Expanding the Dynamic Range of *Methanosarcina acetivorans* through Recombinant Expression of an RNA Polymerase

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Abstract

Methanogens are anaerobic archaea that grow and conserve energy through the production of methane from acetate or C1 carbon substrates such as methanol and carbon dioxide. They play an important role in the global carbon cycle, contributing between 350 and 420 Tg of methane to the atmosphere annually. When used in anaerobic digesters, methanogens ferment organic waste to produce biogas containing methane, which can be used as a renewable fuel source. Due to their use of low-cost feedstocks and their unique metabolism, methanogens can also be a cheap and efficient host organism for the synthesis of organic molecules such as bioplastics and isoprene, a precursor to rubber. One limiting factor for the industrial use of methanogens is a limited understanding of archaeal transcription and translation, and limited genetic tools available for their regulation. This study aims to expand the dynamic range of gene expression of *Methanosarcina acetivorans* through the recombinant *in vivo* expression of an RNA Polymerase. Using *in vitro* transcription, we show that the RNA Polymerase effectively transcribes genes in the presence of *M. acetivorans* lysate. We then demonstrate that the RNA Polymerase produces high levels of transcription in *M. acetivorans in vivo* by expressing the reporter gene *YFAST* under the polymerase's specific promoter. Additionally, we demonstrate a new tool for understanding transcriptional and translational regulation in methanogens. Going forward, a new inducible expression system will be introduced in *M. acetivorans* as well to push its dynamic transcriptional capabilities further.

Research Questions

- Will a heterologous RNA polymerase be inhibited by *Methanosarcina acetivorans*?
- Can the dynamic range of gene expression in *Methanosarcina acetivorans* be increased using a recombinant RNA polymerase system?

Background

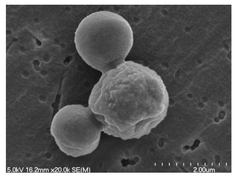


Figure 1. Scanning electron micrograph (SEM) of *Methanosarcina acetivorans.*

Methanogens

- Anaerobic archaea that grow and conserve energy by producing methane.
- *Methanosarcina acetivorans* is a species of methanogen isolated from marine sediment, often used for genetic engineering.
- Have isoprenoid lipids in their cell membrane rather than the fatty acid lipids found in bacteria and eukaryotes.

Applications for Environmental Security

- Methanogens can be used as a renewable fuel source.
- Methanogens have been shown to be a renewable source of isoprene, indicating they could produce terpenes, which are predicted to become a billion dollar industry by 2028.
- Methanogens can synthesize products such as terpenes from cheaper substrates than most bacteria and plants, and are a cleaner source than fossil fuels.

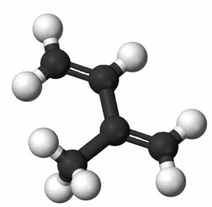


Figure 2. Isoprene, an organic compound that can be synthesized by methanogens, can be used as a precursor to rubber.

Methods

In vitro Transcription

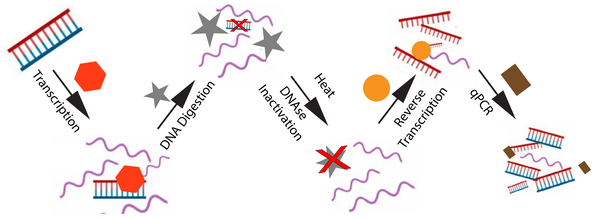
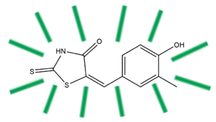


Figure 3. Basic in vitro transcription method outline. Linear plasmid DNA containing the *YFAST* gene under the desired promoter is transcribed with either the recombinant RNA Polymerase or the native *M. acetivorans* RNA Polymerase. DNAse is added to digest the original plasmid DNA then EDTA is added to protect the RNA while the DNAse is heat inactivated. Reverse transcription then occurs using reverse transcriptase, and the resulting ssDNA is amplified through qPCR.

In vivo Expression



YFAST and HMBR

Figure 4. Expression of fluorescence-activating and absorption-shifting tag (YFAST) in *M. acetivorans* will be compared between a native methanogen promoter called *Pmcr* and the *RNAP promoter*. When the fluorogen 4-Hydroxy-3-methylbenzylidene-rhodanine (HMBR), is bound, YFAST fluoresces green. This fluorescence will be measured for the comparison of expression levels between strains.

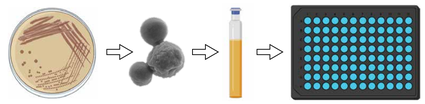


Figure 5. Plasmids with *YFAST* expressed under various promoter systems are transformed into *M. acetivorans*. Cells are grown to mid-log phase, incubated with HMBR, and their fluorescence is measured in a 96-well plate to measure expression levels.

Inducible Expression Design

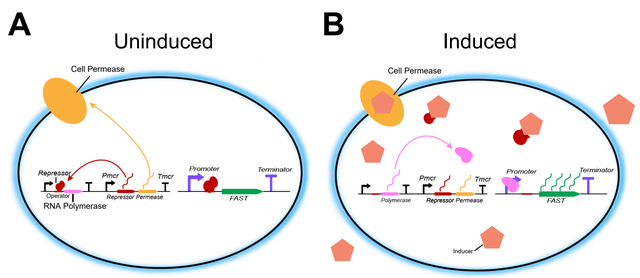


Figure 6. Inducible expression of the *RNA polymerase* gene and the gene of interest (*YFAST*).

A) Genetic elements of pLK28 without the inducer. The permease and operator are constituitively expressed while the *RNA polymerase* gene and gene of interest (*YFAST*) are repressed.

B) Genetic elements of pLK28 with the inducer. The permease, operator, and the *RNA polymerase* gene are all expressed. The RNA polymerase binds to the *RNAP promoter*, allowing *YFAST* to be expressed.

Figure 7. The inducer molecule is used to induce expression of the RNA polymerase and of YFAST. It is imported into cells through a permease and binds to the repressor protein. Once bound, the repressor can no longer bind to the DNA, allowing transcription to occur.

Plasmids

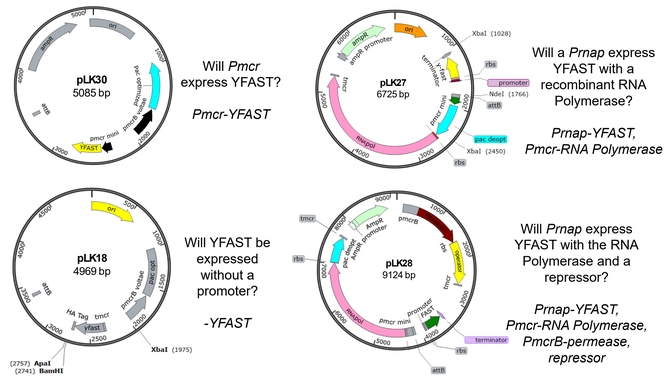


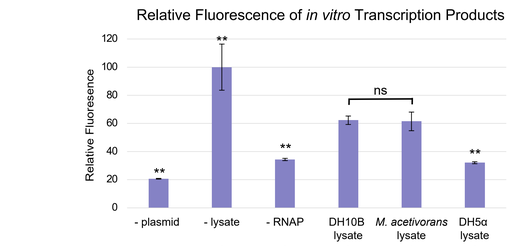
Figure 10. The primary plasmids used for this project. pLK30 expresses *YFAST* under *Pmcr* to test native expression levels from a highly expressed promoter. pLK27 expresses the RNA Polymerase within *M. acetivorans* and uses it to transcribe *YFAST* under *Prnap*. pLK18 has no promoter to act as a promoterless control. pLK28 adds elements needed to establish a repressed system to pLK27 to allow for inducible control of expression from the recombinant RNA Polymerase.

Future Directions

- Compare protein expression levels between strains with *YFAST* under control of *Pmcr* versus *Prnap* using fluorescence assays.
- Detect YFAST expression using Western Blots.
- Further explore tools to help transcription, translation, and their regulation in *Methanosarcina acetivorans* be better understood.
- Establish inducible expression system for tighter control over gene expression.
- Develop fully orthologous gene circuits in *M. acetivorans.*

Results

RNAP is Not Inhibited *in vitro*

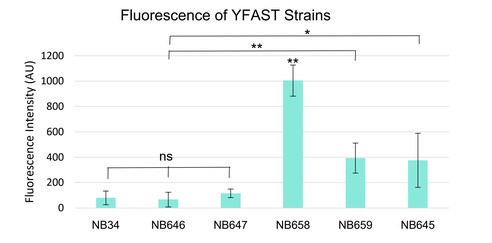


	- plasmid	- lysate	- RNAP	DH10B lysate	*M. acetivorans* lysate	DH5α lysate
plasmid	-	+	+	+	+	+
M. acetivorans lysate	+	-	+	-	+	-
E. coli DH5α F' lysate	-	-	-	-	-	+
E. coli DH10B lysate	-	-	-	+	-	-
RNA Polymerase	+	+	-	+	+	+

Figure 8. qPCR fluorescence shows *M. acetivorans* cell lysate is no less inhibitory than *E. coli* cell lysate. Plasmid DNA was transcribed, reverse transcribed, and amplified with qPCR to compare transcription levels.

E coli DH10B: Protein expression strain
E coli DH5α: Plasmid expression strain
** = p < 0.005
* = p < 0.05
ns = p > 0.05
n = 10, 15, 7, 6, 15, 7

RNAP Expresses YFAST *in vivo*



	NB34	NB646	NB647	NB658	NB659	NB645
YFAST	-	+	+	+	+	+
RNAP Promoter	-	-	+	+	+	-
Pmcr Promoter	-	-	-	-	-	+
RNA Polymerase	-	-	-	+	+	-
Repressor and *Permease*	-	-	-	-	+	-

Strain	Plasmid	Genotype
NB34	parent	parent
NB646	pLK18	*-YFAST*
NB647	pLK11	*Prnap - YFAST*
NB658	pLK27	*Prnap - YFAST, Pmcr-RNAP Polymerase*
NB659	pLK28	*Prnap-YFAST, Pmcr-RNA Polymerase PmcrB-repressor, permease*
NB645	pLK15	*Pmcr-YFAST*

Figure 9. HMBR fluorescence shows *YFAST* is expressed using the RNA Polymerase *in vivo*.

** = p < 0.005
* = p < 0.05
n = 8, 7, 7, 6, 8, 8

Acknowledgements

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