ABSTRACT

MONTOYA, SARAH PRESSLY. Physiological and Genomic Analysis of *Rhodococcus rhodochrous* ATCC 33258. (Under the direction of Dr. Michael R. Hyman).

Acetylene (C_2H_2) is the simplest alkyne and is known to have multiple and diverse effects on microbial enzymes that catalyze important reactions in biogeochemical cycles. The overall aim of the project described in this thesis has been to identify the enzyme responsible for initiating the aerobic catabolism of C_2H_2 in a commercially-sourced C_2H_2-utilizing aerobe, *Rhodococcus rhodochrous* ATCC 33258. Chapter 1 of this thesis provides a summary of the chemical features of C_2H_2 and an overview of prior research into both the physiological and molecular aspects of the anaerobic and aerobic degradation of C_2H_2. Chapter 2 describes the experimental work conducted during this study and includes both physiological studies and genomic analyses of *R. rhodochrous* ATCC 33258. The results suggest that the enzyme responsible for initiating C_2H_2 catabolism in *R. rhodochrous* ATCC 33258 is a monomeric acetylene hydratase that is structurally similar to the functionally equivalent enzyme in the strictly anaerobic C_2H_2-fermenting strain *Pelobacter acetylenicus*. Although prior studies have suggested that acetylene hydratases from aerobes are immunologically distinct from the enzyme in *P. acetylenicus*, our genome analysis indicates that both enzymes likely contain a [4Fe-4S] center and a molybdopterin cofactor. Our results also suggest that the acetylene hydratase from *R. rhodochrous* ATCC 33258 differs from the enzyme characterized in *P. acetylenicus* in that this enzyme likely binds molybdenum rather than tungsten in the molybdopterin cofactor. Although further research is needed to demonstrate that the putative acetylene hydratase identified within the *R. rhodochrous* ATCC 33258 genome can actually catalyze C_2H_2 hydration, the results of this study provide important insights into the aerobic degradation of C_2H_2 and open

several new directions for future research with aerobic C_2H_2-utilizers and their C_2H_2-degrading enzymes.

Physiological and Genomic Analysis of *Rhodococcus rhodochrous* ATCC 33258

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DEDICATION

I would like to dedicate this work to the following:

To my loving husband, Drew Caterinicchio, for his unwavering patience, love, and support. You have been my anchor and I could not have done this without you.

To my parents, Barry and Sara Montoya, who have been a constant source of encouragement, support, and prayers throughout all of my endeavors.

To my brother, Joseph Montoya, without whom I never would have started this journey.

BIOGRAPHY

Sarah Montoya was born to Barry and Sara Montoya in February of 1995 and raised in Summerville, South Carolina. She earned her B.S. degree in Biology with a minor in Health Care Management from Erskine College in 2017. As the daughter of a family physician, Sarah completed most of her undergraduate career with the intention of following in her mother's footsteps and pursuing a career in medicine. During her time at Erskine College, however, her interest in environmental microbiology and the applications it has to offer began to take shape. Following graduation, Sarah worked for a year as a medical assistant while deciding which career path to take. In 2018, she chose to enroll in North Carolina State University to pursue an M.S. degree in Microbiology. She joined the Hyman lab in 2019 to learn about and begin her research on microbes with bio-remediation potential.

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On a personal note, I would like to thank my family (both biological and chosen) for the many ways they have shown me love and support during my journey. I am truly blessed to have so many wonderful people in my life.

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Literature Review

Introduction: The overall aim of this project has been to identify the enzyme responsible for initiating the aerobic microbial degradation of acetylene (C_2H_2) in the bacterium *Rhodococcus rhodochrous* ATCC 33258. The following section provides an overview of the properties of acetylene and summarize the current understanding of the microbial degradation and metabolism of acetylene.

Physical Properties of Acetylene: Acetylene (C_2H_2, IUPAC name: ethyne) is the simplest alkyne and its diverse physical and chemical properties are largely defined by its carbon-carbon triple bond (Table 1.1). At 25ºC, the saturated aqueous solubility of acetylene (~1200 mg/L [~46 mM)) is considerably higher than that of ethane (~60.2 mg/L ([~2.0 mM)) (Yalkowsky, He, & Jain, 2010). Acetylene is also highly soluble in organics such as benzene and chloroform while acetone is used as the solvent for commercial low pressure acetylene gas cylinders. (PubChem Compound Summary for CID 6326, Acetylene). With a pKa of 24, acetylene is also substantially more acidic than either ethylene (pKa = 44) or ethane (pKa = 50) (Pässler *et al.*, 2011).

Chemical Properties of Acetylene: Due to its simple structure and high reactivity, the chemistry of acetylene is diverse and include reduction and oxidation reactions as well as addition reactions such as addition of H_2 to form ethene and addition of water to form acetaldehyde. The acetylide anion ($CH{\equiv}C:^-$) can react with metal cations to form ionic materials (*e.g.* Ag(I) acetylide) and acetylene can also serve as a ligand to transitional metals in both the σ-bonded and π-bonded systems (Hyman & Arp, 1988; Brink, 2014).

Natural Sources of Acetylene: Acetylene is thought to have been abundant in the primordial pre-oxic atmosphere of Earth (Culbertson, Strohmaier, & Oremland, 1988; Oremland & Voytek, 2008), but only trace levels (≤0.1 ppbv) are present in Earth's current atmosphere (Miller, Baesman, & Oremland, 2015). Within the outer Solar System, more significant concentrations of C_2H_2 are generated by the photolysis of methane in the atmospheres of Jovian planets (i.e. Jupiter) and Titan (Oremland & Voytek, 2008). Current sources of atmospheric acetylene on Earth are primarily anthropogenic emissions from partial combustion of fossil fuels (5.0 Tg yr^{-1}) and biomass burning (1.6 Tg yr^{-1}) (Xiao, Jacob, & Turquety, 2007). In the atmosphere, acetylene is one of the longest-lived non-methane hydrocarbons (NMHCs) and is degraded through reactions with OH radicals with a half-life of ~2 weeks (Xiao, Jacob, & Turquety, 2007). Production of acetylene has also been described in several studies involving simple halogenated compounds. For example, pure cultures of methanogens can reduce 1,2-dibromoethene ($C_2H_2Br_2$) to acetylene (Belay & Daniels, 1985) while abiotic reactions between reduced iron-containing minerals such as magnetite (Fe_3O_4), and pyrite (FeS_2) with C_2-chloroethenes also generate acetylene (He *et al*., 2015).

History and Uses of Acetylene: Acetylene was first produced in 1836 by Edmund Davy. The discovery of calcium carbide by Thomas Willson in 1892 led to the widespread use of this compound in production of high-quality steels and the use of acetylene-powered lamps to illuminate mines, homes, and automobiles. Willson's method for calcium carbide production also ultimately led to the formation of the Union Carbide Corporation in North Carolina. Gustaf Dalén was awarded the 1912 Nobel Prize in Physics for developing the porous mass that enables

carbide-derived acetylene to be stored and used as a reliable fuel for lamps in lighthouses, navigation buoys and railroad signals.

Acetylene has long been used for welding and metal cutting due to the high temperature (~3500°C) produced by its flame. Acetylene generated from calcium carbide needs to be purified to remove trace amounts of gases such as hydrogen (H_2) phosphine (PH_3), arsine (AsH_3), ammonia (NH_3), and hydrogen sulfide (H_2S) that contribute to give the crude gas its characteristic garlic-like odor (Schobert, 2014). Purification steps can involve selective absorption of acetylene in water or *N*-methylpyrrolidone through to cryogenic purification processes. Historically important processes for acetylene production other than calcium carbide include coal pyrolysis and thermal cracking of natural gas. Although now largely superseded by the petrochemical industry, industrially important products originally produced from acetylene included acetaldehyde, vinyl chloride, vinyl acetate, acrylic acid, and 1,4-butanediol (Pässler *et al*., 2011; Schobert, 2014).

Effects of Acetylene on Microbial Processes: Acetylene produces multiple mechanistically different inhibitory effects on microorganisms and specific enzymes involved in the biogeochemical cycles of nitrogen and carbon (Hyman & Arp, 1988). For example, acetylene is a substrate for the nitrogen-fixing enzyme nitrogenase and is reduced to ethene. An assay based on this reaction, the acetylene reduction assay (ARA), is widely used for estimating the nitrogen-fixing activity of diverse samples. Acetylene is also a reversible inhibitor of nitrous oxide reductase and causes the accumulation of N_2O in denitrifying systems. The resulting acetylene blockage assay (ABA) can be used to estimate the denitrifying capabilities of a sample and the fluxes of N_2O, a potent greenhouse gas. Acetylene is also a mechanism-based inactivator of

diverse bacterial monooxygenases (Hyman & Wood, 1985; Lan, Smith, & Hyman, 2013). In these cases, acetylene is oxidized into a reactive intermediate that subsequently binds covalently to amino acids in the active site of these enzymes and leads to their irreversible inactivation. The inactivating effect of acetylene has led to the identification of key subunits of these enzymes as well as a fluorescent labeling approach that now enables microorganisms that express these enzymes to be specifically labeled (Hyman & Wood, 1985; Bennett *et al.*, 2016). Acetylene also acts as an inhibitor of other widely distributed enzymes such as H_2-oxidizing (uptake) hydrogenases and nitrate reductases (Hyman & Arp, 1988).

Anaerobic Biodegradation of Acetylene: Degradation of acetylene to CO_2 under anaerobic conditions was first described for estuarine sediments and acetate was found as the major metabolite in these cultures (Watanabe & De Guzman, 1980; Culbertson, Zehnder, & Oremland, 1981; Yeomans & Beauchamp, 1982; Culbertson, Strohmaier, & Oremland, 1988). Multiple strains of acetylene-fermenting anaerobes were isolated from both freshwater and marine sediments after incubation with acetylene (Schink, 1985) and these strict anaerobes were subsequently identified as a new species, *Pelobacter acetylenicus.* Although these strains ferment C_2H_2 to acetate and ethanol, acetaldehyde was suggested as the immediate product of acetylene degradation (Schink, 1985). The initial hydration of acetylene to acetaldehyde is highly exergonic (ΔG = -111.9 kJ/mol), while the subsequent conversion of acetaldehyde to acetate and ethanol is less exergonic (ΔG = -17.3 kJ/mol) (Schink, 1985). Other substrates that support the growth of *P. acetylenicus* include acetoin, ethanolamine, choline, 1,2-propanediol, and glycerol.

Acetylene Hydratase from *Pelobacter acetylenicus*: In *P. acetylenicus*, the enzyme responsible for catalyzing the initial hydration of acetylene to acetaldehyde is acetylene hydratase (AH, EC 4.2.1.112). The enzyme is unique in that it is a tungsten [Fe-S] protein that catalyzes a hydration reaction rather than a redox reaction. Structurally, it is similar to the dimethyl sulfoxide reductase (DMSOR) family of molybdenum (Mo) and tungsten (W) proteins and is structurally distinct from the aldehyde: ferredoxin oxidoreductase (AOR) family and the formyl-methanofuran dehydrogenase family of tungsten-containing enzymes. This enzyme was purified by Rosner & Schink (1995) and shown to be a 73 kDa monomer by SDS-PAGE or to be an 83 kDa monomer, as determined by MALDI-TOF mass spectrometry. The anaerobically purified enzyme contains (per mol), 4.4 ± 0.4 mol Fe, 3.9 ± 0.4 mol of acid-labile sulfur, and 0.5 ± 0.1 mol of tungsten (W), but not molybdenum (Mo). The holoprotein also contains 1.3 ± 0.1 mol/mol molybdopterin guanine dinucleotide. The crystal structure of AH from *P. acetylenicus* has been determined (Seiffert *et al.*, 2007) and the cysteine ligands for a [4Fe-4S] center have been identified as Cys-9, Cys-12, Cys-16, and Cys-46. Another cysteine residue, Cys-141, is thought to be involved in W-binding within the molybdopterin cofactor. The remaining cysteine residues in the protein are not thought to be involved in cofactor or metal binding. The AH from *P. acetylenicus* has been purified both in air and in the absence of O_2. In contrast to the anaerobically purified enzyme which contains a [4Fe-4S] center, aerobically purified AH contains a [3Fe-4S] center but has a similar specific activity to the anaerobically prepared enzyme (Meckenstock *et al.*, 1999). This suggests that either an intact [Fe-S] cluster is not needed for AH activity or that both the [4Fe-4S] cluster and the [3Fe-4S] cluster perform the same reaction. Irrespective of whether the enzyme is purified aerobically or anaerobically, to observe activity in a colorimetric assay following NAD^+ reduction by ADH using acetylene-derived ethanol as substrate, AH has to be

activated by including a strong reductant such as dithionite or titanium (III) citrate in the reaction medium (Rosner & Schink, 1995; Meckenstock *et al.*, 1999). Together, these results suggests that the role of the strong reductant is not reformation of an inactive oxidized [3Fe-4S] center to a functional [4Fe-4S] center as seen with several other well-studied [Fe-S] center-containing hydrolytic enzymes such as aconitase and fumarase (Kennedy *et al.*, 1983; Flint, Emptage, & Guest, 1992).

Recent Studies of Anaerobic Acetylene Degradation: The genomes of three acetylene-fermenting *Pelobacter* strains have recently been sequenced, assembled, and annotated. The novel genomes for *P. acetylenicus* DSM3246, *P. acetylenicus* DSM3247, and *Pelobacter* sp. strain SFB93 each possess an acetylene hydratase encoding *ahy* gene, with strain SFB93 containing two copies of this gene. Genes for the subsequent enzymes in the fermentative pathway, including aldehyde dehydrogenase, phosphate acetyltransferase, acetate kinase, and alcohol dehydrogenase (Schink, 1985), are also present in all three genomes. Furthermore, all three genomes contained gene sequences for five essential genes (*nifHDKEN*) encoding for an iron-molybdenum nitrogenase (N_2ase). Despite having the *nif* genes and fermenting acetylene, no detectable ethylene was produced by DSM3246 cells under experimental conditions. On the other hand, nitrogen fixation growth assay results for strain SFB93 showed acetylene consumption and, in the absence of ammonium ions (which represses N_2ase expression), ethylene production. The co-expression of AH and N_2ase indicates that *Pelobacter* sp. strain SFB93 is capable of transforming acetylene via two separate biochemical mechanisms, acetylene fermentation and nitrogen fixation (Akob *et al*., 2017).

Trichloroethene (TCE) is a toxic chlorinated solvent that is often associated with groundwater contamination. This pollutant can be biodegraded through respiratory reductive dechlorination, where TCE is sequentially reduced to *cis*-1,2-DCE, vinyl chloride (VC), and ethene. While complete reductive dechlorination to ethene is possible, incomplete dechlorination with the accumulation of the more toxic intermediate products such as VC is often observed in the field (Bradley, 2000). The strictly hydrogenotrophic bacteria *Dehalococcoides mccartyi* can reductively dechlorinate TCE to ethene and requires H_2 and acetate to support growth, but this activity in this and similar strains can be inhibited by acetylene (Pon, Hyman, & Semprini, 2003) that can be generated *in situ* from abiotic reactions between acetylene and reduced metal species frequently found in contaminated aquifers (Roberts *et al*., 1996; Butler & Hayes, 1999). In contrast, acetylene utilizing *Pelobacter* strains such as strain SFB93 produce H_2, acetate, and ethanol from acetylene fermentation (Seitz *et al*., 1990; Miller *et al*., 2013). In laboratory constructed co-culture experiments, the inhibitory effect of C_2H_2 on biological reductive dehalogenation of TCE by a mixed TCE-reducing culture has been shown to be overcome by adding an acetylene-fermenting organism to the consortium. *Pelobacter* sp. strain SFB93 supported the reductive dechlorination of TCE by supplying *Dehalococcoides mccartyi* strains with H_2 and acetate fermentation products to be used as an electron donor and carbon source (Mao *et al*., 2017). This coupling of acetylenotrophy with reductive dechlorination can occur within native bacterial communities enriched from chlorinated solvent-contaminated groundwater and amended with C_2H_2 as the sole electron donor and carbon source via fermentation (Gushgari-Doyle *et al.*, 2021). Furthermore, metagenomic analysis identified a novel anaerobic acetylenotroph from the phylum *Actinobacteria* within the community sampled (Gushgari-Doyle *et al*., 2021). These studies, along with future studies on acetylenotrophy, are

valuable to the development of bioremediation strategies, particularly for those environments contaminated with chlorinated solvents like TCE.

Aerobic Biodegradation of Acetylene: Compared to the anaerobic degradation of acetylene, little is known about the aerobic form of this process. The first documented report of aerobic microbial utilization of acetylene described a *Mycobacterium lacticola* strain that grew on a mineral salts medium with acetylene as the sole source of carbon and energy (Birch-Hirschfeld, 1932). Nearly 50 years later, the next aerobic acetylene-utilizing strain was described in the literature (Kanner & Bartha, 1979). This strain, *Nocardia rhodochrous* ATCC 33258 (since renamed *Rhodococcus rhodochrous* ATCC 33258) was isolated from soil sediment and was shown to require the pyrimidine component of thiamine for growth on acetylene. This strain also grows on a wide range of other carbon sources, including acetate, lactate, succinate, ethanol, glucose, glycerol, and sucrose; however, the thiamine requirement for growth on most of these other substrates has not been established.

Another metabolically diverse acetylene-utilizing soil isolate that appears to be closely related to the *N. rhodochrous* strain described by Kanner & Bartha was described contemporaneously by de Bont and Peck (1980). This strain, *Rhodococcus* A1, grows on propyne, ethanol, acetaldehyde, propionaldehyde, and succinate. High levels of AH activity were measured in cell-free extracts from acetylene-grown *Rhodococcus* A1 but this activity was only observed under anoxic conditions, suggesting AH in this aerobic organism is an oxygen-sensitive enzyme (de Bont & Peck, 1980). Additionally, both de Bont and Peck (1980) and Kanner and Bartha (1982) noted elevated levels of acetaldehyde dehydrogenase activity in cells grown on acetylene compared to cells grown on alternative carbon sources. Based on these results, both

sets of authors hypothesized that acetylene is initially hydrated to acetaldehyde which is then further oxidized to acetate (Fig. 1.1). Subsequent DNA hybridization studies revealed the three isolates described above (*Mycobacterium lacticola* (Birch-Hirschfeld, 1932), *Nocardia rhodochrous* (Kanner & Bartha, 1979), and *Rhodococcus* A1 (de Bont & Peck, 1980)) to be highly similar if not identical to one another (de Bont *et al*., 1980; Kanner & Bartha, 1982).

Other aerobic acetylene-utilizing strains have also been described. An acetylene-utilizing *Bacillus* strain isolated from a stream sediment was shown to assimilate $^{14}C_2H_2$ into the washed cell suspensions (Tam, Mayfield, & Inniss, 1983). Another strain *Rhodococcus* (*Nocardia*) *rhodochrous* E5, was isolated from a C_2H_2-consuming agricultural soil. This bacterium grew on acetylene, acetate, acetaldehyde, ethanol, succinate, or glucose and acetylene was transformed to acetaldehyde, ethanol, acetate, CO_2, and biomass in varied proportions. Kinetic calculations of the C_2H_2 conversion to acetaldehyde in acetylene-grown cells showed K_m = 250 μM and V_{max} = 800 nmol · min $^{-1}$ · (mg protein) $^{-1}$. Unlike de Bont and Peck (1980), Germon and Knowles observed growth inhibition by acetaldehyde at higher concentrations (10 mM); however, the addition of acetate relieved this inhibition. Furthermore, inhibition of acetylene consumption by acetaldehyde was seen to a greater extent in the presence of oxygen (Germon & Knowles, 1988).

Subsequently, Rosner *et al*. (1997) investigated AH enzymes in several aerobic acetylene-degrading bacteria. Isolates MoAcy1 (DMS 44186) and TueAcy1 (DMS 44188) were obtained from soil samples and were identified as *Rhodococcus opacus* strains, while isolate TueAcy3 (DMS 44189) was identified as a *Rhodococcus zopfii*, and isolate MoAcy2 (DMS 44187) was identified as a *Gordona* sp. strain. Isolates MoAcy1 and TueAcy1 grew readily on acetylene as a sole source of carbon and energy while isolates MoAcy2 and TueAcy3 both required the presence of yeast extract to grow on this gas. Similar to the anaerobic bacterium *P.*

acetylenicus, AH activity in isolates MoAcy1 and TueAcy1 could only be measured in cell-free extracts in the presence of a strong reducing agent such as titanium (III) citrate and no AH activity could be demonstrated in the absence of a reducing agent or under aerobic assay conditions. Growth of these two isolates on acetylene also depended on the presence of molybdate, but not tungstate, in the growth medium. The specific AH activities measured for isolates MoAcy2, TueAcy3, and *Gordona rubropertincta* (DMS 43197) were found to be the same either in the presence of titanium (III) citrate or under air, indicating a difference between these enzymes and those from *R. opacus* strains. No cross-reactivity was found between cell-free extracts of the aerobic strains and the antibodies raised against purified acetylene hydratase from *P. acetylenicus* (Rosner *et al*., 1997).

Acetylene degradation by other metalloproteins with [Fe-S] clusters: Iron-sulfur proteins are ubiquitous in nature and associated with numerous metabolic activities as proteins containing [Fe-S] clusters catalyze an array of diverse reactions (Beinert & Kennedy, 1989; Beinert, Holm, & Münck, 1997). Although the majority of [Fe-S] clusters are known for their role in electron transfer and redox reactions, several metalloproteins containing [Fe-S] clusters such as aconitase and fumarase catalyze hydrolytic rather than redox reactions. The acetylene-hydrating activity of these enzymes has not been reported, but they do both undergo reversible inactivation in the presence of O_2. This inactivating effect is due to the loss of an Fe from their respective [4Fe-4S] centers and can be reversed by incubating the oxidized enzyme with Fe and a suitable reductant (Kennedy *et al.*, 1983; Flint, Emptage, & Guest, 1992).

More recently, acetylene-hydrating activity has been detected in another [Fe-S]-center-containing enzyme. In the methylerythritol phosphate (MEP) pathway for isoprene biosynthesis,

the final enzyme IspH catalyzes the reductive hydration of (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP) into isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Mössbauer spectroscopic studies indicated that the oxygen-sensitive protein IspH, also known as LytB, contains an unusual $[4Fe\text{-}4S]^{2+}$ cluster in vivo with one iron linked to three inorganic sulfurs from the cluster and to two or three additional non-sulfur ligands (oxygen and / or nitrogen ligands) (Seemann *et al*., 2009). Furthermore, the crystal structure of *Escherichia coli* IspH:HMBPP complex supports a reaction mechanism in which the C4 hydroxyl of HMBPP binds to the unique fourth iron site of the cluster (Gräwert *et al*., 2010; Janthawornpong *et al*., 2013). In addition to the $2H^+/2e^-$ reduction of HMBPP, it has been demonstrated that oxidized IspH can also hydrate acetylenes such as but-3-ynyl diphosphate and pent-4-ynyl diphosphate to aldehyde and ketone products (Span *et al.*, 2012).

CHAPTER 1: Figures

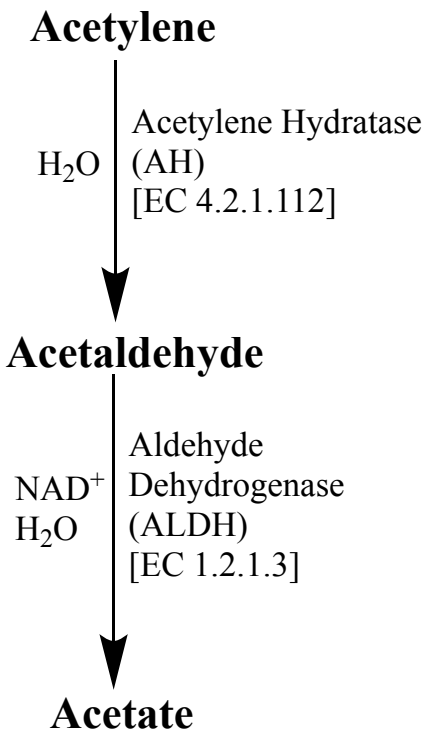

Figure 1.1. First steps from aerobic C_2H_2 catabolism pathway.

CHAPTER 1: Tables

Table 1.1. Properties of Acetylene.

Chemical Formula	C_2H_2
CAS Number	74-86-2
Molecular Weight	26.04 g/mol
C-C bond length	1.20Å
Acidity (pK_a)	24
Solubility in H_2O	47.2 mM (at 20°C, 1 atm) Range of concentrations, (g/L)

CHAPTER 1: References

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CHAPTER 2:

Physiological and Genomic Investigation of Acetylene Hydratase (AH) in *Rhodococcus rhodochrous* ATCC 33258

Section 2.1: Project Background & Rationale

Background: Acetylene (C_2H_2) is the simplest alkyne and is a compound that is known to have multiple and diverse effects on microbial enzymes that catalyze key reactions in the biogeochemical cycles of nitrogen (Hyman & Arp, 1988). For example, acetylene is an alternative substrate for nitrogenases that normally functions to reduce atmospheric nitrogen (N_2) to ammonia (NH_3) and hydrogen (H_2). The nitrogenase-dependent reduction of acetylene (C_2H_2) to ethylene (C_2H_4) is exploited in the acetylene reduction assay that is widely used to estimate rates of biological nitrogen fixation (Hardy *et al.,* 1968). While the competitive inhibitory effect of C_2H_2 on nitrogenase activity is reversible, C_2H_2 irreversibly inactivates ammonia monooxygenase (AMO)(Hyman & Wood, 1985). This membrane-bound enzyme functions to initiate NH_3 catabolism in both aerobic nitrifying bacteria and archaea and oxidizes NH_3 to hydroxylamine (NH_2OH). In this instance, C_2H_2 acts as a mechanism based inactivator and is catalytically activated by AMO and the resulting highly reactive ketene (CH_2CO) covalently binds to active site residues resulting in an irreversible loss of AMO activity (Hyman & Wood, 1985; Gilch *et al.*, 2009). Other C_2H_2 -sensitive microbial enzymes include nitrate reductase, hydrogenases and diverse alkane-oxidizing monooxygenases (Hyman & Arp, 1988; Lan, Smith, & Hyman, 2013).

Acetylene can also serve as growth-supporting sole source of carbon and energy for both anaerobic and aerobic microorganisms. To date, the main research focus has been on anaerobic C_2H_2-utilizing bacteria typified by *Pelobacter acetylenicus*. This anaerobic bacterium ferments C_2H_2 to a mixture of acetaldehyde, ethanol, and acetate (Schink, 1985). Acetylene was abundant

in Earth's early anaerobic atmosphere and this has led to research interest into the potential significance of anaerobic C_2H_2-utilizing prokaryotes (acetylenotrophs) in the early microbial life on Earth and other planets (Culbertson, Strohmaier, & Oremland, 1988; Oremland & Voytek, 2008). More recently, the diversity of anaerobic C_2H_2-utilizers has been examined in studies focused on co-cultures of anaerobic acetylenotrophs with bacteria that dehalorespire environmental pollutants such as trichloroethylene (TCE) (Mao *et al*., 2017; Gushgari-Doyle *et al*., 2021).

The major biochemical interest in anerobic C_2H_2 degradation is primarily directed at acetylene hydratase (AH). This tungsten(W)-containing enzyme initiates C_2H_2 catabolism and is unusual among known W-containing enzymes in that it does not catalyze a redox reaction. This enzyme from *P. acetylenicus* has been purified, crystallized, and spectroscopically-characterized (Rosner & Schink, 1995; Meckenstock *et al.*, 1999; Seiffert *et al.*, 2007). This monomeric enzyme has a mass of 73 kDa and contains a [4Fe-4S] center and a W-binding molybdopterin as cofactors (Rosner & Schink, 1995; Seiffert *et al.*, 2007). The enzyme retains activity after both aerobic and anaerobic purification but in both cases the purified enzyme requires activation using strong reductants (Seiffert *et al.*, 2007; Brink, Schink, & Kroneck, 2011).

In contrast to anaerobic microbial C_2H_2 degradation, much less is known about aerobic C_2H_2-utilizing bacteria and the enzymes responsible for initiating C_2H_2 catabolism in these bacteria. A variety of ostensibly similar aerobic C_2H_2 utilizing bacteria have been described and the most detailed studies of the physiology and enzymology of these bacteria has focused on *Nocardia rhodochrous* (renamed *Rhodococcus rhodochrous*) ATCC 33258 (Kanner & Bartha,

1979; Kanner & Bartha, 1982). This bacterium grows rapidly on C_2H_2 in the presence of thiamine and has been proposed to generate acetaldehyde as an immediate product of C_2H_2 catabolism. This suggests that, like anaerobic C_2H_2-utilizing strains, the initial step in aerobic C_2H_2 catabolism involves the enzymatic hydration of C_2H_2. Following the identification and characterization of acetylene hydratase (AH) in *P. acetylenicus* (Rosner & Schink, 1995), several other aerobic C_2H_2-utilizing strains were isolated and characterized (Rosner *et al.*, 1997). The studies suggest that the AH activity in these isolates is similar to *P. acetylenicus* in that this activity in cell extracts typically required prior activation with strong reductant. However, physiological studies suggested that the aerobic C_2H_2-consuming activity required molybdate (Mo) rather than tungstate (W). Furthermore, studies with polyclonal antibodies raised against the purified AH from *P. acetylenicus* did not cross react with proteins in cell extracts of C_2H_2-grown cells of aerobic C_2H_2-utilizing strains. These latter results suggested the aerobic form of acetylene hydratase is structurally distinct from the enzyme in the obligate anaerobe *P. acetylenicus* (Rosner *et al.*, 1997).

Rationale: The aim of the project described in this chapter was to identify the enzyme responsible for initiating C_2H_2 catabolism in the aerobic C_2H_2-utilizing strain, *R. rhodochrous* ATCC 33258. This project was initiated prior to the start of the COVID-19 pandemic but was greatly impacted by the institutional and personal restrictions this pandemic placed on laboratory research activities. The original research strategy involved two approaches. First, the project aimed to determine specific rates of C_2H_2 utilization by *R. rhodochrous* ATCC 33258 after growth on diverse substrates. The anticipation was that expression levels of C_2H_2-consuming enzymes would be strongly influenced by growth substrate and that these differences could be

explored by simple (SDS-PAGE) and more complex (shotgun proteomics) methods to identify key enzymes involved in C_2H_2 degradation. The second complimentary approach was to determine the genome sequence of this bacterium and to use genomic analyses to identify enzymes involved in C_2H_2 catabolism. While progress was made with both approaches, the results of this study suggest that the genomic approach has led to the identification of a candidate acetylene hydratase in *R. rhodochrous* ATCC 33258 and that this is a similar but distinct enzyme to the equivalent enzyme in the anaerobic C_2H_2-utilizing strain *P. acetylenicus*.

Section 2.2: Materials & Methods

Materials: *Rhodococcus rhodochrous* ATCC 33258 was obtained from the American Type Culture Collection (Manassas, VA). Acetylene gas (C_2H_2) was generated and obtained by reacting calcium carbide (~5g) with water (~100 mL) using a "Burris Bottle"-style two chamber gas generator. The resulting gas was used without further purification.

Maintenance and Growth of Microbial Culture: Freeze-dried *R. rhodochrous* ATCC 33258 was rehydrated according to ATCC instructions and was transferred into several different mediums to determine optimal growth conditions. *R. rhodochrous* was maintained on 1xMv medium agar plates (with or without vitamin added). The 1xMv media contained (per liter) 2.0g of NH_4Cl, 0.075g of $MgCl_2 \cdot 6H_2O$, 0.1g of $(NH_4)_2SO_4$, 0.775g of K_2HPO_4, 0.489g of $NaH_2PO_4 \cdot H_2O$. The medium was supplemented with 2mL of a trace element solution that contained (per liter of water) 50g EDTA, 22g $ZnSO_4 \cdot 7H_2O$, 4.54g of $CaCl_2$, 5.06g of $MnCl_2 \cdot 4H_2O$, 5.0g of $FeSO_4 \cdot 7H_2O$, 1.1g of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.506g $CuSO_4 \cdot 5H_2O$, 1.71g of $CoCl_2 \cdot 6H_2O$. The agar plates were incubated inside a glass desiccator (3 L) at room temperature (about 23°C). Acetylene gas (60 mL) was added to the desiccator chamber headspace through a rubber septum seal using plastic syringes to give a gas phase concentration of ~2% vol/vol.

As needed, the 1xMV medium used to grow suspended liquid cultures or agar plates was supplemented with an aqueous vitamin solution (1:1000 per media volume). The vitamin mix was prepared according to Lidstrom (1988) and contained (per liter, final concentration) biotin (20 mg), folic acid (20 mg), thiamine-HCl (50 mg), calcium pantothenate (50 mg), vitamin B_{12}, (1 mg), riboflavin (50 mg), and nicotinamide (50 mg). Liquid batch cultures were grown on

different carbon sources (liquid or gaseous) in glass serum vials (160 mL) that contained 1xMv media (25 mL). Unless otherwise stated, all cultures were inoculated using cultures previously grown on 1xMv agar plates in the presence of acetylene. The inoculated vials were sealed with sterile butyl rubber stoppers and crimped aluminum seals (Wheaton Scientific, Millville, NJ). Gaseous carbon sources were added to the headspace of the sealed culture vials using Luer Lock plastic syringes fitted with 0.1 μm filters (Durapore PVDF Membrane, Merck Millipore Ltd.). The culture vials were incubated in the dark within a 30°C orbital environmental shaker operating at 150 rpm. For each culture, a sample (50 μL) was streaked onto either Plate Count Agar (PCA) or Luria-Bertani (LB) plates to assess culture purity. Growth of cultures was determined by changes in culture density (OD_{600}) In all cases, samples (0.5 mL) were removed from culture vials using sterile syringes (1 mL). The culture samples were diluted with buffer (50 mM NaH_2PO_4, pH 7.0) and the measured OD_{600} was then multiplied by dilution factor to obtain the actual OD_{600} in the sample. All measurements of optical density were obtained using a Shimadzu 1601 UV-Visible spectrophotometer (Kyoto, Japan).

The ability of *R. rhodochrous* ATCC 33258 to utilize selected primary alcohols as growth substrates was determined using cultures grown in glass serum vials (160 mL) containing 1xMv media (25 mL) supplemented with liquid vitamin mix (25 μL). Each alcohol (0.25 mmol) was added as a neat compound [ethanol (14.5 μL), 1-propanol (18.7 μL), n-butanol (22.9 μL), isobutanol (23.1 μL)]. An additional culture was grown on C_2H_2 (10 mL) as a positive control. All cultures were inoculated with *R. rhodochrous* previously grown on C_2H_2 on 1xMv plates. The inoculated vials were then sealed with rubber butyl stoppers and aluminum crimp seals. Vials were incubated within a dark environmental shaker (30 °C, 150 rpm). Changes in culture densities (OD_{600}) were determined every 24 h.

Gas Chromatography: Consumption of C_2H_2 gas was measured by gas chromatography (GC) by removing samples (10 µL) reaction or culture headspace using a 25 µL gastight glass syringe (Hamilton Co., Reno, NV). The gas samples were directly injected into a Shimadzu GC-14A gas chromatograph (GC) fitted with a flame ionization detector and 30 m Rt Alumina BOND/Na_2SO_4 capillary column (Cat#: 19755; Serial #: 1573860; Restek Co., Bellefonte, PA). The GC was operated with a column temperature of 35 ºC, a detector temperature of 220 ºC, and an injector temperature of 200 ºC. Nitrogen was used as the carrier gas at a constant pressure of 150 kPa.

The GC was calibrated for C_2H_2 using the same sized serum vials (160 mL) used to cultivate *R. rhoodochrous* ATCC 33258 on C_2H_2.Water (25 mL) was added to the vial and the vial was sealed with a butyl rubber stopper and an aluminum crimp seal. The vial was placed into a 30 °C shaking (150 rpm) water bath for ~5 min. Varying volumes of C_2H_2 gas were sequentially added to the headspace of the vial with a plastic syringe. After each gas addition, the serum vial was then returned to the shaking water bath for 1 minute to allow for equilibration of the gas between the gas and liquid phases. After equilibration, samples (10 µL) of headspace were injected into the GC. For each concentration of C_2H_2, three headspace samples (3 x 10 µL) were analyzed with ~2 minutes between each sample injection. The data reported for the calibration are the mean and standard error for these three samples.

Specific rates of C_2H_2 uptake by concentrated *R. rhodochrous* ATCC 33258 cell suspensions: The ability of concentrated resting cell suspensions to consume C_2H_2 was determined by GC analysis of headspace gas from small-scale reactions containing cells of *R.*

rhodochrous ATCC 33258 grown on C_2H_2, as described above. Approximately 15-20 hours before harvesting, the butyl rubber stopper and aluminum crimp seal were removed from the culture vial which was then allowed to re-aerate in a laminar flow hood for 5 minutes. The culture vials were then resealed with a sterile butyl rubber stopper and aluminum crimp seal before C_2H_2 (10 mL) was added. The replenished culture vial was returned to the environmental shaker to incubate at 30° C overnight. Cells were harvested by centrifugation (10,000 x ***g***, 5 minutes, 4°C). After decanting the culture supernatant, the cell pellet was resuspended in buffer (50 mM NaH_2PO_4, pH 7.0) (1 mL) to a final concentration of ~10 mg total protein/mL. The resting cell concentrate was then stored on ice at 4° C and used for experiments within 4h.

Small-scale reactions following C_2H_2 consumption by resting, harvested cells were conducted in glass serum vials (10 mL). The vials contained buffer (0.9 mL) and were sealed with butyl rubber stoppers and aluminum crimp seals. C_2H_2 (100 µL) was added to the headspace and the vials were placed in a shaking water bath operated at 30 °C and 150 rpm to allow equilibration of C_2H_2 between the gas and liquid phases. The reactions were initiated by adding a sample of either concentrated resting cell suspension (100 µL) or buffer (100 µL) using a gastight glass syringe (Hamilton Co., Reno, NV). The reaction vials were then returned to the water bath. Samples of the gas phase (10 µL) were removed at various times and were analyzed for C_2H_2 by GC.

Genomic DNA extraction and sequencing: Genomic DNA extraction from a frozen cell pellet of *R. rhodochrous* ATCC 33258 was performed by SNPsaurus (OR, USA) using the Quick-DNA Miniprep Plus Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol. To increase the DNA recovery from this Gram-positive bacterium, an additional lysozyme

treatment (10 mg/mL final concentration) was applied for 30 minutes at 37° C. The genomic DNA was prepared and converted into SMRTbell libraries using the PacBio SMRTbell® Express Template Prep Kit 2.0 (Pacific Biosciences, Menlo Park, CA). Samples were pooled into a single multiplexed library and size selected using the BluePippin system (Sage Sciences, Beverly, MA) using the recommended 0.75% DF Marker S1 High-Pass 6-kb-10 kb v3 run protocol and S1 marker. A size selection cutoff of 800 (BP start value) was used. The size selected SMRTbell library was annealed and bound according to the SMRT Link Set Up and sequenced on the Sequel II System. Raw PacBio reads were converted to FASTA format with SAMtools then assembled with Flye v2.8 by SNPsaurus (OR, USA).

Contigs with sequences producing significant alignments (BlastN hits) to *Rhodococcus* sp. (Contig_1; Contig_2, Contig_3; Contig_7) were separated from the other remaining contigs, which had been identified as deriving from contaminants due to their BlastN hits and GC content. These four *Rhodococcus* contigs were combined and the resulting *Rhodococcus* genome was annotated using Prokka 1.14.5 (Seemann, 2014) via Galaxy's web-based platform (Afgan *et al*., 2018).

Metabolic and cellular pathways: Metabolic and cellular pathways were predicted using the Kyoto Encyclopedia of Genes and Genomes (KEGG) online tool GhostKOALA (Kanehisa, Sato, & Morishima, 2016). The amino acid FASTA file generated from the Prokka annotation was submitted to the automated server to assign KEGG Orthology (KO) identifiers to the coding sequences (CDS) in the *R. rhodochrous* ATCC 33258 genome. The search was performed against the "c_genus_prokaryotes" KEGG Genes database.

Iron-Sulfur ([Fe-S]) cluster amino acid predictions: The web server MetalPredator (Valasatava *et al*., 2016) was used to search for metal-binding motifs and predict Fe-S clusters within the translated coding genes of *R. rhodochrous* ATCC 33258 (amino acid FASTA file generated by Prokka annotation). The iron-sulfur proteins predicted by the MetalPredator web interface were downloaded and reorganized in terms of ascending ORF. The called amino acid sequences were then analyzed using NCBI's BlastP algorithm to determine if any of the called query sequences shared significant similarity to a previously annotated acetylene hydratase (AH) sequence.

Section 2.3: Results

C_2H_2 GC calibration plot: A calibration plot (Fig. 2.1) for C_2H_2 was determined for gas concentrations up to 11% v/v gas phase, as described in the Methods section. Using the ideal gas equation, the molar volume of C_2H_2 gas was calculated to be 24.876 L for 1 atm and 30°C. A linear relationship ($r^2 = 0.998$) was established between the amount of C_2H_2 gas (in µmoles) and the quantified peak area units detected by the GC.

Thiamine requirement for growth of *R. rhodochrous* ATCC 33258: Kanner & Bartha (1979) originally demonstrated that growth of *R. rhodochrous* ATCC 33258 on C_2H_2 required thiamine in the culture medium. The results in Fig. 2.2 show a clear difference between the time course of C_2H_2 consumption by *R. rhodochrous* inoculated into 1xMv medium with and without added thiamine. For cells grown with thiamine present, the rate of C_2H_2 consumption increased dramatically after 18 hours and the majority of C_2H_2 was consumed within 36 hours. In contrast, minimal consumption of C_2H_2 was observed in cultures grown without thiamine present or uninoculated control culture bottles. The pronounced increase in C_2H_2 consumption in thiamine-containing cultures (Fig. 2.2) coincided with similarly pronounced increases in culture density (OD_{600}) (Fig. 2.3). For example, in the thiamine-containing cultures, an increase in culture density was first observed after 18 h and the maximal culture density was observed after 36 h (average $OD_{600} = 1.3$). In contrast, there was no significant change in the culture densities for the thiamine-deficient cultures and the uninoculated controls at all time points tested.

C_2H_2 uptake by resting *R. rhodochrous* ATCC 33258 cell suspensions: The aim of the physiological/proteomic approach was to identify acetylene hydratase (AH) in *R. rhodochrous* ATCC 33258 by determinations of the specific activity of C_2H_2 uptake by cells grown on different substrates. The expectation was that if growth on specific substrates produced large differences in specific rates of acetylene utilization, SDS-PAGE or shotgun proteomic analyses could then be used to identify candidate hydratases through differences in protein expression patterns. Specific rates of C_2H_2 utilization were to be determined using resting cell incubations in which cells were first grown on individual substrates and then harvested and washed to remove residual growth substrate. These cells would then be incubated with C_2H_2 at high biomass concentrations in small scale reactions. The rate of C_2H_2 uptake would then be determined from the initial rate of C_2H_2 consumption by following changes in the headspace concentration of C_2H_2 using GC analyses.

The results shown in Fig. 2.4 provide an example of this approach with C_2H_2-grown cells of *R. rhodochrous* ATCC 33258. The C_2H_2 added to the reaction vial (100 μL/~4 μmoles) was rapidly consumed within 1 h in reactions containing ~1 mg total cell protein. In contrast, over the same time there was a minimal loss (≤10%) of C_2H_2 from control incubations that lacked cells. Although a calibration plot was not established for C_2H_2 for this reaction configuration, the constant rate of C_2H_2 consumption over the first 30 min of the reaction suggests the initial rate of C_2H_2 consumption can be estimated at ~100 nmoles/min/mg total protein.

Growth of *R. rhodochrous* ATCC 33258 on primary alcohols: The ability of *R. rhodochrous* ATCC 33258 to grow on primary alcohols was tested. The bacterium grew rapidly on all tested alcohols (ethanol, 1-propanol, 1-butanol, and isobutanol (2-methyl-1-propanol)) other than

methanol (Table 2.1). In a separate experiment, the time course of growth was examined for cultures grown on equimolar but carbon-limited concentrations of each alcohol. Apart from isobutanol, the maximum culture density (OD_{600}) observed for each primary alcohol increased with the increasing number of carbons (Fig. 2.5). Growth of *R. rhodochrous* ATCC 33258 on isobutanol (2-methyl-1-propanol) was much slower and the maximum culture density for this substrate was not reached until 168 h after the initiation of the incubation. The proposed measurements of C_2H_2 uptake specific activity with cells grown on alcohols and other substrates were not conducted.

Sequencing and annotation of *R. rhodochrous* ATCC 33258 genome: The results of the PacBio genome sequencing are summarized in Table 2.2. The 7.40 Mb genome was assembled into 4 contigs with an average GC content of 62.3%. Of the total 7,058 genes predicted by the Prokka annotation, 6,984 genes were assigned as protein-coding sequences (CDS). Approximately half of the genes predicted were assigned a non-hypothetical function. A BUSCO analysis indicated a 97.7% completeness of the genome based on a comparison of 743 single copy orthologs from the corynebacteriales_odb10 lineage dataset.

A GhostKOALA KEGG analysis of the draft genome indicated that approximately 41% of the total 6,984 CDS entries could be assigned a KO identifier and classified into a variety of functional categories. The majority of the GhostKOALA KO assignments were classified as part of carbohydrate metabolism, signaling and cellular processes, genetic information processing, and environmental information processing. Amino acid metabolism, metabolism of cofactors and vitamins, energy metabolism, and lipid metabolism functional categories were also represented in the genome to a lesser extent.

Using reconstructed KEGG pathways and modules, the genome of *R. rhodochrous* ATCC 33258 appears to encode complete pathways for glycolysis (Embden-Meyerhof), gluconeogenesis, and both the citrate and pentose phosphate cycles. In terms of energy metabolism, *R. rhodochrous* possesses complete modules for ATP synthesis. The genome also encodes numerous ABC transporters. Some of the complete transport systems identified by KEGG include molybdate (ModABC), taurine (TauABC), sulfonate (SsuABC), sorbitol/mannitol (SmoEFGK), phosphate (PstABCS), glutamine (GlnHPQ), glutamate (GluABCD), D-methionine (MetINQ), biotin (BioMNY), and manganese (MntABC). A few nearly complete ABC transport systems were also noted, including trehalose / maltose (ThuEFG) and ribose (RbsABC). The KEGG analysis also indicates that the bacterium does not possess all the genes necessary for *de novo* thiamine biosynthesis; however, a complete module for the thiamine salvage pathway (M00899) along with the thiminase TenA was noted.

Identification of candidate acetylene hydratase-encoding genes: The predicted amino acid sequences of five previously identified acetylene hydratases (AHs) from previously annotated bacterial genomes (Table 2.3) were individually compared to the entire *R. rhodochrous* amino acid sequence file obtained from the Prokka annotation using BlastP analyses. These analyses consistently identified a putative AH-encoding gene (locus_ID: BHAHFLAH_00032), as well several oxidoreductases that included two molybdenum-containing enzymes (dimethyl sulfoxide [DMSO]/trimethylamine N-oxide [TMAO] reductase and nitrate reductase) from the *R. rhodochrous* genome (Table 2.4). The predicted amino acid sequence identities for the identified genes were generally low (≤25%), although a value as high as 54.26% sequence identity was obtained for one hit for a partial AH sequence. A BlastP analysis of the NCBI database using the

predicted amino acid sequence of BHAHFLAH_00032 identified three proteins of interest. These included a molybdenum-dependent oxidoreductase from a *Rhodococcus opacus* strain and two distantly related acetylene hydratases from different marine organisms (Table 2.5). While the percent amino acid sequence identities for the two AHs were low (~41%), a much higher value (~95%) was obtained for the enzyme from *Rhodococcus opacus*.

Analysis of [Fe-S] center-containing enzymes in *R. rhodochrous* ATCC 33258 genome: Structural analyses of the acetylene hydratase purified from *P. acetylenicus* indicates the enzyme has a tungsten (W) center with a nearby [4Fe-4S] cluster (Rosner & Schink, 1995; Seiffert *et al*., 2007; Brink, Schink, & Kroneck, 2011). MetalPredator is a web interfaced tool that utilizes two libraries of Hidden Markov Model profiles with Pfam domains or metal-binding structural motifs to predict [Fe-S] cluster containing proteins from query amino acid sequences. The 6,984 translated protein coding genes in the Prokka annotation of the draft genome of *R. rhodochrous* ATCC 33258 were therefore surveyed using this application. In total, 128/6984 (1.83%) sequences from were identified that contained putative [Fe-S] clusters. These identified proteins included ferredoxins, rubredoxins, NADH-quinone oxidoreductase subunits (*nuoBEFGI*), glutamate synthase [NADPH] chains, NADPH oxidoreductases/dehydrogenases, and [Fe-S] cluster carrier proteins. Of the five *R. rhodochrous* ATCC 33258 features found to have significant amino acid sequence alignments with previously annotated acetylene hydratases (AHs) (Table 2.4), only three of the sequences were also predicted to contain [Fe-S] centers (Table 2.6A). These included a single [4Fe-4S] center in the putative acetylene hydratase (BHAHFLAH_00032), several different [XFe-XS] centers in the NADH-quinone oxidoreductase

subunit G (BHAHFLAH_01529) and a single [4Fe-4S] center in the periplasmic nitrate reductase (BHAHFLAH_02279).

Cysteine residues and AH alignment: In the case of the putative AH (BHAHFLAH_00032) from *R. rhodochrous* ATCC 33258, the predicted protein contains 795 amino acids with a predicted mass of 89.8 kDa. The protein contains seven cysteine residues (Cys-17, Cys-20, Cys-24, Cys-52, Cys-143, Cys-320, and Cys-736). Four of these residues (Cys-17, Cys-20, Cys-24, and Cys-52) aligned closely with the key cysteine residues that have previously been implicated as ligands to the [4Fe-4S] center (Cys-9, Cys-12, Cys-16, & Cys-46) in the smaller (730 amino acids) AH from *P. acetylenicus* (Seiffert *et al.*, 2007) (Fig. 2.6). Additionally, Cys-143 in the putative AH (BHAHFLAH_00032) aligns with Cys-141 in *P. acetylenicus,* which is believed to coordinate a tungsten (W) atom in the anaerobic AH enzyme (Seiffert *et al.*, 2007; Brink, Schink, & Kroneck, 2011)(Fig. 2.6).

Proteins associated with molybdopterin biosynthesis and molybdenum transport: The AH from *P. acetylenicus* contains a molybdopterin cofactor that binds tungsten (W). Several of the proteins identified in our analyses, including the putative AH (BHAHFLAH_00032) also have distinctive molybdopterin binding motifs (Table 2.6A,B). We therefore examined the draft genome for the presence of molybdopterin biosynthesis genes. Homologs to several of the molybdenum cofactor biosynthesis genes recognized and typically described in *E. coli* were identified in the draft genome (Table 2.7) and the majority of these genes were found closely located to the putative AH-encoding gene (Fig. 2.7; Table 2.7). Importantly, three genes (*modA, modB, and modC*) that encode proteins associated with the transport of molybdate were

identified on contig #3 of *R. rhodochrous* ATCC 33258 genome (Table 2.8). In addition to multiple genes associated with molybdopterin biosynthesis, a gene encoding a putative aldehyde dehydrogenase BHAHFLAH_00019) was also identified close to the AH-encoding sequence (Fig. 2.7).

Section 2.4: Discussion

The results of this study provide molecular evidence that acetylene hydratase (AH) in *R. rhodochrous* ATCC 33258 is similar but not identical to forms of this enzyme found in anaerobic bacteria and that the enzyme most likely contains an [4Fe-4S] center and a molybdopterin cofactor that binds molybdenum rather than tungsten. Our results also suggest that physiological and proteomic studies examining the role of micronutrients, vitamins, and alternative non-alkyne growth substrates may provide important insights into the expression of AH in *R. rhodochrous* ATCC 33258. These major conclusions are discussed in more depth in the following sections.

Identification of putative acetylene hydratase (AH) encoding gene in genome of *R. rhodochrous* ATCC 33258: Several different lines of evidence suggest that the gene BHAHFLAH_00032 encodes AH in *R. rhodochrous* ATCC 33258. First and foremost, this gene was consistently identified through BlastP analyses when the draft genome of *R. rhodochrous* ATCC 33258 was queried using the amino acid sequences of five previously annotated AH enzymes (Table 2.4). Notably, the only well-characterized AH sequence with known C_2H_2-consuming functionality is the first query sequence in Table 2.3. This 730 amino acid sequence (UniProtKB accession Q71EW5.1) is the AH sequence identified in the obligate anaerobe *P. acetylenicus* DSM3246 (recently suggested rename *Syntrophotalea acetylenica*). Although the levels of shared amino acid sequence identity were generally low, this was not unexpected. Rosner *et al*., (1997) demonstrated that there was no cross reactivity between extracts of aerobic isolates grown on C_2H_2 and polyclonal antibodies raised against the anaerobic AH purified from *P. acetylenicus*. More recent evidence pointing to the diversity of anaerobic AH enzymes can be

seen from the results of a survey conducted by Miller *et al*. (2013) on C_2H_2 consumption by anoxic environmental samples. Despite observing C_2H_2 consumption in 21% of anoxic sediment and water samples collected, PCR analyses using primers based on the AH from *P. acetylenicus* only detected genes encoding this enzyme in 63/645 (9.8%) of the environmental samples tested (Miller *et al.*, 2013).

Cysteine Residues & [Fe-S] Clusters: The second line of evidence to support the identity of this putative AH (BHAHFLAH_00032) is the distribution of cysteine residues in the predicted amino acid sequence of the aerobic enzyme. Acetylene hydratase (AH) from *P. acetylenicus* is a monomer of 730 amino acids that folds into a four-domain tertiary structure. Domain I (residues 4-60) contains four cysteine residues (Cys-9, Cys-12, Cys-16, and Cys-46) that act as ligands for the enzyme's [4Fe-4S] center. Although the putative AH from *R. rhodochrous* ATCC 33258 is apparently a substantially larger enzyme (795 amino acids), the N-terminus of the predicted amino acid sequence has a nearly identical distribution of cysteine residues (Fig. 2.7, yellow highlights), suggesting that this enzyme may also contains a similarly positioned [4Fe-4S] center. This conclusion is further reinforced by the crystal structure of AH from *P. acetylenicus* that indicates that Cys-141 is involved in binding tungsten (W) within a molybdopterin cofactor (Seiffert *et al*., 2007). The predicted amino acid sequence of the AH from *R. rhodochrous* ATCC 33258 only contains three additional cysteine residues (Cys-143, Cys-320, and Cys-736) other than the four residues likely involved in [4Fe-4S] center coordination. One of these three cysteines (Cys-143) is also positioned to serve a similar metal-binding role in the enzyme in *R. rhodochrous* ATCC 33258 (Fig. 2.7, blue box).

Molybdopterin Cofactors, Biosynthesis, & Metal Uptake: The third line of evidence supporting the identification of the AH-encoding gene in *R. rhodochrous* ATCC 33258 centers on the role of molybdenum and the biosynthesis of the molybdenum cofactor. Previous studies have shown that expression of AH activity in several aerobes is dependent on molybdate rather than tungstate (Rosner *et al.*, 1997; Rosner & Schink, 1995), suggesting that the enzyme in aerobes may be molybdenum- rather than tungsten-dependent. It has also been suggested that the frequency at which tungsten-containing enzymes are found in extremophilic organisms such as archaea compared to bacteria likely reflects the fact that tungsten is more readily bioavailable than molybdenum under the environmental conditions required by extremophiles and that molybdenum is expected to replace the role of tungsten in enzymes expressed under more benign conditions (Cordas & Moura, 2019). Our sequence-based analyses demonstrated that the amino acid sequence of the putative AH in *R. rhodochrous* ATCC 33258 has a Pfam molybdopterin structural motif (Table 2.6 a & b) and that, like the enzyme from *P. acetylenicus*, shows amino acid sequence similarity with the DMSO reductase (DMSOR) group of molybdoenzymes that all utilize molybdopterin guanine dinucleotide (MGD) cofactors. In addition, the genomic environment of putative AH-encoding gene in *R. rhodochrous* ATCC 33258 contains multiple genes associated with the biosynthesis of MGD (Fig. 2.8, Table 2.7).

Moco Biosynthesis: The biosynthesis of molybdenum cofactor (Moco) has been extensively studied in *E. coli*. In this bacterium, Moco biosynthesis can be divided into four general steps: (i) formation of the cyclic intermediate pyranopterin monophosphate (cPMP, or 'precursor Z') from guanosine-5'-triphosphate (GTP); (ii) conversion of cPMP to MPT by the insertion of two sulfur atoms; (iii) adenylation to MPT-adenosine monophosphate (AMP); (iv) insertion of the metal to

form Mo-MPT and release of AMP. Following insertion of the metal, a variety of additional modification steps can occur to form different variants or derivatives of the molybdenum cofactor. In *E. coli*, five operons have been identified as being directly involved in Moco biosynthesis: *moaABCDE*, *mobAB*, *mocA*, *moeAB*, and *mogA* (Leimkühler, 2020). The proteins encoded by the genes from these operons and their functions are described in more detail below and diagramed in Figure 2.8.

During the first step of Moco biosynthesis, MoaA and MoaC facilitate the conversion of GTP to cyclic pyranopterin monosphosphate (cPMP; originally named 'precursor Z'). Next, MoaD and MoaE form a heterotetrameric MPT synthase which catalyzes the conversion of cPMP to MPT dithiolate, a reaction in which two sulfur atoms are inserted into the C1' and C2' positions of cPMP (Leimkühler, 2020). For new rounds of catalysis, accessory enzymes MoeB, IscS, and TusA are involved in the initial mobilization and transfer of sulfur (using L-cysteine as a donor) and the regeneration of the thiocarboxylate group on C-terminus of the MoaD subunits (Zhang *et al*., 2010; Zupok *et al*., 2019). The role of MoeB in Moco biosynthesis has also been shown to be carried out by the homolog MoeZR in *Mycobacterium* (Voss, Nimtz, & Leimkühler, 2011). The third and fourth steps, which are often merged and described as a single step, rely on MogA and MoeA to synthesize Moco. In bacteria, MogA activates MPT by forming an adenylated intermediate, MPT-AMP, under ATP consumption. In the archaea *Pyrococcus furiosus,* the activity of MogA can be catalyzed by the homologous protein MoaB (Bevers *et al*., 2008). The MPT-AMP intermediate is then transferred to MoeA. MoeA is then thought to mediate the molybdate ligation to the dithiolene sulfurs of MPT, hydrolyzing the intermediate and releasing the AMP moiety. The end product Mo-MPT is a basic form of Moco, which is utilized by sulfite oxidase (SO) family enzymes (Seelmann *et al*., 2020). As this is only one of

four families of tungsten and molybdenum enzymes, many prokaryotes utilize a second enzyme cascade to further modify Moco and produce various forms of the cofactor. The form the molybdenum cofactor utilized by DMSOR family enzymes, such as acetylene hydratase (AH) is the bis-molybdopterin-guanine dinucleotide (bis-MGD). Following the synthesis of two basic Mo-MPT molecules, the MobA protein forms a bis-Mo-MPT intermediate by combining the two MPTs together via a central molybdenum atom. The second step of this modification involves adding two guanine nucleotides (GMP) from GTP to the C4' phosphate of each MPT, thus forming the bis-MGD cofactor. MobA is capable of catalyzing both steps of this modification (Leimkühler, 2020; Seelmann *et al*., 2020).

Based on the GhostKOALA KO assignments, all of the proteins or functional homologs directly involved in the MGD biosynthesis pathway (excluding the sulfur relay system) are found adjacent to the putative AH (BHAHFLAH_00032) in *R. rhodochrous* ATCC 33258 (Fig. 2.7, Fig. 2.8, Table 2.7). Notably, neither the TusA protein nor the MocA protein (MCD biosynthesis) appear to be encoded within the entire genome. While a KO assignment for IscS was found (BHAHFLAH_01803), it is not particularly close to the putative AH.

Metal Uptake: In addition to molybdopterin biosynthesis genes, the draft genome of *R. rhodochrous* ATCC 33258 contains genes that are selective from the transport of molybdate but not tungstate (Table 2.8). Tungstate (WO_4^{2-}) and molybdate (MoO_4^{2-}) are taken up by prokaryotic cells via three primary ATP-binding cassette (ABC) transport systems: ModABC, TupABC, and WtpABC (Grunden & Shanmugam, 1997; Makdessi, Andreesen, & Pich, 2001; Bevers *et al.*, 2006). Each of these systems encodes for a periplasmic binding protein (component A), a transmembrane channel protein (component B), and an ATP hydrolyzing

protein (component C). The three systems differ from each other in both their protein sequences and in their binding affinities. The binding protein ModA binds both molybdate and tungstate with high affinity. TupA specifically binds tungstate with high affinity, whereas WtpA can bind both tungstate and molybdate (Bevers *et al.*, 2006; Bevers, Schwarz, & Hagen, 2011). Reports indicate that WtpA has a higher affinity for tungstate than both ModA and TupA and an affinity for molybdate that is similar to that of ModA (Bevers *et al.*, 2006). In the *R. rhodochrous* ATCC 33258 genome, genes encoding ModA (BHAHFLAH_05637), ModB (BHAHFLAH_05638), and ModC (BHAHFLAH_05639) are found on a different contig (contig 3) from the putative AH (contig 1)(Table2.8).

In the case of *E. coli*, transcription of the molybdate transport genes (*modABC*) are regulated by the transcription factor protein ModE (Grunden & Shanmugam, 1997). Interestingly, the draft genome of *R. rhodochrous* does not appear to encode a ModE regulation homolog. Lack of a ModE molybdenum-responsive regulatory protein is not unheard of, as the archaeal genome of *Pyrococcus furiosus* also appears to lack a ModE homolog (Sevcenco *et al.*, 2010). While the ModABC transport system does not discrimination between molybdate or tungstate, and therefore the exact metal cofactor of acetylene hydratase in *R. rhodochrous* ATCC 3258 can only be speculated upon, none of the genes for the WtpABC or TupABC transport systems with higher affinity for tungstate were found within the *R. rhodochrous* ATCC 33258 genome.

Aldehyde dehydrogenase: A final and more minor line of molecular evidence that supports the identification of the aerobic form of acetylene hydratase is that the cluster of genes in the immediate vicinity of the putative acetylene-hydratase-encoding gene (BHAHFLAH_00032)

also includes a gene that encodes an aldehyde dehydrogenase (BHAHFLAH_00019)(Fig 2.7). Acetaldehyde is the expected immediate product of acetylene hydration and acetaldehyde oxidation to acetate provides a mechanism for directing carbon from acetylene into the major catabolic pathways of the bacterium (Fig. 1.1).

Physiological studies: The results of our physiological studies do not provide direct evidence for the identity of acetylene hydratase in *R. rhodochrous* ATCC 33258, but they do point to potential avenues for future research into the conditions that regulate the expression of this enzyme. For example, our results demonstrate that the uptake of acetylene by both growing and resting cells can be readily detected by gas chromatography (Fig. 2.2 & Fig. 2.4) and that specific activities of acetylene uptake could be readily determined. Although the specific acetylene uptake activities were not determined for cells grown on substrates other than acetylene, the approach has the clear potential to estimate expression levels of this enzyme when the bacterium is grown on different growth substrates or under different growth conditions. This in turn would be expected to enable more direct estimates of enzyme abundance using genome-enabled proteomic approaches such as shotgun proteomics and less direct approaches such as SDS-PAGE analyses of protein expression patterns.

Thiamine: Another area in which physiological studies could contribute to our understanding of acetylene hydratase expression involves the effects of vitamins and micronutrients on the growth and acetylene-utilizing activity of the bacterium. In the case of vitamins, in their original study of *R. rhodochrous* ATCC 33258 (then *Nocardia rhodochrous*), Kanner & Bartha demonstrated a thiamine requirement for growth of this strain on C_2H_2 and indicated that this vitamin is also

required for growth on acetate and fructose. Based on the fact that growth on C_2H_2 was also observed in both filter-sterilized and autoclaved thiamine-containing medium, they speculated the bacterium required only the heat-stable pyrimidine moiety of the otherwise heat-labile thiamine molecule. (Kanner & Bartha, 1979). Our results confirm that growth of *Rhodococcus rhodochrous* ATCC 33258 on C_2H_2 is strongly impacted by thiamine in the mineral salts medium used in this study (Fig. 2.2 & Fig. 2.3).

Thiamine (also known as thiamin or vitamin B_1) is an essential vitamin for nearly all organisms due to its role in glucose and energy metabolism. In its active form, thiamine pyrophosphate (TPP) or thiamine diphosphate (ThDP) acts as a cofactor for several enzymes, such as pyruvate dehydrogenase (PDH), a-ketoglutarate dehydrogenase, and transketolase. These enzymes are vital as they are associated with a variety of central metabolic pathways including glycolysis, the TCA cycle, and the pentose phosphate pathway (Kraft & Angert, 2017). Chemically, thiamine consists of a pyrimidine moiety HMP-PP (4-amino-5-hydroxymethyl-2-methyl-pyrimidine pyrophosphate) and a thiazole moiety HET-P or THZ-P (4-methyl-5-(ß-hydroxyethyl)thiazole phosphate). These thiazole and pyrimidine moieties or precursors are synthesized separately using a number of different enzymes and then joined together by thiamine phosphate synthase (ThiE) to form thiamine phosphate (TP or TMP). Thiamine phosphate kinase (ThiL) then catalyzes the phosphorylation of TP to produce thiamine pyrophosphate (TPP, also known as diphosphate (ThDP)), the active form of the cofactor. In bacteria capable of *de novo* thiamine biosynthesis, HMP-PP (pyrimidine) is synthesized from aminoimidazole ribotide (AIR) by HMP-P synthase (ThiC) and HMP-P kinase (ThiD). HET-P (thiazole) is biosynthesized from 1-deoxy-xylulose phosphate (DXP), glycine (or tyrosine), and cysteine in a more complex oxidative condensation reaction. In bacteria, HET-P *de novo* biosynthesis relies on *thiF, thiS,*

thiG, thiH (or *thiO*), *thiI, iscS,* and *Dxs* gene products to complete this process (Jurgenson, Begley, & Ealick, 2009; Du, Wang, & Xie, 2011).

The genome of *R. rhodochrous* ATCC 33258 does not possess all of the genes required for *de novo* biosynthesis of thiamine from the original precursors. The genes missing from this process are colored red in Figure 2.9. The genome does however possess genes encoding ThiD, ThiM, and ThiE, (Fig. 2.9, blue) which encode a thiamine salvage pathway that enables synthesis of thiamine from the intermediates HMP and HET. Thus, thiamine or the active form of the cofactor (TPP) can be synthesized so long as the HMP and HET intermediates are available. Additional genes encoding enzymes potentially involved in the salvage or recycling of thiamine were also found, including ThiK (thiamine kinase) and TenA (Fig. 2.9). The thiaminase TenA is involved in the salvage of HMP from base-degraded thiamine; however, some reports suggest that the enzyme is also capable of hydrolyzing thiamine itself to HMP and HET (Müller *et al*., 2009). Interestingly, no complete transport systems involved in the uptake of exogenous thiamine, HMP, or HET (Fig. 2.9, purple arrows) were explicitly annotated for the genome. Two genes (BHAHFLAH_04429 and BHAHFLAH_06320) were annotated by Prokka as encoding the substrate-binding component (ThiY) of the ThiXYZ ABC transport system involved in the uptake of HMP; however, neither the ThiX or ThiZ components of the transport channel could be specifically identified (Jurgenson, Begley, & Ealick, 2009). Given the presence of salvage pathway genes and the importance of thiamine for survival, the absence of such transporter genes from the genome annotation may be due to a lack of references or indicative of discoveries yet to come.

Future directions & Concluding Remarks: Although this study has provided multiple lines of evidence to support our conclusions that the acetylene hydratase in *R. rhodochrous* ATCC 33258 is encoded by BHAHFLAH_00032, there are clearly additional step that need to be taken to confirm this. In particular, it is necessary to establish that this putative hydratase can catalyze the expected conversion of C_2H_2 to acetaldehyde. The simplest approach to demonstrate this activity is a conventional purification of the enzyme from whole cells exhibiting high levels of C_2H_2-degarding activity. To this end, further studies investigating the effects of different growth substrates on specific C_2H_2-consuming activities would be useful for maximizing the levels of enzyme expression prior to purification. Given the similarities that appear to exist between the acetylene hydratase in *P. acetylenicus* and *R. rhodochrous* ATCC 33258, initial attempt to purify the enzyme from *R. rhodochrous* ATCC 33258 would be based on the approaches successfully used to purify the enzyme from *P. acetylenicus*. If the purification were successful, considerable structural information about the enzyme could then be obtained from spectroscopic studies involving electron paramagnetic resonance (EPR) to study the role of the putative [Fe-S] center in the catalytic cycle of the enzyme. More modest amounts of purified enzyme could also be used to obtain crystals for X-ray crystallographic analyses that would focus on the 3D structure of the enzyme and confirm the role of specific residue in the bind of the putative [4Fe-4S] and Mo-binding molybdopterin cofactors. As the putative gene sequence for the enzyme is now available, a potential alternative route to confirming the activity of the enzyme could involve heterologous expression of the enzyme and purification using His-tag affinity purification. Potential difficulties with this approach might arise as the host organism would need to be capable of synthesizing and inserting the putative [Fe-S] and molybdopterin cofactors. Potentially more insightful genome-enable analyses would involve shotgun proteomic analyses

that could be used to identify additional enzymes involved in acetaldehyde degradation and molybdopterin biosynthesis whos' expression could be expected to be co-regulated with the putative acetylene hydratase. Similar shotgun proteomic approaches could also be used to further explore regulatory effects of vitamins and metal micronutrients on the expression of acetylene hydratase in this bacterium.

CHAPTER 2: Figures

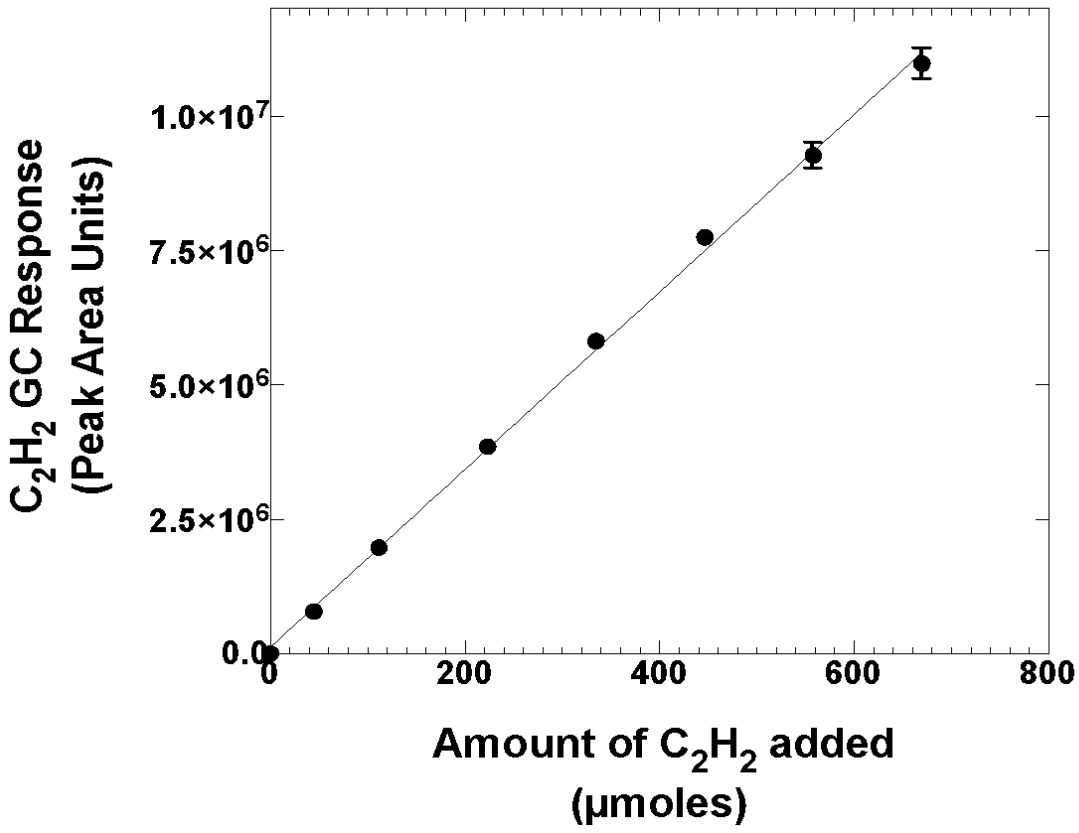

Figure 2.1 C_2H_2 calibration plot. The figure shows the detected GC response (peak area units) plotted versus the amount of C_2H_2 gas added to the 160 mL serum vial (in µmoles). The results show the mean and standard error of triplicate injections (technical replicates).

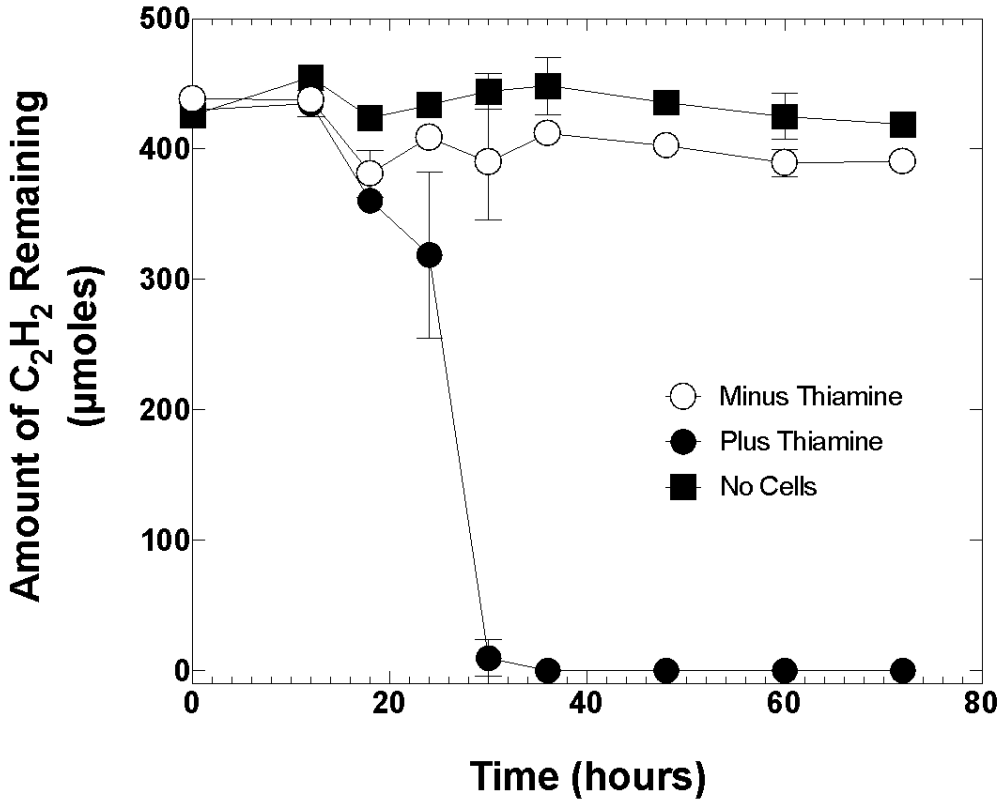

Figure 2.2 Consumption of C_2H_2 by *R. rhodochrous* ATCC 33258. The figure shows the time course of consumption of C_2H_2 from the headspace of culture vials inoculated with *R. rhodochrous* ATCC 33258. The uninoculated (■) and inoculated culture vials contained 1xMV medium with (●) and without (○) thiamine, as described in the Methods section. The amount of C_2H_2 present at each sampling time was determined based on the detected GC response (peak area units) and the C_2H_2 GC calibration plot (Figure 2.1). The results presented are the means and standard errors of three biological replicates.

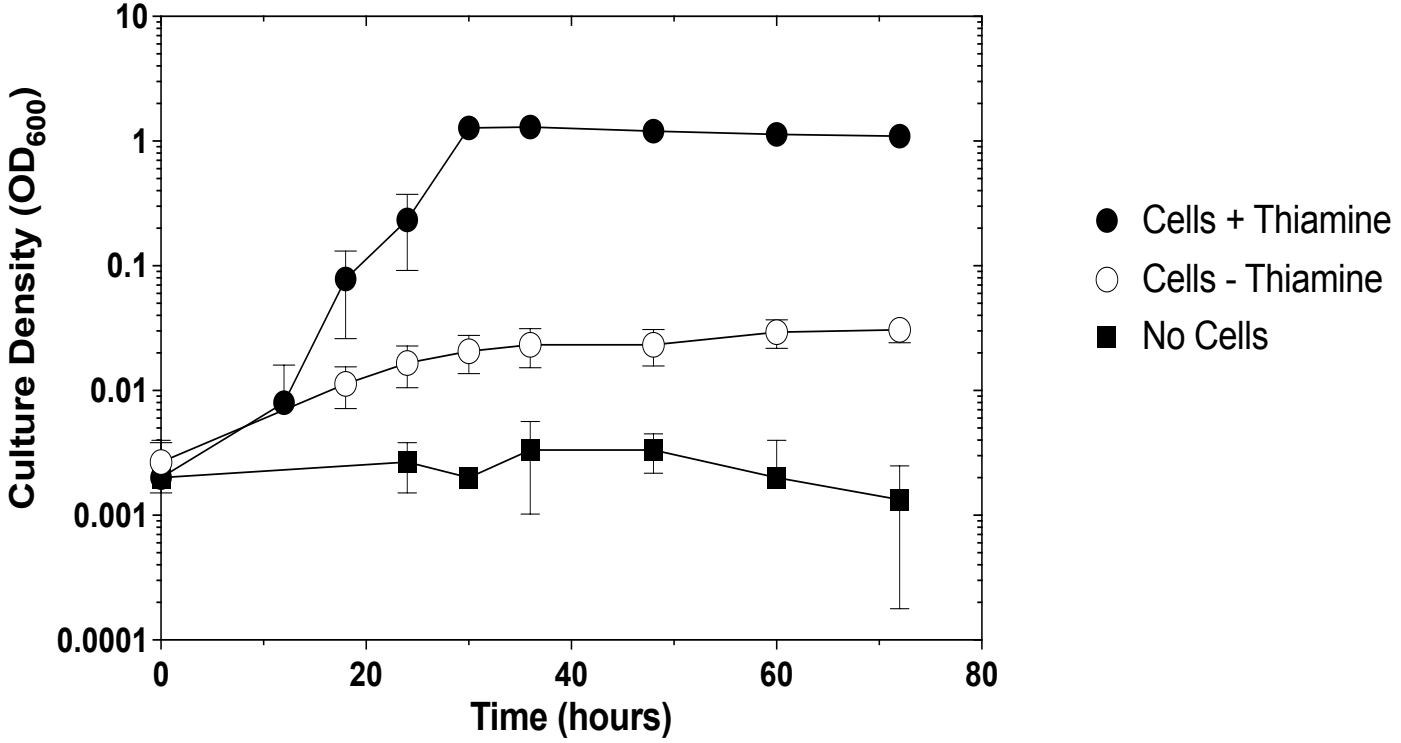

Figure 2.3 Effect of thiamine on growth of *R. rhodochrous* ATCC 33258 on C_2H_2. The figure shows the corresponding changes in culture density (OD_{600}) for the cultures described in Figure 2.2. The maximum culture density for cells supplemented with thiamine (average OD_{600} = 1.3) was observed after 36 h of growth. The results presented are the means and standard errors of three biological replicates.

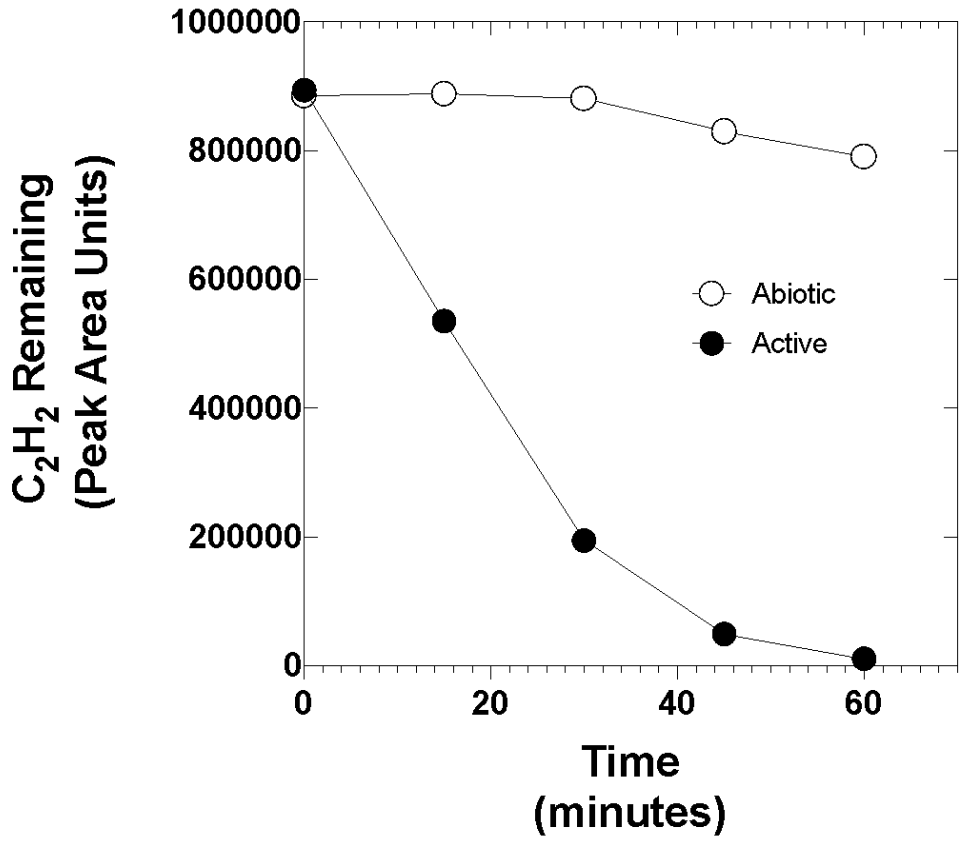

Figure 2.4 C_2H_2 uptake by concentrated cell suspensions of *R. rhodochrous* ATCC 33258. The figure shows the time course of C_2H_2 uptake (~4 µmoles) by concentrated suspensions of C_2H_2-grown *R. rhodochrous* ATCC 33258. The cells were grown, harvested, washed, and incubated with C_2H_2, as described in the Methods section.

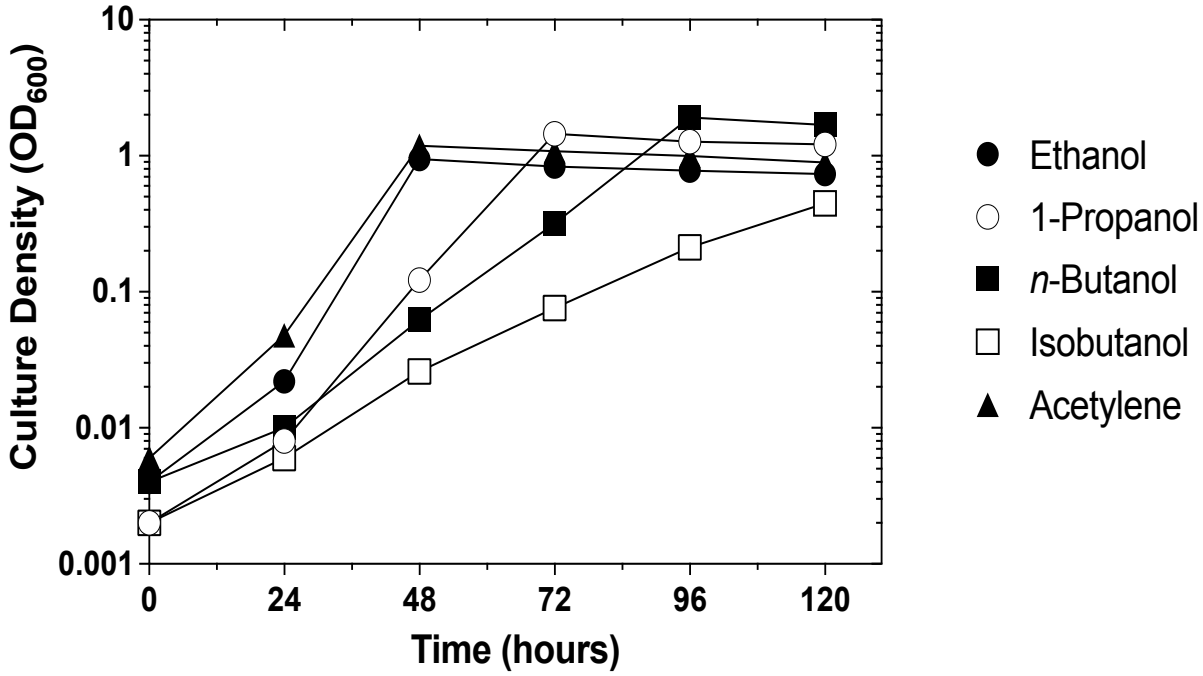

Figure 2.5 Growth of *R. rhodochrous* ATCC 33258 on primary alcohols. The figure shows the measured changes in optical density of cultures during growth on the indicated alcohols (0.25 mmol) or C_2H_2 (44 µmoles). Cultures grown on isobutanol did not reach their maximum culture density (OD_{600}) until 168 hours of growth (not shown).

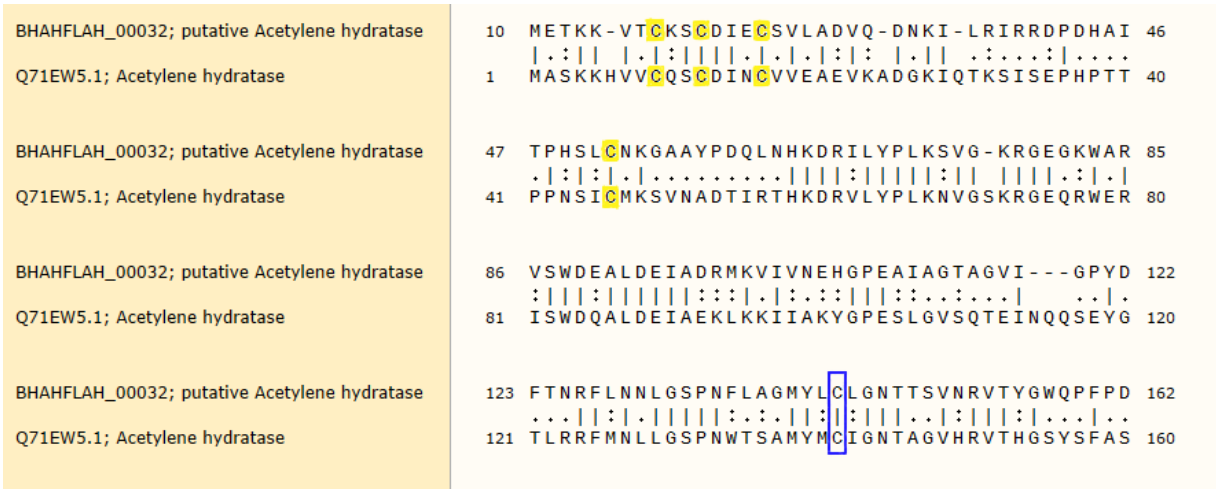

Figure 2.6. Sequence alignment of putative acetylene hydratase (AH) from aerobic *R. rhodochrous* ATCC 33258 with known acetylene hydratase (AH) from *P. acetylenicus.* The figure above shows a partial view of the aligned amino acid sequences for the putative AH (BHAHFLAH_00032) from *R. rhodochrous* ATCC 33258 (top line) and the characterized AH from *Pelobacter acetylenicus* (Accession UniProtKB:Q71EW5.1). The first four cysteines (yellow Cs) show the [4Fe-4S] cluster binding ligands. The next cysteine residue (blue box) is predicted to bind the tungsten (W) metal in the active site. The local alignment of these sequences was performed in SnapGene with their alignment tool (Smith-Waterman).

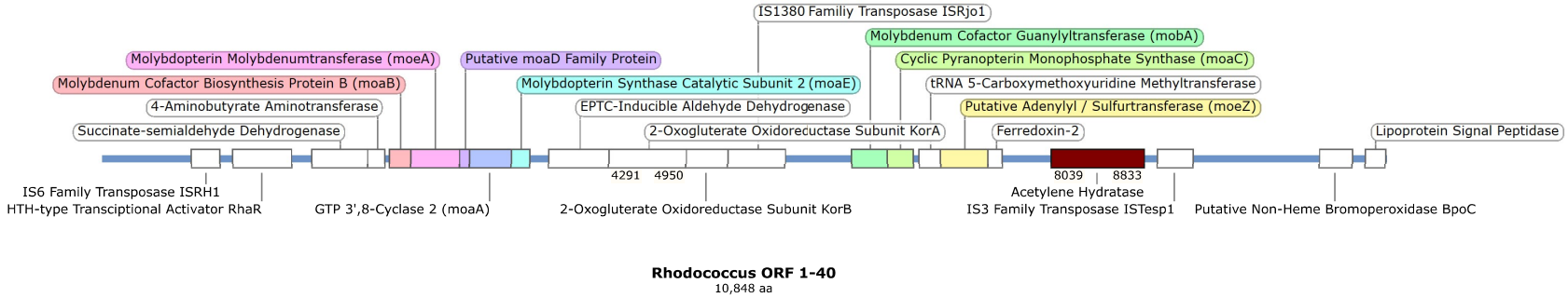

Figure 2.7. Map of acetylene hydratase in relation to molybdenum cofactor (Moco) biosynthesis genes within *R. rhodochrous* ATCC 33258. The image above was created with SnapGene® software (from Insightful Science; available at snapgene.com). The putative acetylene hydratase (AH) is colored dark red while the gene products involved in Moco biosynthesis are indicated by a variety of colors.

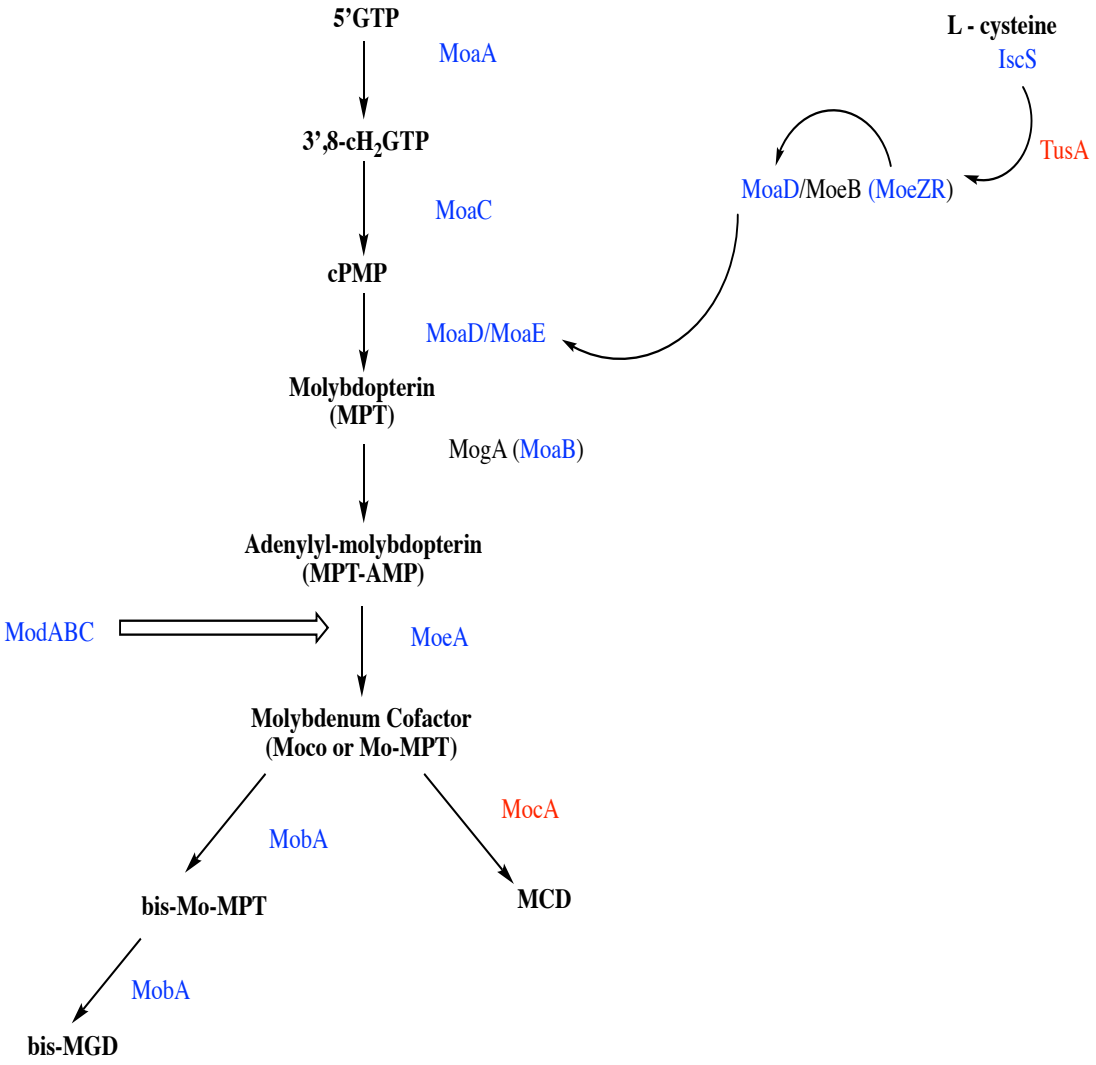

Figure 2.8. Molybdenum cofactor biosynthesis pathway in *R. rhodochrous* ATCC 33258. The blue-colored proteins indicate presence in the genome. Red-colored proteins indicate their absence from the genome. Any proteins in parenthesis are considered homologs of the protein they are beside. The curved arrows on the right of the figure indicate the proteins involved in the sulfur relay system associated with Moco biosynthesis.

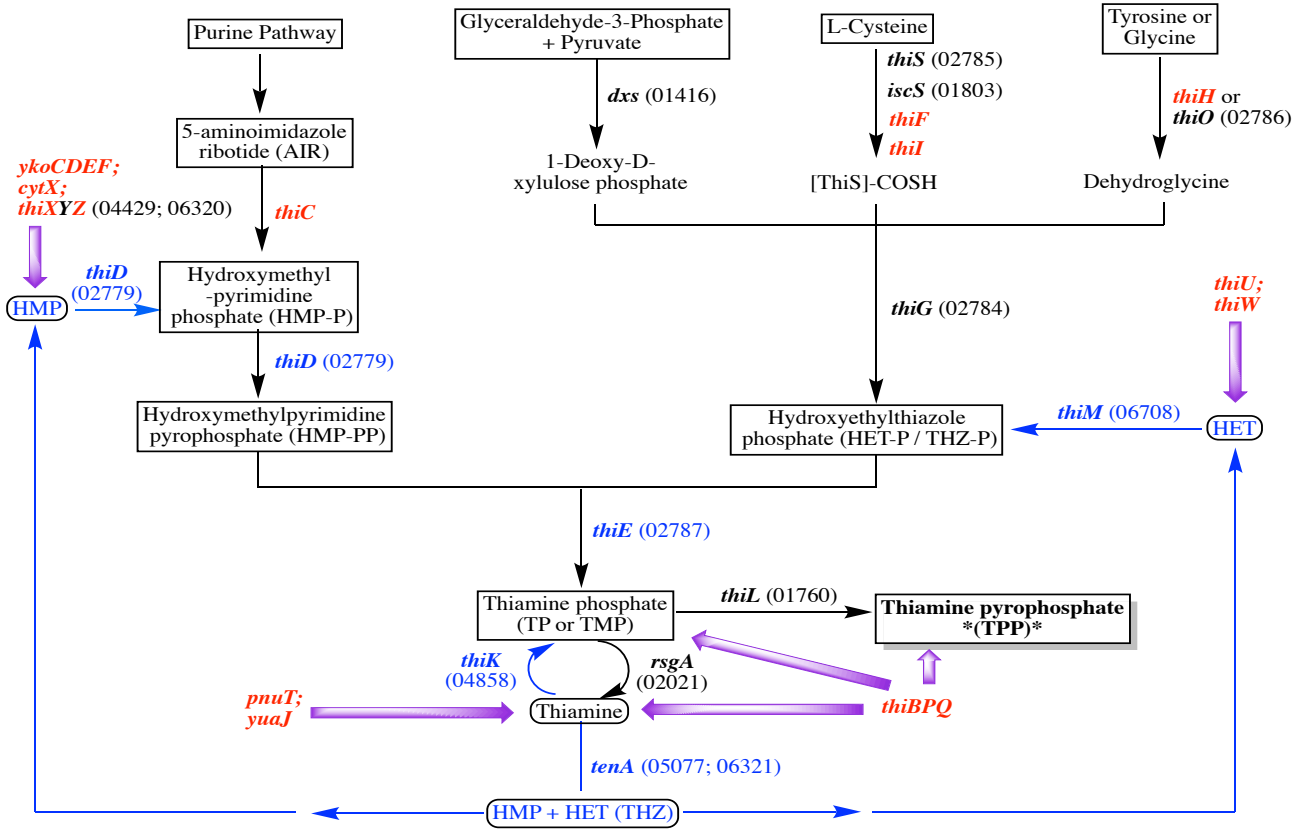

Figure 2.9. Thiamine biosynthesis pathway in *R. rhodochrous* ATCC 33258. The number in parentheses is the locus tag ID (minus the prefix 'BHAHFLAH') that correlates with the protein-encoding gene found within the genome. Pathway genes not identified in the genome are colored red. Blue-colored genes and arrows represent the thiamine salvage portions of the pathway. Putative transporters of exogenous thiamine, TMP, TPP, precursor HMP, and precursor HET are indicated by purple arrows.

CHAPTER 2: Tables

Table 2.1 Growth of *R. rhodochrous* ATCC 33258 on alcohols.

Substrate type	Substrate	OD600 (96hr)	Max OD600	Time (hr) of Max OD
Primary Alcohols	methanol	0.008		
	ethanol	0.776	0.948	48
	1-propanol	1.272	1.45	72
	n-butanol	1.918	1.918	96
	isobutanol	0.212	1.242*	168
Alkyne	acetylene	0.996	1.186	48

Table 2.2 Genome sequence information for *R. rhodochrous* ATCC 33258.

Genome Characteristics	Genome Size (Mbp)	7.40
	No. of contigs	4
	Avg. GC content (%)	62.34
	Total genes	7,058
	CDS	6,984
	No. rRNAs	15
	No. tRNAs	58
	tmRNA	1
BUSCO analysis	Complete & single-copy	97.7%
	Complete & duplicated	2.0%
	Fragmented	0.0%
	Missing	0.3%
	Total no. of groups searched	743

The genome characteristics detailed above are from the Prokka annotation of the *R. rhodochrous* ATCC 33258 genome. The BUSCO analysis was run using the dataset lineage corynebacteriales_odb10.

Table 2.3. Query sequence selections of previously annotated acetylene hydratases (AH) from other bacteria.

Query Order	Source Organism	Definition	Accession	No. AA	Locus_tag
1	*Pelobacter acetylenicus*	Acetylene hydratase	UniProtKB: Q71EW5.1	730	AHY_PELAE
2	*Pelobacter acetylenicus* (strain SFB93)	Putative acetylene hydratase, partial	GenBank: AFP93583.1	94	AFP93583
3	*Gordonia insulae* (strain MMS17-SY073)	Acetylene hydratase	GenBank: AZG47266.1	751	DF316_03874
4	*Rhodococcus* sp. (strain T7)	Acetylene hydratase	GenBank: KAF0966232.1	709	MLGJGCBP_00622
5	*Mycobacterium* sp. (strain THAF192)	Acetylene hydratase (plasmid)	GenBank: QFS94723.1	715	FIV07_28510

The first query sequence selection (UniProtKB: Q7EW5.1)from *P. acetylenicus* is the only well-characterized acetylene hydratase (AH) sequence with known C_2H_2 – fermenting functionality. The rest of the sequences were still selected for comparison as they were annotated as acetylene hydratase (AH); however, their functionality in C_2H_2 consumption within their sourced organism is currently unknown.

Table 2.4 Summary of *R. rhodochrous* ATCC 33258 sequences with significant alignments to selected AH query sequences from Table 2.3.

Locus ID	Prokka Annotation	Order in Hit	E-Value Range	Range % AA Sequence Identity
BHAHFLAH_00032	Acetylene hydratase	1,1,1,2,1 [1]	$2.0E^{-28}$ → 0.0	23.99 → 54.26
BHAHFLAH_02279	Periplasmic nitrate reductase	2,3,2,1,2 [2]	$7.0E^{-4}$ → $2.0E^{-41}$	23.15 → 38.10
BHAHFLAH_01403	DMSO/TMO reductase	3,4,3,3,3 [3]	$5.0E^{-3}$ → $3.0E^{-20}$	22.30 → 26.25
BHAHFLAH_06541	Putative oxidoreductase	4,2,4,4,4 [4]	$4.0E^{-4}$ $1.0E^{-12}$	21.14 → 24.46
BHAHFLAH_01529	NADH-quinone oxidoreductase subunit G	-,-,5,5,- [5]**	$1.6E^{-2}$ $1.8E^{-2}$	0 → 29.11

** Locus ID BHAHFLAH_01529 was only included in the BlastP alignment results for two out of the five selected AH query sequences (GenBank: AZG47266.1 [*Gordonia insulae* (strain MMS17-SY073)] & GenBank: KAF0966232.1 [*Rhodococcus* sp. (strain T7)]

Table 2.5. Relevant standard protein BLAST (BlastP) results from *R. rhodochrous* ATCC 33258 BHAHFLAH_00032 (AA sequence).

Description	Total Score	Query Cover	E - value	Percent Identity	Acc. Length	Accession
Molybdopterin-dependent oxidoreductase [*Rhodococcus opacus*]	1564	98%	0.0	94.40%	786	WP_120661552.1
Acetylene hydratase [*Rhodobiaceae* bacterium]	596	91%	0.0	42.31%	762	AWZ01500.1
Acetylene hydratase [*Halioglobus japonicus*]	587	92%	0.0	41.32%	760	CAA0121193.1

Table 2.6A. *R. rhodochrous* ATCC 33258 gene sequences of interest encoding putative [Fe-S] cluster binding proteins.

Locus ID	No. AA	Prokka Annotation	Pfam Profile Match	E-value	Predicted Ligands
BHAHFLAH_00032	795	Acetylene hydratase	Molybdopterin oxidoreductase Fe4-S4 domain (PF PF04879)	$2.80E^{-12}$	C17-C20-C24-C52
BHAHFLAH_01529	804	NADH-quinone oxidoreductase subunit G	Ferredoxin 2Fe-2S iron-sulfur cluster binding domain (PF15310)	$3.60E^{-23}$	C50-C61-C64-C78
			NADH-ubiquinone oxidoreductase-G iron-sulfur binding region (PF10588)	$2.90E^{-19}$	H112-C116-C119-C125
			Molybdopterin oxidoreductase Fe4-S4 domain (PF04879)	$3.20E^{-6}$	C241-C244-C248-C276
BHAHFLAH_02279	1394	Periplasmic nitrate reductase	Molybdopterin oxidoreductase Fe4-S4 domain (PF04879)	$6.10E^{-14}$	C14-C17-C21-C55

Table 2.6B. All significant Pfam domains identified in BHAHFLAH_00032 of *R. rhodochrous* ATCC 33258.

Pfam Domain	Start	End	E-value
Molybdopterin oxidoreductase	67	509	$1.2E^{-58}$
Molybdopterin dinucleotide binding domain	614	735	$7.5E^{-15}$
Molybdopterin oxidoreductase [4Fe-4S] domain	10	64	$5.3E^{-10}$

Table 2.7. Molybdenum cofactor biosynthesis genes present in *R. rhodochrous* ATCC 33258 genome.

Protein	KO	Locus_IDs
MoaA	K03639	BHAHFLAH_00016; BHAHFLAH_01842
MoaB**	K03638	BHAHFLAH_00013
MoaC	K03637	BHAHFLAH_00026; BHAHFLAH_01844
MoaD	K03636	BHAHFLAH_00015; BHAHFLAH_01841
MoaE	K03635	BHAHFLAH_00017; BHAHFLAH_01845
MobA	K03752	BHAHFLAH_00025
MoeA	K03750	BHAHFLAH_00014; BHAHFLAH_01843; BHAHFLAH_1986; BHAHFLAH_05912
MoeZR**	K21147	BHAHFLAH_00029
IscS	K04487	BHAHFLAH_01803

** MoaB protein in *Pyrococcus furiosus* is responsible for the MPT adenylation step of Moco biosynthesis. This is normally carried out by the MogA protein in *E. coli* (Bevers *et al*., 2008).
**MoeZR protein is homologous to MoeB and its activity in Moco biosynthesis has been shown in *Mycobacterium*. (Voss, Nimtz, & Leimkühler, 2011)

Table 2.8. Molybdate uptake genes in the *R. rhodochrous* ATCC 33258 genome.

Locus ID	Strand	No. AA	Prokka Annotation	GhostKOALA	BLASTp	E-value	% ID BlastP
BHAHFLAH_05637	+	268	Molybdate-binding protein ModA	modA (K02020)	Molybdate ABC transporter substrate-binding protein [Rhodococcus]	0.0	99.25%
BHAHFLAH_05638	+	269	Hypothetical protein	modB (K02018)	Multispecies: ABC transporter permease [Rhodococcus]	$2E^{-180}$	100%
BHAHFLAH_05639	+	361	Molybdate import ATP-binding protein ModC	modC (K02017)	Multispecies: ATP-binding cassette domain-containing protein [Rhodococcus]	0.0	100%

CHAPTER 2: References

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