Engineering energy metabolism in the methanogenic archaeon *Methanosarcina acetivorans*

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Abstract

Methanogens are diverse anaerobic archaea that use the respiratory process of methanogenesis to make methane from various carbon compounds. Methanogens' methane production is highly efficient, and methane can be harvested in a controlled environment (like an anaerobic digestor, a type of bioreactor) and used as a high-energy fuel. Therefore, methanogens are a key area of research for renewable energy synthesis, with inexpensive carbon waste being used for biogas production. Anaerobic digestion has already been implemented in some facilities, but there is high potential for more methanogen implementation in the bioeconomy. However, the ATP yield from substrate of methanogenesis is among the lowest in nature, and if ATP yield was improved, methane production flux and methanogen efficiency could be increased, leading to even more effective biogas synthesis. Methanogens, especially the species *Methanosarcina acetivorans*, are also a good target for genetic manipulation largely due to their flexible metabolism. This study therefore seeks to answer:

1. Can genes be effectively transformed into *M. acetivorans* that are targeted to increase ATP yield when given required inducers?
2. Will this genetic manipulation increase methanogen efficiency?

M. acetivorans will be bioengineered to create two new strains, each expressing one new gene to increase ATP yield. The strains will be characterized to determine expression of the new genes and if their growth rate has changed to make them more efficient for bioreactor use. This project seeks to increase the ability of methanogens to be used as sources of sustainable and renewable energy and lead to further methanogen bioengineering possibilities.

Background

- Though *M. acetivorans* can metabolize diverse carbon sources, this study provides methanol as a carbon source, causing the cells to undergo methylotrophic growth. Methanol is a common carbon source for methanogens and allows for faster growth compared to other substrates.
- There are three objectives for the trajectory of this project:
  - Create plasmids containing "gene 1" and "gene 2" and amplify with *E. coli* transformation.
  - Transform plasmids into *M. acetivorans* and verify transformation.
  - Characterize transformed *M. acetivorans* strains based on "gene 1" and "gene 2" transcription with RT-qPCR and gene impact on methanogen growth with "compound A"-dependent "inducer B"-induced growth rate assays.

Methods and Results

1. Plasmid Assembly

- pMH1 (containing "gene 1") and pMH2 (containing "gene 2") were assembled from a pNB730 backbone using NEBuilder HiFi Assembly, then plasmids were amplified as described in Figure 2.

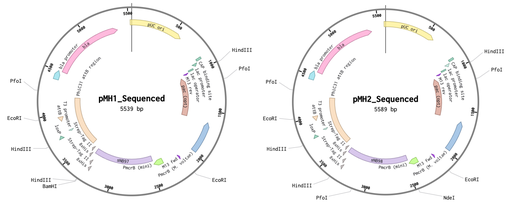


Figure 1. Plasmid maps of pMH1 and pMH2 after sequencing. sNB97 integrated on pMH1 is "gene 1," and sNB98 integrated on pMH2 is "gene 2." Key genes include *bla* (encoding ampicillin resistance), the *attB* and PhiC31 regions, and the *pac* region encoding puromycin resistance.

Amplification of Synthesized Plasmids

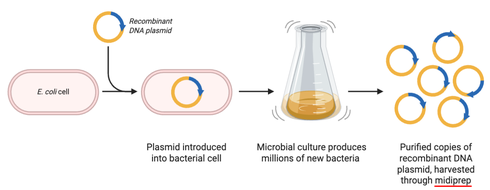


Figure 2. Basic outline of using plasmid transformation into *E. coli* to make and purify many copies of synthesized plasmids. Plasmid assembly mixtures were added into competent cells and SOC and streaked on LB ampicillin plates for incubation, then colonies were picked to LB liquid media and grown up before performing a miniprep. Eventually, purified plasmids were re-transformed into *E. coli* to get a higher yield with a midiprep.

2. *M. acetivorans* Transformation

Figure 3. Overview of using DOTAP liposomal transfection agent to transform purified plasmids into *M. acetivorans* cells of strain NB34. This parent strain has the *hpt* gene deleted, preventing 8-ADP sensitivity. DOTAP incubates first with plasmid DNA and envelops it. Once the mixture is combined with cells, DOTAP combines with the cell membrane and allows the plasmid to enter the cell. After a recovery period of the DOTAP/cell mixture in HS low MeOH + TMA media, cells are spread plated and grown up in a Wolfe incubation chamber, followed by streaking and eventually picking into Balch tubes.

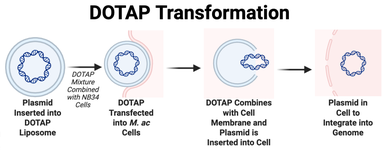
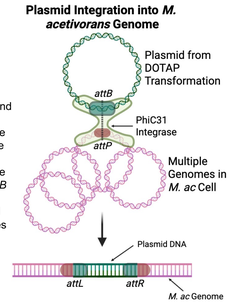


Figure 4. Plasmids transformed into *M. acetivorans* integrate into the genome using *attB/P* integration, and this method also allows for easy screening of successfully integrated transformants. Plasmids have the *attB* region, while the *attP* region is present in the parent strain's genome. The PhiC31 integrase is encoded with the *attB* gene on the plasmid, and once it's translated in the cell, it helps join the *attP* and *attB* regions. The resulting genome includes the plasmid DNA flanked by *attL* (which includes half of *attP* and half of *attB*) and *attR* (which includes the other halves of *attP* and *attB*).
Strain genotypes:
NB786: Dhpt:: PhiC31 int, att:: pMH1
NB787: Dhpt:: PhiC31 int, att:: pMH2



3. gDNA Extractions and Integration Checks

- After colonies from DOTAP transformations were picked into Balch tubes and grown at 35°C, phenol chloroform gDNA extractions were performed on the cultures. After PCR on gDNA samples with primers that either amplified *attP* (meaning they were untransformed) or *attL/R* (meaning they were transformed), gel electrophoresis was performed to determine if transformed cells were present. After confirmation of transformation and integration, a pMH1 culture was saved as strain NB786 and a pMH2 culture was saved as strain NB787.

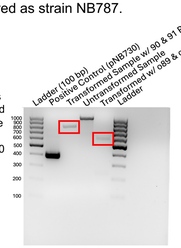


Figure 5. Results of gDNA extraction of one pMH1 culture that had shown transformed cells in previous screening. Banding appeared as expected at 787 bp for the transformed well with oNB90/91 primers and at 580 bp for the transformed well with oNB89/92 primers, and untransformed banding was at 981 bp as expected. pNB730 positive control banding was at 380 bp as expected. The culture had transformed and untransformed cells/genomes.

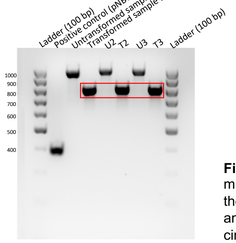


Figure 6. Results from gDNA extractions of three pMH2 cultures. Banding appeared as expected at 787 bp for the transformed wells, at 981 bp for untransformed wells, and at 380 bp for the pNB730 positive control. All three cultures therefore had both transformed and untransformed cells/genomes present.

4. RT-qPCR

- To determine if "gene 1" and "gene 2" are being transcribed and expressed, RT-qPCR was run on the strains to compare transcription of "gene 1" and "gene 2" to a control housekeeping gene, rpoA1 RNA polymerase. First, NB786 and 787, as well as NB34 (the parent strain) and NB565 (a vector-only control with pNB730 integrated) cultures were grown up to mid-exponential phase (OD~0.5), and RNA was extracted from the cultures and treated with DNase. Reverse transcription was used to make cDNA from mRNA, and finally qPCR was run on cDNA extracts to determine gene expression levels.

Figure 7. The process of RNA extractions, starting with a pelleted culture. The pellet is resuspended in TRIzol, after which chloroform is added and the tube is shaken, incubated, and spun down. The aqueous phase is washed with both isopropanol and ethanol, and the ending RNA pellet is resuspended in water. Additionally, the samples went through a DNase treatment and were then resuspended in RNase-free water. Sample purity was verified via nandrop readings.

Figure 8. Using RNA extractions to make cDNA for qPCR. RNA extracts were combined with random hexamers, dNTPs, and reverse transcriptase and incubated to synthesize cDNA. Using the Integra Assist pipetting robot, a 96-well plate was setup with SYBR Green Master Mix, cDNA from each strain, and varying primers that targeted either "gene 1," "gene 2," or rpoA1 expression.

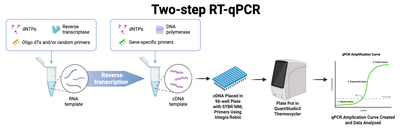
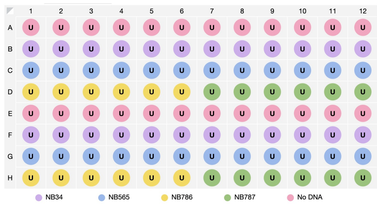


Figure 9. 96-well plate layout for qPCR. Primers for rpoA1 are used with rows A-D, while primers for "gene 1" are used in rows E-H in lanes 1-6 and primers for "gene 2" are used in rows E-H in lanes 7-12. Data analysis is in progress, and conditions are being optimized for future qPCR runs. NB786 data is currently expected to show no "gene 1" expression, as during primer testing it was found that since integration testing, the strain (which previously had fewer transformed cells present) had lost pMH1 integration through passages, with untransformed cells outcompeting transformed cells. Efforts to select for transformed cells in NB786 or create a new strain are underway before future runs.



Future Directions

- Use puromycin pressure to encourage "gene 1" integration in NB786 strain or create new strain from existing pMH1 spread and streak plates.
  - Perhaps, "gene 1" maintenance was unsuccessful because it interfered with key methanogen processes, making it an undesirable candidate for use in bioreactors. "Gene 2," in contrast, could help methanogen metabolism, hence its successful integration and expression.
- Perform growth rate assays on new strains and control cultures to determine if "gene 1" or "gene 2" expression helps improve methanogen growth.

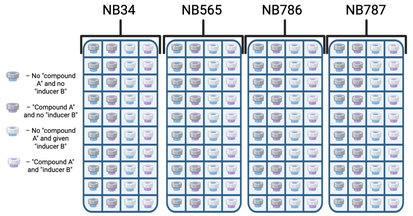


Figure 10. Layout for "compound A"-dependent "inducer B"-induced growth rate assays. 0.1 mL of culture (NB34, 565, 786, or 787) is passaged into 40 maximum MeOH Balch tubes. Half the tubes for each strain are supplemented with 0.02 mL of a 10 mM "compound A" solution, and half the tubes are given "inducer B," so that each strain has 10 tubes with the same circumstances (N=10). The growth rate is determined by measuring OD (optical density) every four hours after inoculation for five days using a spectrophotometer. Assays for NB34, 565, and 787 without "compound A" and with and without "inducer B" are currently in progress to get analysis-ready data.

Acknowledgements

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References

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