Optimizing Genome Engineering of Methanogens for Heterologous α-Humulene Synthase Expression

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Abstract

Genome engineering has become a crucial part of understanding microbial physiology and biotechnological innovation. While genome engineering of eukaryotes utilizing CRISPR-Cas9 has been studied extensively,[1] virtually no archaeal systems have been edited analogously. CRISPR tools would be especially valuable to study archaea due to their unique physiology. Their atypical physiological traits allow for archaea to be utilized in the production of biofuels, synthetic commodities, and in bioremediation efforts otherwise cost-inefficient for other microbes. Optimization of genetic engineering of archaea remains an issue, since each cell can have up to 17 chromosome copies, depending on the carbon source available, that should all receive the same changes.[2] This research project explores three methods for delivering Cas9 into methanogens to facilitate heterologous expression of humulene synthase.

Introduction

- Methanogens belong to the domain archaea and generate methane naturally.
- Methane is a natural gas that is preferred for jet fuels because of its high energy density and its ability to burn cleaner than fossil fuels.
- Methanogens produce methane with efficiencies between 30-90% from simple starting materials often found in waste products,[3,4] whereas yeasts require expensive sugars to ferment into their source of biofuel (ethanol).
- Besides bioenergy, methanogens have potential to synthesize valuable molecules like industrial compounds or drugs.
- Genome editing tools are currently limited for methanogens,[5,6] but previous groups have made progress in using CRISPR-Cas9: overview in **Figure 1**.

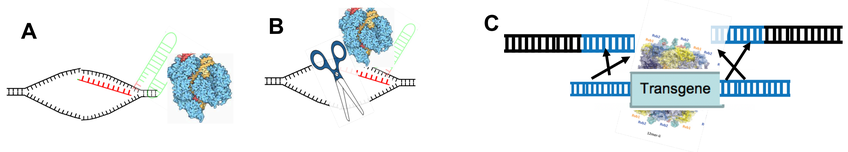

Figure 1. Graphic of CRISPR-Cas9 mediated gene editing process. (A) CRISPR guide RNA hybridizes to genomic DNA and (B) Cas9 endonuclease creates a double stranded break. (C) The transgene embedded in a homology directed repair template (HDR) is stitched into the genome by native cellular machinery.

Can we get methanogens to produce high value products like humulene?

Humulene is a a monocyclic sesquiterpene with applications in scent, flavor, and brewing industries. By inserting the gene that codes for (**Figure 2A**) humulene synthase we hypothesize that methanogens will create (**B**) humulene at (**C**) detectable concentrations and (**D**) transcript levels.

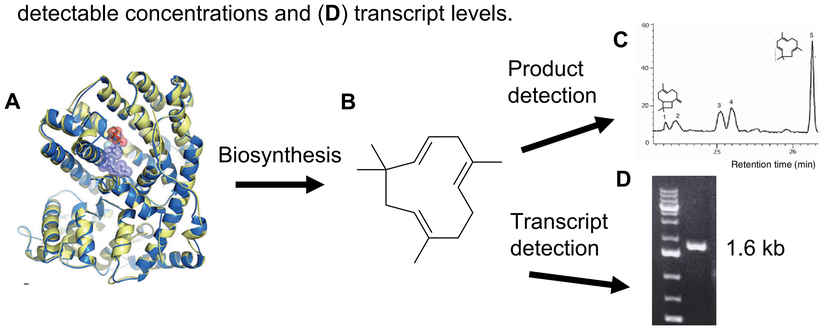

Figure 2. (A) Protein structure of related terpene synthase (α-humulene synthase not available) codes for (B) humulene, which can be detected by (C) gas chromatography.[7] (D) Transcript levels are measured by PCR amplifying cDNA.

Can CRISPR-Cas9 expand the methanogen engineering toolbox?

Plasmids were constructed from six fragments of DNA with homology introduced via PCR amplification (**Figure 3A**). The ligation protocol was carried out using NEBuilder HiFi DNA Assembly Mix (NEB E2621). Once constructed, plasmids were used to transfect *M. acetivorans* with Cas9 in three different ways to compare humulene synthase integration efficiency and humulene production (**B-E**).

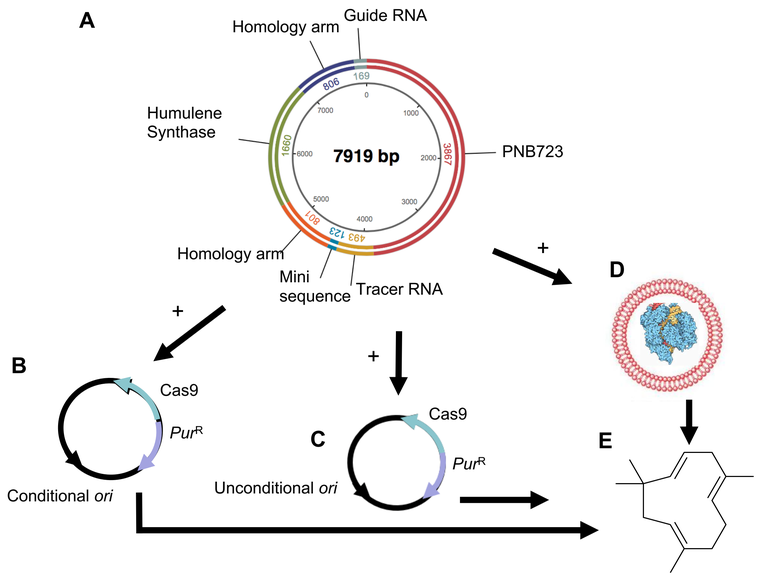

Figure 3. Schematic of CRISPR-Cas9 mediated gene editing strategies for humulene synthase insertion. (A) Plasmid containing HDR template and CRISPR sequences for transfecting *M. acetivorans* along with (B) suicide vector for transient Cas9 expression, (C) replication competent vector for integration for constitutive Cas9 expression or (D) liposomes containing Cas9 protein. (E) Co-transfection of plasmid and Cas9 results in humulene production.

Is α-humulene toxic to cells?

Serial dilutions starting with the theoretical max. of humulene producible by cells could were made and deposited onto freshly inoculated methanogen media. Each of the concentrations of humulene the cells were exposed to resulted in doubling times no different from the control ($p<0.05$). (**Figure 4**). This verifies that humulene is nontoxic and suggests that methanogens could be a suitable platform for its synthesis at least on a small scale.

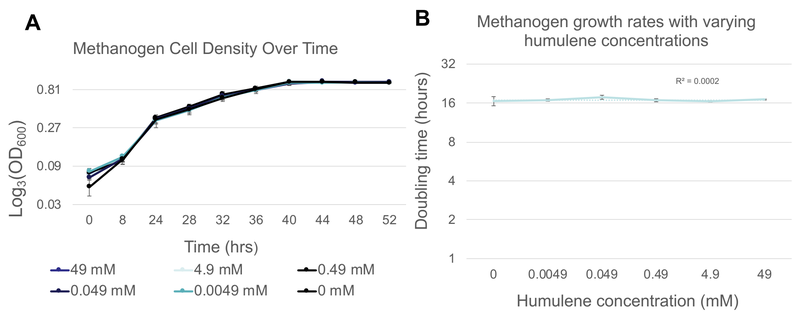

Figure 4. The effect of humulene on cell growth. (A) OD_{600} of methanogen cultures exposed to humulene in oil or oil control. (B) Doubling times of cultures does not vary with humulene concentration. (n=3)

Can co-cultures expand methanogen substrate repertoire?

Co-cultures can be advantageous for creating microbial fuel cells (**Figure 5A**) that can turn CO_2 into biomass and usable carbon for methanogens to make useful products.[3] Co-cultures were made by slowly adapting each microbe to the other's media, then combining in 50/50 media mix (**Figure 5.**)

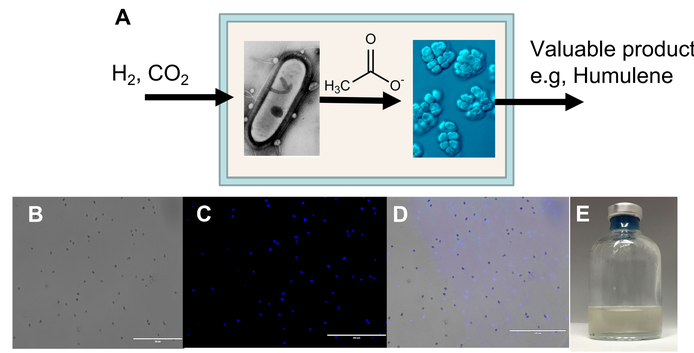

Figure 5. (A) Schematic representation of a microbial fuel cell. Microscopy confirms the coexisting state of adapted *M. acetivorans* and *C. Ljungdahlii* in a co-cultured media. (B) Phase contrast reveals both cell types while (C) DAPI filter only illuminates methanogens. (D) Overlaid phase contrast and DAPI filter. (E) Culture tube with adapted microbes.

Conclusions / Future Directions

- Determine plating efficiency of each Cas9 delivery method.
- Quantify the proportion of chromosomes that received the humulene synthase gene: conduct RT-qPCR to measure the levels of humulene synthase transcript normalized to housekeeping gene *rpoA1.*
- Use optimized gene editing method to insert genes coding for other critical molecules, e.g., anti-malarial drug artemisinin.
- Create a co-culture with edited methanogens: adapt to carbon free media.

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