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. The potential to impact ethylene formation via recently discovered microbial processes 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. The discovery of a novel and genetically regulated anaerobic pathway to produce high levels of ethylene (the DHAP ethylene pathway) impacts the following specific aims:

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. (Tabita and Cannon)

2. Discover effective and active ethylene enzymes encoded in cultured and uncultured organisms from anoxic environments. (Wrighton)

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. (Tabita, Wrighton, and Cannon)

Abstract

We have discovered a novel pathway for recycling distinct byproducts of enzymatic processes requiring S-adenosyl-L-methionine (SAM) as a cofactor [1,2]. This pathway, called the DHAP Shunt, consists of a phosphorylase (MtnP), isomerase (MtnA), and a novel class II aldolase (Ald2) for the salvage of 5'-methylthioadenosine (MTA) (**Fig. 1B**) [1]. A gene cluster for this pathway is widespread in nature (~ 10 % of all bacteria) and this pathway has been verified in *R. rubrum, R. palustris*, *B. thuringiensis* and Extraintestinal Pathogenic *E. coli* strains [1-4]**.** Intriguingly, during *R. palustris* aerobic growth, copious amounts of methionine precursor, methanethiol (CH_3-SH), is produced from (2-methylthio)ethanol (**Fig. 1C,** DHAP-Methanethiol Shunt) [3]. Conversely, **during anaerobic growth we discovered that (2-methylthio)ethanol was further metabolized to methionine, with copious amounts of ethylene gas produced in the process** (**Fig. 1C**, DHAP-Ethylene Shunt) [1]. **This is the first reported anaerobic route to ethylene, involving novel genes and enzymes.** Based on the discovery of this novel and genetically regulated anaerobic pathway to produce high levels of ethylene, the following specific aims are addressed to enhance ethylene production:

Specific Aim 1: Fully probe the catalytic potential of all enzymes of the DHAP ethylene pathway (Tabita and Cannon)**.** SAM is also utilized by Radical SAM and methyltransferase enzymes, producing 5'-deoxyadenosine (5'dAdo) and S-adenosylhomocysteine as byproducts, respectively. We determined the native flux of 5'dAdo and MTA through the DHAP shunt under aerobic and anaerobic growth conditions in *R. rubrum*. During anaerobic growth, 5'dAdo and MTA were metabolized at a rate of ~590 and ~220 nmol/h/g dry cell weight, respectively. During aerobic growth this rate was 100-fold less for both compounds. No metabolism of S-adenosyl-L-homocysteine by the DHAP shunt was observed. This is consistent with subsequent kinetic measurements of purified DHAP shunt enzymes. The DHAP shunt phosphorylase, isomerase, and aldolase were specific for both MTA and 5'dAdo with near equal catalytic efficiency, but not active with S-adenosylhomocysteine (**Fig. 1**) [2]. This indicates that competition of SAM usage between Radical SAM enzymes producing 5'dAdo and those enzymes producing MTA for ethylene production via the DHAP Shunt must be considered for ethylene optimization.

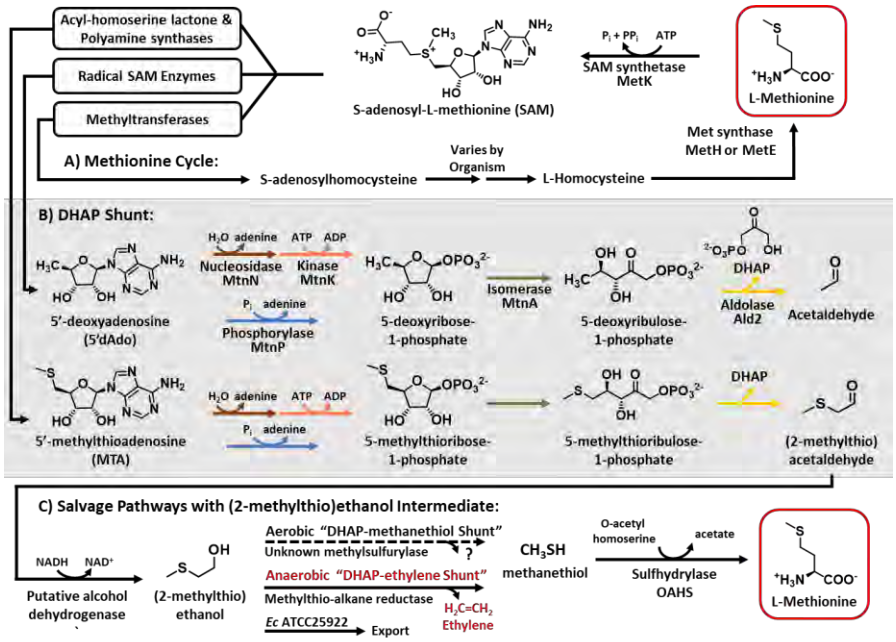


Fig. 1 Salvage of SAM byproducts via the DHAP Shunt Pathways

Understanding catalytic potential through modeling. We are using new physics-based models to evaluate the potential for ethylene production as well. The use of physics-based models are important because whether the end product ethylene is produced in quantity ultimately depends on both catalysis and thermodynamics. The new models are based on the Marcelin-De Donder formulation of mass action kinetics, which differs from the usual kinetic rate laws because it includes both kinetic and thermodynamic terms.

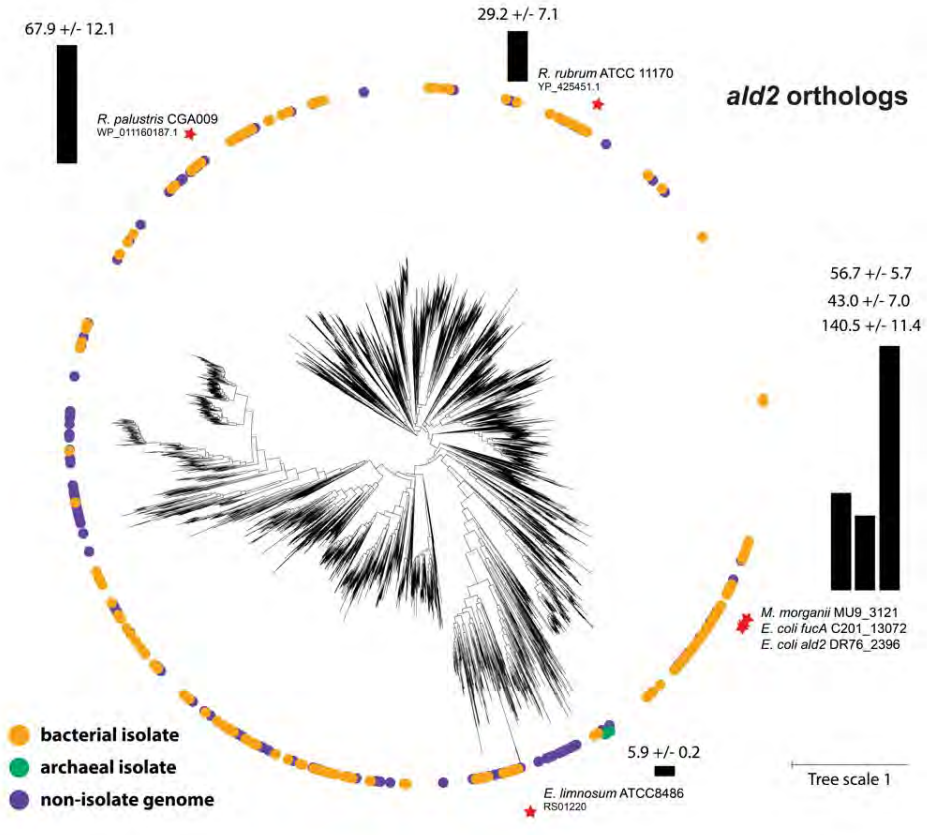
Currently, the metabolic model includes: the Calvin-Benson-Bassham (CBB) cycle, the non-oxidative pentose phosphate pathway, glycolysis, ethanol utilization, the tricarboxylic acid cycle (TCA), the glyoxylate shunt, glutamine and aspartate synthesis, homoserine synthesis, both the S-adenosyl-methionine cycles I and II, methionine synthesis and the DHAP cycle. To date, this is one of the largest thermokinetic models of this kind that have been constructed.

The model correctly fixes CO_2 via the CBB cycle and grows on ethanol in *R. palustris*. Conversion of ethanol to acetaldehyde, acetate and finally acetyl-CoA is highly favored. Acetyl-CoA then feeds into the TCA cycle. The model also predicts that acetate production from *O*-acetyl-L-homoserine via *O*-acetyl-L-homoserine acetate-

lyase is highly favored, despite the fact that this reaction is also predicted to be highly controlled by post-translational regulation.

But acetate is also an end-product of ethylene synthesis in the DHAP pathway. Consequently, because the production of acetate is highly favored from ethanol utilization and O-acetyl-L-homoserine degradation, the driving force for ethylene synthesis is only as strong as the thermodynamic force removing ethylene from the system due to desolvation. It is not clear at this point that this is experimentally the case, as the model is a reduced model of *R. palustris* metabolism. Further improvements to the model are required before any firm conclusions can be drawn regarding the driving force for ethylene production. However, the modeling analysis to-date predicts that the relative amounts of methane, ethane and ethylene are consistent with our experimental data (See abstract and poster *Novel nitrogenase-like C-S lyases link bacterial anaerobic methionine salvage to ethylene and methane production*).

Specific Aim 2: Discover effective and active ethylene enzymes encoded in cultured and uncultured organisms from anoxic environments (Wrighton). Previous studies had indicated that aldolase and isomerase orthologs genes could substantially enhance ethylene levels relative to endogenous genes (1, and unpublished studies). To scale up the search for ethylene-enhancing orthologs we applied targeted functional metagenomics to systematically query genes from the environment. Mining of JGI IMG/M genome and metagenome sequence databases for candidate orthologs to the MTR- 1P isomerase (*mtnA*) and the MTRu-1P aldolase (*ald2*) genes yielded 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 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 (**Fig. 2**).



In order to both ensure recovery of active enzymes and maximize sampling of undefined biochemical diversity, we selected two hundred pairs of candidate *mtnA / ald2* genes considering proximity to high-functioning orthologs and phylogenetic breadth. These genes have been synthesized by the JGI DNA Synthesis Science program for screening via our high throughput lysate activity assay. Optimal orthologs will be further validated via physiological complementation to assess enhanced ethylene production.

References:

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