Iron-Sulfur Cluster Assembly; In vivo Analysis of the Methanogenic SUF System

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IRON-SULFUR CLUSTER ASSEMBLY; IN VIVO ANALYSIS OF THE METHANOGENIC SUF SYSTEM

A Thesis

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Master of Science

in

The Department of Biological Sciences

by

Evan Dunkle
B.S., Temple University, January 2011
December 2019
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<tbody>
<tr>
<td>Fe-S</td>
<td>Iron-Sulfur</td>
</tr>
<tr>
<td>HdrABC-MvhAGD</td>
<td>Heterodisulfide reductase – [NiFe]-Hydrogenase Complex</td>
</tr>
<tr>
<td>FBEB</td>
<td>Flavin-based Electron Bifurcation</td>
</tr>
<tr>
<td>Fmd</td>
<td>Formyl-methanofuran Dehydrogenase</td>
</tr>
<tr>
<td>MFR</td>
<td>Methanofuran</td>
</tr>
<tr>
<td>CODH/ACS</td>
<td>Carbon monoxide decarbonylase/acetyl-CoA synthase complex</td>
</tr>
<tr>
<td>Fe$^{2+}$</td>
<td>Ferrous Iron</td>
</tr>
<tr>
<td>Fe$^{3+}$</td>
<td>Ferric Iron</td>
</tr>
<tr>
<td>S$^{2-}$</td>
<td>Sulfide</td>
</tr>
<tr>
<td>ISC</td>
<td>Iron-Sulfur Cluster Fe-S Assembly System</td>
</tr>
<tr>
<td>SUF</td>
<td>Sulfur Mobilization Fe-S Assembly System</td>
</tr>
<tr>
<td>NIF</td>
<td>Nitrogen Fixation Fe-S Assembly System</td>
</tr>
<tr>
<td>CIA</td>
<td>Cytosolic Fe-S Assembly System</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>Mmp</td>
<td>Methanococcus maripaludis</td>
</tr>
<tr>
<td>Mth</td>
<td>Methanothermococcus thermolithotrophicus</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>IMAC</td>
<td>Immobilized Metal Affinity Chromatography</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>Nickel-Nitrilotriacetic Acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>EPR</td>
<td>Electron Paramagnetic Resonance</td>
</tr>
<tr>
<td>DTH</td>
<td>Sodium Dithionite</td>
</tr>
<tr>
<td>PMS</td>
<td>Phenazine Methosulfate</td>
</tr>
<tr>
<td>6-PGDH</td>
<td>6-phosphogluconate Dehydratase</td>
</tr>
<tr>
<td>NT</td>
<td>Nucleotides</td>
</tr>
<tr>
<td>ampR</td>
<td>Ampicillin Resistance Gene</td>
</tr>
<tr>
<td>kanR</td>
<td>Kanamycin Resistance Gene</td>
</tr>
<tr>
<td>cmR</td>
<td>Chloramphenicol Resistance Gene</td>
</tr>
<tr>
<td>smR</td>
<td>Streptomycin Resistance Gene</td>
</tr>
<tr>
<td>FRT</td>
<td>FLP Recognition Target Sites</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
</tbody>
</table>
ABSTRACT

Iron-sulfur (Fe-S) clusters are among the most ancient and prevalent of all biological cofactors. Their assembly into associated proteins is a tightly regulated process with many organisms employing multiple cluster assembly pathways. Much is known about Fe-S cluster assembly in aerobic organisms such as *Escherichia coli* (*E. coli*), but little is known in regards to cluster assembly in more ancient organisms such as methanogens. Methanogens are members of the domain of Archaea and are defined by their ability to generate methane as a byproduct of their main energy generating pathway. Methanogens also have significantly higher Fe-S cluster content compared to many aerobic based organisms. In methanogens there is one conserved Fe-S cluster assembly system, the SUF (sulfur mobilization) system. Within the *suf* operon there are two genes, *sufB* and *sufC*, that are conserved across all methanogens. Biochemical analysis revealed that the core SUF system in methanogens is made up of a cluster assembly scaffold with the structure of SufB₂C₂ and it appears that the Fe-S cluster assembles on the SufC dimer. *In vitro* analysis revealed that within this complex three conserved cysteine residues within the SufC protein are essential to Fe-S cluster assembly on the scaffold. To evaluate the functionality of the methanogenic SUF system *in vivo* *E. coli* was utilized as a host system. It was found that in addition to the three critical cysteine residues on SufC there were two residues on SufB, a cysteine and histidine residue that were also essential to the scaffold’s functionality *in vivo*. 
CHAPTER 1. INTRODUCTION

1.1. Methanogens

Methanogens are a collection of unique single celled organisms found within the domain of Archaea. All methanogens are members of the phylum *Euryarchaota* and within *Euryarchaota* they are subdivided into four classes, seven different orders and fourteen families.¹-³

Methanogens are defined by their ability to generate methane as a byproduct of their main energy generating biochemical pathway. Methanogens are obligate methane producers, and generate energy through the conversion of specific carbon based substrates to a final product of methane.²,³ A major component of this conversion process is the establishment of a proton gradient on the extracellular side of the plasma membrane.⁴-⁶ This proton gradient is used to generate ATP through the activities of distinct ATPase machinery that involves the movement of protons down the established gradient.⁴-⁶ Currently the substrates for methanogenesis can be divided into three groups (table 1.1). Group one is comprised of most methanogens and in this group the initial electron acceptor in the methanogenesis pathway is CO₂ and the electron donors can be either hydrogen gas, formate, or a few other alcohols.²,³ In the second group the electron donor can be one of a variety of methyl-containing C-1 compounds. Some examples are methanol, methylated amines and methylated sulfides.²,³ Methanogens comprising the second group are found within the orders Methanosarcinales and Methanobacteriales.² In the third group the primary substrate for methanogenesis is acetate. Organisms within this group are able to catalytically remove the methyl group from acetate.²,³ There are currently only two know genera, *Methanosarcina* and
Methanosaeta, that contain methanogens that can perform group three methanogenesis.

Table 1.1. Methanogenesis reaction types and the standard changes in free energy.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>ΔG° (kJ/mol of CH₄)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Type I - CO₂</strong></td>
<td></td>
</tr>
<tr>
<td>4 H₂ + CO₂ → CH₄ + 2 H₂O</td>
<td>-135</td>
</tr>
<tr>
<td>4 HCOOH → CH₄ + 3 CO₂ + 2 H₂O</td>
<td>-130</td>
</tr>
<tr>
<td>CO₂ + 4 isopropanol → CH₄ + 4 acetone + 2 H₂O</td>
<td>-37</td>
</tr>
<tr>
<td>4 CO + 2H₂O → CH₄ + 3 CO₂</td>
<td>-196</td>
</tr>
<tr>
<td><strong>Type II - Methylated C-1 Compounds</strong></td>
<td></td>
</tr>
<tr>
<td>4 CH₃OH → 3 CH₄ + CO₂ + 2 H₂O</td>
<td>-105</td>
</tr>
<tr>
<td>CH₃OH + H₂ → CH₄ + H₂O</td>
<td>-113</td>
</tr>
<tr>
<td>2 (CH₃)₂S + 2 H₂O → 3 CH₄ + CO₂ + 2 H₂S</td>
<td>-49</td>
</tr>
<tr>
<td>4 CH₃-NH₂ + 2 H₂O → 3 CH₄ + CO₂ + 4 NH₃</td>
<td>-75</td>
</tr>
<tr>
<td>2 (CH₃)₂-NH + 2 H₂O → 3 CH₄ + CO₂ + 2 NH₃</td>
<td>-73</td>
</tr>
<tr>
<td>4 (CH₃)₃-N + 6 H₂O → 9 CH₄ + 3 CO₂ + 4 NH₃</td>
<td>-74</td>
</tr>
<tr>
<td>4 CH₃NH₃Cl + 2 H₂O → 3 CH₄ + CO₂ + 4 NH₄Cl</td>
<td>-74</td>
</tr>
<tr>
<td><strong>Type III - Acetate</strong></td>
<td></td>
</tr>
<tr>
<td>CH₃COOH → CH₄ + CO₂</td>
<td>-33</td>
</tr>
</tbody>
</table>

Methanogens are strictly anaerobic organisms that are found primarily in marine and soil sediments. Some species can also be found in the gastrointestinal tracts of certain animals.²³ A wide temperature and pH gradient characterizes the habitats of methanogens with environments ranging from psychrophilic to hyperthermophilic and from acidic to alkaline.² In many of the methanogen’s habitats a complex syntrophic relationship exists between the methanogen and various anaerobic bacteria (fig. 1.1).⁷ This relationship helps provide substrates for methanogenesis while simultaneously
reducing hydrogen concentrations in the environment. Many anaerobic bacteria in these environments are unable to generate adequate energy for their survival when hydrogen concentrations are above a certain threshold.

Methanogens have been implicated in a variety of different industrial applications. Current applications involve waste water treatment and biogas (methane) production from the digestion of organic waste. Both of these current applications involve a syntrophic dynamic with anaerobic bacteria. The bacteria degrade the biopolymers in the organic waste or sewage and through their fermentative activities provide methanogens with the substrates necessary for methane production.

Figure 1.1. Conversion of organic matter to methane through anaerobic food web involving methanogens and anaerobic bacteria.
Recent advances have also implicated methanogens as potential sources for the possible industrial production of certain compounds. For example a group of researchers have shown the ability to manipulate one species of methanogen to produce the compound geraniol, a major component in rose water, in addition to methane.\(^9\) Other researchers have proven the ability to engineer methanogens to produce acetate as well as certain amino acids and vitamins.\(^8,10\) Another key potential use of methanogens is in carbon capture and conversion. Methanogens with their ability to utilize CO\(_2\) for the production of methane are potentially useful as a means of capturing CO\(_2\) emissions from power plants and other high emission industrial facilities.\(^11\) The methane generated from the captured CO\(_2\) could be collected and used to generate electricity or heat creating a form of carbon neutral energy production.

In addition to industrial applications methanogens are also socially relevant due to their contribution to global warming. Methane’s warming potential is approximately twenty five fold greater than that of CO\(_2\).\(^12\) It has also been estimated that methane has contributed to approximately 20% of the earths warming post industrial revolution.\(^13\) Methanogens are the source for the majority all methane emissions.\(^2,13\) Roughly 60% of methane emissions come from human activities such as landfills, the raising of livestock and the cultivation of rice fields.\(^14\) The other 40% of methane emissions are thought to come from natural sources such as wetlands and other marsh environments.\(^14\) As the earth continues to warm it is estimated that the methane emissions from natural sources is expected to increase significantly making the contribution of methanogens to global warming increasingly significant.\(^15\) This makes understanding the ecology of methanogens essential to any attempt to predict or simulate global warming trends.
1.2. Fe-S Clusters

Of all the different types of co-factors present across all biological systems, iron-sulfur (Fe-S) clusters are among the most ancient and are pervasive across a vast range of organisms. Fe-S clusters are unique in that their structure permits the delocalization of electrons across the cluster’s iron and sulfur atoms.\textsuperscript{16,17} This property makes Fe-S clusters well suited for biochemical processes that involve the transfer of electrons. Fe-S clusters are also well suited for certain types of catalysis and for regulating certain cellular processes.\textsuperscript{16,17} Fe-S clusters are essential components to electron transport chains associated with respiration and photosynthesis.\textsuperscript{16,17} They are also involved in a wide range of biochemical processes such as biosynthesis, iron and sulfur storage, transcriptional Regulation and DNA repair.\textsuperscript{16,17} In methanogens they are essential to the methanogenesis pathway, making them critical to methanogen’s ability to produce energy. There are multiple structural forms Fe-S clusters can take. From the simplest structures of [2Fe-2S] and [4Fe-4S] to the most complex structure of [8Fe-8S].\textsuperscript{16} The [2Fe-2S] and [4Fe-4S] clusters are the most common and the more complex cluster types are much rarer and exist in a few types of enzymes such as certain hydrogenases and nitrogenases.\textsuperscript{16} In these enzyme complexes clusters are often associated with other metal atoms. Coordination of Fe-S clusters into proteins is usually accomplished through cluster association with specific cysteine or histidine residues within the protein (fig. 1.2).\textsuperscript{16}
Methanogens have considerably higher Fe-S content when compared to many aerobic based organisms such as *E. coli*. For example genome sequence analysis of *Methanococcus maripaludis*, a model methanogens, reveals that there are a total of 114 different Fe-S proteins present.\(^{18}\) That makes up about 6.6% of the total protein content in *M. maripaludis*.\(^{18}\) That is estimated to be more than double that of *E. coli* which is estimated to have approximately 3% of its protein types associated with Fe-S clusters.\(^{18}\) Also direct measurements of Fe-S cluster content in *M. marpaludis* was found to be approximately twenty times higher than that in aerobically grown *E. coli*.\(^{19}\)

Examples of Fe-S clusters in methanogens can be found in many of the proteins involved in the methanogenesis pathway. Methanogenesis in many group one hydrogenotrophic methanogens is a seven step process with methane generation occurring at the final step (fig. 1.3).\(^{2,5}\) Proton translocation that establishes an extracellular gradient occurs concomitantly with the sixth step of the process.\(^{20}\) The final step in the process involves the enzyme methyl-coenzyme M reductase. This enzyme catalyzes the reaction between coenzyme M and B. The products of the reaction are methane and the heterodisulfide CoM-S-S-CoB. Reducing the disulfide bond between these two coenzymes is essential for their continued use in the methanogenesis pathway.

---

**Figure 1.2.** Three dimensional representations of the most common Fe-S clusters structures and their coordinating amino acid residues

\[ \text{[2Fe-2S]} \quad \text{[3Fe-4S]} \quad \text{[4Fe-4S]} \]
The enzyme complex that catalyzes the reduction of heterodisulfide CoM-S-S-CoB is termed the heterodisulfide reductase –[NiFe]-hydrogenase complex (HdrABC-MvhAGD). In addition to reducing the disulfide bond between coenzyme M and B the HdrABC-MvhAGD complex also reduces ferredoxins that are then used in the first step of methanogenesis, the reduction and fixation of CO₂. Electrons used for the complex’s reductive activities are derived from hydrogen through the MvhAGD hydrogenase portion of the complex. The HdrABC-MvhAGD complex is of particular significance to methanogens because it is critical to both initiating methanogenesis and recycling key coenzymes involved in the process. Electrons transfer through the HdrABC portion of the complex involves a flavin-based electron bifurcation (FBE) pathway. In this system the exergonic reduction of CoM-S-S-CoB is used to provide the means to overcome the energy barrier for the endergonic reduction of ferredoxins. Central to this bifurcation system are Fe-S clusters. The HdrABC-MvhAGD complex in total contains 28 Fe-S clusters, making Fe-S clusters critical to the function of the HdrABC-MvhAGD complex and to methanogenesis. Another Fe-S cluster rich enzyme complex involved in methanogenesis is the formyl-methanofuran dehydrogenase (Fmd) complex. This complex catalyzes the first step of methanogenesis by reducing CO₂ to create formyl-methanofuran (formyl-MFR). The Fmd complex utilizes reduced ferredoxins generated by the Hdr complex as sources of electrons for the reduction of CO₂. The transfer of electrons through the Fmd complex is mediated by Fe-S clusters and there are a total of 46 [4Fe-4S] clusters within the complex.
Figure 1.3. ATP production and the seven steps of hydrogenotrophic methanogenesis in *M. marpaludis*. HdrABC-MvhAGD complex and the formyl-methanofuran dehydrogenase (Fmd) complex are also highlighted to depict components of the process that contain Fe-S clusters.
In addition to being heavily involved in methanogenesis Fe-S clusters are also prevalent in other biochemical pathways within methanogens. The carbon monoxide dehydrogenase/acetyl-CoA synthase complex (CODH/ACS) complex catalyzes the synthesis of acetyl-CoA through the reduction of CO$_2$ and the transfer of a methyl group from the methanogenesis intermediate methyl-Tetrahydromethanopterin (methyl-H$_4$MPT). The catalytic mechanism of both the CODH and the ACS enzyme component of the complex involves a Fe-S cluster associated with a nickel atom. Also the electrons used to reduce CO$_2$ are derived from Fe-S cluster containing reduced ferredoxins. Acetyl-CoA is both a carbon source and an energy source making its availability critical to the well-being of methanogens. Within methanogens Fe-S clusters are also essential to translation. The generation of certain versions of tRNA involves thiolation reactions that incorporate sulfur into the tRNA structure. The enzymes that carry out these thiolation reactions contain a [3Fe-4S] Fe-S cluster that is critical to the enzymes’ function.

1.3. Fe-S Cluster Assembly

Fe-S cluster assembly is a critical process within cells that requires tight regulation and control to ensure successful cluster inclusion into target apo-proteins. Unincorporated Fe-S clusters are labile so cells must assemble and incorporate them into proteins de novo. Fe-S clusters are extremely sensitive to molecular oxygen and when oxidized degrade rapidly. Therefore, protection from oxidative encounters is a critical task that cellular assembly systems must perform. Also most cluster assembly systems need an active mechanism to encourage cluster assembly. The levels of
ferrous iron and sulfide required for clusters to form spontaneously is above all cellular toxicity thresholds.\textsuperscript{19,27} To overcome this dilemma cells need a means to actively encourage Fe-S cluster formation utilizing iron and sulfur sources that are at concentrations bellow cellular toxicity thresholds. To do this cells have developed assembly systems that in addition to incorporating clusters into target apo-proteins, can use cysteine as the sulfur source and an Fe-binding protein as the Fe donor. This gives cluster assembly systems a dual significance to cellular viability in that they are able to provide a critical cofactor essential to many cellular processes and are also able to minimize cellular exposure to potentially toxic ferrous iron and sulfide. Cluster assembly systems as a result need to be exceptionally efficient and well-regulated as assembly system dysfunction can be lethal.

The early earth environment was characterized by significantly lower levels of oxygen and significantly higher levels of ferrous iron (Fe\textsuperscript{2+}) and sulfide (S\textsuperscript{2−}) as compared to today.\textsuperscript{28} The establishment of oxygenic photosynthetic organisms led to an increase in oxygen levels in the earth’s atmosphere. Increasing oxygen levels led to increasing oxidation of ferrous iron.\textsuperscript{28} Ferrous iron (Fe\textsuperscript{2+}) when oxidized becomes ferric iron (Fe\textsuperscript{3+}) and \textit{in vivo} ferric iron will rapidly precipitate as toxic ferric hydroxide.\textsuperscript{27} Anaerobic organisms that initially evolved in an early earth environment were forced to develop mechanisms to deal with ubiquitous iron and sulfide and therefore are not as sensitive to iron and sulfide as more recently evolved organisms.\textsuperscript{28} For example, they lack many targets (cytochromes and unsaturated fatty acids) for sulfide toxicity. In addition to the anaerobic nature of the early earth environment made oxidative stress less of a factor. Early earth anaerobes that utilized Fe-S clusters did not have to
account for oxidative damage and limitations regarding iron and sulfur availability. As a result cluster assembly systems that developed in early earth conditions are more simplistic than assembly systems developed in aerobic conditions where oxidation events are more likely and iron and sulfur availability is more limited.²⁸

Changes in the atmospheric composition led to changes in the Fe-S cluster assembly process. Organisms that evolved in aerobic environments needed mechanisms to protect clusters from oxidation as well as to efficiently utilize the increasingly limited availability of ferrous iron.²⁸ Ultimately changes in the earth’s atmosphere led to the necessity for increasing levels of sophistication in regards to Fe-S cluster assembly. Initial cluster assembly pathways have incurred additional components in more recently evolved aerobic organisms.²⁸ Many aerobic organisms have also incorporated multiple cluster assembly systems. The basis of all systems seems to follow the same general assembly pathway.²⁹ In each assembly system there is a distinct sulfur donor, usually cysteine. The iron source in all systems is still unclear. There is a scaffold component where the cluster is actually assembled and there is a cluster transfer element that moves the assembled cluster from the assembly scaffold to the target holo-protein (fig. 1.4).

![Figure 1.4. General Fe-S cluster assembly pathway common among the various assembly systems.](image)
In prokaryotes there are two main Fe-S cluster assembly systems. The ISC (Iron-Sulfur Cluster) system and the SUF (sulfur mobilization) system.\textsuperscript{30,31} There is also a third system that is termed the NIF (nitrogen fixation) system and is only found in nitrogen fixing bacteria.\textsuperscript{29} The NIF system is only involved in the maturation of different nitrogenases found in these bacteriums.\textsuperscript{29} The mechanism of assembly is very similar in both the ISC and SUF system.\textsuperscript{29-30} Both have a cysteine desulfurase component that extracts sulfur from cysteine. Both have a scaffold component that assembles and releases the Fe-S cluster through ATP hydrolysis associated activity. They both also have distinct cluster carrier proteins. The ISC system has become known as the cellular housekeeping cluster assembly system.\textsuperscript{29} The ISC system has been found to be highly sensitive to reactive oxygen species (ROS) exposure while the SUF system is more resistant to ROS exposure and iron/sulfur limiting conditions.\textsuperscript{30} As a result the SUF system is primarily utilized under conditions of oxidative stress or iron starvation.\textsuperscript{30}

In eukaryotes there are currently three known cluster assembly systems. Eukaryotes have Fe-S clusters present in the cytosol, nucleus and in the mitochondria.\textsuperscript{26} The mitochondria assembly system is also referred to as the ISC system. This system is thought to be derived from a bacterial system that served as the evolutionary ancestor to the mitochondria.\textsuperscript{26} The SUF system is most often found in the plastids of plants and was probably also derived from a bacterial system found in photosynthetic bacteria that likely served as the initial source of this plant organelle.\textsuperscript{26} Eukaryotes also contain a cytosolic assembly system termed the cytosolic Fe-S protein assembly (CIA) system. The substrates for the CIA system are the products of the mitochondrial ISC system and these products must be transported out of the cell's
mitochondria before being utilized by the CIA system. The CIA system performs activities that further mature Fe-S cluster containing proteins so that they can be utilized in the cytosol or within the nucleus of cells.\textsuperscript{26}
CHAPTER 2. SUF SYSTEM MECHANICS IN METHANOGENS

2.1. *E. coli* SUF System

Based upon genome sequencing data all methanogens contain components of the SUF assembly system. Within the methanogenic SUF system two proteins, SufB and SufC, are conserved. These finding suggest that the SUF Fe-S cluster assembly system may have been the initial cluster assembly system to develop.\(^3^2\) The conserved nature of the SufB and SufC proteins across all methanogens suggest that these two proteins together make up the core component of the SUF Fe-S cluster assembly system within these organisms.\(^2^8,3^2\) To gain further insight into the potential mechanism by which a methanogen SufB and SufC assembly system works it is necessary to evaluate previous research into the SUF system of other model organisms such as *E. coli*.

*E. coli* has two distinct Fe-S cluster assembly systems, an ISC system and a SUF system.\(^3^3\) The ISC system serves as the general housekeeping assembly system while the SUF system serves as the assembly system under conditions of stress.\(^3^4\) The *E. coli* SUF system has increased complexity, with four more protein components, than that of genome sequenced methanogens. *E. coli*’s *suf* operon is composed of *sufA*, *sufB*, *sufC*, *sufD*, *sufS* and *sufE* genes.\(^2^9,3^1\) The enhanced complexity of the *E. coli* SUF system is in accordance with the previously described phenomena that more recently evolved aerobic based organisms that have to manage ROS exposure and the limited availability of ferrous iron will have more sophisticated Fe-S cluster assembly systems (fig. 2.1).
The SUF system mechanism of action has been well researched in *E. coli*. In the *E. coli* SUF system the cluster assembly scaffold is made up of the SufB, SufC and SufD protein components. The overall structure is composed of two SufC components, one SufB component and one SufD component (*SufBC2D*). Deletion of any of the scaffold components eliminates the SUF system’s *in vivo* functionality. SufB and SufD are homologs of each other. Sequence analysis reveals that there is a 17% identity and 37% similarity between the two homologs. Both homologs have a structural motif comprised of an N-terminal helical domain, a core domain consisting of a parallel β-helix, and a C-terminal helical domain. Most of the sequence homology between these two proteins occurs at the c-terminal region. SufB is known to accept sulfur and SufD is thought to be involved in iron acquisition. The *sufD* operon seems to be predominately found in aerobic based organism while most anaerobic organism...
lack the *sulD* operon. This suggest that the inclusion of the SufD protein in the SUF system scaffold complex provides a valuable advantage to aerobic based organisms. SufD may provide enhanced protection to oxidation or it may be more efficient at acquiring iron than its SufB homolog. The sulfur acquisition component of the system is made up of SufS and SufE proteins. The SufS component has been shown to function as a cysteine desulfurase and extracts sulfur form L-cysteine. SufE protein has been shown to transfer sulfur from the SufS protein to SufB protein. The iron acquisition system is still poorly understood. SufC has been shown to have ATPase activity and its sequence shows that it is a member of the ABC ATPase superfamily. SufC ATPase activity has been shown to induce conformational changes in SufB and SufD. Within the scaffold complex the two SufC subunits form a dimer upon binding two molecules of ATP and a conformational change is induced in the SufB and SufD subunits when the SufC dimer is formed. It has also been shown that the conformational change induced in SufB and SufD promotes the binding of an Fe-S cluster between the two B and D subunits. Conformational changes in SufB and SufD leads to the necessary positioning of three key amino acid residues thought to be the sites of coordination between the assembled Fe-S cluster and the SUF scaffold complex. Prior to ATP binding on the SufC dimer the coordinating amino acid residues are buried within the interior of the scaffold complex. Upon ATP binding and the subsequent conformational changes these residues are positioned on the outer surface of the scaffold complex. On SufB there are two key cluster coordinating residues, cysteine (C405) and glutamic acid (E434). On SufD the key coordinating residue is thought to be histidine (H360). Once an Fe-S cluster assembles on the scaffold complex it is able to be transferred to
the SufA protein.\textsuperscript{40} SufA acts as an Fe-S cluster transport mechanism and functions to transfer an assembled cluster from the scaffold complex to target apo-proteins (fig 2.2).\textsuperscript{32,40}

\textbf{Figure 2.2.} Assembly and transfer of a Fe-S cluster on the \textit{E. coli} SufBCD scaffold complex
2.2. Methanogen SUF System

The mechanism by which the SUF system functions in methanogens containing the core *suf* operon components of *sufB* and *sufC* has received far less research attention than the SUF system in *E. coli*. It is purported that in a core SUF system, SufB and SufC come together to form the cluster assembly scaffold complex.\(^{32}\) The overall complex in such a system has been purported to be comprised of both a SufB dimer and a SufC dimer (SufB\(_2\)C\(_2\)).\(^{32}\) Evidence in regards to the nature of the scaffold complex’s makeup has been elucidated through several experimental assays. Data and associated figures displayed in section 2.2 were provided by fellow lab member Cuiping Zhao. Two species of methanogens served as the model systems for all experiments carried out in relation to methanogen SUF system research. One organism *Methanococcus maripaludis* (*Mmp*) is a model methanogen and has its entire genome sequenced. *M. maripaludis* is a mesophilic organism found predominately in marsh sediments.\(^{41}\) The other organism utilized was *Methanothermococcus thermolithotrophicus* (*Mth*). *M. thermolithotrophicus* is a thermophilic organism and resides within the same family as *M. maripaludis*, the Methanococcaceae family. Members of the Methanococcaceae family are group one methanogens and carry out hydrogenotrophic methanogenesis through a seven step process.\(^{41}\)

*M. thermolithotrophicus* SufB and SufC proteins were used as a proxy for *M. maripaludis* Suf proteins when *in vivo* experimentation was done in *E. coli*. It was found that the *M. maripaludis* Suf proteins were insoluble when expressed in *E. coli* while *M. thermolithotrophicus* Suf proteins were not. Sequence analysis revealed that over 74% of the *suf* operon sequence is identical between the two species.
In a pull-down experiment it has been shown that SufB protein associates with SufC protein \textit{in vivo}. The SUF system proteins were purified from \textit{Mth} and SufB and SufC proteins were expressed with and without associated Histidine (His) tags. Immobilized metal affinity chromatography (IMAC) utilizing nickel-nitrilotriacetic acid (Ni-NTA) resin was utilized for purification procedures. Ni-NTA resin has specific affinity for histidine residues. Results indicated that when His\textsubscript{6}-tagged SufC was co-expressed with non-tagged SufB it was found that when purified both proteins eluted within the same elution fraction. Likewise when His\textsubscript{6}-tagged SufB was co-expressed with non-tagged SufC both proteins eluted in the same fraction. These results indicate that SufC and SufB proteins associate with one another to form a broader protein complex (fig. 2.3).

![Figure 2.3. SDS-PAGE gel stained by Coomassie blue showing results of a pull-down experiment. Figure shows His\textsubscript{6}-tagged SufC co-expressed with SufB without His tag and His\textsubscript{6}-tagged SufB co-expressed with SufC without a His tag. I represents input of crude extracts; E represents eluant from the nickel chromatography purification](image)
Size exclusion gel filtration chromatography data indicated that SufB and SufC proteins form a stable complex comprised of SufB$_2$C$_2$. Initial elution data indicated the SufC dimer and SufB dimer have different elution points. SufB’s elution peak indicated a molecular weight of 94 kDa. SufC’s elution peak indicated a molecular weight of 69 kDa. When the SufB and SufC proteins were combined and filtered together a single elution peak was found that indicated a molecular weight of 156 kDa corresponding to the combined weight of both the SufC and SufB dimers. This indicates that the SufB and SufC proteins associate together to form a stable complex (fig. 2.4).
Figure 2.4. Size exclusion gel filtration data and cartoons showing SufB and SufC dimers and full SufB$_2$C$_2$ complex. Images of SDS page run with samples from elutants shows the bands of SufB (upper) and SufC (lower)

UV-Visible Spectroscopy and electron paramagnetic resonance (EPR) analysis were used to determine if the SufB$_2$C$_2$ complex can serve as an Fe-S cluster assembly scaffold. UV-vis data for anoxically purified *Mth* SufB$_2$C$_2$ indicated an absorbance peak at 420nm suggesting the presence of a [4Fe-4S] cluster which are known to absorb light at such a wavelength (fig. 2.5 A). When the purified complex was treated with the known iron reducer sodium dithionite (DTH) at 5mM the absorbance peak at 420nm disappeared (fig. 2.5 A). Also the color of the anoxically purified complex was brownish indicating the presence of iron. Treatment with DTH bleached away the brownish color (fig. 2.5 A). EPR analysis of the as-purified *Mth* SufB$_2$C$_2$ complex displayed a peak centered at g ~ 2.01, characteristic of a cubic [3Fe-4S]$^{1+}$ cluster (Stol = $\frac{1}{2}$). The complex when reduced by 5 mM sodium DTH displayed a signal with characteristic of a [4Fe-4S]$^{1+}$ cluster (fig. 2.5 B). It is possible that the [3Fe-4S] cluster found on the non-DTH treated *Mth* SufB$_2$C$_2$ sample was initially a [4Fe-4S] cluster but degraded to a [3Fe-4S] cluster. When the as-purified *Mth* SufB$_2$C$_2$ sample was exposed to an oxygen based environment the absorbance peak at 420nm gradually diminished over time and eventually disappeared (fig. 2.5 C). This data further suggest that a Fe-S cluster is binding the complex as oxygen exposure will lead to Fe-S cluster degradation.
Figure 2.5. Absorbance and Electron Paramagnetic Resonance (EPR) data for SufB$_2$C$_2$ complex purified from *M. thermolithotrophicus*. Images of the purified and DTH treated SufB$_2$C$_2$ products are also embedded.
Investigation of the SufB$_2$C$_2$ complex in *M. maripaludis* yielded similar results to that in *M. thermolithotrophicus*. Again an absorbance peak was found at 420nm and the peak was absent after DTH treatment (fig. 2.6 A). The EPR spectrum of the as-purified *Mmp* SufB$_2$C$_2$ complex did not indicate the presence of an Fe-S cluster (fig. 2.6 B). After DTH treatment there was however a signal indicating the presence of a [4Fe-4S]$^{1+}$ cluster (fig. 2.6 B).

Figure 2.6. Absorbance and Electron Paramagnetic Resonance (EPR) data for SufB$_2$C$_2$ complex purified from *M. maripaludis*. Images of the purified and DTH treated SufB$_2$C$_2$ products are also embedded.
To determine the critical amino acid residues involved in binding an Fe-S cluster to the SufB$_2$C$_2$ complex suf operon sequence comparisons were made between the suf operon in *M. thermolithotrophicus* and *E. coli*. There were two sites in the *Mth sufB* operon that corresponded to known cluster binding sites in *E. coli*’s *sufB* and *sufD* operon. One site corresponded to *E. coli* *sufB* cysteine C405 and the other site corresponded to *E. coli* *sufD* histidine H360. The corresponding sites in *Mth* were cysteine C318 and histidine H346 (fig. 2.7 A). Two other *Mth sufB* sequence sites for cysteine C145 and C175 were found to be conserved in all methanogens of the Methanococci class (fig. 2.7 A). In the *Mth sufC* operon it was found that there are three cysteine residues in a CX18CXXC motif, C218, C237, and C240 that are conserved.
across all group one methanogens and most other Archaea (fig. 2.7 B). Also of note bacterial sufC homologs do not have conserved cysteine residues.

Figure 2.7. Sequence alignment and comparison between the \textit{M. thermolithotrophicus} sufB and sufC operon with the \textit{E. coli} sufB and sufC operon. To evaluate the role the seven amino acids of interest play in cluster assembly point mutations to serine were made. The mutants were then purified anoxically and cluster binding ability was determined through UV-Visible Spectroscopy and EPR. It was found that mutations to the three conserved cysteine residues in SufC led to loss of Fe-S cluster signal as both UV-vis and EPR data did not reveal a cluster's presence (fig. 2.8 A). Mutations to the four key residues in SufB did not lead to lose of cluster signal as UV-vis data displayed an absorbance peak at 420nm. The peak at 420nm disappeared after DTH treatment (fig. 2.8 B). EPR data displayed a signal indicative of a [3Fe-4S]$^{1+}$ cluster and a [4Fe-4S]$^{1+}$ cluster after DTH treatment (fig. 2.8 C) A).
Figure 2.8. Absorbance and EPR data for the SufB2C2 complex containing amino acid point mutations on SufB and SufC purified from *M. thermolithotrophicus*. Images of the purified and DTH treated SufB2C2 products are also embedded. Further analysis of the *Mth* SufC protein component revealed that it appears to have cluster binding capabilities. Anoxically purified *Mth* SufC was evaluated though UV-vis and EPR analysis. The UV-vis data displayed an absorbance peak at 420nm and this peak was eliminated after DTH treatment (fig. 2.9 A). Purified *Mth* SufC protein had an initial brownish color that was bleached upon DTH exposure (fig. 2.9 A). Three individual point mutants were made for each of the conserved cysteine residues in SufC, cysteine was changed to serine. UV-vis data for each point mutant indicated the absence of an absorbance peak at 420nm and all point mutants displayed clear coloration indicating that the point mutants were no longer able to bind Fe-S clusters (fig. 2.9 B). EPR data for purified SufC indicated a signal indicative of a [3Fe-4S]1+ cluster and a [4Fe-4S]1+ cluster after DTH treatment (fig. 2.9 C).
Figure 2.9. Absorbance and EPR data for SufC and SufC protein containing amino acid point mutations. SufC purified from *M. thermolithotrophicus*. Images of the purified and DTH treated SufC products are also embedded.

**Chapter 3. *IN VIVO M. THERMOLITHOTROPHICUS* SUF SYSTEM COMPLEMENTATION IN *E. COLI***

### 3.1. Introduction

Results from pull down and size exclusion gel filtration chromatography analysis indicated that SufB and SufC proteins isolated from a methanogen host associate together to form a complex with the structure of SufB$_2$C$_2$. UV-Visible Spectroscopy and EPR analysis revealed the presence of a Fe-S cluster within the SufB$_2$C$_2$ complex. Gene sequence comparisons between methanogens and *E. coli* further revealed seven amino acid residues that are potentially critical to the functionality of the SufB$_2$C$_2$ complex. Point mutation experimentation revealed that the key residues on SufC are critical to the ability of the complex to bind a Fe-S cluster. In order to evaluate the *in vivo* functionality of the core methanogenic SUF system comprised of SufB$_2$C$_2$ a suitable host system is needed. Most methanogens only have one Fe-S cluster assembly system and knockout mutants made in regards to such a system would be lethal. *E. coli* has two cluster assembly systems, ISC and SUF, and knocking out the SUF system in *E. coli* is non-lethal. Therefore *E. coli* is a suitable host to evaluate the core methanogenic SUF system. The first objective was to create *E. coli* knockout mutants that lacked a functional native SUF system. The *sufBCD* portion of the *E. coli* *suf* operon was chosen a suitable component to knockout. The *E. coli* *sufBCD* operon codes for the cluster scaffold component SufBC$_2$D and likely functions as an analog to the purposed SufB$_2$C$_2$ scaffold in methanogens. Once *E. coli* knockout mutants were
made the methanogenic SUF system operon and variations of it were incorporated into plasmid constructs and then transformed into the *E. coli* knockout mutants.

### 3.2. Experimental Approach

In order to make functional evaluations of the core archaeal SUF system in *E. coli* the *E. coli* ISC assembly system had to be disabled so that there was only one active Fe-S cluster assembly pathway. It is known that *E. coli*’s ISC assembly system becomes disabled and the SUF system upregulated in conditions of oxidative stress and iron starvation.\(^{34}\) This leaves only the SUF system as a means to assemble Fe-S clusters.\(^{34,42}\) Two different strategies were utilized to disable the *E. coli* ISC system. In one strategy oxidative stress was induced in cultures of *E. coli* through the addition of Phenazine methosulfate (PMS) to the growth media (fig. 3.1). Within the cytosol of bacterial cells PMS as well as other phenazine types can be reduced by the oxidation of glutathione and NADH.\(^{43}\) The reduced PMS molecules will then induce oxidative stress through interacting with molecular oxygen, leading to the generation of reactive oxygen species (ROS).\(^{43}\) The other strategy utilized involved inducing iron starvation in the mutant *E. coli* cultures. The compound 2,2’-Dipyridyl is a known iron chelator and is

![2,2’-Dipyridyl](Image)

able to induce conditions of iron starvation when added to the *E. coli* growth media (fig. 3.1).\(^{34}\)

| Phenazine methosulfate | 2,2’-Dipyridyl |
In order to determine the functionality of the \textit{Mth} SUF system in \textit{E. coli} the activity of a specific Fe-S cluster containing enzyme was evaluated. 6-phosphogluconate dehydratase (6-PGDH) is an enzyme that contains an oxygen-labile [4Fe±4S] cluster.\textsuperscript{34} In \textit{E. coli} 6-PGDH is involved in the Entner–Doudoroff biochemical pathway and is essential to carbon assimilation from gluconate.\textsuperscript{34} The strategy was to grow the various \textit{E. coli} types in minimal media with a sole carbon source of gluconate. Gluconate serving as the sole carbon source makes 6-PGDH essential for carbon assimilation and without functional 6-PGDH enzymes \textit{E. coli} will become non-viable. If the SUF system is functionally efficient then 6-PGDH will be assembled properly with intact Fe-S clusters and will be able to effectively process gluconate and supply cells with a source of carbon. Cells will then be viable and evidence of colony formation should be visible. If the SUF system is non-functional then 6-PGDH will not be assembled correctly and cells will subsequently not be able to assimilate carbon from gluconate. Therefore in this scenario 6-PGDH activity is a direct indicator of the functionality of the SUF Fe-S cluster assembly system.

In order to create \textit{E. coli} knockout mutants lacking their native SUF system a specific chromosomal gene disruption procedure was followed (fig. 3.2). This procedure was derived from a research article published in 2000.\textsuperscript{44} To disrupt the chromosomal \textit{sufBCD} operon in \textit{E. coli} the procedure utilizes a specific phage recombinase system. The initial steps in the chromosomal transformation system involve transforming a helper plasmid into stock \textit{E. coli}. There are two different types of helper plasmid that
can be utilized for this procedure, pKD20 or pKD46. The two plasmids differ in that pKD46 contains a native tL3 terminator sequence downstream of the exo region of the plasmid and pKD20 does not contain this terminator sequence. PKD46 was used in this instance. PKD46 contains a phage λ red recombinase operon with an L-arabinose inducible promoter. PKD46 is also a low copy number plasmid which helps minimize competitive inhibition associated with multi-copy plasmids. The plasmid is also curable by growth at 37°C meaning that replication of the plasmid is temperature sensitive. The phage λ red recombinase component is efficient in removing chromosomal genes because it is able to inhibit host efforts in interfering with phage based recombination activities. There are three genes in the phage λ red recombinase system (fig. 3.2 A). The products of the γ gene component inhibits the host RecBCD exonuclease V. RecBCD is an enzyme complex in E. coli that initiates recombination repair from double strand breaks in DNA and will interfere with the other elements of the phage λ red recombinase system. The two other genes termed B and Exo function by accessing DNA ends and promoting recombination. PKD46 has an ampicillin resistance gene (ampR) that allows for selection of successfully transformed colonies.

Initial E. coli cell stocks used were of the K-12 MG1655 cell line. E. coli K-12 MG1655 is a longstanding laboratory cell line that closely approximates wild type E. coli and is believed to contain minimal genetic mutations. Once the pKD46 helper plasmid was successful transformed into the E. coli cell stocks specific PCR products had to be generated prior to the next step. Successful transformation was determined by evidence of bacterial colony viability in ampicillin containing media. The phage λ red recombinase system requires the use of primers that are between 56-70 nucleotides (nt) long. The
primers need to have 36-50 nt homology for the target area of interest on the bacterial chromosome, $sufBCD$ operon in this case, as well as 20 nt sequence homology for a template plasmid termed pKD4, containing a kanamycin resistance ($kanR$) gene flanked by FLP recognition target sites ($FRT$) (fig. 3.2 B). Once PCR products are generated using the described primers and the pKD4 template plasmid the PCR products are transformed into the pKD46 containing $E. coli$ cells through use of electroporation. The phage recombinase system is then induced in transformed cells through addition of L-arabinose to the growth media. Cells whose chromosomes were successfully modified were then selected based on resistance to kanamycin and cell stocks were made. Gene sequencing allowed for further verification that the $sufBCD$ operon was removed and the $kanR$ gene was there in its place. Next another helper plasmid termed pCP20 was utilized to remove the $kanR$ gene from the $E. coli$ transformants. PCP20 Contains a thermally induced FLP recombinase system that acts on the FRT sites flanking the $kanR$ gene and functionally removes the $kanR$ gene leaving behind a single FRT site (fig. 3.2 C). PCP20 contains a chloramphenicol resistance ($cmR$) gene for selecting transformants that have successfully incorporated the pCP20 plasmid. PCP20 also has temperature sensitive replication so it can be cured from transformants through growth at specific temperatures. Once pCP20 was cured additional cell stocks were made.
Figure 3.2. Inactivation of Chromosomal Genes In *E. Coli* K-12 Using PCR Products. **A.** pKD46 transformation into *E. coli*. **B.** Introduction of PCR amplified kanR/frt into pKD46 transformed cells and induction of pKD46 expression. **C.** Transformation of kanamycin resistant cells with pCP20 plasmid and induction of pCP20 expression.

The next step in the *in vivo* evaluation process involved generating plasmids that contained the various *M. thermolithotrophicus (Mth) sufBC* operon constructs. Two plasmid constructs generated previously were utilized for this process. The foundation of each construct is based on the pQE2 plasmid vector line. All pQE2 plasmid constructs utilized contain a lac operator component and lac operon repressor gene. Expression of SUF system components is induced through exposure to Isopropyl β-D-1-thiogalactopyranoside (IPTG). IPTG induces expression by binding to the lac operon.
repressor protein and induces the lac repressor to detach from the lac operator. The lac repres-

sor when bound to the operator blocks DNA polymerase from binding to the promotor element so its removal allows DNA polymerase to bind and transcription to begin. The plasmid termed “pQE2-Nhis-MTHSufCB-PING-spr” was used as the source for the complete \textit{Mth sufBC} operon. The plasmid “pQE2-Nhis-sufbcd\_MG1655” served as the source for the native \textit{E. coli sufBCD} operon. The pQE2-Nhis-sufbcd\_MG1655 plasmid was used as a positive control to evaluate the degree to which growth defects induced through PMS or 2,2’-dipyridyl could be relieved through the expression of genes within the various pQE2 plasmid constructs. All of the pQE2 plasmids utilized except for the control plasmid contain a spectinomycin and streptomycin resistance gene (\textit{smR}). The control plasmid contains an ampicillin (\textit{ampR}) resistance gene. To evaluate the previously identified key amino acids on SufB and SufC four additional plasmid constructs were made with the codons for key amino acids within the \textit{sufBC} operon changed to codons for serine. One construct contained the \textit{sufC} operon with three codons of key cysteine residue, C218, C237 and C240, changed to codons for serine. Another construct contained the \textit{sufB} operon with codons of two key amino acids, C318 and H346, changed to serine. A third construct contained the \textit{sufBC} operon with codons for the three previously discussed cysteine residues on \textit{sufC} and the two key residues on \textit{sufB} changed to codons for serine. The fourth construct contained the codon for C218 on \textit{sufC} changed to the codon for serine and on \textit{sufB} the codons for C318 and H346 were changed to codons for serine. To generate plasmid constructs that contained these point mutations in the \textit{sufBC} operon Gibson assembly procedures were utilized. Gibson assembly allows for the joining of multiple DNA fragments into a
single linear fragment. Often when using Gibson assembly to construct plasmids one of the DNA fragments is termed the “recipient” and the other the “insert”. The “recipient” is the larger of the two fragments. In order to guide the annealing process Gibson assembly requires that the fragments to be joined have overlapping ends. Specific PCR primers are often used in amplifying the DNA fragments of interest to ensure that the fragments have the necessary overlapping feature. The primers are often designed such that they contain a 5’ end that is identical to an adjacent segment and a 3’ end that anneals to the target sequence (fig. 3.3).

Figure 3.3. Primer design for creating DNA fragments that can be utilized in Gibson Assembly. Two different primer sets are utilized and for each the 3’ end of the primer is designed so that it anneals to a given target sequence.

Gibson assembly involves incubating the DNA fragments to be joined in a reaction mixture containing three enzymes. The T5 Exonuclease enzyme removes nucleotides from the 5’ end of the DNA. This leaves 3’ end over hangs that serve as a template for fragment annealing. After fragments anneal Phusion DNA Polymerase will then add missing fragments to the annealed DNA strands. Finally, Taq DNA Ligase
covalently links the annealed complementary DNA fragments and removes any nicks and creates an adjoined DNA fragment. For the plasmid constructs containing *sufBC* point mutations pQE2-Nhis-MTHSufCB-PING-spr served as the recipient element and the various inserts were derived from previously made plasmids containing the *sufBC* operon with corresponding point mutations. Once the Gibson assembly procedure was complete the newly constructed plasmid constructs were transformed into the previously made *E. coli* *sufBCD* knockouts using a heat shock transformation procedure. Transformed cells were chosen based on antibiotic resistance to spectinomycin. Cell stocks were then made and samples were sent out for genetic sequencing to confirm the presence of the anticipated DNA sequence. Once sequencing results confirmed the expected sequences *sufBC* expression was verified in the different *E. coli* mutants (fig. 3.4). Once all necessary verifications were completed the *in vivo* evaluation could begin.

*In vivo* analysis was performed using 15% agar plates made of M9 minimal media with 1M CaCl$_2$ and 1m MgSO$_4$ added per the manufactures instructions. VWR was the manufacturer of the M9 minimal media used and Sigma-Aldrich was the manufacturer of the CaCl$_2$ and MgSO$_4$ used for the *in vivo* analysis. Plates also contained 0.2% gluconate and 0.5mM IPTG and some also contained varying concentrations of PMS or 2,2’-dipyridyl. PMS and 2,2’-dipyridyl were added to the agar mixture post autoclaving and after the agar mixture had cooled bellow 50°C to avoid degradation of the two compounds. Stock solutions of PMS and 2,2’-dipyridyl were sterile filtered prior to addition to minimize the risk of contamination. Both PMS and 2,2’-dipyridyl are light sensitive compounds and will degrade over time when exposed to UV radiation so solutions of both types were stored in opaque containers and exposure to
UV-light was kept to a minimum. On the initial day of the experiment samples from cell stocks of each of the *E. coli* types to be evaluated were streaked over antibiotic containing agar plates. The plates were allowed to incubate overnight at 37°C and then on the following day a single bacterial colony from each plate was inoculated into 5ml of overnight pre-culture containing M9 minimal media with 1M CaCl2 and 1m MgSO4 added per the manufactures instructions. The pre-cultures were formulated without antibiotics and also contained 0.2% gluconate and 0.5mM IPTG. Inoculated pre-cultures were allowed to incubate overnight for approximately nineteen hours in a shaker at 250rpm and 37°C. On the third day the ODs of all the overnight pre-cultures were normalized to an OD of 0.25 at 600nm in M9 minimal media with the same formulation as before. Once normalizations were made the pre-cultures were further diluted 100x in M9 minimal media and then 10ul were spotted in triplicate on the various agar plates. As the plating was being performed cell suspensions were periodically mixed to maintain fully heterogeneous mixtures. Afterwards plates were given 20min to dry and care was taken to minimize light exposure to the plates. After the drying period plates were placed in a 37°C incubator. Plates were checked every twenty four hours for a period of four days.
3.3. Results

Before *in vivo* experimentation could begin a confirmation was needed to ensure that the core plasmid construct utilized for all the different subtypes allowed for successful expression of both the SufB and SufC proteins. Cell samples of each type were grown up to an optical density (OD) of 0.6 at 600nm light wavelength and then IPTG was added to the growth media to induce *suf* operon expression. Cells continued to grow and then were harvested at a later time. All plasmid constructs utilized contained a histidine tag element associated with the *sufC* gene. As a result when the SufC protein is translated it will have a series of histidine residues associated with its N-terminal. Immobilized metal affinity chromatography (IMAC) utilizing nickel-nitrilotriacetic acid (Ni-NTA) resin, specific for his tagged proteins, was used for purification.
For in vivo experimentation utilizing phenazine methosulfate (PMS) agar plates with PMS concentrations of 2.5uM, 5uM, 10uM, 15uM, and 20uM were utilized (fig. 3.5). All plates including the control contained the same agar based formulation and only varied in regards to PMS concentration. The wild type (WT) sample was taken directly from frozen E. coli K-12 MG1655 cell line stock. The positive control used involved E. coli K-12 MG1655 knockouts for sufBCD containing the pQE2-Nhis-sufbcd_MG1655 plasmid encoding the native E. coli sufBCD operon.

Results for the PMS plating indicate that for PMS concentrations ranging from 0uM to 5uM all E. coli sample types were viable as evident by clearly visible and continuous colonies at the 48 hour mark (fig. 3.5). At the 10uM concentration there begins to be a loss of viability in some of the samples as evident by fragmentation and
loss of continuity within the bacterial colonies (fig. 3.5). The negative control and the point mutant samples were the first to lose viability. The WT, positive control and unaltered MTHsufBC sample type were all still viable. At 15uM PMS the WT and positive control are still viable and the MTHsufBC sample is semi-viable (fig. 3.5). All of the other sample types are nonviable. At 20uM only the WT and positive control are viable (fig. 3.5). Plating results at all concentrations tested are consistent across all three sample replicates.
Figure 3.5. Phenazine methosulfate plating results after 48 hours and at seven different concentrations of PMS. Eight different sample types were utilized including wild type (WT) *E. coli* and both a positive control and a negative control. Other sample types contained various point mutations within the *sufBC* operon. MG1655sufBCD in the figure denotes the positive control lane. (Caption cont’d.)
MTHsufBC denotes the *E. coli* sufBCD knockouts containing a plasmid construct with the full unmodified *Mth* sufBC operon. The lanes labeled ΔsufBCD identifies the *E. coli* sufBCD knockouts that lack plasmid complementation. This served as a negative control. The remaining four lanes contain *E. coli* mutants with various different point mutations within the *Mth* sufBC operon. For figure notations regarding the samples containing point mutations within the sufBC operon C denotes cysteine and S denotes serine. The amino acids that were mutated are listed within brackets after the gene in which the point mutations were made. For example in MTHSufC(C218/237/240S)B, cysteine 218, 237 and 240 were changed to serine and all the mutations are within the sufC gene.
2,2'-dipyridyl Plating

For *in vivo* experimentation utilizing 2,2'-dipyridyl agar plates with 2,2'-dipyridyl concentrations of 100uM, 200uM, 300uM and 400uM were utilized (fig. 3.6). All plates including the control contained the same agar based formulation and only varied in regards to 2,2'-dipyridyl concentration. All sample types were the same as the PMS plating experiment and are denoted the same way.

At the 96 hour mark all sample types were viable from concentrations of 0uM to 100uM 2,2'-dipyridyl (fig. 3.6). At 200uM the WT sample is viable and the positive control is mostly viable. The MTHsufBC sample type is semi-viable as evident by faint colony formation. All the other sample types are nonviable. There are two discrepancies for the MTHsufCB(C318/H346S) and MTHsufC(C218/237/240S)B(C318/H346S) sample types as one of the three replicates shows the presence of a semi viable colony (fig. 3.6). It is possible that these two outliers are the results of external bacterial contamination. The other two replicates show no evidence of colony formation. At 300uM the WT is viable while the positive control is semi-viable (fig. 3.6). All other sample types are nonviable. At 400uM all sample types are nonviable (fig. 3.6).
Figure 3.6. 2,2′-dipyridyl plating results after 96 hours and at seven different concentrations of 2,2′-dipyridyl. Eight different sample types were utilized including wild type (WT) *E. coli* and both a positive control (MG1655sufBCD) and a negative control (ΔsufBCD). Other sample types contained various point mutations within the *sufBC* operon. All sample types are identical to those used in the PMS platting experiment (fig. 3.5).
CHAPTER 4. DISCUSSION

Methanogens and their ancient origins provide an invaluable window into the origins of essential biochemical processes that are prevalent across a wide variety of organisms. Fe-S clusters are among the most ancient and ubiquitous cofactors in all of cell biology. Their involvement in a wide range of cellular activities makes them an essential component to the wellbeing of many organisms. As the earth’s atmosphere has changed throughout history a unique challenge has arisen. Rising oxygen levels coupled with decreasing levels of ferrous iron have made maintaining Fe-S clusters increasingly challenging. The development of multiple cluster assembly systems that involve increasing levels of complexity is a testament to the value of these co-factors and the commitment organisms are willing to make to maintain them. The robust connection between methanogens and Fe-S clusters creates a dynamic through which much can be learned about the origins of Fe-S clusters and their assembly. The sulfur mobilization (SUF) system is the core Fe-S cluster assembly pathway in methanogens and within the SUF system products of two genes, sufB and sufC, appear to makeup the core structure of the system. In addition to being found within methanogenic Archaea the SUF system and variations of it can also be found in other prokaryotes as well as in eukaryotes. The SUF system is E. coli has been well researched and contains several components that are not found in methanogens. In E. coli the cluster assembly scaffold is made up of SufBC_2D. In addition to the scaffold component of the system E. coli also has a cysteine desulfurase and sulfur transfer component, SufS and SufE, that are absent in methanogens. The E. coli SUF system also contains a Fe-S cluster transport protein termed SufA. In methanogens it appears the SufC and SufB proteins
associate together and form the cluster assembly scaffold with a structure of SufB$_2$C$_2$. Within this scaffold there is evidence from absorption and EPR data that a [4Fe-4S] cluster assembles within the SufB$_2$C$_2$ scaffold complex. Sequence analysis comparisons of the suf operon between different methanogens and E. coli revealed seven amino acids in the core methanogenic suf operon that may be critical to Fe-S cluster assembly within the scaffold. Two sites on sufB correspond to the know Fe-S cluster assembly site in the E. coli SufBC$_2$D scaffold. Two other amino acid sites on SufB are conserved in all methanogens of the Methanococci class. In sufC it was found that there were three cysteine residues conserved across all group of methanogens. When point mutations for these sites were made absorbance and EPR data indicated that the three conserved cysteine residues on SufC appear to be critical to Fe-S cluster assembly. When changed from cysteine to serine both absorbance and EPR data indicated a loss of Fe-S cluster signal. This is distinct from the assemble site in E. coli where the Fe-S cluster is known to assemble between the SufB and SufD subdomains. In methanogens the cluster assembly site being on SufC suggest that initial SUF system assembly scaffold may have been comprised of only the SufC protein.

In vivo experimentation indicated that the methanogenic SUF system can partially complement the E. coli SUF system with cellular viability able to be maintained under a certain threshold of both oxidative stress and iron starvation. In order for the methanogenic SufB$_2$C$_2$ scaffold to partially complement the E. coli system both SufB and SufC proteins are needed and all the amino acids residues of interest within the SufB$_2$C$_2$ scaffold are essential. Even the residues on SufB contrasting the in vitro analysis which indicated that the SufB residues of interest were not necessary for
cluster assembly within the scaffold complex. Potentially the SufB element provides structural stability or potentially enhances the cluster binding efficiency of SufC. This may work to elevate the rate in which the scaffold can transfer assembled clusters to their target sites. In this way SufB may be a modifier element that works to influence the central component of the complex. This dynamic may be essential for effective scaffold function \textit{in vivo}, especially under conditions of stress where the rate at which the scaffold works is critical to whether or not cells can overcome conditions of stress.

Uncovering the secrets behind Fe-S clusters and their origins has broad implications that reach beyond cell biology. The ubiquitous nature of Fe-S clusters makes research efforts to study them consequential to numerous fields. In medicine developing effective methods to target Fe-S cluster viability and assembly is a potential new pathway to treating infectious disease. In bioengineering, manipulation of Fe-S cluster containing proteins can be central to developing and enhancing new processes that can be utilized for the manufacturing of different organic compounds including examples from pharmaceuticals to aromatic compounds used in cosmetics. In the fight against climate change Fe-S cluster containing proteins could play a central in engineering organisms that can be involved in carbon capture and conversion technology. Fe-S clusters can also play a central role to infrastructure by being heavily involved in organisms that are utilized in the treatment of waste water and to the generation of electricity through anaerobic digestion. As the knowledge based regarding these crucial cofactors continues to expand manipulation of Fe-S cluster containing proteins may become one the most crucial ingredients in engineering the future of life.
WORKS CITED


VITA

Evan Dunkle was born in Montgomery County, Maryland in 1987. Evan attended Linganore High School in Frederick, Maryland and graduated in 2005. He initially attended the Georgia Institute of Technology in Atlanta, Georgia and then later transferred to Temple University in Philadelphia, Pennsylvania. Evan graduated from Temple University in January 2011 with a Bachelor of Science Degree in Biology. After graduating from Temple University Evan worked for several years in the biotechnology and pharmaceutical industries. Prior to attending LSU Evan was living and working in Baltimore, Maryland. Evan’s mother and father live in Frederick, Maryland. He also has four siblings; a younger sister who lives and works in Maryland, a younger brother who lives and works in Washington D.C. and two step sisters who both live and work in Pennsylvania.