THE EVOLUTION OF MICROBIAL ELECTRON TRANSFER ON EARTH

By

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Abstract of the Dissertation

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All life on Earth is dependent on biologically mediated electron transfer (*i.e.,* redox) reactions that are not at thermodynamic equilibrium. Biological redox reactions originally evolved in prokaryotes and ultimately, over the first ~ 2.5 billion years of Earth's history, formed a global electronic circuit. To maintain the circuit on a global scale requires that oxidants and reductants be transported; the two major planetary electron conductors that connect global metabolism are geological fluids - primarily the atmosphere and the oceans. Because all organisms exchange gases with the environment, the evolution of redox reactions has been a major force in modifying the chemistry at Earth's surface. First, a review is given of the discovery and consequences of redox reactions in microbes with a specific focus on the co-evolution of life and geochemical phenomena. With the larger picture in mind, the focus is then directed specifically to one of the earliest metabolic pathways on Earth. The reduction of elemental sulfur. is an important energy-conserving pathway in prokaryotes inhabiting geothermal environments, where sulfur respiration contributes to sulfur biogeochemical cycling. Despite this, the pathways through which elemental sulfur is reduced to hydrogen sulfide remain unclear in most microorganisms. We integrated growth experiments using *Thermovibrio ammonificans,* a deep-sea vent

thermophile that conserves energy from the oxidation of hydrogen and reduction of both nitrate and elemental sulfur, with comparative transcriptomic and proteomic approaches, coupled with scanning electron microscopy. Our results revealed that two members of the FAD-dependent pyridine nucleotide disulfide reductase family, similar to sulfide-quinone reductase (SQR) and to NADH-dependent sulfur reductase (NSR), respectively, are over-expressed during sulfur respiration. Scanning electron micrographs and sulfur sequestration experiments indicated that direct access of *T. ammonificans* to sulfur particles strongly promoted growth. The sulfur metabolism of *T. ammonificans* appears to require abiotic transition from bulk elemental sulfur to polysulfide to nanoparticulate sulfur at an acidic pH, coupled to biological hydrogen oxidation. A coupled biotic-abiotic mechanism for sulfur respiration is put forward, mediated by an NSR-like protein as the terminal reductase.

Prior Publications

Several sections of this publication have been published elsewhere.

Chapters 2 and 3 reproduced work from the following 3 publications:

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CHAPTERS 2 AND 3

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CHAPTER 4

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Dedication

This work is dedicated to my daughter Tallulah Rose Jelen, now 5 years old, for whose entire life I have been working toward this dissertation. My primary motivation for undertaking this journey was to better understand the effect we have on our home; now I hope to apply this knowledge to bring out the ways we can understand and live in better harmony with the natural cycles we came from.

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CHAPTER 1

Thesis Introduction

Energy intricately links physics, chemistry and biology. In biology, the fundamental transduction of energy from the abiotic to the biotic realm is based on the controlled transfer of electrons. In energetic terms, lifeforms can be split into three groups, those that oxidize inorganic chemicals "chemoautotrophs," those that obtain energy from light "photoautotrophs" and those that obtain energy from ingesting previously fixed organic carbon, "heterotrophs." In common to all of these mechanisms of transduction is biological electron transfer. For chemotrophs and heterotrophs, the "downhill" transfer of electrons is from a molecule of high reduction potential to low reduction potential. These electron transfers power a proton (or sodium)-motive force across a membrane, which in turn powers a cell. For phototrophs, the electron is first excited by a photon to a higher energy level before its downhill slide can be used to create proton-motive force across the membrane.

The active players in transferring electrons are proteins, nanomachines that evolved in prokaryotes in Pre-Cambrian time. Classed into six overarching functional "EC" groups by the enzyme commission classification system, "EC1," the oxidoreductases, are responsible for catalyzing electron transfer, or "redox" reactions. One of the smallest known proteins of this type, ferredoxin, has a long history of research behind it. Margaret Dayhoff, an early pioneer of bioinformatics, published a seminal paper with her colleague Richard Eck in 1966, "Evolution of the structure of ferredoxin based on living relics of primitive amino acid sequences." (Dayhoff and Eck 1966). By analyzing the primary sequence of ferredoxin found in today's organisms, they proposed a reconstruction of its evolutionary

history, that it had evolved by the doubling of a shorter protein, this one consisting of only eight of the simplest amino acids. Indeed, these first studies into protein sequence examined what is now seen as a ubiquitous trend in protein evolution - repeatable modules, with variations in how a relatively small number of parts are structured to give rise to a spectacular array of functions. This was not only an important insight into how proteins are structured today, but also into their evolution through geological time. Nitrogenase, existing today as a large, multi-subunit protein, evolved from smaller and less complex units (Boyd, Hamilton et al. 2011). Copper-binding cytochrome *c* oxidase, responsible for reduction of oxygen across the aerobic divisions of life, did not exist in its current form for at least half of Earth's history. Rather, through trial and error in an expanding chemical and changing environmental context, genes encoding the useful modules in the appropriate arrangements were preserved. Today's robust workhorses are the result of billions of years of evolution, of an interplay between environmental factors, geological and solar energy, and life's widening catalogue of energy-transducing enzymes and pathways.

How microbes could possibly co-evolve with geological processes to change planetary chemistry is poorly understood. The seminal ideas were born from Winogradsky and Vernadsky's groundbreaking work in the 19th and early 20th centuries and have been supported by numerous accounts of how Earth's surface environment has co-evolved with microbial life throughout its past. We now understand that half way through Earth's history, microbes from anaerobic beginnings gained the ability, using energy from the Sun, to split water, in turn producing the very oxygen on which all advanced life now depends, while also drastically altering Earth's chemistry. But many questions remain at the deep-time and molecular level. How were the original protein domains structured? Which

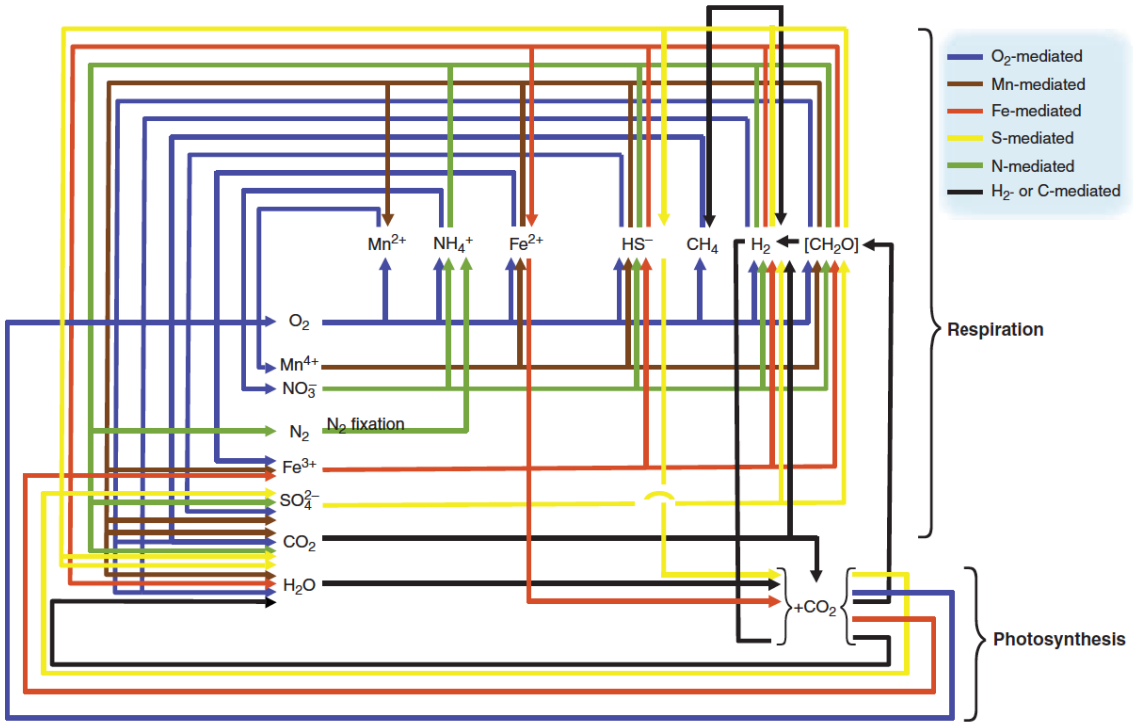
cofactors did they use in their active sites? How many truly original modules did the landscape of electron transfer begin with and what were their first forms?

To make the "planetary-level biogeochemical electron transfer network" less of an abstraction, a map, or "circuit diagram" is useful, and a marvel in its relative simplicity. The diagram from (Falkowski, Fenchel et al. 2008), reproduced below (Figure 1.1), highlights life's cyclical nature, depicting today's biological electron flows, their effect on the major elements of life, and how the distinct biological pathways are necessary to sustain one another. Chapter 2 of this thesis begins by annotating this map with the EC1 enzyme homologues directly responsible for its operation, describing their core structures and use of cofactors. In this way, a "parts list" is sketched out, and insights made into protein evolution through coupling with geological history. Many fascinating connections between the evolution of the geosphere and biosphere, unified by evolving planetary redox potential, are expanded on in Chapter 3. Starting from a reduced state, Earth has become progressively oxidized, not just from photosynthetic oxygen production but from hydrogen escape and other factors. Life has had to respond. Just as the layered properties of a Winogradsky column are an emergent property of microbes responding to a redox gradient that they create, so there is a trend of these emergent layers seen in life's network of metabolism, through Earth history.

Almost all work in molecular biology, whether it be single-cell genomics, metagenomics or proteomics, now relies heavily on previously described and biochemically characterized examples, to which sequence comparisons can be made, inferring identical or similar function. Describing the electron transfer network in terms of its 400 different gene homologs or looking back in time at protein evolution *in silico* are no exceptions to this dependence. Chapter 4 of this dissertation focuses on a specific

pathway, specific environment, and single organism (*Thermovibrio ammonificans*), to understand better which genes are responsible for its respiration of sulfur. Investigating a microbe that lives at the hydrothermal vents with no light, respiring an electron acceptor that was likely ubiquitous in the Archean is a way of peering far back into geological time, using a living fossil. Many deep-branching Archaea and Bacteria living in hydrothermal or geothermal environments conserve energy by sulfur respiration, substantiating the importance of this pathway on the early Earth. However, with very few exceptions, the molecular basis of sulfur respiration remains unknown, despite the fact that it may be one of the most ancestral respiratory pathways. In this work we identify the genes and enzymes involved in sulfur respiration in the deep-branching, anaerobic thermophile, *T. ammonificans*, we propose a model in which this bacterium conserves energy, and we discuss its evolutionary implications.

Figure 1.1 Earth's electron marketplace (Reproduced from (Falkowski, Fenchel et al. 2008))



A schematic diagram depicting a global, interconnected network of the biologically mediated cycles for hydrogen, carbon, nitrogen, oxygen, sulfur, and iron (Falkowski, Fenchel et al. 2008).

CHAPTER 2

The Role of Microbial Electron Transfer on Earth

2.1 Brief History of the Discovery of Biological Electron Transfer Reactions

In 1863, four years after the publication of *The Origin of Species,* George G. Stokes, Lucasian Professor of Mathematics at Cambridge University, published a paper "On the Reduction and Oxidation of the Colouring Matter of the Blood." Inspired by descriptions of remarkable spectra created by sunlight shone through dilute blood (Hoppe 1862), Stokes envisioned a way to test his supposition that the characteristic color change from bright red, arterial, to deep purple, venous, blood was accomplished by reduction of the coloring pigment, now known to be hemoglobin (Stokes 1863). Using blood of sheep or oxen supplied by his butcher, Stokes carefully chose reducing agents (*e.g.,* ferrous sulfate) compatible with an alkaline solution (acidic solutions decomposed the pigment) and recreated the reversible spectral changes in a tube, using chemical oxidation and reduction.

Of course, before the discovery of the electron, the terms "oxidation" and "reduction" had a more literal meaning than the electron-transfer formalizations of today. In its earliest descriptions by Antoine Lavoisier, reduction was the loss of molecular oxygen, while oxidation was its addition (Lavoisier 1774). By Stokes' time, electricity had been discovered and the thermodynamic relationship between electricity and chemistry would soon precisely be defined (Nernst 1889). Concomitantly, biochemical transformations at the microbiological level were being studied in such labs as those of Koch and Pasteur. Their pioneering research, which recognized the importance of oxidation-reduction reactions, was largely limited to isolated cultures and carbon-based transformations (*e.g.*, fermentation). The recognition that oxidation-reduction reactions were critically important

in changing environmental chemistry came about largely through the insight of Sergei Winogradsky (Dworkin and Gutnick 2012). Early in his career, Winogradsky was intent on understanding the role of the large sulfur granules accumulated by the sulfur oxidizing bacterium *Beggiatoa* in natural environments. His experiments culminated in describing an organism able to derive energy solely from sulfur compounds, an entirely new type of metabolism, which is now called *chemolithotrophy*. Subsequently, Winogradsky isolated nitrifying bacteria and confirmed the biological ability to fix inorganic carbon using energy from the redox transformations of inorganic (nitrogen) compounds (Winogradsky 1888). The knowledge that non-photosynthetic microbes could obtain energy and carbon not only from organic compounds (*e.g.*, carbohydrates) but also from inorganic molecules (*e.g.*, H_2S, NH_3, Fe^{2+}, CO_2) effectively was the beginning of a new field of science — microbial ecology. Indeed, Winogradsky was the first to isolate a free-living diazotroph (nitrogen-fixing organism), thereby proving that microbes could not only oxidize ammonium but could also reduce N_2 gas from the atmosphere. His work led to the notion that biological electron transfer reactions involving carbon, nitrogen and sulfur form a network of interdependent elemental cycles mediated solely by microbial metabolism (Schlesinger and Bernhardt 2013).

Fundamentally, however, the microbiologists of the time failed to appreciate that microbial metabolism could change Earth's chemistry on a global scale. By the end of the 19th century, the Russian-Ukrainian geochemist Vladimir Vernadsky was formulating a broad theory of Earth history, with the fundamental hypothesis that life itself was a geological "force" that had shaped the planet. His book "The Biosphere" was published in Russian in 1926, but the full English translation was not published until 1997 (Vernadsky 1997, Piqueras 1998). Consequently, Vernadsky's ideas were largely isolated from

European and American scientists. Vernadsky understood that neither individual cycles, nor biology and geology operated independently but were in constant interaction, the planetary steady state being balanced by positive and negative feedbacks. In the latter part of the 20th century, Vernadsky's ideas were echoed and eloquently elaborated upon by James Lovelock and Lynn Margulis (*i.e.*, the "Gaia" hypothesis; (Lovelock and Margulis 1974)). The coupling of the sulfur, carbon, iron and other cycles through time has since been supported by analysis of sedimentary isotopic records (Petsch and Berner 1998).

Like most geologists at the time, Vernadsky believed that Earth had always been in a steady state and he never considered explicitly the origin and evolution of biogeochemical cycles. We now understand (albeit still incompletely) that biogeochemical cycles largely result from an interaction between biological electron transfer reactions (with or without protons) that, over geological timescales, can profoundly change the chemistry of Earth's surface. The emergent network of elemental cycles is far from thermodynamic equilibrium and the primary source of external energy is solar radiation. This charged, electron-transfer network can be thought of as a global electrical circuit board, driven by solar power that is transduced by enzymes able to catalyze electron flow. At this scale, the connections between the biological "transistors" are the atmosphere and oceans, which act as global electron conducting media, ferrying oxidants and reductants across the planet with variable resistances dependent on the spatial and temporal scales being considered.(Mitchell 1961, Falkowski, Fenchel et al. 2008)

2.2 Materials and Methods:

A list of the taxa presented in Figure 2.1 and the sources used to survey their physiology and metabolism are given in Supplementary Table 2. To annotate Figure 1.1

with the EC1 proteins (oxidoreductases) that catalyze the necessary redox reactions, a comprehensive list of Earth's biogeochemical pathways was created (Supplementary Table 1). These include all biochemical pathways transferring electrons between environmental substrates and cells for the purpose of energy transduction, or redox transformations enabling biological fixation and incorporation of elements. Subsequently, the Kyoto Encyclopedia of Genes and Genomes (KEGG database), specifically the KEGG "module" pathway maps (https://www.genome.jp/kegg/module.html) were found for each pathway. These maps lay out the reactions needed for a specific metabolic process and give sets of Kegg Orthology (KO) groups that are capable of catalyzing each reaction. By collecting these KO groups, a comprehensive group of orthologous proteins that carry out biogeochemical electron transfer on Earth was put together. For pathways not found in KEGG, a manual literature search for the oxidoreductases needed was carried out. The resulting data are in Supplementary Table 1. Figure 2.2 uses Supplementary Table 1 to annotate a redrawn version of Figure 1.1. The numbers in boxes on each pathway represent number of EC1 homologs responsible for redox reactions within metabolisms.

2.3 The Oxidoreductases: Core Structures and Cofactors

The enzymes responsible for electron transfer are called oxidoreductases (Enzyme Commission Class 1; *i.e.*, EC1; (Tipton and Boyce 2000)). These proteins, major players in the core machinery of metabolism, are ubiquitous across the tree of life (Figure 2.1). Biologically driven electron-transfer reactions, dependent on Earth's two external energy sources, solar radiation and chemical reductants resulting from geothermally derived heat, are the primary energy-transduction processes across the tree of life.

Structural analysis of the EC1 class reveals a relatively small subset of key protein folds, many of which contain transition metals at their active sites (Ragsdale 2006, Harel, Bromberg et al. 2014, Senn, Nanda et al. 2014). Indeed, the entire network of contemporary global electron transfer reactions (Falkowski, Fenchel et al. 2008) employs fewer than 400 homologous genes (Figure 2.2; Supplementary Table 1), all of which evolved in prokaryotes in Precambrian time. These catalysts are necessary for the emergence of nonequilibrium biogeochemical cycles on the planet's surface. At the origin of life, it is possible that the redox chemistry made possible by oxidoreductases was coupled to a small set of the earliest metabolic processes. Although the evolutionary history of the primary amino acid sequences in the oxidoreductases has been obscured by gene duplication and horizontal gene transfer, the evolution of the folds in the catalytic sites and the transition metals employed can potentially be used to infer the history of these enzymes.

Analysis of the metal-containing oxidoreductases reveals that approximately 60% contain iron in the active site. Of iron-containing oxidoreductases, more than half use iron-sulfur clusters while the remaining members mostly employ hemes (Harel, Falkowski et al. 2012). The role of iron-sulfur minerals in catalyzing prebiotic reactions has long been recognized as a potential pathway leading to the formation of simple organic molecules and chemical bond energy required for the origin of life (Williams 1981, Wächtershäuser 1988, Wächtershäuser 1990, Russell and Martin 2004). Available during the early Archean Eon (Hazen 2013), these mineral motifs appear to have been appropriated into short peptide folds very early in the origin of metabolism (Dayhoff and Eck 1966). In a prebiotic world, short peptides, condensed from amino acids, could have sequestered iron-sulfur clusters from the environment to form protoferredoxins (Gibney, Mulholland et al. 1996, Russell 2007). Indeed, the Fe_4S_4 cluster in the active site of ferredoxins is bound to a very highly

conserved protein fold consisting of a CxxCxxC…C peptide sequence (Mulholland, Gibney et al. 1999, Kim, Rodriguez-Granillo et al. 2012). Notably, early in the evolution of iron-sulfur containing proteins, there appears to have been very strong selective pressure for specific folds. Iron-sulfur clusters themselves are achiral. However, an analysis of 3D structures of iron-sulfur containing oxidoreductases in the protein data bank (PDB, http://www.rcsb.org) reveals that all folds around the metal centers are right handed. Given life's early selection of L amino acids in all structural proteins, right-handed protein folds are favored over left-handed folds around iron sulfur centers due to an increased number of hydrogen bonds stabilizing the cluster's entatic state (Vallee and Williams 1968, Kim, Rodriguez-Granillo et al. 2012). The ubiquitous right-handed fold around iron sulfur centers across the tree of life emphasizes how mineral availability, mineral electronic structure (Hazen and Sverjensky 2010), and contingency during selection of chiral amino acids played critical roles in shaping oxidoreductases. Ferredoxins and other iron-sulfur containing proteins are found throughout life, both alone and as domains of larger enzymes (Page, Moser et al. 2003, Krishna, Sadreyev et al. 2006). The ferredoxin fold underwent gene duplication and variation leading to a wide set of iron-sulfur containing oxidoreductases (Rees and Howard 2003), such as NiFe hydrogenase, the evolution of which allowed microbes access to free hydrogen as a source of reductant (Kim, Senn et al. 2013). Following iron, oxidoreductases contain metals in the following order of relative abundance: Cu, Mn, Ni, Mo, Co, V, W (Kim, Senn et al. 2013).

The ability to synthesize organic molecules from inorganic precursors was a critical invention in the early stages of evolution of life and ultimately required redox reactions. Early heterotrophic life may have preceded autotrophy but depletion of amino acids and other organics in a primordial soup context would have given autotrophs a selective

advantage. Six different pathways of carbon fixation are known (Fuchs 2011). While the Calvin-Benson-Bassam cycle is the most prominent in the extant biosphere (Hügler and Sievert 2011), other pathways have long been studied because, among other things, they provide clues to understanding how carbon metabolism evolved (Falkowski 2007). The last universal common ancestor may have used a combination of parts from different extant pathways (Braakman and Smith 2012), though it is possible the original pathway for carbon fixation no longer exists (Nitschke and Russell 2013). The reductive acetyl-CoA pathway (also known as the Wood-Ljungdahl (WL) pathway; (Ljungdhal 1986, Ragsdale 2008)) is found in life's most deeply branching lineages, such as methanogens (Fuchs 2011). Due to its small energy requirements and its capacity to incorporate different one-carbon compounds and carbon monoxide of geothermal origin, this linear pathway potentially served as a primordial carbon fixation and energy metabolism pathway. The EC1 enzyme central to the WL pathway, the bifunctional carbon monoxide dehydrogenase/acetyl CoA synthase, as well as early nickel or iron containing hydrogenases all have active sites with structures bearing resemblance to the "ready made" metal centers of minerals such as greigite, mackinawite and millerite (Russell and Martin 2004). As with ferredoxins, it is possible that in a prebiotic world, short peptides condensed from amino acids could have appropriated existing mineralogical (Fe,Ni)S catalytic activity to kick-start autotrophic pathways (Volbeda and Fontecilla-Camps 2006).

Bioinformatic network analyses suggest that ferredoxin-like Fe_2S_2 and Fe_4S_4 domains share high evolutionary connectivity to those binding hemes (most often containing an iron atom bound within a porphyrin ring) (Liu and Hu 2011, Harel, Bromberg et al. 2014). Hemes are used across the tree of life in the transfer of single electrons without undergoing protonation. Heme containing molecules such as cytochromes are often interspersed

between electron carriers that also undergo protonation, such as quinones. This results in a hopscotch-type pattern in which protons are ferried on one carrier and then ejected into a confined space while the electrons continue to be carried through the membrane to an oxidant (*i.e.*, the Q-cycle)(Trumpower 1990). By conserving energy derived from a vectoral flow of protons, life evolved to access energy released from the oxidation and reduction of a large variety of electron donor-acceptor pairs (Richardson 2000). The result is that virtually identical molecular motors transducing energy throughout the tree of life are flexibly fueled.

Moreover, in the course of early evolution, life became increasingly dependent on a more reliable source of energy - light. As hemes evolved, several sets of porphyrin derivatives emerged with large effective photon absorption cross sections. Amongst these are bacteriochlorophylls, phycobilins and chlorophylls. Heme and chlorophyll are functionally related as well as being two end products of the same biosynthetic chain (Granick 1965). Bacteriochlorophylls, in association with carotenoids, allowed for efficient collection of dilute solar energy and its conversion to chemical bond energy under anaerobic conditions. These molecules are distributed solely within the bacterial domain where they are coupled to the reduction of inorganic carbon using a variety of electron donors including H_2, H_2S, organic matter and Fe^{2+}. (Bacterio)chlorophylls themselves are modified tetrapyrroles with an extra ring (chlorins) and contain a covalently bound magnesium atom (Mauzerall 1998). Despite their structural complexity, bacteriochlorophylls are more deeply rooted than chlorophylls (Xiong, Fischer et al. 2000). In both iron-containing and magnesium-containing porphyrins and chlorins, photon induced oxidation-reduction reactions do not lead to displacement of the metal center, meaning that they are not sacrificial but reversible and catalytic. The evolution of

chlorophyll-protein complexes ultimately allowed access to H_2O as a virtually limitless electron source, leading to formation of free O_2 gas as a waste product. Oxygenic photosynthesis, which evolved in a single bacterial clade, the *Cyanobacteria* (Figure 2.1), would ultimately result in almost complete oxidation of Earth's surface, and would profoundly transform the very mineral chemistry from which it evolved.

How life originally converted light to chemical energy is not known, but the solar excitation of certain minerals can drive electron transfer reactions far from thermodynamic equilibrium even without life (Cairns-Smith 1978, Braterman, Cairns-Smith et al. 1984, Mercer-Smith and Mauzerall 1984, Zhang, Ellery et al. 2007, Jie, Nita et al. 2013). Photogeochemical, abiotic formation of high-energy chemical products is sacrificial rather than catalytic, however these reactions potentially provided prebiotic substrates for the subsequent evolution of biological catalysis. For example, siderite ($FeCO_3$) was likely an abundant sedimentary mineral in shallow waters of the early Archean Eon, when soluble ferrous iron was available, oxygen concentrations were extremely low, and carbon dioxide concentrations were presumably very high. Exposure of siderite to UV radiation under anoxic conditions leads to the irreversible, photochemical oxidation of iron and the reduction of protons to form molecular H_2 (Kim, Yee et al. 2013). The effective cross sections for the photochemical oxidation of siderite in the UV potentially supplied electrons for life in the Archean oceans at fluxes comparable if not exceeding that from hydrothermal vents (Tian, Toon et al. 2005, Kim, Yee et al. 2013). Key steps in the reductive tricarboxylic acid cycle (one of the six extant carbon fixation pathways) have been carried out abiotically with photo-driven ZnS catalysis (Zhang, Ellery et al. 2007, Guzman and Martin 2009). These and other photogeochemical reactions involving minerals almost certainly played a significant role in fueling the earliest life forms on Earth

as well as anticipating the emergence of photosynthesis (Schoonen, Smirnov et al. 2004, Mulkidjanian and Galperin 2009).

The ability to harvest chemical energy, to reduce inorganic carbon and to access the continuous flux of solar energy reaching the planetary surface can be considered key tipping points in altering Earth's redox state and pushing the planet even further from thermodynamic equilibrium. Currently, microbes are still the major players in Earth's elemental cycling.

2.4 Oxidoreductases and the Tree of Life: A Diversified Investment

The molecular machines that capture energy from the Sun, from redox gradients, or from consuming organic matter (phototrophy, chemotrophy and heterotrophy) all share homologous components, using electron transfer chains built from appropriately poised redox enzymes (Kim, Senn et al. 2013). The energy for life is transduced exclusively from electron flow (Lane 2015) through a series of far-from-equilibrium chemical reactions catalyzed by a small set of EC1 proteins operating, ultimately creating a vectoral flux of protons coupled to the formation of ATP (Falkowski 2007). In every organism, electrons are stripped from the geosphere at a high energy (or excited to a higher energy by light), used to power intracellular processes, then either deposited back to the geosphere or incorporated into organic molecules (Trefil, Morowitz et al. 2009, Lane and Martin 2012).

Nitrate respiration is the use of nitrate as a terminal electron acceptor to generate metabolic energy when oxygen is absent and has been described in a taxonomically diverse set of microbes, including members of the alpha-, beta-, gamma-, and epsilon-proteobacteria, gram positive bacteria, and Archaea (Philippot 2005). Organisms characterized as capable of sulfate reduction can so far be split into five Bacterial and two

Archaean lineages (Muyzer and Stams 2008). These metabolisms are widespread, their host organisms found in a broad range of environmental conditions such as shallow marine and freshwater sediments, oil wells and hydrothermal vents. They are also present in the digestive systems of higher living organisms. Sulfur respiration can also be found in both Bacteria and Archaea, while methanogenesis is a metabolism confined to the Euryarchaeota (Figure 2.1). The ability to fix nitrogen is spread widely, though paraphyletically, across the Bacterial and Archaeal domains (Raymond, Siefert et al. 2004), while anoxygenic photosynthesis can be found in Bacterial groups such as the *Acidobacteria, Heliobacteria,* green sulfur bacteria and purple non-sulfur bacteria.

Contemporary oxidoreductases have had billions of years to evolve and radiate across multiple lineages, often via HGT. The age and mode of evolution often obscures the origin of individual proteins. However, even distantly related genes have been required to conserve aspects of protein structure and function through geological time (Illergard, Ardell et al. 2009, Senn, Nanda et al. 2014). Considering only genes encoding EC1 proteins, it becomes clear that a small number of highly conserved geometries and cofactors have been selected as life's redox catalysts. We estimate that only about 400 gene homologs are part of complexes directly participating in electron transfer for energy metabolism (Figure 2.2; Supplementary Table 1). Despite the extraordinary diversity of life, the core machines responsible for energy metabolism are of a strikingly small set. One reason for this parsimony was described by Eck and Dayhoff decades ago in a paper discussing the evolution of the structure of ferredoxin: “We explain the persistence of living relics of this primordial structure by invoking a conservative principle in evolutionary biochemistry: The process of natural selection severely inhibits any change in a well-adapted system on which several other components depend.” (Dayhoff and Eck

1966). Due to the interaction of other enzymes with ferredoxin, iron-sulfur clusters are central to biology, irreplaceable even if iron is often a limiting nutrient (Fontecave 2006). The availability of crucial transition metals like iron and nickel, along with the negative redox potentials associated with methanogenesis, sulfur reduction, sulfate reduction and anoxygenic photosynthesis (–500 to 0 mV) in the reducing conditions of the Archaean oceans resulted in these transition metals being necessary for the basis of early biological electron transfer. That specific systems have evolved to transport nickel and synthesize enzymes using it as cofactor suggests that it has been evolutionarily favored for specific reasons, even though there are redox cofactors that could, and often do, replace it (Maroney 1999).

Another reason that such a small set of proteins plays such a large role in the Earth's elemental cycles is that the various electron-transport chains found ubiquitously throughout life share common components. For example, chemoautotrophic, anaerobic and aerobic respiration, as well as anoxygenic and oxygenic photosynthesis all use similar proton-coupled electron-transport schemes. The basis of the scheme is separation of protons from electrons across a membrane. The return flow of the protons is coupled to enzymes such as ATP synthase, which conserve the electrochemical energy (i.e. the proton motive force) as chemical bond energy. The peripheral components of these proton-coupled electron-transport reactions have been selected for reaction with specific substrates and products, but the basic architecture of all the core pathways shares similar protein structures and ligands, including iron–sulfur clusters, pterins, haems and quinones. These interchangeable structures and ligands have evolved into a metabolic network with overlapping functions across the tree of life (Figure 2.3). Although components like nitrate reductases or photosystems do not directly work together, the intermediate components (quinones,

cytochromes) and carbon metabolisms (*e.g.* Glycolysis, TCA cycle) enable a modular system whereby these metabolisms, though different, draw on some of the same components. Many biological energy-transduction systems share a small subset of metabolic pathways such as glycolysis (the Embden–Meyerhof, the Entner–Doudoroff, including Archaean modifications of these pathways) or the reverse TCA cycle (Braakman and Smith 2012). Thus, metabolism, using a variety of electron donors and acceptors, draws catalysts from a core set of similar components and pathways to enable a flow of electrons and protons. This modular approach to metabolism has bestowed great flexibility on a relatively small number of EC1 genes.

Once evolved, genes for an entire metabolic pathway can spread readily, even between the kingdoms *Bacteria* and *Archaea* (Nelson, Clayton et al. 1999). Acquisition between lineages is primarily via HGT (Baymann, Lebrun et al. 2003), with genome analyses pointing to prolific early gene sharing (David and Alm 2011). Many of the genes that evolved and spread during this punctuated period of biological innovation (in the early to mid-Archaean eon, which coincides with rapid diversification of bacterial lineages), are likely to be involved in electron-transport and respiratory pathways. For example, genes for sulfite reduction are well distributed through *Bacteria* and *Archaea* while there is strong support for their monophyletic origin (Klein, Friedrich et al. 2001). The core genes for Earth's redox network are spread across multiple microbial lineages (Figure 2.1), revealing life's investment in oxidoreductases as highly diversified. Individual organisms certainly die, branches on the tree of life have gone extinct, but even during mass extinctions, instructions for catalyzing proton-coupled electron flow between diverse substrates are robustly conserved, ready to re-expand as conditions allow. There are interesting exceptions to this pattern, with some pathways for energy metabolism more evenly

distributed than others. For example, while glycolysis can be found in all three kingdoms of life, nitrification and nitrogen fixation (Boyd and Peters 2013) are not found in eukaryotes. Methanogenesis is so far confined to the *Euryarchaeota*, a phylum of *Archaea* (although recent environmental genome reconstructions suggest the potential for methanogenesis in other uncultured archaeal groups (Evans, Parks et al. 2015)). The reasons for these limitations are not well understood. It may be that complex metabolic pathways built on a large number of genes are difficult to transfer "in bulk" unless via endosymbiosis (Blankenship, Sadekar et al. 2007). Eukaryotic life, the most morphologically diverse and complex, is built upon redundant biochemistry (heterotrophy and oxygenic phototrophy) inherited completely from prokaryotes. Due to this highly limited metabolic capability, eukaryotes are ultimately dependent on the prokaryotic metabolic pathways for (among other things) key nutrients and recycling of wastes.

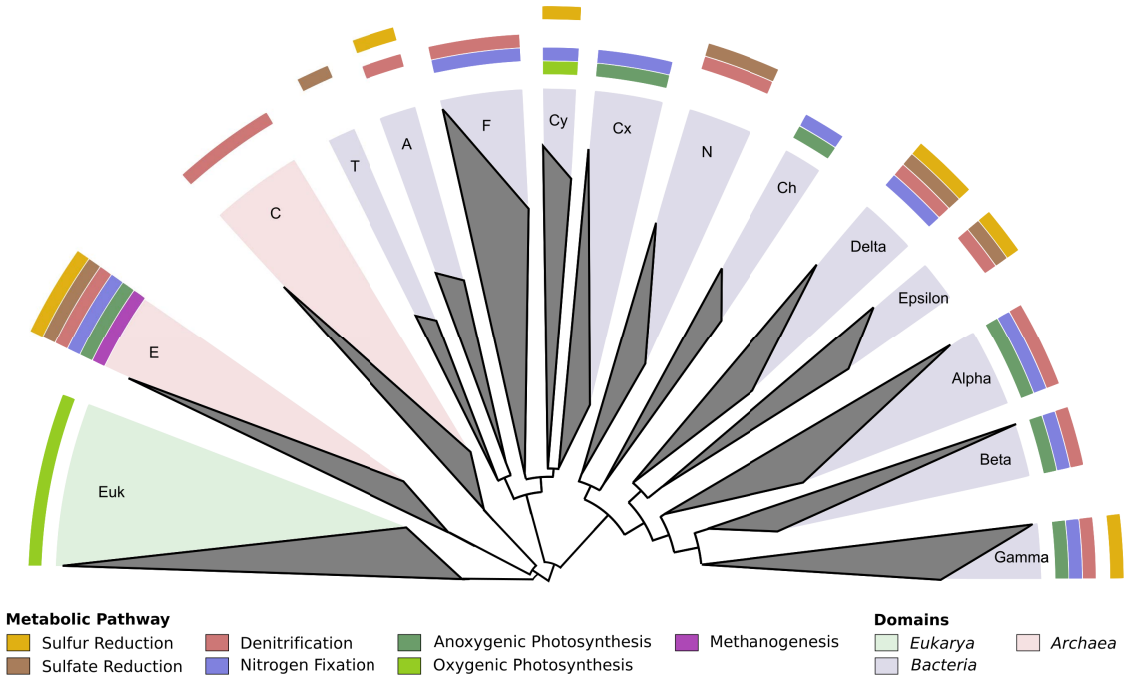
2.5 Conclusions

The progressive oxidation of Earth's surface with time is analogous to the ordered layers of metabolism in a Winogradsky column. In effect, the latter is a spatially organized structure that represents the sequential evolution of microbial metabolism and, in effect, recapitulates geochemical oxidation states over Earth's history. The layered properties of a Winogradsky column are emergent; that is, the microbes self-organize along a redox gradient that they create, the metabolism of each group of organisms coupled within the column via the exchange of diffusible gases and ions of CHNOS, as well as of the reducible metals Fe and Mn. While this coupling occurs on a local scale over months a Winogradsky column, it also occurs on a time scale over months and years to geologic time. In both

cases, the reactions are far from thermodynamic equilibrium and are ultimately powered by solar energy.

The presence in every organism of a redox pathway as part of this balanced, yet decoupled scheme (Figure 2.2) (Falkowski, Fenchel et al. 2008) has ultimately pushed and effectively sustained Earth far from thermodynamic equilibrium. The function-bearing 3D structures responsible for catalyzing redox reactions have evolved to transduce energy from the Sun (as well as geothermal sources) and, via a highly replicative biological framework, now cover practically the entire planetary surface, leaving Earth the only planet that we know of with an atmosphere so far from thermodynamic equilibrium that life must be invoked to explain the distribution of the gases. Microbes, generating gases such as CH_4, N_2O, and NO, as well as O_2, have had significant impact on atmospheric composition, setting the scene for complex multicellular evolution. Many of these gases have absorption spectra that make them potential targets for remotely sensing the potential of life on extra-solar planets (Beichman, Woolf et al. 1999). False-positive scenarios, in which detected gases may not be of biological origin, must be separated from scnearios that could truly signal life. Distinguishing the role that chance played in the development of Earth's redox network from the inevitable outcomes based on appropriate (amenable to life) conditions will factor into the statistics and stages of life existing elsewhere (Morowitz, Kostelnik et al. 2000, Kelley, Baross et al. 2002, Nealson and Berelson 2003, Nitschke and Russell 2009, Wong and Lazcano 2009).

Figure 2.1 Distribution of selected metabolic pathways on the 16S rRNA tree of life.



The tree (constructed with ARB; (Quast, Pruesse et al. 2012)) was edited for clarity and shows selected bacterial and archaeal taxa. The area of each branch is proportional to the total number of 16S rRNA sequences present in the database. Metabolic pathways were assigned based on physiological data (Supplementary Table 2). Distance of domains from first organism not intended to reflect evolutionary timescale.

Euk – *Eukarya*; E – *Euryarchaeota*; C – *Crenarchaeota*; T – *Thermodesulfobacteria*; A – *Aquificae*; F – *Firmicutes*; Cy – *Cyanobacteria*; Cx – *Chloroflexi*; N – *Nitrospirae*; Ch – *Chlorobi*; Delta – *Deltaproteobacteria*; Epsilon – *Epsilonproteobacteria*; Alpha – *Alphaproteobacteria*; Beta – *Betaproteobacteria*; Gamma – *Gammaproteobacteria*;

Figure 2.2 Earth's electron marketplace (annotated).

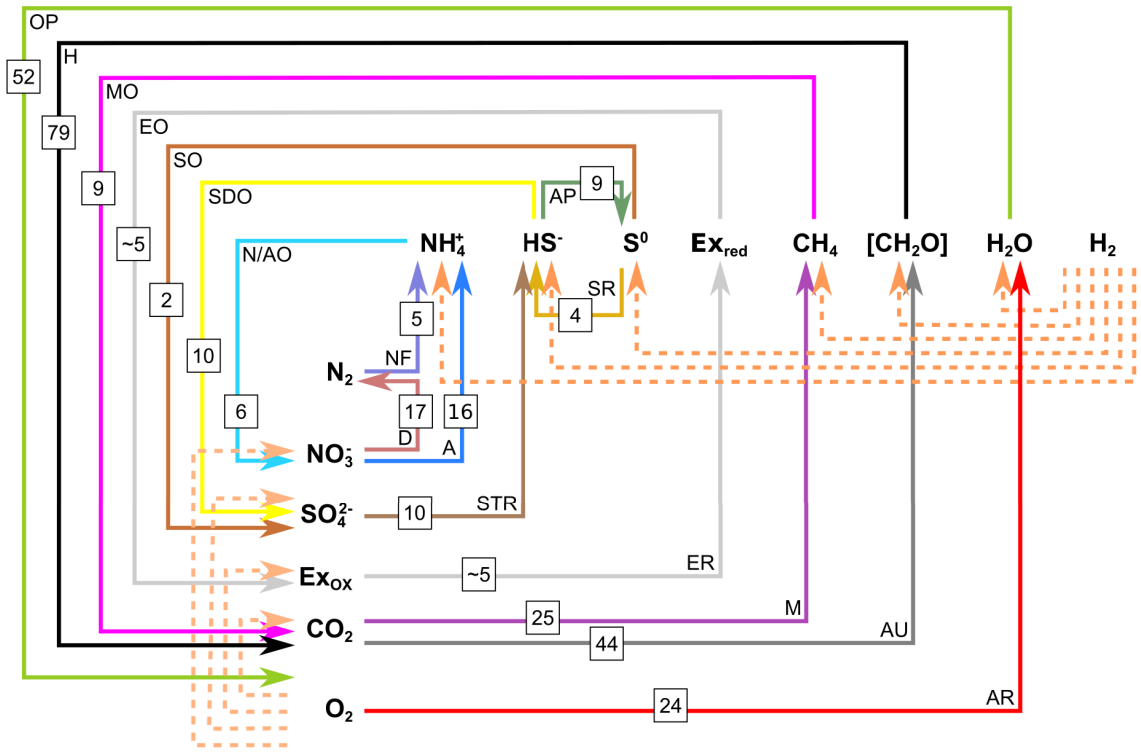
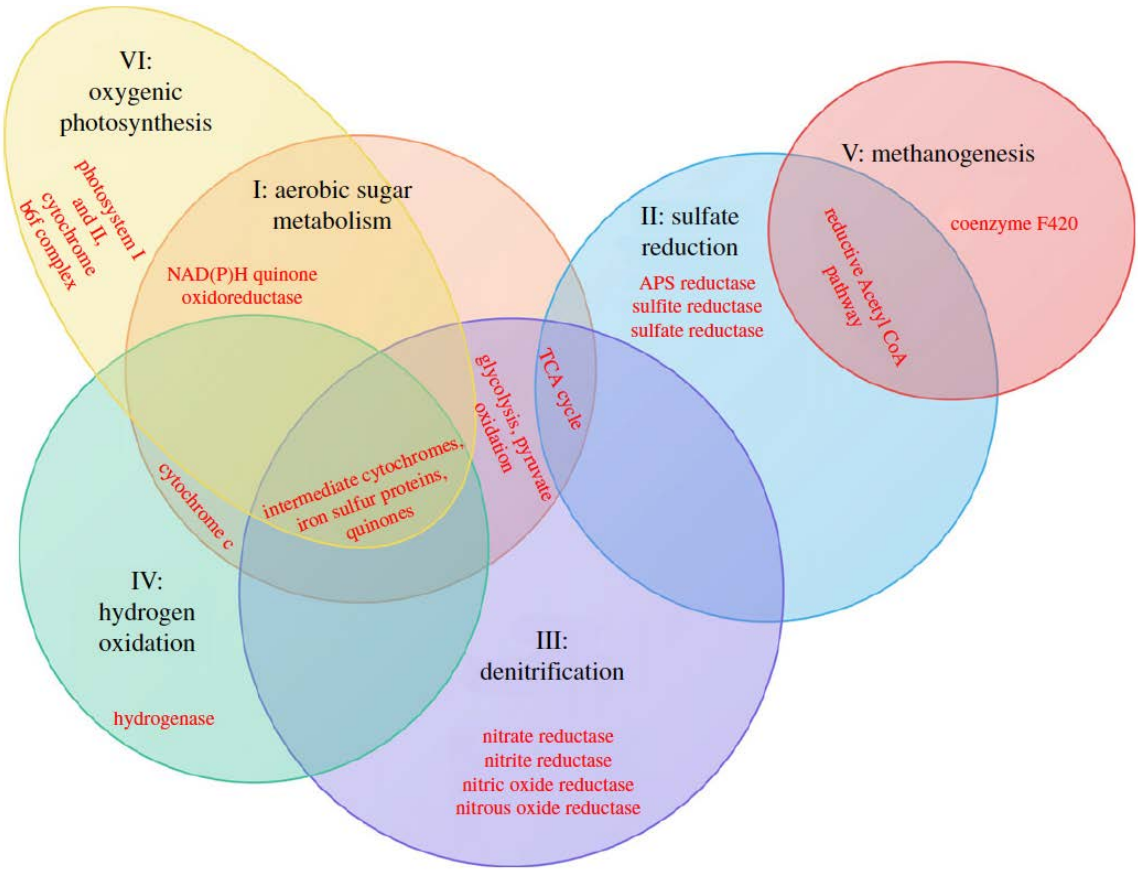


Figure adapted from (Falkowski, Fenchel et al. 2008). Numbers in boxes represent number of EC1 homologs responsible for redox reactions within metabolisms. Intracellular EC1 homologs omitted. The complete list of the 398 identified homologs is available as Supplementary Table 1. Arrows in top-left portion of figure represent oxidative reactions, arrows in the bottom-right represent reductive reactions. Solid lines connect redox couples as presented in Figure 3.1b. Dashed lines imply physical participation of O_2 or H_2 (H^+) rather than redox chemistry. OP – oxygenic photosynthesis; H – heterotrophy; MO – methane oxidation/methanotrophy; EO – other elements oxidation; SO – sulfur oxidation; SDO – sulfide oxidation; N/AO – nitrification/ammonia oxidation; AP – anoxygenic photosynthesis; SR – sulfur reduction; NF – nitrogen fixation; D – denitrification; A – ammonification; STR – sulfate reduction; ER – other elements reduction; M – methanogenesis; AU – autotrophy; AR – aerobic respiration

Figure 2.3 Venn diagram of the basic metabolic pathways



The metabolic pathway groups I to VI (large font) are shown to have redox components of electron-transport chains as well as auxiliary pathways in common (small font). Pathway group I: 'aerobic sugar metabolism' has components such as NAD(P)H reductases, as well as membrane-localized cytochromes and quinones, in common with pathway group VI: 'oxygenic photosynthesis'. Pathway group II: 'sulfate reduction' utilizes oxidized sulfur compounds as terminal electron acceptors and carry out carbon metabolism via reductive acetyl CoA pathway or the TCA cycle. Pathway group III: 'denitrification' uses unique oxidoreductases to reduce nitrates and other oxidized nitrogen compounds but still requires the common components of the electron-transport chain as well as glycolysis and the tricarboxylic acid (TCA) cycle as a source of reductant. Pathway group IV: 'hydrogen oxidation' uses an electron-transport chain but is not dependent on glycolysis or the TCA

cycle for producing reductant. Pathway group V: ‘methanogenesis’ does not use an electron-transport chain and is the only pathway using coenzyme F420. Size of circles is irrelevant.

CHAPTER 3

Co-Evolution of Microbial Metabolism with the Geosphere

3.1 Biogeochemical Cycles: Microbially Mediated Redox Reactions and the Geosphere

Although more than twenty-five elements can be found in biomolecules, six elements are most common (Da Silva and Williams 2001, Schlesinger and Bernhardt 2013). These are carbon, hydrogen, nitrogen, oxygen, phosphorus and sulfur, the so-called CHNOPS set of elements. Among these, phosphorus is the only element that exists primarily in a single oxidation state under most biological conditions (Pasek 2008) (Footnote 1). Considering biological redox reactions as a means of decoupling protons from electrons to generate the proton motive force necessary for energy transduction (*i.e.,* generation of ATP), all elements can be divided in two categories: carriers of both electrons and protons (hydrogen molecules) and carriers of only electrons. Of the major elements, CNOS are hydrogen carriers, with carbon and nitrogen higher in hydrogen density (*i.e.*, methane and ammonia respectively). In this context, all other redox elements of biological significance such as selenium, arsenic, iron and manganese are electron carriers only, and are minor players in the contemporary global electron marketplace (Nealson and Saffarini 1994, Lovley 2002). Using redox chemistry, biology sequesters CNOS into reduced oxidation states for construction of organic macromolecules (Kluyver and Donker 1926).

While multicellular eukaryotes contribute to a small number of elemental cycles (*e.g.,* organic carbon mineralization, photosynthesis), biogeochemical cycling of all other elements is largely under prokaryotic control (Vernadsky 1997, Whitman, Coleman et al. 1998, Wackett, Dodge et al. 2004, Falkowski, Fenchel et al. 2008, Offre, Spang et al. 2013,

Rousk and Bengtson 2014). The extremely high abundance of microorganisms on Earth (most recent estimates suggest a total of between 9 and 31 × 10^{29} cells) (Kallmeyer, Pockalny et al. 2012) highlights their quantitative importance in influencing the biogeochemical cycling of elements. Microbes (including eukaryotic algae) are responsible for up to 45% of carbon fixation and hence oxygen inputs to the atmosphere (Field, Behrenfeld et al. 1998) and about 95% of its consumption during the remineralization of organic matter. Because they are responsible for about half of N_2 fixation on Earth (Fowler, Coyle et al. 2013), microbes are responsible for approximately 50% of N_2O emissions, humans being responsible for the remaining 50% originating mostly from industrial N_2 fixation (Ravishankara, Daniel et al. 2009), albeit the N_2O emissions still from a microbially mediated process in an industrial setting (e.g. wastewater treatment or agriculture) (King, Kirchman et al. 2001) (Fowler, Coyle et al. 2013). In terms of global methane emissions, biogenic methane (including through microbial activity in industrial settings) accounts for 71%, whereas thermogenic (natural seeps and fossil fuel leaks) accounts for 23% and pyrogenic (incomplete combustion of biomass or biofuels) for about 6% of total emissions (Kirschke, Bousquet et al. 2013).

A detailed overview of individual biogeochemical cycles is beyond the scope of this review (see (Falkowski, Scholes et al. 2000, Gruber and Galloway 2008, Knittel and Boetius 2009, Canfield, Glazer et al. 2010, Fuchs 2011, Hügler and Sievert 2011)), however, a better understanding of the emergence and function of the electron marketplace as a whole is required to understand the direction and magnitude of ongoing changes (Bardgett, Freeman et al. 2008, Doney 2010). While single cycles are often described in separate models, microorganisms have evolved to couple specific cycles of CHNOS within their energy transduction schemes (Figure 2.2). For example, the oxidation of reduced

sulfur species (*e.g.,* sulfide, thiosulfate, elemental sulfur) is often coupled with the respiration (reduction) of oxygen or nitrate. The resulting energy obtained from these reactions is used to fix carbon dioxide within the same organism. The coupling of different CHNOS half-cells within an organism effectively interconnects the major biogeochemical cycles across time and space. This intimate connection between separate cycles can also be seen at the ecosystem level, with processes like denitrification able to limit the carbon cycle on large scales (Handoh and Lenton 2003, Falkowski and Godfrey 2008, Godfrey and Falkowski 2009). The individual species responsible for these reactions is largely irrelevant – as long as the metabolic processes are transferred across geologic time (Falkowski, Fenchel et al. 2008).

As discussed in section 2.3, a set of bacteriochlorophylls, phycobilins and chlorophylls evolved from hemes, allowing life to access the Sun as a practically unlimited energy source (Granick 1965). The first phototrophs were anoxygenic, similar to those found in today's anaerobic photic zones. Anoxygenic phototrophs accept electrons from Fe^{2+}, H_2 or H_2S to either Type I (FeS center) or Type II (pheophytin/quinone complexes) reaction centers (RCs) (*e.g.,* RCs of green sulfur bacteria and purple bacteria, respectively) (Blankenship 2010). The requirement for exhaustible electron donors, however, sets a metabolic limit on these autotrophs and their ability to fix carbon. The emergence of oxygenic photosynthesis, lifting limits on electron supply, is incompletely understood (Blankenship and Hartman 1998), but the ability to access a surfeit of electrons from unlimited H_2O greatly boosted the energy available to phototrophs. The active site responsible for oxidizing H_2O and releasing O_2, the so-called water oxidizing complex (WOC), is comprised of a unique tetramanganese complex, held in a specific orientation by the D1 protein of photosystem II. Phylogenies of cyanobacterial D1 sequences suggest

that the WOC evolved from an anoxygenic Type II photosystem (Cardona, Murray et al. 2015). The only extant prokaryotic phylum capable of oxygenic photosynthesis (Figure 2.1), the *Cyanobacteria*, express both Type I and Type II reaction centers. There are competing hypotheses for how *Cyanobacteria* obtained both RCs. The two major hypotheses are that the two reaction centers evolved independently and subsequently were incorporated into one organism *via* horizontal gene transfer (HGT), versus parallel evolution of both RCs in one organism *via* gene duplication followed by mutation and selection (Blankenship 2010). All photosynthetic eukaryotes share a common cyanobacterial ancestor acquired by endosymbiotic engulfment that resulted in the formation of plastids (Blankenship and Hartman 1998). The evolution of oxygenic photosynthesis and the transfer of its molecular machinery to eukaryotes massively enhanced the primary productivity of the planet (Canfield, Rosing et al. 2006).

3.2 Materials and Methods:

Panel A from Figure 3.1 was modified after (Lyons, Reinhard et al. 2014), with relative availabilities of important electron donors and acceptors overlaid (Anbar and Knoll 2002, Anbar 2008)(text size of molecule is proportional to inferred availability). A representative list of enzymes involved in catalyzing the redox pairs from panel B of Figure 3.1 was assembled, and the midpoint potentials of redox centers were investigated through literature search and given in Panel C of Figure 3.1. The list of enzymes and the sources for the midpoint potentials of their corresponding redox centers is given in Supplementary Table 3.

3.3 Life's Co-Evolution with Environmental Redox State

The intimate connection between the biosphere and geosphere can be inferred from geochemical proxies and physical fossils over the history of Earth (Dobretsov, Kolchanov et al. 2008, Moore, Jelen et al. 2017)(Figure 3.1a). The most intensively studied change occurred between 2.3 and 2.4 Gya and is called (somewhat euphemistically) the Great Oxidation Event (Farquhar, Bao et al. 2000, Canfield 2005, Holland 2006, Lyons, Reinhard et al. 2014). The oxidation of Earth's atmosphere is itself a consequence of the coordination of biologic and geologic processes (Falkowski and Isozaki 2008, Canfield 2014). Oxygenic photosynthesis appears to have evolved only once in prokaryotes (Figure 2.1), and led to the formation of molecular oxygen as a waste product. This process was necessary but not sufficient to lead to net accumulation of oxygen in Earth's atmosphere. On geologic time scales, net accumulation of oxygen in the atmosphere and ocean can only be achieved by the burial of organic matter (*i.e.,* the "global" half cell reductant) in the lithosphere and its sequestration by tectonic activity, either on cratons or deep into the mantle by subduction (where it is not reachable by heterotrophs) (Falkowski and Isozaki 2008, Canfield 2014, Manning 2014).

The initial accumulation of oxygen following the GOE appears to have been relatively modest, perhaps leading to only 1% of the atmospheric volume or less (Figure 3.1a), but it was sufficient to oxidize many transition metals on the surface of the planet (Williams 1981, Anbar and Knoll 2002). A second, and larger rise of oxygen occurred circa 750 Mya (Falkowski, Katz et al. 2004, Reinhard, Planavsky et al. 2013, Canfield 2014), and is attributed to the rise to ecological prominence of eukaryotic algae (Knoll 2014), which are larger and sink faster than cyanobacteria. The subsequent burial of carbon fixed by eukaryotic algae brought oxygen closer to its present atmospheric level (Lenton, Boyle et

al. 2014, Lyons, Reinhard et al. 2014), facilitating creation of further oxidized mineral species. As a consequence, approximately 2,800 new mineral species were subsequently formed, making biological processes responsible for a majority of the 5,000 mineral species estimated on the planet today (Hazen, Papineau et al. 2008)(rruff.info/ima).

Life occupies a large range of environmental redox states, reflected in the >1.6 volt range in midpoint potentials of the oxidoreductases (Figure 3.1c). The early metabolisms discussed in Chapter 1 (e.g. methanogenesis, sulfur reduction, sulfate reduction, hydrogen oxidation, working with half-cells in the range of –500 to 0 mV, formed the initial core of the global network of electron-transfer reactions and set the stage for metabolisms that would access half-cells with higher redox potential. Oxygenation of the oceans increased the usage of transition metals or cofactors able to access higher redox potentials, like heme and copper. A Winogradsky column classically demonstrates how metabolism can be coupled in a relatively small spatial scale across this range in potentials. The anaerobic organisms at the bottom of the column derive energy from the redox pairs available to fix carbon chemoautotrophically. To accomplish this, their enzyme cofactors possess appropriately low midpoint potentials that oxidize hydrogen and reduce low-potential substrates such as SO_4^{2-}. The facultative or aerobic organisms living above the anaerobes in the column occupy an increasingly oxidized environment. The cofactors at active sites of their redox enzymes show increasingly higher midpoint potentials, necessary to reduce, for example, NO_3^- (Figure 3.1c). At these active sites, enzymes rely on bound transition metals (*i.e.,* Fe, Ni, Mn, Co, Mo, W, Cu, V) or other cofactors like NAD or FAD that can readily switch between oxidation states at physiological pH ranges. The electrons are guided through protein structures via cofactors by outer-sphere electron transfer (Da Silva and Williams 2001). Cofactors have been selected for based on, among other factors, their

redox properties. Iron, for example, provides access to low reduction potentials, with examples in the Fe-binding active sites of hydrogenases, the enzymes oxidizing one of the strongest environmental reductants, H_2. Hemes, due in part to their delocalized π-bonds, can reach higher potentials. Finally, Cu gives access to relatively high midpoint potentials like that in cytochrome *c* oxidase, reducing one of the strongest biologically accessible environmental oxidants, oxygen (Castresana, Lübben et al. 1994, Liu, Chakraborty et al. 2014).

An electron transfer pathway is most efficient with cofactors in the pathway ordered by redox potential (Da Silva and Williams 2001), with centers distanced approximately 14 Å from each other (Page, Moser et al. 1999), both highly conserved structural features of EC1 enzymes. Cofactors generally coordinate with specific ligands, allowing a "fine tuning" of their native redox potentials (Hosseinzadeh and Lu 2015). The redox tuning is strongly related to the protein environment (Choi and Davidson 2011) and is generally accomplished by one of three broad mechanisms. The first is by controlling degree of solvent exposure; burying a redox center into a hydrophobic protein center will increase its reduction potential. The second is by adjusting the electrostatic environment of the center. For example, the addition or removal of nearby polar or charged amino acids can vary potentials by 100 to 200 mV (Liu, Chakraborty et al. 2014). Lastly, the hydrogen bonding networks around metal center ligands can also affect the redox potential, and have been shown to be primarily responsible for the differences in ferredoxin, rubredoxin and HiPIP (High Potential Iron Proteins) reduction potentials.

Midpoint potentials for Fe (E'_m) bound by different proteins range from –500 to +400 mV (Moffet, Foley et al. 2003), providing a large range in redox functionality (Fontecave 2006). A recent analysis of the secondary structure directly surrounding metal

centers in EC1 structures (*i.e.,* their tuning environment) illustrated an evolutionary transition from centers with high loop character, (implying higher rates of evolution and low catalytic specificity) to centers with either distinct **α**-helix or **β**-sheet character, bestowing high catalytic efficiency (high substrate specificity) at the expense of evolvability and locking these enzymes into their current form (Caetano-Anollés and Caetano-Anollés 2003, Kim, Senn et al. 2013). Nitrogenases and sulfite and nitrite reductases exemplify relatively promiscuous enzymes in terms of substrate specificity, implicating them as more evolvable and ancient. An illustration of the relationship between oceanic redox state, availability of redox couples, and the progression of metal center midpoint potentials is given in Figure 3.1.

The primary inputs of transition metals into the ocean environment include continental weathering processes, aeolian transport and output of hydrothermal fluids, with the main sinks being the formation of insoluble mineral oxides or sulfides. The oxidation state of the oceans has had profound effects on transition metal availability. Transition metal containing minerals come in two main “flavors” at Earth's surface: sulfides and oxides. Under mildly reducing conditions, iron, for example, primarily occurs as soluble Fe^{2+}. In the oceans of the Archean Eon, it is estimated that soluble, ferrous, iron ion concentrations were several micromolar (Anbar 2008). Following the GOE, ferrous iron became oxidized to insoluble ferric iron and precipitated as iron ferrihydroxides (rust). Similarly, manganese was extremely soluble as Mn^{2+} under reducing conditions and precipitated as manganese oxides following the GOE (Saito, Sigman et al. 2003). In contrast, copper and molybdenum were relatively insoluble under reducing conditions, and would have been found as sulfide mineral deposits in marine sediments. Upon oxidation of the ocean, these two would become mobilized as soluble ions, and the metals became increasingly available for

biological reactions (Anbar 2008) (Footnote 2). The change in the oxidation state of the oceans contributed to a change in the selection of metals incorporated into the active sites of oxidoreductases. Thus, the second most abundant metal found in EC1 proteins is copper.

As global oxygenation expanded, copper became widely exploited in aerobic pathways, including the direct access of O_2 as an electron sink for respiration (Ochiai 1983, Canfield 2014, Jelen, Giovannelli et al. 2016). Proteins containing copper generally have high midpoint potentials (Liu, Chakraborty et al. 2014) with functions including electron transfer, O_2 binding, O_2 activation and reduction to water, NO_2– and N_2O reductions, and substrate activation such as hydrogen atom abstraction (Klinman 1996, Solomon, Chen et al. 2001, Solomon, Heppner et al. 2014). Reduction potentials for type 1 copper proteins can range from +183 to +800 mV (Liu, Chakraborty et al. 2014). This highly positive range is not possible for iron redox chemistry to achieve. The availability of copper allowed access to the highest-redox-potential half-cells used by life, and therefore the capability to extract more energy than ever from carbon metabolites (Falkowski 2015). Even before the GOE, in the late Archean, it is possible that local pockets of oxidative weathering reactions were present in biological soil crusts and aquatic microbial mats (Crowe, Døssing et al. 2013, Riding, Fralick et al. 2014, Lalonde and Konhauser 2015). These localized oxygen-producing systems could have increased the availability of copper and potentiated early metabolisms involving aerobic oxidation of CH_4 and NH_4^+.

Proteome analyses have revealed a fundamental evolutionary constant, that the number of metal-binding proteins within a proteome scales with proteome size (Dupont, Yang et al. 2006). Given a relatively constant proteome size across the tree of life (within an order of magnitude), the selection of a different metal often leads to tradeoff of an existing metal. This musical-chairs evolutionary selection system has allowed each superkingdom's use of

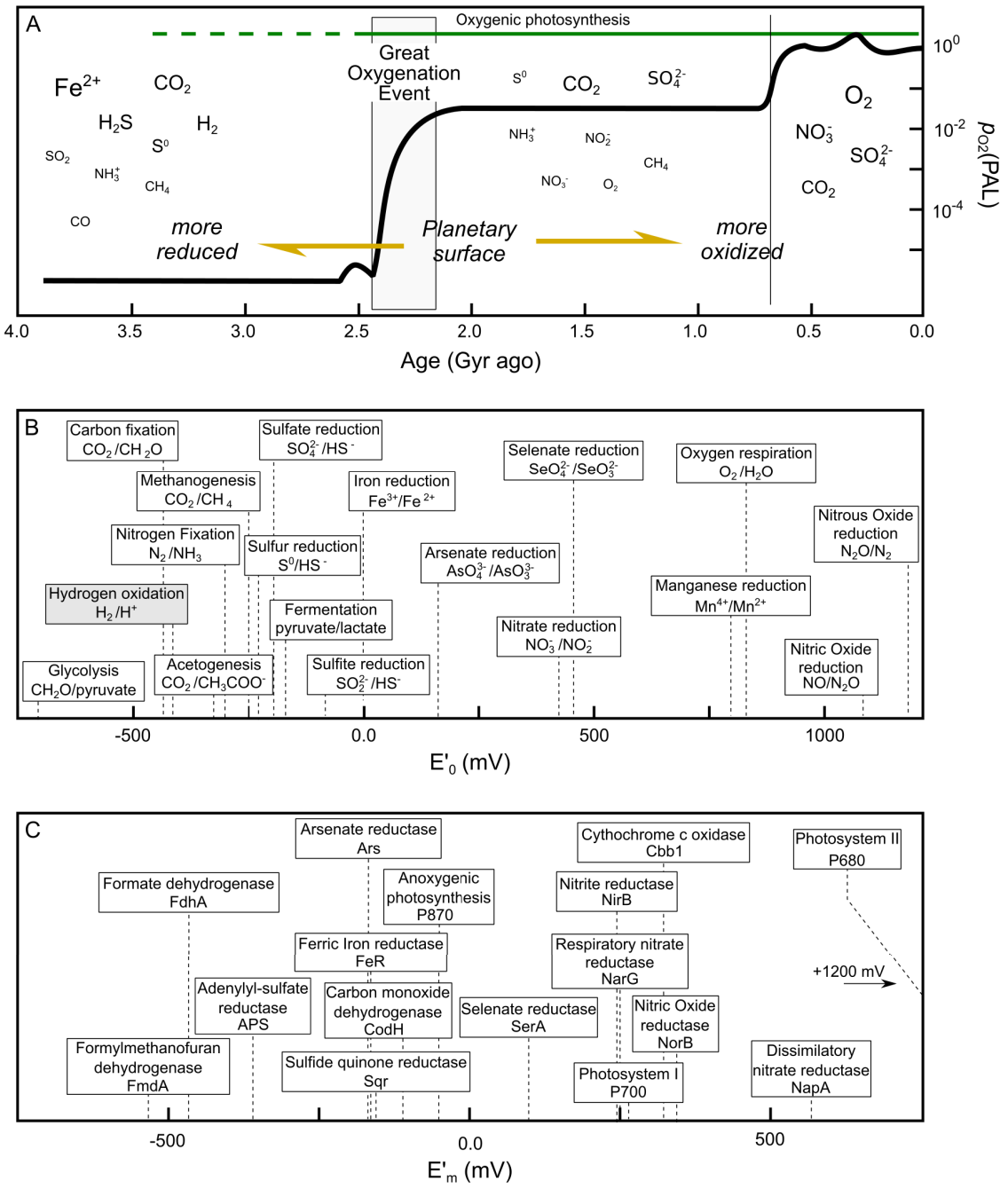
Fe, Mn, Zn and Co to evolve and correspond to the metals' bioavailabilities in the chemical environments at the time of their origin (Dupont, Yang et al. 2006). When manganese and copper became available as a result of increased oxidative weathering, folds evolved to bind these elements, driven by availability as well as selective pressure from an increasingly oxidizing environment. As organisms evolved in increasingly higher redox potential environments, folds that incorporated the Cu-binding domain of cytochrome *c* oxidase emerged, with the more reducing folds, once required by the reducing Archean environment, lost from the sequences encoded in their genomes. It is becoming increasingly compelling to geochemists and microbial biologists that biology has not only caused global redox trends (Falkowski, Fenchel et al. 2008, Canfield 2014) and expanded mineral evolution (Hazen, Papineau et al. 2008), but that the evolution of microbial genomes has responded to these trends through changes in the number and identity of metal binding folds (Dupont, Yang et al. 2006).

During the GOE, change was brought to much of the planet's anaerobic life in the form of oxygen, poisonous to metal centers, especially iron, in many anaerobic enzymes. Branches of life unable to evolve were forced to retreat into anoxic environments, in deep sediments and near ocean vents (Imlay 2006). While some of the evidence and specific mechanisms leading to the GOE are still debated (Canfield 1998, Arnold, Anbar et al. 2004, Kaufman, Johnston et al. 2007, Ducluzeau, Van Lis et al. 2009), sequestration of reduced carbon into the lithosphere uncoupled oxidative and reductive pathways, resulting in accumulation of oxidants (primarily O_2, NO_3^-, and SO_4^{2-}) at the planetary surface (Figure 3.1a), prompting the emergence of still higher midpoint potentials for catalytic centers like nitrate reductase and cytochrome *c* reductase (Figure 3.1c) (Castresana, Lübben et al.

1994). These molecules, accessing high-energy reactions made possible by free oxygen, significantly altered the landscape of biological metabolism (Raymond and Segrè 2006).

Through synthesis of the geochemical record with biological metal utilization and metabolism, the early Earth's biogeochemical network and the links between global redox state, metal availability, metabolic pathways and microbial evolution can be reconstructed (Moore, Jelen et al. 2017). In the reducing Archaean ocean at 3.8 to 3.4 Ga, sulfur reduction, sulfate reduction, methanogenesis and anoxygenic photosynthesis were discovered and spread amongst early prokaryotes. The appearance of nitrogen fixation between 3.2 and 2.9 Ga (or earlier) supported the expansion of microbial communities by providing a reliable source of fixed nitrogen (Moore, Jelen et al. 2017). As the ocean became oxidized, from 3.0 to 2.45 Ga, microbial electron transfer expanded to use positive redox potentials, allowing for the exploitation of new metal cofactors and electron transfer reactions. The emergence of oxygenic photosynthesis in cyanobacteria, between 3.0 and 2.7 Ga (Battistuzzi, Feijao et al. 2004, Battistuzzi and Hedges 2009, Bosak, Liang et al. 2009, Sim, Liang et al. 2012) altered the availability of other key metals, like copper, through the oxygen generated. The subsequent evolution of metabolic pathways closed key biogeochemical cycles and formed a complete circuit of biological electron transfer as exists today (Moore, Jelen et al. 2017).

Figure 3.1 Co-evolution of the geosphere and biosphere



Composite figure showing the co-evolution of geosphere and biosphere through time as depicted by change in planetary redox state, availability of redox couples and midpoint potential of EC1 enzymes. A – Schematic representation of oxygen's rise throughout Earth history modified after (Lyons, Reinhard et al. 2014). The two main oxygenation events are

presented together with the availability of relevant electron donors and acceptors (size proportional to the inferred availability; (Anbar 2008)). B – Standard reduction potential at pH 7 (E'_0) of biologically relevant redox pairs. Redox half reactions given represent the reductive side (*i.e.,* terminal electron acceptor) of given pathways. Hydrogen oxidation represents the only exception given as reference. C – Midpoint potential (E'_m) of key EC1 enzymes involved in selected energy metabolism pathways presented in B. The midpoint potential of the catalytic redox center is reported. A list of enzymes and the sources for the midpoint potentials of their corresponding redox centers is given in Supplementary Table 3.

CHAPTER 4

Elemental Sulfur Reduction In The Deep-Sea Vent Thermophile, *Thermovibrio Ammonificans*

4.1 Introduction

The evolution of geobiological electron transfer enzymes plays a large role in the co-evolution of Earth's geosphere and biosphere (Falkowski, Fenchel et al. 2008). Shuttling of electrons between life's macro-elements has had an evolutionary history at least as long as life itself, culminating in the tightly linked global metabolic network seen today. The genes encoding the enzymes responsible for oxidation and reduction reactions evolved, duplicated and mutated, and have been shared across many branches on the canonical tree of life (David and Alm 2011, Raanan, Pike et al. 2018). An overarching driver for the evolution of life's electron transfer machinery has been the progressive oxidation, both abiotic and biological, of Earth's oceans and atmosphere (Catling, Zahnle et al. 2001, Jelen, Giovannelli et al. 2016). The biogeochemical cycling of sulfur is of special interest in the evolutionary history of electron transfer reactions; energy transduction using sulfur cycling began early in Earth's history, well before the large-scale appearance of free oxygen in the environment (Philippot, Van Zuilen et al. 2007). Some of the oldest existing versions of electron transfer machinery may be those seen in deep-branching microbes reducing environmental sulfur compounds for energy conservation, such as *Thermovibrio ammonificans*. This bacterium is found today in geothermal habitats. It belongs to the deep branching lineage, Aquificae (family Desulfurobacteriaceae), and its genome includes slowly-evolving vestiges of the earliest microbial metabolisms as well as recently acquired traits (Giovannelli, Sievert et al. 2017).

Under normal environmental conditions, sulfur shares with nitrogen the widest range of stable redox states for elements involved in biogeochemical cycling, readily switching between the -2 to +6 oxidation states as well as stable states (*e.g.* S^0 or SO_2). Elemental sulfur (S^0 or $S_8{}^0$) is an oxidant that would have been bioavailable before the rise of oxygen. Its primary source is volcanism. Indeed, hydrothermal vents provide a habitat around which microbial communities capable of metabolizing sulfur thrive (Canfield and Raiswell 1999). Many chemical and biological oxidations of sulfides are incomplete, holding at S^0, rather than sulfur's highest oxidation state, sulfate, allowing elemental sulfur, whose solubility and reactivity at temperatures below 100 °C is low, to accumulate in sediments and geological deposits (Rabus, Hansen et al. 2013). Sulfur derivatives, mostly in the form of SO_2, are one of the most abundant components in volcanic gases, second in dry mass only to CO_2 (Stoiber 1995, Montegrossi, Tassi et al. 2001). Both can easily react with each other to form deposits of S^0 (SO_2 is usually more abundant in younger, active volcanoes) (Stoiber 1995). The direct precipitation from S^0 vapors and the oxidation of H_2S with metal ions in solution or with oxygen are other mechanisms of S^0 deposition (Steudel 1996, Xu, Schoonen et al. 2000). Sulfidic ores in exposed rock can be mobilized either chemically or by microbiological attack. As a consequence, S^0 and sulfur compounds are the most abundant sources both of electron acceptors and electron donors in volcanic environments and are used by a plentitude of microorganisms to support growth (Blöchl, Burggraf et al. 1995, Schönheit and Schäfer 1995, Stoiber 1995, Xu, Schoonen et al. 1998, Xu, Schoonen et al. 2000, Amend and Shock 2001, Price and Giovannelli 2013). In the context of Archean life at the vents, reduction of S^0 may have played a central role in the production of biomass. As opposed to nitrate or even sulfate, respiration of sulfur could have been among the earliest pathways of microbial respiration (Philippot, Van Zuilen et al. 2007), but much

about the genes and mechanisms behind the pathways for biological sulfur reduction remains unknown.

Microbial respiration of elemental sulfur occurs in a diverse set of organisms (Rabus, Hansen et al. 2013) via at least two distinct mechanisms. The metabolic strategy is almost ubiquitous among hyperthermophilic archaea, and not uncommon in bacteria. The two types of sulfur respiration pathway that have been studied in detail are those of the rumen bacterium *Wollinella succinogenes* (class Epsilonproteobacteria, phylum Proteobacteria) and the thermophilic archeon *Pyrococcus furiosus* (class *Thermococci*, phylum Euryarchaeota). *W. succinogenes* oxidizes H_2 via a [NiFe]-hydrogenase, with electrons transferred, via menaquinones, to a periplasmic membrane-bound polysulfide reductase, PsrABC (Kletzin, Urich et al. 2004). PsrA is responsible for polysulfide reduction to H_2S, at a molybdopterin active site, PsrB is an [FeS] electron transfer protein and PsrC is a quinone-containing membrane anchor (Jormakka, Yokoyama et al. 2008). In addition, a polysulfide transferase (Sud) protein is believed to increase the availability of sulfide from protons and sulfur (Lin, Dancea et al. 2004). Homologous mechanisms to *W. succinogenes* for energy-conserving sulfur respiration, terminating in a molybdopterin oxidoreductase, have been found in a variety of phylogenetically diverse organisms (Schut, Bridger et al. 2007, Liu, Beer et al. 2012), including the archaea, *Acidianus ambivalens* (Laska, Lottspeich et al. 2003), *Pyrodictium abyssi* (Dirmeier, Keller et al. 1998) and *Pyrodictium brockii* (Pihl, Black et al. 1992) and in the bacterial genus *Shewanella* (with the exception of *S. denitrificans* (Burns and DiChristina 2009, Warner, Lukose et al. 2010)).

The archaeon *P. furiosus* grows by fermentation of carbohydrates, with acetate, CO_2 and H_2 as end products. With S^0 added to a growing culture, production of H_2 decreases dramatically and H_2S can be detected. An up-regulated membrane-bound oxidoreductase

(MBX) cluster is believed to oxidize ferredoxin and reduce the sulfane sulfur of polysulfide (Wu, Schut et al. 2018). An up-regulated NAD(P)H elemental sulfur oxidoreductase (NSR) was thought to have oxidized NAD(P)H produced by MBX, reducing S^0 and releasing H_2S (Schut, Bridger et al. 2007). However, NSR knockouts in *P. furiosus* were still able to grow with S^0 as electron acceptor (Bridger, Clarkson et al. 2011), and now MBX, renamed MBS, is thought to directly reduce polysulfides, with the di and tri-sulfides produced spontaneously converting to S^0 and H_2S (Wu, Schut et al. 2018). This mechanism has so far only been found in members of the heterotrophic *Thermococci* class.

The pathway of sulfur respiration in several strictly anaerobic deep-sea vent microbes, including members of the Aquificae and of the Epsiloproteobacteria, is unclear. Homologs to PsrABC are present in the genomes of S^0-reducing Epsilonproteobacteria, but they are missing, along with the MBX cluster, in the Aquificae (Campbell, Smith et al. 2009, Giovannelli, Ricci et al. 2012). Instead, within the *T. ammonificans* genome, homologs to the genes coding for NSR and for the sulfide quinone reductase (SQR) have been identified (Giovannelli, Sievert et al. 2017). SQR, commonly involved in the oxidative conversion of sulfide to S^0, has been characterized in anoxygenic phototrophs (Arieli, Shahak et al. 1994).

The study of deep-branching microorganisms has both evolutionary and ecological relevance. From an evolutionary perspective, the genomes of these bacteria, including *T. ammonificans*, hold important clues to early microbial metabolism and its evolution. In such genomes, distinguishing between the set of core, or vertically inherited genes and the set of acquired, or horizontally transferred genes, can help distinguish the microbe's ancestral state from recent adaptations to the modern Earth (Giovannelli, Sievert et al. 2017). *T. ammonificans* and its close relatives are also critically important from an ecological standpoint. First, these bacteria fix CO_2 of geothermal/magmatic origins in the

absence of oxygen and therefore they represent the primary producers in marine geothermal environments; second, they conserve energy by nitrate and S^0 respiration, contributing to the cycling of nitrogen and sulfur. The genes and pathways involved in the respiratory metabolism of *T. ammonificans* are only partially known. The first step in nitrate respiration is catalyzed by a periplasmic nitrate reductase (NapA; (Giovannelli, Sievert et al. 2017)), but the genes involved in S^0 respiration are unknown. The purpose of this study is to identify them. Our findings are relevant to understanding the *T. ammonificans* pathway of S^0 reduction as well as teasing apart its core (S^0 reducing) and acquired (nitrate reducing) respiratory metabolism. This is key to aligning biological data with geochemical predictions of Archean environments (Shock, McCollom et al. 1995, Moore, Jelen et al. 2017). Knowledge of life's earliest electron transfer proteins will help in understanding the overall evolution of electron transfer machinery on Earth (Jelen, Giovannelli et al. 2016).

4.2 Materials and Methods

4.2.1 Genome analysis

T. *ammonificans* is known oxidize dissolved H_2 and to respire nitrate or sulfur, but not sulfate or thiosulfate (Vetriani, Speck et al. 2004). The genome of *T. ammonificans* was previously sequenced and annotated (Giovannelli, Ricci et al. 2012). Of the 1888 genes predicted in the genome, putative genes involved in hydrogen oxidation and sulfur respiration were identified by BLAST using previously characterized gene sequences (*e.g.,* polysulfide reductase from *Wolinella Succinogenes*, sulfide quinone reductase from *Oscillatoria limnetica*) or by ontology using functional search terms (*e.g.,* hydrogenase, sulfur, polysulfide).

4.2.2 Culture conditions and calculation of growth parameters

Cultures of *Thermovibrio ammonificans* strain HB-1 were grown in modified SME medium, as previously described (Vetriani, Speck et al. 2004). The Na_2S titrated to ensure an anoxic culture resulted in a slightly sulfidic (<1mM) medium at pH 5.5. Cultures were incubated at 75°C with two different electron-acceptors. For nitrate reduction, anoxic batch cultures were grown at a starting concentration of 20 uM nitrate in the medium. For S^0 reduction, 3% solid sulfur, by weight, was added to modified SME medium without nitrate. In both cases, the headspace consisted of H_2/CO_2 (80%:20%) at 30 psi. The headspace of the S^0 reducing cultures was flushed and replaced every 2 hours, and the tubes were shaken vigorously. To test for sulfur disproportionation, cells were incubated over a two-week period in stoppered tubes containing sulfur-amended SME medium in which the H_2/CO_2 headspace was replaced with N_2/CO_2 (80%:20%). To allow for greater partitioning of H_2S into the gas phase and to maintain favorable thermodynamic conditions for sulfur disproportionation, the medium to headspace ratio was increased from 1:2.5 to 1:4 (Amenabar, Boyd et al. 2018). Growth curves for both nitrate and sulfur reducing cultures were obtained from direct counts of cells sampled during exponential growth. For fluorescence microscopy, cells were fixed with 25% v/v gluteraldehyde and stained with 0.1% w/v acridine orange as previously described (Vetriani, Speck et al. 2004). Direct cell counts were performed on an Olympus BX 60 epifluorescence microscope with an oil immersion objective (UPlanF1 100). Growth rates (μ; h^{-1}) were estimated as: $\mu = (\ln N_2 - \ln N_1)/(t_2 - t_1)$, where N_2 and N_1 are number of cells ml^{-1} at time (in h) $t_2 - t_1$. Generation times (t_g; h) were calculated as: $t_g = (\ln 2)/\mu$ (Vetriani, Speck et al. 2004).

To investigate the necessity of cellular access to the bulk sulfur for growth, 50 mL aliquots of SME medium were supplemented with the elemental sulfur sequestered within 6-8 kilodalton dialysis tubing and inoculated with cells of *T. ammonificans*. Briefly, approximately 15 cm of dialysis tubing (6-8 kD MWCO, 10 mm Flat-width) was cut, one end was sealed with a weighted dialysis tubing closure, and 0.75g of elemental sulfur was carefully added to the inside of the tubing through the remaining opening. The tube was sealed with a second closure, then rinsed and soaked in deionized water for an hour to ensure removal of any external sulfur. The dialysis tubing was then introduced into the culture vessel. Cultures grown with sulfur exposed fully to the medium (no dialysis membrane) were used as positive controls, while sterile, uninoculated media containing sulfur sequestered in dialysis membranes, but without *T. ammonificans* cells were used as negative controls. The supernatant was periodically tested for cells and sulfide production over the course of two weeks. Sulfide concentration was assessed using a photometric $CuSO_4$ assay previously described (Cord-Ruwisch 1985). Scanning electron microscopy (SEM) micrographs of the inside and outside of tubing were obtained on a Phenom ProX scanning electron microscope at 10kV, once cultures had been sacrificed at two weeks. The same instrument was used at 15kV to perform Energy Dispersive X-Ray Spectroscopy (EDX) for elemental analysis of particles in the samples.

4.2.3 Cell harvesting for transcriptomic and proteomic analyses

All cultures of *T. ammonificans* were harvested during the late exponential growth phase. Nitrate-reducing cultures (80 mL for RNA extractions, 100 mL for protein extractions) were cooled, shaken and collected by centrifugation for 20 min at 12,000 g at 4°C. The cell pellet was resuspended and transferred to 2 mL microcentrifuge tubes to consolidate the biomass into a single tube. Sulfur-reducing cultures (600 mL total for RNA extractions, 200 mL for protein extractions) were cooled and sonicated three times on ice to detach cells from sulfur granules (30 seconds of sonication followed by vigorous shaking each time). After final shaking, large sulfur particles were let to settle for about a minute. The resulting supernatant was transferred to centrifuge tubes, with care taken to avoid transferring the solid sulfur at the bottom or floating at the top of the culture. Cells were collected by centrifugation, the pellet was re-suspended and cells were consolidated into single 2 mL microcentrifuge tubes by successive centrifugations.

4.2.4 RNA extraction

Total RNA was extracted from cells of triplicate cultures of *T. ammonificans* grown under nitrate-reducing and sulfur-reducing conditions. The cell pellets were frozen in liquid N_2 and thawed three times, supplemented with 7 µL of β-mercaptoethanol, 100 µL of lysozyme solution (3mg/mL), vortexed and incubated at 37°C for 15 minutes. Subsequently, 50 µL 10% SDS were added and the mix was incubated at 37°C for 10 min, with brief vortex every 2-3 minutes. An equal volume of phenol (pH 4.3) was added to the mix, the tubes were inverted by hand 100x, centrifuged for 3 minutes at 10,000 × g and the aqueous phase was retrieved This extraction was repeated three times followed by an extraction with an equal volume of chloroform/isoamyl alcohol (24:1). The aqueous phase

was precipitated with 0.1 volumes of 3.0M Na-acetate and 0.6 volumes of 100% isopropanol overnight at -20°C. The pellet was recovered by centrifugation, washed with 500 µL 80% EtOH and air dried. The pellet was then re-suspended in DEPC-treated sterile H_2O. DNA digestion was performed using the Ambion DNA-Free DNAse treatment; 5 µL RNA, 5 µL Buffer, 39 µL DEPC-H_2O and 1 µL DNAse were incubated for 30 minutes at 37°C and inactivated for 5 minutes at room temperature. To rule out carryover of genomic DNA, an aliquot of the DNAse-treated RNA was subjected to a PCR amplification of the 16S rRNA gene using primers Bact 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and Univ 1517R (5'-ACGGCTACCTTGTTACGACTT-3'). The product of the PCR reaction was then inspected on an agarose gel to ensure that no amplification had occurred and that no carryover DNA was present in the RNA samples. Samples were stored at -80°C and used for RT-qPCR and for transcriptomic analyses. The Nanodrop ND-1000 spectrophotometer was used with the RNA-40 setting to quantitate RNA throughout the extraction and purification processes.

4.2.5 Transcriptomic analyses

To lower the amount of rRNA, each RNA preparation was subjected to subtractive hybridization using the RiboZero kit (Bacteria) by Illumina following the manufacturer protocols. This kit was selected since the genome sequence of *T. ammonificans* was tested in silico by Illumina and the bacterium is listed as a compatible species for the subtractive hybridization. rRNA-depleted RNA was reverse transcribed using the First Strand cDNA synthesis kit by Invitrogen following the manufacturer protocol. Obtained cDNA was sequenced on an Illumina HiSeq 2500 sequencing platform (Molecular Research LP, Shallowater, TX), resulting in ca. 10 million 2x150bp paired end reads for each sample.

Obtained reads were QC/QA using FastX toolkit (Gordon and Hannon 2010). Low quality reads, reads with low-quality nucleotide scores and reads shorter than 50 bp were removed. Kallisto (Bray, Pimentel et al. 2016) was used to map the quality checked reads against the *T. ammonificans* genome and estimate transcript abundances were expressed as transcripts per million (TPM), which indicates the proportion of transcripts in the pool of RNA (Li, Ruotti et al. 2009). The accompanying R tool for RNA-seq analysis, Sleuth (Pimentel, Bray et al. 2017) was used to investigate the results and calculate differential expression of genes for the different conditions and evaluate intra- and inter-condition variability. Up- and down-expressed genes were selected using a cutoff of $p<0.01$, representing a high confidence cut off for differential expression analysis.

4.2.6 Protein extraction and proteome analyses

In order to further investigate the response of *T. ammonificans* to different electron-acceptor regimes, triplicate comparative proteomic experiments between cultures grown under nitrate reducing vs. sulfur reducing conditions were performed. The protein fraction was extracted from cell biomass. 50 µL of 2X Laemmli buffer was added to each sample, which was then sonicated, heated at 60 °C for 10 min, and centrifuged, with supernatant saved. 50 µL of 8M urea was added, sonicated and the supernatant from previous step added back. Sample was then centrifuged and supernatant saved. 100 µL of 2X Laemmli buffer was added, then the sample was sonicated, frozen, thawed and frozen at -80C overnight. The supernatant from the previous step was added back, the sample centrifuged, and protein concentration measured at 660 nm using a colorimetric assay (Pierce™ 660nm Protein Assay Reagent; Thermofisher, Waltham, MA). SDS-PAGE gel purification, 29 µg/sample, in duplicate was then performed. Label-free LC-MS was carried out on the six

samples, and open-source X! Tandem software was used to match spectral data to peptide sequences within the *T. ammonificans* genome. Spectral counts for each of the six groups were modeled using a binomial distribution, with the respective group total as a denominator (thus normalizing the six datasets). For each protein, the two conditions were compared using logistic regression, a type of regression based on binomial distributions that allows for covariates. The overdispersion (between-replicate variability) was corrected for, resulting in a coefficient estimate, a standard error, a z-value (which is the ratio of the two), and finally a p-value. The p-value permissive results ($p<0.01$) were ordered by z-value to infer the most differentially expressed proteins between nitrate reducing and sulfur reducing conditions.

4.2.7 RT-qPCR

Primers for genes putatively involved in sulfur and nitrate respiration (Table 4.1), as well as for the 16S rRNA gene, were designed using Perl Primer for amplified fragments of about 200bp, appropriate for RT-qPCR. Primer sequences are as follows: *napA*_forward GCATACCGCTACAATGACC, *napA*_reverse TAAACGAAGACCATACCCTCC, *nsr*_forward TGACAATATCCTTGCCGCC, *nsr*_reverse AAAGCCGAAGAACCTGCC, *sqr*_forward CAATGTTTATACCCGCAACCA, *sqr*_reverse GATTCTCGCCATAACTTCGG, *16S rRNA*_forward GCTGAAACTCAAAGGAATAG, *16S rRNA*_reverse GACTTAACCCAACACCTC.

A Stratagene MX3000P qPCR instrument was used for RT-qPCR, according to the reaction and cycling protocols set forth by the iTaq Universal SYBR Green One-Step Kit. Each 50µL PCR tube contained 0.5µL of each forward and reverse primer, 25µL of

Mastermix (containing Taq DNA polymerase, dNTPs, $MgCl_2$ and reaction buffer), 2µL SuperScript III reverse transcriptase(RT), 21µL of DEPC-treated H_20 and 1µL of RNA sample (in experimental triplicate), or for controls (in duplicate), 1µL DEPC-treated H_20. After a 30min RT phase at 55 °C, RT was deactivated for 2min at 94 °C, and then 35 cycles of qPCR were performed, 15 sec at 94 °C, 30 sec at 45 °C and 1min at 68 °C for each cycle. Melt curve analyses were performed on all PCR products. Primer efficiencies were calculated for each primer pair by performing qPCR on dilutions of genomic DNA, from 1x to 10,000x dilution. Efficiencies (Eff) were calculated from the slope resulting from Ln(DNA conc.) vs. CT (crossing threshold) and using $Eff=10^{(-1/slope)}$ according to (Pfaffl 2001). The relative quantifications of target gene transcripts in comparison to a reference transcript (16S rRNA) were calculated according to Equation 1 from (Pfaffl 2001). The RT-qPCR experiments were compliant with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin, Benes et al. 2009).

4.2.8 Phylogenetic analyses

Protein sequences homologous to the NSR-like and SQR-like proteins of *T. ammonificans* were collected by searching the NCBI non-redundant database by protein BLAST search. SQR and NSR sequences from a close relative to *T. ammonificans, Phorcysia thermohydrogeniphila* (Pérez-Rodríguez, Grosche et al. 2012), were included (NCBI accession numbers MH010574 and MH010575, respectively). Sequences were aligned using muscle (Edgar 2004) and the alignment manually refined in Seaview (Galtier, Gouy et al. 1996). A primary neighbor-joining phylogenetic tree of over 200 SQR-like homologs, including the SQR-like *T. ammonificans* pyridine nucleotide reductase, was used to identify Type VI sequences, to which the *T. ammonificans* SQR-like sequence belongs (Marcia, Ermler et al. 2010). Phylogenetic trees of the resulting Type VI SQR and

the NSR alignments were constructed using the Maximum Likelihood algorithm with Phylo-ML (Guindon and Gascuel 2003), using the LG+I+G model chosen by ProtTest (Abascal, Zardoya et al. 2005), and aLRT scores calculated.

4.3 Results

4.3.1 Genome analysis

While *T. ammonificans* is able to reduce elemental sulfur to hydrogen sulfide, its S^0 reduction pathway remains unclear. We searched the genome of *T. ammonificans* for proteins that showed similarity to known sulfur respiration enzymes. Two protein sequences from the *T. ammonificans* genome showed similarity to NADH-dependent sulfur reductase (NSR) from *P. furiosus* (WP_011012327.1): Pyridine nucleotide-disulfide oxidoreductase WP_013537753.1 and pyridine nucleotide-disulfide oxidoreductase WP_013538070.1 (32% identity, eval 6E-43 and 31% eval 3E-40 respectively). A NiFe-dependent hydrogenase large subunit (WP_013537242.1) showed similarity to *P. furiosus* sulfhydrogenase alpha (WP_011012029.1; 37% identity, eval 2E-83) and a NADH ubiquinone oxidoreductase (WP_013537243.1) was homologous to *P. furiosus* sulfhydrogenase delta (WP_011012028.1; 40%, e-val 7E-59). No genes with similarity to the SurR sulfur response regulator (Lipscomb, Schut et al. 2017) were found in the *T. ammonificans* genome. The *T. ammonificans* sequence WP_013537594 is annotated as a Sulfide Quinone Reductase (SQR) by KEGG (Kyoto Encyclopedia of Genes and Genomes), though the function of this sulfide oxidizing gene in a sulfur-respiring thermophile is not clear.

Using the PsrA (polysulfide reductase) subunit from *W. succinogenes* (CAA46176.1), two low identity hits were found within the *T. ammonificans* genome, a molybdopterin

oxidoreductase (WP_013537181.1) (23% identity, eval 2E-27) and a molybdopterin dinucleotide-binding region protein WP_013537976.1 (24% identity, eval 9E-20), respectively. Blasting PsrB (CAA46177.1) against *T. ammonificans* gave a molybdopterin dinucleotide-binding region protein WP_013537976.1 (33% identity, eval 1E-25) and [4Fe-4S] ferredoxin WP_013537752.1 (34% identity, eval 1E-15). No genes with significant similarity to PsrC or sulfide dehydrogenase were found.

T. ammonificans can also use nitrate as a terminal electron acceptor, reducing it to ammonium. The periplasmic nitrate reductase catalytic subunit NapA has been previously identified, and is encoded by the gene *napA* with locus Theam_0423 (WP_013537181.1) (Giovannelli, Sievert et al. 2017).

4.3.2 Growth kinetics

T. ammonificans was grown under nitrate- and sulfur-reducing conditions, by supplementing batch cultures with either one of the two terminal electron acceptors, under otherwise identical conditions. Non-flushed sulfur grown cultures reached dissolved sulfide concentrations of up to 3mM by the late exponential phase. Calculations of growth kinetics revealed doubling times of 0.61 h (± 0.16 h) in nitrate-reducing cultures and 1.8 h (± 0.63 h) in regularly flushed and shaken sulfur-reducing cultures (non-flushed sulfur culture had a doubling time of 6.33 h). Sulfur-reducing cultures consistently grew more slowly than nitrate-reducing cultures, but similar cell densities could be achieved after 18 h ($>1\times10^9$ cells/mL) when the headspace was flushed with H_2/CO_2 every 2 h to reduce the accumulation of H_2S. The short rod morphology and size of the cells was similar under the two experimental conditions. Cultures grown on elemental sulfur appeared to have produced an extracellular polymeric substance, clearly visible as remaining in the non-

aqueous layers after nucleic acid extractions. These substances were not seen during extractions of cultures grown with nitrate as electron acceptor.

4.3.3 Scanning Electron Microscopy

To investigate cellular access to solid bulk sulfur, *T. ammonificans* cultures and sterile controls (in triplicate) were imaged by scanning electron microscopy after 24 h and 48 h of incubation. Micrographs revealed that the sulfur granules had a pitted appearance once *T. ammonificans* had partially reduced them as terminal electron acceptor for growth (Figure 4.1 d-f). After 24 h growth, small pits appeared, from 0.5 to 2 μm in diameter, with larger pits appearing sporadically. In cultures grown for 48h, deeper pits up to 7 μm wide were ubiquitous and overlapping. In comparison, the surface of the sulfur granules in the sterile controls appeared smooth (Figure 4.1 a-c).

Samples of cultures prepared by filtration onto 0.2 μm filters and subsequent freezing on the Phenom cryo-stage, were examined by SEM to further visualize the cells and their physical relationship with bulk sulfur. Images from sulfur cultures revealed the presence of bright mineral nanoparticles, only about 40-100 nm across, ubiquitous throughout the culture and appearing in clusters or strings (Figure 4.2a). While we attempted to confirm that the nanoparticles consisted of sulfur using Energy Dispersive X-Ray Spectroscopy, the particles were below the size threshold for detection. Cells did not seem to be attached to bulk sulfur or directly associated with the nanoparticles. The nanoparticles were absent from the nitrate-reducing cultures and sulfur sterile controls (not shown).

4.3.4 Incubation of T. ammonificans cells with sulfur sequestered in dialysis tubes

In order to investigate cellular need for direct access to bulk sulfur, elemental sulfur was sequestered in dialysis tubing with a 6-8 kDa pore size. Cultures of *T. ammonificans* in which sulfur was directly accessible to the cells were carried out as positive controls. Sulfide levels measured in positive controls (direct contact with bulk sulfur allowed) were higher than 10 mM after 4 days, a clear indication that cells reduced sulfur to hydrogen sulfide.

Direct cell counts, taken over the same period, did not indicate net growth in cultures with bulk sulfur sequestered in dialysis tubes, and the concentration of sulfide did not increase. Positive controls grew to about 10^8 cells ml^{-1} at two weeks, whereas no cells were detected in negative controls. Dialysis tubing from two-week old cultures were opened and revealed dark-colored sulfur around the inside surface of the tubing, suggesting gradual reduction of the sequestered sulfur through the dialysis tubing. However, bright yellow, oxidized sulfur remained in the deeper interior of the bag and no color change was observed in dialysis tubing in the no-cell controls. SEM images from the inside surface of the dialysis tubing of inoculated cultures revealed the presence of sulfur nanoparticles in a large range of sizes (Figure 4.2 b-c). These nanoparticles were completely absent in dialysis tubing from the negative controls (not shown).

4.3.5 Sulfur Disproportionation

T. ammonificans has been characterized as a strict hydrogen-oxidizing chemolithoautotroph. However, its ability to disproportionate elemental sulfur coupled to H_2S production had not been previously investigated. To this end, cells were incubated over a two-week period in stoppered tubes containing sulfur-amended SME medium in

which the H_2/CO_2 headspace was replaced with N_2/CO_2. Under these conditions, neither growth nor production of hydrogen sulfide occurred.

4.3.6 Comparative transcriptomic analyses of sulfur vs. nitrate-reducing cultures

To identify the genes and proteins involved in respiratory S^0 reduction in *T. ammonificans*, we carried out comparative transcriptomics and proteomics analyses of cultures grown under sulfur- and nitrate-reducing conditions. We hypothesized that cells grown with sulfur as the terminal electron acceptors would differentially express genes necessary for sulfur respiration compared to nitrate-reducing cells.

To compare gene transcript abundance between nitrate- and sulfur reducing-cultures, each culture was grown in triplicate and samples were collected during the late exponential growth phase. Among the gene transcripts putatively involved in S^0 reduction, the *sqr*-like gene (encoding for WP_013537594) was one of the most differentially expressed overall, while the *nsr*-like gene (encoding for WP_013538070) showed no significant differential expression between the two conditions sampled (Table 4.1).

Principal component analysis between samples showed that the variability within replicates of the same condition was minimal, with the exception of five transcripts that showed significant variability: RNAse (THEAM_RS09515), 23S Ribosomal RNA (THEAM_RS00860; THEAM_RS05970), 16S ribosomal RNA (THEAM_RS05985), and one hypothetical protein.

Of the 1888 genes predicted in the genome of *T. ammonificans*, 312 were differentially transcribed in sulfur reducing vs. nitrate reducing conditions with a conservative p value < 0.01. Of these 312 transcripts, 129 were downregulated, and 183 were upregulated during growth on sulfur vs. nitrate. The top ten up and down-regulated

genes are reported in Table 4.2. Along with the *sqr*-like oxidoreductase of the FAD-dependent pyridine nucleotide-disulfide family (WP_013537594), the top upregulated gene transcripts included those encoding a general function peptidase U32 (WP_013537275), a signal transduction protein (WP_013537978), an iron (metal) dependent repressor (WP_013537721), a MerR family transcriptional regulator (WP_013537823) and several hypothetical proteins. The ten most down-regulated gene transcripts included those encoding the isocitrate dehydrogenase (WP_013537776), two subunits of the NADH-ubiquinone dehydrogenase (WP_013538235; WP_013538236), NADH dehydrogenase (WP_013538241), a transport system permease protein (WP_013536952) and a [4Fe-4S] ferredoxin (WP_013537752). All raw transcriptomic data is available in the ncbi SRA (sequence read archive) database, accession SRP131525.

4.3.8 RT-qPCR analyses of gene transcripts of sulfur and nitrate respiration genes

In order to verify differential transcription of the genes putatively involved in sulfur, we performed RT-qPCR on three selected genes encoding: the SQR-like oxidoreductase of the FAD-dependent pyridine nucleotide-disulfide family (WP_013537594; highly upregulated in the transcriptomic analyses), the NSR-like oxidoreductase (WP_013538070), also related to the FAD-dependent pyridine nucleotide-disulfide family, and the catalytic subunit of the periplasmic nitrate reductase, NAP (WP_013537180; Table 4.1). The transcripts encoding for the NSR-like protein (WP_013538070) showed no significant variations between the two conditions, whereas the *sqr*-like gene was significantly up-regulated during growth on sulfur (2.4 fold change, standard deviation 1.10; Table 4.1). RT-qPCR results indicated that the transcripts for the NapA nitrate reductase were significantly downregulated in sulfur-reducing conditions as compared to

nitrate reducing cultures; however, the transcriptome data did not show a statistically significant downregulation of the *napA* transcripts (Table 4.1).

4.3.8 Comparative proteomic analyses of sulfur vs. nitrate-reducing cultures

Proteomic analyses were carried out on triplicate nitrate- and sulfur-reducing cultures of *T. ammonificans* and proteins that were both up and down regulated were identified. Proteomic data for the putative sulfur-reducing genes of interest from the genome analysis are highlighted in Table 4.1. The SQR-like (WP_013537594) and NSR-like (WP_013538070) proteins were significantly over-expressed in sulfur-reducing cultures, with a fold change relative to the nitrate-reducing conditions of 8.76 and 3.95, respectively (Table 4.1).

Between the two growth conditions, 111 proteins were differentially expressed with a conservative p value of $p<0.01$. Of these, 74 were more highly expressed during growth on sulfur vs. nitrate, and 37 were less expressed. The top ten most highly up and downregulated proteins are listed in Table 4.3. The ten most upregulated proteins included the Hyn hydrogenase subunit described above (WP_013537874) and the SQR-like protein (WP_013537594). The 10 most downregulated proteins included the molecular chaperone GroEL (WP_013537971), carbon monoxide dehydrogenase (WP_041440014), desulfoferrodoxin (WP_013537205), NADH dehydrogenase (WP_013538242), ABC transporter ATP-binding protein (WP_013537203), SufBD protein (WP_013537204), NADH dehydrogenase subunit I (WP_013538240) and a carbohydrate porin (WP_013538274).

Comprehensive tables of differentially transcribed genes and differentially expressed proteins with a $p<0.01$ confidence from the triplicate samples are given in Tables 4.2 and

4.3, respectively. A consensus heatmap of top differentially transcribed genes and differentially expressed proteins (sulfur vs. nitrate reducing conditions) in *T. ammonificans* is shown in Figure 4.3.

4.3.9 Phylogenetic and sequence analyses of the SQR and NSR-like oxidoreductases

Phylogenetic analyses of the SQR-like and NSR-like oxidoreductases of *T. ammonificans* were carried out to reconstruct their evolutionary history (Figures 4.4 and 4.5). The closest relative to the SQR-like enzyme from *T. ammonificans,* at 86% identity, was the homolog from *Phorcysia thermohydrogeniphila* (Pérez-Rodríguez, Grosche et al. 2012), a close relative that also conserves energy by sulfur and nitrate respiration. The next closest relatives to the SQR-like enzyme from *T. ammonificans* were found in sulfur-oxidizing members of the genus *Hydrogenobaculum*, although the amino acid sequence identity dropped to about 57% (Figure 4.4). Phylogenetic tree reconstruction indicated that the NSR-like enzyme of *T. ammonificans* forms a discrete lineage with the *P. thermohydrogeniphila* homolog (48% identity), only distantly related to the enzymes from other sulfur-reducing archaea and bacteria, with 35% identity to the enzyme of the archaeon *Thermococcus kodakarensis* and 33% identity to the enzyme of the bacterium *Thermosipho atlanticus* (Figure 4.5).

The primary amino acid sequence of the NSR-like enzyme from *T. ammonificans* was aligned with those of homologous enzymes that were biochemically characterized and shown to reduce S^0 to H_2S. This analysis confirmed that the cysteine at position 43, necessary for S^0 reduction in the enzyme from *Thermococcus kodakarensis* (Kobori, Ogino et al. 2010), is conserved in the active site of the NSR homolog from *T. ammonificans* (Figure 4.6). Cys43 is also conserved in *Pyrococcus horikoshii* and *Shewanella loihica.*

4.4 Discussion

4.4.1 Growth under different electron accepting conditions

In the reduction of S^0 by H_2, only 2 electrons are transferred; in contrast, the reduction of NO_3^- requires 8 electrons. The standard free energy of reduction of S^0 by H_2 is -32.8 KJ/mol e^-, whereas a much higher ΔG^0 of -594.4 KJ/mol e^- is obtained from the reduction of NO_3^- by H_2. Although energy gained from a reaction does not necessarily lead to higher growth rates, in our experiments, *T. ammonificans* grew approximately three times faster by respiring nitrate, over sulfur. The higher doubling time associated with non-flushed and stationary batch culture is likely related to either lower H_2 partial pressure or accumulation of toxic sulfide than in flushed tubes as well as less access to bulk sulfur than in shaken cultures, discussed below. *T. ammonificans* was unable to grow by sulfur disproportionation, as shown by the lack of growth with sulfur as the sole electron source (no H_2 in the medium headspace). While we can't exclude that, under the conditions tested, sulfur disproportionation was thermodynamically unfavorable, it should be noted that no members of the family Desulfurobacteriaceae have been shown to be able to disproportionate sulfur.

4.4.2 Direct access of T. ammonificans cells to elemental sulfur is required for growth

SEM micrographs of sulfur-respiring cultures, with and without bulk sulfur sequestered inside dialysis tubing, suggest that energy conservation by elemental S^0 reduction in *T. ammonificans* occurs via a transition of bulk sulfur to dissolved polysulfide to nanoparticulate sulfur (via hydrolysis of polysulfide under acidic conditions), driven by H_2S release from biological hydrogen-dependent S^0 reduction. Since polysulfides are not stable at the acid pH of the SME medium (5.5), and sulfur has very low solubility in water

(although its solubility increases at higher temperature), the physico-chemical form of sulfur actually taken up and reduced by *T. ammonificans* in culture is unlikely to be solid sulfur, but rather nanoparticulate sulfur produced by the biotically driven (metabolic H_2S release from S^0 reduction) transition of bulk sulfur to nanoparticulate sulfur.

Following growth, the sulfur granules have a "pitted" appearance (Figure 4.1), a possible signature of microbial attachment or of chemical interaction at close proximity. In contrast, no “pitting” was observed on the sulfur granules sequestered in the dialysis tube. Therefore, we can conclude that direct access to elemental sulfur appears to be necessary for growth of *T. ammonificans*. However, in the presence of cells of *T. ammonificans*, reduced sulfur was present inside the dialysis membrane, even though we could not measure an increase of sulfide and in cell biomass. Sulfur granules from inside dialysis tubes showed no signs of pitting. Taken together, these observations implicate that, in *T. ammonificans*, direct access to bulk sulfur is necessary for growth but also suggest that, in the presence of metabolically active but not dividing cells of *T. ammonificans*, S^0 reduction and production of sulfur nanoparticles occurred over a two-week period.

Both mechanisms have been reported in sulfur-reducing archaea: for instance, the genus *Pyrodictium* (meaning "fire-network") is so named because of the cobweb-like networks grown around bulk sulfur (Stetter, König et al. 1983), showing a preferred attachment to the mineral. However, it has been shown that direct attachment is not necessary for growth by *Pyrodictium brockii* (Kelly 1990), which still grows with bulk sulfur sequestered by dialysis tubing. Similarly, the ability to grow without direct access to sulfur was demonstrated in *Acidolobus sulfurireducens* (Boyd and Druschel 2013) and in *Acidianus* sp. strain DS80 (Amenabar, Boyd et al. 2018). It is possible that the extracellular

polymeric substances produced during growth on sulfur in *T. ammonificans* are involved in attachment, either to bulk sulfur or to walls of a hydrothermal vent.

Sulfide in the original medium (<1mM), as well as that produced by *T. ammonificans* during growth, interacts with the bulk elemental sulfur to produce nanoparticles. While biologically produced H_2S is necessary to perpetuate the cycle in laboratory batch conditions, naturally occurring H_2S at a deep-sea vent would increase the availability of S^0 nanoparticles; biologically produced H_2S from growing *T. ammonificans* cells could additionally enhance the local availability of nanoparticulate sulfur necessary for growth. Sulfur and other metal-sulfide nanoparticles have been shown to be abundant at deep-sea hydrothermal vent sites (Yücel, Gartman et al. 2011, Gartman, Findlay et al. 2014), with nanoparticulate S^0 constituting up to 44% of total elemental sulfur (Findlay, Gartman et al. 2014). This self-perpetuating system produces sulfide, further solubilizing the bulk elemental sulfur into dissolved polysulfides. Polysulfides are more highly soluble in an aqueous environment than elemental sulfur and may allow a microbe better access to the electron acceptor. For instance, *Wolinella* grown at pH 7.2 (Macy, Schröder et al. 1986), or even pH 8.5, likely uses polysulfide as its electron acceptor at a periplasmic membrane site (Klimmek, Kröger et al. 1991). However, polysulfides are stable only at pH values of ≥8 and readily dissociate to nanoparticulate sulfur and sulfide at more acidic pH (Gun, Modestov et al. 2004). In the acidic medium at which the *T. ammonificans* culture is grown, polysulfides become unstable and are cleaved, resulting in the precipitation of nanoparticulate sulfur. The same is true at the slightly acidic pH (between pH 5 and 6) of *T. ammonificans*' natural habitat.

Sulfur nanoparticles appeared solely in inoculated medium and in increasingly large sizes behind the dialysis membranes during the sulfur sequestration experiment (Figure 4.2

b-c). With the nanoparticles themselves soluble and neutrally charged, we propose that these may diffuse through the cellular lipid membrane and act as a cytoplasmic terminal electron acceptor for the upregulated NSR-like enzyme. A similar mechanism of S^0 reduction through dialysis tubing was described for *Acidilobus sulfurireducens,* where the authors also showed that the growth rate of sulfur nanoparticles, and thus the size distribution, was influenced by the pH of the medium as well as presence of organic carbon (Boyd and Druschel 2013).

4.4.3 NSR is expressed during respiratory sulfur reduction in T. ammonificans

Comparative proteomic and transcriptomic analyses between sulfur and nitrate-reducing conditions revealed differences in the expression as a result of growth on the two different electron acceptors (Figure 4.3). On the oxidative side of the process, growth on sulfur upregulated one of the *T. ammonificans* membrane-bound hydrogenases (Hyn), while downregulating a second membrane-bound hydrogenase (Ech), (Tables 4.2 and 4.3). One of the cytoplasmic hydrogenases (Hyd) was also upregulated in sulfur respiring conditions. This differential regulation of hydrogenases implies that separate pathways of electron transfer are active in *T. ammonificans*, influenced by electron acceptor availability (Giovannelli, Sievert et al. 2017). Existence of separate and differentially regulated dissimilatory electron transfer pathways is also supported by a dramatic downregulation of genes for various NADH dehydrogenases on sulfur growth vs. nitrate (Tables 4.2 and 4.3). Of the possible sulfur-respiration genes delineated in the prior genome analysis (Giovannelli, Sievert et al. 2017), two showed significant upregulation, the SQR and NSR-like proteins. In particular, the NSR-like enzyme (WP_013538070.1), though not showing a significant difference in mRNA transcripts, was detected at significantly higher levels in the triplicate sulfur-reducing culture proteomes than the nitrate-reducing ones (Table 4.1).

Taken together, these data suggest that, when growing on sulfur, *T. ammonificans* appears to be re-directing NADH reducing power, as well as sourcing electrons for the transport chain from an upregulated membrane-bound hydrogenase. With NADH dehydrogenases across the genome being heavily downregulated in sulfur-respiring conditions (Tables 4.2 and 4.3, Figure 4.3), the up-regulated NSR could be a possible sink for the NADH, and a mechanism for reducing S^0. Further, NSR could potentially contribute to the overall proton gradient of the cell, donating intracellular H^+ to the released HS^- (Figure 4.7). The discrepancy between the NSR-like mRNA transcripts (not upregulated in sulfur conditions) and proteomic data (significantly upregulated in sulfur) could be a consequence of toxic sulfide accumulation in the culture medium. Despite flushing with fresh H_2/CO_2 every 2 hours, sulfide levels from growth build up quickly in the batch culture, inducing the expression of detoxifying enzymes (*e.g.,* SQR; see following discussion). However, with proteins having a higher half-life than mRNA transcripts, the NSR-like enzyme could still be detected in significantly higher levels in sulfur cultures by the late exponential phase.

The NSR-like protein could function as NSR does in *P. furiosus*, to reduce zero-valent sulfur using electrons sourced from NADH (Figure 4.7). Kinetics studies of the *P. furiosus* NSR showed that the rate of sulfide production from colloidal sulfur was linear, without the lag phase that would be expected if polysulfide had to be generated from the sulfur first. This evidence further supports nanoparticulate sulfur as a possible substrate for the enzyme (Schut, Bridger et al. 2007). NSR-like enzymes belong to the pyridine nucleotide disulfide oxidoreductase (PNDOR) family. These enzymes use the FAD coenzyme as an electron shuttle to transfer reducing equivalents from NAD(P)H to a cysteine (Cys) residue within a redox-active disulfide bridge. The substrate is then reduced by the reduced disulfide.

In addition to the NSR from *P. furiosus*, several other characterized enzymes show enough similarity, in sequence or structure, to infer a putative function for the NSR-like enzyme in *T. ammonificans.* An NAD(P)H oxidase from *Thermococcus kodakarensis*, with 99% coverage and 35% sequence identity, was shown to have elemental sulfur reductase properties in anaerobic settings. A conserved Cys43 residue was found to be necessary for the sulfur reductase activity in the *T. kodakarensis* enzyme (Kobori, Ogino et al. 2010); this residue is thought to be redox active and involved in a cys-S intermediate during the catalytic cycle. The NSR comparative sequence alignment shown in Figure 4.6 includes the homologous enzyme from *Shewanella loihica* (Warner, Lukose et al. 2010), which has been experimentally demonstrated to reduce polysulfide, as well as the NSR from *P. furiosus* (Schut, Bridger et al. 2007), able to reduce sulfur in vitro. The Cys43 residue is conserved in both enzymes and in the *T. ammonificans* NSR-like protein. Interestingly, the CoA persulfide/polysulfide reductase from the archaeon, *Pyrococcus horikoshii* (Herwald, Liu et al. 2013), shares close structural similarity with the NSR-like enzyme from *T. ammonificans,* contains a cysteine residue at position 43, and was shown to be capable of NAD(P)H-dependent polysulfide reduction (Figure 4.6). Similarly, the bacterium *Nautilia profundicola,* able to respire sulfur through a yet unknown pathway, encodes an NSR homolog, with the conserved Cys43 (Smith, Campbell et al. 2008). In contrast, *Hydrogenobacter thermophilus* (Kawasumi, Igarashi et al. 1984) and *Thermocrinis ruber* (Huber, Eder et al. 1998), despite encoding a NSR homolog, are not able to conserve energy by sulfur respiration, and their enzymes do not have the conserved Cys43 (Figure 4.6). *Aquifex aeolicus*, without a clearly conserved Cys43, has been shown to respire sulfur via a different sulfur reducing complex (Guiral, Tron et al. 2005) using three proteins, SreA,

SreB and SreC (Guiral, Prunetti et al. 2012). Overall, these observations strongly suggest *T. ammonificans* NSR-like enzyme's direct involvement in S^0 reduction.

The overall mechanism proposed for sulfur respiration in *T. ammonificans* is summarized in Figure 4.7. The end product of metabolism, H_2S, diffuses from the cell and then serves to transition bulk sulfur to dissolved polysulfide. Polysulfides are hydrolyzed under the acidic growth conditions, forming sulfur nanoparticles. Cytoplasmic access to sulfur nanoparticles is either mediated by an unidentified transporter or directly by nanoparticle mobility across the membrane. Reduction of sulfur and H_2S diffusion out of the cell results in a proton gradient. Cellular access to bulk sulfur aids in the efficiency of the overall process by keeping the constituents in close proximity, ultimately increasing growth rate.

4.4.4 The SQR of T. ammonificans may be more versatile than previously known

The SQR-like pyridine nucleotide-disulfide oxidoreductase showed clear upregulation in sulfur vs. nitrate medium, in both the transcriptome and proteome at high confidence levels (Tables 4.1, 4.2 and 4.3; Figure 4.3). Further, RT-qPCR for the pyridine nucleotide-disulfide oxidoreductase gene showed between a 2 and 3.5 fold induction of the gene in sulfur respiring conditions, validating the transcriptome results (Table 4.1). However, the induction of SQR under sulfur reducing conditions was unclear, as known SQRs work in the oxidative direction, *i.e.* oxidizing sulfide to reduce a quinone. Both anoxygenic photolithotrophs and chemolithotrophs take advantage of sulfide as an electron source in this way (Arieli, Shahak et al. 1994, Griesbeck, Hauska et al. 2000).

Therefore, we envisioned two possible scenarios to explain the overexpression of the SQR-like enzyme in sulfur-grown cultures of *T. ammonificans*: in the first scenario, the

enzyme works in the oxidative direction, oxidizing H_2S and simultaneously reducing the quinone pool. In this case the main function of this enzyme would be sulfide detoxification.

In the second scenario, the SQR-like enzyme works in the reductive direction, oxidizing the quinone pool and contributing to the reduction of elemental sulfur. With electron carriers (*e.g.,* menaquinones) in the membrane of *T. ammonificans* poised at the appropriate potentials, it possible to hypothesize that an electron originally sourced from hydrogen, via one of the upregulated hydrogenases, is subsequently used to reduce sulfur to sulfide by the SQR-like FAD-dependent pyridine nucleotide-disulfide reductase. The function of SQR in the reductive direction has been proposed also for the homologous enzyme of *Sulfurovum* sp. NBC37-1 (Figure 4.4), a mesophilic hydrogen- and sulfur-oxidizing Epsilonproteobacterium (Yamamoto, Nakagawa et al. 2010).

An example of life's flexible response to an increasingly oxidized environment is demonstrated by the reversal of electron flow through certain oxidoreductases or even parts of metabolic pathways, depending on context. For instance, hydrogenases, found in all three domains of life, catalyze the reversible interconversion of H_2 to protons and electrons. The tungsten- and the molybdenum-containing aldehyde oxidoreductases from *Clostridium formicoaceticum* are well characterized, and reversible (Huber, Caldeira et al. 1994). Recently, a reversible form of the tricarboxylic acid cycle (TCA) was discovered in *Thermosulfidibacter takaii*, a deep branching member of the Aquificae (Nunoura, Chikaraishi et al. 2018). In this bacterium, the switch from oxidative to the reductive form of the TCA occurred with the reverse reaction of citrate synthase (Nunoura, Chikaraishi et al. 2018).

4.5 Concluding Remarks

The sulfur metabolism of *T. ammonificans*, a deep-branching thermophile isolated from active deep-sea vents, was investigated. Biological hydrogen oxidation drives sulfide production by *T. ammonificans*, generating dissolved polysulfides and nanoparticulate sulfur, the substrates for the terminal reductase. Biologically produced H_2S from *T. ammonificans* cells plays a part in the sulfur cycle, by additionally enhancing the local availability of nanoparticulate sulfur, via polysulfide production, and by further reduction of sulfur. A homolog to the NADH-dependent sulfur reductase (NSR) in *P. furiosus*, shown to have sulfur-reducing activity, is found in the *T. ammonificans* genome and is significantly induced during growth on sulfur vs. nitrate as electron acceptor. Phylogenetic tree reconstruction and sequence alignment of NSR, found commonly in sulfur-reducing prokaryotes, further support its function as an NADH dependent sulfur reductase in *T. ammonificans*. The PNDOR family of enzymes, to which the *T. ammonificans* NSR-like enzyme belongs, conserve protein folds that bind the flavin dinucleotide (FAD) cofactor and enable it to use sulfur as substrate. Transition metals have been found to have an important place in shaping the earliest catalysis and protein structure on Earth (Moore, Jelen et al. 2017) but our work demonstrates that organic cofactors such as FAD, as well as transition metals, played an important part in shaping the earliest electron transfer machinery on Earth. Life has evolved several solutions to catalyzing the respiration of sulfur, an element with many naturally occurring allotropes. Through more work like that on *T. ammonificans* and NSR, we can better understand how the thermodynamic stability of a dynamic substrate like sulfur can drive biological evolution.

Table 4.1 Summary of proteome, transcriptome and RT-qPCR results for sulfur- and nitrate-reducing transcripts and proteins.

			RT-qPCR (S^0 vs. NO_3^-)		Transcriptome (S^0 vs. NO_3^-)		Proteome (S^0 vs. NO_3^-)	
Annotation	**NCBI gene locus**	**NCBI protein accession**	**avg. fold change**	**st. dev.**	**b value**	**p value**	**z-val**	**p value**
SQR-like *	THEAM_RS04245	WP_013537594	2.41	1.1	3.05	8.33E-17	8.76	1.99E-18
NSR-like **	THEAM_RS06650	WP_013538070	0.77	0.57	not significant	p > 0.01	3.95	7.89E-05
NapA***	THEAM_RS02185	WP_013537180	0.29	0.03	not significant	p > 0.01	not significant	>0.05

NCBI protein product annotation:

* pyridine nucleotide-disulfide oxidoreductase
** pyridine nucleotide-disulfide oxidoreductase
*** periplasmic nitrate reductase

Positive values for z-val or b-val indicates higher expression in sulfur-reducing cultures compared to nitrate reducing. Negative values indicate lower expression in sulfur-reducing cultures.

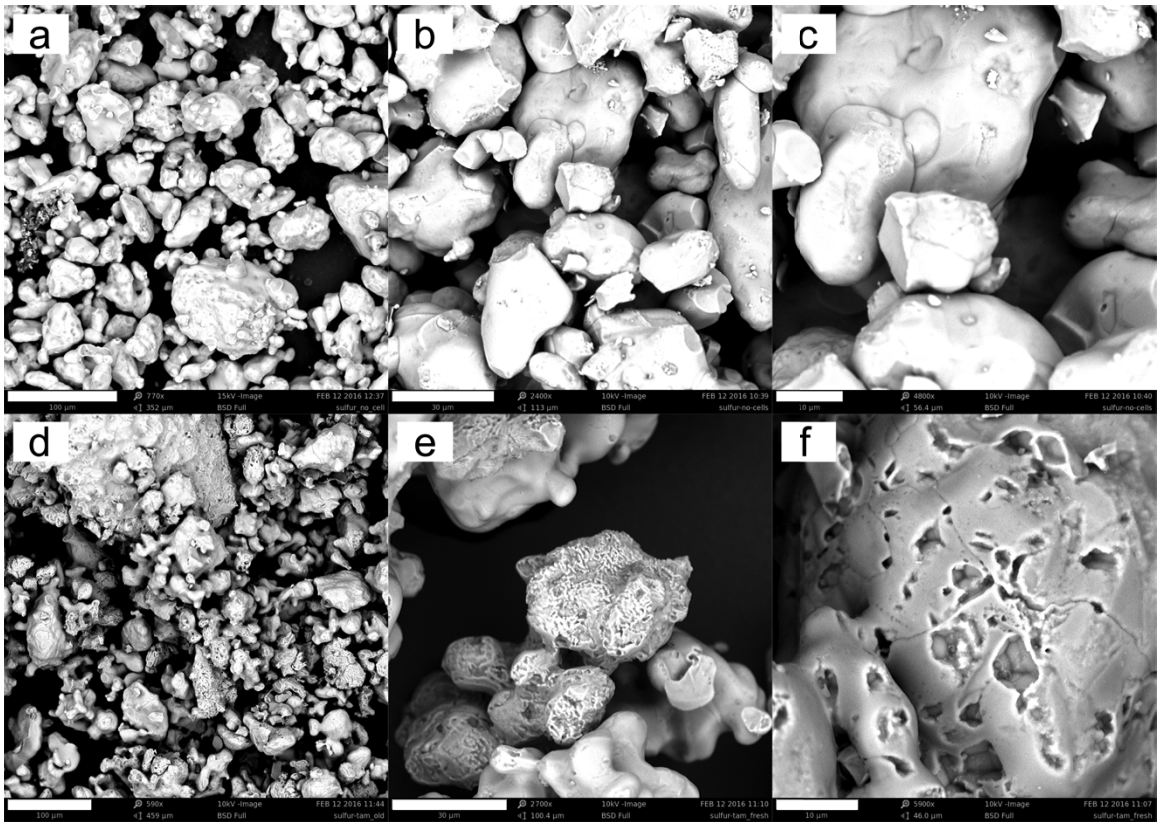
Table 4.2 Proteins encoded by the top up- and down-regulated gene transcripts in the transcriptome of batch cultures of *T. ammonificans* grown in sulfur-reducing conditions.

Annotation	NCBI gene locus	NCBI protein accession	fold change (S^0 vs. NO_3^-)	*p* value
Proteins encoded by the top 10 up-regulated genes				
hypothetical protein	THEAM_RS02645	WP_013537274	36.2	4.75E-07
hypothetical protein	THEAM_RS05760	WP_041439483	33.0	6.15E-20
none	THEAM_RS06745	WP_013538087	30.2	7.37E-07
signal transduction protein	THEAM_RS06185	WP_013537978	24.3	3.59E-04
peptidase U32	THEAM_RS02650	WP_013537275	23.6	5.97E-14
hypothetical protein	THEAM_RS08400	WP_013538393	21.6	1.50E-03
FAD-dependent pyridine nucleotide-disulfide family (Sqr-like)	THEAM_RS04245	WP_013537594	21.0	8.33E-17
iron (metal) dependent repressor	THEAM_RS04875	WP_013537721	20.9	1.04E-16
MerR family transcriptional regulator	THEAM_RS05370	WP_013537823	19.7	2.80E-08
hypothetical protein	THEAM_RS05955	WP_013537937	18.1	5.36E-05
Proteins encoded by the top 10 down-regulated genes				
hypothetical protein	THEAM_RS08575	WP_013538424	-14.0	1.04E-03
hypothetical protein	THEAM_RS08580	WP_013538425	-12.6	6.70E-06
isocitrate dehydrogenase	THEAM_RS05140	WP_013537776	-10.4	2.27E-04
NADH-ubiquinone oxidoreductase	THEAM_RS07510	WP_013538235	-9.1	1.20E-10
transport system permease protein	THEAM_RS00995	WP_013536952	-9.0	1.31E-06
4Fe-4S ferredoxin	THEAM_RS05020	WP_013537752	-8.8	3.03E-05
NADH dehydrogenase	THEAM_RS07540	WP_013538241	-7.2	5.81E-03
biopolymer transporter protein ExbD	THEAM_RS02985	WP_013537346	-7.0	1.05E-03
NADH-quinone oxidoreductase subunit M	THEAM_RS07515	WP_013538236	-6.7	1.44E-06
phosphoglycerate mutase	THEAM_RS06760	WP_013538091	-6.6	6.96E-08

Table 4.3 List of the top up- or down-regulated proteins in batch cultures of *T. ammonificans* grown in sulfur-reducing conditions.

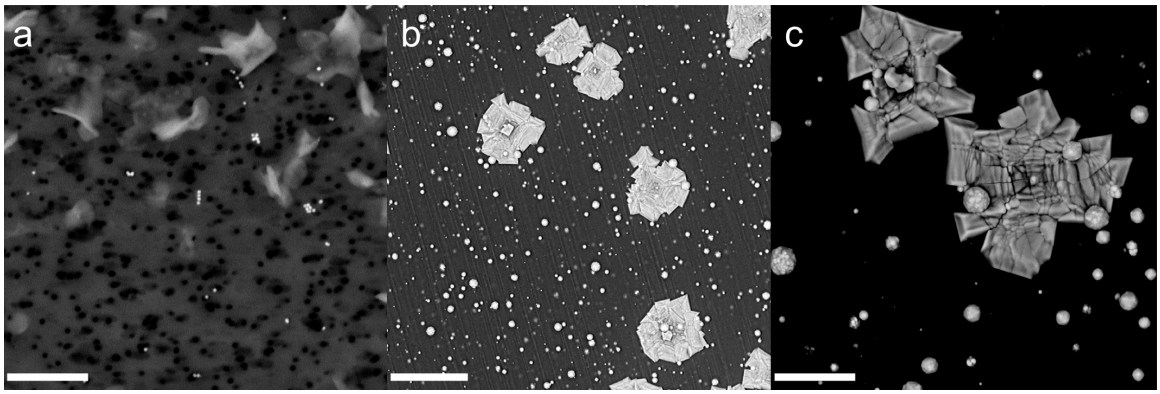
Annotation	NCBI gene locus	NCBI protein accession	z-val (S^0 vs. NO_3^-)	*p* value
Top 10 up-regulated proteins				
hydrogenase 2 large subunit	THEAM_RS05630	WP_013537874	11.17	3.66E-4
pyridine nucleotide-disulfide oxidoreductase (Sqr-like)	THEAM_RS04245	WP_013537594	8.76	1.99E-18
Type II secretion system protein E	THEAM_RS09170	WP_013524959	8.01	1.17E-15
hypothetical protein	THEAM_RS03980	WP_013537539	6.93	4.18E-12
glutamine synthetase	THEAM_RS03195	WP_013537387	6.47	2.93E-3
hypothetical protein	THEAM_RS09120	WP_013524950	6.26	3.82E-10
carbamoyl phosphate synthase large subunit	THEAM_RS07915	WP_013538311	6.16	7.07E-10
cytosol aminopeptidase	THEAM_RS00835	WP_013536922	6.11	1.00E-09
hydantoin utilization protein A	THEAM_RS05660	WP_013537880	5.73	4.60E-3
transporter	THEAM_RS06420	WP_013538025	5.70	1.17E-08
Top 10 down-regulated proteins				
molecular chaperone GroEL	THEAM_RS06150	WP_013537971	-11.05	2.16E-28
carbon monoxide dehydrogenase	THEAM_RS06740	WP_041440014	-9.51	6.84E-4
desulfoferrodoxin	THEAM_RS02305	WP_013537205	-9.49	2.24E-21
NADH dehydrogenase	THEAM_RS07545	WP_013538242	-7.48	7.70E-14
ABC transporter ATP-binding protein	THEAM_RS02295	WP_013537203	-7.10	2.10E-3
SufBD protein	THEAM_RS02300	WP_013537204	-6.64	2.68E-3
NADH dehydrogenase subunit I	THEAM_RS07535	WP_013538240	-6.36	2.06E-10
membrane protein	THEAM_RS07695	WP_013538273	-6.23	3.39E-3
carbohydrate porin	THEAM_RS07700	WP_013538274	-5.65	4.83E-3
glutamate synthase	THEAM_RS06765	WP_013538092	-5.14	6.81E-3

Figure 4.1 Scanning electron micrographs of sulfur granules in the growth medium of un-inoculated blanks and batch cultures of *T. ammonificans* grown under sulfur-reducing conditions.



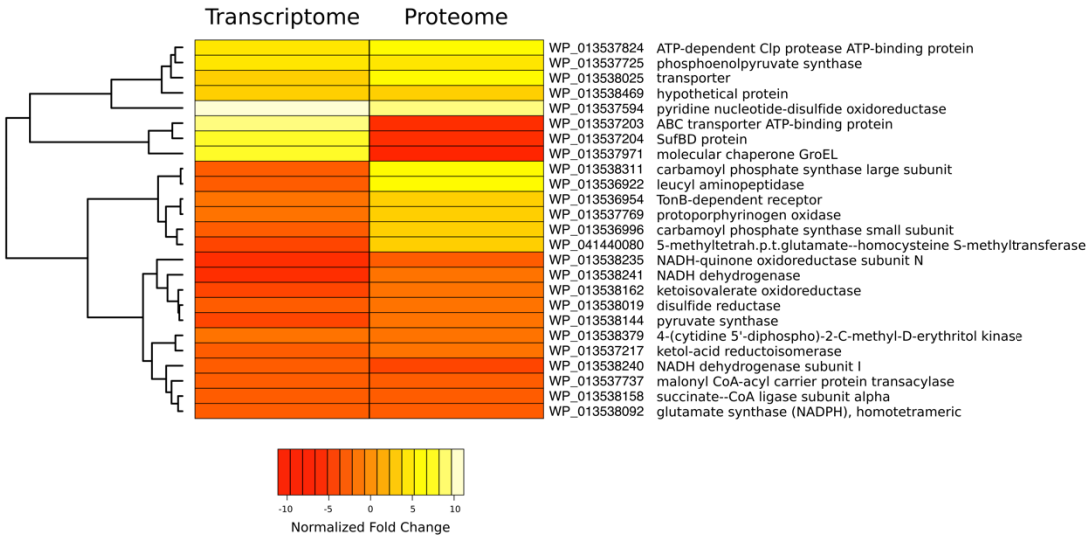
a, b and c - appearance of sulfur granules in un-inoculated medium; d, e and f - appearance of sulfur granules in *T. ammonificans* batch cultures after 24 hours (d) and 48 hours (e,f). Size bars: a, d: 100 µm; b, e: 30 µm; c, f: 10 µm.

Figure 4.2 Scanning electron micrographs of sulfur spherical micro and nanoparticles observed in the growth medium of batch cultures of *T. ammonificans* grown under sulfur-reducing conditions.



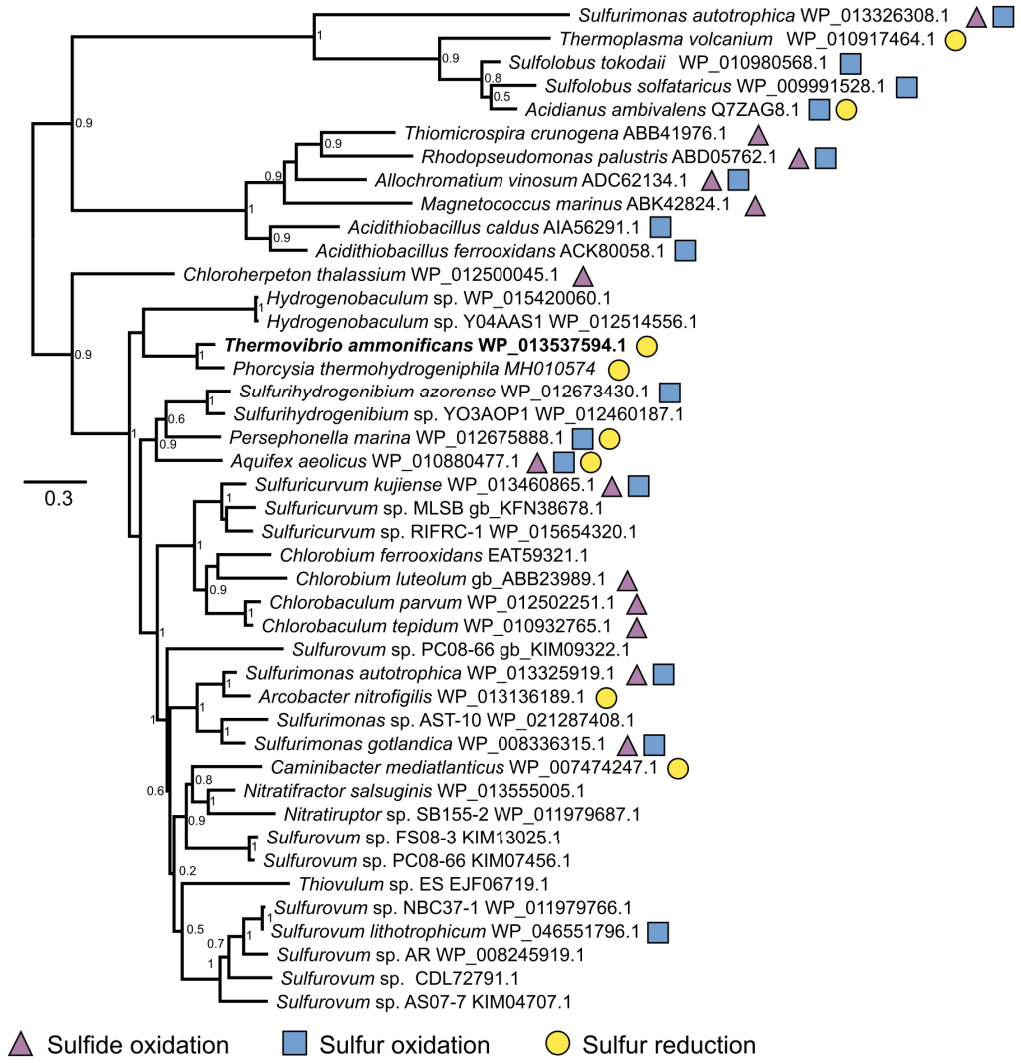
a - Nanoparticles of sulfur in batch cultures of *T. ammonificans* in direct contact with the sulfur; *b* - Nanoparticles of sulfur observed in the interior surface of the dialysis bag used to separate batch cultures of *T. ammonificans* from sulfur particles during growth. The larger angular crystals are consistent with deposits of NaCl present in the medium, as confirmed by Energy Dispersive X-Ray Spectroscopy; *c* - close up of *b*. Scale bar 3 µm (*a*), 30 µm (*b*) and 10 µm (*c*).

Figure 4.3 Consensus heatmap of the top differentially transcribed genes and differentially expressed proteins (sulfur vs. nitrate reducing conditions) in *T. ammonificans* batch cultures.



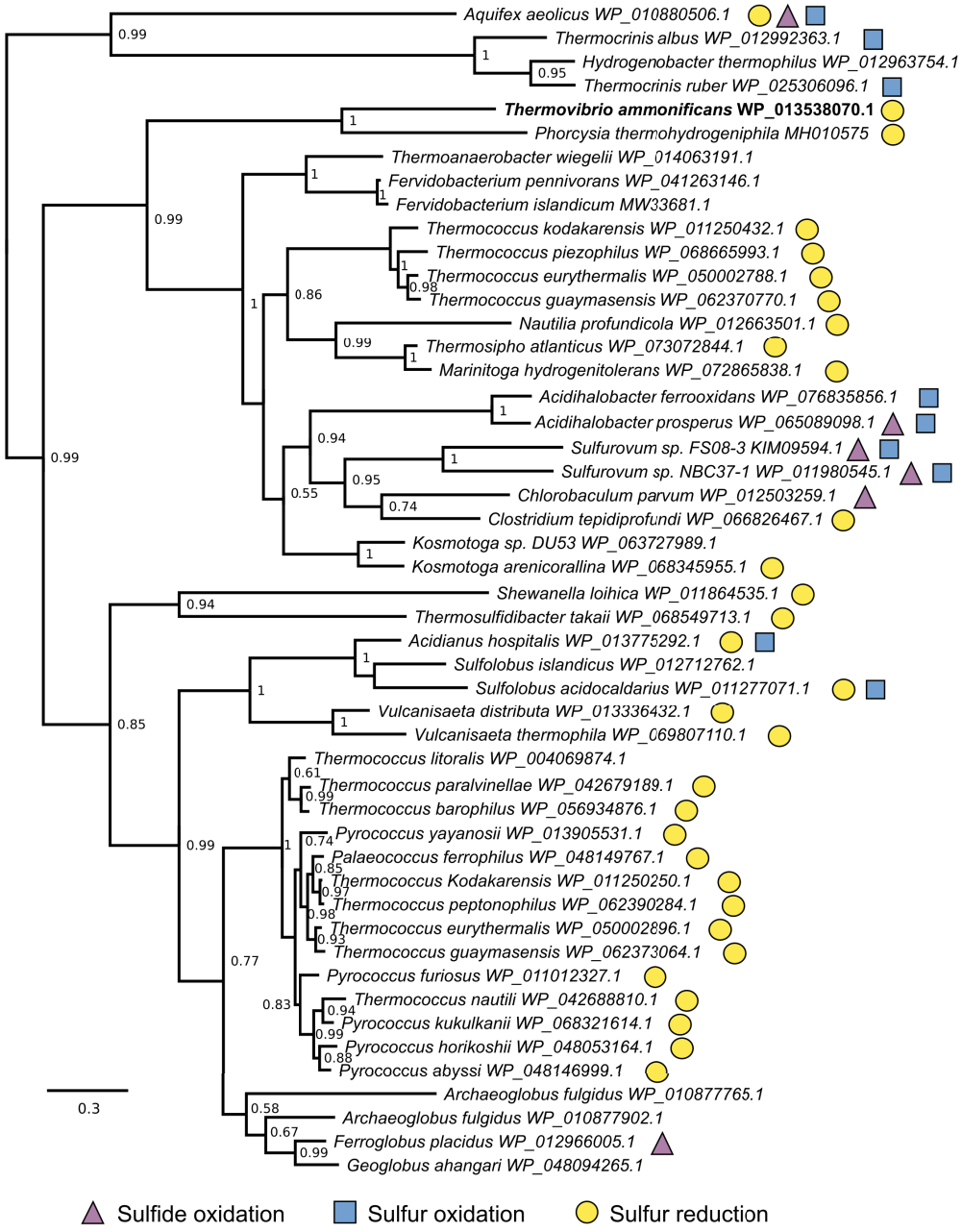
The top 25 hits are shown. The dendrogram groups genes and proteins showing up-regulation in both the transcriptome and proteome, up-regulation in the transcriptome but down-regulation in proteome (or vice versa) and those genes showing down-regulation in both transcriptome and proteome for sulfur respiring conditions.

Figure 4.4 Maximum-likelihood phylogenetic tree showing the position of *T. ammonificans* SQR-like oxidoreductase relative to the SQR type VI cluster.



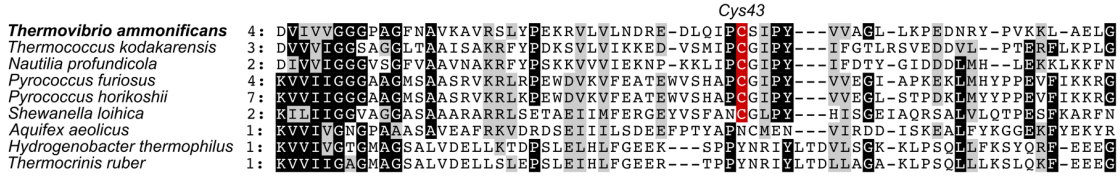
Sulfur energetic metabolism of key cultured strains is shown in the figure. The aLRT probability is shown at each node; SQR proteins of the type V cluster were used as outgroup.

Figure 4.5 Maximum-likelihood phylogenetic tree showing the position of *T. ammonificans* NSR-like oxidoreductase relative to closely related sequences from NCBI.



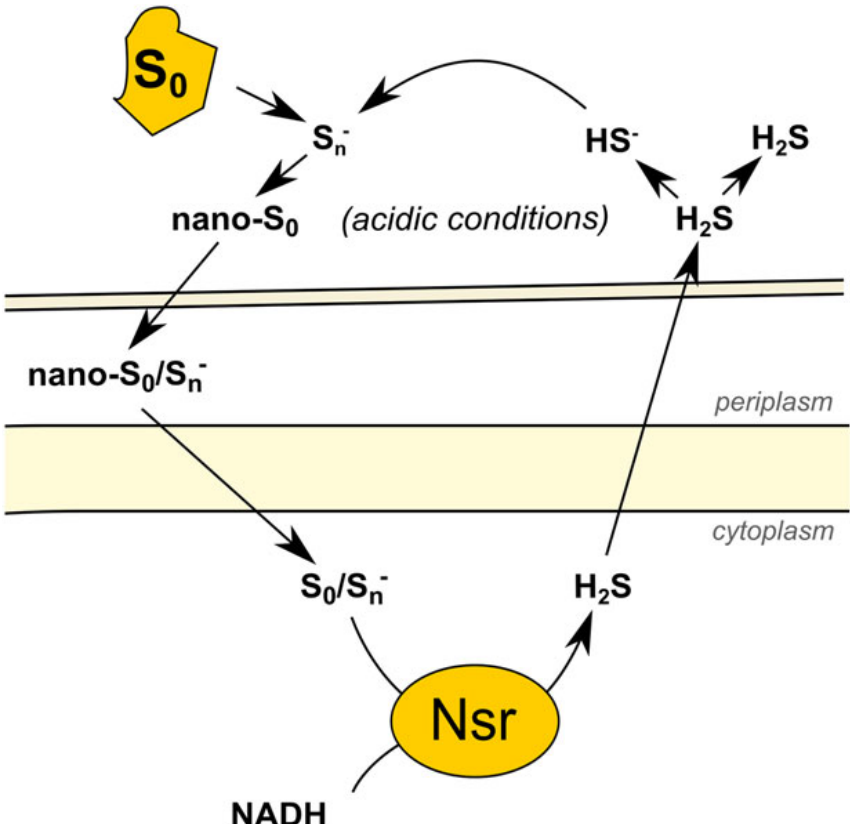
Sulfur energetic metabolism of key cultured strains is shown in the figure. The aLRT probability is shown at each node.

Figure 4.6 Multiple sequence alignment of the active site of NSR-like proteins from different bacteria and archaea.



The NSR from *Pyrococcus furiosus*, *Thermococcus kodakarensis* and *Shewanella lohica* were biochemically characterized as having S^0 reduction functionality. The Cys43 residue necessary for S^0 reduction is conserved in the NSR-like enzyme of *T. ammonificans*.

Figure 4.7 Proposed model of sulfur respiration in *T. ammonificans*.



Sulfide ions promote the dissolution of elemental sulfur and the formation of polysulfide (S_n^-). Due to the acidic pH, the polysulfides hydrolyze, precipitating into elemental sulfur nanoparticles, providing substrate for the cytoplasmic NSR-like oxidoreductase. Access to sulfur nanoparticles is either mediated by an unidentified transporter or directly by nanoparticle mobility across the membrane.

CHAPTER 5

Conclusions: Future Progress In Understanding Life's Role In Earth Science And Elsewhere

5.1 Closure

At the heart of this dissertation is an effort to better understand the network of biotic and abiotic mechanisms that maintain global elemental cycling, *i.e.* biogeochemistry, specifically how biological electron transfer co-evolved with planetary chemistry and geology to set the scene for the Earth and life as it can be seen today. It can be easy to take for granted the long legacy of evolution that brought about human life, so it comes as no surprise that, before our persistent scientific inquiry during the last few centuries, most considered humans and the rich diversity of lifeforms around us to be placed here by intelligent design. Many can now understand that the long neck of a giraffe so suited to reaching the tall trees, or the slick body of a fish able to dart through the ocean, can be attributed to natural selection. But evolution runs far deeper than explaining the physical attributes of the organisms around us. Looking out into a parking lot and seeing cars, trucks, motorbikes and more, all of different colors and styles, each suited to a particular use, one only has to peek under the hood to see that the parts composing them are all the same. An engine, oil, gears, axles, wheels: all modular parts that can be disassembled and reassembled into varying and sometimes improved forms. These basic parts, copied, distributed and assembled in different ways across the parking lot are analogous to the machinery that microscopic organisms, through the same process of natural selection, discovered and tested to provide the basis of all life, giraffe, colored fish, or otherwise. These are the molecules and machines that store and copy genetic code, the cell

membranes, transporters, and more built from these instructions, and, central to this dissertation, the proteins that work together in converting energy provided by the outside world into the chemical energy directly used by life.

5.2 Organic Cofactors and Network Analysis

One such protein, the NSR enzyme described in Chapter 4, has at its active center an organic cofactor, FAD. The work in Chapters 2 and 3 focused on enzymes incorporating the transition metals available - for an early enzyme, those like iron and nickel. However, only half of EC1 enzymes characterized in the PDB contain a metal at the active site, the rest use an organic cofactor derived from simple amino acids or a hybrid – an organic scaffold holding a metal like molybdenum (Schwarz, Mendel et al. 2009) or iron in place. Transition metals have clearly played a role in the evolution of EC1, and due to the possibility that we can reconstruct their environmental availability through Earth history, they are an attractive choice for studying how biological electron transfer matured. However, the opportunity to add EC1 enzymes like NSR, using organic cofactors such as FADH, NADH, CoA and quinones to this research is important, as well as those hybrids described, like molybdenum pterins and iron hemes. Network analysis of transition metal active sites has shown us that the proximity of similar modules within a protein is a result of their repeated duplication and diversification (Raanan, Pike et al. 2018), and it is likely that the modules were of polyphyletic origin from the Archean eon (Harel, Bromberg et al. 2014). By adding cofactor centers into the frame that these network analyses provide, a more complete understanding of how biological electron transfer arrived to its modern state can be realized.

5.3 Metagenomics, Metatranscriptomics and Biogeochemical Networks

Now that “omics” of all types have established themselves in scientific inquiry, metagenomics deserves mention for its current use and future potential in illuminating how microbial communities participate in elemental cycling. For a system-level understanding of biogeochemistry, looking at individual genomes of each known organism in a sample is not nearly as revealing as sequencing the entire community. Community sequencing, or metagenomics, allows for examination of an environmental sample's entire biochemical capacity rather than individual pieces of the community's metabolic puzzle. By using databases of known genes with annotated function, the sequenced genome of a given microbe can quickly infer biochemical capacity by homology of its computationally derived open reading frames (ORF), to known genes (Brown 2002). Not all microbes of importance can be isolated and cultured, so not all have been individually sequenced, but for those fully sequenced organisms, lifestyles are revealed. Each genome indicates heterotrophy or autotrophy, whether an organism feeds on fixed carbon from the environment or is able to fix its own from CO_2. If the organism is capable of fermentation, the substrates it is capable of metabolizing can be detected. If the organism uses an electron transport chain, the redox pairs it has the capacity to use for energy transduction are uncovered. The ability to photosynthesize or fix nitrogen can quickly be highlighted, information critical to understanding an organism's place in biogeochemical cycling.

A service such as the Kyoto Encyclopedia of Genes and Genomes (KEGG) or JGI’s Integrated Microbial Genomes (IMG) is able to link a genome (or metagenome) in its database to a list of genes, which is subsequently linked together to show pathways and biogeochemical capacity. In KEGG, these genes are represented by KO IDs, or

orthologous groups of genes, which are in turn organized into pathway modules (Kanehisa, Goto et al. 2011). KEGG pathway maps are organized in a hierarchical fashion to organize pathway modules into computationally friendly and biologically meaningful networks. After condensing mass amounts of genetic information into KEGG metabolic pathway maps, an example (Figure 5.1) shows that the total number of gene families carrying out denitrification, a major energy transduction in biogeochemical cycling, is a highly limited set. The theme encountered here with denitrification is repeated in the remaining biogeochemical pathways; they are controlled by a small core set of genes. Adding the KO groups that are directly involved in Earth's known biogeochemistry reveals a surprisingly small set, around 400 orthologous groups, as described in Chapter 2.2.

Many ORFs in a typical metagenome are annotated with unknown or hypothetical function, genes either homologous to a gene of unknown function or those without a known homolog. A typical new genome contains 20-30% genes of unknown function. This lack of complete information, though representing a significant area for improvement in genomics in general, is not as big of a problem when studying biogeochemistry, where relevant pathways are represented by a core set of well-known genes. Though new electron-transfer pathways based on favorable redox couples have been predicted, they have yet to be found in nature. Novel metabolism will be discovered, but only so many options are available to the microbial community (Shock, McCollom et al. 1995, McCollom and Shock 1997).

For measuring the immediate genetic expression of a sample, metatranscriptomics, or the sequencing of a community’s active mRNA transcripts, reveals the current biochemical state of the sample (Moran 2009). Perturbations to an environment may

result in different metatranscriptomic profiles from its microbial community, uncovering clues as to the response of the system to any kind of change. Modeling future states of planetary biogeochemistry will be greatly aided by compiling and comparing metatranscriptomic and other "omics" data (proteomics, metabolomics) from various environments under different stresses. The biogeochemical models resulting from these data will not only be applicable to our own planet, however.

5.4 Exoplanets and Biosignatures

As all living organisms exchange gases with their environment (Vernadsky 1997), biological electron transfer exerts great control over Earth's inventory of gases, in turn playing a great role in phenomena like global climate change. The notion that life affects atmospheric inventory is not, however, limited to Earth. With the atmospheric makeup of extra-solar planets now inferred from their absorption spectrum seen here on Earth with space telescopes like Darwin (European Space Administration) and Terrestrial Planet Finder-TPF (NASA), a grand application of reconstructing co-evolving biological and planetary elemental cycles here on our planet is to aid in remotely sensing the potential of life on exoplanets (Des Marais, Harwit et al. 2002, Des Marais, Nuth III et al. 2008). This is a complex area of research, where true atmospheric biosignatures, based on their composition being far out of thermochemical redox equilibrium, must be carefully distinguished from false positives (Seager, Deming et al. 2010, Schwieterman, Kiang et al. 2018). Earth, our only concrete example of a planet harboring life, has an atmosphere of 21% oxygen gas and a resultant ozone layer, far from the expected levels of these gases under equilibrium conditions, *i.e.* without a constant source of oxygen, such as water-oxidizing photosynthesis (Selsis, Despois et al. 2002). Presence of oxygen at 10^{-3} –

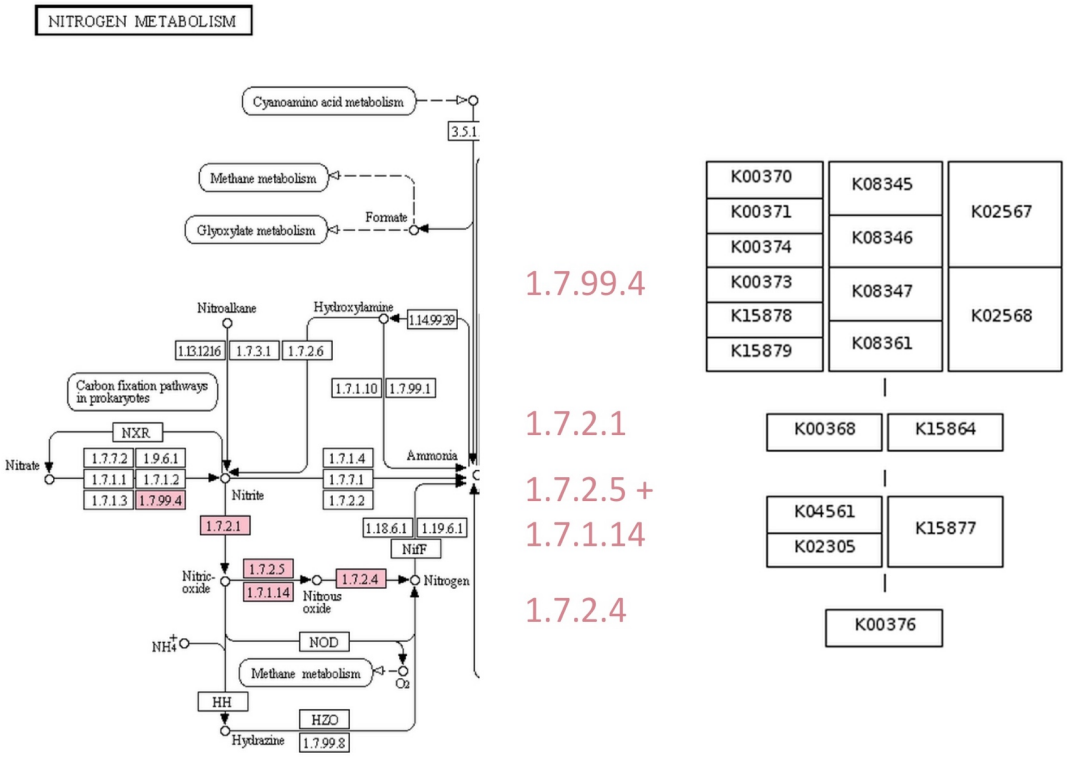
10^{-4} of present atmospheric levels on Earth could be, however, a signal of a runaway greenhouse, due to photolysis of water vapor and hydrogen escape (Seager, Deming et al. 2010). Through modeling terrestrial planet atmospheres, ways to discriminate between biologically and photolytically produced oxygen have been used (Segura, Meadows et al. 2007).

5.5 Novel Plastic Degradation by Microbes and the Future

The study of the evolution of biological electron transfer is one that extends back to the origin of life and forward to Earth's future, providing important insights as to how our planet will respond to anthropogenic perturbations. This is not a finished story. Microbes, for example, have evolved a pathway to degrade poly(ethylene terephthalate) (PET) plastic, the type commonly used for disposable water bottles. Humans have been synthesizing PET from petrol products for less than a century, and due to the lack of enzymes able to degrade the polymer into its innocuous monomer components, it has become persistent and ubiquitous in a distressing number of natural environments (Eriksen, Mason et al. 2013, Van Cauwenberghe, Vanreusel et al. 2013, Cózar, Echevarría et al. 2014, Wilcox, Van Sebille et al. 2015). The 2016 discovery and isolation of *Ideonella sakaiensis* from sediments at a PET bottle recycling site revealed two enzymes responsible for degradation of PET. The organism synthesizes a PETase and MHETase that it uses to break down PET polymers into monomers that it is able to use for carbon assimilation (Yoshida, Hiraga et al. 2016). By further interrogation into their closest relatives, as well as their 3D structure, more can be known about how and when these novel enzyme combinations specializing in plastic degradation may have appeared. This story certainly illustrates, however, that gene duplication, followed by

further mutation and selection is happening, potentially creating new nanomachines to add to and influence the electron marketplace. These novel innovations, once spread through Earth's microbial populations, have been the building blocks of how biology harnesses the energy of natural electron flow. Considering, finally, that anthropogenic additions of fixed nitrogen to the environment have easily overtaken all biological nitrogen fixation in the oceans (Gruber and Galloway 2008), or that de-novo proteins capable of electron-transfer are now being synthesized (Kim, Pike et al. 2018), the human impact on this network, and its potential, is obvious. Our actions, influencing the nitrogen cycle with fertilizers that have sustained massive human population growth, influencing the carbon cycle by burning ancient fossil-fuels, or adding plastics to natural environments, have had such far-reaching effects through the biogeochemical network that we have left our own geological mark on the planet and have ushered in a new era, the Anthropocene.

Figure 5.1 KEGG pathway map for nitrogen metabolism.



EC#s highlighted in pink are expanded to their KO definitions, representing the complete catalog of genes needed for denitrification, a small, conserved set (Kanehisa, Goto et al. 2011).

APPENDIX 1

ACRONYMS AND DEFINITIONS LIST

Craton – A large, stable block of Earth's crust, forming the nucleus of a continent.

E'_0 – Standard reduction potential (the measure of the tendency of a chemical species to acquire electrons and thereby be reduced) at pH 7.0

E'_m – Standard midpoint potential (group is half reduced/half oxidized) at pH 7.0

Entatic state – A specific electronic state of an atom or group, defined by the surrounding protein environment.

Ferredoxin – Low molecular weight iron sulfur protein that acts as a multifunctional electron carrier in biological redox reactions.

Gene Homologs – Genes duplicated from a common ancestor, related by their sequence identity.

Protein active site – Region of an enzyme where substrates bind and undergo chemical reaction

α-helix, **β**-sheet and loops – Elements of protein secondary structure, local repetitive conformations of peptide chain.

Protein Domain – Conserved portion of a protein sequence and/or tertiary structure able to exist independently of the whole protein.

Protein Fold – The way the secondary structure elements of the structure are arranged relative to each other in space.

Proteome – The entire set of proteins expressed by a genome at a particular time.

Redox – Abbreviation for "Oxidation/Reduction," as in a redox reaction.

Transduction – In biophysics, the conversion of one form of energy (i.e. light or chemical) to another, usually a transmembrane electric potential.

APPENDIX 2

RELATED RESOURCES

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2. Falkowski PG. 2015. *Life's Engines: How Microbes Made Earth Habitable.* Princeton University Press. Princeton, NJ, USA

3. Johnson S. 2008. *The Invention of Air: A Story of Science, Faith, Revolution, and the Birth of America*. Penguin. New York, NY, USA

4. Lane N. 2015. *The Vital Question: Energy, Evolution, and the Origins of Complex Life*. WW Norton & Company. New York, NY, USA

5. Vernadsky VI. 1997. *The Biosphere*. Copernicus, an imprint of Springer-Verlag New York. New York, NY, USA

FOOTNOTES

Footnote 1: The discovery that anaerobic microbes can produce reduced phosphorous as phosphine gas, and that phosphine is a global constituent of Earth's atmosphere is prompting reconsideration of phosphorous biogeochemistry (Roels and Verstraete 2001).

Footnote 2: Archean copper concentrations in the ocean are reconstructed from geological lines of evidence as well as chemical modeling. In contrast to the perspective highlighted here, literature can be found showing little change in copper concentrations on either side of the GOE (Fru, Rodríguez et al. 2016), and that modeled Cu(I) concentrations in the Archaean were significant, at levels that would not limit modern phytoplankton (Saito, Sigman et al. 2003).

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