtgt caggtcgttg ctcttaattt gagtgaagta gaaaatgaac gcgaccgaaa aatgaatgag gaccagggtc ttgaccatct tataacagtt gttgatggta gctttgaaga aattccctat cccgatttct ctttcgatgt ggtgtggtcc caggatgcaa tccttcatag tggtaatcgt gaacaggtta tcaaagaagt tgctcgtgtg ctgaaaagtg gcggagattt tgtatttact gatccgatgc agactgacga ttgtccggaa ggggtcttac aaccaatact ggaccgtatc catctggaaa ccctcggttc acccggtttt

cttgcccgtt tcaaatatga attctcggat ggctcagtct acaatctcaa tatgttcccc ttgagaaaag attacaccag gcaacttctg catgaagttg gtttccagga aataaacacc ctgggggact ttaaagagac ctacaaagag gatgagcctg atttcttcct ccatgttgca gaaaaaaatt aa	tatcgggaat ctgccaaaaa gtatggtatg aaggaaattg aatttgagaa acatgcttcc cagctgccga cccattatgg aagagtgttg aaagaaactg aaagtcagga agatgagctc tccaaagtag tcagtcagaa ctacatcaat aatatgaaac agggccttaa ccactgggta aacggtggca ataacggata tctgacctgg ggtatattcc atttacgtaa aaaataa

Figure 2: (Below) Figure 2 below, shows a gel electrophoresis that was done on the isolated colonies from the plasmid+PCR plate in figure 2a.

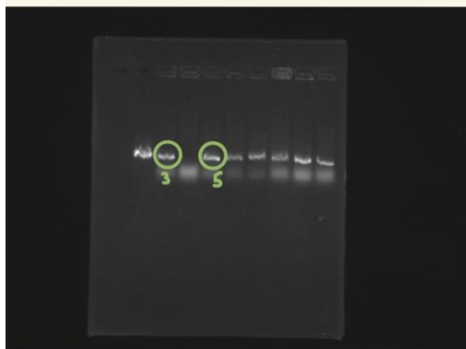


Figure 2a: (Below) The plasmid plate should have had less colonies that the plasmid+PCR plate but this would not have made the results less accurate.

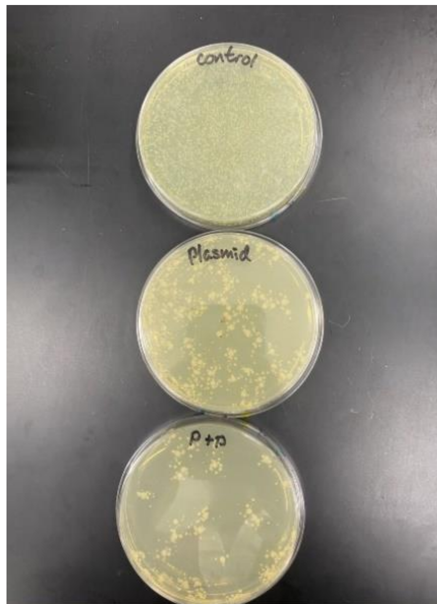


Figure 3: (Below) Figure three shows a gel containing the DNA size ladder, colony 3, colony 5, pUHE21, pB121, GSMT, GSMT/SDMT, and SDMT. The GSMT, GSMT/SDMT and SDMT reactions worked the best on this gel. This gel is the reason indications of a misread of colonies 3 and 5 began to arise.

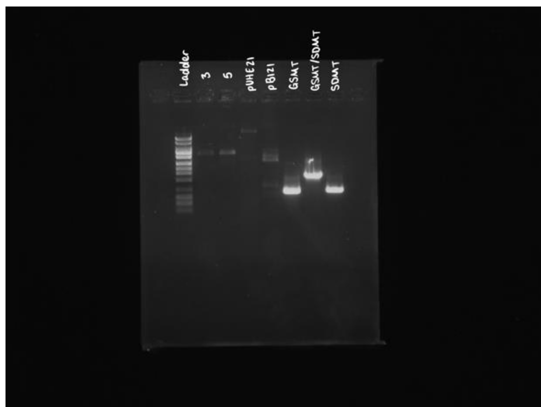


Figure 4: (Below) Figure 4 is the gel containing a DNA size ladder, 4 GSMT/SDMT colonies, 3 SDMT colonies, and 2 GSMT colonies that were isolated off a streaked plate of freshly transformed competent cells. GSMT/SDMT-3, SDMT-1 and GSMT-2 were chosen for future experiments and tests due to the clarity of the bands on the gel.

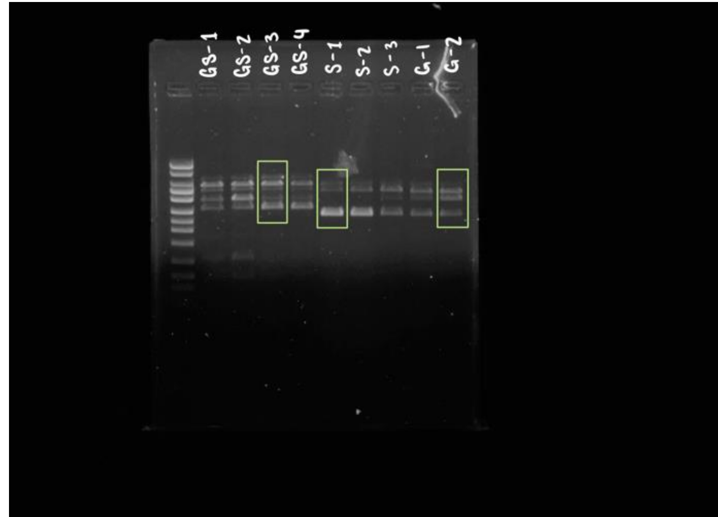


Figure 5: (Left below) Figure 5 shows a rerun of GSMT/SDMT-3, SDMT-1, GSMT-2 with pTRC99A and a DNA size ladder to reference DNA sizes. The gel shows an accurate size representation of GSMT-2 and GSMT/SDMT-3. The lane containing SDMT-1 does not have two bands which is indication of a restriction site the may be impeding the desired results.

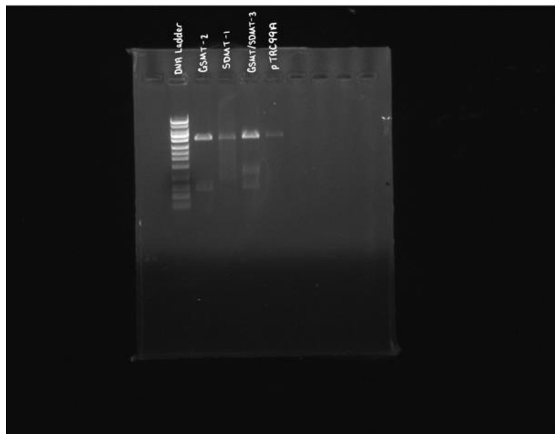


Figure 6: (Right below) Figure 6 is a gel from an digest done with the Xba1 enzyme to check the Xba1 restriction site in all of the isolated colonies of GSMT, SDMT, GSMT/SDMT, and pUC19.

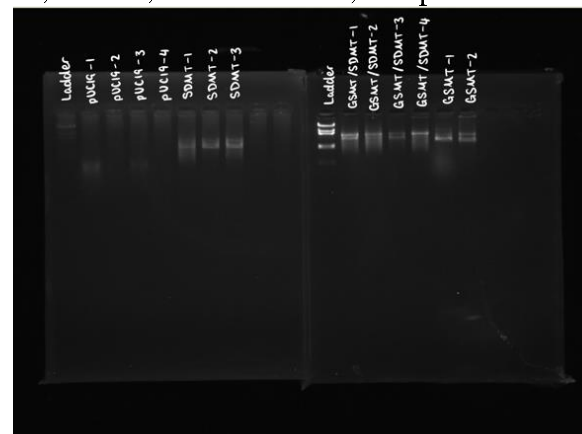


Figure 7: (Left Below) The gel shown is the gel electrophoresis results from a digest done with EcoRV enzyme to check the orientation of the cleavage sites in the GSMT, SDMT, GSMT/SDMT, and pUC19.

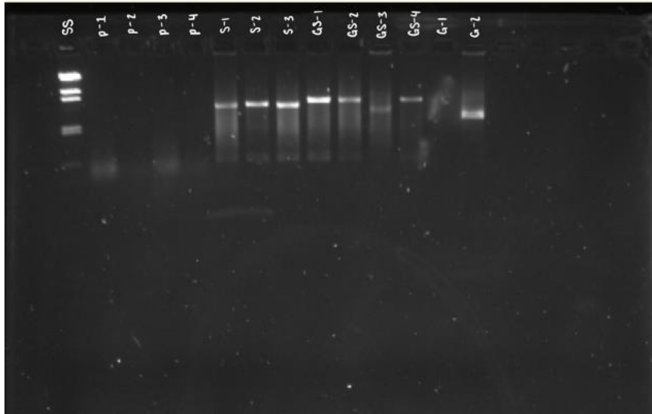


Figure 8: (Below) The figure shown below shows the mechanism in which the gene insertion will proceed. Starting with cloning the PCR product of GSMT/SDMT into pUC19, then using the restriction sites, cutting out the GSMT/SDMT product with HindII and BamHI to be able to clone to product into pTRC99A.

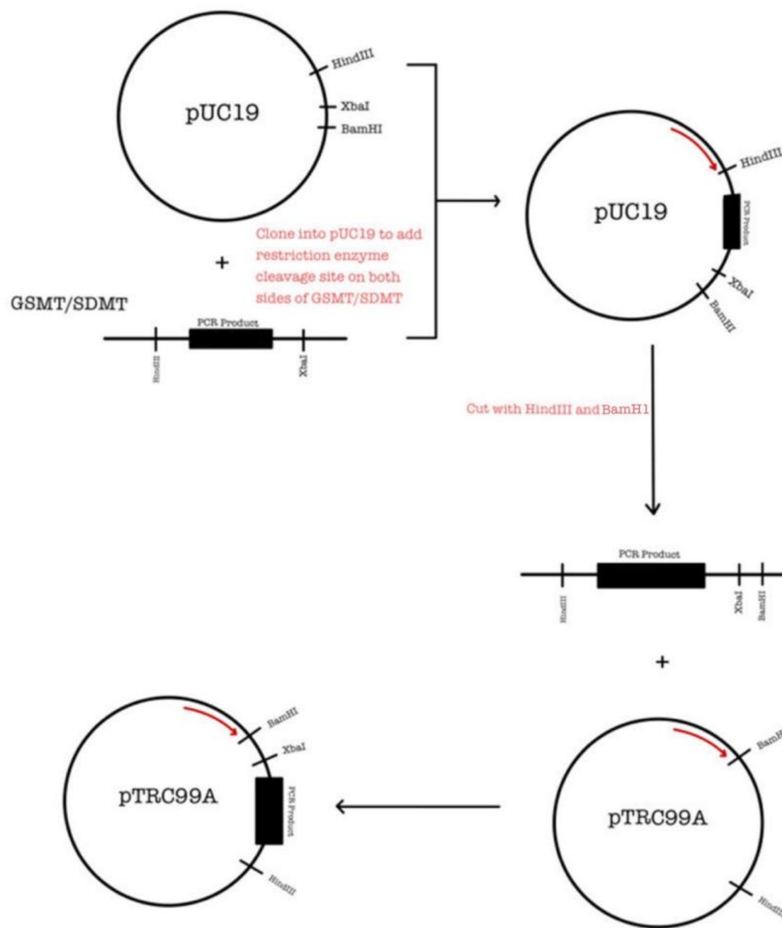
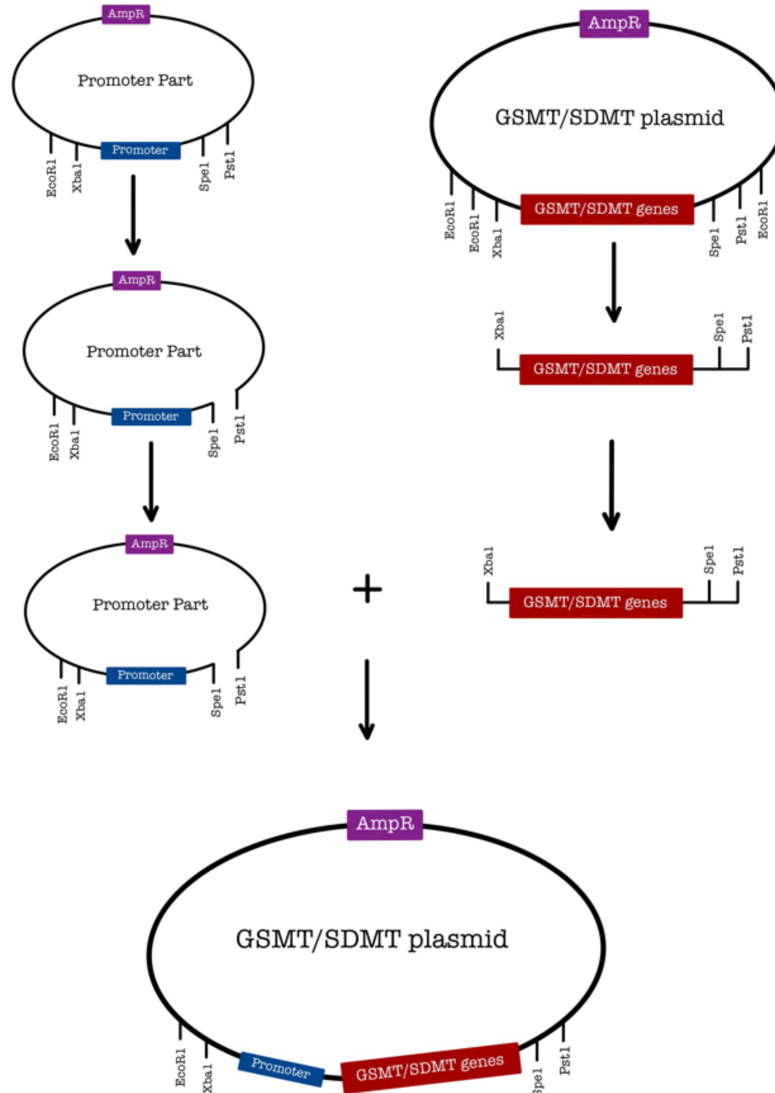


Figure 9: The figure shown below is the mechanism used to verify if *B. japonicum* could express GSMT/SDMT gene cluster.



Discussion

The progression of the project is slow, and some results are inconsistent due to many factors including, old solutions, concentrated buffer, and other circumstances that cannot be avoided most times. There were many experiments that did not work, including using magnesium chloride mixed into the first initial PCR reactions to influence the primer-template annealing temperature, affinity, specificity and yield. The results compared to PCR with just sterile water were not drastic enough to continue the experiment. Another issue was with the

colonies 3 and 5 that were isolated from the plasmid+PCR plate. After months of attempting to get the DNA to show affirmative results, it was decided to take many steps back and make fresh dilutions of everything, including making fresh competent cells, fresh media, and purifying plasmids from that. After doing the first digest with the new GSMT, SDMT, and GSMT/SDMT isolates, the results seemed to conclude that old solutions and strains were the main underlying issue.

The point this project has reached is to replace the plasmid purification kit due to the materials possibly getting too old to work effectively. For the plasmids, the next steps will be checking the orientation of the cleavage sites on the GSMT, SDMT, and GSMT/SDMT isolates to see if any of them could be used to clone into pUC19. This is done because the restriction sites much be in a certain orientation for the genes to be expressed properly when they are inserted into pTRC99A. Once the orientations are defined, isolates will also be chosen from the Xba1 digest, isolates that have the Xba1 restriction site that is needed for cloning. The plasmid pUC19 is also resulting in insufficient bands on the gels that are being run, which is the reason the plasmid kit will be replaced with a newer one, to see if the kit is the issue or the plasmid itself.

This project was worked on consistently for almost 18 months now, so a great deal of progress has been made and will continue to be made throughout the year up until December and possibly a few months after if another student will take over this project.

Acknowledgments

Dr. David Westenberg was there through every failed experiment and positive result. I fell in love with microbiology fairly late into my academic career, so I was nervous about my lack of expertise when starting this research. After asking Westenberg if he had any research openings available, I started this project right away! I have loved every minute of working in the lab and learning invaluable knowledge and techniques when it comes to genetics and general lab work. There are just some things you can’t learn when sitting in a lecture and I have had to privilege of learning many concepts hands on and seeing them happen in real time. I cannot thank Dr. Westenberg enough for the opportunities he has opened up for me.

I would also like to thank Dr. Mei-Chin for providing the plasmids to Dr. Westenberg for this project. The plasmids contained our target gene needed to be inserted into *B. japonicum.*

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OURE Reflection

While working on lab work, I had the opportunity to learning many lab techniques including PCR reactions, dilutions, plasmid purification, T-vector cloning and much more. During my time working on my project, I took detailed notes on every technique and experiment that was done. If an experiment failed, then notes would be taken on what to do next to make it work or why it failed in the first place. For my first project, I was able to test various bioactive glass sent to us from companies against three common bacteria known to cause skin infections during surgery.

I started off by making plates of *E. coli*, *S. epidermidis*, and *Ps. Aeruginosa* and incubating. Broth cultures were made from these plates for future testing. A mixture was then made with saline, bacteria, and a hole punched sample of each bio active glass in a 2ml centrifuge tube to test the growth inhibitability of each material. There were 5 materials total that were tested again three different bacteria so 15 tubes were made for each test that was done. Once incubated overnight, the solution was spread onto agar plates labelling corresponding to each tube and incubated overnight once more. To show bacterial growth inhibition, this same technique was done with dilutions of the bacteria in order to be able to count the colonies. Times incubations were then done after this to show growth inhibition over time with the two material that worked best in the previous tests.

This project demonstrated two specific materials working better than the others that were test and we developed a hypothesis that stated the material that the two products were made of, making a webbed matrix when exposed to moisture, is the cause of increased bacterial growth inhibition during suspension. I was able to use this project to show deep understanding of experimental design as well as trail and error focused experiments.