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Exploring Nitrogen Fixation in Methanosarcina acetivorans

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Exploring Nitrogen Fixation in *Methanosarcina acetivorans*

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

By

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Introduction

Methanogens are abundant anaerobic archaea and are the only organisms capable of producing methane as a part of their energy metabolism. Methane is an essential intermediate in the global carbon cycle, an important greenhouse gas, and a valuable biofuel. In addition to producing methane, methanogens are the only diazotrophic archaea meaning they are able to reduce inert dinitrogen (N_2) to biologically available ammonia (NH_3) so that it can be used in the cell.[1] The only organisms able to fix nitrogen are bacteria and methanogens, while eukaryotes rely on external sources such as fertilizer or symbiotic relationships with nitrogen fixers. This process, called biological nitrogen fixation, is completed by the enzyme nitrogenase and serves as an essential contributor to the global nitrogen cycle by producing approximately two-thirds of the fixed nitrogen on Earth.[2]

Nitrogenase functions uniquely to complete electron reduction reactions of inert molecules (eg. N_2 or CO_2) and can be categorized into three distinct types in diazotrophs: molybdenum-nitrogenase, vanadium-nitrogenase, and iron-nitrogenase (Table 1). Molybdenum-nitrogenase is the most researched and commonly used complex by diazotrophs while vanadium-nitrogenase and iron-only nitrogenase serve as alternatives when Mo is unavailable.[2] In order to produce functioning nitrogenase enzymes, several factors are required. The Mo-, V-, and Fe-only nitrogenases are related structurally, mechanistically, and phylogenetically, but vary genetically in the encoding gene clusters.[3] The structure of Mo-nitrogenase is encoded by *nifH*, *nifD*, and *nifK* genes. V-nitrogenase is encoded via *vnfH, vnfD, vnfG, and vnfK* genes, and Fe-only nitrogenase is produced by *anfH, anfD, anfG, and anfK* genes. The gene clusters are grouped as *nif*, *vnf*, and *anf* respectively; the H subunit encodes the iron protein and requires ATP to donate electrons to the subunits D and K which contain the enzyme catalytic site. The E and N subunits act as the scaffold where the active site cofactor is assembled before being transferred to subunits D and K.[4] The process of nitrogen fixation is dependent on large amounts of ATP and electrons varying based on the catalytic cluster. The preferred Mo-nitrogenase consumes 16 ATP, while V-nitrogenase uses 24 ATP and Fe-only nitrogenase requires a minimum of 40 ATP molecules.[2]

Table 1. The three genetically distinct nitrogenase systems.

	Components		
System	Reductase	Dinitrogenase	Reaction
Mo	NifH	NifDK (MoFe)	$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP$
V	VnfH	VnfDGK (VFe)	$N_2 + 12H^+ + 12e^- + 24ATP \rightarrow 2NH_3 + 3H_2 + 24ADP$
Fe	AnfH	AnfDGK (FeFe)	$N_2 + 20H^+ + 20e^- + 40ATP \rightarrow 2NH_3 + 7H_2 + 40ADP$

The model methanogen used for this research is *Methanosarcina acetivorans* due to its diverse metabolic pathways and robust genetic system. *M. acetivorans* has the *nif* operon to encode molybdenum-nitrogenase as well as the *vnf* and *anf* operons to encode V- and Fe-only nitrogenase alternatives, making it one of the few methanogens to have complete gene clusters for all three nitrogenases (Figure 1).[1] Since the fundamental goal of any organism, *M. acetivorans* included, is to grow and divide as efficiently as possible, nitrogenase is strictly regulated and only produced in the absence of a usable nitrogen source such as ammonia (NH_3).[1]

As of 2016, the genomes of over sixty species of methanogenic archaea have been sequenced.[4] Research on the genomes of methanogens such as *M. acetivorans* led to the characterization of numerous genes with unknown functions. Genes similar to those of nitrogenase have been shown to possess functions unrelated to nitrogen fixation, such as F_{430} biosynthesis genes.[5,6,7] My research focused on the uncharacterized gene clusters *ma2032-33* and *ma1631-33* (Figure 1). These genes have conserved nitrogenase domains: *ma2032* has fused *nifH* and *nifD* domains, while *ma2033* has a *nifD* domain. The *ma1631-33* gene cluster contains two nitrogenase homologs and one iron protein homolog. I hypothesized the *ma2032-33* and *ma1631-33* clusters are not involved in the process of nitrogen fixation in *M. acetivorans* because of the numerous nitrogenase-like genes that have unrelated functions.

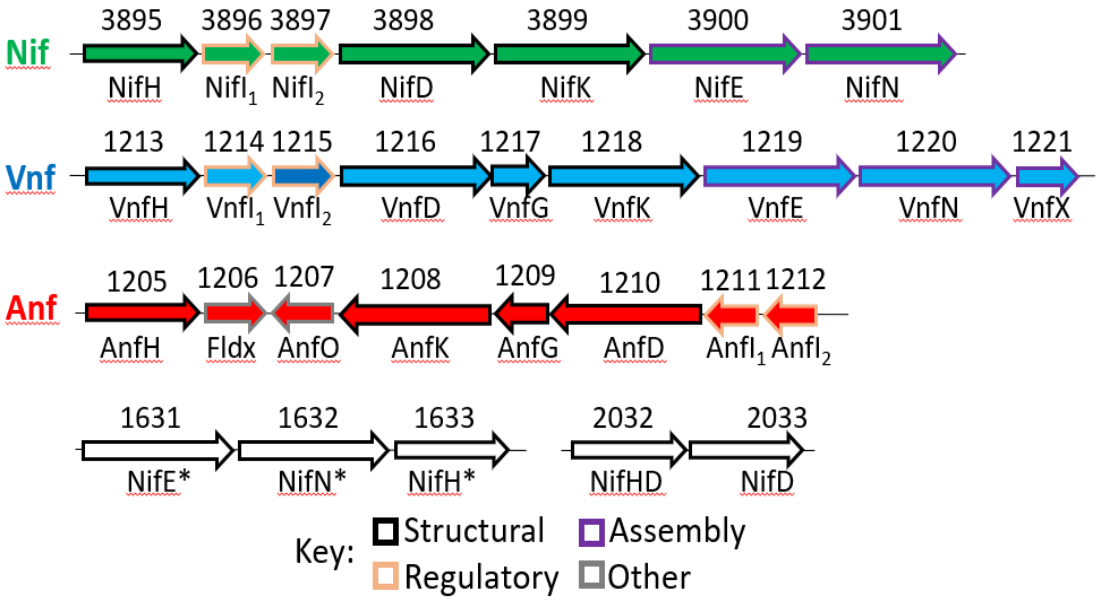


Figure 1: The *M. acetivorans nif, vnf,* and *anf* gene clusters compared to the nitrogenase-like gene clusters *ma1631-33* and *ma2032-33*. Arrows represent genes, and the outline of the arrow indicates predicted functions of the genes. The numbers above each gene are the locus tags and below is the predicted protein. This figure shows the similarities of our targeted genes to those encoding the structural components of Mo-nitrogenase.

To test this hypothesis, I used the recently developed CRISPRi-dCas9 system to generate *M. acetivorans* strains in capable of expressing either *ma1631-33* or *ma2032-33*. The system is similar to the CRISPR-Cas9 system, in which a short RNA sequence (a guide RNA, or gRNA) targets the Cas9 endonuclease to a complementary DNA sequence which is subsequently cleaved.[8] Essentially, dCas9, a catalytically dead Cas9 that cannot cleave DNA, creates a CRISPRi-dCas9 system able to block transcription of targeted genes and operons in *M. acetivorans*.[1] Generating customized gRNA coupled dCas9 for *ma2032* and *ma1631* respectively allows the ability to assess the importance of these genes.

Materials and Methods

Overall Components of Creating Knockdown Strains

Step One: Generate a plasmid containing dCas9 and gRNA to target each gene

Step Two: Use Gibson assembly to insert gRNA-containing gBlocks into CRISPRi-dCas9 plasmid

Step Three: Transform *E. coli* and select colonies using chloramphenicol resistance

Step Four: Perform colony PCR to screen for gBlock insertion into the plasmid

Step Five: Purify plasmid from *E. coli* and confirm correct plasmid sequence

Step Six: Transform *M. acetivorans* and select colonies using puromycin resistance

Step Seven: Perform colony PCR to screen for plasmid integration into the chromosome

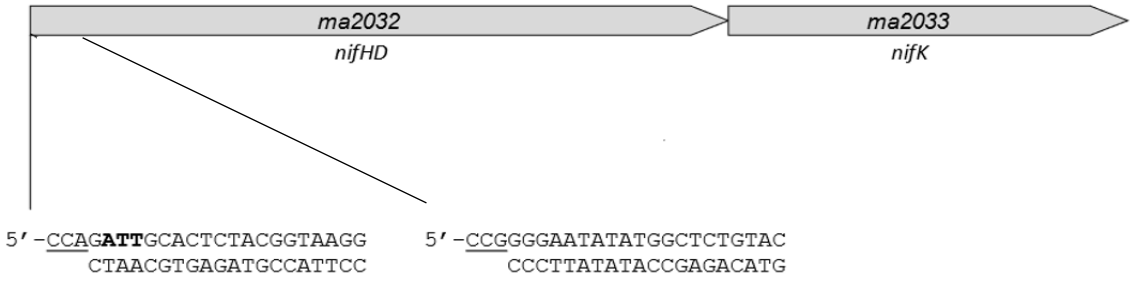
Step Eight: Use quantitative PCR to assess repression of target gene

Step Nine: Use knocked down *M. acetivorans* strains for phenotype analysis

CRISPRi-dCas9 Plasmid Construction

We generated two plasmids that contain dCas9 and a guide RNA to target *ma2032-33* and *ma1631-33* to repress transcription, designed using Geneious Prime. It is likely that *ma2032-33* and *ma1631-33* are operons, indicating that targeting the first gene should stop transcription of the whole gene cluster (Figures 2 and 3). During our research, a single gRNA was unable to completely silence the gene clusters, so a second gRNA was added to the first plasmids to form a dual-knockdown strain for both *ma2032-33* (Figure 2) and *ma1631-33* (Figure 3). Synthetic DNA oligos, or short single-stranded

DNA, were designed for assembly (IDT) with CRISPRi-dCas9 plasmid pDL734. We linearized 1 μg of plasmid pDL734 (7.52 μL) using 2 μL 10x CutSmart buffer, 1 μL restriction enzyme AscI, and 9.48 μL H_2O.



ma2032 gRNA 1: CCTTACCGTAGAGTGCAATC with activity score ~ 0.154

ma2032 gRNA 2: GTACAGAGCCATATATTCCC with activity score ~ 0.806

Figure 2: picture of *ma2032-33*. Bold ATT indicates the start codon, and the underlined CCA and CCG indicate the Protospacer adjacent motif (PAM) which is required for Cas nuclease to know where to cut. gRNA #1 and gRNA #2 are approximately 400 base pairs apart.

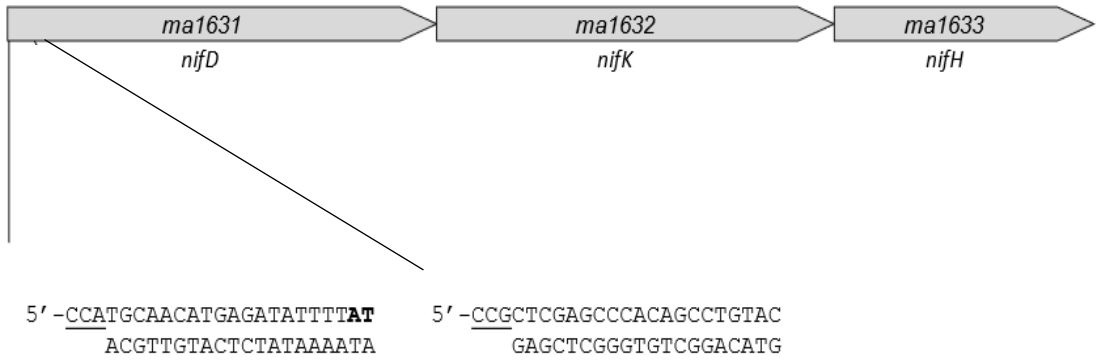


Figure 3: picture of *ma1631-33*. Bold AT indicates the start codon, and the underlined CCA and CCG indicate PAM sequence.#1 and gRNA #2 are approximately 250 base pairs apart.

ma1631 gRNA 1: ATAAAATATCTCATGTTGCA with activity score ~ 0.150

ma1631 gRNA 2: GTACAGGCTGTGGGCTCGAG with activity score ~ 0.683

Table 2: Primers Used

Primer Name	Sequence 5' – 3'
PCR Amplification of gBlock	
gBlock_HDR_For…….	GTTTAAACCTGCAGGCGCGCCAACAACATCAGTCA
gBlock_HDR_Rev……	AGTGAGTCGTATTAATTAAGCGGCCGCGATCG
Hpa.promoter.F ……	GACAAAATACCTGGTTACCCAGGCCGTGCCGGCA CGTTAACAACAACATCAGTCACCTA
Hpa.terminator.R……..	CTCGACAGCGACACACTTGCATCGGATGCAGCC CGGTTAACTACATGAGGGCTGAAAAGC
Confirmation of Gibson assembly	
CasHDRSeq.F …….	cag cta gct aac gcg tat taa agg
Cas9NHEJ.R…….	GCCATTTTTCTTCTCACCGGGGAGCTGAGC
Hpa.promoter.F (as listed) used to screen for gBlock integration at the 2nd site	
Hpa.terminator.R (as listed) used to screen for gBlock integration at the 2nd site	
Sequencing	
CasNHEJ_seq_rev……	TATCGATCGCGCGCAGATCCGAACG
Hpa.terminator.R (as listed)	
Conformation of Plasmid Integration	
C2Achr2……	ttg att cgg ata ccc tga gc
Plscreen3……...	gca aag aaa agc cag tat gga
Quantitative PCR	
ma2032_qPCR_For…..C	TCGGTGACCTTGAGCTTGA
ma2032_qPCR_Rev….	GCAGAGGGAACAGAGCTCC
ma1631_qPCR_For….	CGCCCTCAACGAAAGCCA
ma1631_qPCR_Rev….	CCGGAACAGCAGCAAGAAAG
16sRNA.qPCR.F……..	GGTACGGGTTGTGAGAGCAA
16sRNA.qPCR.R…….	CTCGGTGTCCCCTTATCACG

Table 3: Plasmids and Strains Used

Plasmids used in this study

pDL734 – CRISPRi-dCas9 backbone plasmid

pDL540 – pDL734 with single gRNA targeting *ma1631*

pDL542 – pDL734 with single gRNA targeting *ma2032*

pDL544 – pDL734 with dual gRNAs targeting *ma2032*

pDL545 – pDL734 with dual gRNAs targeting *ma1631*

pDL535 – plasmid used as positive control for transformation

Strains used in this study

WWM73 – *Methanosarcina acetivorans* parent strain

DJL 72 – Control strain with no gRNA

DJL 121 – Contains integrated pDL540 (targets *ma1631*)

DJL 123 – Contains integrated pDL542 (targets *ma2032*)

DJL 125 – Contains integrated pDL544 (targets *ma2032*)

DJL 126 – Contains integrated pDL545 (targets *ma1631*)

Gibson Assembly

Double-stranded synthetic DNA fragments, called gBlocks, and AscI-digested pDL734 were assembled using Gibson assembly Ultra Kit (Synthetic Genomics). Gibson assembly combines multiple DNA fragments using 5' exonuclease to chew back the fragment ends, DNA polymerase to fill in the gaps, and DNA ligase to seal the nicks.[9] Gibson assembly reaction mixes were used to transform *Escherichia coli* as described below.

After an unsuccessful attempt, we did PCR to amplify the *ma2032* gBlock ends in an attempt to troubleshoot Gibson assembly. The 24 μL PCR master-mix was created using 15.75 μL H_2O, 5 μL 5x Q5 buffer, 0.5 μL 10 mM dNTP, 1.25 μL 10 μM gBlock

HDR forward, 1.25 μL 10 μM gBlock HDR reverse, 1μL gBlock, and 0.25 μL Q5 polymerase. 1 μL of plasmid pDL535 was used as the positive control. PCR cycled at 98°C for 30 seconds, the thirty cycles of 98°C for 10 seconds, 67.1°C for 30 seconds, and 72°C for 20 seconds, followed by a cycle of two minutes at 72°C . After PCR, we purified the DNA sample using New England BioLabs Monarch Nucleic Acid Purification Kit with no major changes to the provided instructions in an attempt to wash away enzymes for a more effective Gibson assembly. We repeated AscI plasmid pDL734 digestion by the methods listed above and repeated Gibson assembly using a new Gibson assembly Ultra Kit from Synthetic Genomics and the new PCR product.

In making the dual-knockdown strains, we first did PCR to change the gBlock ends for plasmid integration into the HpaI site of pDL734 by allowing overlap with pDL540 and pDL542 after *HpaI* digestion. A 24 μL master-mix was created for each of three reactions, one with the new gBlock, one positive control, and one negative control. We used 15.75 μL H_2O, 5 μL 5x Q5 buffer, 0.5 μL 10mM dNTP, 1.25 μL 10mM hpa promoter F, 1.25 μL 10 mM hpa terminator R, 0.25 μL Q5 polymerase to make the master mix and added 1 μL gBlock. PCR cycled at 98°C for 30 seconds, the thirty cycles of 98°C for 10 seconds, 72°C for 30 seconds, and 72°C for 20 seconds, followed by a cycle of two minutes at 72°C.

To create the dual-knockdown strains using the Gibson assembly Ultra Kit from Synthetic Genomics, we created four individual Gibson assembly reactions with 5μL provided master mix, 0.73 μL pDL734, 1 μL of gBlock for *ma2032* or *ma1631*, and 3.27 μL H_2O. Two reactions used 1 μL pDL540 and pDL542, so H_2O was adjusted to 3 μL to create a 10 μL reaction. The four reactions were set up as pDL734 + *ma1631*, pDL734 +

ma2032, pDL 540 + *ma1631*, and pDL542 + *ma2032*. To improve Gibson assembly transformation, we used a pellet paint co-precipitant.

First, we thawed the pellet paint and sodium acetate to room temperature and inverted the pellet paint to suspend it. We added 2 μL of pellet paint and 1.5 μL sodium acetate to a tube before adding 44 μL EtOH. This incubated at room temperature for two minutes, then we centrifuged 16,000 g for five minutes. After this step, a bright pink pellet was observed in the tube. We removed the supernatant using a pipette and rinsed the pellet with 60 μL 70% EtOH then vortexed and spun again. We again removed the supernatant before repeating the rinse with 100% EtOH. We allowed it to air dry to remove residual EtOH, then resuspended the pellet in 10 μL H_2O and added all 10 μL to 50 μL of competent cells.

Transformation of *E. Coli*

The assembly mix was used to transform *Escherichia coli* strain WM4489 using the heat shock method.[10] This method allows plasmid DNA to be inserted to the *E. coli* competent cells by incubating a mixture of cells and DNA on ice. We then heat shocked the mixture by placing them at 42°C for 45 seconds, then putting them back on ice. We added SOC media to the transformed cells and incubated them at 37°C for thirty minutes. After this step, we plated the cells on LB media with 34 μg/mL chloramphenicol antibiotic to select for colonies containing the plasmid.

PCR Screening & Sequencing

E. coli transformants were screened using PCR and 1% agar gel electrophoresis of PCR products to confirm the gBlock was integrated into the plasmid. We performed

colony PCR using 16.7 μL H_2O, 2 μL 10x Taq buffer, 0.4 μL 10 mM dNTP, 0.4 μL 10 μM Cas HDR seq, 0.4 μL 10 μM Cas NHEJ rev, 0.1 μL Taq polymerase, and the transformed colonies alongside a positive and negative control. PCR included 94°C for five minutes, thirty cycles of 94°C for 30 seconds, 57°C for 45 seconds, and 72°C for thirty seconds, then a seven-minute cycle at 72°C . Gel electrophoresis used 5 μL ethidium bromide, 1x TAE buffer, and agar.

Once gBlock insertion into the plasmid in *E. coli* was confirmed by PCR screening of transformants, the plasmids were purified from *E. coli* using New England Biolabs Monarch Plasmid Miniprep Kit and DNA Cleanup Kit, then sent to be sequenced (Eurofins Genomics). This confirmed that the sequences were correct, and the plasmids could be used to transform *M. acetivorans* to create knockdown strains targeting *ma2032* and *ma1631*.

Transformation of *M. acetivorans*

We used plasmids pDL540 and pDL542 with the correct sequence to transform *Methanosarcina acetivorans* using the liposome-mediated method to create a knockdown strain for each gene cluster.[11] 10 mL WWM73 was harvested and resuspended in 1 mL bicarbonate-buffered sucrose. DNA-liposome complexes were formed by a mixture of 25 μL DOTAP in 100 μL of bicarbonate-buffered sucrose with 2 μg of plasmid DNA in 50 μL bicarbonate-buffered sucrose. This incubated for 60 minutes at room temperature, then 1mL of resuspended cells were added to the DNA-liposome complex and incubated for four hours at room temperature. Cells were recovered in high-salt medium overnight at 37°C , then were plated on selective media with 2 μg/mL puromycin antibiotic to ensure only our transformant cells grew.

The media was incubated at 35°C until colonies formed, which were then grown in liquid media. We performed colony PCR to screen for plasmid integration into the chromosome of *M. acetivorans* by previous methods using 16.3 μL H_2O, 2 μL10x Taq buffer, 0.4 μL 10 mM dNTP, 0.4 μL 10 μM primer c2Achr2, 0.4 μL 10 μM primer Plscreen3, and 0.1 μL Taq polymerase for the master mix per reaction. 0.4 μL cells were obtained from resuspending 500 μL pellet into 20 μL H_2O. This cycled at 94°C for five minutes, thirty cycles of 94°C for 30 seconds, 52°C for 45 seconds, and 72°C for 30 seconds, followed by 72°C for three minutes.

Growth of *M. acetivorans*

We grew all *M. acetivorans* in Balch tubes prepared with 10mL HS medium[12] containing 125 mM methanol and 0.025% sodium sulfide and maintained it with 2 μg/mL puromycin as needed. To evaluate the roles of each gene cluster in nitrogen fixation, *M. acetivorans* strains were grown in NH_4Cl-free medium. 18 mM NH_4Cl was added to growth experiments as needed using sterile syringes. Growth was measured using a spectrophotometer to record optical density at 600 nm.

To assess involvement of *ma2032-33* and *ma1631-33* in nitrogen fixation, we grew our modified *M. acetivorans* strains in the presence and absence of fixed nitrogen. The reduced nitrogen source in this study was NH_4Cl, and the positive control was DJL72 with no gRNA. We used twenty-four tubes to make four biological replicates per strain per nitrogen source. The media used was HS, DTT-reduced, NH_4Cl free, and we added 50 μL 125 mM MeOH, 100 μL 0.025% N_2S, and 2 μL of cells to each tube, and 100 μL of 1 mg/mL NH_4Cl to half of the tubes. The tubes were incubated at 37°C and growth was recorded two times per day for four days using a spectrophotometer to

measure optical density at 600 nm, and the data was analyzed in Microsoft Excel (Figure 12).

RNA Extraction for cDNA Synthesis

To confirm each strain was knocked down using qPCR, we first purified and extracted RNA for cDNA synthesis. Cultures were grown in biological triplicate to an OD_{600} of approximately 0.4 (mid-log phase). 4 mL were harvested anaerobically, resuspended in 1 mL Trizol and frozen at -80˚C. We used the ZYMO Research Direct-zol RNA Miniprep Kit to purify and extract RNA according to the provided instructions for *ma2032* and *ma1631*. To ensure all DNA was gone, we used one additional DNase treatment (Invitrogen DNA-free kit) to further degrade the DNA. We checked the RNA concentrations using the Thermo Scientific NanoDrop 2000 spectrophotometer. Our ten-reaction master mix for DNase digestion was created using 10 μL of DNase and 50 μL buffer. Based on the recorded concentrations of RNA, we calculated varying amounts of H_2O and RNA to create nine 50 μL reactions that included 5 μg RNA, 6 μL master mix, and H_2O. DNase was removed using inactivation reagent.

For cDNA synthesis to use in qPCR, we first created two master mixes (BioRad). The first was a ten-reaction Reverse Transcriptase (RT) master mix using 10 μL of RT, 40 μL of reaction mix, and 20 μL of primers. The second master mix acted as a negative control and contained no Reverse Transcriptase (NRT). The NRT mix used 16 μL of reaction mix and 8μL of random primers. We created nine individual 20μL reactions using 7 μL RT master mix, H_2O, and 300 ng of RNA using the concentrations from the Thermo Scientific NanoDrop 2000 spectrophotometer. Three 20 μL reactions were created using 6 μL of NRT master mix, 300 ng of RNA, and H_2O.

Quantitative PCR Analysis of Target Genes

Using a 50-fold dilution of cDNA, we performed qPCR to assess gene regulation changes between carbon sources MeOH, TMA, and NaOAc. Each reaction used 2.75 µL of H_2O, 1.65 µL of cDNA, 0.6 µL of primer, and 5 µL of supermix (BioRad). Overall, we created enough master mix for twenty-six 16s reactions, twenty *ma2032* reactions, and twenty *ma1631* reactions to have two technical replicates for each combination of primers and carbon sources. For qPCR of nitrogen sources, our mixes included 55 µL H_2O, 12 µL of 1 µM primers, and 100 µL of supermix with a 50-fold dilution of cDNA. qPCR data were analyzed using the comparative threshold cycle ($\Delta\Delta Cq$) method[13].

Results & Discussion

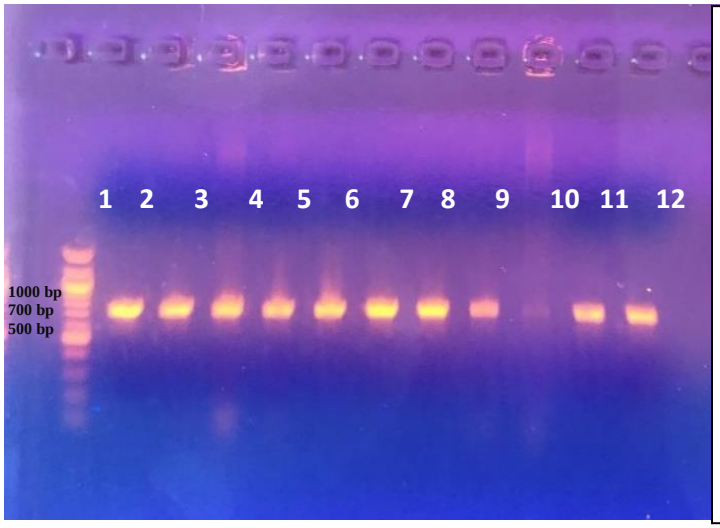


Figure 4: Colony PCR products confirming gBlock insertion into the plasmid in *E.coli* for the first gRNA. From left to right: ladder, lane 2-5 strains irrelevant to this project, lanes 6-7 two transformed *E. coli* colonies containing pDL540 (targeting *ma1631*) and lanes 8-9 *E. coli* colonies containing pDL542 (targeting *ma2032*). Lanes 10-12 are irrelevant to this study.

Figure four shows the gel electrophoresis of PCR products from chloramphenicol selected *E. coli* colonies after plasmid pDL734 (used to build knockdown strains) was AscI digested and Gibson assembly was performed to insert the gBlock containing the first gRNA sequence into each plasmid. We generated two plasmids, pDL540 and

pDL542, that contain dCas9 and a guide RNA to target *ma2032-33* and *ma1631-33* respectively to repress transcription. Positive bands in the gel confirmed the gBlock was inserted into the plasmid, indicating the plasmid could be purified from *E. coli* and sent to be sequenced.

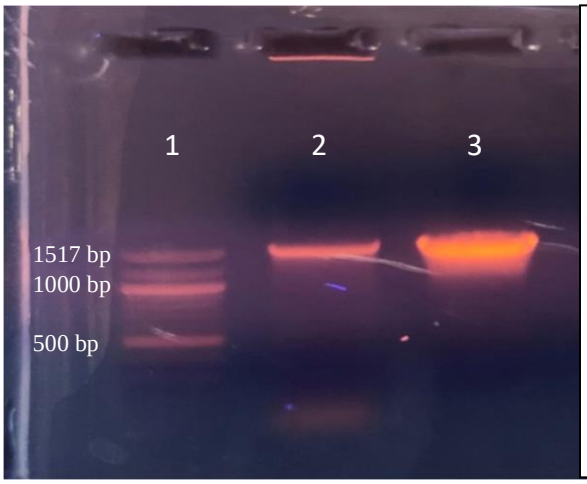


Figure 5: Products of Colony PCR after transformation and growth of *M. acetivorans* for strains containing the plasmid with the first gRNA. From left to right: lane one is the ladder, lane two is a *M. acetivorans* colony of strain DJL121 targeting *ma1631*, and lane three is a *M. acetivorans* colony of strain DJL 123 targeting *ma2032*.

Figure five shows the gel electrophoresis of PCR products from *M. acetivorans* colonies selected from growth on puromycin. The positive bands in the gel indicate the plasmid is integrated into the chromosome in both *M. acetivorans* strain DJL121 (gRNA targeting *ma1631*) and *M. acetivorans* strain DJL123 (gRNA targeting *ma2032*). These results indicate that the modified strain can now be used in growth studies to assess phenotypic changes. In our study, qPCR of these *M. acetivorans* strains showed that, though the plasmid was integrated into the chromosome, the genes were not silenced (Figure 6). Potential explanations for why the first gRNA was unable to silence the genes could be the lower activity score (relative to the second gRNA) or a suppressor mutation to prevent the binding of dCas9 in our system.

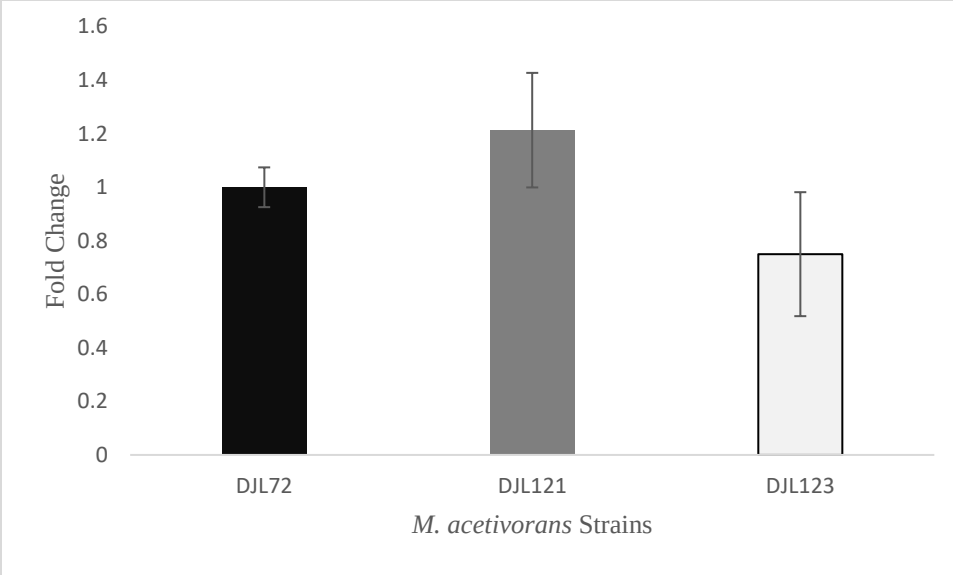


Figure 6: qPCR results of single gRNA strains DJL121 (targeting *ma1631*) and DJL123 (targeting *ma2032*) compared to control strain DJL72. Results show neither strain has the targeted gene silenced. p-values are not significant.

Since a single gRNA was ineffective in silencing gene clusters *ma2032-33* and *ma1631-33*, we made the decision to create a second gRNA and attempt to use both in a dual-knockdown strain of *M. acetivorans* silencing each gene. We generated two plasmids, pDL544 and pDL545, that contain dCas9 and dual guide RNAs to target *ma2032-33* and *ma1631-33* respectively to repress transcription.

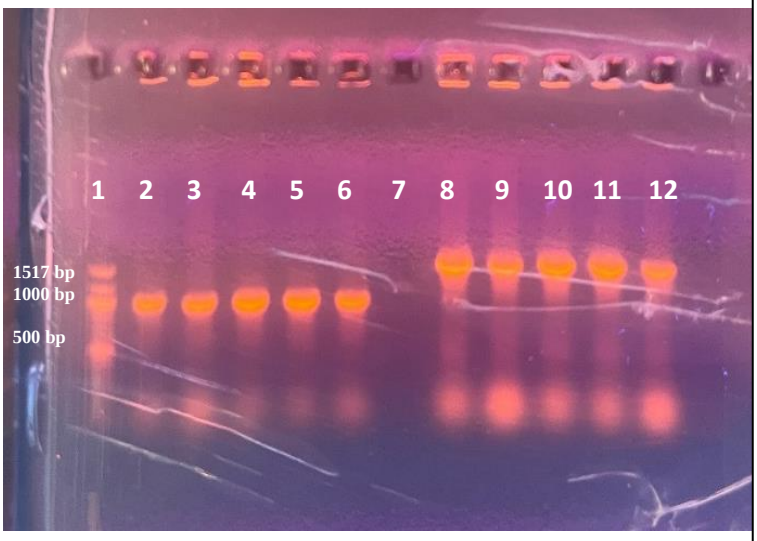


Figure 7: Colony PCR confirming gBlock integration into the plasmid in chloramphenicol selected *E. coli* colonies containing the second gRNA. From left to right: ladder, lanes 2-6 are five *E.coli* colonies containing pDL544 (plasmid with dual gRNAs targeting *ma2032*) and lanes 8-12 are five *E. coli* colonies containing pDL545 (plasmid with dual gRNAs targeting *ma1631*.)

Figure seven shows the gel electrophoresis of PCR products from chloramphenicol selected *E. coli* colonies after Gibson assembly and transformation. Positive bands indicate that the second gRNA-containing gBlock is integrated into the plasmid to create the dual gRNA knockdown plasmid. pDL544 was the plasmid pDL734 with dual gRNAs targeting *ma2032*, and pDL545 was the plasmid pDL734 with dual gRNAs targeting *ma1631*. Since these results confirmed the gBlock was integrated into each plasmid, the plasmid was purified from *E. coli*, sequenced, and used to transform *M. acetivorans* to create the knockdown strains.

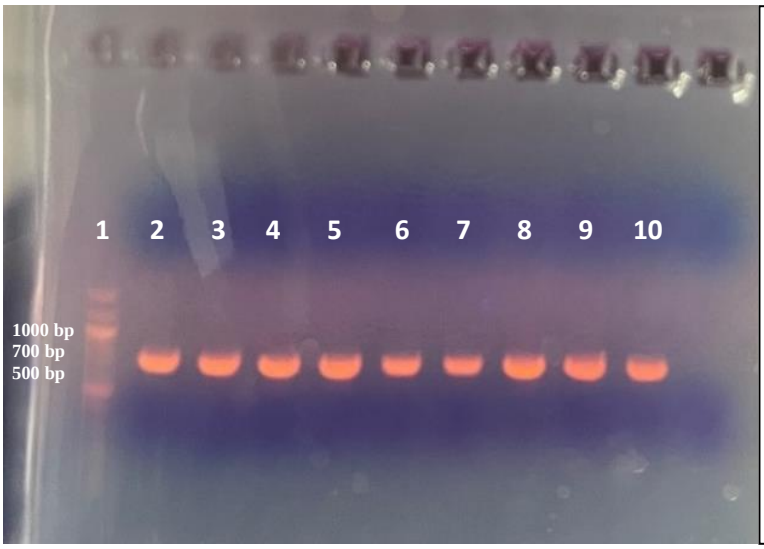


Figure 8: Colony PCR products confirming plasmid integration into the chromosome in *M. acetivorans* dual knockdown strains. From left to right: lane one is the ladder, lanes 2-4 are three colonies of DJL123 (*M. acetivorans* knockdown strain containing only the first gRNA targeting *ma2032)*, lanes 5-7 are three colonies of DJL125 (*M. acetivorans* dual gRNA knockdown strain targeting *ma2032*,) and lanes 8-10 are 3 colonies of DJL126 (*M. acetivorans* dual gRNA knockdown strain targeting *ma1631*.)

Figure eight shows the gel electrophoresis of PCR products from puromycin selected *M. acetivorans* colonies containing our dual gRNA knockdown system. Lanes 5-7 show plasmid integration into the chromosome of knockdown strain DJL125 targeting *ma2032*. Plasmid pDL544 was used in the dual gRNA knockdown targeting *ma2032* in strain DJL125, and plasmid pDL545 was used in the dual gRNA knockdown targeting *ma1631* in strain DJL126 shown in lanes 8-10. These results indicate that the knockdown *M. acetivorans* strains could then be used in phenotypic analysis studies, such as our growth

experiment with varying sources of nitrogen. Since the plasmid was confirmed in the chromosome, we performed qPCR on both DJL125 (targeting *ma2032*) and DJL126 (targeting *ma1631*) which confirmed both genes were knocked down (Figure 9).

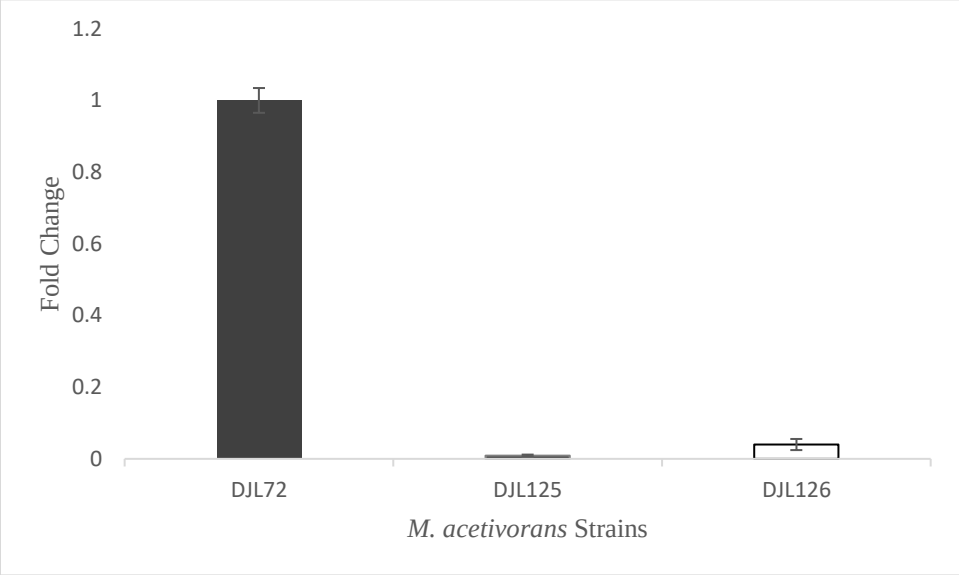


Figure 9: qPCR results of dual gRNA strains DJL 125 (targeting *ma2032*) and DJL 126 (targeting *ma1631*) compared to control strain DJL72. Results confirmed both strains are silenced. P-values are significant at 0.000199 (DJL125) and $1.017*10^{-6}$ (DJL 126).

Figure 9 shows qPCR confirmed a decrease in the relative expression of *ma2032* and *ma1631* in dual gRNA-*ma2032* and gRNA-*ma1631* strains compared to the control, indicating a successful knockdown using the dual-gRNA methods. Figures 10 and 11 compare the fold change between the single gRNA knockdown and dual gRNA knockdown compared to the control strain for *ma2032* and *ma1631* respectively.

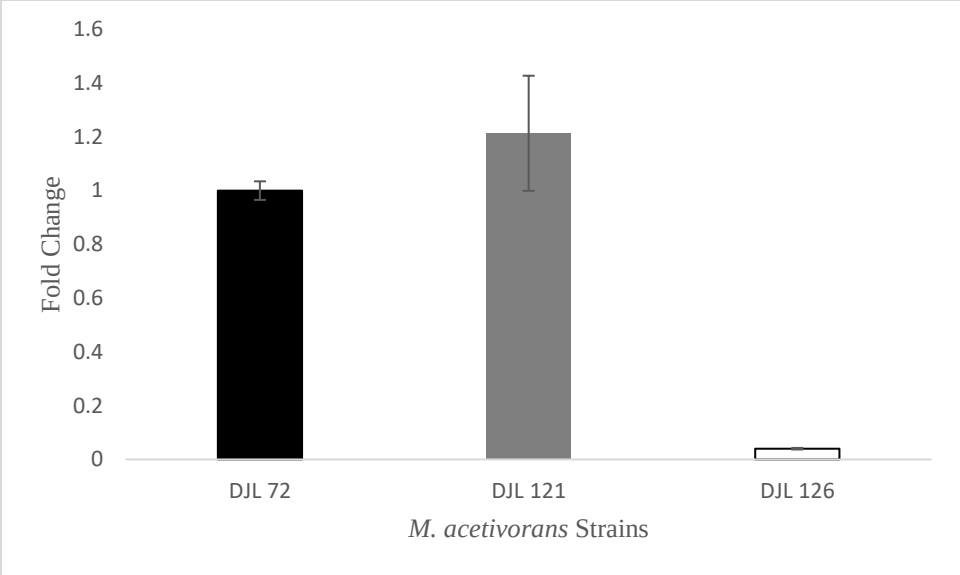


Figure 10: qPCR results of single gRNA strain DJL121 and dual gRNA strain DJL126 each targeting gene *ma1631* compared to control strain DJL72 with no gRNA.

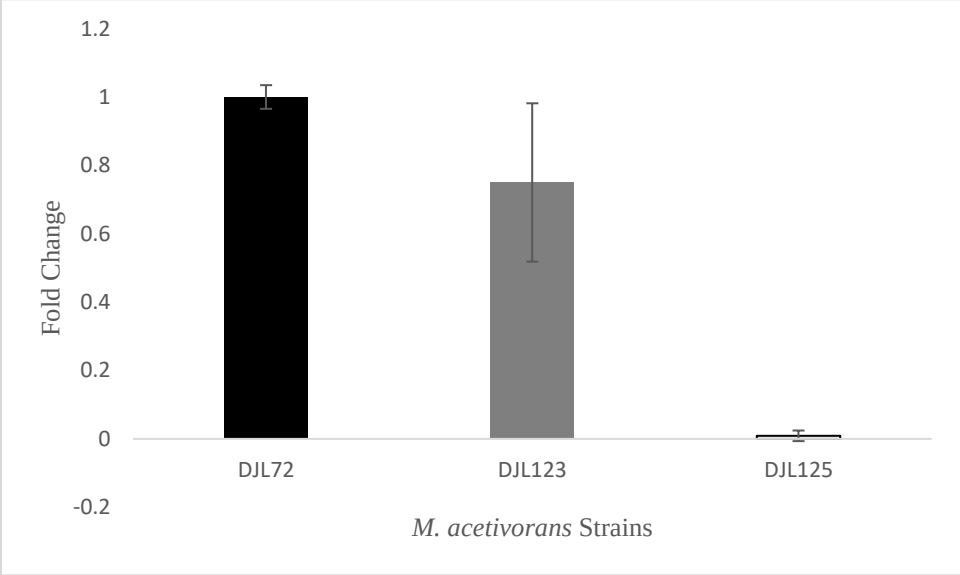


Figure 11: qPCR results of single gRNA strain DJL123 and dual gRNA strain DJL125 each targeting gene *ma2032* compared to control strain DJL72 with no gRNA.

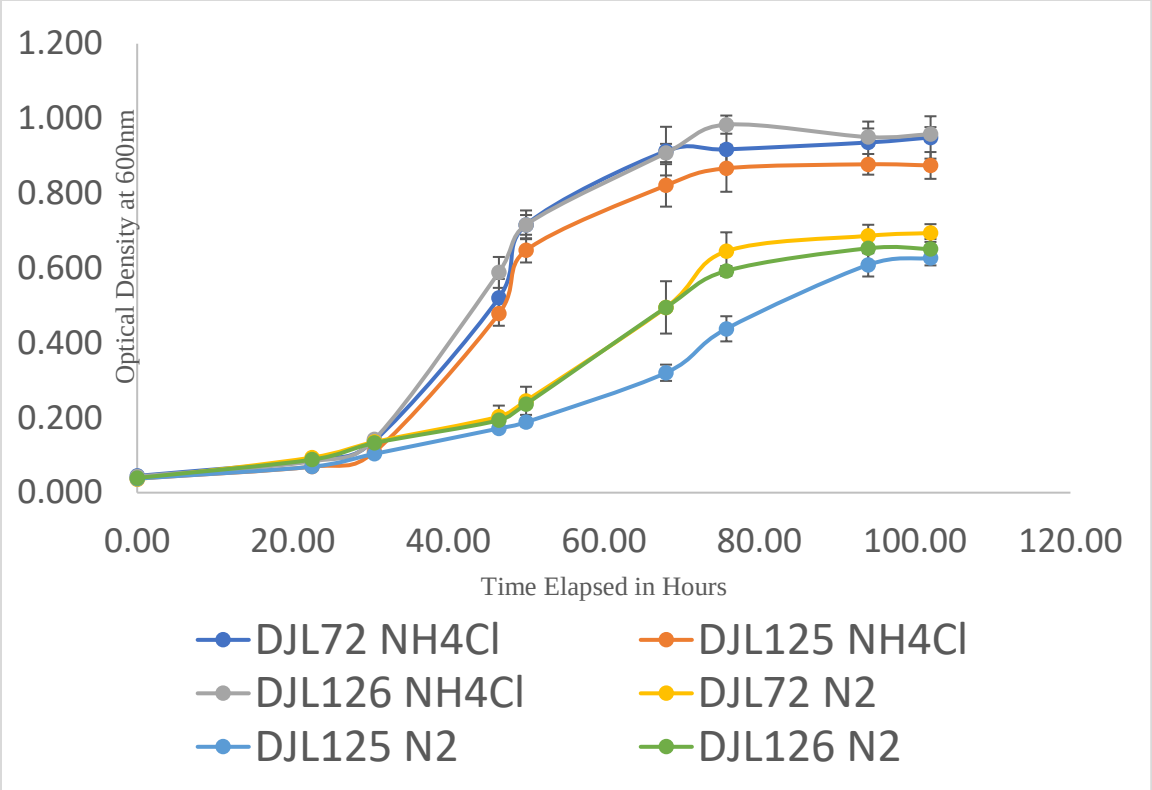


Figure 12: Growth curve of DJL125 (dual knockdown dCas9 targeting *ma*2032), DJL126 (dCas9 targeting *ma* 1631), and DJL72 (gRNA-free, expressing dCas9) in HS media with and without 1 mg/mL NH_4Cl to assess nitrogen fixation over a period of

Figure 12 indicates that genes *ma2032* and *ma1631* are not required for nitrogen fixation, but there may be a small effect on overall growth in DJL125 targeting *ma2032*. Further research could benefit from growth studies on varying carbon sources, specifically acetate, due to the upregulation of genes during qPCR of carbon sources. Repeating our methods in creating the knockdown strain of *ma2032-33* and *ma1631-33* using only the second gRNA (opposed to a dual knockdown) could potentially indicate if a dual-knockdown was necessary to completely silence the gene cluster, or if the first gRNA's activity score was simply too low to efficiently bind by itself, as the second gRNA chosen had a much higher activity score. The results shown in figures 10 and 11

indicate using a second gRNA to target a second site in the original sequence can greatly improve the knockdown efficiency compared to using one gRNA. This alludes to further research necessary to understand the CRISPR system and using multiple gRNA to target a number of sites when one gRNA is inefficient.

Our knockdown methanogen strains could further be grown in molybdenum-free media to assess the role of *ma2032-33* and *ma1631-33* in supporting molybdenum-independent nitrogen fixation using V- and/or Fe-nitrogenases. The two gene clusters may act as reductases to partner proteins, which could play a role in tetrapyrrole reduction based on their proximity to methyltransferase genes in the genome. Tetrapyrroles are a type of molecule that include four pyrrole-like compounds linked together by methine bridges, such as coenzyme F_{430}.[14] Coenzyme F_{430} is the cofactor required by methyl-coenzyme M reductase, which catalyzes the reduction of methyl-coenzyme M to methane in the last step of methanogenesis.[15] Further research on both gene clusters is necessary.

In addition, the CRISPRi-dCas9 system acts as an efficient method of determining if a gene is essential to the organism. As previously mentioned, the genomes of over sixty species of methanogenic archaea have been sequenced, uncovering numerous genes with unknown functions.[4] Mapping essential genes in *Methanosarcina acetivorans*, alongside countless other species, may open more opportunities to research and characterize unknown pathways and functions of genes. Understanding which genes are essential could indicate possible involvement in essential processes such as nitrogen fixation or methane production. The CRISPRi system can be used to silence genes more easily, and

genes that are unable to silence even after attempts with dual-knockdown strains and gRNA with high activity scores are likely essential.[16]

A potential effect of methanogen research at the macro level is the possibility of creating agronomically and economically sustainable fertilizer.[2] The current agricultural fertilization practice uses chemical nitrogen as an ingredient which raises many concerns about the negative consequences to the environment. The ability to understand methanogens' simpler nitrogenases poses the possibility of reforming crop plants substantially. In addition, methanogens ability to fix nitrogen also plays a crucial role in the methane production of wetlands, especially with limited nitrogen, which contributes 20% of the annual global methane emissions.[17] Thus, continuing research on *Methanosarcina acetivorans* working to characterize unknown gene clusters and further understand each of the three nitrogenases present in this species has big implications for our global wetlands and current fertilizer practices.

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