Novel Microbial Routes to Synthesize Industrially Significant Precursor Compounds

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Project Goals

Ethylene is the most widely employed organic precursor compound in industry. Using recently discovered efficient microbial anaerobic ethylene synthetic processes, the potential to impact ethylene formation is tenable using plentiful lignocellulose and/or CO_2 feedstocks. The overall long-term objective is to develop an industrially compatible microbial process to synthesize ethylene in high yields. Based on the discovery of a novel and genetically regulated anaerobic pathway to produce high levels of ethylene (the DHAP ethylene pathway), the following specific aims will be addressed:

1. Fully probe the catalytic potential of all enzymes of the DHAP ethylene pathway and determine the regulatory mechanism of DHAP-ethylene pathway gene expression. Model the thermodynamics and kinetics of ethylene synthetic pathways.
2. Discover effective and active ethylene enzymes encoded in cultured and uncultured organisms from anoxic environments.
3. Construct a modular set of optimized genes (from Aims 1 and 2) on a DNA fragment containing specific regulatory elements that will allow high level gene expression in model organisms that have been flux optimized.

Abstract

While studying the role of RubisCO and the RubisCO-like Protein (RLP) in sulfur metabolism, we discovered novel pathways for the metabolism of the key compound 5-metthylthioadenosine (MTA). We discovered distinct anaerobic MTA pathways in *Rhodospirillum rubrum* and the related organism *Rhodopseudomonas palustris* (1). **During the course of elucidating these novel anaerobic MTA metabolic pathways, we found that one of these routes resulted in the production of copious quantities of ethylene (1) (Fig. 1A), the first reported anaerobic route to produce ethylene, involving novel genes and enzymes.** Ethylene synthesis was shown to be highly regulated, with significantly high concentration produced under optimum conditions (~ 100 µmol/L/culture OD) (1) using either CO_2 or organic carbon growth substrates. A search of publically available genomic databases for other organisms that possess the newly discovered genes encoding anaerobic ethylene synthesis enzymes indicated this pathway was widespread in genomes from multiple phyla of industrially relevant Proteobacteria, Firmicutes (including lignocellulose degrading strains, Fig.1B) and a few archaea (1).

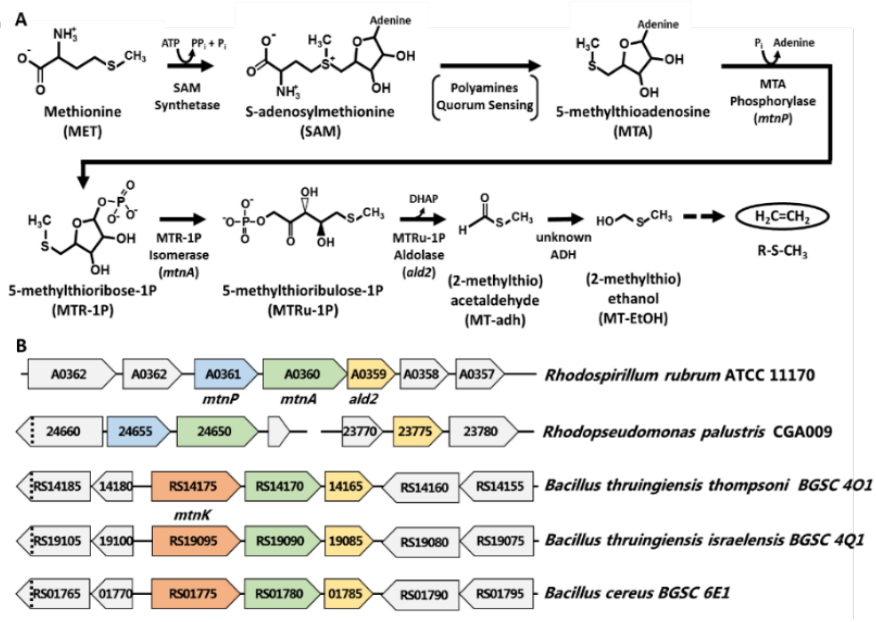

Fig. 1. Anaerobic ethylene pathway, enzymes, and genes. (A) Anaerobic ethylene-forming pathway from *R. rubrum* and *R. palustris* (from ref. 1). (B) Alignment of *R. rubrum* and *R. palustris* gene cluster Rru_A0359 – Rru_A0361 for bioethylene production with genes from representative lignocellulose degrading *Bacillus* spp. (Blue) *mtnP*; (Red) *mtnK*, 5-methylthioribose kinase, which in conjunction with *mtnN* (MTA nucleosidase) replaces *mtnP* in *Bacillus sp.*; (Green) *mtnA*; (Yellow) *ald2*.

Based on the discovery of a novel and genetically regulated anaerobic pathway to produce high levels of ethylene (1) (Fig. 1), the following specific aims will be addressed to enhance ethylene production:

<u>Specific Aim 1</u>: Analysis and Characterization of Ethylene Biosynthetic Pathways (Tabita, Cannon). In this aim, we will characterize the ethylene biosynthetic pathway with respect to its (A) biochemistry, (B) regulation and (C) thermodynamics & kinetics.

<u>Specific Aim 2</u>: Discover effective and active ethylene enzymes encoded in cultured and uncultured organisms from anoxic environments (Wrighton).

<u>Specific Aim 3</u>: Construct a modular set of optimized genes (from Aims 1 and 2) on a DNA fragment containing specific regulatory elements that will allow high level gene expression in model organisms that have been flux optimized (Tabita, Wrighton, Cannon)).

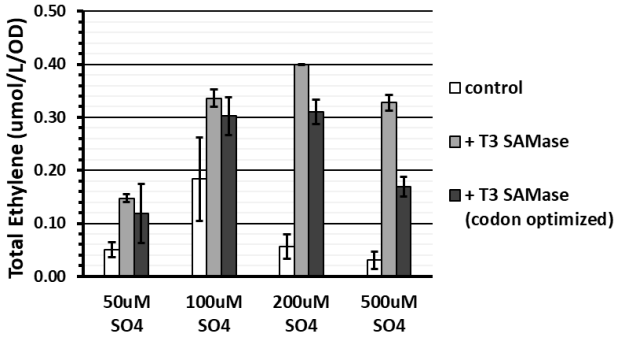

Fig. 2. Total ethylene produced by *R. palustris* when grown anaerobically in 0.5 % ethanol / 0.1 % bicarbonate minimal media supplemented with the indicated amount of ammonium sulfate. When either the native T3 coliphage SAM hydrolase (T3 SAMase) or the codon optimized gene for *R. palustris* was expressed, total ethylene production was enhanced up to 10-fold and suppression by non-limiting sulfate levels (≥ 200 µM) was overcome.

Recent Progress/Current Studies:

Aim 1: (A) Enhanced ethylene synthesis: Under conditions where cellular MTA formation is minimal, subsequent ethylene production is low. To circumvent this rate limiting formation of MTA, we have employed a novel viral enzyme, SAM hydrolase, from the T3 coliphage. This highly active enzyme (kcat ~ 300 / s) directly catalyzes SAM conversion to MTA and homoserine, and it is naturally used by the coliphage to evade the *E. coli* SAM-dependent restriction complex. When heterologously synthesized in *R. palustris*, the SAM hydrolase enzyme not

only enhanced ethylene production by up to 10-fold due to the specific conversion of native cellular SAM to MTA, it also overcame the regulatory effects of non-limiting extracellular sulfate levels on ethylene production (Fig. 2).

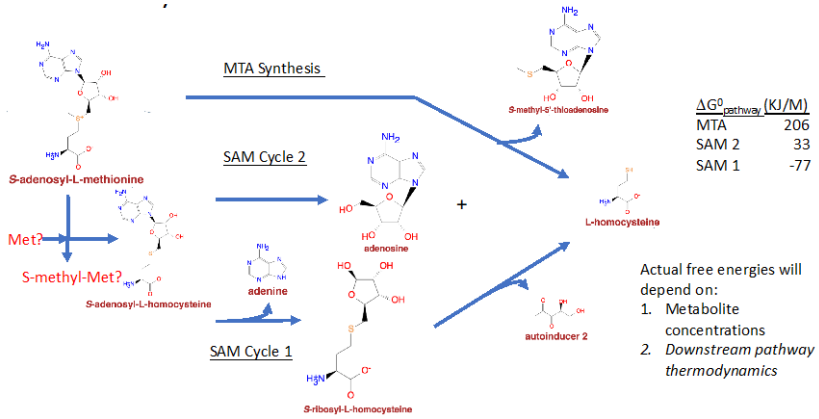

Fig.3. Development of kinetic and thermodynamic model for *R. palustris* metabolism.

(B) Computational Models: Computational models will be used to evaluate the thermodynamics of ethylene production and predict strategies for optimizing these conditions. Possible bottlenecks are first the redirection of SAM flux away from biomass production and towards MTA and subsequently ethylene production (see above). A second possible bottleneck may be the final steps in the ethylene pathway, which have not been characterized yet. We have already started developing a full kinetic and thermodynamic model of *R. palustris* metabolism including these pathways to evaluate the kinetics and thermodynamics (Fig. 3).

Aim 2: Discovery of more efficient enzymes of the anaerobic ethylene pathway: Previous studies had indicated that more effective aldolase ans isomerase genes could replace endogenous genes and substantially enhance ethylene levels (1, and unpublished studies). To scale up the search for ethylene-enhancing orthologs we apply targeted functional metagenomics to systematically query genes from the environment. Candidate genes will be synthesized for expression in our *R. palustris* genetic system and assayed in (i) a high throughput lysate activity assay and (ii) via physiological complementation to identify orthologs that enhance ethylene production. We have mined JGI IMG/M genome and metagenome sequence databases for candidate orthologs to the MTR-1P isomerase (*mtnA*) and the MTRu-1P aldolase (*ald2*) genes in collaboration with JGI scientists. The initial search identified 1,371,813 and 96,049 candidate genes for *mtnA* and *ald2*, respectively. Subsequent filtering based on synteny, phylogeny, and sequence homology with experimentally validated enzymes has yielded over 2500 candidate orthologs for each target gene, from multiple metagenomic samples covering a wide variety of environments including wetlands, forest soils, rhizosphere, and bioreactors. Candidates clustering with sequences from experimentally validated representatives, having both *mtnA* and *ald2* on the same contig, or having metatranscript data are ranked higher as synthesis targets than those identified by gene sequence alone. This approach should both ensure recovery of active enzymes and maximize sampling of undefined biochemical diversity.

References

1. North, J. A., Miller, A. R., Wildenthal, J. A., Young, S. J., and Tabita, F. R. Microbial pathway for anaerobic 5'-methylthioadenosine metabolism coupled to ethylene formation. Proc. Natl. Acad. Sci. U S A. 2017 Nov 28;114(48):E10455-E10464. doi: 10.1073/pnas.1711625114. Epub 2017 Nov 13.