Eat Methane

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Introduction

Climate change and global warming is internationally recognized as a danger, threatening the current world's existence. The main cause of this change is greenhouse gas emissions (*What Is Climate Change?* | *United Nations*, n.d.). Greenhouse gases trap solar heat, increasing temperatures around the planet. The three most significant greenhouse gases are carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) (Króliczewska, 2023). However, the warming impact of methane is 86 times stronger than that of carbon dioxide, per unit of mass. Additionally, methane remains in the atmosphere for 12 years, harming climate, health, agriculture, and economy far faster than carbon dioxide emissions (*Methane | Climate & Clean Air Coalition*).

Furthermore, methane is a major health hazard, causing numerous health problems and premature deaths annually. A 40% reduction in methane emissions could avoid 540,000 emergency room visits, 180,000 premature deaths, and 11,000 hospitalizations every year (Climate and Clean Air Coalition, 2021). In the U.S. alone, over 6 million tons of methane annually, resulting in nearly 1 billion usd of loss. Additionally, an annual cost of 10 billion usd is spent as a result of methane's links to respiratory diseases strengthens the case for its rapid reduction (Climate and Clean Air Coalition, 2021). damage to economy and human well-being (Rutherford & Myers, 2024). Methane comes from a variety of sources, including livestock, oil drills, and landfills. Landfills in particular contribute to 16% of all methane emissions (*Estimates of Methane Emissions by Segment in the United States*, 2024). In 2022, methane emissions from landfills overcame emissions from 24 million gasoline-powered passenger vehicles (*Basic Information About Landfill Gas* | *US EPA*, 2024).

As high concentrations of methane harm both humans and the environment, landfills are required to remove methane.

To combat these methane emissions, landfills commonly use systems of perforated piping to transfer the landfill gas (or LFG) to sites where it can be purified and finally to the landfill's flares. These flares burn the methane, releasing carbon dioxide into the atmosphere without generating electricity for the facility. We believe that this solution is not sufficient and could be significantly enhanced through synthetic biology (*Basic Information About Landfill Gas* | *US EPA*, 2024).

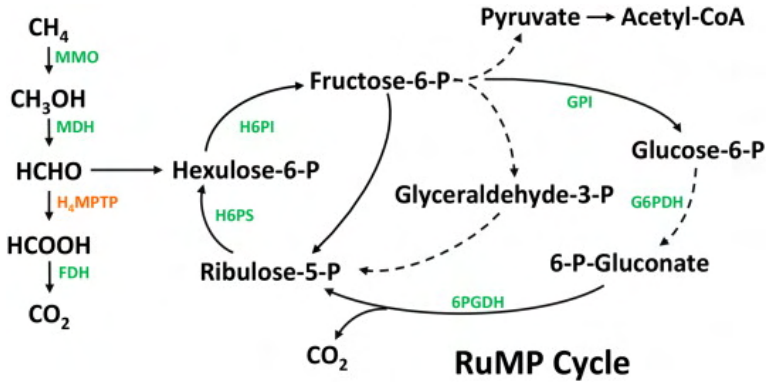

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Fig 1: The ribulose monophosphate cycle found naturally in methanotrophs. B. *subtilis* lacks the first two steps; the conversion of methane to methanol, and methanol to formaldehyde.

To mitigate the emissions of methane from landfills, we will use the ribulose monophosphate cycle (RuMP), which occurs naturally in B. *subtilis*, which processes formaldehyde into fructose, a usable sugar. We can convert methane into formaldehyde by first converting it into methanol using a methane monooxygenase enzyme, and then converting methanol into formaldehyde via the methanol dehydrogenase enzyme. This methane pathway will work with the first enzyme being produced the slowest to prevent the accumulation of the hazardous chemicals methanol and formaldehyde (Wu et. al, 2023). We hypothesize that genetically edited microbes can remove methane from landfills. We hypothesize that genetically editing B. *subtilis* to contain methane monooxygenase will allow it to convert methane into methanol. We plan to investigate if it is possible to utilize the RuMP cycle, methane monooxygenase, and methane dehydrogenase to make B. *subtilis* remove methane from a system.

Materials and Methods

All in *vivo* experimentation was conducted with *B. subtilis* strain 168. Fragments of DNA homologous to the amylase gene were copied from PDR110. Both the strain and the plasmid were graciously sent by Leah McKinney at the University of Wisconsin Madison. Unless otherwise mentioned, Spectinomycin plates are at a concentration of 50 ug/ml.

First a linear construct based on (Wu et al., 2019) and advice from Leah Mckinely was designed using snapgene.

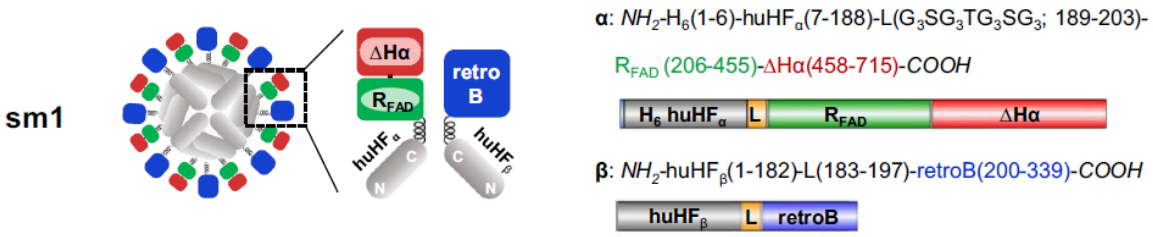


Fig 2: Diagram of mini methane monooxygenase from (Wu et al., 2019). Note the presence of only two subunits.

Historically, methane monooxygenase has been a very challenging protein to work with. Proper enzyme function requires several different parts to fold and assemble correctly. Successes in getting the enzyme expressed and functioning properly in any organism have been rare. We chose to base our work on a new paper published in May 2024 by a group from the University of Korea (Yu et al). In this paper, the authors design a miniature version of the enzyme soluble methane monooxygenase, aka mini-sMMO. This mini-sMMO has a number of advantages. Firstly, the mini-sMMO is only composed of two subunits, alpha and beta, in comparison to the nine subunits of the original sMMO. Secondly, sMMO requires protein chaperones to assemble, whereas the mini-sMMO is designed to self-assemble. Lastly, the mini-sMMO has been successfully expressed in *E. coli* and found to be more efficient than normal methane monooxygenase.

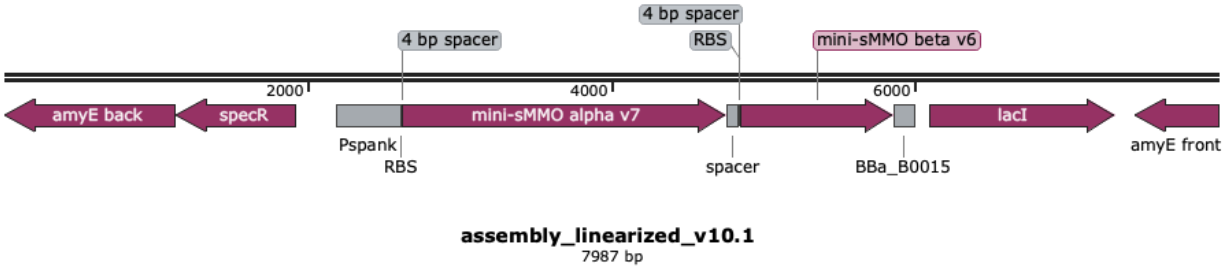


Fig 3: Our linearized construct, further described below. The Pspank and lacI assembly create an IPTG inducible promoter. This allows trigger methanol production to be triggered only when we are ready to assay.

Our DNA construct is primarily based on pDR110, a B. *subtilis* amyE integration vector, and the mini-sMMO (Yu et al.). The ends of our DNA construct have homologous regions with B. subtilis amylase gene, allowing for homologous recombination to occur and replacing the amylase gene in B. subtilis with the mini-sMMO gene. Inserting at this location has the added benefit of allowing us to perform the iodine-starch test to confirm that our gene has been successfully transfected. Amino acid sequences of the mini-sMMO were taken from the paper and codon optimized for B. *subtilis*. They were then assembled with 70 bp noncoding spacers, B. subtilis specific ribosome binding sites and a promoter. Our DNA construct also included the specR gene and the lacI operon. The spectinomycin resistance gene specR allows us to screen for transformed colonies. Since methanol, the product of mini-sMMO acting on methane, is toxic to B. subtilis, we also included the lacI operon, so that mini-sMMO will be expressed only when IPTG is added.

Amino acid sequences were taken from the paper and codon optimized using Integrated DNA technologies condon optimizer for B. *subtilis,* while avoiding intrinsic terminators. They were then assembled with 70 bp noncoding spacers to avoid translational coupling.

As the construct was nearly 8 kbp, it was divided into four segments. The two homologous ends were sourced from PDR110 via linear PCR using NEB followed by PCR purification, and the two fragments coding for a modified sMMO were ordered from Integrated DNA Technologies and Twist Biosciences. To confirm the oligonucleotides ordered, a PCR was conducted using New England Biolabs Q5 Hot Start High-Fidelity 2X Master Mix for 12 cycles and a gel was run.

Both overlap extension PCR and Gibson assembly using NEB's NEBuilder HiFi DNA Assembly Master Mix were tested. While overlap extension was successful in combining two fragments, it struggled with all four, and had many products of incorrect length. An attempted assembly with a combination of overlap extension and gibson links failed to create transformants. The assembly was put through PCR and sent for sequencing to Plasmidasourus. NGS sequencing results revealed an amplified section of PDR110 with no additional inserted genes.As such a new reaction with all fragments combined using Gibson assembly was used in our transformation.

Bacillus was cultured from glycerol stock at 37C, 140 rpm for 12-14 hours in a LB broth, with a 1:10 ratio of Broth to airspace. 5 ml of this liquid culture was added into a solution of 100 ml of 0.2 um filter sterilized Spizizen salt solution, 20 mg of casamino acids, and 100 mg of yeast extract split across two 500 ml flasks. The culture was incubated for 4 hours at 37 C, 90 rpm.

Spizizen salts - 1x (for 1 L)

$MgSO_4$ x $7H_2O$	0.2 g	▢
K_2HPO_4	14 g	▢
KH_2PO_4	6 g	▢
Trisodium citrate x $2H_2O$	1 g	▢
$(NH_4)_2SO_4$	2 g	▢

Fig 4: Spizizen salt solution recipe from iGEM team Brno _Czech_Republic)

A new filter sterilized solution solution was made with 90 ml Spizizen salt solution, 2 mg calcium chloride and 21.4 mg magnesium chloride. 0.5 ml of the 4 hour culture was added to this solution in a 1L flask, which was then incubated at 37 C for one hour and thirty minutes at 90 rpm resulting in competent cells (protocol adapted from iGEM team Brno _Czech_Republic).

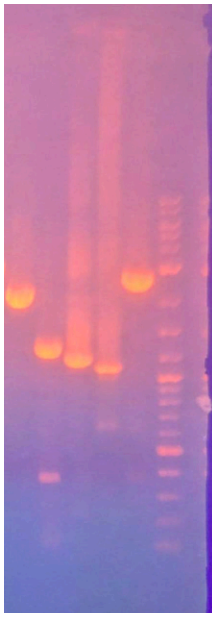
Following this, 1:10 serial dilutions of 500 ng of DNA were added to a 500 ul culture of competent cells, and incubated for an hour at 37 C, 250 rpm. The mixture was streaked onto prepared Spectinomycin plates. An iodine starch test was performed on all colonies.

The colony from the undiluted plate was then collected and re-streaked onto an LB plate. Three colonies from this plate were used to inoculate standard LB culture media in 3 ml tubes. They were cultured for 3 hours at 37 C. Cultures were stored in glycerol stock -80 C, by combining 600 µL culture and 400 µL 50% glycerol in a cryovial.

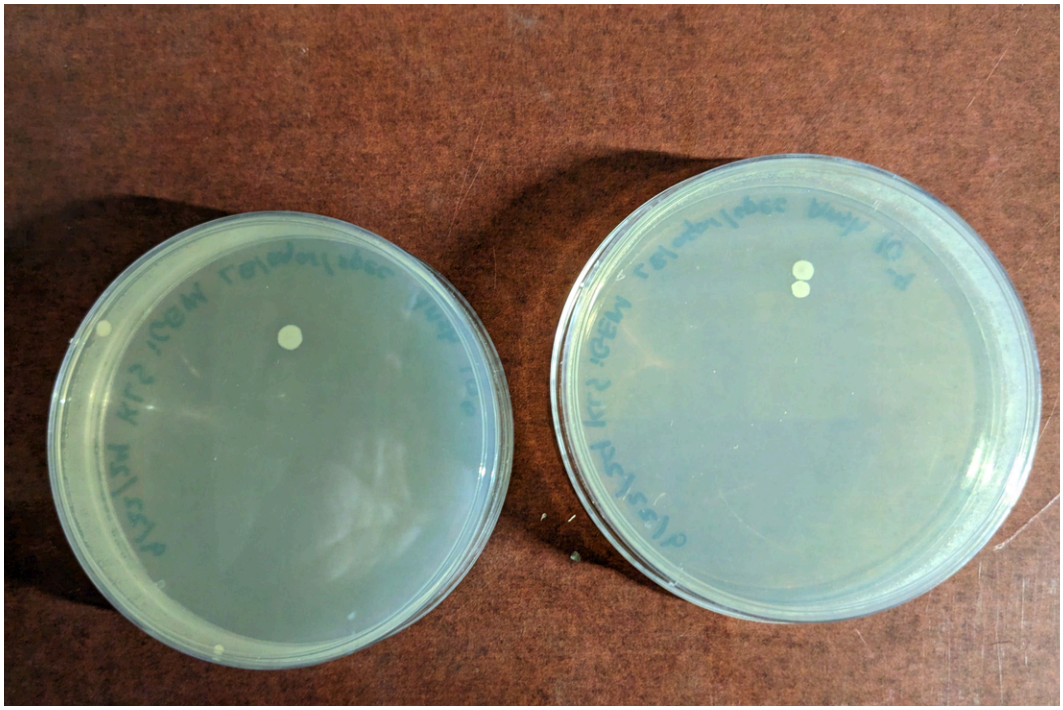
There were three colonies found in total, one on the undiluted plate, and two on the 1:10000 plate. Additionally there was a colony on the negative control plate.

Considering this, we performed an Iodine starch test to check for the knockout of the amylase gene.

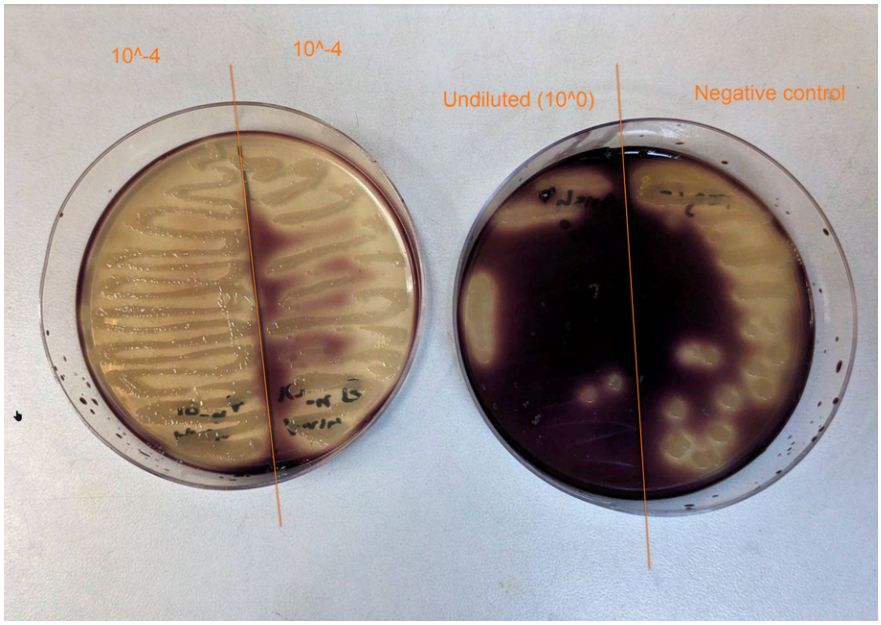
Results



From right to left: Ladder, frag 1, frag 2a, frag 2b, frag3, frag 4



(Fig. 5) 10^{-4} serial dilution colonies on the right and 10^{0} serial dilution colonies on the left



(Fig. 6) Left side petri dish is a 10^{-4} serial dilution of the edited *B. Subtilis*. Right side, left half is undiluted 10^{0} *B. Subtilis*, and right side, right half is negative control.

Our results from the iodine starch test in Figure 6. The negative control with untransformed bacteria had colonies, which was unexpected since the bacteria there should not have spectinomycin resistance. As such they were plated in the iodine starch test, and show presence of the amylase gene with a clear halo.

The left side petri dish contains edited *B. Subtilis*, that's been through a 10^{-4} serial dilution. This petri dish shows a minute purple coverage with large halos. This indicates these colonies also have the amylase gene. The left half of the right petri dish contains a 10^{0} serial dilution of the edited *B. Subtilis* that is significantly more purple than the 10^{-4} serial dilution, leaving the possibility of a knocked out amylase gene.

Conclusion

The iodine starch test showed a few promising results. We expected that the genetically edited *B. Subtilis* would not be able to consume the starch, resulting in the iodine reacting with the starch and producing a purple hue. In the case that the *B. Subtilis* consumed the starch, we would expect that the iodine would remain yellow. The 10^{0} undiluted *B. Subtilis* petri dish showed more purple coverage when compared to the 10^{-4} diluted strain. This result was reassuring as it indicated that the genetic modification had been implemented correctly, leading to the iodine reaction in the undiluted strain and that its expression varied correctly across different levels of dilution. However, we are still uncertain whether the edit actually occurred due to the unexpected purple coverage on the control side.

Our project contributes research into genetically editing *B. Subtilis* to consume methane and convert it into a sugar, fructose, that it can use. Current research published in the *Journal of Biotechnology* has edited *B. Subtilis* to process methanol into formaldehyde, a compound that *B. Subtilis* can already convert into fructose (Gao et al.). Our project enables *B. Subtilis* to process methane into methanol, which combined with the edit from the research mentioned above and *B. Subtilis's* already present mechanism will allow *B. Subtilis* to convert methane into fructose, making our research the final step in the process to convert methane into fructose.

The methane monooxygenase we are utilizing has been implemented successfully in *e coli*. In addition, the next step in our process to convert methanol into methane has been successfully implemented in other bacillus, leaving us with high hopes that our implementation will be successful. Unfortunately, our results are currently inconclusive. While three colonies were present on the test plates after transformation in our study, there was also a colony on the negative control plate. The iodine starch test shown in figure 6 is not conclusive evidence of a successful transformation. As a next step, we plan to run colony PCR to ensure the genetic edit occurred. In the case the edit was successful, there still remains the question of checking to confirm the enzyme is translated correctly and is reasonably efficient at methane removal. Another limitation is that prior to potential implementation, the risk that genetically engineered bacteria could out compete natural bacteria in the soil must be addressed. Our team has developed a theoretical solution to address this. By integrating a Toxin-Antitoxin system, where bacteria continually produces a toxin and only produce a antitoxin when near Silicic acid due to an inducible promoter, containment is possible. Silicic acid is commonly present in landfills, a large source of methane, due to industrial waste production of semi soluble sodium silicates, meaning additional application is not necessary.

Methane is nearly 86 times more of a potent greenhouse gas than carbon dioxide per unit of mass, and can remain in the atmosphere for up to 12 years, damaging climate, health, agriculture, and the economy faster than carbon dioxide emissions (*Methane | Climate & Clean Air Coalition*). The research presented in this paper would enable the development of large-scale modified *B. Subtilis* strains. The wide-spread usage of these methane-consuming *B. Subtilis* strains could help significantly in reducing global atmospheric methane concentration and play a role in mitigating the effects of climate change.

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