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Optimizing Genetic Manipulation of Methanogens through Faster Cloning Techniques

Merrisa Jennings
University of Arkansas, Fayetteville

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Merrisa Jennings

Biological Engineering Program

Biological and Agricultural Engineering Department

College of Engineering

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04/25/2018 (Signature and Date)

Brian E. Haggard

4/25/2018 (Signature and Date)

Jin-Woo Kim

04/27/2018 (Signature and Date)

Dan Lessner

4/25/2018 (Signature and Date)

Departmental Honors Coordinator or Department Head

Abstract

Methanogenesis is the biological production of methane. Only anaerobic archaea known as methanogens are capable of such a metabolic feat. They have strict living conditions and substrate sources which determine their rate of metabolism. This is of particular importance from a greenhouse gas reduction perspective or biogas capturing perspective. One of the best ways to optimize methanogen methane production is via genetic manipulation. The current procedures are timely though, therefore a faster cloning processes should be developed. The objective of this study was to optimize a premade genetic transformation kit known as the Gibson Kit. The Gibson Kit was supposed to ease the work of genetic manipulation by combining several linear chunks of DNA together at once via the use of sequence overhangs. The resulting plasmid from the Gibson could then be transformed into *E. coli* where *E. coli* is supposed to replicate the plasmids extremely quickly. Afterwards, that plasmid could then transform methanogens. Several baseline PCR (polymerase chain reaction) transformations were performed to linearize and amplify the desired plasmid of pNB72.3. PCR amplifications of the desired gene segments which would be added to the plasmid were also performed. The desired gene segments being assembled were supposed to take out the production of Cytochrome C within the methanogen electron transport system by deleting the ccmf gene. Several PCR experiments were carried through without success. The cause of the failures included the primers “hair pinning” at the recommended annealing temperatures, primers being too nonspecific, and primers unable to perform efficiently at the recommend annealing temperatures. After several tries of no success, the Gibson Kit was tested without PCR linearization of the circular plasmid. Instead, standard digestion was relied on. With standard electro competent cell transformation and antibiotic screening, the Gibson product was then tested for successful transmission of the plasmid to the *E. coli.* Results were negative; therefore, the optimization of faster cloning techniques was unsuccessful, but it will help guide future efforts.

Introduction

Microorganisms provide many beneficial services to humans and the environment. For example, they are necessary in water and waste water treatment (e.g. nitrification in streams, Haggard, et al., 2005), food production (e.g. cheese, Hugenholtz, 1999), biofuel production (e.g. biomass conversion, Peralta-Yahya, 2012), and medicine production (e.g. antibiotics, Gust, 2004). Microorganisms have the potential to be genetically engineered to perform their processes more efficiently; they can even be engineered to perform desired tasks unnatural to their normal function (McDaniel, 1999). For example, genetically altered microorganisms can produce products they would not typically produce like insulin (Williams, 1982). Recent advances in genetic engineering allow microorganisms to be manipulated in a more streamlined and regulated manner (e.g. Gibson Kit, SGI-DNA, 2016). This allows for an increase in both human and environmental services by the genetically improved microorganisms.

One particular group of microorganisms that are of interest are methanogens. As their name implies, methanogens are methane producing prokaryotes. They are of interest because their methane by-products can be of benefit to society if collected properly. The captured methane can be used as a biofuel, potentially offsetting energy produced from fossil fuels (Luthey-Schulten, 2015). The flip side is that any uncollected methane could lead to environmental harm by contributing to the rising greenhouse gas accumulation in the atmosphere (Ashish Kumar Pandey, 2015). Since methanogens are anaerobes, they have strict living qualifications which limit their growth and overall methane production (Elsevier, 2018). Through careful genetic manipulation of methanogens, these limits can be overcome to adjust methane production.

Several studies have already been conducted to understand methane production within methanogens. It has been determined that methane is produced through three major pathways: (1) reduction of carbon dioxide, (2) fermentation of acetate and (3) dismutation of methanol or methylamines (Lessner, 2009). Each of these reactions rely on key enzymes, such as coenzyme M, coenzyme B, methyl–coenzyme M, and

heterodisulfide reductases. Therefore, enzyme translation correlates to methane production. Specific strains of methanogens have been found to be more aerobic tolerant due to their high number of putative antioxidant and repair proteins (Sheehan, 2015). Both the enzymes and the proteins are transcribed from specific genes, some of them of known genomic sequences. Thus, genetic manipulation of those sequences results in regulated methanogen growth and methane production.

Through careful and controlled experimental manipulations, methanogens have the potential to be engineered more easily for faster methane production and overall human benefits. The goal of this study was to optimize the genetic manipulation of methanogens. Since methanogens are relatively difficult to culture due to their specific anaerobic environmental requirements and long doubling time, *E. coli* is partially substituted for methanogens during genetic studies (Bertani, 1987). First the targeted gene sequence is assembled into a plasmid. Then that plasmid is transformed into *E. coli,* where *E. coli* then replicate the plasmid to optimal concentrations. Finally, the plasmid is purified and transformed into methanogens. That is when the gene manipulation can be accessed via phenotype changes. Current methodology of cloning is long and strenuous though, with much time and resources spent towards DNA cloning instead of the actual genetic study. Thus, one objective of this study is to streamline the cloning method for *E. coli* to lead to faster genetic manipulation of methanogens. Possible ways include the use of the Gibson Assembly Kit and improving other prepared DNA cloning techniques such as the PCR.

An enzyme of particular interest for genetic manipulation is Cytochrome C. Cytochrome C is found in the electron transport methane production site of methanogens and is crucial for the fermentation of acetate. Methanogenesis starting with acetate appears to take a longer time than that of starting with methanol. Perhaps, this has to do with the necessity of Cytrchrome C. The overall bases of this study stems from the exploration of Cytochrome C's impact on methanogenesis and acetate uptake. The plasmid utilized in this study correlates to the removal of the ccmf gene which is responsible for the production of Cytochrome C. in methanogens.

Literature Review

Methanogens make up a unique anaerobic subset of the archaea domain. They are the only microbes capable of reducing various forms of short chained organic substrates into methane or other carbon-based products (Lessner, 2009). There are five total orders of methanogens. Four out of the five (Methanobacteriales, Methanococcales, Methanomicrobiales and Methanopyrales) are 'obligate carbon dioxide-reducing' species. This means that they are only capable of producing methane from carbon dioxide reactants. The last order of methanogens (known as Methanosarcinales) are more flexible in that they can produce methane from additional methylotrophic substrates such as acetate, methanol, and methylamines. Each of these orders have the terminal metabolic pathways in common (Figure 1). Step 1 is methyltransferase. Step 2 is then methyl–coenzyme M reductase or the demethylation of methyl–CoM to methane. Lastly step 3 is heterodisulfide reductase or the reduction of CoM-S-S-CoB to the sulfhydryl forms of the cofactors as catalysed by methyl–CoM and heterodisulfide reductases.

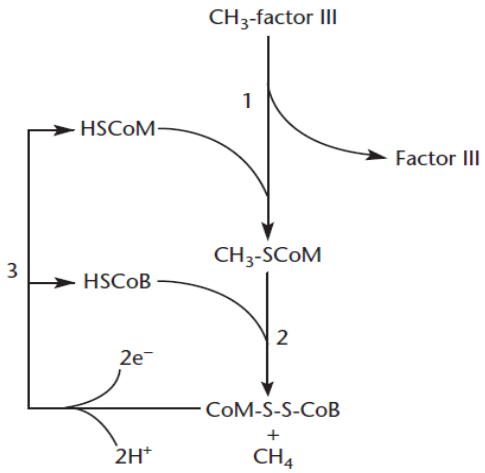

Figure 1: Common Reactions/ Reagents in Methanogenesis (copied with permission from Lessner's work March 30, 2018)

Therefore, the different methane producing pathways are determined based on the method of obtaining the methyl group for methyltransferase. These methods are classified into three main metabolic pathways:

> **"(1) reduction of carbon dioxide**: Carbon dioxide is reduced to a methyl group with electrons derived from the oxidation of electron donors (primarily hydrogen or formate) which are also the source of electrons for the reduction of CoM-S-S-CoB.
>
> **(2) fermentation of acetate**: Acetate is cleaved to provide the methyl group and a carbonyl group for oxidation to carbon dioxide, providing electrons for reduction of CoM-S-S-CoB.
>
> **(3) dismutation of methanol or methylamines:** Four molecules of substrate are demethylated to provide the methyl groups, with one oxidized to carbon dioxide to provide electrons for reduction of CoMS- S-CoB." (Lessner, 2009)

Even though the order called Methanosarcinales is capable of following the first method of metabolic pathways, it is found that the reduction of carbon dioxide is responsible for only 30% of the biologically produced methane. The majority of biological methanogenesis (approximately 70%) actually comes from the second metabolic pathway- the cleaving of acetate into a methyl group. Furthermore, only two genera within this order, *Methanosarcina* and *Methanosaeta*, are capable of even using acetate as an energy source in this metabolic pathway. Therefore, this study focuses on these two specific genera as the main methane producers.

Specific living requirements must be met in order for the methanogens to grow properly. All pathways utilize metalloenzymes containing iron, molybdenum, tungsten, cobalt, zinc and nickel. Nickel is especially important because it is necessary for methyl–coenzyme M (CoM) reductase, or the enzyme in the last step of all methanogenic pathways (see Figure 1). Therefore, access to these metal cofactors is crucial to the vitality of these organisms. Since they are anaerobes, oxygen must also be eliminated for their survival.

Alongside environmental control, genetic manipulation of these species is of particular importance because it has allowed researchers to find significant metabolic mechanisms and regulations. By removing or adding specific genetic codes, one can compare the original to the mutants in order to determine how that code connects to the overall metabolic function. These analyses have been conducted through genetic manipulation processes such as proteomics and transcriptomics.

Current bacterial genetic manipulation calls for the inclusion of common antibiotic markers with the desired genetic code in order to screen for the transformed mutants versus the wild type organism (Atomi, 2012). Since many archaeal species, specifically methanogens, have been determined to be resistant against those common antibiotics, the conventional screening process has been hindered for methanogen genetic manipulation. Alongside the antibiotic resistance, methanogens require specific living requirements that are difficult to inexpensively include in the screening process in a lab, such as an anaerobic environment. Thus, a method for studying methanogen genetics without using methanogens has been essential to continue the advancements. Pseudo-studies of the methanogens have been conducted through the use of prokaryotic bacteria, such as *Escherichia coli*. The desired gene of the methanogen is transcribed onto a shuttle vector (usually a DNA plasmid) with an antibiotic marker. This plasmid would then be exposed to *E. coli* in which it could be absorbed under antibiotic pressure during the screening process. If successful, then the mutated *E. coli* could express what was desired from the methanogen genetic code.

Despite the hindrances, genetic studies of methanogens using only methanogens is possible. Bertani and Baresi (1987) performed the first ever DNA-mediated transformation for a methanogen called *Methanococcus voltae*. It was determined that puromycin and the puromycin transacetylase (pac) gene from the bacterium *Streptomyces alboniger* and its derivatives were useful as the methanogenic antibiotic and the resistance marker genes. The initial integration shuttle vectors used the pac gene to transform the *M. voltae* and *Methanococcus maripaludis.* Other screening processes based on histidine

auxotrophy/prototrophy using the hisA gene as a marker have also been used. Many of the selection processes utilized for one Methanogen have been proven to be successful in other methanogen species.

Other mutation-causing techniques have also been achieved to lead to genetically altered methanogens. Irradiation with UV and gamma rays have led to mutations related to histidine, purine, and vitamin B12 in methanogens (Bertani, 1987). Other techniques utilized resistance to 2-bromoethanesulfonate and to 5-methyl-DL-tryptophan. Work determining which genetic manipulation is best for methanogen alteration is still being continued.

In the end though with limits in environmental restraints and antibiotic markers, the cheapest option is to continue using the shuttle vectors to pseudo-study methanogen genetics in other bacteria (such as *E. coli*). In order to make this process even quicker, premade DNA transformation kits could be utilized. One possible option is the Gibson Kit, which should be tested and optimized in this particular situation. Optimization of this process should be conducted in order to test the kit's use in genetic alteration.

General Methods

The type of plasmid DNA that was chosen to transform the *E. coli* was the combination of plasmid pNB72.3 along with Upstream CcmFgibsUs and Downstream CcmFgibsDs. They were of importance because the Upstream and Downstream segments contain the genetic sequence coding for the methanogens' electron transport chain but excludes the ccmf gene which produces cytochrome C protein. Since the objective of the Gibson Kit NEB is to combine multiple linear fragments of DNA sequences in one simple step, pNB72.3 had to be transformed into a linear plasmid first. Several PCR experiments were conducted to complete this task. Plasmid digestion could have been used, but this process is not as reliable as PCR.

A PCR is comprised of free nucleotides, primers, DNA template, the DNA replicating enzyme- DNA polymerase, and DNA denaturing and annealing temperatures. The idea is to replicate double stranded DNA in a relatively short amount of time. PCR can also be used to transform circular plasmids into linear plasmids by replicating only certain fragments of the plasmid. In this particular case, it was the whole plasmid fragment with no sticky ends, thus resulting in a linear product. The process of a PCR is as follows (see Figure 2). First, high "denaturing" temperatures separate the complimentary double strands of DNA. Afterwards, a temperature drop forces the separate strands to anneal (stick together), but the larger strands have a harder time finding the right base pair matches, so the shorter segments of single stranded DNA, called primers, attach to the larger strands instead. During extension, the enzyme polymerase and free nucleotides fill in the gaps of the now partially double stranded DNA (large original strand + primer). This results in a fully replicated double strand of DNA. IMPORTANT NOTE: Enzymes are always added last to the ingredient list to reduce the chance of protein denaturation.

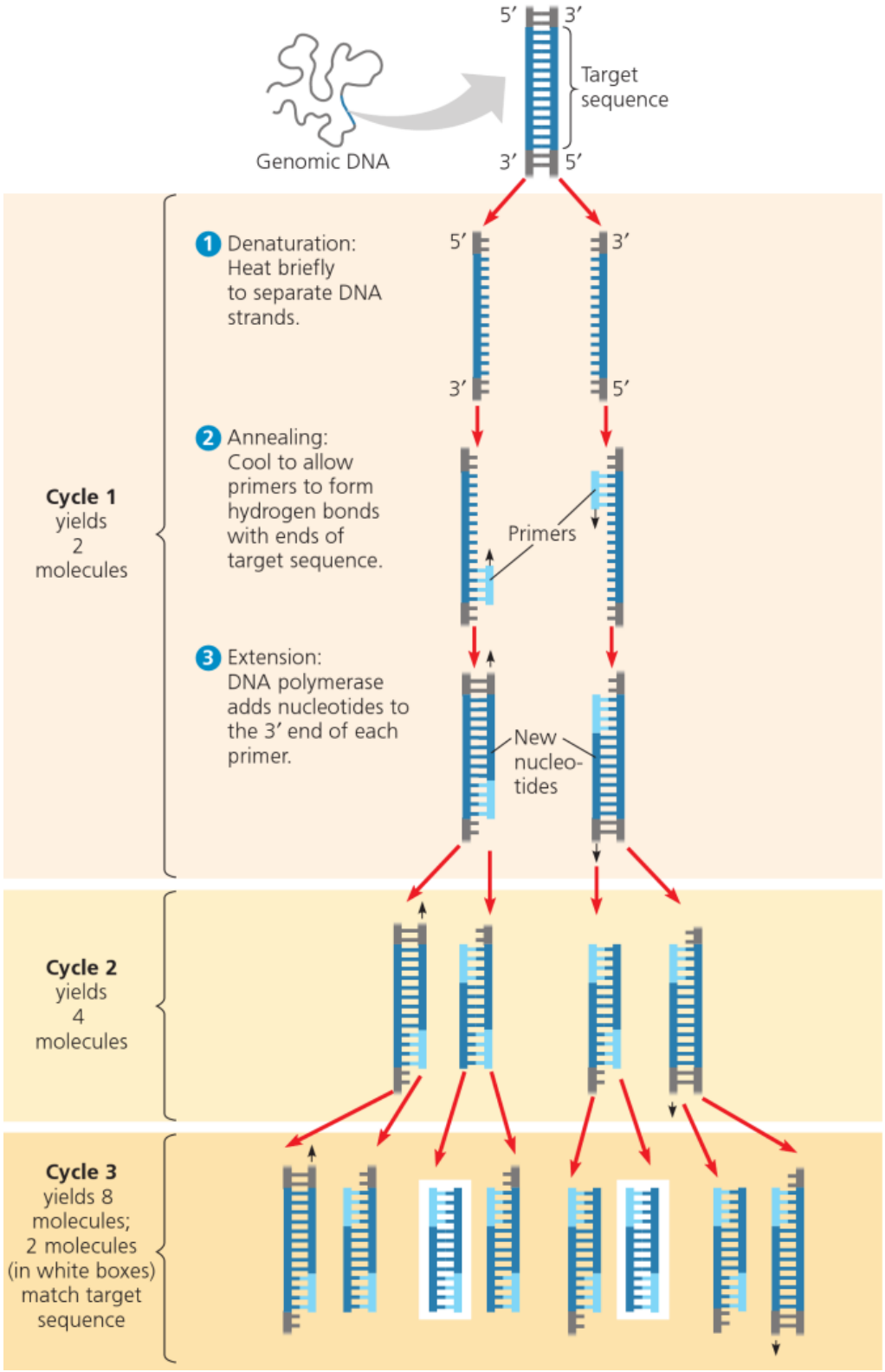

Figure 2: A Break Down of How PCR Works (copied from from LaboratoryInfo.com on March 30, 2018)

Before running the PCR, the concentration of the original plasmid and Upstream/Downstream segments were needed to be found. This was because the template DNA has to be roughly 1 ng in order to run a PCR. If more, the PCR could gunk up with excess template DNA instead of the desired amplified target sequence. The concentration of the plasmid was found to be 293 ng/μl using the standard nucleic acid analysis in the UV-Vis spectrometer with both wavelengths of 260 nm and 280 nm, a dilution factor of 30, and base pair unit of 4400 Daltons. The concentration for the Upstream and Downstream segments were also found via the same manner. The final concentration for CcmFgibsUs was made to be 30 ng/μl and CcmFgibsDs to be 24 ng/μl. Due to discrepancies, different dilutions of the plasmid were required for each PCR.

After PCR amplification, the PCR products would then ran on a 0.5% SDS gel to check for the correct plasmid length of 4000-4500 base pairs (bp) and Upstream and Downstream segments at 1000 bp. If seen at the appropriate markers, plasmid purification from the PCR product would be necessary before future use because the PCR may have resulted in a range of DNA fragments, depending on DNA shearing, primer-to-primer annealing, and many other factors. With the help of a gel clean-up kit, the PCR linearized plasmid and segments at those specific base pairs were able to be extracted from the gel. The samples could then be further checked on a 0.5% SDS gel in order to verify again that the correct band markers were still present.

After the following phases proved successful, the linear plasmid and desired gene fragments were then planned on being combined in the Gibson Assembly Kit. This kit is advantageous compared to traditional gene cloning because it combines multiple fragments of DNA to a vector in one setting, saving both time and resources. In order to complete a comparative analysis, two separate reactions would be performed using the Gibson Kit. The first reaction was just with the plasmid and desired Upstream and Downstream gene segments. The second reaction tested the reliability of the Gibson mix materials via us of a positive control. This positive control was included in the Gibson Kit.

Each reaction would then be further checked in order to see if the product contained the DNA markers on a 0.5% SDS gel. Those products could then be individually transferred to electro competent *E. coli* cells using the standard electro competent method. Electro competent cells are cells which are hardy enough to withstand pulses of electricity. After electrotransfomation, these cells would then grow in standard *E. coli* broth inside a constantly shaking incubator. After substantial growth had occurred (cloudiness within the broth), the cells would lastly be spread on an antibiotic selective agar plate. Growth of cells would confirm genetic transformation.

Experimental Methods and Results

EXPERIMENT 1

The first experiment's goal was to make linear DNA from plasmid pNB72.3 via PCR for use in the Gibson Kit. It was believed that PCR would be better to linearize plasmids then plasmid digestion. Therefore, a prep PCR was assumed to be necessary before use of the Gibson Kit.

A single PCR tube was assembled with the ingredients listed in Table 2, and the PCR machine was set to the correct temperatures for each cycle as seen in Table 1. To run the PCR, the plasmid template was diluted through serial dilution to obtain a final concentration of 0.97 ng/μl. Afterwards, the completed PCR material was tested on a 0.5% SDS gel against a NEB 1Kb ladder and the original pNB72.3 DNA template to see if the PCR was successful in creating more of the linear DNA template. The expected band should have appeared at 4500 bp. The results were negative though because there was no visible band in the gel for the PCR material, thus the process did not accurately linearize and amplify the plasmid.

Possible thoughts as to why this experiment failed include the following:

- improper annealing temperatures- if inaccurate, the DNA will not stick to the primer and allow the extension step to begin;

- not enough enzyme- perhaps replication did occur, but not enough enzyme to replicate a noticeable amount via gel;
- low enzyme activity- low replication rates for enzymes calls for longer times during extension step;
- not enough GC enhancer- GC nucleotide combos result in higher energy bonds between base pairs than AT, as they contain 3 hydrogen bonds instead of 2. Thus, primers with higher GC contents will anneal at higher temperatures than primers with lower GC contents because it takes less energy to bond them with something, meaning they will stick too soon to the DNA template strands and mess up the extension period. GC enhancer works to stabilize the bond energy of GC base pairs to that of AT base pairs to avoid improper annealing times;

dilution of DNA template was improperly performed, so not enough DNA present during replication in PCR.

Table 1: Polymerase Chain Reaction (PCR) Settings for All Experiments (Exp) with a Repeat Factor for the Cycle Denaturation, Primer Annealing and Extension Procedure Steps

	EXP 1	**EXP 2**	**EXP 3**	**EXP 4**	**EXP 5**	**EXP 6**	**EXP 7**	**EXP 8**
INITIAL DENATURATION (℃**)**	98	98	98	98	98	98	98	98
(MIN:SEC)	0:30	0:30	0:30	0:30	0:30	0:30	0:30	0:30
CYCLE DENATURATION (℃**)**	98	98	98	98	98	98	98	98
(MIN:SEC)	0:10	0:10	0:10	0:10	0:10	0:10	0:10	0:10
PRIMER ANNEALING (℃**)**	65	65, 98	65.5-68.2	61.2, 63.8	70.8-78	72	68.5-71.9	71.2
(MIN:SEC)	0:15	0:15	0:15	0:20	0:30	0:30	0:30	0:30
EXTENSION (℃**)**	72	72	72	72	72	72	72	72
(MIN:SEC)	1:40	2:00	3:10	0:20	1:00	1:10	1:10	1:10
REPEAT FACTOR	30X	30X	35X	30X	35X	38X	38X	38X
FINAL EXTENSION (℃**)**	72	72	72	72	72	72	72	72
(MIN:SEC)	2:00	2:00	4:00	2:00	2:00	2:00	2:00	2:00
HOLD (℃**)**	4	4	4	4	4	4	4	4
(MIN:SEC)	∞	∞	∞	∞	∞	∞	∞	∞

Table 2: Polymerase Chain Reaction (PCR) Compounds and Reagent Volumes for All Experiments (Exp)

	EXP 1	EXP 2	EXP 3	EXP 4	EXP 5	EXP 6	EXP 7	EXP 8
5X Q5 REACTION BUFFER (µL)	10	10	10	10	10	10	10	10
10 µM DNTPS (µL)	1	1	1	1	1	1	1	1
10 µM FORWARD PRIMERS (µL)	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
FORWARD PRIMERS		CcmFgibDsFor		CcmFUS-GibFW, CcmFgib-DsFor		CcmFUSGibFW	CcmFUS-GibFW, CcmFUSFW	CcmFUSFW
10 µM REVERSE PRIMERS (µL)	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
REVERSE PRIMERS		CcmFgibUsRev		CcmFDS-GibRV, CcmFgib-UsRev	CcmFDS-GibRV	σ NB302	σ NB302, CcmFDSRV	CcmFDSRV
TEMPLATE DNA (µL)	1	1-2	1	1	1	1	1	1
TEMPLATE DNA	0.97 ng/µl *pNB72.3*	0.25-1.25 ng/µl *pNB72.3*	0.25 ng/µl *pNB72.3*	*C2 Genomic DNA*	*Gibson*	0.5/0.25 *Gibson*	*Gibson/ N2*	*G2/ gel G2*
GC ENHANCER (µL)	10	10	10	0	0	0	0	0
DMSO (µL)	0	0	0	0	5	0	0	0
DEIONIZED WATER (µL)	22.5	22.5-21.5	22	33.5	27.5	32.5	32.5	32.5
Q5 DNA POLYMERASE (µL)	0.5	0.5	1	0.5	0.5	0.5	0.5	0.5

EXPERIMENT 2

The second experiment tested for reagent quality by changing the reagent volumes and PCR settings within 8 different samples (as seen in Tables 1 & 2). The DNA template was diluted through serial dilution to obtain a final concentration of 0.125 ng/μl. In order to determine if DNA concentration affected the

results, some samples were given a higher concentration. Two large batch master mix of PRC reagents were created to supply ingredients to those 8 PCR tubes without the DNA mixed in to it (4 samples with 1 μl of DNA template and 4 samples with 2 μl of DNA template). This allowed for an ample amount of mixing for all the ingredients.

This experiment also did not show any band markers when ran on a 0.5% SDS gel. The expected band should have appeared at 4500 bp. Therefore, none of the PCR tubes were successful in replicating the desired DNA. A third experiment was then ran looking more into the variation of annealing temperatures and annealing times.

EXPERIMENT 3

The third experiment again changed the reagent volumes and PCR settings within 6 different samples (as seen in Tables 1 & 2). The rationale behind these changes was to ensure that the temperatures were not disrupting the PCR process. Perhaps the primers were unable to anneal to the DNA because they have extremely different annealing temps. Since the third experiment also did not show any band markers when ran on a 0.5% SDS gel, none of the PCR tubes were successful in replicating the desired DNA. The expected bands should have appeared at 4500 bp. This lead us to look into the nature of the primers themselves. The NEB website resource suggested an annealing temperature around 65℃ for the primers. But upon further investigation, it appears that those specific primers "hair pinned" close to that temperature. "Hairpin" refers to single stranded DNA (in this case, our primers) folding in on itself and attaching to its own nucleotides (Nature Education, 2014). Those primers were supposed to attach to the DNA template strands to start the replication process of a particular gene sequence with the PCR. Therefore, the "hairpin" obstructed that process. Thus, new primers were selected.

EXPERIMENT 4

This next experiment used the PCR to amplify the desired gene targets for the Gibson Kit reaction. These gene fragments relate to the deletion of the ccmf gene. The gene fragments were labeled Upstream and

Downstream (as a symbolism of which region the segments would be added to the linearized plasmid). Each segment was made up of two primers. The names for each primer are listed below.

- CcmFgibUsRev -> Upstream Reverse
- CcmFUSGibFW -> Upstream Forward
- CcmFDSGibRV -> Downstream Reverse
- CcmFgibDsFor -> Downstream Forward

To increase the concentration of each segment, an external standard was performed with the PCR using methanogen chromosomal DNA (see Tables 1 & 2). An external standard PCR just replicates genes by using an extensive piece of chromosomal DNA template. For the external standard DNA template, a standard methanogen C2 genomic DNA was selected. The two PCR results with a NEB 1Kbp Ladder were then ran on a 0.5% SDS gel (see Figure 3). The expected bands should have appeared close to 1 kbp. This gel proved successful amplification of those segments as since they appeared at the appropriate markers. These combined PCR samples were then saved and renamed to the following:

- 1) Upstream segment-> CcmFgibsUs
- 2) Downstream segment-> CcmFgibsDs

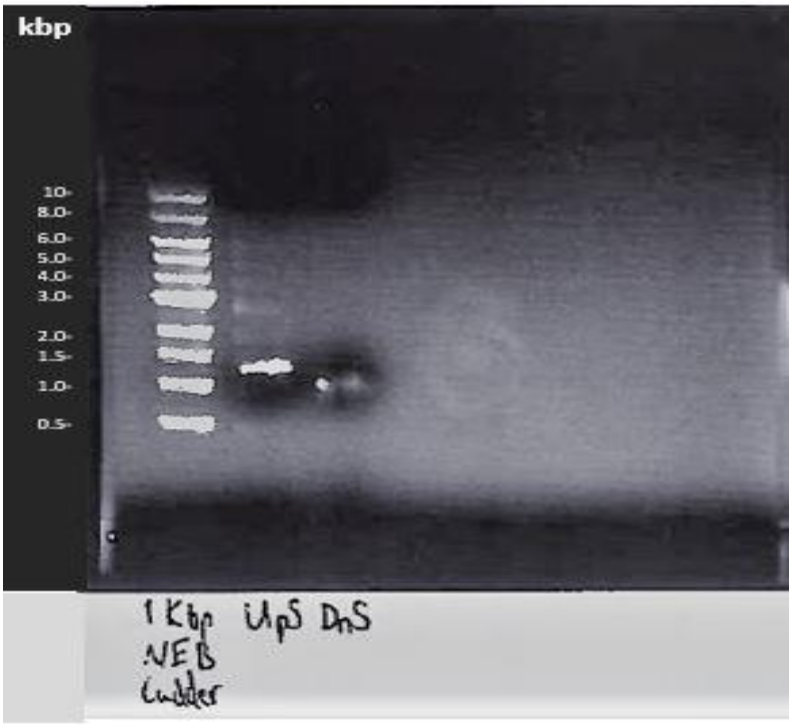

Figure 3: Fourth Polymerase Chain Reaction (PCR) Experiment on 0.5% SDS Gel with 1Kbp NEB Ladder, Upstream CcmFgibsUs Product (UpS), and Downstream CcmFgibsDs Product (DnS)

EXPERIMENT 5

After determining the appropriate dilution factor for the amplified gene segments, these segments were combined with the plasmid PNB72.3 using the Gibson kit. The plasmid underwent digestion, but this does not ensure linearization. The common rule of thumb followed was preparing twice the quantity of segments for every one plasmid vector. Thus, the final concentration for CcmFgibsUs was made to be 30 ng/μl and CcmFgibsDs to be 24 ng/μl (found via running standard nucleic acid analysis as previously described). The Gibson tube was made with 0.8 μl pNB72.3, 1 μl CcmFgibsUs, 1 μl CcmFgibsDs, 10 μl Gibson Master Mix, 7 μl DI Water. The idea was to create a transferable DNA template with the desired fragments without having to use the PCR to linearize the plasmid. The resulting Gibson tube was then placed in the thermocycle at 50 °C for 1 hour. The Gibson product was used to transform the electro competent cells.

A PCR of the newly created Gibson product was performed to amplify the DNA concentration of the combined primers and plasmid using various annealing temperatures (see Table 1). The polar aprotic solvent DMSO was added to counterbalance any polarity which may hinder the mixing of ingredients in the PCR (see Table 2).

The experiment did not work in transferring the appropriate DNA sequences because none of the cells withstood the antibiotic screening. This could have been predicted as the gel screenings did not show the appropriate markers when the Gibson product ran on a 0.5% SDS gel, as stated due to no markers showing up.

EXPERIMENT 6

The next course of action was to try nesting primers. Nesting primers results in nested PCR, which is a designed to improve sensitivity and specification within the results (Carr, 2010). It does so by running two sequential PCR sets. The first DNA sequence replicated is actually primers for the second and final DNA sequence amplification. Thus, the primers are nested in the products of the first PCR run.

Two PCR tubes were made, the difference being the amount of template DNA (see Tables 1 & 2). The tube labeled N1 contained ¼ the original concentration of the DNA template, whereas the tube labeled N2 contained ½ the concentration. The product from the Gibson mix previously made in experiment 5 was selected as the template DNA. Even if the Gibson Assembly Kit didn't combine the segments with the plasmid, the hope was that the two segments at least combined. This PCR could then amplify the Downstream + Upstream segment combination. There were results on the 0.5% SDS gel (see Figure 4), but the results were not specific enough. Only one band around 2 kbp should have showed up. Too wide of bands or too many bands imply variation in DNA base pair lengths. The final product N2 of this PCR was saved, to be used as template DNA in future experiments.

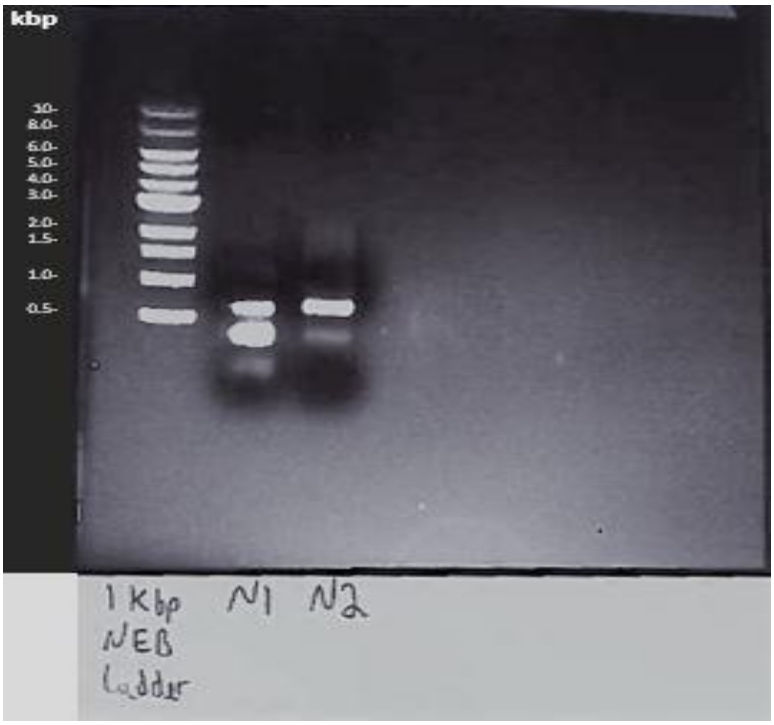

Figure 4: Sixth Polymerase Chain Reaction (PCR) Experiment on 0.5% SDS Gel with 1 Kbp NEB Ladder, ¼ Template DNA Product (N1), and ½ Template DNA Product (N2)

EXPERIMENT 7

The next trial then looked at using different primers (see Tables 1 & 2) to amplify the appropriate sequence designated to remove the ccmf gene. Three PCR tubes were made containing the following primers.

- G1 = CcmFUSGibFW & σ NB302
- G2 = CcmFUSGibFW & CcmFDSRV
- N3 = CcmFUSFW & CcmFDSRV

The PCR products were then placed on a 0.5% SDS gel with a NEB 1Kbp Ladder (see Figure 5). The PCR product from G2 appeared to contain the appropriate DNA band marker, thus this PCR was successful in replicating the appropriate DNA sequence (and linearizing the plasmid). The only issue was that the G2 bands were a little smeary (meaning that the PCR products were nonspecific and multiple lengths of DNA sequences were created). The next step was to try and reduce the amount of smearing.

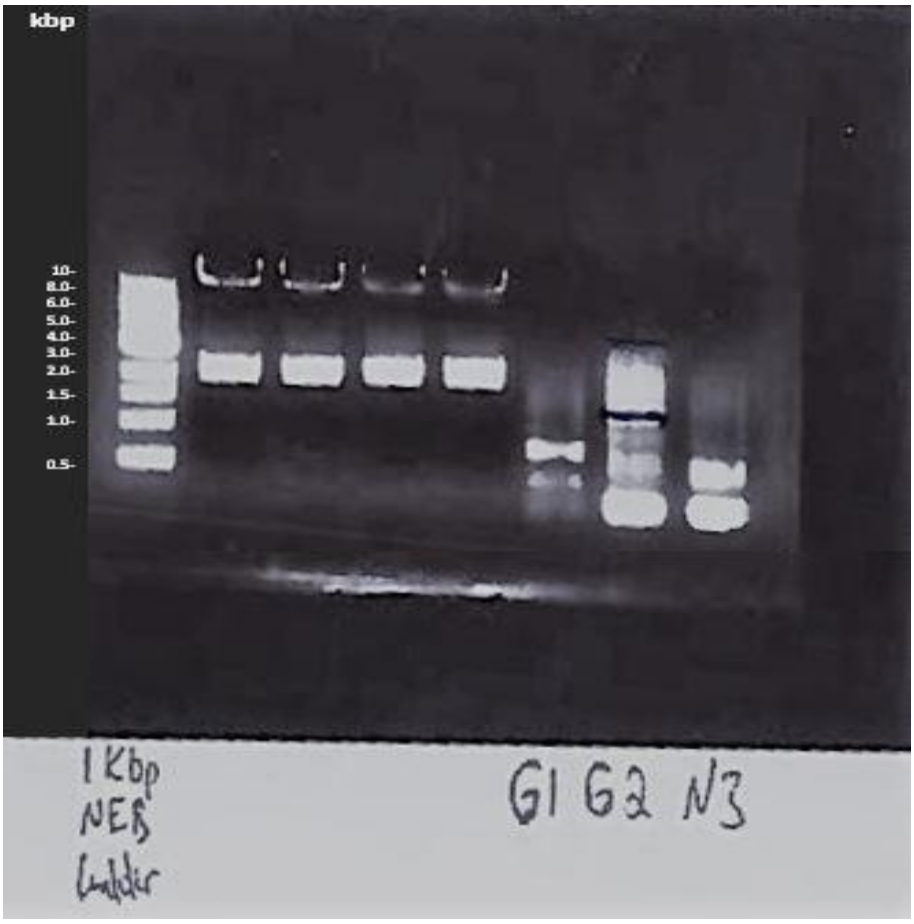

Figure 5: Seventh Polymerase Chain Reaction (PCR) Experiment on 0.5% SDS Gel with 1Kbp NEB Ladder, Primers CcmFUSGibFW & σ NB302 Product (G1), Primers CcmFUSGibFW & CcmFDSRV Product (G2), and Primers CcmFUSFW & CcmFDSRV Product (N3)

EXPERIMENT 8

The next PCR trials tested for the same DNA marker of 2 kbp, but it used the G2 PCR product as the template DNA (see Tables 1 & 2). Two PCR tunes were made, changing the source of the G2 PCR template DNA.

- G3 = G2 PCR template product
- G4 = smear G2 from 0.5% SDS gel of Experiment 7 (this was completed via a standard gel cleanup kit)

The PCR products were then placed on a 0.5% SDS gel with a NEB 1Kbp Ladder (see Figure 6). The appropriate band marker of 2000 base pairs was not present, so this trial was also unsuccessful in replicating the correct DNA sequence.

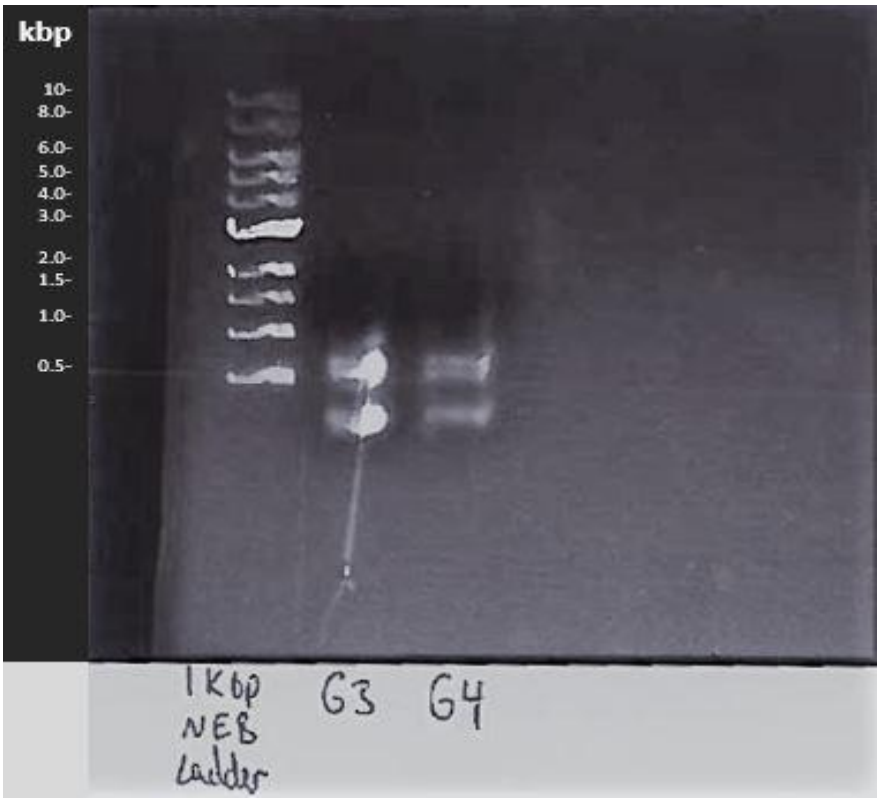

Figure 6: Eighth Polymerase Chain Reaction (PCR) Experiment on 0.5% SDS Gel with 1Kbp NEB Ladder, PCR Product using Previous PCR Product as DNA Template (G3), and PCR Product using 0.5% SDS Gel Band Marker of Previous PCR Product as DNA Template (G4)

EXPERIMENT 9

Since none of the PCR samples could linearize the plasmid or amplify the correct DNA sequences, a second trial of the Gibson Kit was performed. Again, the idea was that perhaps the Gibson mix could use a combine these fragments with a digested plasmid after all, and the initial PCR step of linearization could be avoided.

The new Gibson mix was made with 3.4 μl pNB72.3, 1.79 μl CcmFgibsUs, 3.67 μl CcmFgibsDs, 10 μl Gibson Master Mix, 1.14 μl DI Water. The resulting Gibson tube was then placed in the thermocycle at 50 °C for

1 hour. A positive control was also created to assess if the Gibson mix materials were degraded or contaminated. This positive control was made with 10 μl + Control and 10 μl Gibson Master Mix.

The product from this Gibson experiment was then transferred into electro competent *E. coli* cells using the standard protocol for electro competent cellular transformation. The antibiotic used during the screening process was ampicillin. At first lots of growth occurred on the selection plate. In order to ensure that this was due to gene transformation and not from lack of ampicillin exposure (as the other mass of dead cells could have blocked the antibiotic from the living cells), the colony was spread onto another screening plate. Only three colonies grew this time, so they were spread onto another screening plate again. This time nothing survived. This showed that this Gibson trial was unsuccessful because none of the desired genes were transferred to the *E. coli* via these trials.

Discussion and Future Opportunities

Overall, PCR prep of the plasmid is necessary before using the Gibson Kit. The Gibson Kit could still be viable in combining multiple DNA fragments in a shorter amount of time than regular methods, though, as long as the Gibson Kit materials have not degraded or been contaminated and the plasmid ahs been linearized. Buying the materials in bulk is recommended to avoid this issue. By increasing the volume, you are able to dilute any contaminations. Also, the freeze-thaw impact on the individual enzymes as they are transferred in and out of the freezer between experimental sets would be reduced as the increased volume of solution would act as an insulative barrier. If in doubt of enzymatic activity, add more enzymes to the Gibson mixture as a comparative analysis. If the end result is the same regardless of enzyme concentration, then the limiting reagent is one of the other Gibson mix components. Lastly, knowledge of proper primers and annealing temperatures is crucial in linearizing plasmids successfully. In conclusion, further testing should still be conducted for determining better optimization of the genetic cloning procedures for methanogens via *E. coli*.

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