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Understanding Cytochrome *c* Maturation in Anaerobic Archaea

An Honors Thesis submitted in partial fulfillment
of the requirements for Honors Studies in
Biological Sciences

By

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Spring 2020
Biological Sciences
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Table of Contents

Page Number

2: **Acknowledgements**

4: **Abstract**

5-8: **Introduction**

9-20: **Cloning of *Methanoperedens nitroreducens* Cytochrome *c* Protein**

9-10: Introduction

10-16: Materials and Methods

16-19: Results

19-20: Discussion

20: Further Experiments

21-30: **CRISPR/dCas9-Mediated Silencing of *trx3*, *trx6*, and *trx7***

21-23: Introduction

23-25: Materials and Methods

26-28: Results

28-29: Discussion

29-30: Further Experiments

31-32: **References**

Abstract

Methanoperedens nitroreducens (MPEBLZ), an archaeal methanotroph and close relative of *Methanosarcina acetivorans*, contain numerous cytochrome *c* proteins. However, difficulty in using these organisms as a model for cytochrome *c* research has created a pressure to express cytochrome *c* proteins in an organism that is much easier to work with. A putative cytochrome *c* protein (MPEBLZ_04274) from *M. nitroreducens* was attempted to be cloned into a model methanogen *M. acetivorans* as well as *Escherichia coli*. Cytochrome *c* proteins are important for many metabolic processes within anaerobic archaea. In order for a mature cytochrome *c* to be formed heme must be translocated out of the cell and covalently attached to thiol bonds within CXXCH binding motifs within the protein. Preliminary data suggests that thioredoxin system components, which catalyzes redox reactions within the cell, may be important for the insertion of heme in cytochrome *c* proteins. To investigate this, CRISPR-dCas9 targeted repression of *trx3*, *trx6*, and *trx7* was carried out to elucidate the potential impact that these thioredoxins have on the maturation of cytochrome *c* proteins as well as the ability of the cell to overcome general oxidative stressors.

Introduction

The largest producers of environmental methane, methanogens, are crucial to the carbon cycle. Methane is approximately twenty-five times more potent than carbon dioxide as a greenhouse gas (1). Rising global temperatures and increased mobilization of environmental carbon means understanding more about methanogens will become crucial if we hope to limit greenhouse gas production in the future. Most of this methane is produced from acetate (2). Acetate is a common byproduct of anaerobic fermentation, which is the method many organisms use to generate ATP in an environment devoid of oxygen. Without methanogens, many of these environments would develop toxic levels of acetate and create an environment unsuitable for many organisms. Not only are methanogens important for maintaining healthy ecosystems, including those important to industry such as wastewater treatment and anaerobic digestion, but methanogens are also important for the production of methane as a biofuel, a cleaner energy source.

Methanosarcina acetivorans is a commonly used organism for research involving methanogens. This is because *M. acetivorans* contain the machinery for all three (methylated compounds, CO₂, and acetate) methanogenic metabolic pathways (2). The genome is fully sequenced with tools developed for genetic related work to be conducted more easily. *M. nitroreducens* is an ANME-2 clade methanotroph and close relative of *M. acetivorans* (3). Methanotrophic archaea occupy many of the same environments methanogens are commonly found in due to their metabolic usage of methane as a carbon energy source. *M. nitroreducens* utilizes nitrate as an electron acceptor and reduces it to nitrite, with the transfer of electrons being facilitated by cytochrome *c* proteins (3).

Cytochrome *c* proteins are important for some methanogens and methanotrophs to grow and carry out metabolic processes. In *M. acetivorans* cytochrome *c* proteins are especially important when growing with acetate (4). These proteins have been shown to play a crucial role in the transfer of electrons into the cell from fermenting bacteria, and also out of the cell to an oxidized metal(5). Cytochrome *c* proteins have also been implicated in the ability for some prokaryotes to participate in direct interspecies electron transfer (DIET). This is the process of transferring electrons in a syntrophic relationship. Some species accomplish this through the use of an e-pili, which is an electrically conductive pili bridge formed between the electron donor and the electron recipient (5). There is evidence to suggest that cytochrome *c* proteins are able to form bridges through the stacking of proteins that allow for electron transfer in this bridge-type manner(5). Despite the metabolic importance many of the processes as to how these proteins mature in anaerobic archaea are unknown (4). Cytochrome *c* maturation is well studied in bacteria but none of the machinery in archaea has yet to be biochemically characterized. There are even cytochrome *c* proteins in methanogens that have yet to be identified, let alone characterized.

Cytochrome proteins are a family of heme-containing proteins. There are many types of cytochrome proteins (6). Although these proteins all play a similar electron transfer role in the cell, the way their heme cofactor is attached differs among the subtypes. Cytochrome *c* proteins are unique in the fact they covalently bind to heme. So far five cytochrome *c* maturation genes (*ccm*) have been identified in the genome of *M. acetivorans*, with those being *ccmABCEF* (2). These proteins are predicted to translocate

the heme to the extracellular surface of the membrane and attach it to the thiols of cysteines within a CXXCH motif of apo-cytochrome *c* proteins.

In addition to *ccm* genes, the thioredoxin system may have some effect on cytochrome *c* maturation. This system is important for oxidative stress response within the cell. *M. acetivorans* contains eight thioredoxin system proteins (Trx) one of which being a NADPH-dependent reductase, and the remaining exhibiting disulfide reductase capabilities (7). This system allows for the maintenance of a reduced intracellular environment, as well as the ability to reduce disulfide bonds both within the membrane as well as extracellular proteins. Due to the nature of heme insertion into cytochrome *c* proteins, it is theorized that thioredoxin may play a role in preparing the thiol bonds found within the CXXCH motif of cytochrome *c* for heme insertion. This ability would confer a higher cytochrome *c* maturation efficiency by helping to prevent indiscriminate metal binding to the cysteine residues found within the CXXCH-heme binding motif.

By searching for proteins with a CXXCH motif, it is predicted that *M. nitroreducens* contains >40 cytochrome *c* proteins. These putative proteins cannot be studied in the native methanotroph due to extremely slow growth and low cell yields. The first goal of this project was test if the predicted cytochrome *c* proteins of *M. nitroreducens* are recognized as cytochrome *c* proteins in surrogate hosts. A single putative cytochrome *c* gene from *M. nitroreducens* was cloned into both *E. coli* and *M. acetivorans*. This was done in an attempt to see if *E. coli* and *M. acetivorans* could recognize the protein, and whether the cytochrome *c* maturation genes each organism possess allowed for mature cytochrome *c* proteins to be made. Cloning into these model organism would also allow for an efficient method of future cytochrome *c* research, as *M.*

nitroreducens is unable to be isolated for pure culture and long growth periods make cytochrome *c* research using the organism challenging. The second goal of this project was to test whether thioredoxin components are involved in cytochrome *c* maturation in *M. acetivorans*. Thioredoxin knockdowns were constructed using CRISPR-dCas9 to assess the roles of three thioredoxin proteins (Trx1, Trx3 and Trx7) on the ability of cells to overcome oxidative stress and also to see if there was a change in levels of heme insertion into cytochrome *c* proteins. A depiction of these projects can be seen below (Figure 1). A better understanding of the machinery behind cytochrome *c* synthesis could lead to novel ways to control methane production, for energy or for the environment, or with the adaptation of metabolic pathways for the production of bio-batteries.

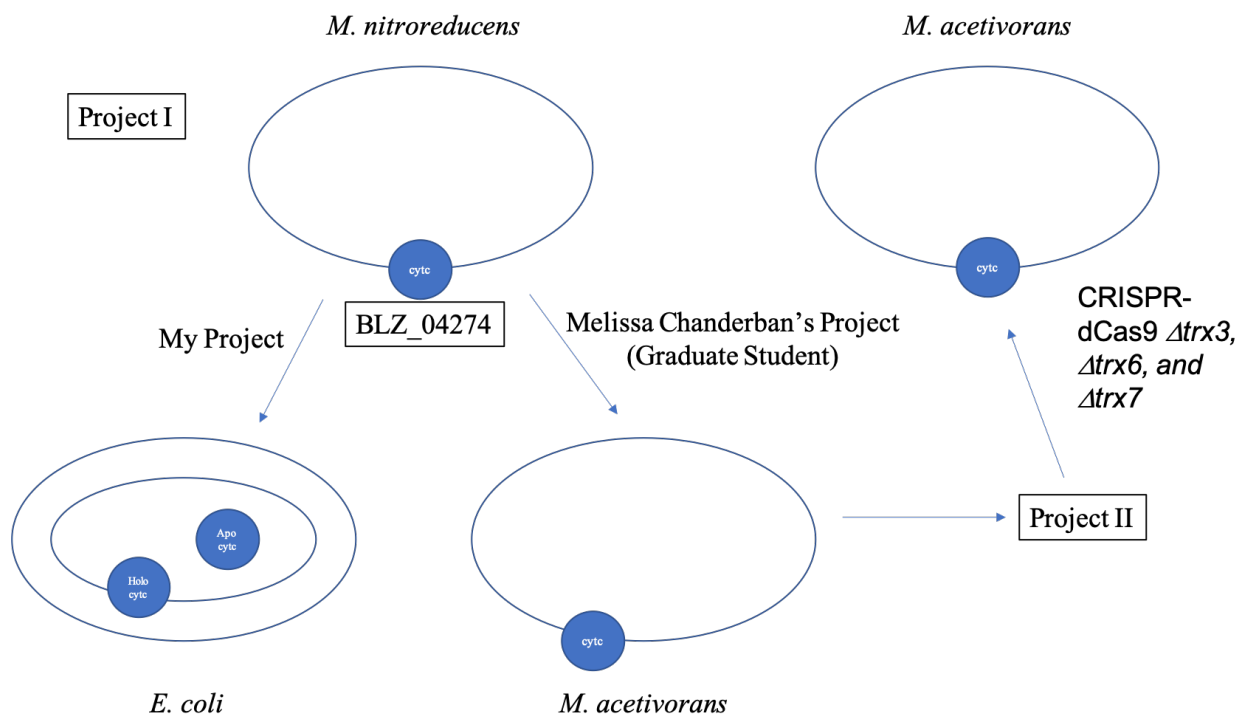


Figure 1. Chart depicting the main focus of projects

Project I:
Cloning of *Methanoperedens nitroreducens* Cytochrome *c*
Protein

Introduction

Methanoperedens nitroreducens (MPEBLZ), an ANME-2 clade methanotroph, is a close relative of our model organism *Methanosarcina acetivorans*. ANME-2 clade methanogens encode a high number of *c*-type cytochromes, with *M. nitroreducens* encoding 43 of these proteins (3). Due to these two factors it became the organism of choice for use in standard cloning procedures. The metabolic pathway of *M. nitroreducens* is very similar to that of *M. acetivorans* so much so that it is described as “reverse-methanogenesis” (8). Methane is consumed in a process that utilizes many of the same metabolic enzymes present in *M. acetivorans*, because of these similarities it was believed that *M. acetivorans* would be able to recognize, and produce the mature the cytochrome *c* protein from *M. nitroreducens*.

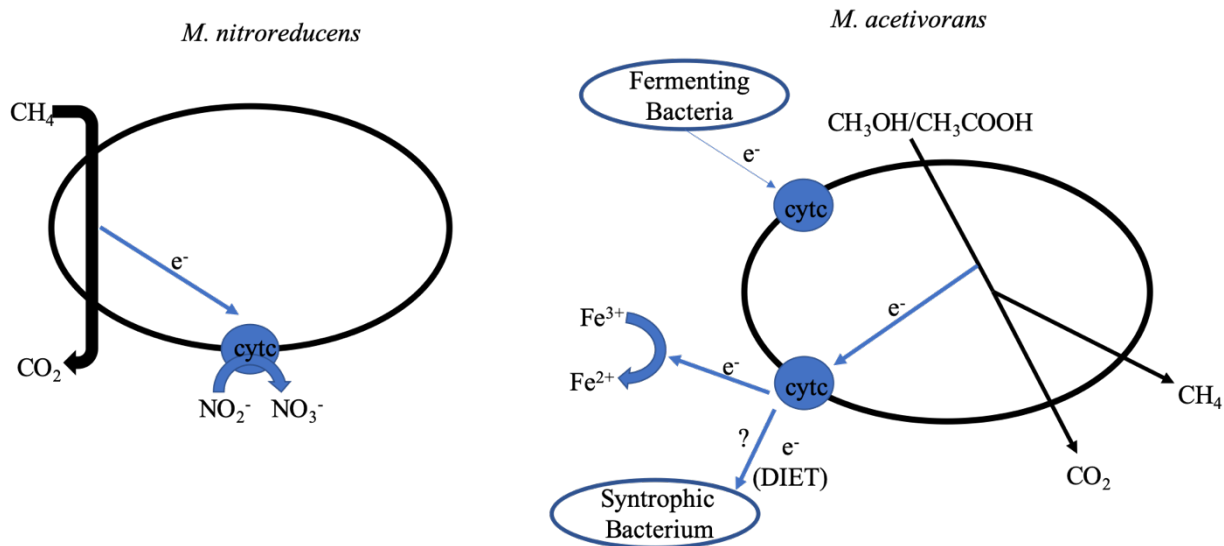


Figure 2. Basic metabolic pathways for *M. nitroreducens* and *M. acetivorans*. Showing the methods in which cytochrome *c* is used differently among the phylogenetically close relatives

M. nitroreducens is hard to grow. So far it has not been able to be isolated and grown in pure culture, it also exhibits extremely slow growth (8). This is the reason that the MPEBLZ *cytc* gene was chosen to be moved and expressed in *M. acetivorans* and *E. coli* instead of working with the methanotroph directly. MPEBLZ_04274 was the selected gene; this is a suspected multi-heme cytochrome *c* protein with 4 CXXCH heme-binding domains (3). The cloning of this gene would not only allow for cytochrome *c* to be studied in an organism that is much easier to work with, but it also would provide conclusive biochemical evidence that the gene selected was a cytochrome *c* protein. It is hypothesized that MPEBLZ_04274 is a gene that encodes a cytochrome *c* protein, and that the protein will be able to be cloned into and expressed within *E. coli*.

Materials and Methods

Cloning of MPEBLZ gene into *E. coli* pET-28a (+)

The theoretical cytochrome *c* genomic sequence of MPEBLZ_04274 was first examined and compared against other possible targets for cloning.

```
>KPQ41182.1 hypothetical protein MPEBLZ_04274 [Candidatus Methanoperedens sp. BLZ1]
MTNKMILIGIAIAAIGLVALPQTLLFAGQHNFYDTIGAGAASVPCEKCHADVFSSELSQPGSVNAIHMAQ
GCEGCHVTTAPTKEGLTQGGALGSDFHAAALPLCLDCHSGTGPGLDARSILSGPAEAHVAFAGQANDSLL
LRGSNEACISCHTHVAVDINWTKAYKISFNAMENATTGLHEWTVNNFATEGTVQIQTYGNRSGEINSATE
PVVSISPTPLGFDPANP
```

Figure 3. Hypothetical cytochrome c protein MPEBLZ_04274 with signal peptide highlighted

The genetic code was then optimized for use in *M. acetivorans*, ensuring the codons are frequently used in the genome of *M. acetivorans*.

```
> MPEBLZ_04274 coding sequence optimized for M. acetivorans
ATGACCAACAAGATGATCCTCATCGGCATCGCAATCGCAGCAATCGGCCTCGTCGCACTCCCTCAGACCTCGCACT
CTTCGCAGGCCAGCACAACTTCTACGACACCATCGGCGCAGGCGCAGCATCCGTCCCTTGCAGAAAAGTGCCACGCAG
ACGTCTTCTCCGAACTCTCCAGCCTGGCTCCGTCAACGCAATCCACATGGCACAGGGCTGCGAAGGCTGCCACGTC
ACCACCGCACCTACCAAGGAAGGCCTCAGCAGGGCGGCGCACTCGGCTCCGACTTCCACGCAGCAGCACTCCCTCT
CTGCCTCGACTGCCACTCCGGCACCGGCCCTGGCTCGACGCAAGGTCCATCCTCTCCGGCCCTGCAGAAGCACACG
TCGCATTCGCAGGCCAGGCAACGACTCCCTCCTCCTCAGGGGCTCCAACGAAGCATGCATCTCCTGCCACACCCAC
GTCGCAGTCGACATCAACTGGACCAAGGCATACAAGATCTCCTTCAACGCAATGGAACGCAACACCGGCCTCCA
CGAATGGACCGTCAACAACTTCGCAACCGAAGGCACCGTCCAGATCCAGACCTACGGCAACAGGTCCGGCGAAATCA
ACTCCGCAACCGAACCTGTCTCTCCATCTCCCTACCCCTCTCGGCTTCGACCTGCAACCCCTGACTACAAAGAC
GATGACGACAAGTGA
```

Figure 4. MPEBLZ_04274 genetic code optimization for *M. acetivorans*

Three primers were then ordered from IDT: BLZ_04274For which contained a 5' NdeI restriction enzyme site, BLZ_04274nSPFor which would create an insert lacking a signal peptide and also contained a 5' NdeI site, and BLZ_04274RevBamHI which contained a 3' BamHI site. These primers are listed below:

Primer Name	Primer Sequence
BLZ_04274For	ggtggtcatATGACCAACAAGATGATCCTCATCGGCATC 5' NdeI
BLZ_04274nSPFor	ggtggtcatATGCTCTTCGCAGGCCAGCACAACTTCTACG 5' NdeI
BLZ_04274RevBamHI	ggtggtgatccTCACTTGTCGTCATCGTCTTTGTAGTCAG 3' BamHI

Four PCR reactions were then carried out to amplify BLZ_04274 with and without the signal peptide using pDL215, the plasmid containing the MPEBLZ gene of interest,

according to New England Biolab's (NEB) protocol for use with Q5® High Fidelity DNA Polymerase. The PCR reactions were created as follows:

Reaction Number	Nucleic Acid Containing Components
1	BLZ_04274For, BLZ-0474Rev, and pDL215
2	BLZ_04274nSPFor, BLZ-0474Rev, and pDL215
3	BLZ_04274For, BLZ-0474Rev (Control)
4	BLZ_04274nSPFor, BLZ-0474Rev (Control)

PCR reactions were then placed in the thermocycler and ran using the protocol as outlined by NEB for use with Q5® polymerase. Following PCR, a small amount of PCR products was run on a 1% agarose gel utilizing 120 V for 25 minutes. After verifying positive results from the agarose gel, the remaining PCR products were cleaned using Promega Wizard Plasmid Prep Kit. Next, double-digests were created utilizing restriction enzymes NdeI as well as BamHI (NEB) for inserts BLZ_04274, BLZ_04274nSP, and for the vector pET-28a(+).

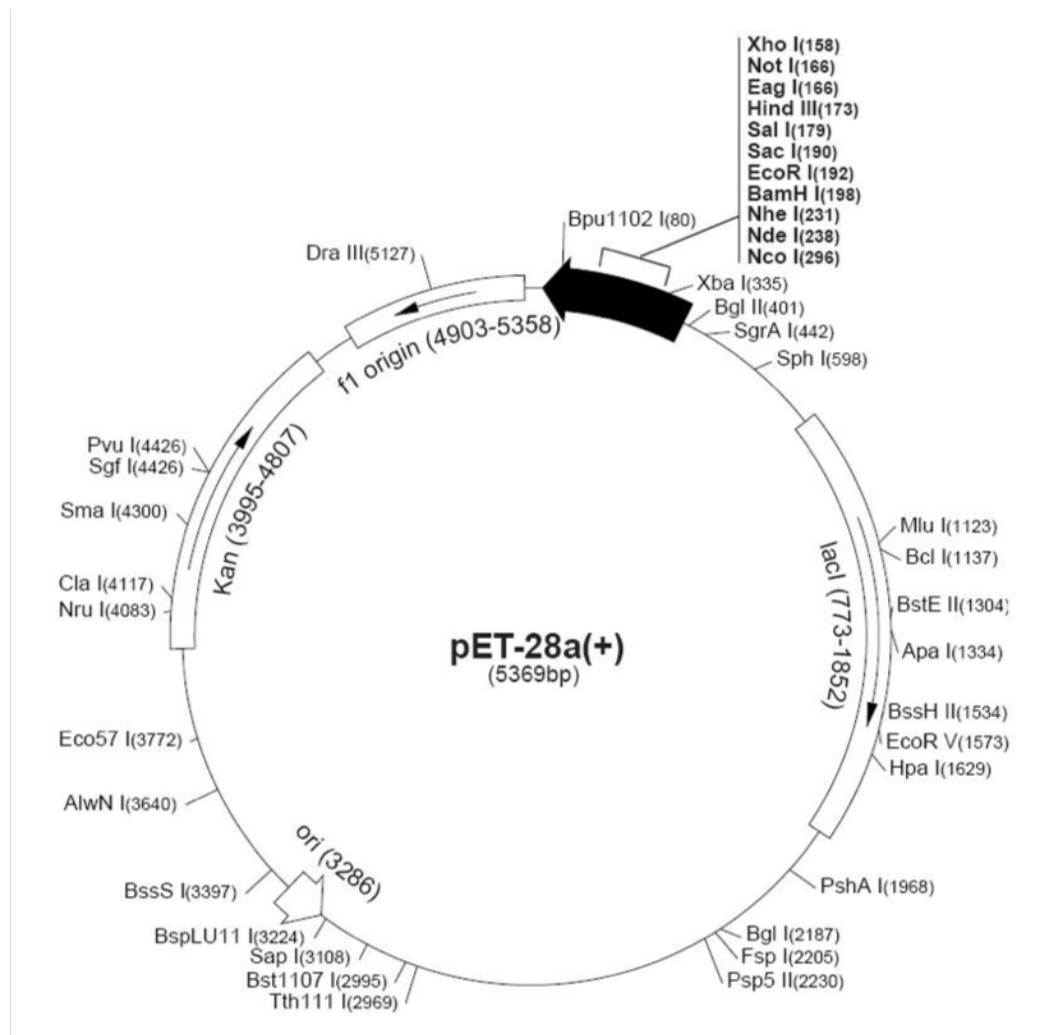


Figure 5. pET-28a(+) showing restriction enzyme sites. Image comes from the plasmid manufacturer New England Biolabs (NEB)

The digests were allowed to run overnight at 37°C. The next day the digestion products were then cleaned using Promega Wizard PCR Cleanup Kit. The cleaned digested products were then run on a 1% agarose gel to verify size. After verifying that the inserts and vector were of the correct size, they were quantified using a spectrophotometer using the double-stranded DNA setting, and absorbance was measured at 260/280nm to find the concentration of DNA as well as if there was protein contamination. Following quantification, a ligation reaction was created, ensuring to use the recommended amount

of both vector and insert as specified by NEB for use with T4 DNA Ligase. The ligation reactions were then placed in the thermocycler overnight at 16°C.

Transformation into DH5 α Competent Cells

At the end of the ligation step, two plasmids were generated, pDL221 and pDL222. pDL221 contained the BLZ_04274nSP gene inserted into pET-28a(+), and pDL222 contained BLZ_04274 gene. Next, the plasmids were transformed into DH5 α competent cells. Transformations were carried out as outlined by NEB protocol. Transformants were spread on LB agar plates supplemented with kanamycin at 50 μ g/mL. Two different amounts of transformants were used: 50 μ L as well as 200 μ L. Plates were then incubated at 37°C and monitored for growth. After growth was observed BLZ_04274nSP transformed colonies were screened via colony PCR. BLZ_04274 transformed colonies were screened using restriction digestion. After confirmation of successful plasmid transformation, cultures were started and plasmids harvested. These plasmids were sent for sequencing to ensure proper insertion had occurred.

Protein Expression in Rosetta[™] DE3

Following confirmation of correct sequence insertion into pDL221 and pDL222, plasmids were transformed into Rosetta[™] DE3 competent cells following NEB protocol. These competent cells were selected due to their ability to overexpress protein via the *lac* operon found within pET vectors with induction from Isopropyl- β -D-thiogalactopyranoside (IPTG). All of the plates containing transformants showed significant amounts of growth. Because of the difficulty in finding isolated colonies a streak plate was prepared using the transformation plates as a starter. Streak plates were made for Rosetta[™] DE3+ pDL221 and Rosetta[™] DE3+ pDL222. Cells were taken from

the streak plate to create freezer stocks for future use. Protein expression was started by inoculating 10 mL of LB media with the freezer stocks generated above. Cultures were allowed to grow overnight, with 1 mL of the starter culture being transferred to 10 mL of LB media. These were allowed to shake at 200 rpm at 37°C until a OD₆₀₀ of between 0.6-.08 was reached. The cultures were then split into 5 mL quantities, and one 5 mL culture for both pDL221 and pDL222 were induced with IPTG given at 500 µM concentration. Cultures were allowed to shake at 100 rpm for 22 hours at 16°C. After 16 hours 1 mL was pulled from each culture, spun down, and the cell pellet was frozen for future use. Frozen cell pellets were thawed on ice and resuspended with 1 mL of Tris buffer (50 mM Tris+ 150 mM NaCl). Each sample was sonicated, and then SDS buffer with BME was added. The samples were then boiled for 10 minutes. The samples were then loaded onto a SDS-PAGE gel which was ran for 40 minutes at 170 V. After the SDS-PAGE gel finished, the gel was removed and stained with Coomassie Blue. The gel was submerged in the stain for 1 hour with light shaking. After 1 hour the gel was rinsed with destain, and allowed to shake gently for 2 hours. At this point the destain was removed and images of the gel were taken.

Anaerobic growth of *E. coli* Rosetta™ DE3 strains

Anaerobic LB media was made, 1 M NaNO₃, as well as 50% (w/v) glycerol solution. Four growth tubes were made which all contained the following: 0.1 mL of cell culture, 20 mM nitrate solution, 0.1 mL of glycerol solution as well as kanamycin/chloramphenicol at 50 µg/mL and 17 µg/mL respectively. Four growth conditions were utilized, pDL221 and pDL222 were allowed to grow at 37°C while

shaking at 250 rpm, and pDL221 and pDL222 were placed in the incubator at 37°C with no shaking.

Results

The results from the agarose gel which contained a small amount of PCR product from the insert sequence amplification showed the generation of sequences that were of the correct size BLZ_04274nSP is 684 base pairs in length and BLZ_04274 is 708 base pairs in length. The digestion reactions were also run on an agarose gel to ensure the correct size and that the digestions proceeded as expected. The agarose gel showed that the digestion occurred as it should with inserts BLZ_04274 being 708 base pairs, BLZ_04274nSP being 684 base pairs, and with the vector pET-28a(+) being approximately 5 kb in length. Due to the location of restriction enzyme sites, NdeI and BamHI approximately 40 base pairs were excised from pET-28a(+). The vector also showed that it was in-fact linearized due to it running true to its size (Figure 6).

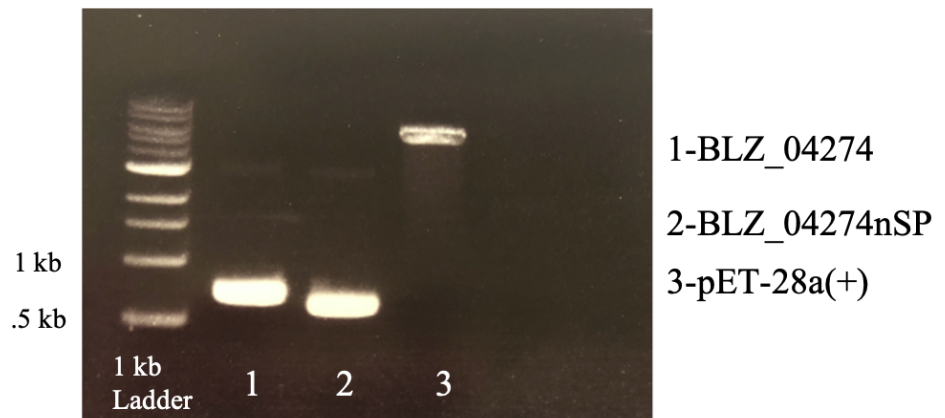


Figure 6. Results from Digestion Reaction

Following confirmation of correct digestion, ligation reactions were carried out. After transformation, the generated colonies were checked to see if the plasmid had been successfully transformed. Figure 7 shows the results of colony PCR, as indicated by the

band at around 0.6 kb successful incorporation of plasmid pDL221 had occurred in colony A. None of the other five colonies showed successful incorporation of the plasmid. Figure 8 shows the results from a restriction digestion of plasmids coming from colony cultures. When visualizing the agarose gel, bands of the correct size were seen in both colonies F and H, when printing the image the upper half of the gel did not print well. However, successful incorporation can be seen clearly in colony H (Figure 8).

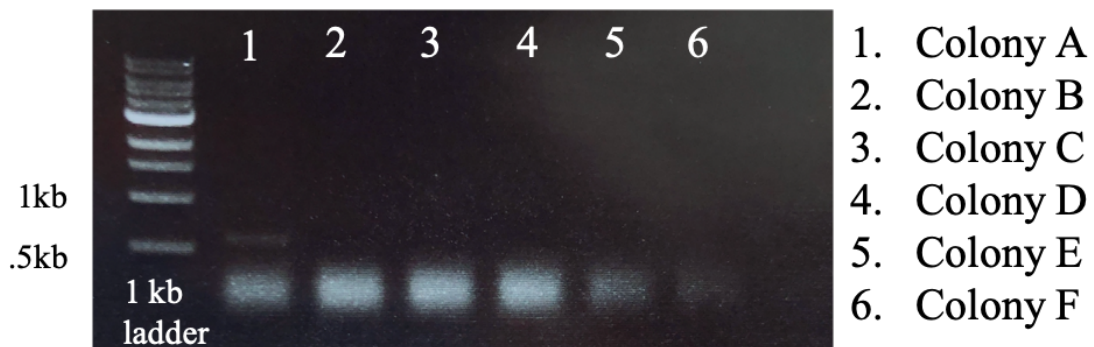


Figure 7. Results from Colony PCR screening for BLZ_04274nSP

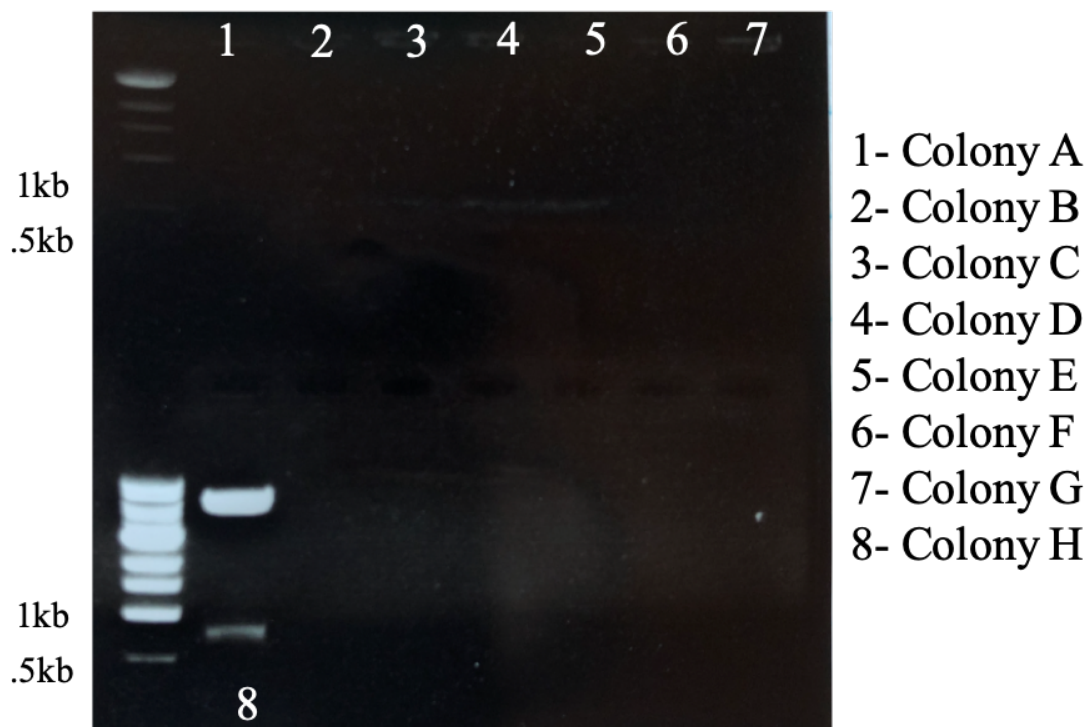


Figure 8. Mini Colony Digest Screening for BLZ_04274

Plasmids pDL221 and pDL222 were sent for sequencing and the inserted sequences were confirmed. After the SDS-PAGE gel was run, the protein of interest was not seen. The 235 amino acid residue theoretical cytochrome *c* protein should have resulted in a pronounced band at approximately 25 kDa (Figure 9).

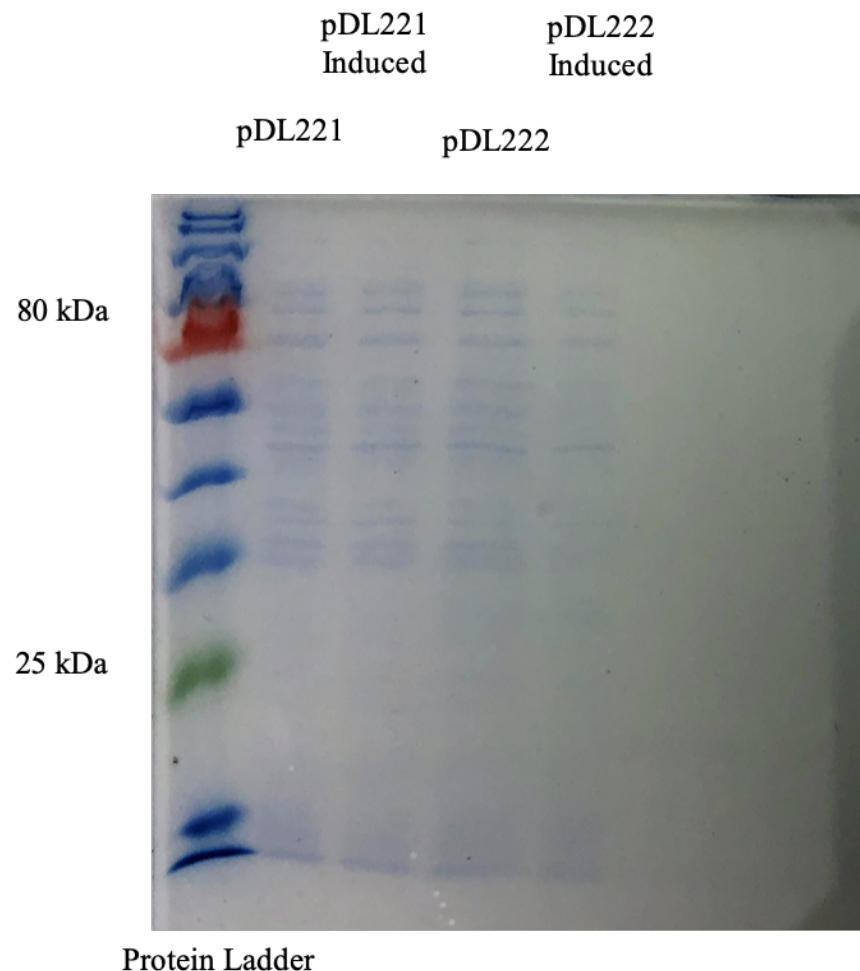


Figure 9. SDS-PAGE showing results from protein overexpression of pDL221 and pDL222 in Rosetta™ DE3 cells.

It was believed that proteins lacking the signal peptide would appear in similar quantities to those that contained the signal peptide, but that the differences between the two would appear when using a heme strain. It was thought that BLZ_04274 expressed from pDL222 would show higher heme content, when compared to the heme content of

BLZ_04274nSP expressed from pDL221. It has been shown that *E. coli* utilizes cytochrome *c* when growing on anaerobically in the presence of nitrate. Because of this culture were grown in anaerobic LB media with nitrate to try and stimulate cytochrome *c* maturation genes. Due to lack of viable growth when subjected to anaerobic conditions in the presence of nitrate no protein expression study was carried out.

Discussion

Although successful insertion of BLZ_04274For, as well as BLZ_04274nSPFor, occurred as evident through agarose gel visualization and gene sequencing, the protein was not able to be generated by the *E. coli* strains. After induction with IPTG of Rosetta DE3 cells carrying plasmids with the genes of interest with and without the signal peptide, no overexpressed protein was produced, as shown by the lack of bands on the SDS-PAGE gel. The strain carrying BLZ_04274nSP may have had protein toxicity issues, since theoretically the protein would have been produced in large amounts but not able to be targeted outside of the cell. This may have resulted in proteolysis to prevent cell death. However, lack of protein overexpression in the signal peptide containing strain showed there is likely some other factor preventing the protein from being produced. Subsequent anaerobic growth also produced no positive results, with cell cultures not growing. The lack of cell growth prevented the possibility for another SDS-PAGE gel to be run because there was inadequate cell yield of the anaerobically grown cultures. In addition, this lack of protein expression may have been caused by the difference in *E. coli* cytochrome *c* maturation genes. CcmH is an necessary gene for cytochrome *c* maturation in *E. coli* that is absent in archaea (9). There are also slight alterations in the primary amino acid of CcmE between archaeal and bacterial System I-*ccm* (9). It is possible that

because of this, it was not feasible to express a methanotroph archaeal cytochrome *c* protein in *E. coli*. Another possibility was regulation behind cytochrome *c* production. *E. coli* does not produce cytochrome *c* in detectable amounts while growing aerobically but does utilize these proteins while undergoing anaerobic respiration (10). The type of electron acceptor is important in the production of cytochrome *c* proteins, it has been previously shown that *c*-type cytochrome expression occurs while being grown in the presence of either nitrate or nitrite (10). This differential usage under very specific environmental conditions may mean that its production under normal growth conditions is unfavorable for the cell. This would put the cell at a disadvantage energetically, because energy would be expended on trying to either repair the protein or synthesizing new ones if constitutively expressed.

Further Experiments

Although focus shifted away from this project, due to difficulty in expressing BLZ_04274 in *E. coli* as well as *M. acetivorans*. Work conducted in the methanogen model by Melissa Chanderban did not produce positive results, with no protein being overexpressed either. Due to the lack of successful results in both model organism; other projects were sought out. Further work could involve improving the method of anaerobic growth with the *E. coli* strain. If cytochrome *c* protein was able to be harvested in appreciable levels then heme stain experiments could be conducted. This would allow for BLZ_04274 and the protein lacking the signal peptide to be examined for different levels of heme-bound cytochrome *c*.

Project II: CRISPR/dCas9 Silencing of *trx3*, *trx6*, *trx7*

Introduction

The thioredoxin system plays a crucial role in the maintenance of a reduced cellular environment, and the reduction of oxidized proteins. In addition, recent findings by the Lessner lab have shown that in particular, overexpression of two thioredoxin proteins, Trx3 and Trx6, were shown to change levels of heme-bound cytochrome *c* protein in the methanogen model *Methanosarcina acetivorans* (7). Trx7 may be the intracellular thioredoxin supplying electrons to the cytochrome *c* synthase module (7). Trx3 and Trx6 are targeted to the cell membrane, whereas Trx7 is found in the cytosol (7). Based on the ability of thioredoxin to participate in the reduction of proteins, and the findings that altering the expression of Trx3 and Trx6 changed levels of heme-bound cytochrome *c*, there is reason to believe that the thioredoxin system may interact with the thiol bonds contained within the cytochrome *c* heme-binding domain. *M. acetivorans* encode a System I-type cytochrome *c* maturation pathway which contains the genes *ccmABCEF*. The *ccm* system is responsible for heme translocation ubiquitously across phyla that utilize this *ccm* pathway (9). It has been shown that in *E. coli* CcmH was an essential protein for cytochrome *c* maturation, and functions as a disulfide oxidoreductase (9). This reductase activity reduced the heme-binding motif, thus preparing the protein for heme insertion (9). However, CcmH is absent in *M. acetivorans* and other archaeons, further necessitating that there must be another source for preparing cytochrome *c* for the incorporation of the heme cofactor. Figure 10 shows the purposed interaction between the *ccm* and thioredoxin systems as it pertains to the maturation of cytochrome *c* proteins. Heme is translocated outside the cell and comes in contact with reduced apo-cytochrome

c proteins, which was previously oxidized upon exiting the cell and subsequently reduced once in close proximity to heme.

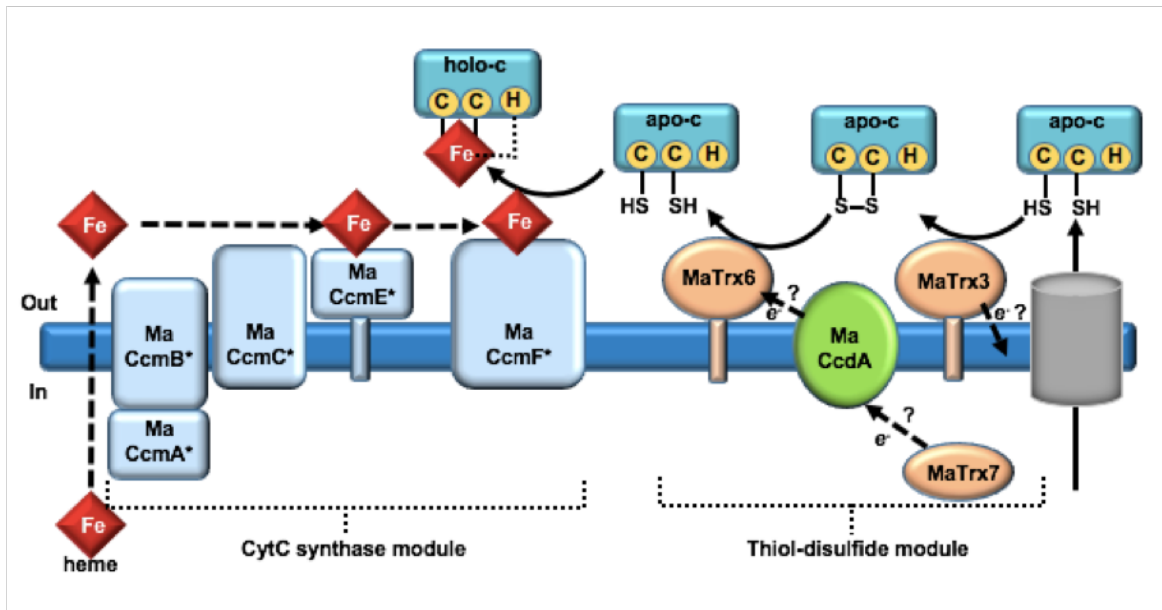


Figure 10. Theoretical role of thioredoxin in cytochrome *c* maturation, as well as method of cytochrome *c* production within *M. acetivorans* obtained from Dr. Daniel Lessner via personal communication

A second project was started, looking into how thioredoxin proteins may impact cytochrome *c* maturation and the ability for cells to overcome oxidative stressors. Deletions of Δ trx3, Δ trx6, and Δ trx7 were going to be made via CRISPR-Cas9. The CRISPR-Cas9 (Clustered Regulatory Interspaced Short Palindromic Repeats) system is an immune defense utilized by many organisms (9). This system is naturally used to target foreign DNA inserted into a genome. The system consists of repeating sequences of DNA coding with spacers that contain remnants of the genetic code from previous foreign DNA insertions, so this allows the CRISPR system to quickly recognize these sequences if they appear again in the genome of the organism. The short spacer sequences can be transcribed into RNA which guides the Cas9 enzyme to initiate a double-stranded break of the DNA helix. These RNA sequences can also be synthesized

in the lab, termed guide RNAs (gRNA), and these sequences target genes of interest for genome editing based on repair mechanism (11). The double-stranded break can be repaired in two ways, non-homologous end joining (NHEJ) or through homology-directed repair (12). Until recently CRISPR-Cas9 has not found extensive use with archaea. The Metcalf group generated a chimeric strain of *M. acetivorans* using NHEJ machinery from *Methanocella paludicola* (12). Due to the promising results from the usage of the CRISPR system in *M. acetivorans* already, and the previous results that changing levels of thioredoxin proteins altered heme-bound cytochrome *c* levels a hypothesis was made. It was hypothesized that by altering the presence of thioredoxin proteins that the amount of holo-cytochrome *c* proteins will change and that cells be less able to overcome oxidative stressors.

Materials and Methods

Generating $\Delta trx3$, $\Delta trx6$, and $\Delta trx7$ Gene Knockouts via CRISPR-Cas9

CRISPR-Cas9 utilizing NHEJ repair was originally planned for use to generate $\Delta trx3$, $\Delta trx6$, and $\Delta trx7$ gene knockouts. However, after transforming plasmids containing the machinery to generate the gene deletions, we found that cultures were not viable. The cells exhibited extreme difficulty in growing, and cultures grew very frothy and displayed unusual growth patterns. This then caused us to switch from relying on NHEJ and instead focus our attention on the homology-directed repair mechanism. Homology arms were generated to help provide a template for double-stranded break repair. Despite having all the fragments needed for the creation of a viable CRISPR-Cas9 plasmid and the production of homology arms, Gibson assembly repeatedly failed to produce viable plasmids which could then be transformed. One final method was utilized,

CRISPR-dCas9, which uses a catalytically “dead” Cas9 enzyme which is unable to produce double-stranded breaks at the target site. This then allows the dCas9 enzyme to act as a site-directed repressor, decreasing the number of transcripts produced.

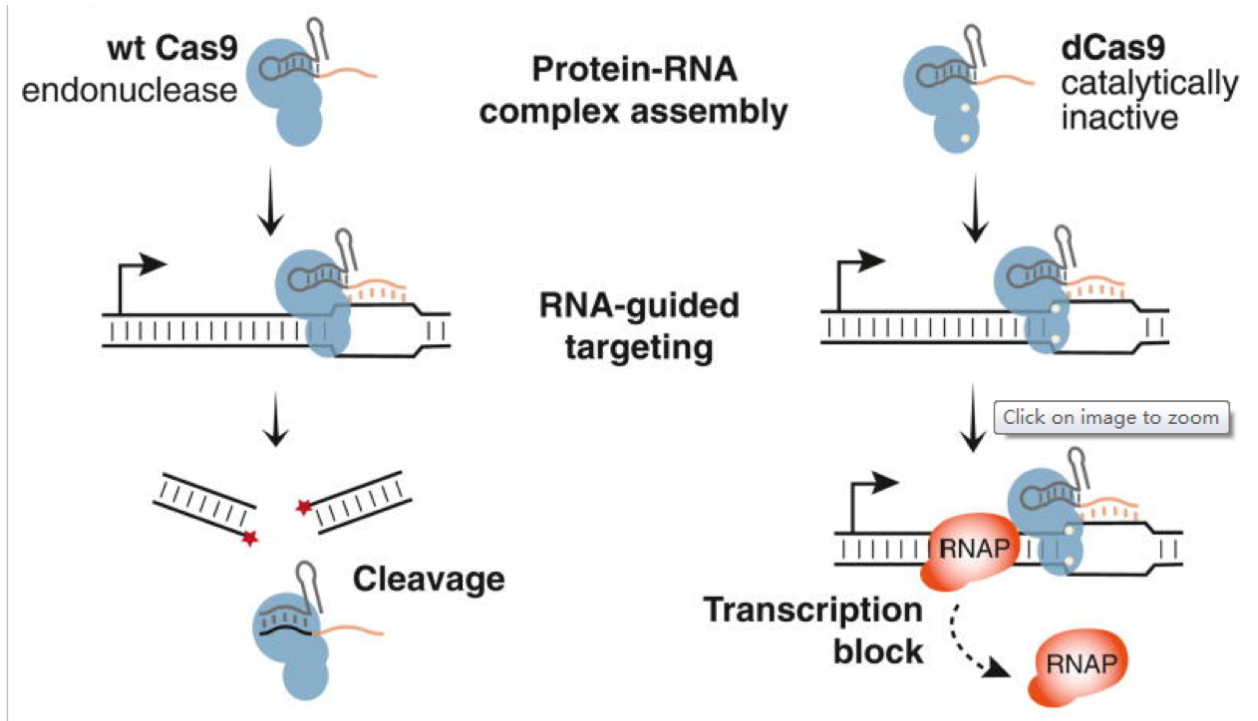


Figure 11. General overview of CRISPR-Cas9 vs CRISPR-dCas9. CRISPR-dCas9 contains mutations that prevent the enzyme from being able to catalyze double-stranded breaks at the target-site. Figure obtained from iGEM parts registry (Part:BBa_K1051800)

CRISPR-dCas9 plasmids were constructed for targeting *trx3*, *trx6* and *trx7*.

Strains involved in the following experiments are listed in the table below.

Strain	Description
DJL72	pDL734 plasmid integrated, dCas9 without guide RNA
DJL86	pDL536 plasmid integrated, dCas9 targeting coding sequence of <i>trx6</i>
DJL87	pDL537 plasmid integrated, dCas9 targeting coding sequence of <i>trx7</i>

DJL90	pDL533 plasmid integrated, dCas9 targeting upstream of <i>trx3</i>
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Oxidative Challenge Assay of *trx3*, *trx6*, and *trx7* Knockdowns

A previously developed challenge assay was used to test the survivability of the thioredoxin knockdown strains in comparison to pseudo-wildtype strain DJL72 that lacks a gRNA but expresses dCas9 (13). Cultures were grown to an OD₆₀₀ of between 0.4-0.75. At this point, the cells were harvested anaerobically for use in the assay. The cells were resuspended in media which did not contain cysteine or resazurin to a volume that would generate equivalent OD₆₀₀ for all the cultures. This media was used because cysteine could act as a reductant, preventing the oxidant from fully affecting the cells, and resazurin can exacerbate the oxidative stress. The cells were then tested in varying concentrations of hydrogen peroxide for 30 minutes. At this point, the cells were transferred into a microtiter plate following a serial dilution pattern to standard HS media which contained methanol and sulfide. The plates were put in a gasket sealed jar and allowed to incubate at 37°C aerobically while being monitored for growth.

Results

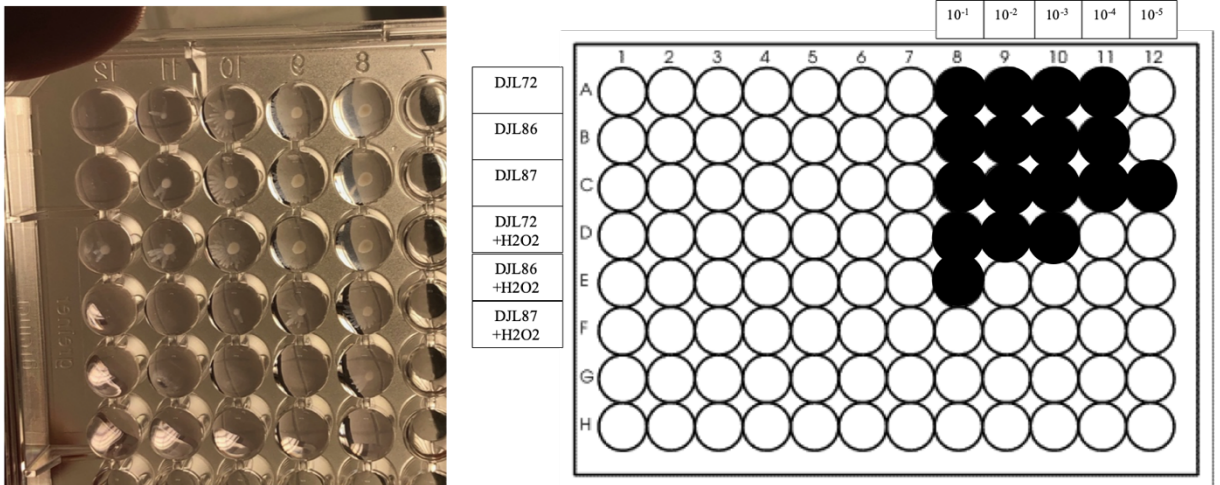
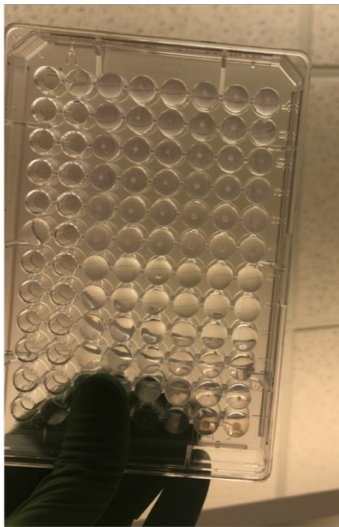


Figure 12. Depiction of growth observed on the microtiter plate 7-days post challenge, the microtiter place is seen on the right and an animated diagram of the plate showing black dots to represent the wells with growth. Challenge included exposure to H_2O_2 at a concentration of 3mM

Cells were harvested at late-log phase (between 0.7 and 0.9 OD_{600}) due to difficulty in timing of culture growth, and subjected to 3 mM H_2O_2 . Growth was analyzed 7 days post-challenge with diminished survivability in the groups exposed to hydrogen peroxide. The figure above shows the wells in which growth occurred, with a black dot representing growth. The unexposed group showed roughly similar survivability with regards to the control strain DJL72. When examining the test group, the thioredoxin knockdown groups were unable to overcome the oxidative challenge as well as the control strain did.



DJL 72, 0mM H ₂ O ₂
DJL 86, 0mM H ₂ O ₂
DJL 87, 0mM H ₂ O ₂
DJL 72, 1mM H ₂ O ₂
DJL 86, 1mM H ₂ O ₂
DJL 87, 1mM H ₂ O ₂

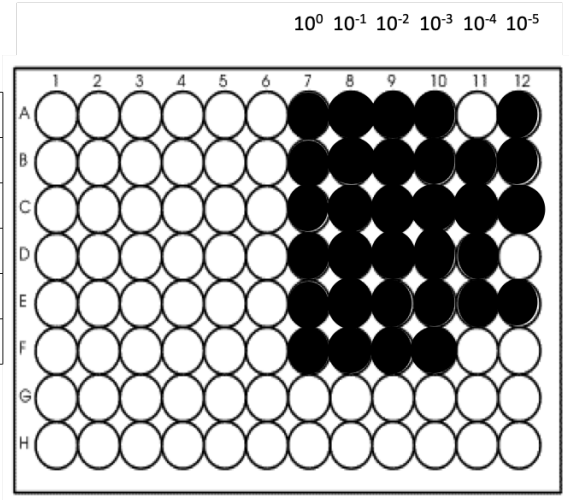


Figure 13. Depiction of growth observed on the microtiter plate 7-days post challenge, the microtiter place is seen on the right and an animated diagram of the plate showing black dots to represent the wells with growth. Challenge included exposure to H₂O₂ at concentrations of 0, 1, 2, and 3mM.



DJL 72, 2mM H ₂ O ₂
DJL 86, 2mM H ₂ O ₂
DJL 87, 2mM H ₂ O ₂
DJL 72, 3mM H ₂ O ₂
DJL 86, 3mM H ₂ O ₂
DJL 87, 3mM H ₂ O ₂

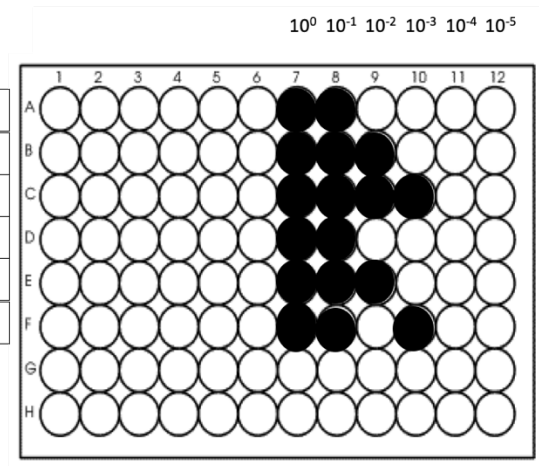


Figure 14. Depiction of growth observed on the microtiter plate 7-days post challenge, the microtiter place is seen on the right and an animated diagram of the plate showing black dots to represent the wells with growth. Challenge included exposure to H₂O₂ at concentrations of 0, 1, 2, and 3mM.

This growth study expanded on the effect of varying H₂O₂ concentrations on the survivability of thioredoxin knockdowns. Four concentrations of hydrogen peroxide were used; 0 mM, 1 mM, 2 mM, and 3 mM. Groups that were exposed to a higher concentration of hydrogen peroxide were not as able to overcome the oxidative challenge as those which were either not exposed to hydrogen peroxide or were exposed to 1 mM.

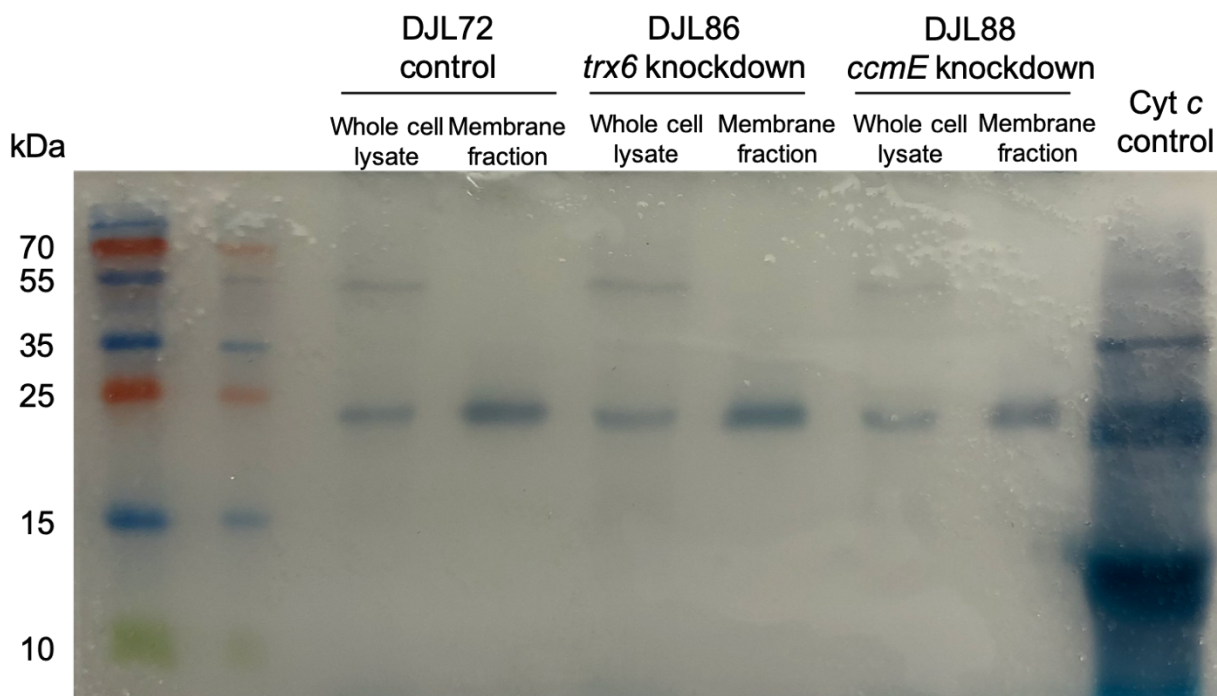


Figure 15. Results of preliminary heme staining conducted using *trx6* knockdowns. Image received from Melissa Chanderban via personal communication

A heme stain was done by Melissa Chanderban, a graduate student also working on the thioredoxin system. Trx6 was hypothesized to be important for the final reduction of cysteine residues in cytochrome *c* proteins just prior to heme insertion. However, based on the results of the heme staining no difference in heme content was seen between the *trx6* knockdown and the control stain DJL72.

Discussion

The challenge assays showed varying results. The first assay completed supported our hypothesis, with thioredoxin knockdowns showing less survivability when exposed to hydrogen peroxide as compared to the control strain. However, the subsequent assay showed sporadic growth, with the control strain DJL72 growing as well, or poorer than the thioredoxin knockdowns. This could have been the result of several factors. First, it was difficult to get similar growth across all the strains. It took several passes, and

several overgrown cultures before similar timed growth occurred among all the strains. Due to this difficulty, it was not uncommon to test strains of which one was just beginning logarithmic growth, and one that was about to plateau. Due to the difference in growth phases, different proteins could be in production that may help, or hinder, a cultures' ability to handle the oxidative burden presented by hydrogen peroxide. Another possibility is an experimental error. Some of the wells on the second challenge assay turned pink, which is the result of the chemical indicator resazurin being oxidized. This change occurred days after the microtiter plates were placed in the incubator. This likely means that oxygen made its way into the gasket-sealed jar. Oxygen exposure would have created another oxidative challenge that may have skewed the survivability. One final thing that could have happened is that due to the passing of the strains several times, a mutation occurred that prevented repression of the thioredoxin genes. This would result in growth similar to the control strain DJL72.

Further Experiments

There is much more to be accomplished when examining thioredoxins' potential role in oxidative stress and cytochrome *c* maturation. Progress has since occurred in increasing the efficiency in Gibson assembly through the use of a smaller vector. The best course of action is to utilize this protocol to generate marker-less deletions of *Δtrx3*, *Δtrx6*, and *Δtrx7*. In the meantime, qPCR will need to be used to ensure that transcriptional knockdown is occurring in the dCas9-targeted thioredoxin genes. If marker less deletions are able to eventually be made further experiments to elucidate thioredoxins' potential role in cytochrome *c* maturation and oxidative stress could occur.

Strains of *Δtrx3*, *Δtrx6*, and *Δtrx7* knockouts could be grown, and the cells harvested and used with a heme stain to visualize heme incorporation relative to a control strains.

References

1. Enzmann, F., Mayer, F., Rother, M., & Holtmann, D. (2018). Methanogens: biochemical background and biotechnological applications. *AMB Express*, 8(1). doi: 10.1186/s13568-017-0531-x
2. Galagan, J. E., Nusbaum, C., Roy, A., Endrizzi, M. G., Macdonald, P., FitzHugh, W., & Calvo, S. et al (2002). The Genome of *M. acetivorans* Revels Extensive Metabolic and Physiological Diversity. *Genome Research*. 12, 532-542.
3. Arshad, A., Speth, D. R., Graaf, R. M. D., Huub J. M. Op Den Camp, Jetten, M. S. M., & Welte, C. U. (2015). A Metagenomics-Based Metabolic Model of Nitrate-Dependent Anaerobic Oxidation of Methane by Methanoperedens-Like Archaea. *Frontiers in Microbiology*, 6. doi: 10.3389/fmicb.2015.01423
4. Wang, Mingyu, Jean Francois Tomb, and James G. Ferry 2011. Electron Transport in Acetate-Grown *Methanosarcina Acetivorans*. *BMC Microbiology* 11.
5. Lovley, D. R. (2017). Syntrophy Goes Electric: Direct Interspecies Electron Transfer. *Annual Review of Microbiology*, 71(1), 643–664. doi: 10.1146/annurev-micro-030117-020420
6. Liu, Jing, Saumen Chakraborty, Parisa Hosseinzadeh, et al. 2014. Metalloproteins Containing Cytochrome, Iron-Sulfur, or Copper Redox Centers. *Chemical Reviews*. American Chemical Society.
7. McCarver, A. C., Lessner, F. H., Soroeta, J. M., & Lessner, D. J. (2017). *Methanosarcina acetivorans* utilizes a single NADPH-dependent thioredoxin system and contains additional thioredoxin homologues with distinct functions. *Microbiology*, 163(1), 62–74. doi: 10.1099/mic.0.000406

8. Guerrero-Cruz, S., Cremers, G., Alen, T. A. V., Huub J. M. Op Den Camp, Jetten, M. S. M., Rasigraf, O., & Vaksmaa, A. (2018). Response of the Anaerobic Methanotroph “Candidatus Methanoperedens nitroreducens” to Oxygen Stress. *Applied and Environmental Microbiology*, 84(24). doi: 10.1128/aem.01832-18
9. Allen, J. W., Harvat, E. M., Stevens, J. M., & Ferguson, S. J. (2006). A Variant System I for Cytochrome *c* Biogenesis in Archaea and some Bacteria has a Novel CcmE and No CcmH. *FEBS Letters*, 580(20), 4827–4834. doi: 10.1016/j.febslet.2006.07.073
10. Thöny-Meyer, L., Fischer, F., Künzler, P., Ritz, D., & Hennecke, H. (1995). *Escherichia coli* Genes Required for Cytochrome *c* Maturation. *Journal of Bacteriology*, 177(15), 4321–4326. doi: 10.1128/jb.177.15.4321-4326.1995
11. Doudna, Jennifer A., and Emmanuelle Charpentier 2014. The New Frontier of Genome Engineering with CRISPR-Cas9. Science. American Association for the Advancement of Science.
12. Nayak, Dipti D., and William W. Metcalf 2017. Cas9-Mediated Genome Editing in the Methanogenic Archaeon *Methanosarcina acetivorans*. Proceedings of the National Academy of Sciences of the United States of America 114(11). National Academy of Sciences: 2976–2981.
13. Horne, A. J., & Lessner, D. J. (2013). Assessment of the oxidant tolerance of *Methanosarcina acetivorans*. *FEMS Microbiology Letters*, 343(1), 13–19. doi: 10.1111/1574-6968.12115