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Elevated levels of external cysteine inhibit nitrogen fixation by the methanogenic archaeon Methanosarcina acetivorans

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Elevated levels of external cysteine inhibit nitrogen fixation by the methanogenic archaeon *Methanosarcina acetivorans*

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Introduction

Nitrogen is one of the most limiting nutrients on earth yet vital to life as it is the building block for DNA, RNA, and proteins (1). There is an abundance of nitrogen; however, only a small portion is available for consumption. Fixed nitrogen sources are forms of nitrogen that are biologically available and can readily be used for an organism's metabolism, and one of the most common fixed sources of nitrogen is ammonia (NH_3) (2). Although the atmosphere is composed of nearly 80% nitrogen, it is in the form of dinitrogen (N_2) which is not a fixed source and cannot be readily used by majority of organisms (1). To combat this, in the field of agriculture, commercial fertilizers are used to provide nitrogen to the crops which has a significant negative impact on the environment. With increased usage of fertilizers, there is damaging amounts of nitrate residues left in the soil that leads to pollution of water and the atmosphere via leaching and gentrification (3). The key to combating limiting nitrogen in agriculture may be through the study of nitrogen-fixing prokaryotes. If crops had the genetic tools to create fixed nitrogen, there would be no reliance on synthetic fertilizers to provide the plants with ammonia.

Certain bacteria and archaea play a vital role in the earth's nitrogen cycle through their ability to convert the abundant nitrogen in the atmosphere (N_2) into a usable form through a process called nitrogen fixation. As of now, this is only found in prokaryotes. Nitrogen fixation takes dinitrogen from the atmosphere, transforming it into ammonia at the cost of a minimum of 16 ATP ($N_2 + 16ATP + 8e^- + 8H^+ \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$) (4). This process is possible due to a specialized enzyme called nitrogenase. There are three nitrogenases with different metals, molybdenum (Mo), vanadium (V), and iron

(Fe) in the active site which are used depending on the environment and particular metal availability. The corresponding genes to these different metal cofactors and nitrogenase are *nif* for Mo-, *vnf* for V-, and *anf* for Fe-only nitrogenase (**Figure 1**)(5). Mo-nitrogenase is considered the main form of nitrogenase, with V- and Fe- nitrogenases being considered alternative nitrogenases.

Mo-Nitrogenase is referred to as the canonical nitrogenase because it is found in all

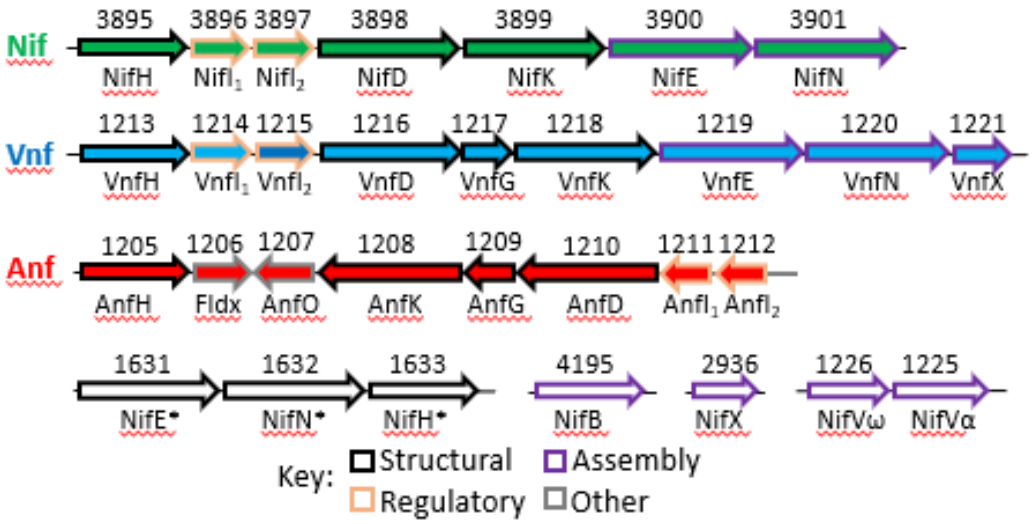

Fig. 1. The *nif*, *vnf*, and *anf* gene clusters and other putative nitrogen fixation genes in *M. acetivorans*. Numbers above arrows (genes) are the locus tags and below is the predicted protein. Arrow outline denotes predicted function as shown in the key.

nitrogen fixing organisms, and it results in more efficient growth than the alternative nitrogenases (6). Growing by nitrogen fixation, dizotrophically, is a very energy intensive process, and although, it is not as efficient as growing with a fixed nitrogen source, it provides organisms with a means of surviving under nitrogen poor conditions. It is primarily species of bacteria capable of nitrogen fixation; however, nitrogenase is believed to have originated in the archaeon, the methanogen (7).

Methanogens are the only archaea capable of fixing nitrogen, and they produce methane as a catabolic end-product (2). They are commonly found in swamps, freshwater

sediments, landfills, and the stomachs of ruminants where they grow in anaerobic conditions (4). Because of the harmful impact that commercial fertilizers and fossil fuels have on the environment, methanogens are a key organism of interest because of their ability to fix nitrogen as well as their production of methane which can be used as a biofuel. Through genetic engineering, there is the possibility to integrate nitrogenase and other supporting genes into crops, so that they would not have to rely on added fixed nitrogen from sources such as fertilizer. The primary research on this subject has been completed on nitrogen fixing bacteria; however, eukaryotes have been proven to be more genetically similar to archaea than bacteria (8). Other indicators of future success in genetically engineering eukaryotes with nitrogenase genes are found in methanogen studies that show different enzymes are used in methanogenic nitrogen fixation compared to bacteria (2). Although *Methanosarcina* grow more slowly than other species of methanogens, they have all three nitrogenase enzymes, and their metabolic versatility makes them an ideal model to study nitrogen fixation by methanogens (9).

To investigate the mechanisms and regulations of nitrogenase and nitrogen fixation further, *Methanosarcina acetivorans* was used because it contains all three

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)	$N_2 + 20H^+ + 20e^- + 40ATP \rightarrow 2NH_3 + 7H_2 + 40ADP$

nitrogenases, and little is currently known about the regulation of its nitrogen fixation by metal and nitrogen availability (**Table 1**).

To learn more, we searched to define all nitrogen sources that *M. acetivorans* can use by testing for alternative fixed nitrogen sources. In order to do so, we conducted growth experiments with a mutant strain incapable of nitrogen fixation due to repression of the *nif* operon. While analyzing the results of the alternative fixed nitrogen source experiments, it became apparent that cysteine was causing issues in proper cell growth and inhibited the ability of the mutant strain to overcome repression of the *nif* operon. Because cysteine is a known sulfur source in methanogens and a common sulfur source for eukaryotes, it became important to look further into such abnormal growth. The majority of the project then shifted towards the understanding of why cysteine is inhibiting growth in the knockdown strain of *M. acetivorans*.

Materials and Methods

CRISPRi-dCAS9

In order to test that the *M. acetivorans* cells were using the nitrogen provided and not growing by fixing dinitrogen, an *M. acetivorans* strain, DJL74, was used which is incapable of nitrogen fixation due to CRISPRi-based repression of the *nif* operon (10). Transcription of the *nif* operon is blocked by a gRNA that guides dCas9 to the *nif* operon (**Figure 2**) (10). With dCas9 blocking transcription of the *nif* operon, cells are unable to produce nitrogenase and therefore only grow when provided a usable fixed nitrogen source.

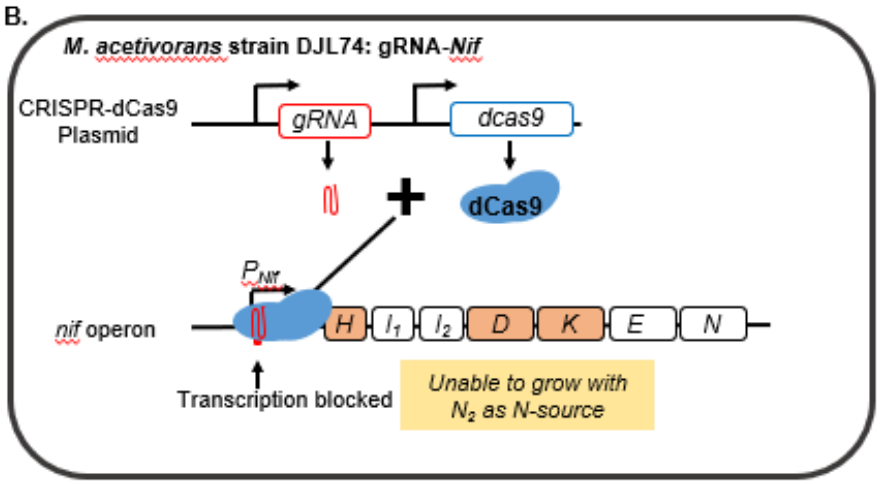

Figure 2 **(10)**
The transcription of nitrogenase is blocked from a *nif* operon specific gRNA leading the dCas9 to the promoter.

Growth Experiments

All experiments used media that was prepared under sterile conditions in an N_2-CO_2 atmosphere in the anerobic glove bag. The CO_2 level remained between 18-20% and H_2 gas around 5%. The water base for the medium was supplemented (g/L) with the following: NaCl, 23.4; $NaHCO_3$, 3.8; KCl, 1.0; $MgCl_2 \cdot 6H_20$, 11.0; $CaCl_2 \cdot 2H_2O$, 0.3. The media is reduced by DTT (0.231). A 1% trace elements solution and a 1% vitamin solution were added as well. Resazurin (0.0001% total concentration) is present in the media as an indicator for the presence of oxygen. At a pH of 7.4 and a final concentration of 0.005M, KH_2PO_4 was added to the media (11). No cysteine or ammonium chloride was originally present and was only supplemented when needed. In the last experiment looking at growth using nitrogenase with different metal cofactors, media was specifically made molybdenum free. For the conditions testing Mo-nitrogenase, molybdenum was added to the corresponding tubes rather than already being present in the media. The media was made anaerobically and placed in sealed Balch tubes (11).

Methanol (CH_3OH), a final concentration of 250mM, and sodium sulfide (Na_2S), a final concentration of 0.025%, were added at the beginning of each experiment as a carbon and sulfur source, respectively. The positive control was created by using ammonium chloride, a confirmed fixed nitrogen source for methanogens, to a total concentration of 10mM. The negative control for most studies was done by adding 200 µl of water which cannot be used as a nitrogen source, demonstrating how the cells grow without a nitrogen source. Three to five replicates were used for each condition tested to ensure accuracy and consistency within the results. All amino acids and inorganic acids were tested at 10mM unless otherwise specified. After all the chemicals were added to create the appropriate conditions, 200 µl of cells were added from a standard medium high salt medium that contained excess ammonium chloride (NH_4Cl). Because of the excess NH_4Cl, there was an initial jump in growth to an optical density (OD) ca. 0.2 due to a transfer of some of the ammonium chloride. The tubes were then gently shaken and cleaned before being placed in the spectrophotometer where an OD reading was taken at 600nm. The OD was recorded two to three times a day for approximately a week and a half until they reached a plateau for each growth study. Cells were incubated at 34˚C in between readings for optimal growth. Cell growth for each condition was charted with time in hours across the x-axis and the OD at 600 nm on the y- axis. In the nitrogen source experiments, acids that resulted in cell growth similar to the positive control of ammonium chloride meant that it could be considered a potential fixed nitrogen source for *Methanosarcina acetivorans*. Conditions that grew identical to the negative control (water) are considered not a fixed nitrogen source for the cells.

The substances tested as possible fixed nitrogen sources were cysteine ($C_3H_7NO_2S$), methionine ($C_5H_{11}NO_2S$), alanine ($C_3H_7NO_2$), urea ($CO_2(NH_2)_2$), glutamine ($C_5H_{10}N_2O_3$), glutamate ($C_5H_8NO_4$), trimethylamine ($N(CH_3)_3$), and sodium nitrate ($NaNO_3$). Cysteine, thiosulfate (S_2O_3), and sodium sulfide (Na_2S) were used when comparing the effect of known sulfur sources on the growth of the knockdown strain, DJL74. When further testing the effects of cysteine, a pseudo-wildtype was used (DJL72). DJL72 is identical to DJL74, except there is no gRNA to guide a dCas9 to block transcription of the *nif* operon. Therefore, the cells grow by fixing dinitrogen when ammonium chloride is not present.

Dot Blot

A dot blot was performed to observe whether Mo-nitrogenase was being transcribed in the strain DJL74. Samples were taken from tubes of two conditions, 0mM cysteine and with 5 mM cysteine. There was also a tube with ammonium chloride that acted as a control for cells that are not actively transcribing the *nif* operon. Samples from the cysteine tubes were taken when the 0mM cysteine tubes were at 0.205 and 0.202 OD at 600 nm; a resuspension volume of 43 µl was used. The 5 mM cysteine tubes were at an OD of 0.131, and there was a resuspension volume of 28 µl. The resuspension buffer consisted of 50 mM Tris, 150mM NaCl, 1 mM PMSF (protease inhibitor), and 1mM Benzamidine. All 10 mL of the culture were centrifuged in a 15 mL conical tube (8500 x g, 4˚C, 10 min.) and then resuspended. After transfer to a microcentrifuge, they were allowed to freeze at -80 ˚C. Samples were thawed on ice then freeze-thawed using cold ethanol and lukewarm water; this was repeated a total of four times. DNase (20x 1 mg/mL) was added then incubated at 37 ˚C for 20 minutes. Four microliters of lysate

were dotted onto nitrocellulose membranes and then aired dried in the fume hood. Membrane was rinsed with 1x Tris-buffered saline Tween (TBST) and 5% milk for 25 minutes. Primary antibodies were added with a 1:5,000 dilution for the NifD antibody, and then incubated overnight. The following day they were rinsed three times with TBST before adding the secondary antibodies (1:10,000) in TBST and milk. After incubating for one hour, it was rinsed three more times, and then incubated for 5 minutes with 0.5 mL Biorad ECL reagent. The image was created using the Alpha imager for 1-3 minutes.

Results/Discussion

Alternative Fixed Nitrogen Sources Growth Studies

On average, the replicates for the positive control, ammonium chloride (NH_4Cl), grew to an OD of approximately 0.95 before it reached a plateau in growth which is shown in **Figure 3**. The negative control, DJL74 with water, had an initial growth up to 0.12 and then remained stagnant until around 150 hours of incubation where a second growth occurred up to 0.49. Cells given alanine, urea, methionine, and cysteine remained at a low OD around 0.1. Similar to the negative control, there was a spike in growth between five and six days after inoculation for all of the nitrogen sources tested in this study except for cysteine conditions which remained at its low OD. Because there was no significant growth by any of the substances tested, none of them appear to be a fixed nitrogen source for the methanogen.

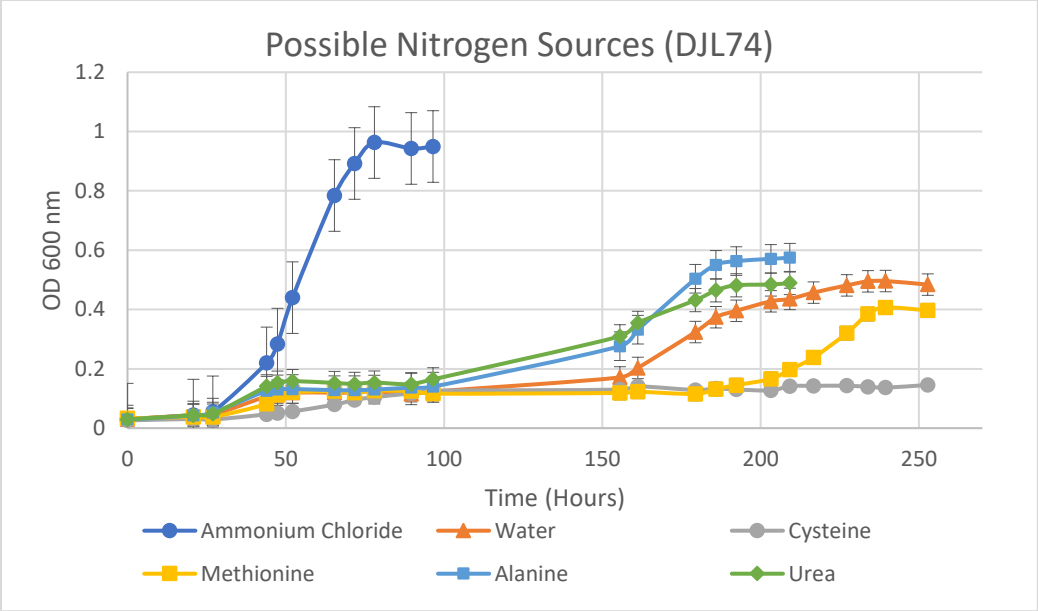

Figure 3
The first set of possible fixed nitrogen sources tested all showed growth similar to the negative growth control, except for cysteine which had little to no growth.

Figure 4 shows the second set of possible nitrogen sources where glutamine, glutamate, trimethylamine, and sodium nitrate were tested. The positive and negative controls grew nearly identical to that of the first study. *M. acetivorans* supplied with glutamate and sodium nitrate did not grow, but similar to the negative control, there was a spike in growth around 140 and 120 hours, respectively. In conditions with glutamine, growth started at the same time as the positive control, ammonium chloride, but only grew, on average, to around an OD of 0.52. This is approximately half of the maximum growth of the positive control. Although the growth is not as efficient as the positive control, the growth is significant in a manner that suggests that glutamine could be used as an alternative nitrogen source by *M. acetivorans*. When trimethylamine was provided, growth started at the same time as tubes containing ammonium chloride and glutamine and reached a slightly higher maximum growth than ammonium chloride. *M. acetivorans*

cells with trimethylamine approached its plateau of growth around one day after ammonium chloride. Because DJL74 grew efficiently with trimethylamine present, it is a worthy candidate for a fixed nitrogen source. Trimethylamine can also be used as a carbon source by *M. acetivorans* and releases NH_3 when trimethylamine is consumed which is likely why growth exceeded that of the positive control.

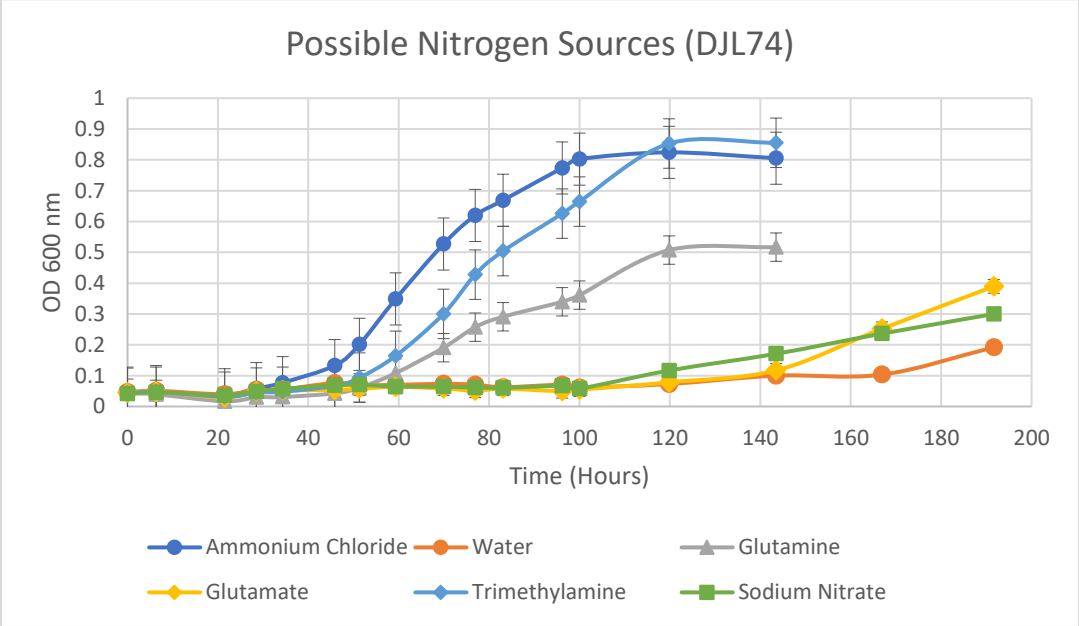

Figure 4
The second set of possible fixed nitrogen sources showed that trimethylamine grew very similar to the negative control, and glutamine grew to half the maximum OD of the positive control. Other sources tested were identical to the negative control.

In the future, experiments should be repeated to look for consistent results, and an alternate study with different strains of *M. acetivorans* such as the wildtype or pseudo-wildtype should be performed as well. It is also important to continue the study and investigate further into other amino acids and inorganic acids that could work. For example, based off of the observed growth with glutamine, lysine and asparagine should be tested due to their similarity in structure.

When the DJL74 strain was originally tested to ensure there was no transcription of the *nif* operon, the results showed that the CRISPR-dCas9 system was effective in blocking transcription (11). However, the results from the nitrogen growth experiments showed that even when a nitrogen source was not present (water), the cells were still able to growth around a week after inoculation. Theoretically, the cells should not be growing if the *nif* operon is blocked by the dCas9 because nitrogen is a vital nutrient, and they cannot fix nitrogen and there is no nitrogen source readily available to them. Yet, they eventually grew to almost half the OD of the positive control. The two hypotheses for the cause of this growth were that the cells are somehow overcoming the dCas9 suppression over time or that the cells are adapting by using either vanadium or iron nitrogenase rather than the blocked molybdenum nitrogenase. A dot blot, shown in **Figure 5**, was performed using samples of DJL74 from the growth study. The results showed that molybdenum nitrogenase was detected after 150 hours when growth began, and no nitrogenase was found in the control where the cells were given a fixed nitrogen source. This confirms that the cells did not adapt by producing alternate nitrogenases, and they are in fact still producing nitrogenase.

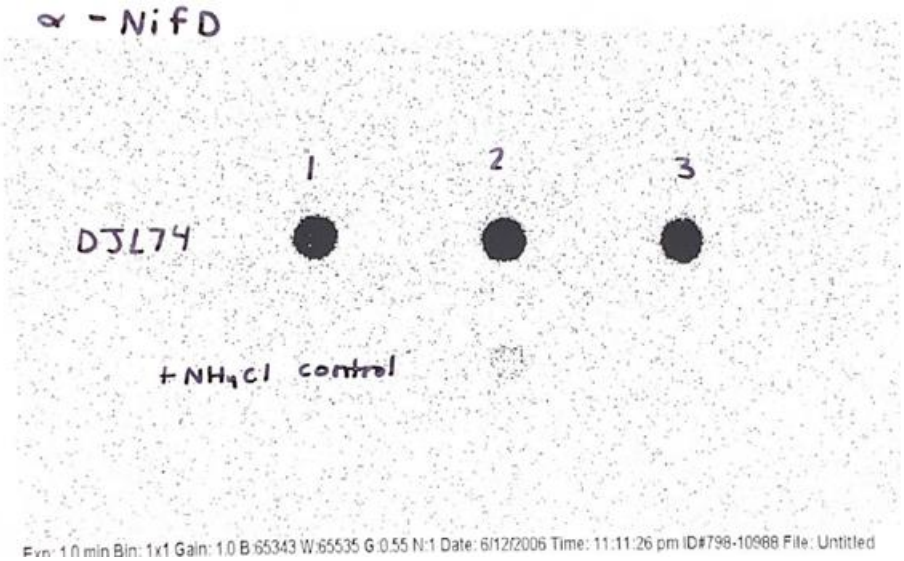

Figure 5
The dot blot results show that nitrogenase is present in the knockdown strain when *nif* is supposed to be repressed in comparison to the control of normal growth without nitrogen fixation and added ammonium chloride where there is no detection of nitrogenase. This indicates that the cells are overcoming suppression over time.

The other abnormal observation from these experiments is the lack of growth in cells when 10mM cysteine is present, even well after the other conditions began growing by overcoming transcriptional suppression. Cysteine is a known sulfur source for *Methanosarcina acetivorans*, as well as a major sulfur source for eukaryotes as well. When cysteine is used as a sulfur source in other experiments, the typical concentration is 3mM, but by increasing the concentration to 10mM, the differences became significant. Looking into the cause of this lack of growth is important because when considering genetic engineering nitrogenase genes into crops in the future, we must assess any challenges or limitations that may occur.

Sulfur Source Growth Studies

To assess whether or not there is an issue with other sulfur sources as well, the next experiment was performed looking at thiosulfate, cysteine, and sodium sulfide. **Figure 6** shows the growth of the known sulfur sources with the knockdown DJL74 where the positive control, ammonium chloride, grew to maximum OD around 0.85. All of the conditions containing 10mM cysteine showed no growth even when paired with thiosulfate or sodium sulfide. Conditions supplying thiosulfate and sodium sulfide alone did not grow until 150 hours after inoculation. Once growth was initiated, they reached a plateau around an OD of 0.5 at 175 hours. When 0.5 mM cysteine was paired with

sodium sulfide, growth was nearly identical to that of thiosulfate of sodium sulfide alone with just a slightly slower rate of growth. Cells given cysteine at 3mM eventually grew to the same OD as thiosulfate and sodium sulfide alone; however, the doubling time was almost 1.5 times slower.

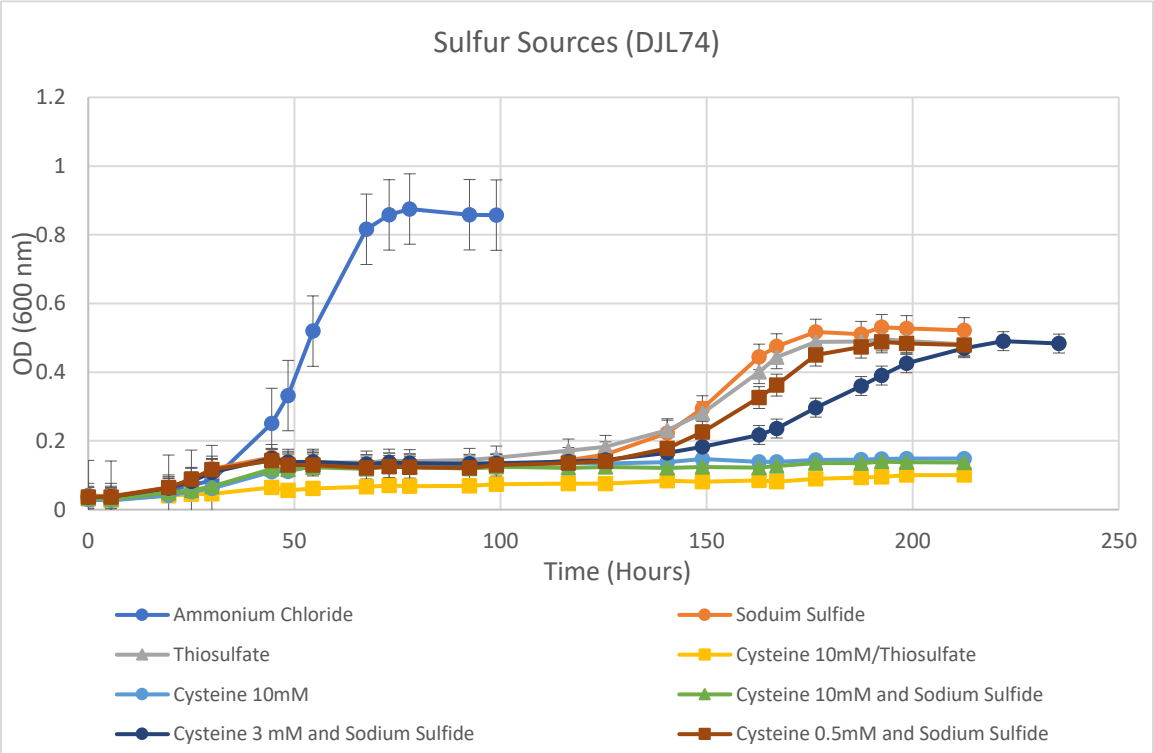

Figure 6
The results of the growth study for the effect of sulfur sources on diazotrophic growth of DJL74. Growth with 10mM cysteine had no growth, while growth at lower concentrations grew but at a slower rate.

The results showed that only cysteine caused growth issues in DJL74, and this is the first evidence that the effect is concentration dependent. With only 0.5mM cysteine, there was only a minor delay in growth, and at 3mM the changes were more significant. All conditions of 10mM cysteine had a complete lack of growth for the entirety of the study. Therefore, the higher the concentration of cysteine, the greater the negative impact

on growth. To pinpoint the concentration of cysteine where the effects turn drastic from a decrease in growth to no growth, a larger array of concentrations were tested next.

Cysteine Growth Studies

Figure 7 shows the results from the study comparing the effects of different concentrations of cysteine on the knockdown strain DJL74. Out of the concentrations tested without ammonium chloride, 10, 7.5, and 5mM, none grew past an OD average of 0.15. When ammonium chloride was present, the cells grew rapidly; however, the higher the concentration of cysteine, the slower the rate of growth and the lower the maximum OD was. The differences were minimal. The condition of no cysteine nor ammonium chloride grew identical to that of the negative controls in the alternative fixed nitrogen source study. There was no growth until around 150 hours where the cells multiplied to an OD of 0.45.

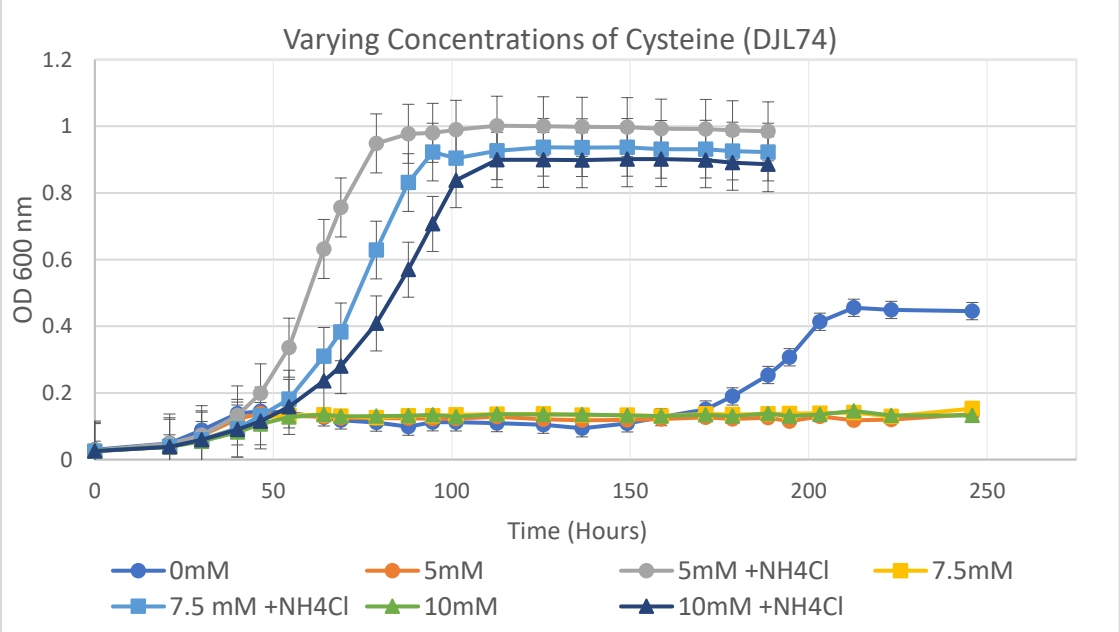

Figure 7
The results of the different concentrations of cysteine on diazotrophic and non-diazotrophic growth. The effects were minimal when ammonium chloride was present, but when fixing nitrogen, the cells did not grow when cysteine was present.

When 5, 7.5, and 10mM cysteine were tested with *M. acetivorans*, all showed no growth in diazotrophic conditions. When ammonium chloride was present, there is still sufficient growth but as the concentration of cysteine increased, rate of growth was slower and did not reach as high. Because there was some growth in the previous study at 3mM, and there was no growth at 5mM, we can conclude that there is a threshold close to 5mM where the effect of cysteine goes from a decrease in growth to complete inhibition of diazotrophic growth. Due to the small change in growth in non-diazotrophic conditions, we cannot conclude that cysteine is only affecting the process of nitrogen fixation. To test further, a second strain of *M. acetivorans* was used, DJL72, which is the same as DJL74 except there is no gRNA to lead the dCas9 to blockage of molybdenum nitrogenase transcription. DJL72 is referred to as the pseudo-wildtype. By comparing DJL74 and DJL72, we can locate whether the cause of the problem is rooted in nitrogen fixation of a bigger metabolic issue.

When 5mM cysteine is added to the pseudo-wildtype (DJL72), there is still a visible effect on diazotrophic growth. **Figure 8** shows there is no significant difference between growth when 5mM cysteine is present along with ammonium chloride in comparison to only ammonium chloride. The condition with cysteine even grew to a slightly higher OD than ammonium chloride alone. Under nitrogen fixing conditions, cysteine had a large delay on growth and reached its plateau approximately one day later than the control for normal diazotrophic growth.

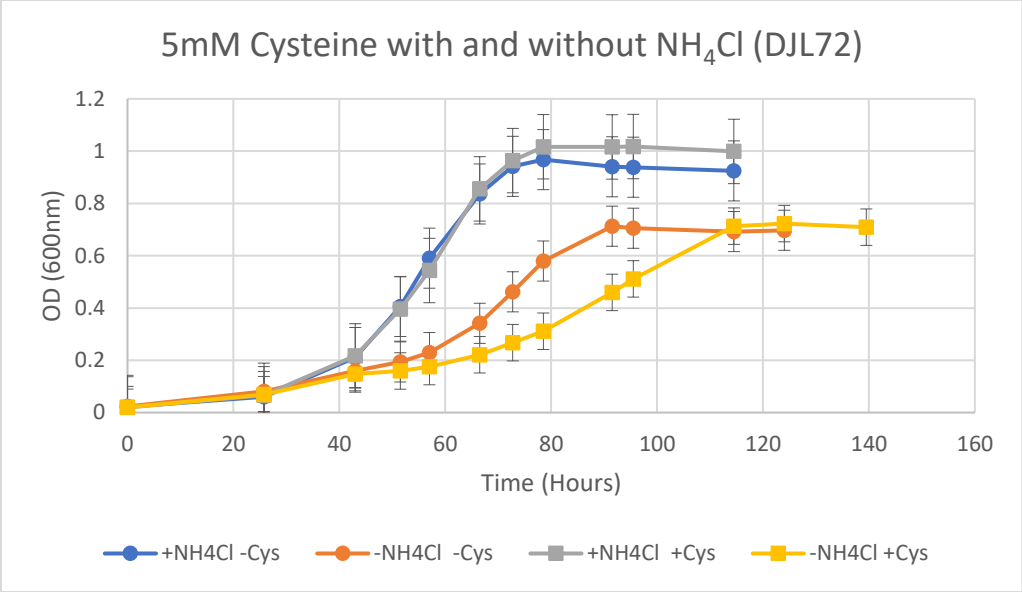

Figure 8
The results of the study comparing the effects of cysteine on growth of the pseudo-wildtype. There is no significant different in growth with ammonium chloride, but there is delayed growth with cysteine when growing by nitrogen fixation.

There was no difference in the rate of growth when the cells were not fixing nitrogen, and the condition with cysteine actually had higher growth than the condition with only ammonium chloride. However, compared to the control for normal growth by molybdenum nitrogenase, when only cysteine was added to the cells' environment, growth was significantly delayed. This provides more evidence to the hypothesis that elevated levels of cysteine are affecting not just overall growth, but specifically, nitrogen fixation and possibly nitrogenase function.

In all previous experiments, only molybdenum nitrogenase has been utilized when testing nitrogen fixation, so it is important to test whether there will be a similar effect when *M. acetivorans* is using the alternate metal cofactors, vanadium and iron. The growth study comparing growth of DJL72 and cysteine when nitrogen fixation is occurring by the main and alternative nitrogenases is shown in **Figure 9**. The iron and

vanadium conditions with ammonium chloride both grew to an OD of almost 1.1 in 80 hours. Cells given molybdenum and ammonium chloride grew at the same rate but only grew to an OD of 0.96. When 5mM cysteine is present along with ammonium chloride and its designated metal, growth was approximately 20 hours slower than its corresponding condition of only ammonium chloride with molybdenum, vanadium, or iron. The growth of the controls for nitrogen fixation (no ammonium chloride nor cysteine) showed molybdenum growing to the highest OD (0.7) the fastest, then vanadium (0.5), and then iron-only (0.4). Cells with molybdenum and cysteine took around 150 hours to grow to an OD of 0.67 while growth with vanadium nitrogenase and cysteine took 187 hours to reach 0.55. Lastly, iron-only with cysteine present reached nearly 0.4, 211 hours after inoculation.

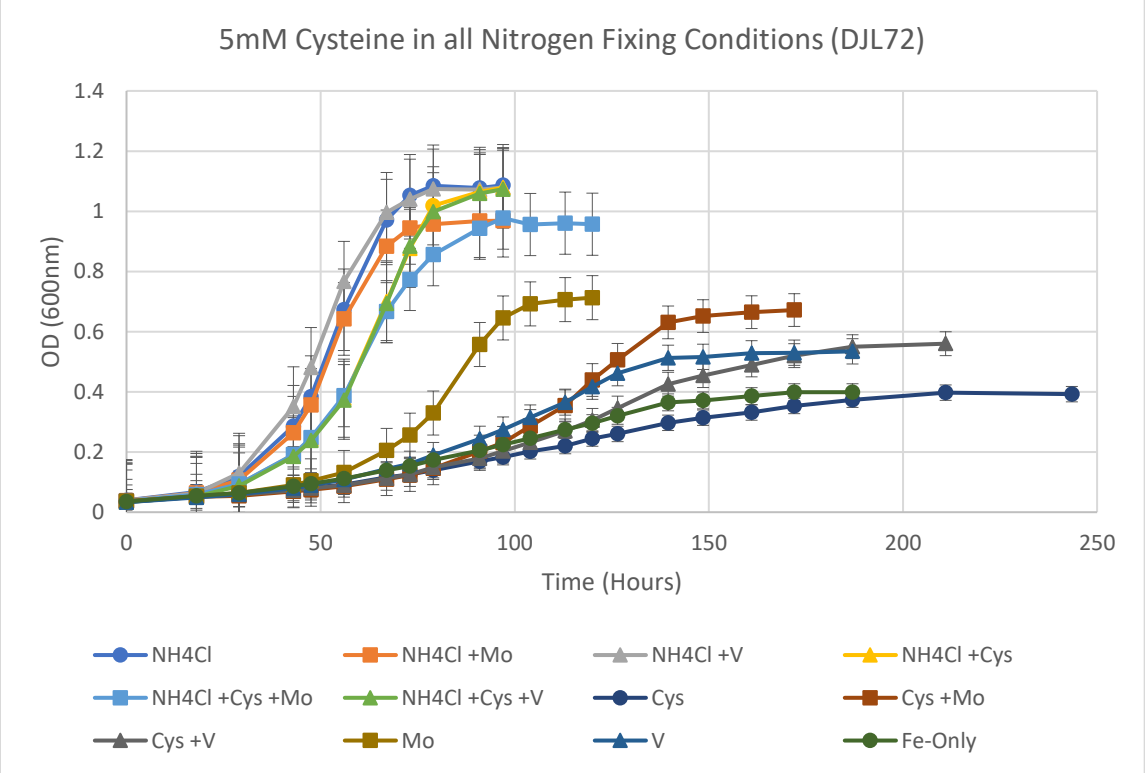

Figure 9
The results from the growth study comparing growth with cysteine on nitrogen fixation with different metal cofactors for nitrogenase. Cysteine present in all conditions results in a slower rate of growth, but the difference is greater in molybdenum environments.

Although there was a slight delay in the rate of growth in non-diazotrophic conditions with 5mM cysteine, the most noticeable differences were when the cells were fixing nitrogen using the molybdenum nitrogenase. Molybdenum nitrogenase is the most energy efficient when it comes to fixing nitrogen, so it was expected that molybdenum, then vanadium, and then iron only would grow from most efficient to least. Currently, it is unknown why molybdenum and cysteine are affecting growth of *M. acetivorans* so greatly when the cells are growing diazotrophically.

Because the results are the most dramatic with media containing molybdenum and when the cells are growing dizotrophically, any further experiments should focus on these conditions. One option to investigate the direct cause for the observed change in growth is to measure the transcription levels of *nif* operon at different levels of cysteine. Another hypothesis is there may be an indirect relationship between elevated levels of cysteine causing an increase in sulfide production, and sulfide is known to be toxic to most forms of life (12). Therefore, in the future, an assay of sulfide production with 5mM cysteine or higher would be beneficial in order to observe if higher levels of sulfide result in slower or lack of growth.

Nitrogen fixation is a vital metabolic process to our earth's nitrogen cycle, and it has incredible potential in the development of our agriculture. Nitrogen fixing microorganisms are commonly free-living but some form symbiotic relationships with plants such as legumes and provide them nitrogen; however, this is very environment specific (13). If plants were able to integrate nitrogenase and fix nitrogen themselves, the

negative effects of our current agricultural industry could be curbed. Because there is not much known about nitrogen fixation in model organisms such as *Methanosarcina acetivorans*, research should be conducted to fill in our gaps of knowledge on the mechanisms and regulations of methanogenic nitrogenase.

References

1. Zerkle AL, Mikhail S. 2017. The geobiological nitrogen cycle: From microbes to the mantle. *Geobiology*. 15(3); 343-352. doi:10.1111/gbi.12228.

2. Raymond J, Siefert JL, Staples CR, Blankenship RE. 2004. The Natural History of Nitrogen Fixation. *Mol. Biol. Evol*; 21(3); 541-554. doi:10.1093/molbev/msh047.

3. Sheoran S, Kumar S, Kumar P, Meena RS, Rakshit S. 2021. Nitrogen fixation in maize: breeding opportunities. *Theor. Appl. Genet.*; 134(5), 1263-1280. doi: 10.1007/s00122-021-03791-5.

4. Thauer RK, Kaster A, Seedorf H, Buckel W, Hedderich R. 2008. Methanogenic archaea: ecologically relevant differences in energy conservation. *Microbiol*. 6(8); 579-91. doi: 10.1038/nrmicro1931.

5. Hu Y, Ribbe MW. 2015. Nitrogenase and homologs. *J Biol Inorg Chem.* 20, 435–445. doi: 10.1007/s00775-014-1225-3.

6. Bellenger JP, Xu Y, Zhang X, Morel FMM, Kraepiel AML. 2014. Possible contribution of alternative nitrogenases to nitrogen fixation by asymbiotic N_2-fixing bacteria in soils. *Soil Biol. Biochem*; 69;413-420. doi: 10.1016/j.soilbio.2013.11.015.

7. Boyd ES, Garcia Costas AM, Hamilton TL, Mus F, Peters JW. 2015. Evolution of Molybdenum Nitrogenase during the Transition from Anaerobic to Aerobic Metabolism. *J Bacteriol*; 197(9); 1690-1699. doi:10.1128/jb.02611-14.

8. Leigh JA, Albers S-V, Atomi H, Allers T. 2011. Model organisms for genetics in the domain Archaea: methanogens, halophiles, *Thermococcales* and *Sulfolobales*. *FEMS Microbiol. Rev*; 35(4); 577-608. doi:10.1111/j.15746976.2011.00265.x.

9. Allers T, Mevarech M. 2005. Archaeal genetics – the third way. *Nat Rev. Genet*; 6(1); 58-73. doi: 10.1038.nrg1504.

10. Dhamad AE, Lessner DJ. 2020. A CRISPRi-dCas9 System for Archaea and Its Use to Examine Gene Function during Nitrogen Fixation by *Methanosarcina acetivorans*. *Appl. Environ. Microbiol*; 86(21). doi:10.1128/aem.01402-20.

11. Sowers KR, Baron SF, Ferry JG. 1984. *Methanosarcina acetivorans* sp. nov., an Acetotrophic Methane-Producing Bacterium Isolated from Marine Sediments. *AEM*; 47(5); 971-978.

12. Caffrey SM, Voordouw G. 2010. Effect of sulfide on growth physiology and gene expression of Desulfovibrio vulgaris Hildenborough. *ALJMAO*; 97(1); 11-20. doi:10.1007/s10482-009-9282-y.

13. Peters G, Burgess BK. 1980. Biological Nitrogen Fixation: A Process. Antioch Rev; 38(4); 479-492.