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Establishing Methanogenic Archaea as an Alternative for Use in Synthetic Biology

A Demonstrative Advancement in Foundational Synthetic Biology through Production of Geraniol

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Abstract

Modern synthetic biology provides an opportunity to address urgent environmental problems using novel green technology. Here, we explore the utility of the methanogenic archaeon, *Methanococcus maripaludis* as an alternative host for biosynthesis of biofuels and high-value chemicals from inexpensive growth substrates of H_2/CO_2 or formate. The two major goals of this study are: 1) demonstrating the utility of archaea in synthetic biology through production of geraniol and 2) improving the genetic tools for synthetic biology available in archaea. Geraniol is a 10-carbon monoterpene-alcohol naturally occurring in plants such as rose and lemongrass. Due to its rose-like scent, it has a long history of usage approved by FDA in the fragrance and food industry. In recent years, potential usages of geraniol start expanding to biofuel, organic insect repellent and tumor suppressor. Currently, geraniol is commercially available through extraction mainly from plants. However, it is an inefficient model for geraniol production, as geraniol represents only a small portion of the plant biomass. In this study, a synthesized gene encoding geraniol synthase was expressed in *M. maripaludis*, and geraniol production was quantified as 0.2% of lipid dry weight using GC/MS. In order to optimize the gene expression and geraniol production, we are attempting to make new tools for *M. maripaludis*, including a protein expression and regulation tool through use of fluorescence protein, a ribosome-binding-site library enabling variable levels of expression, and a complete flux balance model of the lipid biosynthesis pathway.

Methanococcus maripaludis

M. maripaludis is a methanogenic archaeon native to salt marshes. It utilizes CO_2 and H_2 or formate through a process called methanogenesis, a form of anaerobic respiration that results in the production of methane. *M. maripaludis'* relatively simple genome, cheap substrates and mesophilic living conditions make it an ideal organism for synthetic biology. *M. maripaludis* creates isoprenoid lipids to make up its membrane, and these lipids can be precursors to high value chemicals.

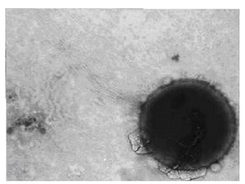
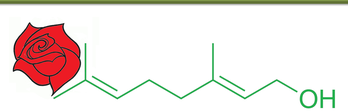


Fig. 1 *Methanococcus maripaludis* [1]

Fig. 2 *M. maripaludis* is an obligate anaerobe, so we must use an anaerobic chamber to handle cultures

Geraniol



- Geraniol is an acyclic monoterpene-alcohol
- Has a higher energy density than ethanol and a heat of combustion similar to diesel
- Can be used as a botanical insect repellent
- Has many uses in fragrances and flavorings
- Has been shown to inhibit the growth of pancreatic, prostate, and colonic cancers

Geraniol Production

Idea: Successful transformation of a plasmid containing a gene for geraniol synthase (GS) will enable a methanogen to synthesize geraniol.

Step 1: A vector (Registry #Bba_k1138000) containing a codon optimized GS gene was transformed into *M. maripaludis* , thereby extending its natural isoprenoid pathway (**Fig 3**). Cultures containing this plasmid were tested for geraniol at various stages of growth in both extracellular and intracellular content.

Step 2: The extraction of geraniol from *M. maripaludis* cultures was accomplished using hexanes as an organic solvent. The extractions were concentrated via evaporation under a stream of N_2 gas, and the samples were evaluated by Gas Chromatography/Mass Spectrometry (GC/MS).

Results: The two chromatographs shown in **Fig. 4** are the results of running a geraniol standard and an extracted *M. maripaludis* sample. Mass Spec confirmed the peak in both of these instances to be the presence of geraniol ((*trans*)-3,7-Dimethyl-2,6-octadien-1-ol). A wild type *M. maripaludis* culture was also extracted using hexanes and run through GC/MS and no peak was present. We were able to calculate the production of geraniol in our model as 0.2% of lipid dry weight by plotting the concentration of various standards against the integral area of the peak created and obtaining a linear relationship.

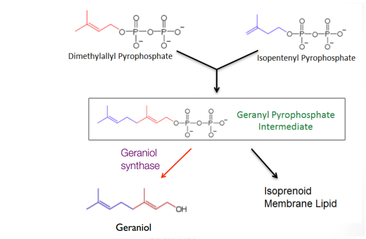


Fig. 3 Geraniol is synthesized from the compound geranyl pyrophosphate which occurs naturally in *Methanococcus* along the isoprenoid lipid pathway. A *Methanococcus* cell that successfully expresses GS will be able to catalyze the reaction producing geraniol.

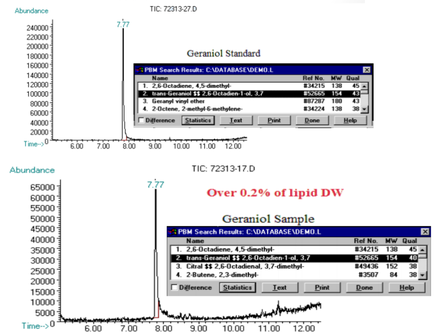


Fig. 4 Geraniol was shown to be present in the extracellular content of a GS expressing *Methanococcus* culture. The chromatographs are partnered with their respective mass spec analyses, confirming these peaks at identical retention times to be caused by the presence of geraniol.

Metabolic Flux Model

Idea: To increase geraniol yield by mapping the entire lipid biosynthesis pathway in *M. maripaludis*, performing a genome-scale flux balance analysis and developing strategies for the overproduction of isoprenoid compounds.

Results: We designed a metabolic model for isoprenoid biosynthesis based upon the complete pathway, including stoichiometric values of acetyl-CoA, ATP, and other compounds required for cell growth. The Biocyc, KEGG, IMG, and SEED databases were used to identify candidate genes/enzymes responsible for each reaction (**Fig. 5**).

In progress: Complete a genome-scale flux balance analysis using OptFlux™ and develop strategies for the overproduction of isoprenoid compounds. OptFlux™ is an in-silico metabolic engineering software that provides the capability to run simulations given any mutation or environmental condition. We are now using the software to optimize the production of a specific biomass by determining candidates for efficient knock-out strains or beneficial environmental conditions (**Fig. 6**).

Outook: Build a regulatory model for isoprenoid biosynthesis in *M. maripaludis*, allowing further model optimization. Using primary literature, we will identify enzyme kinetics and effectors, missing genes/enzymes and regulation upon each individual reaction.

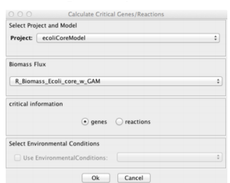


Fig. 6 Excerpt from OptFlux ™ software exemplifying how a user may calculate critical genes/reactions for their biomass flux of interest. [2]

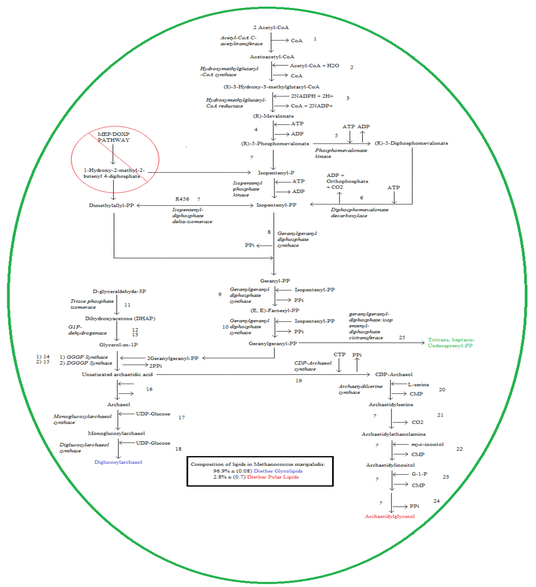


Fig. 5 Isoprenoid biosynthesis pathway in *M. maripaludis* . This pathway begins with the product of autotrophic CO_2 fixation, acetyl-CoA, and proceeds through the mevalonate pathway. No experimental evidence has been shown that *Methanococcus* utilizes the MEP/DOXP pathway

Protein Expression/Quantification Tool

The vector below has been optimized for expression in *M. maripaludis* and uses the red fluorescent protein, mCherry, as a fluorescent tag. The use of BioBrick™ standards allows researchers to insert any gene of interest that will be covalently linked to mCherry. Fluorescent screening of transformed cultures will effectively quantify expression of the gene of interest in at least a 1:1 ratio.

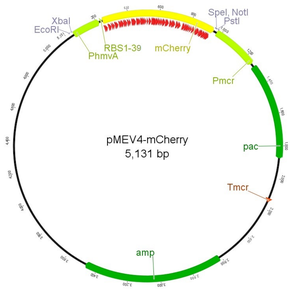


Fig. 7 pMEV4-mCherry vector. The red fluorescent protein, mCherry, was codon optimized (shown in red) and cloned into the vector. The restriction sites just downstream of mCherry allow us to covalently link a protein of interest to mCherry. This vector contains selective marker genes for replication in both *E. coli* and *M. maripaludis*.

Ribosome Binding Site Library

Idea: The ribosome binding sites of archaea are not well characterized. A ribosome binding site (RBS) with higher affinity for ribosomes will result in increased translation efficiency.

The pMEV4-mCherry vector mentioned above (**Fig. 7**) will allow us to characterize a library of RBS for use in methanogens. In the pMEV4-mCherry vector, the region labeled 'RBS 1-39' flanking the mCherry gene is where our varying RBS sequences will be cloned.

Results: Primers were designed for a mutation of each base along a 12 base-pair mutation region (**Fig 8**). This region includes the RBS, spacer, and first base of the start codon in variants 34-36. Additionally, two primers (37 & 38) were designed from 16S data to create a 'perfectly' bound RBS and an 'negatively' bound RBS. After successful cloning, these RBS sequences were transformed into *M. maripaludis*.

In progress: Transformed cultures are being screened for fluorescence and evaluated for relative strength of expression per RBS.

Native RBS/Spacer	CAGGTAGCGCT
Variant 1	GAGGTAGCGCT
Variant 2	TAGGTAGCGCT
Variant 3	AAGGTAGCGCT
Variant 4	CGGGTAGCGCT
Variant 5	CTGGTAGCGCT
Variant 6	CCGGTAGCGCT
Variant 7	CAAGTAGCGCT
Variant 8	CATGTAGCGCT
Variant 9	CACGTAGCGCT
Variant 10	CAGATAGCGCT
Variant 11	CAGTTAGCGCT
Variant 12	CAGCTAGCGCT
Variant 13	CAGGAAGCGCT
Variant 14	CAGGGAGCGCT
Variant 15	CAGGCAGCGCT
Variant 16	CAGGTGGCGCT
Variant 17	CAGGTTGCGCT
Variant 18	CAGGTCGCGCT
Variant 19	CAGGTAACGCT
Variant 20	CAGGTATCGCT
Variant 21	CAGGTACCGCT
Variant 22	CAGGTAGGGCT
Variant 23	CAGGTAGTGCT
Variant 24	CAGGTAGAGCT
Variant 25	CAGGTAGCACT
Variant 26	CAGGTAGCTCT
Variant 27	CAGGTAGCCCT
Variant 28	CAGGTAGCGGT
Variant 29	CAGGTAGCGTT
Variant 30	CAGGTAGCGAT
Variant 31	CAGGTAGCGCG
Variant 32	CAGGTAGCGCC
Variant 33	CAGGTAGCGCA
Variant 34	CAGGTAGCGCTG
Variant 35	CAGGTAGCGCTT
Variant 36	CAGGTAGCGCTC
Variant 37	GGAGGTGCGCT
Variant 38	CCTCCAGCGCT

Fig. 8 Variants 1-33 are mutations in the RBS/Spacer region and 34-36 are mutations in the start codon. Variant 37 is the "perfect" RBS and 38 is the "negative" RBS

Environmental Applications

Biofuel: Recent studies on geraniol have revealed its potential as a biofuel to be worth considering due to its high energy density and heat of combustion. Additionally, if geraniol is taken one reaction further to geraniol acetate by the enzyme geranyl acetyltransferase, its biofuel properties become even more promising.

Insect Repellent: Studies have also shown geraniol to be a leading natural insect repellent. In fact, Cartersville, Georgia's own BugBand® is a company that manufactures and sells geraniol-based insect repellent products as a natural alternative to harmful chemicals such as DEET.

However, an issue arises for these uses in that the current method for obtaining geraniol is an inefficient model - through extraction of plant biomass. A methanogen with the ability to catalyze the reaction from geranyl pyrophosphate to geraniol provides a new opportunity to biologically synthesize geraniol in a much more efficient model. Further improvements upon geraniol synthase expressing methanogens such as those being done in this study, may lead to significantly increased yield.

Biogas: Methanogens play a vital role in anaerobic digestion/biogas production. Biogas, being comprised mostly of methane and carbon dioxide, can be burned to generate heat and electricity. Generating biogas via anaerobic digestion of biomass and organic waste is one of the few proven, cost-effective, scalable bioenergy strategies [3]. In this study, we are establishing the basic genetic tools that allow researchers to work more effectively with methanogens towards practical applications in these industries. Together with new tools developed by others, scientists may one day be able to not only convert waste into high-value chemicals such as geraniol, but generate biogas as a renewable energy source in parallel. These technologies would contribute to establishing the next generation of commercial models that help build a cleaner and more sustainable world.

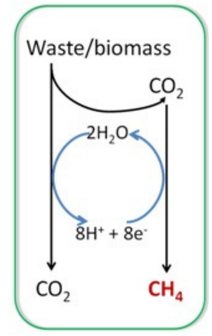


Fig. 9 Conceptualized super cell that converts idealized organic matter ($2CH_2O$) directly into biogas [3].

Future Work

- Improve upon geraniol extraction methods from *M. maripaludis* cultures
- Combine knowledge accumulated in this study to further optimize geraniol production, such as using high strength of RBS for better expression of GS, making mutants and changing cultivating conditions that may increase metabolic flux towards geraniol.
- Further extension of isoprenoid pathway to geranyl acetate and possibly other high-value chemicals having potential applications in environment.

References

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