Designing a Binary Plasmid that Allows Inducible Non-Antibiotic Elimination of the Host

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Title: Designing a Binary Plasmid that Allows Inducible Non-Antibiotic Elimination of the Host

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Designing a Binary Plasmid that Allows Inducible Non-Antibiotic Elimination of the Host Bacterium

Tia Camarata

Abstract

Antibiotic usage is not always a feasible method for controlling bacterial growth in a research setting. Specifically, the soil bacterium *Agrobacterium tumefaciens* is useful in genetically engineering plant tissue, but there are a limited number of antibiotics that effectively kill this species. Therefore, the goal of this project is to develop a method of engineering *Agrobacterium* with the potential to be eliminated without the use of antibiotics. We are currently in the process of synthesizing a plasmid that has three major components: a pCambia backbone, a rhamnose-inducible promoter, and the *E. coli* gene *hok*.

pCambia was selected to be the backbone of the proposed synthesized plasmid because it is a binary vector and has been successful in previous plant transformation experiments. Binary vectors are useful because they have a broad host range origin of replication, meaning that they can be used in several species of bacteria with successful replication, namely *E. coli* and *Agrobacterium*. A rhamnose-inducible promoter was obtained that operates best in the presence of low levels of rhamnose and can be suppressed in a solution of 0.2% glucose. Hok is a host-killing peptide that degrades the plasma membranes of bacteria and is naturally present in *E. coli* and is used by the bacterial cell itself during apoptosis.

Utilizing the *hok* gene with a rhamnose-inducible promoter, bacterial death should be able to be controlled (provided the promoter is not leaky). Thus, the growth of an engineered strain of *Agrobacterium* will be controlled by simply changing the growth medium; the colonies will thrive in glucose but will be effectively killed off upon the introduction of rhamnose. This should allow elimination of the bacterium after it has delivered its genetic payload to the plant cell.

Introduction

Purpose

As is the case in most circumstances, there are shortcomings that are associated with any methodology and the genetic engineering of plants is no exception. Historically, plants were mainly modified through selective breeding over multiple generations to eventually reach a desirable product. Crops such as corn and wheat are prime examples of this practice, where the current phenotype no longer resembles the original plant from which these crops came. Today, a greater understanding of genetics and the advent of new technology has allowed for the development of new methods for altering plant tissue. The genetic modification of plants may include microprojectile bombardment, electroporation, microinjection, and transposable elements, but our area of focus for our project was the use of microbial vectors, primarily *Agrobacterium tumefaciens* (NRC 2004).

Agrobacterium tumefaciens is a gram-negative bacterial species that lives in the soil and infects plant tissue in many dicotyledonous plants. *A. tumefaciens* is like tumor viruses in that it enters wounds on a plant that are closest to the soil and introduces part of its genome into plant cells, causing the cells in the infected area to divide rapidly. The bacterial genes present in the host plant's chromosome cause a heightened release of the plant hormones cytokinin and auxin. These hormones cause an even greater level of cell division, ultimately resulting in a tumor-like structure called a crown gall. *A. tumefaciens* lives within the gall and is released when the affected tissue is broken down by other opportunistic pathogens (Collins 2001).

While it is true that *A. tumefaciens* is a plant pathogen, this very aspect makes *A. tumefaciens* a prime candidate for genetic research. Gene transfer is a necessary part of the lifecycle of *A. tumefaciens*, and in a controlled setting this ability can be used to one's advantage

when introducing a desired gene into a plant species susceptible to *A. tumefaciens* infection. As a result, *A. tumefaciens* is widely used in both research and commercial settings for this very purpose. Once the gene has been successfully introduced into plant tissue, the bacteria must then be eliminated using antibiotics. Antibiotic use however, may lead to new issues over time.

First and foremost, bacteria can become resistant to antibiotics over time. Of course, this issue is more pressing in the medical field, where the development of resistant bacterial strains such as Methicillin-resistant *Staphylococcus aureus* (MRSA) can seriously impact survivability rates. All the same, it cannot be negated in the discussion of microbial vector genetic engineering because if an antibiotic becomes less effective over time, control over the vector is lost.

Another issue is the availability of an effective antibiotic may change over time, and a prime example of this problem is Timentin. While Timentin used to be a common antibiotic in veterinary medicine and was the industry standard for any experiments involving the elimination of *Agrobacterium*, Timentin is no longer commercially available as Smith Kline Beecham discontinued its production. Timentin is a combination of the antibiotics Ticarcillin and Clavulanic Acid. The components can be purchased from chemical supply companies but the prices of this drug have gone well past the point where the monetary cost outweighs its benefit in research. Combining unstable availability with the possibility of resistance makes the use of antibiotics seem less attractive when considering the outcomes of a tissue engineering protocol. Thus, engineering a strain of *Agrobacterium* that can be destroyed via other means is particularly useful.

The ultimate goal of my thesis research was to create a strain of *Agrobacterium* that would be capable of destroying itself, without the use of conventional bacteriocidal methods. This not only included completing a plasmid that contains the desired genetic sequence, but to

also use the synthesized plasmid to transform bacteria. *E. coli* was used first to easily replicate the plasmid and was tested to confirm that bacterial death can be induced. This process is still in progress, but once the plasmid has been completed and copied, it will be used to transform *Agrobacterium*. Upon reaching this stage, the self-destructing *Agrobacterium* strain will be transferred to tobacco (*Nicotiana tabacum*) to test its practical use.

Plasmid components

Plasmids are small, circular pieces of DNA that are separate from a cell's chromosomal DNA. They are naturally found in bacteria, and while plasmids do not contain genes that are necessary for survival of the organism, they may contain genes that are advantageous such as those that confer antibiotic resistance. Plasmids are very useful for genetic research because they are easy to manipulate in a laboratory setting. Typically, plasmid "backbones" are available for purchase and contain two essential parts. The first is a multi-cloning site, which is flanked upstream and downstream on the DNA sequence by restriction sites. Restriction sites are sequences of the DNA recognized by restriction enzymes, which sever the DNA at that specific site. Once the backbone is cut by specific restriction enzymes, the circle is opened and the desired gene can be inserted into this site through a process called ligation. The other essential component of a plasmid backbone is a selection marker, which is used to confirm that bacteria have been transformed with the proper synthesized plasmid. Usually, the selection marker is a gene allowing for resistance to a certain antibiotic so that all other bacterial cells are killed in culture and only the transformed bacteria can grow.

pCambia was selected to be the backbone of the proposed synthesized plasmid because as a binary vector of the *pPZP* family, it has been successful in previous plant transformation experiments (Hajdukiewicz *et al.* 1994). Binary vectors are useful in these types of experiments

because they have a broad host range origin of replication, meaning that they can be used in several species of bacteria with successful replication, namely *E. coli* and *Agrobacterium*. The selection marker for pCambia is resistance to chloramphenicol.

Hok is a host-killing peptide that degrades the plasma membranes of bacteria and is naturally present in *E. coli* (Pecota *et al*. 2003). Holins are proteins produced by bacteriophages that function in a similar manner. Such a protein is used to create pores in the membrane of a bacterial cell so that hydrolytic enzymes called lysins can degrade the peptidoglycan of the bacterial cell wall, allowing the newly-constructed bacteriophages to be released by the cell (Wang *et al*. 2000). Unlike holins however, hok is used by the bacterial cell itself during apoptosis (Hayes 2003). The translation of *hok* mRNA is normally blocked by the antisense inhibition of *hok* from RNA of the gene *sok* (Pecota *et al*. 2003). Until conditions are met where apoptosis is induced, *sok* acts a safeguard in *E. coli* that suppresses *hok* until it is needed (Hayes 2003). When *hok* is separated from the checks and balances that exist due to the presence of *sok*, the cells that synthesize the hok protein product will be ultimately destroyed. However, the expression of *hok* can still be controlled by using an inducible promoter.

Cardona and Valvano (2005) synthesized the plasmid pSCrhaB2 using *E. coli* and *Burkholderia cepacia*. pSCrhaB2 features a rhamnose-inducible promoter that operates best in the presence of low levels of rhamnose and can be suppressed in a solution with glucose concentrations as low as 0.2% (Cardona and Valvano 2005). Cardona and Valvano generously supplied samples of the plasmid pSCrhaB2 for my research. For the purposes of this thesis, the uncut version obtained from Cardona and Valvano will be referred to as pSCrhaB2, while the cut version will be called *rhaB* because the rhamnose-inducible promoter is the sequence of interest. *RhaB* offers a unique opportunity to regulate the expression of a gene of interest. In our case, that gene is *hok*.

Utilizing the *Hok* gene with a rhamnose-inducible promoter, bacterial death can be controlled. As demonstrated by Cardona and Valvano (2005), a solution of 0.2% glucose is capable of suppressing the promoter, while low concentrations of rhamnose allow for transcription of *hok* downstream. Thus, the growth of the engineered strain of *Agrobacterium* will be controlled by simply changing the growth medium; the colonies will thrive in glucose but will be effectively killed off by the synthesis of the hok protein upon the introduction of rhamnose. Figure 1 has been included at the end of the introduction to act as a summary of the plasmid components mentioned above and as a point of reference for the rest of this thesis.

Not only does a self-destructing bacterial cell line have a myriad of uses in the laboratory, it would also have a beneficial impact on animal health. Many attempts to create genetically modified organisms by artificially introducing new genes using a bacterial vector involve the use of antibiotics. First, antibiotics are the most common selection marker for the transformed bacterial cells, where the cells that contain the new plasmid are resistant to a certain antibiotic and persist in its presence. Once these bacteria are introduced to their plant host, they must eventually be killed off using another antibiotic that they are not resistant to. The presence of antibiotics in food products can have negative health impacts on both animals and humans alike when this food is consumed. The proposed cell line of *Agrobacterium* has the potential to allow for the bacterial cells to die in the presence of rhamnose instead of an antibiotic. As a sugar, rhamnose is harmless to both plants and animals. Genetically modified food sources that utilize this method will thus act as a much safer and healthier alternative.

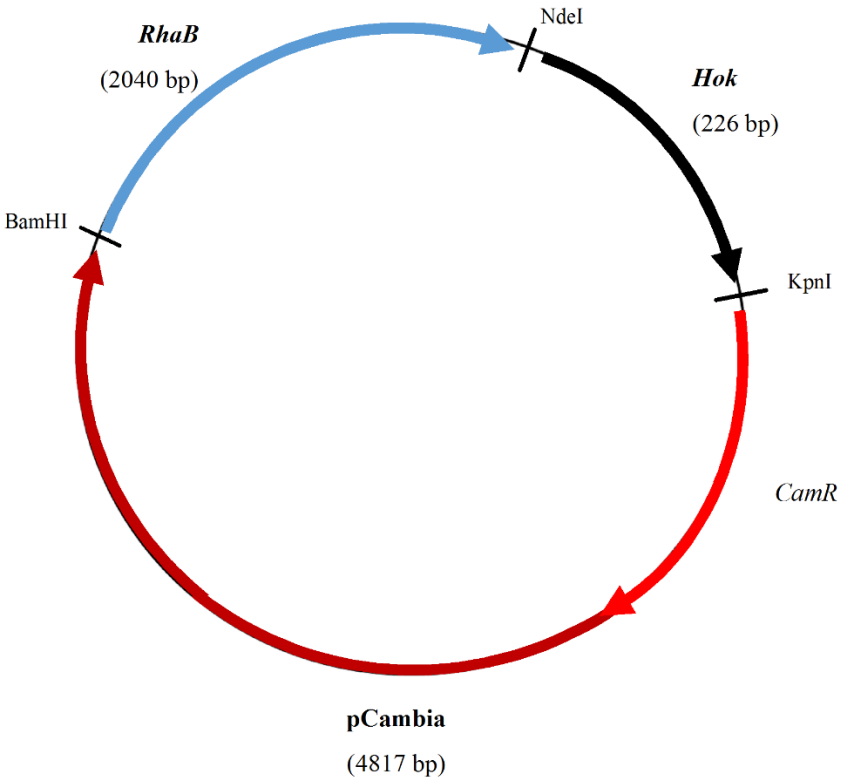


Figure 1. A vector map of the proposed plasmid, composed of a pCambia backbone featuring chloramphenicol resistance (*CamR*), a rhamnose-inducible promoter (*RhaB*), and the host-killing gene *hok*. Cut sites and relative sizes of each fragment are included for reference.

Part I

Methods

My independent study this past spring semester involved the preparation of the individual plasmid fragments that will be ultimately introduced to the desired bacteria via transformation. *E. coli* strain DH5α was transformed with the plasmid backbone, pCambia, using chloramphenicol for selection. The other fragment, a portion of the plasmid pSCrhaB2, was used to transform DH5α with trimethoprim for selection. A minipreparation was performed on the resulting bacterial colonies to isolate the plasmid DNA from the chromosomal DNA of the

bacteria. The products of the Miniprep were then amplified using PCR with the proper primers. The primers for pCambia corresponded with BamHI and KpnI cut sites, while the primers for pSCrhaB2 corresponded with BamHI and NdeI cut sites.

The PCR products were run through a gel for assurance that the sequences were the correct sizes. A gel elution procedure was followed to reclaim the DNA from the gel. The gel elution resulted in low yields each time, so multiple rounds of PCR had to be performed in order to have high enough concentrations to continue the project.

When adequate yields were reached, pSCrhaB2 was digested using NdeI and BamHI. The purchased *hok* sample was rehydrated and then digested using NdeI and KpnI at the same time that pSCrhaB2 was digested. Any remaining fragments left over from the digest that were undesirable were removed from the solution using a PCR column cleanup kit, which yielded 50 µl of each product. DNA yields were determined using a Qubit 2.0 Fluorometer to ascertain how much of each product was needed to use for ligation. After the rhamnose promoter and *Hok* were ligated together for one hour at room temperature, the ligation product was run through a gel to confirm that it was the proper size.

The proper band was cut out of a gel and a gel elution procedure was performed. When the product was quantified, the yield was too low to get a reading on the fluorometer. In response, the sample was desiccated using a speed vacuum. In considering the low yields from both the gel electrophoresis and the gel elution, it was decided that all three components of the plasmid would be ligated at once.

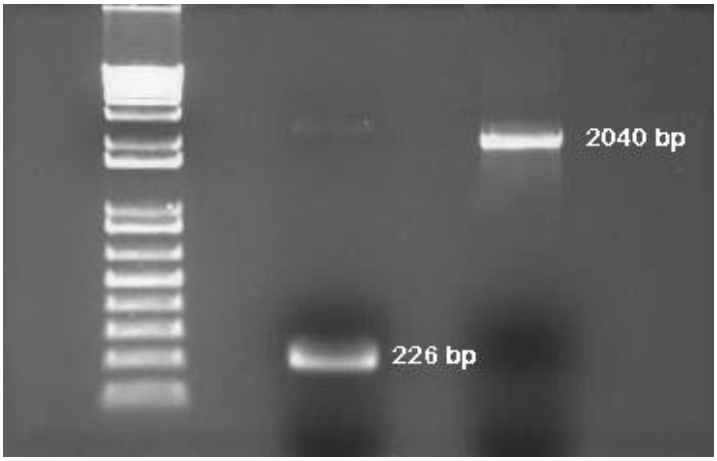
A simultaneous ligation reaction needed to involve the proper amounts of each component. 20 ng of pCambia was combined with 60 ng of total insert, such that *hok* and *rhaB*

were in a 1:3 ratio. The ligation progressed for one hour at room temperature and then the reaction tube was placed in a 37°C water bath to heat-inactivate the ligase.

Using One Shot TOP10 competent cells, *E. coli* was transformed with 5 µl of the ligation product. 50 µl and 100 µl of the transformation reaction was plated onto plates made with Luria broth, 1% glucose and 50 µg/ml chloramphenicol. Colonies were picked and used to inoculate Luria broth (LB) tubes with 1% glucose and 50 µg/ml chloramphenicol to create a stock solution of the transformed *E. coli* colonies. New tubes containing 1% glucose were inoculated with 100 µl of the corresponding stock solution in addition to tubes containing LB, 2% rhamnose, and chloramphenicol. This process involved touching a sterile wooden stick to a colony on the agar plate. The stick would be dipped into a glucose tube before being placed into a rhamnose tube such that each colony had cells in both glucose and rhamnose solutions.

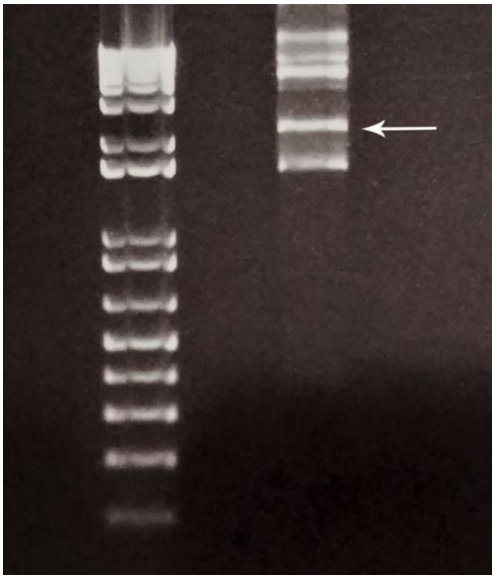
Results

A gel electrophoresis of the *hok* and pSCrhaB2 restriction digests confirms that the reactions yielded the proper size fragments. *hok* is the first band after the ladder and is 226 base pairs in size, while pSCrhaB2 is 2040 bp (Figure 2). The faint band at 2266 bp observed from the gel electrophoresis of the ligation of *Hok* and pSCrhaB2 yielded a concentration of DNA that was too low to be read by the fluorimeter after gel elution (Figure 3). Eventual inoculation of 1% glucose LB tubes and 2% rhamnose LB tubes resulted in bacterial growth in all tubes (not pictured).



1 2 3 4 5 6 7

Figure 2. Size confirmation for the products of a restriction digest of *hok* and pSCrhaB2. From left to right, lane two contains 5 µl of 1 KB+ ladder, lane three contains *Hok*, and lane five contains *rhaB*. Lanes one, three, five, and seven do not contain any samples. Data collected 1 February 2017.



1 2 3 4 5

Figure 3. Gel electrophoresis with 5 µl of 1 KB+ ladder in lane two and 20 µl of the ligation of *hok* and *rhaB* in lane four. Arrow indicates band for proper ligation product of 2266 bp. Data collected 10 October 2016.

Discussion

The growth observed in the 2% rhamnose solution despite transformation with our plasmid indicates an issue with the plasmid itself. pCambia was certainly present in the transformed *E. coli* because the cells were still resistant to chloramphenicol and were able to grow on the plates and in both glucose and rhamnose. This result led us to believe that pCambia had re-ligated with itself without the *Hok* and rhamnose promoter insert ligating to the backbone. We determined that our first effort to prevent this potential re-ligation of pCambia would be to alter the ligation procedure and observe any changes in bacterial growth.

Part II

Methods

More pCambia was digested using KpnI and BamHI and the PCR purification kit once again helped to prepare the digest product. The previously digested *Hok* and rhamnose products were dried down using the speed vacuum to increase the available concentration of DNA for ligation. The new ligation procedure involved a sequential method of each of the components, where the first reaction contained 1 µl ligase, 2 µl 5x buffer, 4 µl *Hok*, and 3 µl rhamnose product. This reaction progressed for one hour at room temperature and then the solution was immediately used in the second reaction with pCambia. The 10 µl of the previous ligation were combined with 7 µl of the digested pCambia, 1 µl ligase, and 2 µl 5x buffer for a total of 20 µl. After another hour at room temperature, the reaction tube was placed in a 37°C water bath for 20 minutes to heat-inactivate the ligase.

5 µl of the ligation product was used to transform *E. coli* using One Shot TOP10 competent cells. 50 µl, 100 µl, and 150 µl of cells were plated on Luria agar plates with 1% glucose and chloramphenicol. After growing overnight at 37°C, colonies were selected using

sterile sticks as described in part I. Twelve tubes of LB glucose and twelve tubes of LB rhamnose were inoculated in total. The tubes grew overnight at 37°C in a shaking incubator.

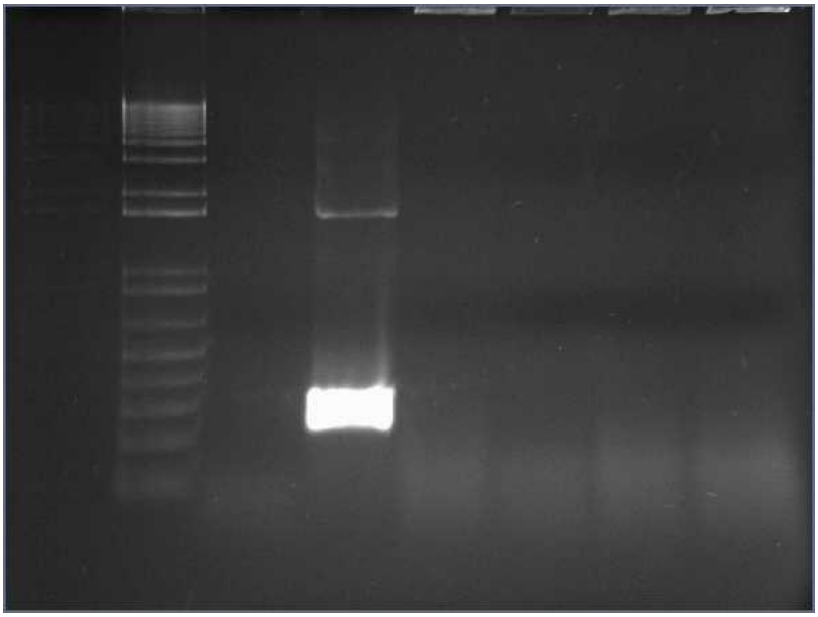
Four glucose tubes were selected at random to test for the presence of *Hok* using colony PCR with Takara SapphireAmp Fast PCR Master Mix. 1 µl of each colony was used as the template DNA for that reaction, along with 0.5 µl of the *Hok* forward primer and 0.5 µl of the reverse. The positive control was a sample of *Hok* that had already been digested and purified. Gel electrophoresis was performed using all of the PCR samples.

A minipreparation was performed on two of the colonies checked with PCR to better separate the plasmid DNA from the chromosomal DNA of the bacteria. 10 µl of each of the isolated DNA samples were digested using NdeI, KpnI, and BamHI and then run through a gel to visualize the effects of the digest. To further confirm the results observed from the gel electrophoresis, PCR was performed on the two samples. The DNA from the two colonies was used for three separate reactions. Each reaction tested for a different plasmid component. For example, 1 µl of the miniprep DNA from one colony was combined with 1 µl of the rhamnose Bam primer, 1 µl of the rhamnose Nde primer, and 22 µl of nuclease free water. Gel electrophoresis was again used to visualize the results of the PCR.

Results

Both glucose and rhamnose tubes exhibited growth for each colony, as was observed in part I. The gel electrophoresis of the colony PCR products indicated that *Hok* was absent in the colonies selected, where the only visible band was from the positive control lane of the gel electrophoresis. All experimental lanes did not have a band of 226 bp like the positive control (Figure 4). *Hok* appeared absent in both colonies that were tested, but *rhaB* and pCambia were present (Figure 5). *RhaB* is 2040 bp and is represented by band C and pCambia is 4817 bp and is

represented by band A. pCambia was further digested into a separate fragment of 2489 bp, as represented by band B. Band D is currently of unknown origin (Figure 5). The presence of pCambia and *rhaB* is again confirmed in figure 3, with a pCambia band of 4817 bp and a *rhaB* band of 2040 bp. *Hok* was found to be present in minute amounts with a band at 226 bp. The bands of 678 bp and 452 bp were determined to be the result of multiple *Hok* fragments ligating together (Figure 6).



1 2 3 4 5 6 7 8

Figure 4. Gel electrophoresis to confirm absence of *Hok* in randomly selected transformed colonies. Lane two contains 5µl of 1 kB+ ladder, lane three contains the negative control, lane four contains the positive control, and lanes five through eight contain the results from colony PCR. Data collected 17 February 2017.

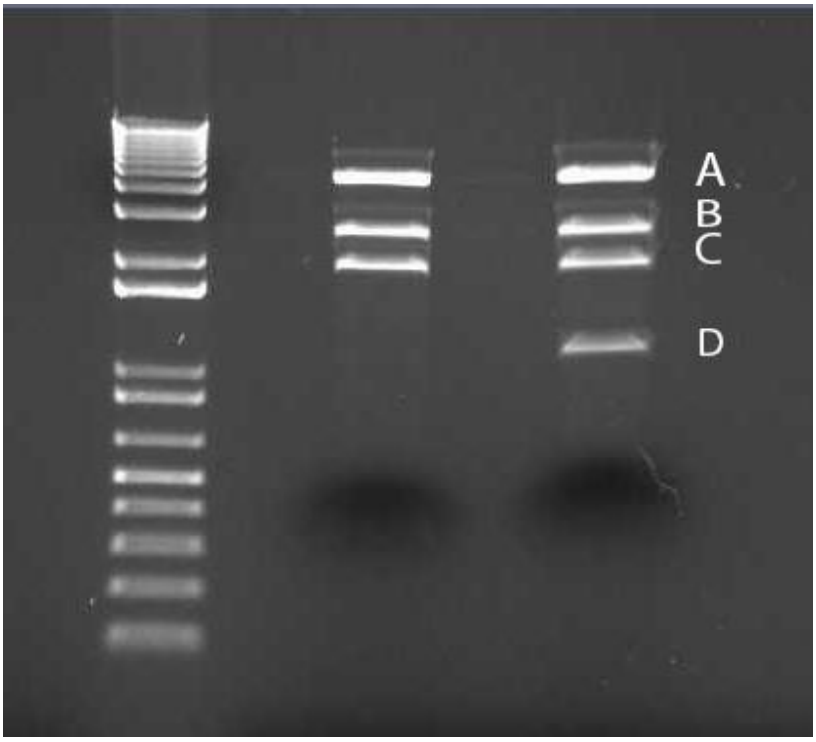
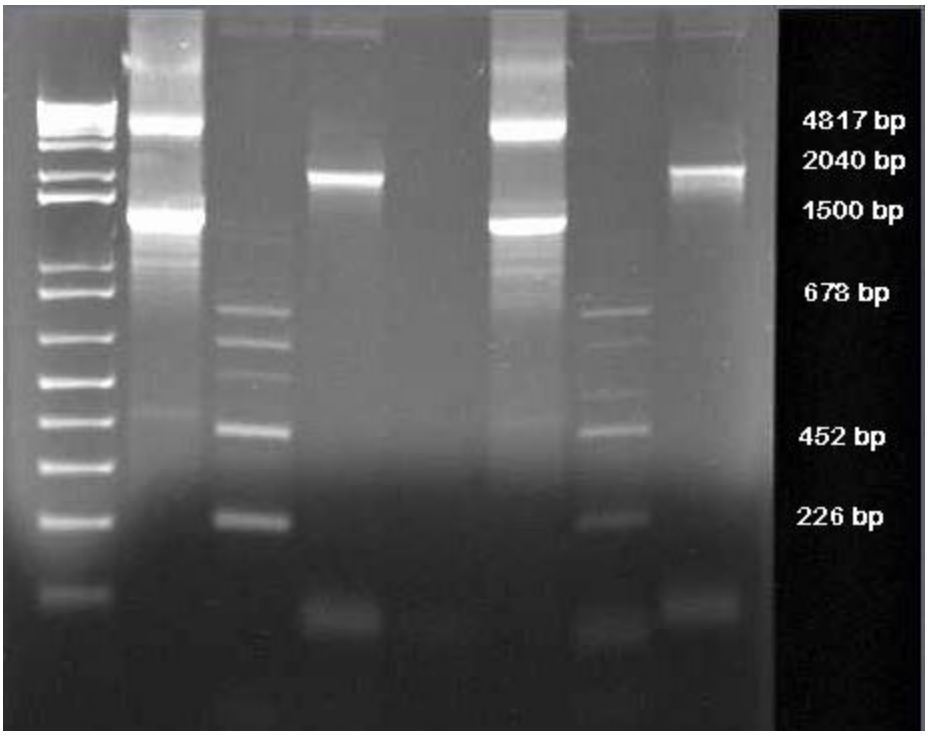


Figure 5. Gel electrophoresis of the two colonies checked with PCR digested with NdeI, KpnI, and BamHI. Lane two contains the ladder and lanes four and six contain the DNA from the two colonies. Band A has a size of about 5000 bp, band B is about 2500 bp, band C is 2000 bp, and band D is about 1200 bp. Lanes one, three, five, and seven do not contain any samples. Data collected 22 February 2017.



1 2 3 4 5 6 7 8

Figure 6. Gel electrophoresis of the two colonies after PCR with primers for each plasmid fragment. Lane one is the ladder. Lanes two through four contain samples from one colony, testing for pCambia, *Hok*, and *rhaB* respectively. Lane five is the negative control. Lanes six through eight contain samples from the other colony, testing for pCambia, *Hok*, and *rhaB* respectively. Data collected 3 March 2017.

Discussion

All tests for *hok* were negative until PCR was performed on selected bacterial colonies using the forward and reverse *hok* primers. Until that point, *hok* must have been in concentrations that were too low to visualize on a gel. We had previously thought that the reason for the bacterial growth in a rhamnose solution despite changing the ligation protocol was due to leakiness of the rhamnose-inducible promoter. However, the literature describes the promoter as being tightly regulated, so it seemed conflicting to have such a result (Cardona and Valvano

2005). The result from figure three shows that multiple pieces of varying size were present after PCR using the *hok* primers. This indicates that the *hok* fragments are ligating together to form concatemers. If *hok* were to be in the wrong orientation when this occurred, then there would be no gene product to kill the cells when the promoter was induced in the presence of rhamnose. Currently, the formation of concatemers seems to be the most likely explanation for why the plasmid is not effective in killing the bacteria.

Part III

Methods

The remaining digest products of the plasmid components that were prepared in Part II were used for another sequential ligation. However, for this reaction *hok* was treated with calf-intestinal alkaline phosphatase (CIAP). One µl of CIAP was incubated with the digested *hok* product in 2 µl of a 10x dephosphorylation buffer for 10 minutes at 37°C, the recommended temperature for DNA with a 5' overhang. Heat inactivation of CIAP occurred at 65°C for 15 minutes.

The sequential ligation as described in Part II was repeated with the *hok* treated with alkaline phosphatase. As before, the ligase was heat-inactivated for 20 minutes at 37°C. The transformation of *E. coli* used 5 µl of the ligation product. 50 µl, 100 µl, and 150 µl of the transformed cells were plated on Luria agar plates containing chloramphenicol and 1% glucose. Solutions of 1% glucose and 2% rhamnose were inoculated with the four colonies that grew using sterile wooden sticks. The degree of growth was noted in each tube after a 24-hour incubation at 37°C.

Results

Bacterial growth was observed in both the glucose and rhamnose solutions like the transformations performed previously, despite treating *hok* with alkaline phosphatase. As shown by the images in figure seven, both glucose and rhamnose solutions exhibited similar bacterial growth, indicating a malfunction in one or more of the plasmid components such that the bacteria survived in the rhamnose solution.

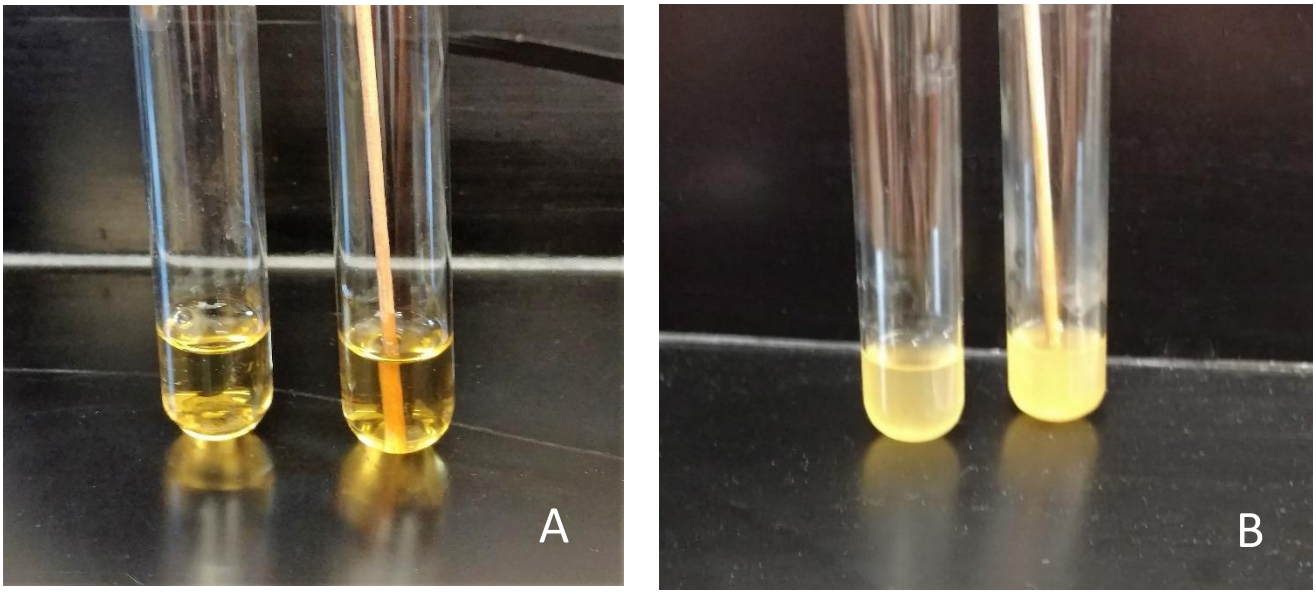


Figure 7. Bacterial growth in glucose and rhamnose solutions before and after incubation at 37°C. Image A shows the glucose solution on the left and the rhamnose solution to the right. No bacterial growth occurred yet when the photograph was taken. Likewise, image B shows the glucose and rhamnose tubes, with similar bacterial growth after incubation. Data collected 29 March 2017.

Discussion and Future Plans

Treatment with alkaline phosphatase prevented the concameters from forming with *hok*, but growth still occurred in the 2% rhamnose solution. However, it was observed that only four individual colonies grew between the agar plates inoculated after the transformation from Part III. In contrast, parts I and II had many colonies that grew on each plate individually. This

observation suggests that the formation of concameters had been an issue previously, but was not the only issue. *Hok* was assumed to be functional after it was ensured that only one copy of the gene was ligating to the rest of the plasmid instead of multiple pieces but growth still occurred despite the introduction of rhamnose. This leads us to believe that the rhamnose-inducible promoter is not as tightly regulated as we believed it to be. A potential experiment to test this postulate would be inserting the gene for green fluorescence protein (GFP) downstream from the promoter to visualize the promoter's regulation of gene transcription.

The logical next step to continue this research in the future would be to try an alternative to the *rhaB* promoter. In addition, *hok* could potentially be replaced instead. It is possible that *hok* is non-functional in our current system, but an alternate explanation is that *hok* is simply higly effective in killing the transformed bacteria. When a highly-lethal gene is combined with a leaky promoter, the bacteria with the proper plasmid would be killed off before reaching the rhamnose-induction stage. In this case, instead of replacing the promoter, *hok* can be interchanged with a new killing gene that is not as effective and allows for the bacteria to tolerate low levels of the enzyme without dying too soon. One such peptide is barnase, a ribonuclease (RNAse) (Hartley 1988).

Despite the shortcomings of this project, the concept of a plasmid that induces self-destruction still remains as a useful alternative to relying on antibiotics. A plasmid such as this has much potential in the field of genetic research. It is now just a question of what components will work well together to make the plasmid functional.

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