Enhancing Growth and Bioproduct Output in Engineered Methanogens

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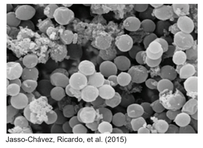
Abstract

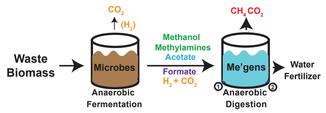
Methanogens are obligately anaerobic archaea noteworthy for producing methane from C1 compounds and acetate. Their ability to convert low-energy, otherwise inaccessible carbon into methane is a result of their highly efficient central respiration, which accounts for approximately 99% of the chemistry in the cell. A result of this respiratory strategy is a high substrate:product conversion ratio which is industrially relevant for the production of biomethane, and may also be harnessed for the production of value-added commodities through strain engineering and synthetic biology. One area of interest are terpene compounds, as methanogen membranes are composed 5% by dry weight of isoprenoid lipids and flux through the isoprenoid biosynthetic pathways is naturally high in Archaea compared to Eukarya and Bacteria. To assess the metabolic plasticity of methanogens, our laboratory has engineered *Methanosarcina acetivorans* to produce the hemiterpene isoprene. We found that engineered methanogens directed up to 4% of total carbon substrate towards isoprene with increased overall biomass. Optimization of isoprene synthesis by archaea will require developing large-scale process conditions to capture methane and isoprene. While methanogens are routinely grown at large scale in municipal and agricultural anaerobic digesters for biogas, process conditions for scaling up pure cultures on defined culture medium have not yet been optimized. We are systematically evaluating bioreactor conditions including gas exchange and nutrient flow rates to improve methanogen growth in pure culture. To date we have increased final OD600 in batch 1.5L scale from 0.165 to 0.705 while trapping 5.58mg isoprene in a 1.5ml oil trap accounting for 0.16% of substrate carbon. Future experiments will assess growth in continuous culture and effects of process parameters on isoprene yield while optimizing isoprene capture.

Methanogens are highly efficient microbes



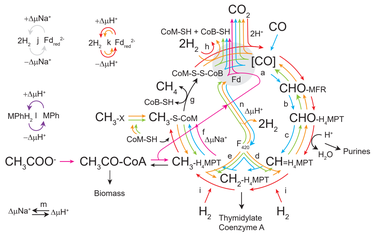
Jasso-Chávez, Ricardo, et al. (2015)

Methanosarcina acetivorans
- Obligate anaerobe
- 5.75 mbp genome
- Mesophilic
- Widest metabolic capacity of a methanogen
- Capable of autotrophic and heterotrophic growth on CO, methanol, methylamines, methyl sulfides and acetate
- NOT capable of growth on H_2:CO_2
- Genetically tractable



Methanogens in wastewater treatment. Methanogens consume terminal degredation products and play an essential role in wastewater treatment and carbon cycling

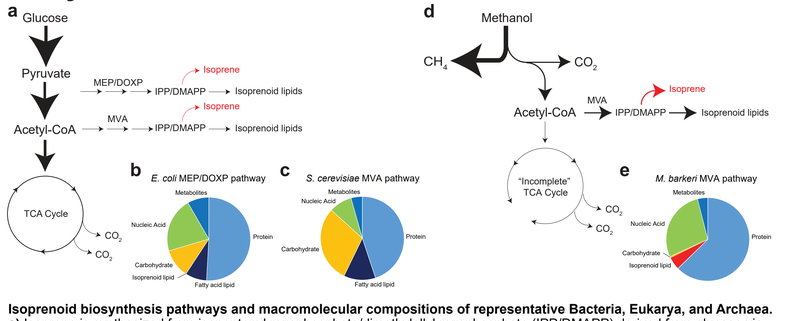
Methanogen metabolism



The Wolfe Cycle of Methanogenesis. (Buan 2018)
Primary pathways:
- All methanogenesis
- Hydrogenotrophic pathway
- Methyl respiration
- Methylotrophic pathway
- Acetoclastic
- Carboxydotrophic pathway

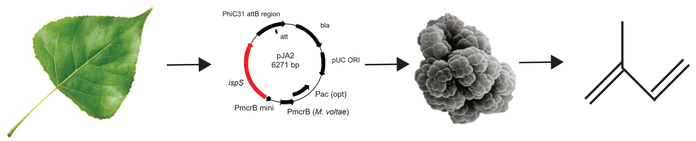
All pathways produce <1 ATP per mol of substrate

Methanogen have a high flux towards isoprenoid lipid biosynthesis



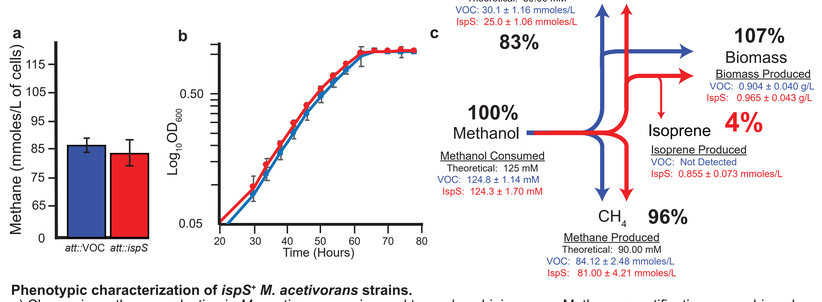
Isoprenoid biosynthesis pathways and macromolecular compositions of representative Bacteria, Eukarya, and Archaea. **a)** Isoprene is synthesized from isopentenyl pyrophosphate/dimethylallyl pyrophosphate (IPP/DMAPP) derived from glucose via the methylerythritol phosphate/deoxy xylulose phosphate (MEP/DOXP) pathway in bacteria or mevalonate (MVA) pathway in eukarya. **b & c)** relative amounts of macromolecules in E. coli bacterium (Egan & Vollmer, 2013) and S. cerevisiae yeast (Yamada & Sgarbieri, 2005), respectively. d) isoprenoid lipids are synthesized from IPP/DMAPP by the archaeal MVA pathway in methanogens. **e)** isoprenoid lipids in methanogens comprises 5% biomass dry weight. (Feist et al., 2006) Arrow sizes and line widths depict published carbon fluxes through each pathway. One or more genes is required for most organisms to produce isoprene monomer (red arrows).

Engineering methanogens to produce non-native metabolites



General overview of engineering a isoprene producing methanogen.
The gene for isoprene synthase was selected from *Populus alba*. The gene was optimized for expression in *Methanosarcina* species and is cloned into a plasmid containing an archaeal antibiotic resistance marker. The plasmid is transfected into *M. acetivorans* and selected for under antibiotic stress. Confirmation of gene insertion of attained by PCR and expression is confirmed via reverse transcription.

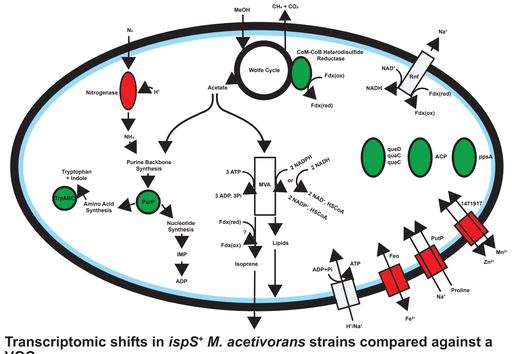
Engineered *Methanosarcina acetivorans* diverts carbon from CO_2 to isoprene



Phenotypic characterization of *ispS*⁺ *M. acetivorans* strains.
a) Change in methane production in *M. acetivorans* engineered to produce bioisoprene. Methane quantification was achieved via GC-FID. Blue bars indicate methane production by a vector only control whereas the red bars indicate *ispS*⁺ strains. b) Growth rates of engineered *M. acetivorans* strains. Growth rates of *ispS*⁺ and VOC strains were measured in HS+MeOH medium via absorbance at 600n,. There was no significant variance in growth rate between the two strains. c) Mass balance of *ispS*⁺ and VOC strains of *M. acetivorans.* Methanol consumption as well as methane, CO_2, and isoprene production was measured by GC-FID. Cultures used for this experiment were desiccated and measured for dry weight. It was found that the carbon utilized for isoprene production was not diverted from biomass but rather CO_2 production.

Transcriptomic analysis of engineered *M. acetivorans* reveals energetic adaptations and links to amino acid biosynthesis

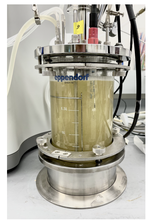
Significant differentially expressed genes in isoprene producing *M. acetivorans*					
Symbol	Base Mean	log2Fold Change	pvalue	padj	Annotation
MA_RS22160	33694.16	-6.52	5.01E-32	5.80E-29	NifB/NifX family molybdenum-iron cluster-binding protein
MA_RS00110	25853.32	-6.11	9.93E-81	1.53E-77	metal-dependent transcriptional regulator
MA_RS22925	80806.74	-5.80	0.00124	0.03425	cobalamin-dependent protein
MA_RS18185	1363.29	-5.58	3.47E-18	1.46E-15	ferrous iron transport protein A
MA_RS00115	71109.92	-4.97	8.0E-101	1.85E-97	zinc ABC transporter substrate-binding protein
MA_RS18180	540.8029	-4.90	5.08E-05	0.00252	ferrous iron transport protein A
MA_RS22145	41764.52	-4.84	1.09E-18	5.06E-16	4Fe-4S binding protein
MA_RS24210	995.4087	-4.31	1.90E-04	0.00793	MotA/TolQ/ExbB proton channel family protein
MA_RS22155	31497.83	-4.04	1.98E-13	4.82E-11	NifB/NifX family molybdenum-iron cluster-binding protein
MA_RS20610	7740.685	-3.75	7.26E-15	2.10E-12	cyclopropane-fatty-acyl-phospholipid synthase family protein
MA_RS18175	6346.078	-3.65	4.96E-08	5.10E-06	ferrous iron transport protein B(FeoB)
MA_RS00120	28452.21	-3.00	1.29E-31	1.19E-28	ABC transporter ATP-binding protein
MA_RS00125	17828.53	-2.95	2.31E-28	1.78E-25	metal ABC transporter permease
MA_RS18165	1289.199	-2.56	1.25E-04	0.00541	FeoC-like transcriptional regulator
MA_RS04620	48910.11	-2.09	1.53E-04	0.00649	phosphate ABC transporter substrate-binding protein PstS family protein
MA_RS21905	3447.194	2.15	8.65E-07	7.15E-05	6carboxytetrahydropterin synthase QueD(queD)
MA_RS16350	6555.411	2.31	1.65E-06	1.30E-04	4Fe-4S dicluster domain-containing protein
MA_RS16345	6402.436	2.34	2.38E-06	1.69E-04	CoB--CoM heterodisulfide reductase iron-sulfur subunit B family protein
MA_RS17205	9024.879	2.35	5.56E-07	4.77E-05	sulfopyruvate decarboxylase subunit beta
MA_RS04950	5306.457	2.38	6.54E-08	6.57E-06	iron ABC transporter substrate-binding protein
MA_RS16355	26508.23	2.41	8.60E-09	1.02E-06	FAD-dependent oxidoreductase
MA_RS11535	3618.26	2.55	1.81E-09	2.71E-07	tyrosine-type recombinase/integrase
MA_RS07365	4911.462	2.64	4.11E-06	2.80E-04	energy-coupling factor transporter transmembrane protein EcfT
MA_RS17200	19106.75	2.65	9.40E-07	7.63E-05	cysteate synthase
MA_RS07360	11748.58	2.91	3.59E-08	3.86E-06	energy-coupling factor ABC transporter ATP-binding protein
MA_RS21910	8313.924	2.92	1.76E-11	3.88E-09	7-carboxy-7-deazaguanine synthase QueE
MA_RS01605	283.0543	3.15	2.31E-19	1.19E-16	methyltransferase domain-containing protein
MA_RS21920	12705.21	3.20	6.64E-11	1.28E-08	7-cyano-7-deazaguanine synthase QueC
MA_RS15690	12811.78	3.59	5.62E-09	7.22E-07	indole-3-glycerol-phosphate synthase
MA_RS15685	14395.35	4.21	9.73E-04	0.02868	tryptophan synthase subunit beta



Transcriptomic shifts in *ispS*⁺ *M. acetivorans* strains compared against a VOC.
Differential expression analysis of isoprene producing M. acetivorans against a vector only control yielded 153 significantly (p>0.05) differentially expressed genes (62 downregulated, 91 upregulated). Filtering for genes with log2 fold-change greater than 2 revealed 3 highly significant genes (Left table). Isoprene producing *M. acetivorans* showed a decrease in genes associated with membrane permeability (red) and an increase in genes associated ith amino acid biosynthesis and lipid biosynthesis (green). Most notable is a decrease in metal and sodium transporters which would force ions through the energy generating ATPase pumps. Genes associated with acyl transport (ACP) and lipid metabolism (ppsA) were upregulated. Additionally, genes associated with indole biosynthesis (TrpABC) were upregulated alongside formate-phosphoribosyl-aminoimidazolecarboxamide ligase, PurP, the enzyme responsible for routing purine backbones towards amino acid biosynthesis or energy producing nucleotides.

Scaling pure-culture methanogen growth

Bench-scale growth
- 10mL volume
- Sealed vessel
- Fixed C+E
- Pressurized
- No pH control
- Easy headspace sampling
- Manual measurements
- Inexpensive replicates



Bioreactor growth
- 1-2L volume
- Open vessel
- Variable gas mixes
- Variable C+E
- Variable agitation
- pH control
- Automated measurements
- Multiple growth conditions

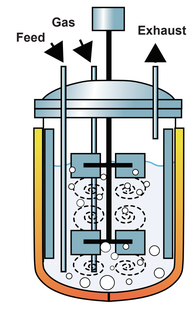
Establishing optimum methanogen bioreactor culture conditions

Major determining variables:
Media composition
- Can be separated into stable and expendable components
  - Stable: Salts, minerals, trace elements
  - Expendable: C+E sources, vitamins, sulfur, bicarbonate
- Replenishment of expendable components increases growth

Anaerobic conditions
- Sparging to remove O_2
- Reducing chemical components

Gas diffusion
- Gas composition (CO_2%, N_2%)
- Sparge rate
- Impeller location
- Agitation speed

Exhaust
- Narrow exhaust increases pressure within vessel and improves gas solubility



Optimization of *Methanosarcina acetivorans* mono-culture in Eppendorf BioFlo 320 reactor										
Reduction time	Inoculation Volume (ml)	pH control (6.8)	Agitation (RPM)	CO_2 Sparge (SLPM)	Volume (Liter)	MeOH (mmol/min)	Additional Supplement	Lag time (Hours)	Pressure (PSI)	Final OD
Before	10	Y	150	0.1	1	1.2	N/A	NM	NM	**0.165**
Before	10	N	50	0.1	1.5	1.2	N/A	NM	NM	**0.033**
Before	40	N	50	0.1	1.5	0	N/A	NM	NM	**0.042**
After	50	N	50	0.1	1.5	0	N/A	NM	NM	**0.062**
After	50	Y	50	0.05	1.5	1.2	N/A	NM	NM	**0.404**
After	100	Y	50	0.05	1.5	1.2	N/A	NM	NM	**0.456**
After	100	Y	150	0.1	1.75	1.2	N/A	NM	NM	**0.532**
After	100	Y	150	0.1	1.75	1.2	N/A	NM	NM	**0.520**
After	200	Y	200	0.15	1.75	1.2	N/A	NM	NM	**0.705**
After	200	Y	200	0.15	1.75	2.4	N/A	48	1.1	**0.602**
After	200	Y	250	0.15	1.75	2.4	45mM $NaHCO_3$	48	4.2	**0.588**
After	200	Y	250	0.15	1.75	2.4	45mM $NaHCO_3$	36	1.5	**0.622**

Increasing *M. acetivorans* growth in a bioreactor.
Methanosarcina acetivorans was grown in an Eppendorf BioFlo320 bioreactor in HS+MeOH medium. A large starting inoculum is required to overcome temporary exposure to O_2 during the transfer from a starter culture to the fermentation vessel. The presence of CO_2 in the gas mixture is required for M. acetivorans growth, even though the cells lack ecH and are incapable of utilizing H_2/CO_2 for growth. Placing the impeller height at the liquid-gas interface improves CO_2 solubility and without needing to increase sparge rate. The addition of a continuous bicarbonate drip and choking the exhaust reduces lag time and prevents oxygen contamination. The addition of the carbon source, MeOH, allows the culture to survive longer before crashing but is not the limiting factor in maximum OD. Further experiments are necessary to relieve further metabolic bottlenecks as well as improving isoprene harvest.

Future Directions

- **Refine bioreactor growth conditions**
- **Quantify relationship between available CO_2 and growth yield**
- **Develop supplement cocktail to maxize methanogen growth**
- **Improve isoprene capture**
- **Apply optimum culture conditions on mutant strains**

Recent publications

- Aldridge, J., **Carr, S.**, Weber, K. A., & Buan, N. R. (2021). Anaerobic prodution of isoprene by engineered methanosarcina species archaea. *Applied and environmental microbiology*, 87(6), e02417-20.
- Carr, S., Aldridge, J., & Buan, N. R. (2021). Isoprene production from municipal wastewater biosolids by engineered archaeon M*ethanosarcina acetivorans. Applied Sciences,* 11(8), 3342.
- Carr, S. Buan, N. R. (2022). Insights into the biotechnology potential of *Methanosarcina. Frontiers in Microbiology.* DOI 10.3389.fmcib.2022.1034674

Funding sources

This work was supported by the Nebraska Public Power Disctrict throuhg the Nebraska Center for Energy Sciences Research at the University of Nebraska-Lincoln

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