Novel nitrogenase-like C-S lyases link bacterial anaerobic methionine salvage to ethylene and methane production

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Project Goals

Ethylene is the most widely employed organic precursor compound in industry. The recently discovered efficient microbial anaerobic ethylene synthetic processes was previously partially characterized, but the terminal enzyme(s) responsible for ethylene production remained unknown. The long-term goal of this project is to identify, isolate, and characterize the specific enzyme(s) that catalyzed anaerobic microbial ethylene synthesis. This is part of a larger project to develop an industrially compatible microbial process to synthesize ethylene in high yields. The specific goals in elucidating the unknown ethylene-producing enzyme system are as follows:

1. Identify the genes and gene products in *Rhodospirillum rubrum* responsible for anaerobic ethylene synthesis (Tabita, Hettich, and Wrighton)
2. Determine the regulation and control of identified genes required for anaerobic ethylene synthesis (Tabita and Wrighton)
3. Characterize the enzymes and the reaction they catalyze that directly generates anaerobic ethylene (Tabita and Cannon)

Abstract

Our previous work identified a novel anaerobic microbial pathway (DHAP-Ethylene Shunt) [1] that recycled 5'-methylthioadenosine (MTA) back to methionine with stoichiometric amounts of ethylene produced as a surprising side-product. MTA is a ubiquitous byproduct of polyamine synthesis and homoserine lactone-based quorum sensing in bacteria. The initial steps of the DHAP-ethylene Shunt are catalyzed by a phosphorylase (MtnP), isomerase (MtnA), a novel aldolase (Ald2), and an alcohol dehydrogenase sequentially convert MTA to dihydroxyacetone phosphate (DHAP) and (2-methylthio)ethanol (Fig. 1). **The (2-methylthio)ethanol serves as a direct precursor to ethylene and methionine via an unresolved process involving unknown genes and enzymes** [1]. **This is the first reported anaerobic route to ethylene, involving novel genes and enzymes.** Here we report the identification of the genes and putative gene products specifically responsible for this anaerobic ethylene process and the regulatory system controlling gene expression for the pathway.

1. Genes and gene products in *Rhodospirillum rubrum* responsible for anaerobic ethylene synthesis: We sought to identify the genes and proteins responsible for ethylene production via proteomics and specific gene deletion studies. Ethylene production from (2-methylethio)ethanol is highly regulated by the presence of exogenous sulfate. Therefore, cells were grown under sulfate replete (ethylene suppressing) and sulfate limiting (ethylene inducing) anaerobic growth conditions. Cells were harvested and differential proteome analysis via HPLC-MS/MS was performed to identify proteins that increased in abundance during ethylene inducing conditions. Surprisingly, proteins with the highest increase in abundance during production of ethylene from (2-methylthio)ethanol corresponded to novel nitrogenase-like proteins of unknown function and previously characterized O-acetyl-L-homoserine sulfhydrylases [2] (Fig. 1; MarBHDK and OASH).

Deletion of the genes corresponding to nitrogenase-like proteins (*mar*BHDK), with increased abundance during ethylene inducing conditions, rendered *R. rubrum* incapable of growth or ethylene production utilizing (2-methylethio)ethanol. Reintroduction of these genes expressed *in trans* from a plasmid restored both growth and ethylene production (Fig. 2A). Cells were also grown under inducing conditions for *bona fide* molybdenum nitrogenase (NifHDK) (glutamate as N-source). While maximal acetylene reduction (nitrogenase activity) was observed, nitrogenase was unable to perform the methylthio-alkane reductase activity of MarBHDK to convert (2-methylthio)ethanol to ethylene. **This is the first indication of a nitrogenase-like complex responsible for the reduction of a carbon-sulfur bond, and the first observation of a nitrogenase-like complex involved in sulfur (methionine) metabolism** (Fig. 1).

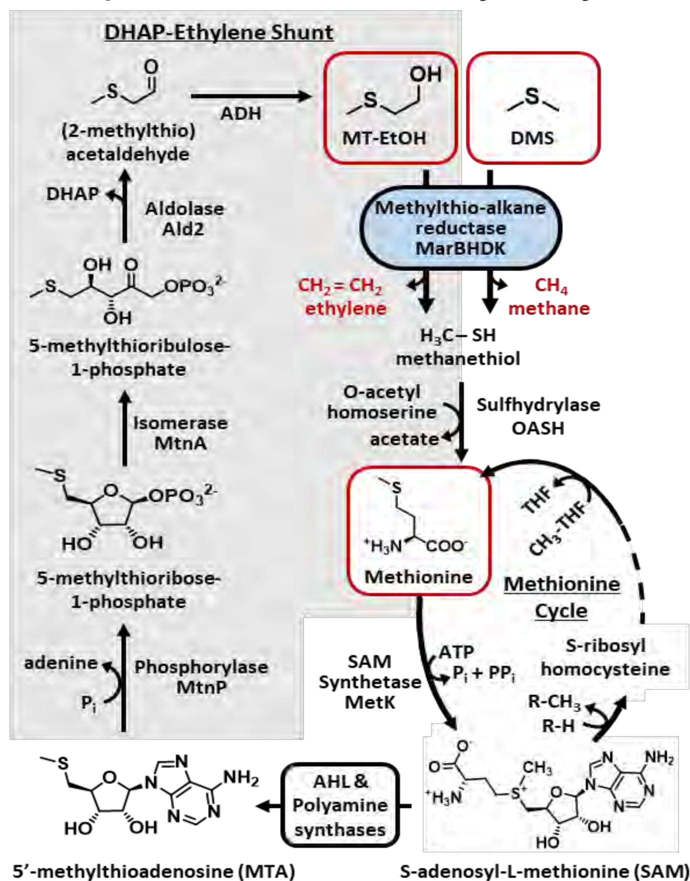

Fig. 1. Full elucidation of the DHAP-ethylene Shunt pathway reveled involvement of novel nitrogenase-like methylthio-alkane reductases (MarBHDK)

We further probed the substrate specificity for this novel methylthio-alkane reductase process. Indeed, other small volatile organic sulfur compounds (VOSCs) required the nitrogenase-like gene products in order to be utilized as a sulfur source by *R. rubrum* for growth and methionine metabolism (Fig. 2A). Utilization of dimethyl sulfide, the most abundant VOSC in the environment, resulted in stoichiometric production of methane, and ethylmethyl sulfide led to stoichiometric production of ethane gas. Utilization of larger VOSCs such as (3-methylthio)propanol did not require the nitrogenase-like gene products and no gaseous hydrocarbons were detected from growth and methionine metabolism from larger VOSCs. Each putative reaction to produce a gaseous hydrocarbon from its corresponding VOSC was modeled to determine the change in standard Gibbs free energy for the overall reaction in solution (Fig. 2B). Standard free energies were calculated with *NWChem*, a computational chemistry electronic structure software developed under BER funding. The standard free

energies of reaction predict that ethylene formation will be the most favorable. Likewise, the relative difference in gas production observed experimentally between wildtype and Δ*mar*BHDK also suggests, like the standard free energies, that ethylene production from (2-methylthio)ethanol is the most favored catalytic product.

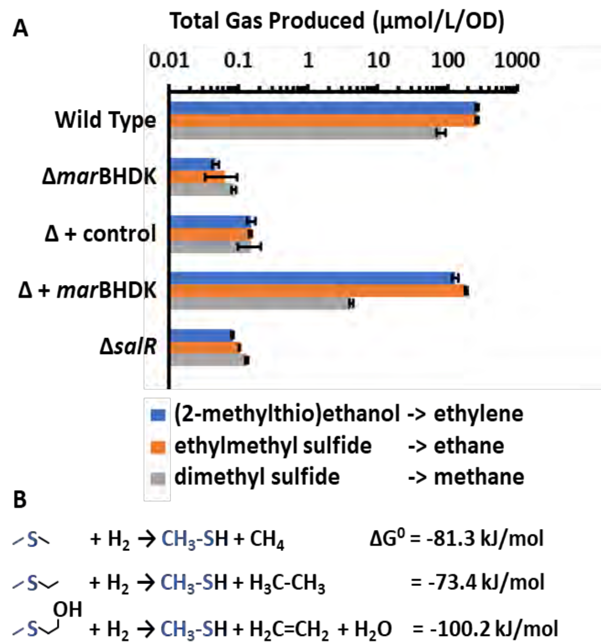

Fig. 2. Proposed reactions catalyzed by nitrogenase-like methylthio-alkane reductases (A) Total ethylene, ethane, or methane produced upon feeding *R. rubrum* with (2-methylthio)ethanol, ethylmethyl sulfide, or dimethyl sulfide, respectively. **(B)** Free energy calculations for each proposed reaction.

2. Regulation of *marBHDK* gene expression: A random mutagenesis screen of *R. rubrum* identified multiple isolates in which an integrated transposon was inserted into a putative LysR family regulator gene *(salR)* located 7 genes away from the *marBHDK* operon. Such mutants were unable to grow or utilize (2-methylthio)ethanol, ethylmethyl sulfide, or diemethyl sulfide as sulfur source for growth or produce any corresponding hydrocarbon gasses (Fig. 2A; Δ*salR*). However, they were still able to grow normally using sulfate as sole sulfur source. Subsequent RNA-seq differential gene expression analysis revealed that the *marBHDK* gene cluster and O-acetylhomoserine sulfhydrylase genes for methionine synthesis (Fig. 1) were upregulated during ethylene inducing conditions (limiting sulfate) only when a functional *salR* gene was present. No changes in expression levels of these genes were observed in the Δ*salR* deletion strains. **Therefore, this LysR-like regulator is termed SalR for Sulfur Salvage Regulator of genes responsible for sulfur salvage from VOSCs and other organic sulfur compounds.**

3. Other Organisms with Methylthio-alkane Reductases:

The *R. rubrum* MarHDK gene products are homologous to nitrogenase proteins NifH (nitrogenase reductase iron protein), and NifDK (nitrogenase catalytic subunits α/β) and are part of the Group IV Nitrogen Fixation-Like clade (Fig. 3). While the *bona fide* nitrogenases (Groups I-III) catalyze nitrogen fixation, the Nitrogen Fixation-Like members of known function catalyze reduction of other distinct compounds containing double bonds such as porphyrin ring reduction during bacteriochlorophyll and F430 cofactor biosynthesis (Fig. 3; Groups V and IV-B). However, save for IV-B and a single group IV member from *Endomicrobium proavitum* (Fig. 3; Group IV-A) [5], which can also catalyze N_2-fixation, the function of the remaining Group IV members is unknown. The homologous methylthio-alkane reductase proteins form their own clade that is distinct from other nitrogenase and nitrogenase-like sequences, suggesting other organisms can utilize environmental VOSCs, producing ethylene and methane in the process (Fig. 1; Group IV-C). Organisms with putative methylthio-alkane reductases include members from alpha-proteobacteria, particularly rhodospirilla and rhizobia, and firmicutes, particularly selenomonads and ruminal clostridia.

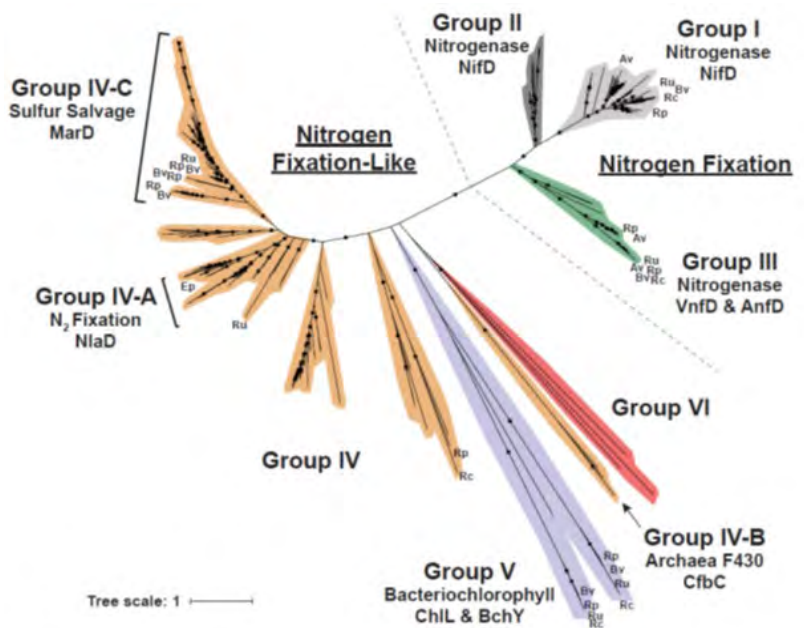

Fig. 3. Phylogenetic tree of NifD superfamily based on an LG+R6 evolution model. The scale bar represents the number of substitutions per site. UFBoot support values of 95% or greater are shown as black circles on branches. Clade coloring and numbering follows Raymond [3] and Méheust [4].

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