ABSTRACT

COUNTS, JAMES AARON. Determinants for Hot Acid Microbial Lifestyles: Chemolithoautorophic Physiology of the Extremely Thermoacidophilic Archaeal Lineage Sulfolobales. (Under the direction of Dr. Robert M. Kelly).

The Crenarchaeal order *Sulfolobales* consists exclusively of extremely thermoacidophilic organisms from terrestrial geothermal environments. This niche requires numerous adaptive features to tolerate harsh degrees of acidity (<4.5) and temperatures (>65˚C). Further, mixing with subsurface environments causes dramatic fluctuations in the above-mentioned stressors and provides capacious energy in the form of reduced inorganic sulfur compounds (RISCs) – primarily hydrogen sulfide, mineral sulfides, and zero-valent sulfur – and dissolved and mineral-bound transition metals (primarily iron). To better understand thermoacidophily, nine type-strains from culture repositories were sequenced, leveraging recent advances in third-generation sequencing technologies. This includes *Acidianus ambivalens, Acidianus brierleyi, Acidianus infernus, Acidianus sulfidivorans, Metallosphaera hakonensis, Metallosphaera prunae, Stygiolobus azoricus, Sulfolobus metallicus,* and *Sulfurisphaera ohwakuensis.* These organisms are largely chemolithotrophic, include several facultative anaerobes and the only obligate anaerobe from the Sulfolobales; they span 5 named genera, encapsulating a broad picture of the order. Analysis of these and 14 additional type-strain Sulfolobales genomes identifies a core genome of approximately 1,100 genes and an open pan genome.

The addition of these genomes also provides resolution for poor branching in existing taxonomy compiled with earlier methods. Through the use of a Thermoprotei orthologous core of 97 proteins and average amino acid identity (AAI), a proposal is made for several taxnomical shifts within the order Sulfolobales. Proposed is the reclassification of *Sulfolobus metallicus* as *Cocturalobus metallicus,* including unnamed species *Sulfolobus sp.* JCM16833 and JCM16834, representing an extremely acidophilic (pH~2), thermophilic (65-70˚C) genus of obligate chemolitotrophic (*coctura,* ore smelting) aerobes. *Sulfolobus islandicus spp.* and the newly characterized *Sulfolobus sp.* A20 should be reclassified in the recently named genus *Saccharolobus* Finally, evidence suggests that the newly isolated, sequenced *Sulfodiicoccus acidiphilus* be reclassified in a new family: Sulfodiicoccaceae, which may represent a distantly branched group of sulfur-inhibited, chemoheterotrophs.

Deeper Sulfolobales genome analysis suggests there are numerous well-conserved pathways for processing inorganic compounds. This includes the 3-hydroxypropionate/4-hydroxybutyrate (near complete excluding *S. acidiphilus*), the heterodisulfide reductase complex, thiosulfate:quinone oxidoreductase (except *S. acidocaldarius*) and sulfide:quinone

oxidoreductase. Unsurprisingly, there are sulfur-related genes of relative rarity, including the sulfur oxygenase reductase (SOR), sulfur-reductase (Sre), and tetrathionate hydrolase (TetH), largely limited to facultative and obligate anaerobe(s), but also appearing in the most acidophilic Sulfolobales. Interesting are several genes closely linked with sulfur and acidophily, including an uncharacterized TetH paralogue, NiFe hydrogenase, and a novel (within the order) cytochrome bd-like oxidase. This last enzyme complex may represent a crucial element of pH homeostasis maintenance, given that these cytochromes usually have exceptionally high oxygen affinity and intransigence to strong reductants (e.g. hydrogen sulfide). Further, the putative cytochrome bd, as well as doxBCE (a universal Sulfolobales oxidase) are present even in the strict anaerobe *Stygiolobus azoricus*.

Within this environment, iron-biooxidation is another prevalent phenotype. *Metallosphaera sedula* was the most proficient iron-biooxidizer with constitutively-expressed ferrous iron oxidation "fox" genes. Herein is demonstrated the activity of this complex (isolated from *M. sedula*) as well as identification of its constituents. Confirmation of its conservation among select members of the order cements its role in extremely thermoacidophilic iron biooxidation. Further, iron oxidation rates appear to relate to *fox* gene expression, particularly FoxABCDW, which likely constitute the active complex. Evolutionary analysis of the primary oxidase subunit FoxA suggests the speciation of cytochrome c-like oxidases follows the speciation of Crenarchaeal aerobes of the lineage Sulfolobales and the recently identified candidate phylum Marsarchaeota. Further, examining multiple genomes, a urease-like complex and a peroxiredoxin from the OsmC lineage suggest the importance of pH homeostasis and free-radical formation in these acidic, metal-laden environments.

Determinants for Hot Acid Microbial Lifestyles: Chemolithoautorophic Physiology of the Extremely Thermoacidophilic Archaeal Lineage Sulfolobales

by
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BIOGRAPHY

James Aaron Counts was born in Orlando, Florida in 1989 and spent his entire childhood in the western Orlando community. He attended West Orange High School, where he was a National Honor Society member, Percussionist, and long-time Oboist. Upon graduation, he attended the University of Florida studying Chemical Engineering, where he was a member of the engineering honor society Tau Beta Pi and took part both professionally and voluntarily in numerous K-12 STEM and academic outreach programs. While interning at a local pharmaceutical and contract research company in his final year, he decided to pursue post-secondary education, enrolling at North Carolina State University in chemical and biomolecular engineering.

Since arriving at North Carolina State, James settled into the Hyperthermophiles lab of Robert M. Kelly, studying questions of extreme metabolic and physiological evolution, as well as origins of microbial life, working with novel thermoacidophilic enzymes and expanding our understanding of microbial life at the limits of the habitable universe. While studying at NC State, he completed a traineeship in the NIH Molecular Biotechnology Training Program and worked as an intern in process optimization at Novozymes NA, Inc. In addition to a love of science, he has a love of running, embarking on a secret career as an amateur long-distance runner, culminating in the completion this fall of his first road marathon in 3:00:15 and coaching middle school cross-country in Durham County Public Schools, where he resides.

He hopes to continue research in the areas of environmental microbiology, evolution and ecology focusing on extreme/underexplored habitats, eventually moving into an academic lab at a research institution in the same field.

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Abstract

The current upper thermal limit for life as we know it is approximately 120 ˚C. Microorganisms that grow optimally at temperatures of 75 ˚C and above are usually referred to as 'extreme thermophiles' and include both bacteria and archaea. For over a century, there has been great scientific curiosity in the basic tenets that support life in thermal biotopes on earth and potentially on other solar bodies. Extreme thermophiles can be aerobes, anaerobes, autotrophs, heterotrophs, or chemolithotrophs, and are found in diverse environments including shallow marine fissures, deep sea hydrothermal vents, terrestrial hot springs—basically, anywhere there is hot water. Initial efforts to study extreme thermophiles faced challenges with their isolation from difficult to access locales, problems with their cultivation in laboratories, and lack of molecular tools. Fortunately, because of their relatively small genomes, many extreme thermophiles were among the first organisms to be sequenced, thereby opening up the application of systems biology-based methods to probe their unique physiological, metabolic and biotechnological features. The bacterial genera Caldicellulosiruptor, Thermotoga and Thermus, and the archaea belonging to the orders Thermococcales and Sulfolobales, are among the most studied extreme thermophiles to date. The recent emergence of genetic tools for many of these organisms provides the opportunity to move beyond basic discovery and manipulation to biotechnologically relevant applications of metabolic engineering.

Introduction

Extreme thermophiles are distinct from other organisms due to their ability to subsist optimally at temperatures in excess of 75 ˚C. Their survival in these harsh environments piqued the interest of curious microbiologists as far back as the turn of the 20th century. In fact, one of the earliest reports of thermophiles occurred in 1903 describing bacterial samples taken from pools in Yellowstone NationalPark.[1] Although this drew interest and debate about the limits of life and our evolutionary history, the study of thermophiles did not begin in earnest until the 1960s. Around this time, extensive sampling projects in Yellowstone lead to the isolation of *Thermus aquaticus*[2] (an aerobic bacterium with optimal growth between 70 and 75 ˚C), known for its DNA polymerase that revolutionized the field of molecular biology through its use in the polymerase chain reaction (PCR). This enzyme in particular represented one of the earliest uses of thermally stable enzymes for a biotechnological application. The next few decades yielded the discovery of thermophiles around the globe in extremely diverse environments, ranging from volcanoes and calderas to deep sea smoker vents to terrestrial mud pools.[3–7]

The apparent diversity and novelty of these microbes likely drove early research in this field to uncover the molecular machinery central to their survival. In fact, some of the earliest sequenced genomes were extremophiles,[8–10] furthering efforts to under-stand the molecular and genetic basis for thermophily and the evolution of life. However, a lack of genetics tools has impeded the extensive study of these organ-isms by traditional approaches (i.e., gene deletions to understand the consequences of loss of function). In lieu of more traditional methods, the availability of genomic data for many extreme thermophiles supported 'omics'-based approaches to ascertain the function of specific genes and their roles in the unique biochemistry of these organisms. As such, the merger of systems biology (e.g., transcriptomics and genomics), traditional microbiological studies, and newly emerging genetic systems[11] is opening the door for metabolic engineering opportunities to bring extreme thermophiles into the technological lime-light. This will allow for these organisms to be utilized as sources of uniquely functioning enzymes, optimized niche industrial strains, and novel metabolic engineering platforms. Such opportunities for biotechnological application are already being pursued for members of the bacterial genera *Caldicellulosiruptor* and *Thermotoga* and for archaea belonging to the orders Thermococcales and Sulfolobales. Here we present a brief overview of these extremely thermophilic organisms, with the intention of highlighting potential biotechnological applications, which exploit their distinctive metabolisms.

Sulfolobles

Perhaps the most distinctive subject matter for this review focuses on the extreme thermoacidophiles from order Sulfolobales. The Sulfolobales comprise an order of archaea taxonomically defined within the class Thermoprotei, within the phylum Crenarchaeota. These organisms inhabit environments characterized by both extreme temperatures (65–90 ˚C) and low pH (1.0–3.5), such as terrestrial solfatara or mud pools, often closely associated with volcanic activity and laden with inorganic materials.[12] In fact, the first species of the order to be isolated, *Sulfolobus acidocaldarius*(from Locomotive Spring in Yellowstone National Park), was reported to oxidize sulfur to fuel autotrophic growth, leading to the name Sulfolobales.[7] However, this phenotype has not been observed in the currently studied *S. acidocaldarius* type strains, although many isolates from the genera *Sulfolobus*, *Metallosphaera* and *Acidianus* utilize S^0 as an electron donor.[5,13–21] Thus, reports that *S. acidocaldarius* strains from culture collections cannot[5] oxidize S suggests that repeated passages on rich media have led to the loss of this ability or that inherent difficulties exist in isolating pure cultures from environmental enrichments. Beyond sulfur oxidation, several species, especially those from the genera *Acidianus*, *Sulfurisphaera*, and

Stygiolobus, are capable of sulfur reduction, and often utilize hydrogen to produce hydrogen sulfide as a metabolicend-product.[13,18,19,22–24]

While many members of the order grow lithotrophically, most known species exhibit modes of either strict heterotrophy or mixotrophy. Most members of the genera *Sulfolobus* and *Metallosphaera* are capable of utilizing protein-rich substrates, such as yeast extract or tryptone, under aerobic conditions. Furthermore, several species, such as *Sulfolobus solfataricus*, *Sulfolobus shibitae*,[25] and *Sulfolobus islandicus*,[26] use a wide variety of sugars in catabolic metabolism. In addition, members of the order, particularly in the genera *Metallosphaera* and *Acidianus*, are capable of oxidizing metal sulfides: a trait that is particularly useful for bioleaching of base, precious and strategic metals from mineral ores.[27–30] Finally, some members of the genus *Acidianus* are capable of using ferric iron as an electron acceptor under anaerobic conditions.[21,31]

Carbon Dioxide Fixation

Interestingly, the natural habitats of many Sulfolobales (solfatara/calderas) are limited or devoid of complex carbon sources, necessitating the process of autotrophy. The ability of organisms to fix carbon dioxide from the atmosphere is considered by many to explain the early formation of the multi-carbon molecules required to fuel life, explaining their retention in species across all three domains of life.[32,33] As it stands, 6 major routes exist for the fixation of carbon dioxide: the Calvin-Benson cycle (present in most plants), the reductive citric acid cycle (green sulfur bacteria), the reductive acetyl-CoA cycle (acetogens/methanogens), the hydroxypropionate bi-cycle (Chloroflexus), and the 3-hydroxypropionate/4-hydroxybutyrate (3-HP/4-HB) or dicarboxylate/4-hydroxybutyrate pathways (both from the Crenarchaeota).[34] The habitats from which the Sulfolobales were isolated, unlike many other organisms, are characterized by copious oxygen and inorganic electron donors.[12] While the reactions driving this cycle are some of the most energy-demanding for autotrophic carbon assimilation, their advantage may lie in their relative insensitivity to oxygen, avoidance of side-reactions, direct utilization of bicarbonate, and thermal stability.[34,35]

Briefly, the 3-HP/4-HB cycle has two major products that enter into cellular metabolism. The first portion involves the addition of two bicarbonate molecules to acetyl-CoA to form succinyl-CoA, which is subsequently reduced in the second half of the cycle to 4-hydroxybutyrate and eventually dissociated to two molecules of acetyl-CoA (**Figure 1-1**).[36] The key enzyme in the cycle is a biotinylated acetyl-CoA/propionyl-CoA carboxylase, that is, bi-functional and efficient in substrate turnover.[37] Metabolic analysis of the cycle has revealed that the major product of the cycle is not acetyl-CoA (as originally hypothesized), but rather succinyl-CoA (roughly two-thirds

of the carbon flux), yielding malate and oxaloacetate in subsequent oxidationreactions.[38] This requires a turn and a half of the cycle to maintain acetyl-CoA levels and generate succinyl-CoA.

From an application-oriented point-of-view, it may be possible to use this pathway to sustainably produce high-value specialty chemicals, such as 3-hydroxyproprionate (3-HP) or succinate. The former is used industrially in polymer production and the latter is used to produce solvents and polymers.[46] For this reason, several attempts have been made to utilize these genes in metabolically engineered hosts. For instance, the first three enzymes have been expressed in *P. furiosus* to introduce a temperature-shift-responsive metabolic mode for the production of 3-HP.[47] Further work with this metabolically engineered strain has demonstrated that the addition of a biotin protein ligase can improve 3-HP titers more than eight-fold.[48] This dramatic improvement is likely due to the presence of a biotinylated subunit on the key acetyl-CoA carboxylase enzyme from the cycle.[36] Thus, this well-studied pathway has opportunities to be utilized and improved upon.

Sulfur Utilization

In contrast to carbon metabolism, sulfur metabolic pathway discovery is hampered by the tendency of elemental sulfur and its derivatives to react non-enzymatically, masking the true identity of an enzyme's substrate or products.[49,50] Because S^0 is sparingly soluble in water under standard conditions, the true substrate for microbial growth on sulfur is likely soluble polysulfides and polythionates, introduced by nonenzymatic reactions.[51–53] However, these solubilizing reactions only occur at near-neutral pH, since under acidic conditions the equilibrium strongly favors elemental sulfur.[50] In order to over-come these solubility issues, it has been proposed that acidophiles may physically associate with sulfur particles or that at elevated temperatures sulfur becomes sufficiently soluble to support growth.[53] Regardless, these organisms have a suite of proteins capable of manipulating the initial elemental sulfur from the environment, as well as many of its derivatives.

The sulfur oxygenase reductase (SOR), first identified in a member of the Sulfolobales, *Acidianus ambivalens*,[54] appears to be key to acidophilic sulfur oxidation. This intracellular enzyme is active on elemental sulfur, indicating transport of elemental sulfur or one of its derivatives to the cytoplasm by some still unidentified mechanism. SOR appears to be limited to the Sulfolobales and a few extremophilic bacteria;[55] this makes sense given that other sulfur lithotrophs grow closer to neutral pH where more soluble sulfur species are abundant. SOR acts on elemental sulfur by disproportionating it equally between oxidized (sulfite; SO_3^{2-}) and reduced (hydrogen sulfide; H_2S) products. Further, the production of thiosulfate observed in early studies

of the SOR[54] is now believed to be the result of a nonenzymatic reaction.[56] SOR requires oxygen to function but uses no additional co-factors, suggesting its ability to conserve cellular energy. Instead, it 'activates' long, unwieldy hydrophobic sulfur chains into smaller intermediates that can be used by other enzymes to generate energy.

Acidianus ambivalens has served as the model organism for sulfur metabolism studies in the Sulfolobales, since measurements of sulfur-active enzymes from cell extracts were used to construct a conceptual model of its sulfur oxidation pathways.[57] While the SOR enzyme has been the most thoroughly characterized enzyme with respect to sulfur metabolism,[54,55,58] studies of enzymes purified or detected from *A. ambivalens* cell extracts provide some insights into the complete oxidation pathway. The *A. ambivalens* genome remains unsequenced, so many of its enzymes are identifiable only by their activity in cell extracts. This presents a challenge for systems biology-based efforts to understand the details of sulfur oxidation in other Sulfolobales, or even how this process contributes to *A. ambivalens* energetics and metabolism. Regardless, there appears to be two parallel processes by which *A. ambivalens* (and presumably the other sulfur-oxidizing members of the Sulfolobales) gain energy while oxidizing elemental sulfur to sulfate (SO_4^{2-}). One pathway uses the membrane-associated oxidoreductases, TQO and SAOR, to reduce an electron carrier (such as quinone),[59] thereby generating proton motive force via the terminal oxidase,[60,61] while the other pathway produces one high-energy phosphate bond (ADP from AMP) by direct substrate level phosphorylation via APSR and APAT, generating sulfate in the process[57] (**Figure 1-2**). While the sulfur metabolism has been examined in bioleaching applications (see next section), the ability of *S. metallicus* to remove toxic H_2S from high-temperature gas streams represents a potentially important technological use of sulfur oxidation.[62]

A. ambivalens has also served as the model system for anaerobic sulfur reduction among the Sulfolobales.[63] The enzyme pathway for sulfur reduction in *A. ambivalens* appears to be much simpler than for oxidation, possibly involving only two closely-associated membrane complexes. A membrane hydrogenase passes electrons (via a quinone molecule) from H_2 to a sulfur reductase, where they are used to reduce elemental sulfur to H_2S. The cycling of quinones between the two enzymes — forming a 'redox loop' similar to the one used in *Escherichia coli* during growth on nitrate[66] — is likely the way protons are transported across the membrane, coupling sulfur reduction to energy conservation (**Figure 1-2**).

Metal Oxidation

Along with interest in sulfur metabolism, some of the earliest work in determining the mechanism of metal oxidation in the Sulfolobales (and acidophiles, in general) involved the

spectroscopic identification of unique cytochromes from iron-oxidizing cultures.[67] This original research led to the intensive study and eventual development of a model in the mesoacidophile *Acidithiobacillus ferroxidans*, involving the shuttling of electrons from the outer membrane of the cell to the inner membrane, driving a terminal oxidase to maintain pH homeostasis and the production of reducing power for intracellular metabolic needs.[68] Not surprisingly, the spectroscopic data from *S. metallicus* demonstrated early on that key differences exist between bacterial and archaeal metal oxidation, particularly in the presence of cytochromes and the roles of various protein complexes in transporting electrons.[67,69] However, some of the same systems-based approaches were utilized to detect the transcriptomic response of known iron-oxidizers, including *S. metallicus*,[64] *M. yellowstonensis*,[70] and *M. sedula*,[65] in the presence of iron. Interestingly, these experiments suggest the importance of merging several systems biology techniques in order to ascertain new pathways and information. While all three species contain the fox stimulon (an assortment of ferrous-responsive genes A–J) and key related genes (such as rusticyanin and the cystathionin-β-synthase subunits A and B), their regulation varies dramatically among the species with both constitutive and inducible expression observed during iron supplemented growth.[64,65,70] Yet, the merger of this transcriptomic data and genomics analysis yielded a hypothesized pathway for metal biooxidation in these organisms (**Figure 1-2**), which relies on a cytochrome b (as opposed to a cytochrome c), bifurcating rusticyanin(s), and two terminal complexes: an NADH dehydrogenase (generating reducing power) and a putative cytochrome c oxidase (driving pH homeostasis). Although similarities exist between the two systems in *A. ferroxidans* and *Metallosphaera*/*Sulfolobus* spp., distinctive co-factors and apparent differences in organization suggest that these systems are evolutionarily divergent modes of biooxidation.[71]

These differences, as well as the major phenotypical differences between these two classes of metal-mobilizers, relates to their use in metal bioleaching applications. For example, many mesophilic organisms are ill-suited to bioleaching of highly gangue (i.e., high sulfur content) ores due to the extremely exothermic nature of sulfur oxidation chemistry. The build-up of heat can be problematic in large heap operations that rely on mesophiles alone.[72,73] This physiological trait cannot be undervalued given that the removal of elemental sulfur can improve cyanidation (a form of chemically-driven solubilization), which is commonly used in gold mining. Furthermore, extreme thermophiles appear to have some niche advantages over mesophiles for bioleaching of several specific types of copper ores, including the enhanced dissolution of copper from recalcitrant primary ores (such as chalcopyrite),[74–76] selective mobilization of copper over molybdenum in copper-bearing molybdenite,[77,78] and the unassisted mineralization of arsenic in

the form of arsenate from enargite ores.[79] Bioleaching operations targeting copper have increased dramatically and currently account for more than 15% of the global output.[80] Thus organisms that present an inherent propensity for copper solubilisation such as *A*. *brierleyi*, *S. metallicus*, or *M. sedula* deserve more investigation for their potential industrial application.

Pyrococcus furiosus

Pyrococcus furiosus, the type strain of the genus, was first isolated in 1986 from a hydrothermal vent off of the coast of Vulcano Island (Italy) and has been one of the most studied hyperthermophiles to date, due to its intriguing phenotypical characteristics.[81] Exhibiting optimal growth at 100 ˚C and a pH near 7, it was the second genus, after the autotrophic sulfur-oxidizing *Pyrodictium*, capable of growth at temperatures at or above 100 ˚C.[81] As a heterotrophic organism, *P. furiosus* is capable of utilizing hexoseoligosaccharides such as cellobiose and laminarin,[82] chitin,[83,84] and peptides.[85] Efforts over the past three decades have elucidated many unique features of this organism, including various novelties in metabolic pathways, regulatory mechanisms and proteins and enzymes.

Central Glycolytic Metabolism

P. furiosus grows well on disaccharides (maltose and cellobiose) and glucans (laminarin and starch), but not on monosaccharides nor pentoses.[86] The reasons for this anomaly are unknown but monosaccharides may not be available externally in these high-temperature environments as they are susceptible to the Maillard reactions, in which sugars react with available amino acids to form glycosylamines; this problem is especially exacerbated in peptide-rich media. Disaccharide and polysaccharide transport may also be more efficient energetically. P. furiosus derives no net substrate level phosphorylation from glucose to pyruvate conversion, unlike the traditional Embden-Meyerhof (EM) pathway that provides two ATP per glucose and the Entner-Doudoroff (ED) pathway which yields one ATP per glucose (see **Figure 1-3**). Thus, the only net substrate level phosphorylation gains are a result of ATP-forming hydrolysis of acetyl-CoA, produced from pyruvate via pyruvate oxidoreductase and acetyl-CoA synthetase.[87] *P. furiosus* contains an untraditional variation of EM glycolysis, in which glucokinase and phosphofructokinase utilize ADP as the phosphoryl group donor, generating AMP.[86] In the early 1990s, these were the first reported ADP-dependent kinases.[86,88] The absence of an energy-conserving step in the glycolytic pathway is due to the absence of a 1,3-biphosphoglycerate intermediate, which is found in both the EM and ED pathways. As shown in **Figure 1-3**, this direct conversion from glyceraldehyde-3-phosphate (GAP) to 3-phosphoglycerate (3PG) does result in

production of a reducing equivalent in the form of reduced ferredoxin, but does not result in substrate-level phosphorylation. The phosphate group is released via hydrolysis without capture of this high-energy bond. The enzyme responsible for the conversion of GAP to 3PG, GAP ferredoxin oxidoreductase (GAPOR), is unusual in that it requires tungsten, an element rarely found in biology.[89] The absence of other, more traditional glycolytic enzymes makes GAPOR's function critical to sugar utilization. Thus, tungsten levels have a significant impact on the growth of *P. furiosis* in the presence of maltose.[89]

Fermentation Pathways

P. furiosus produces reduced ferredoxin through central glycolysis and, as an obligate anaerobe, must have a route to dispose of any excess reducing power. Two primary routes exist for this purpose depending on hydrogen partial pressures, the availability of elemental sulfur and nitrogen, and other regulatory factors. The primary route of regenerating oxidized ferredoxin is through a membrane-bound hydrogenase (MBH) that produces an ion gradient that allows ATP production via ATP synthase.[90] The hydrogenase is thought to exchange the proton gradient generated by hydrogen production for a Na^+ gradient and this is utilized by a Na^+-dependent ATP synthase.[91] This energy-conserving hydrogenase therefore constitutes a single-step electron transport chain, and has been proposed as an evolutionary precursor to the complicated, multi-step electron trans-port chains that are common in present day microbes.[87] While the exact mechanism coupling proton transfer and hydrogen production is unknown, it is estimated that 0.3–0.4 molecules of ATP are produced per two electrons transferred.[87] Thus approximately 1.2 moles of ATP are produced for every mole of glucose converted to acetate via glycolytically produced reducing equivalents. Given the low energy production resulting from glycolysis, this fermentative process is particularly critical.[87]

When elemental sulfur is present, *P. furiosus* produces hydrogen sulfide rather than hydrogen gas.[92] As with H_2 production, a proton gradient is formed by a membrane-bound oxidoreductase (MBX).[93] MBX is highly homologous to the MBH and is thought to oxidize ferredoxin.[93] However, it is not known if MBX reduces sulfur directly or generates NADPH that is then used by a cytoplasmic NADPH- and CoA-dependent enzyme.[93] The reason for the preference for sulfur over proton reduction is not clear but it is strong since the switch from H_2 to H_2S production begins only minutes after the addition of sulfur to a growing culture.[94] The shift is mediated by SurR, a redox-responsive transcriptional regulator that has been well characterized.[95,96]

Another method for disposing of reductant during fermentation involves the transformation of pyruvate to alanine with the addition of available nitrogen.[97] This results in a major energetic penalty, however, as the pyruvate is not used to produce acetyl-CoA, which is responsible for the majority of ATP production. Thus, alanine pathway is only utilized when sulfur is absent and the hydrogen partial pressures are high.[97]

Applied Biocatalysis and Metabolic Engineering

Prior to detailed knowledge of the *P. furiosus* genome and development of genetic manipulation methodology, early work focused on characterizing its novel enzymes, with an eye toward industrial applications. While the DNA polymerase from *Thermus aquaticus* (Taq) is the most widely known and utilized thermo-stable polymerase in PCR reactions, the *P. furiosus* DNA polymerase is considerably more thermostable and of higher fidelity, yet two- to three-fold lower processivity. Owing to its 3' to 5' exonuclease proofreading activity, the polymerase exhibits a 10-foldreduction in error rate compared to the Taq polymerase.[98] Additionally, the NADP(H)-dependent hydrogenase (SH1) from *P. furiosus* is extremely thermostable and has a temperature optimum of 95 ˚C.[90] It has been utilized in a renewable H_2 production *in vitro* system in which sugars are completely oxidized to CO_2 and H . A combination of pure enzymes comprising the pentose phosphate pathway (PPP) were used to convert sugars to CO_2 and the NADPH that is then produced is oxidized and H_2 is produced by SH1.[90] Many other enzymes of interest from *P. furiosus* have been purified and characterized,[99] and include carbohydrate hydrolyzing enzymes (e.g.,α-amylase,[100] amylopullulanase,[100] endoglucanase,[101] and β-glucosidase,[102] and chitinase[83]) and proteases.[103,104]

P. furiosus now has a facile genetic system which has led to efforts directed at metabolic engineering.[105] Earlier work on a related extreme thermophile, *Thermococcus kodakarensis*, a member of the same order as *P. furiosus*, the Thermococcales, paved the way for the *P. furiosus* genetic tools.[106] For *P. furiosus*, its high growth temperature and tolerance to cold shock opens up its use for hosting metabolic pathways from much less extremethermophiles.[107–109] In fact, a novel temperature-shift strategy has been demonstrated that minimizes *P. furiosus* metabolism at sub-optimal temperatures to direct energy to heterologous product formation.[110] As mentioned above, *P. furiosus* produces soluble hydrogenases, which can regenerate reducing equivalents from hydrogen gas.[47] These hydrogenases could allow metabolically engineered *P. furiosus* to use electrons from H to produce highly reduced chemical products.[47] The insertion of three genes from the *M. sedula* 3-HP/4-HB carbon fixation cycle into *P. furiosus* demonstrated production of 3-HP utilizing sugars and sequestering carbon dioxide for a portion of the molecule.[47] Another

heterologous pathway expressed in *P. furious* utilized genes from three thermophilic organisms with optimal temperatures ranging from 65 to 75 ˚C for the production of n-butanol.[111] With this alcohol pathway, significant diversion to ethanol was shown due to promiscuity of the aldehyde dehydrogenase enzymes.[111] The use of less thermophilic enzymes in heterologous biosynthetic pathways has provided insights into *P. furiosus* native metabolism at lower temperatures. For example, at 70–80 ˚C, acetoin is produced as a major metabolic product. Along these lines, it was shown that the removal of acetolactate synthase in *P*. *furiosus* generates small amounts of ethanol as a metabolic end product, as pyruvate was directed toward acetate and eventually ethanol rather than acetoin.[109] In addition to its native abilities to utilize sugars, *P. furiosus* was engineered with a 16 gene cluster to oxidize carbon monoxide to carbon dioxide, producing H_2 and energy in the process.[107] Overall, the ability to engineer utilization of unique energy sources, manipulate temperatures to optimize enzyme activities, and insert genes from a variety of organisms with different optimal growth temperature provide tools not typically available in model mesophilic hosts.

Caldicellulosiruptor spp.

Caldicellulosiruptor is a bacterial genus containing the most thermophilic, cellulolytic microorganisms known to date. Isolated worldwide and having optimal growth temperatures between 70 and 78 ˚C, these Gram-positive, a sporogenic, obligate anaerobes have the ability to degrade unpretreated lignocellulosic biomass, a highly sought after phenotype for consolidated bioprocessing of fuels and chemicals. Many well-studied cellulolytic microbes are known to either secrete individual enzymes or large cellulolytic enzyme complexes (e.g., the cellulosome[112]) into their environment. In contrast, *Caldicellulosiruptor* species instead use an array of multi-modular enzymes to breakdown plant biomass.[113–118] These carbohydrate-active enzymes (CAZymes) are composed of both catalytic [e.g., glycoside hydrolases(GH)] and noncatalytic [e.g., carbohydrate binding module (CBM)] domains. All *Caldicellulosiruptor* species are able to utilize fructose, galactose, glucose, xylose, and pectin via a classical EMP pathway.[119–121] However, arabinose, rhamnose, and fucose utilization, is not conserved throughout all species.[119,121–124] Some of the sugars, such as xylose and arabinose, are broken down via the nonoxidative PPP and then piped into the EMP pathway as intermediates.[125] Although *Caldicellulosiruptor* species lack the oxidative PPP, which generally is responsible for NADPH production, they are still capable of generating NADPH; the exact enzymatic mechanism for this process is currently unknown.[121] Members of the *Caldicellulosiruptor* genus also contain an incomplete tricarboxylic acid (TCA) cycle,

consisting of a reductive branch leading to fumarate and an oxidative branch producing succinyl-CoA.

Carbohydrate Utilization

Caldicellulosiruptor spp. produce many highly versatile and efficient multi-modular carbohydrate-degrading enzymes, made up of combinations of GH and CBM domains. For example, CelA, a lignocellulosic CAZyme, is composed of five carbohydrate-specific domains: GH9-CBM3-CBM3-CBM3-GH48 connected by linker regions.[126–128] These different segments allow proteins to have multiple functions: simultaneously binding to its substrate (via the CBM3s), as well as cleavage of specific bonds (via the GH9 and GH48 domains); these GH9 and GH48domains are capable of endo- and exoglucanase activity. CelA is present in only the most cellulolytic species of the genus and its two GH domains provide these species with a unique 'drilling' mode of action during biomass deconstruction.[126] While CBMs allow the CAZyme complex to adhere to the bio-mass, surface layer homology (SLH) domains are also found in the *Caldicellulosiruptor* multi-modular scheme.[129,130] Instead of being freely transported out of the cell, CAZymes with SLH domains are tethered to the cell's S-layer. As such, the enzymes can break-down and bind, if they contain CBMs, substrates in close proximity to the microbe, providing better access to available sugar moieties. It also has been recently found that *Caldicellulosiruptor* species have a novel method of attaching themselves to crystalline cellulose. Structurally unique proteins, called tapirins, are expressed on the cell surface, and contain a binding domain specific to insoluble cellulose.[131] Present in every member of the genus and highly expressed, tapirins are thought to play an important role in how plant matter is deconstructed by microbes in this genus.

Both methods of attachment, along with the large inventory of glycolytic enzymes, give this genus its impressive ability to degrade a wide variety of lignocellulosic substrates.[112] *Caldicellulosiruptor* species are capable of breaking down cellulose and hemicellulose (hexoses and pentoses), both as simple monosaccharides and complex biomasses.[114–116,122,132–136] Unlike many cellulolytic organisms, they do not exhibit car-bon catabolite repression, a process by which certain sugars are preferentially metabolized, while excluding the usage of others.[112,117,120] This is especially advantageous in an industrial process involving lignocellulose conversion to fuels and chemicals, as these microbes can utilize multiple sugars simultaneously, with numerous points of entry to central carbon metabolism (see **Figure 1-4**). Although *Caldicellulosiruptor saccharolyticus* was shown to grow well on a variety of sugars (arabinose, fructose, galactose, glucose, mannose, and xylose) simultaneously, the extent of which each monosaccharide was digested varied, with fructose being the most utilized.[117] In the absence of an apparent carbon

utilization regulatory system, variation in sugar utilization among *Caldicellulosiruptor* species is likely due to presence or absence of certain metabolic pathways, for example, the oxidative PPP, and/or essential transporters; the latter has only recently been better understood for a few *Caldicellulosiruptor* species with transcriptomics analysis of growth on substrates, such as simple sugars, crystalline cellulose (Avicel), and complex biomasses like switchgrass.[113,114,117,121,137]

One option to improve degradation of lignocellulose is to increase the CAZyme inventory of a microbe. While generally highly conserved in the *Caldicellulosiruptor* genus, the SLH domain xylanase from *Caldicellulosiruptor kronotskyensis*, Calkro_0402, is not present in *C. bescii* and, thus, was inserted into the genome to improve its ability to utilize xylan.[130] The manipulated strain successfully expressed the protein on the S-layer of the cell and improved xylan utilization significantly by doubling xylose release into the supernatant from oat spelt xylan. Growth on washed and unwashed birch xylan was improved, while dilute acid-pretreated switchgrass solubilization remained unaffected; this indicated that there are still other hurdles to lignocellulosic degradation that must be overcome. However, on substrates with high xylan content, the engineered strain showed improved solubilisation, possibly due to increased substrate attachment.

Fermentation

The major fermentation products of the *Caldicellulosiruptor* genus are hydrogen, carbon dioxide, and acetate.[122,138,139] Lactate production has also been measured, but only a trace amount of ethanol has been detected in wild-type cultures.[119,123,124,140] By far, the most studied product of all of these is molecular hydrogen, especially with *C. saccharolyticus*.[120,121,141–154] H_2 generation is completed via hydrogenases utilizing reducing equivalents (Fd_{red} and NADH) from central carbon metabolism (see **Figure 1-4**). *C. saccharolyticus*, along with several other extreme thermophiles, is considered to be an ideal 'biohydrogen factory', as reported yields are close to the so-called 'Thauer limit' of 4 moles H_2 per mol glucose.[120,122,155] Decreased H_2 production results in the accumulation of NADH and Fd_{red}, while increased H_2 can instead push metabolic flux toward lactate production. At high levels of molecular hydrogen, product inhibition occurs via increased dissolved H_2 levels.[156] NADH and Fd_{red} are simultaneously oxidized by a bifurcating [FeFe]-hydrogenase, which uses both electron donors at the same time,[157] while Fd_{red} is also oxidized by a membrane-bound [NiFe]-hydrogenase that is related to that found in *P. furiosus*.[91]

With the recent development of a genetic engineering system in *Caldicellulosiruptor bescii*, based on auxotrophic selection-targeted manipulations of the *Caldicellulosiruptor* genome and consequently metabolism are now possible.[158–161] The first directed demonstration of these

methods actually involved the deletion of the single lactase dehydrogenase (ldh—Athe_1918) present in *C. bescii* to halt lactate production.[162] While the wild type and parent strains produced less hydrogen than the well-studied *C. saccharolyticus* (1.8 and 1.7 versus 2.5 mol H_2/mol of glucose, respectively), the ldh knockout produced significantly more H_2 on switchgrass, closer to the theoretical goal (3.4 mol H_2/mol of glucose). As lactate formation ceased, acetate production increased by 38–40% over the wild type and parent strains.

More recently, ethanol production was demonstrated in *C. bescii* through the addition of an NADH-dependent alcohol dehydrogenase gene from *Clostridium thermocellum* (adhE—Cthe_0423) into the strain lacking lactate formation;[163] *C. bescii* does not possess a native alcohol or acetaldehyde dehydrogenase, and thus a representative gene was recruited from another thermophilic Firmicute. Owing to the lower thermostability of the protein, growth of the engineered strain was done at a maximum of 65 ˚C. Strain growth on cellobiose, Avicel and switchgrass, and resulted in 14.8 mM, 14 mM, and 12.8 mM ethanol, respectively. Acetate production was also lowered, ranging from ~4 to 5 mM compared to the wild type (~6 mM) and parent (~8–9 mM) on all tested substrates. Another attempt at ethanol production was completed by individually inserting two bi-functional alcohol dehydrogenase genes from *Thermoanaerobacter pseudethanolicus* 39E, *adhB* (Teth39_0218) and *adhE* (Teth39_0206), into the *C. bescii* lactase dehydrogenase knockout.[164] Growing the modified strain at 75 ˚C with cellobiose, ethanol was produced at reported levels of 1.4 mM and 2.9 mM, acetate at 15.5 mM and 14.1 mM, and H_2 at 23.2 mM and 22.5 mM for the AdhB and AdhE knock-in strains, respectively; similar ethanol levels were also measured on Avicel and switchgrass.

With a genetic system now in place, gene 'knockouts' in *C. bescii* can be strategies to understand *Caldicellulosiruptor* metabolism and physiology. For instance, an uncharacterized [Ni-Fe] hydrogenase maturation gene cluster (hypABFCDE—Athe_1088-Athe_1093) was deleted from the aforementioned, modified ethanol-producing *C. bescii* strain containing *adhE* from *C. thermocellum.*[165] The resulting strain produced 20% less H_2 than its parent, yet its H_2 yield per mol of cellobiose increased 63% (3.58 versus 2.19 mol H_2/mol cellobiose). Fermentation patterns on Avicel, cellobiose, and switchgrass showed that the engineered strain also produced acetate (1.6–5.7 mM—34% less than parent) and ethanol (1.9–2.7 mM—73% less than parent). Additionally, the knockout had reduced growth and a longer lag phase, which could result from the deleted gene acting as an ATP-generating protein pump. Other genetic manipulations with *C. bescii* include the deletion of CelA (Athe_1867),[128] a predicted pectin lysase, and a putative AraC family transcriptional regulator genes (pecABCR—Athe_1853–1856).[166] Continued efforts with

the newly established genetics tools in *C. bescii,* and eventually other *Caldicellulosiruptor* species, will help reveal the basis for its ability to grow on lignocellulosic substrates.

Thermotoga spp.

The bacterial genus *Thermotoga* contains nine named species that are obligate anaerobes capable of growth at optimal temperatures between 65 and 80 ˚C, mostly isolated from submarine geothermal features.[167–169] These rod-shaped, Gram-negative, eubacteria were originally identifiable by their disinctive 'toga'-like outer sheath and absence of an outer membrane. Beyond their unique appearance, the species in the genus *Thermotogas* have a remarkably large number of homologs (roughly 24% of the genome) with sequenced archaea.[170] This curious result has led some research into the evolutionary divergence/convergence of this bacterial lineage, suggesting the genomic features that may be critical in defining thermophily, such as the discovery of genes associated with biosynthesis of di-myo-inositol-phosphate, which may serve as a critical thermoprotectant compatible solute.[171] Further phylogenetic analysis has even suggested that mesophily may have developed from thermophily (within the order Thermotogales), given the ancestral sequence reconstruction of more thermally stable myo-inositol-phosphatesynthase (MIPS)[172] and emergence of 'mesotoga' species.[173] Also of interest is the presence of a system for catabolizing myo-inositol that provides utilization of compatible solutes but cannot provide a complete source for carbon utilization.[174] Within the genus, *Thermotoga maritima* has served as a model species for studying evolution, biomass deconstruction, and biohydrogen production.[175]

Carbohydrate Utilization

All *Thermotoga* species are chemoheterotrophs, although the range of substrate usage varies and includes numerous pentoses, hexoses, disaccharides, and polysaccharides, as well as yeast extract, acetate, methanol, and pectin.[168,169,176–180] This ability to utilize a broad array of carbohydrates appears to be supported by bioinformatics and transcriptomics suggesting a substrate-specific regulation and function of large numbers of ABC-transporters,[181] as well as many α- and β-GH.[118] Intriguingly, *T. maritima* grows faster on complex carbohydrates than on monosaccharides, suggesting an adaptation to the breakdown of biomass in their natural environments.[182] The metabolism of carbohydrates by these organisms results not only in the formation of some typical fermentation products, such as acetate, carbon dioxide, and lactate, but also the generation of molecular hydrogen, and small amounts of ethanol, butanol, and butyrate.[183] It is worth noting that *Thermotoga* species utilize the traditional EMP and ED

pathways[184,185] for carbon utilization. However, they also contain, in some cases, unique enzymes that are adapted to optimizing the use of reducing power and energy generated from biomass deconstruction for the synthesis of fermentation products.

The initial genome annotation of *T. maritima* suggested a prevalence of mono- and polysaccharide utilization proteins (as much as 7% of identified genes).[170] In contrast to organisms that produce large complexes for carbohydrate degradation (i.e., cellulosomes), *T. maritima* utilizes a broad array of both extra- and intra-cellular GH, which have been detailed in previous reviews.[118] More recent examination of the pan genome, as well as transcriptomic data, suggests that *Thermotoga* species vary with respect to specific ABC sugar transporters and GH.[186] Overall, the preponderance of thermally stable, polysaccharide-degrading enzymes makes members of the genus and their enzymes intriguing candidates for the deconstruction of complex carbohydrates in industrial applications.[187] However, one of the limiting factors is the absence of any apparent capacity for growth on crystalline cellulose, suggesting a lack of cellulolytic enzymes in *Thermotoga* species.[188] In fact, to address this issue, efforts were directed at the ectopic expression of cellulases from *C. saccharolyticus* fused with *T. maritima* signal peptides. The resulting plasmids were used for *Thermotoga sp*. strain RQ2 transformations, where enhanced exoglucanase activity was observed, but eventually was lost due to poor plasmid maintenance.[189] However, a stable genetic system for *T. maritima* and *T. sp.* RQ7 was recently reported, based around a cryptic plasmid isolated from the latter.[190]

Fermentation

Of the major fermentation products from *Thermotoga spp.,* H_2 production is particularly interesting from a biotechnological perspective. High yields (3.8 mol H_2/mol glucose) reported by *Thermotoga neapolitana* under anaerobic and microaerobic growth conditions[191–193] approach the Thauer limit.[155] The production of H_2 is most efficient when the balance of fermentation products is skewed toward acetate production as compared to lactate production, given that the enzymes identified in acetate production, phosphate acetyl-transferase and acetate kinase, avoid the re-oxidation of NADH and instead produce Fd_{red} and ATP, respectively. In contrast, the production of lactate is driven by a lactate dehydrogenase that uses reducing equivalents (NADH) generated in the glycolytic process. Another possible key to efficient hydrogen production in these organisms, as in the *Caldicellulosiruptor*, is the coupling of Fd_{red} and NADH oxidation by a bifurcating, [FeFe] hydrogenase,[194] in which Fd_{red} likely drives the less favorable oxidation of NADH and improves the overall thermodynamics for producing hydrogen. This enzyme complex, first identified in *T. maritima,* appears to have a homolog in *T. neapolitana,* which has the highest

reported H_2 yields within the genus. There is also evidence that the build-up of molecular hydrogen and a possible inhibition mechanism can be alleviated through the co-culturing of *T. maritima* with *Methanococcus jannaschii*; the latter oxidizes H_2 and generates methane.[195] This results in significant upregulation of CAZymes and growth-phase enzymes, as well as denser cultures[196] (see **Figure 1-5**).

Besides molecular hydrogen, several species have been reported to produce ethanol as a fermentation product.[177,197] This result was not expected given the lack of detectable pyruvate decarboxylase activity. However, more recent work has identified the presence of both an alcohol dehydrogenase (from *Thermotoga hypogea*)[198] and a bi-functional pyruvate ferredoxin oxidoreductase-pyruvate decarboxylase enzyme.[199] Additionally, butyrate (an odiferous organic used primarily as a perfume or food additive) production has been linked with hydrogen biosynthesis in studies involving T. neapolitana[200]; however, the mechanism of butyrate synthesis is still unknown in these organisms.

Thermus spp.

The genus *Thermus* was among the first bacteria to be studied with respect to thermophily, and the DNA polymerase from *Thermus aquaticus*[2] was used in early efforts with the PCR. While not all *Thermus* species (at least 11 have been named and characterized) grow optimally at 70 ˚C and above (extremely thermophilic), there are some that meet this thermal threshold. Thermus species are typically non-motile, nonsporulating, and are not naturally capable of fermentation. Efforts directed at under-standing *T*hermus *thermophilus* metabolism revealed that this bacterium uses glycolysis and the TCA cycle to drive carbon flux and bioenergetics.[202,203] Molecular genetics tools were developed for *Thermus thermophilus*,[204] based on its natural competence, which opened up opportunities for it to be examined as a model thermophile. In fact, the relative stability of thermophilic enzymes and early interest in the genus sparked the undertaking of crystallization projects aimed at characterizing recombinant versions of all the identified coding ORFs from *T. thermophilus*.[205–209] The overall goal of such projects was to provide a comprehensive database of structural characteristics that aid in the determination of protein function and domain architecture representing all of the major classes of proteins identified to date.

Enzyme and Metabolic Engineering Efforts with Thermus

Although more thermophilic microorganisms have become available, *Thermus* species can be sources of thermostable enzymes for biotechnological applications. For example, enzymes from *Thermus* were included in an *in vitro* pathway that converted glucose into n-

butanol[210] and a xylose isomerse from this species was used to enable a recombinant *Saccharomyces cerevisiae* strain to grow on xylose.[211] Although genetics are relatively facile for these organisms, metabolic engineering pursuits have been limited. One of the earliest examples of metabolic engineering of the organism involved the transfer of nitrification genes among two members of the genus, allowing an aerobic *Thermus* species to grow anaerobically.[212] Additionally, a few attempts have been made at overexpression of native genes for the purpose of biotechnological applications involving specific enzymes such as DNA polymerase and Mn-dependent catalases.[213,214] More recently, a strain of *T. thermophilus* HB8 was generated that could co-utilize xylose and glucose at temperatures up to 81 ˚C, with a view towards processing lignocellulose, although this strain could not deconstruct biomass nor ferment the C5/C6 sugars.[215]

Conclusion

Although the study of extreme thermophiles has only gained traction in the past few decades, there are numerous metabolic and physiological features that distinguish these organisms from the other major groups of life and justify continued research endeavors. Much of this information has been ascertained via the use of genomics, transcriptomics, and proteomics in conjunction with traditional microbiological/biochemical techniques. Furthermore, this synthesis has led to the development of metabolic and physiological models in extreme thermophiles that are beginning to rival better characterized mesophilic systems. With the advent of next-generation sequencing technologies, it seems likely that previous work will be furthered by large-scale comparative genomics and metagenomics projects; this should further the discovery of novel metabolic features (i.e., enzymes and native biological pathways) with vital importance to our fundamental understanding of biology.

Beyond the scientific merit of studying extreme thermophiles, numerous opportunities exist to utilize these organisms for biotechnological advancement. As previously emphasized, the extreme conditions under which these organisms subsist has led to evolutionarily distinct metabolic and physiological features. In general, thermally stable proteins and heat-tolerant metabolic hosts could provide a major economic benefit to industrial processes. In the case of upstream processes, it may be possible to eliminate or minimize the energy costs associated with cooling or sterilizing bioreactors; while downstream processes may benefit from simple techniques—such as heat pre-treatment—to select for thermophilic enzymes produced recombinantly in mesophilic hosts, eliminating costly purification steps. Additionally, the increase in available genetic systems in these organisms will open many avenues for metabolic

engineering. In fact, these organisms could have vital roles in the future of bioprocessing ranging from sustainable biochemical engineering to specialty chemical production to the deconstruction of inorganic and organic raw materials and even the recovery of base, precious and strategic metals.

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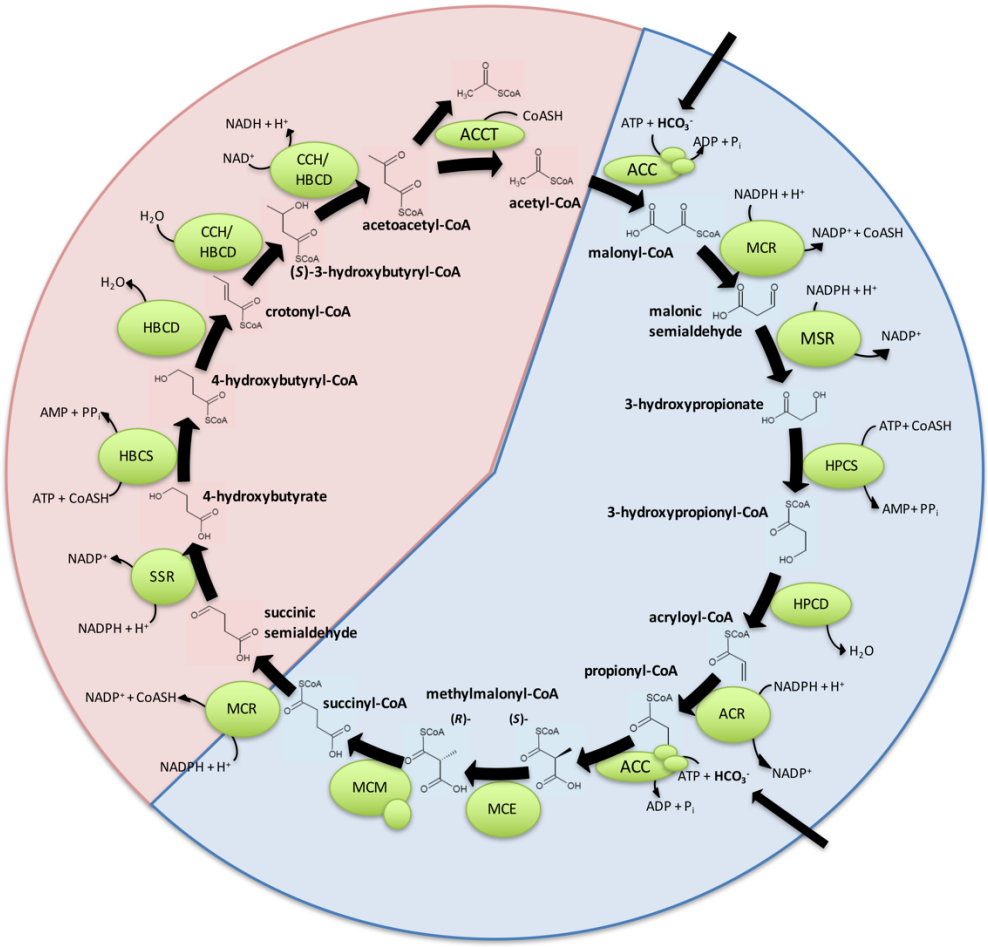

Figure 1-1. 3-Hydroxypropionate/4-hydroxybutyrate (3-HP/4-HB) cycle from Metallosphaera sedula.

The cycle consists of two major portions: carbon incorporation (via bicarbonate) occurs in the first half (blue) of the cycle and is followed by subsequent reduction and reformation of two acetyl-CoA molecules in the second half (red). Enzymes listed and their references: acetyl-coA carboxylase[38,40] (ACC), acetoacetyl-CoA β-ketothiolase[37] (ACCT), acryloyl-CoA reductase[41] (ACR), crotonyl-CoA hydratase[42] (CCH), 4-hydroxybutyrate-CoA dehydratase[37] (HBCD), 4-hydroxybutyrate-CoA synthase[43] (HBCS), 3-hydroxypropionate-CoA dehydratase[41] (HPCD), 3-hydroxypropionate-CoA synthase[44] (HPCS), methylmalonyl-CoA epimerase[45] (MCE), methylmalonyl-CoA mutase[45] (MCM), malonyl-CoA/succinyl-CoA reductase[46] (MCR), malonate semialdehyde reductase[46] (MSR), and succinate semialdehyde reductase[46] (SSR).

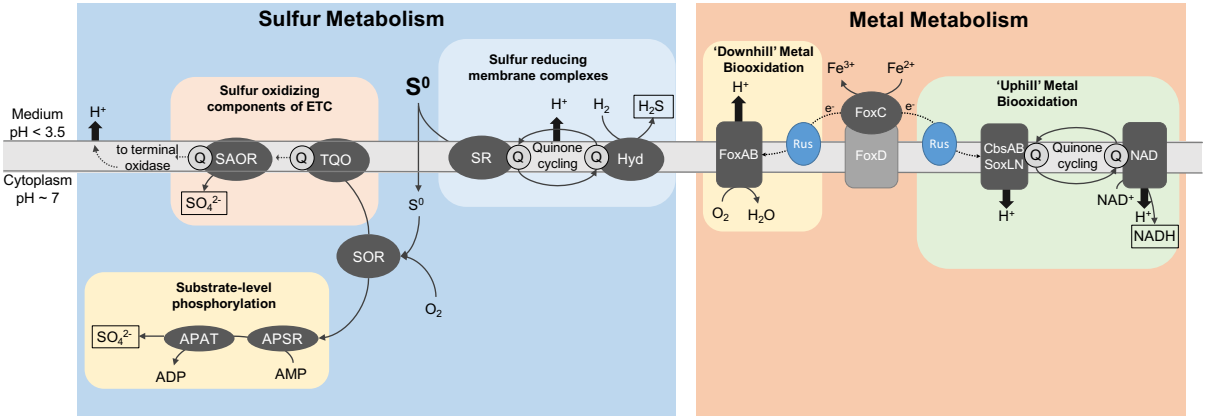

Figure 1-2. Chemolithotrophic pathways in the Sulfolobales.

The first half of the figure (blue) shows the hypothetical pathways for sulfur utilization in the Sulfolobales, including both oxidizing and reducing pathways, beginning with elemental sulfur. Sulfur reducing complexes: hydrogenase (Hyd), sulfur reductase (SR).[64] Sulfur oxidizing enzymes: sulfur oxygenase reductase (SOR),[55] thiosulfate:quinone oxidoreductase (TQO),[60] Sulfite:acceptor oxidoreductase (SAOR), adenylylsulfate reductase (APSR), adenylylsulfate:phosphate adenyltransferase (APAT).[58] The second panel shows a hypothetical pathway for the oxidation of ferrous iron using several *fox* stimulon proteins as well as some iron-responsive respiratory proteins. Ferrous-oxidation (Fox), rusticyanin (Rus), cystathionine-β-synthase containing protein subunits A and B (CbsAB), sulfur oxidation (Sox), NADH dehydrogenase (NAD).[30,65,66]

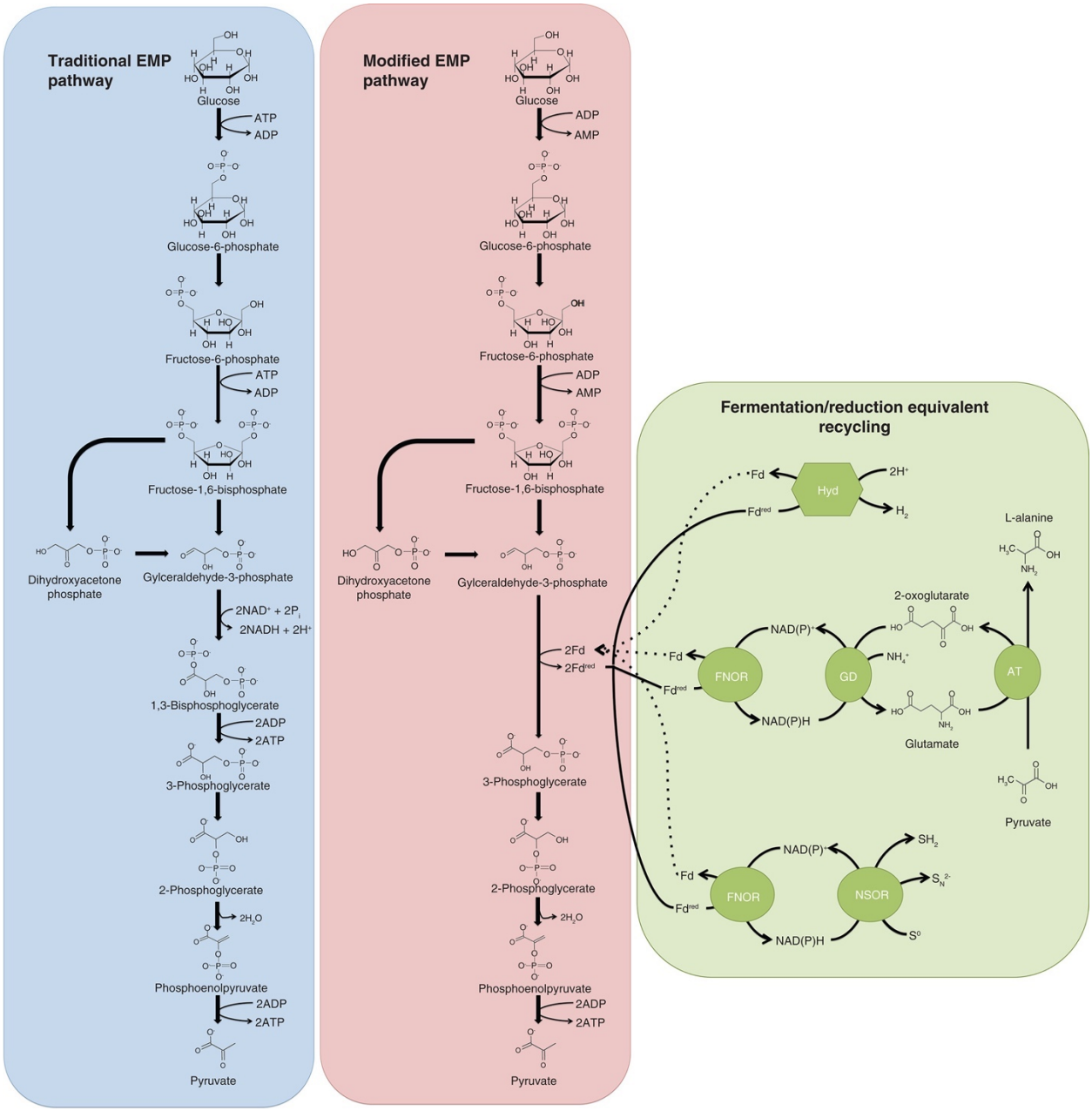

Figure 1-3. Comparison of traditional Embden-Meyerhof-Parnas pathway with the modified pathway in the archaeon *P. furiosus*.

Included are three fermentative pathways which utilize the reduced ferredoxin produced via glycolysis. Enzyme abbreviations: hydrogenase (hyd), ferredoxin:NADP oxidoreductase (FNOR), glutamate deaminase (GD), alanine aminotransferase (AT), and NADP:sulfur oxidoreductase (NSOR).

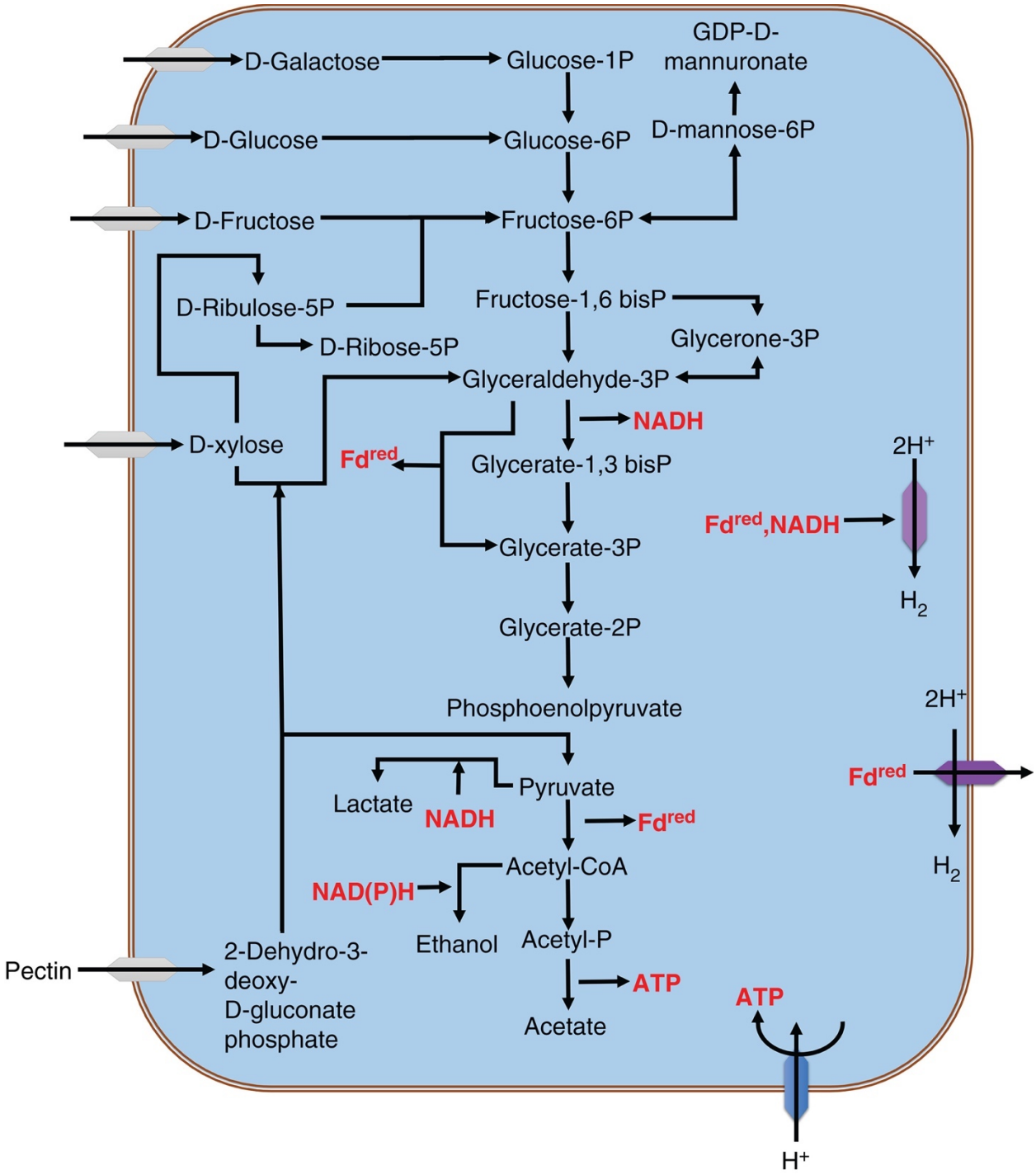

Figure 1-4. Conserved metabolic pathways in all *Caldicellulosiruptor* species.

This includes sugar uptake, glycolytic, and fermentative pathways. The figure includes only the major steps, or start and end products. P is the abbreviation for phosphate, NADH for reduced nicotinamide adenine dinucleotide, ATP for adenosine triphosphate, Fd^{red} for reduced ferrodoxin, and GDP for guanosine diphosphate.

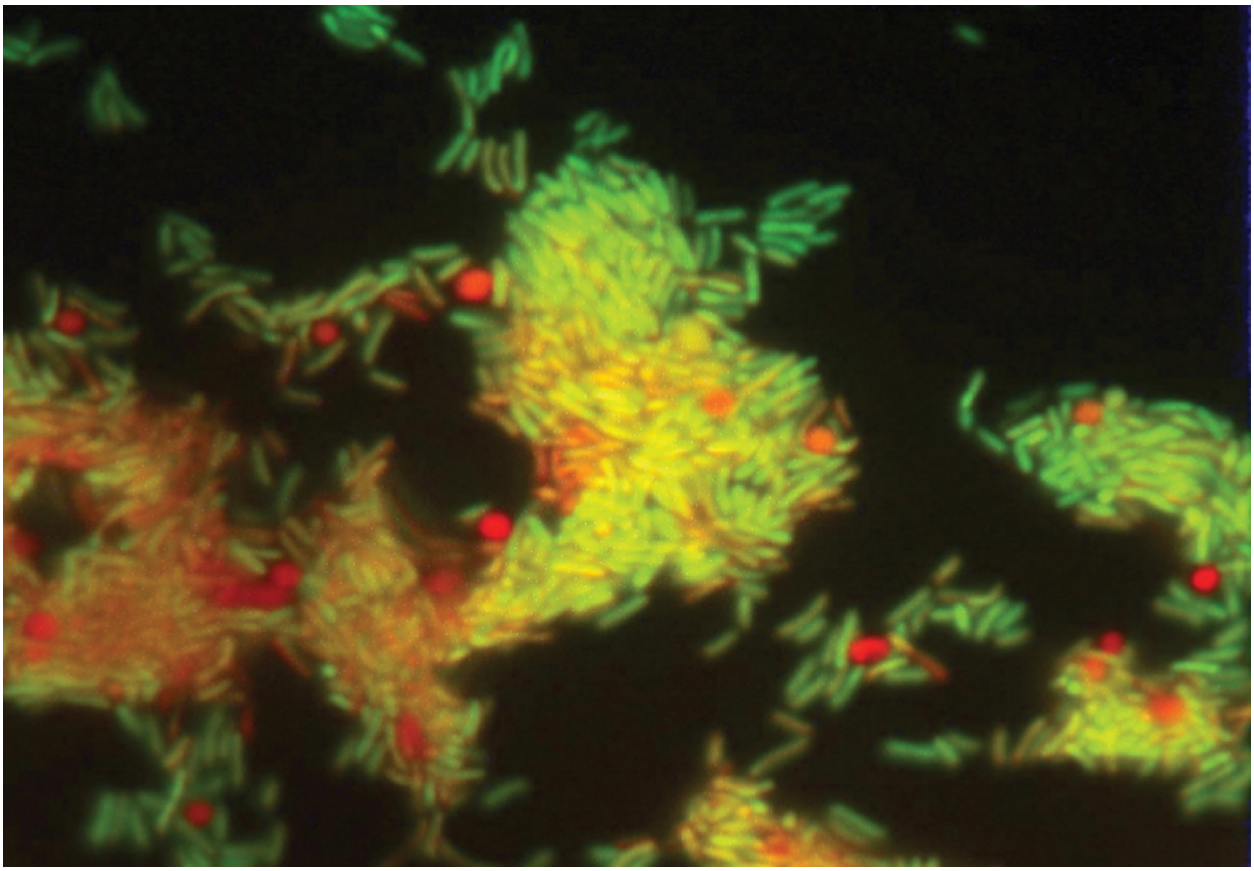

Figure 1-5. Co-culture of *Thermotoga maritima* (yellow/green rods) and *Methanocaldococcus jannaschii* (red cocci)[197]

CHAPTER 2: The Exceptionally Extremophilic Sulfolobales: An Archaeal Order Dominating Exceedingly Hot, Acidic, and Inorganic Terrestrial Springs

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Abstract

The archaeal order Sulfolobales are among the few inhabitants of extremely acidic, terrestrial geothermal features. These environments are often carbon-limited, and yet teaming with life driven by inorganic materials percolating from the depths below where volcanic activity mingles with mineral-rich fissures. These primary producers conserve the energy pouring from the core of the Earth, using electrons from inorganic sulfur, iron, and acid-driven proton motive force to fix carbon dioxide and generate biomass. While their discovery has shifted our understanding of extremophily, their continued study is producing models for essential biological processes, as well as novel sources of biotechnologically-adaptable enzymes and hosts. Further investigation in the current genomic age will likely unravel mysteries of evolutionary origins, extraterrestrial analogues, and highly niche-driven biodiversity. Already a number of these organisms have been isolated from locations spanning the globe. However, newer species with intriguing phenotypes are still being discovered, suggesting that there is more to find, while their diversity and global habitats remain to be explored.

Introduction

The archaeal order Sulfolobales (Archaea; TACK Group; Crenarchaea; Thermoprotei; Sulfolobales) is composed of strictly thermoacidophilic organisms (T_{opt} > 60 ˚C and pH_{opt} < 4.5) originating from terrestrial thermal springs throughout the world[1,2]. This group was one of the first discovered from the third domain of life in the 1960's, paving the way for a dramatic reconfiguration of microbial taxonomy and challenging many of our preconceived notions surrounding the limits of life and our own evolutionary roots[3]. From the pioneering work of microbiologists like Thomas Brock[3], Wolfram Zillig[4-7], and Karl Stetter[8-13], a modest repository of representative species has developed over the past few decades and several species, such as *Sulfolobus acidocaldarius, Saccharolobus solfataricus,* and *Sulfolobus islandicus*, have emerged as model, genetically-tractable organisms[14-16]; these model organisms bridge the knowledge gap between the divergence of bacteria and eukaryotes, explaining the emergence of complex molecular machineries (e.g. polymerases/ribosomes), elucidating the role of proteins in regulating gene expression, and eradicating the divide between the inorganic, primordial life and the modern, organic paradigm[1].

Currently, the order Sulfolobales contains six named genera (*Acidianus, Metallosphaera, Sulfolobus, Stygiolobus, Sulfurisphaera, Saccharolobus,* and *Sulfodiicoccus*), mostly defined by the sequencing of 16/23S rDNA sequences and physiologically-distinguishing traits. These traits span the range of oxygen tolerance: *Stygiolobus azoricus* is the only obligate anaerobe[13], while

Acidianus spp. behave as facultative aerobes[12] and *Metallosphaera, Sulfolobus, Saccharolobus, Sulfodiicoccus,* and *Sulfurisphaera* are believed to behave as obligate aerobes or microaerobes. In addition to this, there are many species capable of carbon dioxide incorporation, while others are limited to the apparent catabolism of amino acids and saccharides[1]. Also, there is a broad spectrum of capabilities to utilize sulfur species and metal species as energy sources to power respiratory and anabolic metabolisms[2].

In the past, these organisms have mainly been studied for their eccentricities, from sulfur reduction and concomitant oxidation in *Acidianus ambivalens* to carbon fixation in *Metallosphaera sedula* to the initial characterization of metal biooxidizing complexes in *Sulfolobus metallicus*. More recently, genetic engineering has emerged, particularly for *Sulfolobus acidocaldarius,* which has emerged as a model system for the study of DNA replication, regulation, and repair[1], carbohydrate utilization, biofilm formation[17], and archaeal motility[18]. While this has opened the door for bioengineering applications, the inability to expand the ease of genetic manipulation to Sulfolobales with more eccentric physiologies (iron oxidation, sulfur biotransformation, and carbon assimilation) has stymied the utilization of the breadth and depth of extreme thermoacidophily[19]. Additionally, many of these organisms were isolated in the pre-genomics era, requiring painstaking effort to validate genetic determinants and isolate novel proteins and leading to an eventual dormancy in research endeavors. Thus, the future of the Sulfolobales is one in which the genetic-tractability of hosts is expanding. New, comprehensive genetic information complements earlier research focusing on chemolithoautotrophic capabilities and revealing the depth of identified diversity among the existing organismal catalogues. These guardians of the geothermal acidic springs serve a critical role in the regulation of escaping materials and energy from our inner planet, particularly in sulfur-rich geothermal vents which are ubiquitous throughout the world.

Acidic Thermal Springs: An Inorganic *Tour de Force*

Aqueous geothermal features connect the inner Earth and the terrestrial and marine crust, serving as a throttle between the expulsion of inorganic innards into our cooler, oxygen-rich, organic biotope. At these intersections, subsurface fluids can expel as either steam, due to rapid expansion (fumaroles), or collect in thermal pools (springs). In either case, hyperacidic features can form in the presence of strong acid-rich magmatic exhausts, and tend to contain copious levels of sulfuric acid, likely produced by the oxidation of hydrogen sulfide, sulfidic ores, and colloidal sulfur[20]. However, the kinetics of sulfur-species oxidation in oxic and anoxic conditions often cannot account for the large buildup of sulfate via slow, abiotic processes[21,22], implicating

microbial life (orders of magnitude faster) in this biotransformation[23]. Although the abiotic process of sulfur oxidation is relatively slow, there is an abundance of energy to be gleaned from the complete oxidation of sulfide species (over 500 kJ/mol)[24]. Thus, the formation of intermediate/oxidized species of sulfur provoked the use of the names *Sulfolobales*, *Sulfolobaceae*, and *Sulfolobus*, which all pay homage to the role of sulfur-driven energy conservation in these organisms.

There are, however, other major energy sources in these environments, such as soluble metal species, primarily iron. In acidic environments, metals are more soluble, particularly ferrous and ferric iron, which are capable of hydration coordination, keeping them active in aqueous forms. In turn, the continued decrease in pH appears to improve the difference between the reduction potential of ferric and ferrous iron (E0' Fe^{3+}/Fe^{2+} = 0.77V) versus oxygen and water (E0' O_2/H_2O = 1.12V)[25,26]. Further, the kinetics of autooxidation (by oxygen) of ferrous iron are dramatically slower at pH 2 versus circumneutral conditions, creating a reservoir of iron reductant for respiratory iron biooxidation, a more wide-spread phenomenon in hyperacidic environments[27]. In general, this translates into increased mobilization of numerous industrially-relevant, metal-oxidizing species that do not require the addition of exogenous energy[28-30], largely driven by biotic-abiotic iron cycling[31]. Additionally, iron can serve as an electron sink in anoxic environments where reduced sulfides, produced through the conservation of energy from hydrogen or from the disproportionation of sulfur from sulfur trioxide, can reduce ferric iron[32,33], and potentially mitigate the cellular damage from highly reductive sulfur species. Furthermore, ferric iron can also be recycled through photoreduction[34,35], and potential remixing could provide a ready source of ferrous iron.

Finally, carbon dioxide and hydrogen are readily available in geothermal settings. Interestingly, the availability of hydrogen (a powerful reductant[36]) is plentiful in these features, where copious outflows are measurable[22,37]. Further, while hydrogenogenic organisms may be present in these environments, acidic springs seem to favor the use of hydrogen as a primary source of energy to produce biomass[38,39]. In particular, these acidic environments appear to be driven by the expulsion of vapor-phase and not liquid-phase vents[38,40]. In fact, this source of energy may be a primary driver of autotrophy; this would be a critical process in supporting life since little organic carbon enters from geothermal activity, whereas carbon dioxide is readily available from magmatic gas flows[41,42]. Thus, highly acidic geothermal environments may contain heterotrophic organisms, but their presence is likely dependent upon the presence of primary producers utilizing inorganic energy and carbon inputs.

Origins and Evolution of Extreme Thermoacidophily

The study of extremophilic environments from thermal springs to glaciers, and brine pools to desert sands have often focused on the elucidation of one particular type of niche adaptation. The capability to subsist and thrive in these environments is not often limited to a particular domain of life and varying degrees of biodiversity can be observed in a multitude of extreme environments[43-45]. In the case of thermophily[46] and acidophily[47,48], there are studies that demonstrate each variable has a marked impact on microbial diversity, particularly the latter. However, the convergence of evolutionary constraints unexpectedly serves as a strict throttle on the proliferation of biodiversity. In the case of hot acid springs, there is growing evidence that the archaea are both prolific and dominant inhabitants of these concomitantly demanding niches[47,49,50].

This presents a question of whether these environments represent an ancient holdover of primordial life: Did the domain Archaea form in the most extreme but energetically rich environments, allowing diversity to flow outward to the remainder of the uninhabited planet? The name itself, Archaea, originating from the same Greek word that gives us ancient, suggests the thinking of early evolutionary microbiologists and the poetic input of evolutionary philosophers such as Carl Woese[51]. However, recent analysis of the uncultured world has begun to challenge the clean delineation of the three domains, the basis of our last four decades of evolutionary philosophizing[52-54]; the root of our evolutionary expanse is now in question, not by reckless hypothesis but through the preponderance of data suggesting reconsideration. This is in addition to an expanding archaeal domain, not of intransigent ancient inhabitants, but unculturable ecological participants, particularly in the TACK Superphylum[55-57] and the newly identified Asgardarchaeota[53,58,59].

Furthermore, DNA sequencing techniques are exponentially expanding the repository from which evolutionary inference can be made. In the case of thermoacidophily, growing evidence suggests that Archaea dominate these niches[47,50,60]. Further, aerobic respiration seems to play a critical role in the metabolism of the organisms present, suggesting the importance of high energy redox transformations[61]. This suggests that extreme thermoacidophiles may be a recent addition to the tree of life, most likely originating within the last 0.85 Ga from multiple convergent evolutionary trajectories[47]. This only became possible when phototrophic oxygenesis, exclusive of thermoacidophilic environments[62], dramatically altered the Earth's atmospheric composition[63].

While various forms of acid conjugates exist in these environments, there is a clear dominance of sulfuric acid[64], suggesting that sulfate is a primary product of the organisms in these

environments[64,65]. Thus, thermoacidophilic springs could be an environmental niche created through evolutionary processes (niche construction[66]), occurring after the great oxygenation events that have limited further diversification, particularly given the limited potential for backflow of genetic information to these environments. In all, this suggests that extremely thermoacidophilic Thermoprotei lineages, particularly the Sulfolobales, may represent recently differentiated orders within the broader evolutionary history of microbial life.

The Current Taxonomical Landscape of Order Sulfolobales

The Sulfolobales were first identified in the 1960's through the pioneering work of Thomas Brock[3] in Yellowstone National Park (WY, USA), identifying numerous strains of high temperature (>70 ˚C), high acid-thriving (pH < 3.0), lobed organisms capable of heterotrophic and autotrophic modes of life. Even early on, there was an acknowledgement that these organisms are distributed in terrestrial and aquatic geothermal acid springs across the world (isolation sites in **Figure 2-1**). Since the initial characterization of the order, there have been numerous characterized and deposited strains, such that by the early 2000's there were five named genera (*Acidianus*[12], *Metallosphaera*[10], *Stygiolobus*[13], *Sulfolobus*[3], and *Sulfurisphaera*[67]), largely organized via DNA-DNA hybridization, G+C content, and 16S rRNA sequencing.

However, when whole genome sequencing techniques first arrived, *Sulfolobus solfataricus* P2 became the first assembled Sulfolobales genomes that represented a new model for understanding life in hot acid[68]. Since then, several more species have been sequenced from the order, including members of the genera *Acidianus*[69-71], *Metallosphaera*[72-74], *Sulfolobus*[75,76], and *Sulfurisphaera*[77]. These additional genomes have largely preserved the taxonomical boundaries defined in the pre-genomics era and have further revealed many of the determinants of metabolic capabilities and physiological traits from across the extreme thermoacidophilic landscape.

Recently, there has been a push to begin reorganizing the order into new groupings such as the re-named genus *Saccharolobus*[78], and the existing genus *Sulfurisphaera*[79]. Additionally, new isolates continue to arise from the sampling of hot acid springs and within the last decade there have been calls for two new genera, *Aramenus* and *Sulfodiicoccus,* based on the isolation of two new organisms *Aramenus sulfurataquae*[80] and *Sulfodiicoccus acidiphilus*[81,82]. Finally, some of these newer inferences appear conclusive with the examination of even a single-locus (such as 16S rDNA alignment in **Figure 2-2**), *e.g. Saccharolobus, Sulfurisphaera,* and *Sulfodiicoccus* are relatively deep monophyletic groupings. In contrast, this is the taxonomical space around the current genera *Metallosphaera* and *Acidianus*, the latter of which is not well-supported as

monophyletic or the current *Sulfolobus metallicus* group, which is sharply removed from the location of the genus type strain *Sulfolobus acidocaldarius*. Further, there are numerous species that will likely require reclassification as the evidence provided in multi-locus alignments become available, such as the data presented in **Chapter 6**, herein.

Acidianus

The name *Acidianus* (Lat. *acidius* and *Ianus*) pays homage to the facultative nature of this genus, where members are often capable of shifting metabolically between an anaerobic life of hydrogen sulfide production and an aerobic life of sulfur biooxidation[12]. Most of the named species to date are strict chemolithoautotrophs, transforming sulfur in the presence of hydrogen or oxygen as a means of generating reducing power and a circumneutral cytoplasmic pH, for carbon assimilation[12,83,84]. While sugar utilization is observed in some members of the genus (*A. brierleyi, A. manzaensis, A. copahuensis*)[12,85,86], obligate autotrophy appears to be a widespread feature among many members (*A. ambivalens, A. infernus, A. sulfidivorans*)[6,12,83]. Interestingly, metal-biooxidation (iron, copper, molybdenum, lead) appears to be another wide-spread but not requisite metabolic mode for these organisms[83,85,87]. From a physiological perspective, this genus also contains some of the most acidophilic Sulfolobales (pH_{range} 0.8-2.5), with the most acidophilic (pH_{opt} 0.8) *Acidianus sulfidivorans*[83].

Easily the most well-studied metabolic pathways in the genus *Acidianus* involve the biotransformation of sulfur species. As mentioned above, many of these organisms are capable of growing in the presence of carbon dioxide and hydrogen with supplementation of sulfur. This process involves the transfer of electrons from hydrogen, a strong reductant, to soluble sulfur, via the interaction of two membrane complexes: hydrogenase and sulfur reductase[88], producing volatile hydrogen sulfide. Furthering this process is the ability of the organisms to re-oxidize sulfide via a membrane sulfide:quinone oxidoreductase[89], recapturing some of the energy conserved in highly reduced sulfur species, and potentially circumventing the toxicity of such a labile reductant[90]. Additionally, many of these organisms have membrane soluble tetrathionate hydrolases[91], as well as thiosulfate:quinone oxidoreductases[92] that provide a means to conserve energy from soluble thiosulfates. These electron flows are conserved and pushed downward to oxidases (in aerobic environments), such as the DoxBCE[93,94].

Perhaps, the most curious enzyme in the sulfur transformation process for many of these organisms is the sulfur oxygenase reductase (SOR), first isolated from *A. ambivalens*, but also characterized in *A. brierleyi* and *Acidianus tengchongensis*. The complex is believed to catalyze the disproportionation of sulfur into sulfide and sulfite, in the presence of oxygen[95]. Intriguingly,

this process is believed to occur in a cytoplasmic complex composed of 24 SOR monomers, with a reduced iron monoheme around -268 mV, making sulfur reduction possible[96,97]. Further, apolar structure of the complex appear to promote the entry of polysulfides at four-fold symmetry channels[98] and the exit of polar molecules at separate tri-fold symmetrical pores[99]. This cytosolic entry of polysulfide is intriguing because no mechanism to-date has been observed for transmembrane sulfur trafficking in these organisms.

Metallosphaera

The genus *Metallosphaera* originated at the isolation of *Metallosphaera sedula* by Huber in 1989 from the Pisciarelli Solfatara in Italy[10]. The name (Latin *metallum* and *sphaera*) was given due to the prolific biooxidation of metal species when originally isolated in the lab. The genus has since grown to include the species *Metallosphaera cuprina*[100], *Metallosphaera hakonensis*[101], *Metallosphaera prunae*[9], *Metallosphaera yellowstonensis*[102]. The temperature and pH optima of the organisms are in excess of 70 ˚C and below pH 3.0. Unlike the *Acidianus spp.,* the *Metallosphaera spp.* are strict aerobes, incapable of utilizing solid sulfur as a substrate but still capable of carbon fixation and limited utilization of organic carbon. Intriguingly, they have some of the highest G+C fractions of Sulfolobales species (>40%), with the exception of the newly discovered *S. acidiphilus* (around 52.0%)[82].

As the name suggests, metal species have been a driver of much of the research around this genus. After the initial identification of iron-induced respiratory genes in *Sulfolobus metallicus,* the putative genes for iron biooxidation ("fox" locus) were further identified in *Metallosphaera sedula*[103] and *Metallopshaera yellowstonensis*[104]*.* The proposed mechanism centers on the biooxidation of a putative cytochrome b-containing complex, feeding an oxygen-respiratory terminal complex, which could provide proton pumping action. Although, the genes do not appear in the genome of *Metallosphaera cuprina*[73]*,* the putative pathway has yet to be confirmed, suggesting a possible alternative source of biooxidation or another competing mechanism altogether. In fact, a recent study of molybdenum and vanadium biooxidation in *M. sedula* and *M. prunae*, suggests that a separate Rieske protein complex may be induced in the presence of these redox-active metals, although evidence exists for the importance of iron in driving copious metal biooxidation in these organisms[105].

In any case, metals appear to play a crucial role in the physiology of these organisms. Beyond simply biooxidizing these molecules for benefit, the *Metallosphaera spp.* also harbor several systems for the regulation of metal species and circumvention of deleterious effects originating from metals in their environments. For example, the management of copper toxicity is

closely linked to the CopA (Cu^{2+} efflux pump) and PitA (Phosphate importer)[106,107], which may work to sequester intracellular divalent cations. Further, the isolate *M. prunae* originated from a smoldering uranium slag-heap and was discovered to resist the toxic effects of uranium biooxidation through the initiation of a dormant state[108] that was tied to a putative network of toxin-antitoxin systems[109], similar to those seen in antibiotic resistant bacteria[110,111].

Finally, *M. sedula* served as the principle model for carbon fixation in the Sulfolobales, via the 3-HP/4-HB cycle, which cycles acetyl-CoA and generates larger carbon molecules through the addition of bicarbonate via a promiscuous propionyl-CoA carboxylase[112]. The greater pathway involves about 13 enzymes that have all been characterized either *in vivo* or *in vitro*[113-115]. Also, the interconnection of this pathway to central carbon metabolism has been demonstrated through the exit of succinate (roughly 2/3 of the carbon assimilated) into the TCA cycle[116]. Further, many of these genes appear to be conserved in the order, suggesting a conserved pathway for carbon assimilation throughout the order that may or may not be functional in currently studied species[117].

Sulfolobus

This genus was the original type genus of the order and has over the years accommodated a number of species with a similar 'lobus' morphology and low G+C content with phylogenetic similarity to the other Sulfolobales. However, with recent reclassifications, it now composes just a few named species: *S. acidocaldarius, Sulfolobus shibatae*[7], *Sulfolobus costariquensis* A20[76], *S. islandicus*[118], and *S. metallicus*[11]. The former three are strict heterotrophs. Despite the initial characterization of *S. acidocaldarius* as a chemolithoautotroph[3], later evidence has questioned these observations, suggesting that neither sulfur oxidation nor carbon fixation are capable in the current repository strain; recent attempts to reconstruct these pathways suggest that this may be a more recent loss of function[117]. In contrast to *S. acidocaldarius,* as well as the other *Sulfolobus spp.* named thus far, *S. metallicus* is a strictly chemolithoautotrophic organism capable of growth on either elemental sulfur or sulfidic ores (such as pyrite). In fact, it was the initial source for investigations into the iron biooxidative capacity of extreme thermoacidophiles[26], and the induction of "fox" genes[119].

Stygiolobus

Originating from the Island of Sao Miguel (Azores, Portugal), *Stygiolobus azoricus* represents the sole obligate anaerobe of the order, subsisting entirely through the reduction of sulfur via hydrogen gas[13]. Its high optimum temperature (80 ˚C), low pH optimum (2.5), and

requirement for pressurized H_2/CO_2 gas may imply it subsists deeper than other species, in the anoxic zone of hot springs. Although it appears to deviate so dramatically from the other isolated Sulfolobales, phylogenetic analysis has suggested that *S. azoricus* may be closely related to *S. acidocaldarius*[8].

Sulfurisphaera

This particular lineage consists of organisms that are strictly extremely thermophilic (T_{opt} > 80 ˚C) facultatively aerobic, moderate to extreme acidophiles (pH_{opt} 2.0-4.0). Thus far, their isolates have only been identified in the Asian pacific islands, including, Japan and Indonesia[67,79,120]. They are capable of utilizing sulfur as a substrate in the presence of hydrogen for anaerobic growth, as well as utilizing complex carbon molecules for aerobic growth, although their utilization of sugars is often incapable of sustaining growth.

Sulfodiicoccus

This recently isolated genus consists of just a single isolate from Japan, *Sulfodiicoccus acidiphilus,* which is a strict aerobe with a relatively low optimum temperature (65-70 ˚C) and mild acidophily (pH_{opt} 3.0-3.5)[82]; also, the organism has the highest G+C content (52.0) of any Sulfolobales species to-date. Interestingly, the species' growth is inhibited by the presence of solid sulfur[81], suggesting it lacks many of the components involved in detoxifying transient species formed abiotically from solid sulfur. In contrast, the organism appears to strictly utilize complex carbon sources and sugars as primary metabolic inputs, and lacks the molecular machinery present in other Sulfolobales to fix carbon dioxide[81,82]. Interestingly, it appears that a previously unnamed species *Sulfolobus sp* MK5[35], may be fairly similar. Thus, there may be another species which could be characterized to see if these traits mark a stringent evolutionary deviation for this particular genus.

Conclusion

The Sulfolobales are ubiquitous inhabitants and dominant biogeochemical transformers of extremely thermoacidophilic environments across the globe. Their metabolic flexibility and physiological adaptiveness suit the excessively harsh confluence of two extremophilies, temperature and acidity, which to varying degrees have been shown to strongly hamper diversification of microbial species. Further, the order is largely composed of aerobic lineages, which lends credence to the argument that thermoacidophily, unlike thermophily or acidophily, is

a more recent evolutionary branching. Curiously, the branching of thermoacidophily, distinctive from thermophily, occurs at several points in the Crenarchaea, suggesting that convergent evolutionary trajectories resulted in these niche organisms. This might further support the idea that Sulfolobales, along with other polyextremophilic organisms, may not have simply diversified into their own niche but may have even constructed their own environment, erecting an evolutionary barrier that requires a strong activation energy to overcome. Within this order rests a number of physiological traits and specialized enzymes capable of transforming energy-laden inorganic materials, which could serve a role in developing novel and sustainable biotechnological approaches to dealing with waste recovery and synthetic bioprocessing.

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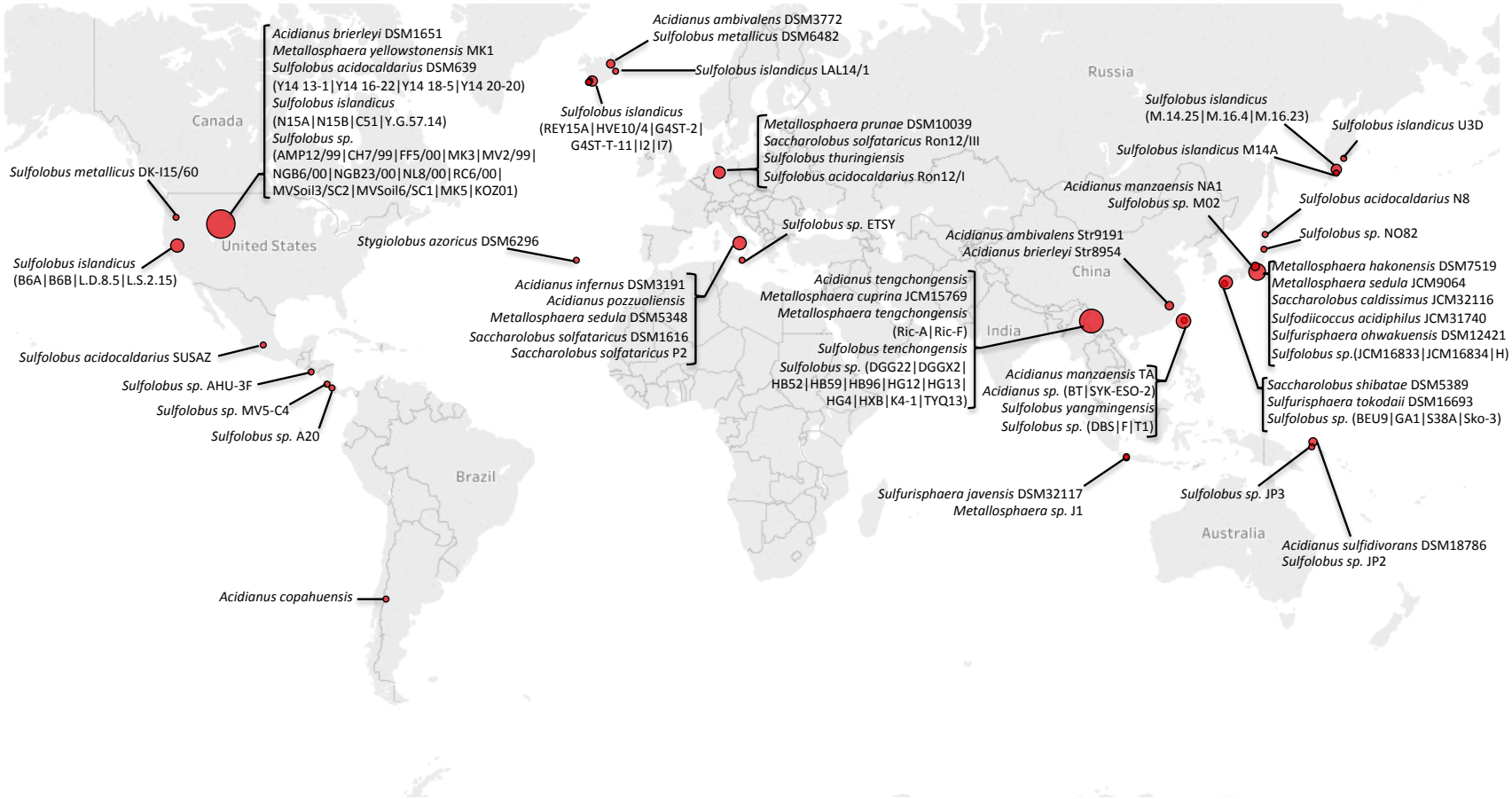

Figure 2-1. Map of reported isolation sites for Sulfolobales.

Isolation sites were determined based on references reported with NCBI records of 16S rRNA deposited in the "gene" (part of a whole genome assembly) or "nucleotide" (individual submissions) databases. This map was constructed in the R-based platform Tableau using OpenStreet geographical data. For unknown locations, GPS coordinates from the nearest municipality were used. Pipes separate strain designations of the same/undefined genus designation. The size of geographical points is based upon the number of isolates from a given site.

Figure 2-2. 16S rDNA Phylogenetic Reconstruction.

Sequences were derived from the "Gene" and "Nucleotide" databases of NCBI, filtering for uncultured, poorly reported, and redundant deposited sequences. Alignment was performed using MUSCLE and trimmed to exclude regions with under 50% coverage and identity. The outgroup for the alignment included type strains from the other 4 orders of the class Thermoprotei: (Acidolobales, Desulfurococcales, Fervidicoccales, Thermoproteales). Finally, a tree was reconstructed using FastTree with the general time-reversible (GTR) algorithm and gamma optimization.

CHAPTER 3: Complete Genome Sequences of Extremely Thermoacidophilic Metal-Mobilizing Type Strain Members of the Archaeal Family Sulfolobaceae, *Acidianus brierleyi* DSM-1651, *Acidianus sulfidivorans* DSM-18786, and *Metallosphaera hakonensis* DSM-7519

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Abstract

The family Sulfolobaceae contains extremely thermoacidophilic archaea that are found in terrestrial environments. Here, we report three closed genomes from two currently defined genera within the family, namely, *Acidianus brierleyi* DSM-1651T, *Acidianus sulfidivorans* DSM-18786T, and *Metallosphaera hakonensis* DSM-7519T.

Main

Members of the crenarchaeal family Sulfolobaceae are exclusively extreme thermoacidophiles, given that their optimal growth temperatures exceed 65˚C and their optimal pH levels are below 3.5. Thus, they inhabit the most inhospitable environments on earth (e.g., volcanic solfatara fields, geothermal mud springs, etc.)[1]. Three species, *Acidianus brierleyi*[2], *Acidianus sulfidivorans*[3], and *Metallosphaera hakonensis*[4], were identified as candidates for sequencing given their reported abilities to utilize metal substrates as electron donors and support chemolithoautotrophic growth. The family Sulfolobaceae, within the order Sulfolobales, was originally named for its members' perceived ability to utilize, and thrive in, sulfur-rich thermal environments; newer evidence, however, shows that, in fact, many members of this taxonomical clade fail to utilize sulfur and/or other sources of electron donors, including metals and their ores, as well as simple and complex carbohydrates. The source locations and known optimal growth conditions for these species are summarized in **Table 3-1**.

To date, there is limited genomic information available for extreme thermoacidophiles, partly due to difficulties in assembling their genome sequences. As an example, a previous sequencing of *M. hakonensis* via the Ion PGM platform resulted in an assembly of 129 contigs (total size, 2,387,907 bp; largest contig size, 269,819 bp; N50 contig size, 59,396 bp; RefSeq assembly accession number GCF_001315825.1). Here, single-molecule real-time (SMRT) technology was used to overcome previous assembly limitations.

Each of the species, whose genome sequences are reported here, was obtained from the Leibniz-Institut DSMZ GmbH. Cultures were grown at their optimal conditions (as specified by DSMZ), and genomic DNA was isolated using phenol-chloroform-isoamyl alcohol separation and propanol precipitation. Samples were sequenced using a PacBio RS II system with one SMRT cell per organism. The assembly was created using CLC Genomics version 11.0.1 with the proprietary Genome Finishing Module, which uses a de Bruijn graph algorithm tailored to PacBio data to improve the final output[5]. Long read data (with coverage in excess of 300) were corrected with the software (100 coverage retained) and used for the assembly, after which the contigs were joined via an iterative process of manual curation (mapping, correcting, extending, and aligning).

All parameters, except the percentage of reads to retain during the correction step, were default values. A summary of the final statistics is given in **Table 3-1**. Once closed, the assemblies were annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP)[6].

Overall, the assembled genome sequences represent both new additions to the available genome sequence information on species representing two of the genera within the family Sulfolobaceae and an improvement on the previously reported assembly of *M. hakonensis*.

Data availability

The genome sequence information reported here has been deposited in DDBJ/ENA/GenBank under the accession numbers given in **Table 3-1** and under the BioProject ID PRJNA463410.

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Table 3-1. Characteristics of sequenced species and genome sequence assembly statistics

Species	DSM/JCM designation	Isolation site	T_{opt} (°C)	pH_{opt}	G+C content (mol%)	Genome size (bp)	No. of CDSs	No. of tRNAs	No. of rRNAs	GenBank accession no.
A. brierleyi	1651/8954	Acidic hot spring, Yellowstone NP, USA	70	1.5–2.0	31 (2)	2,947,156	3,120	46	3	CP029289
A. sulfidivorans	18786/13667	Solfatara, Lihir Island, Papua New Guinea	74	0.8–1.4	31.1 (3)	2,287,077	2,312	46	3	CP029288
M. hakonensis	7519/8857	Geothermal field, Hakone NP, Japan	70	3.0	46.2 (7)	2,544,018	2,570	45	3	CP029287

[a]Abbreviations: T_{opt}, optimal temperature for growth; pH_{opt}, optimal pH level for growth; CDSs, coding sequences; NP, national park.

CHAPTER 4: Genome Sequences from Five Type Strain Members of the Archaeal Family Sulfolobaceae: *Acidianus ambivalens*, *Acidianus infernus*, *Stygiolobus azoricus*, *Sulfolobus metallicus*, and *Sulfurisphaera ohwakuensis*

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Abstract

The archaeal family Sulfolobaceae is composed of extreme thermoacidophiles (T_{opt} > 65 ˚C, pH_{opt} <3.5). This project greatly expands the genomic data available (five closed, one near-complete) for a metabolically diverse set of extremophiles, spanning five genera; this includes obligate and facultative, aerobes and anaerobes, sulfur-reducing and oxidizing, metal-mobilizing, autotrophic, and heterotrophic microbes.

Main

Extreme thermoacidophilic Sulfolobales (Archaea, Crenarchaeota, Thermoprotei, Sulfolobales, Sulfolobaceae) are ubiquitous among extremely acidic geothermal environments around the world. These organisms subsist in inorganic-laden environments ranging from calderas to mud pits to sulfur pools, often acting as some of the first microbial actors on energetic materials percolating from subsurface terrestrial activities. The genomes presented here come from the species *Acidianus ambivalens* DSM-3772, *Acidianus infernus* DSM-3191, *Stygiolobus azoricus* DSM-6296, *Sulfolobus metallicus* DSM-6482, and *Sulfurisphaera ohwakuensis* DSM-12421. The impetus for discerning these genomes sequences is their diverse metabolic capabilities, as well as their global distribution, which are detailed in **Table 4-1**. This work represents a second stage in sequencing that complements a previous work focused on the metal-biooxidizing species of the genera *Metallosphaera* and *Acidianus* (1).

All of the species presented here were obtained from isolates deposited by various researchers and curated by the Deutsche Saamlug von Mikroorganismen und Zellkultern GmbH (DSMZ). All of the cultures were cultured as recommended by DSMZ; further, the *Acidianus spp.* and *S. azoricus* were cultured in anaerobic conditions with a hydrogen/carbon dioxide headspace. Organic solvent-based DNA extraction was performed with phenol, chloroform, and isoamyl alcohol, as well as a propanol precipitation.

Single Molecule Real Time (SMRT) sequencing was performed with continuous long-read libraries for all samples on Pacific Biosciences (Menlo Park, CA, USA) sequencers. Additional short-read data were collected in either single-end 151 bp reads or paired-end 250 bp reads on an Illumina (San Diego, CA, USA) sequencer. Assemblies were performed with a recent repeat graph assembler designed for long error-prone reads, Flye (2) [v2.4.2], using raw long-read data. All of the sequencing and assembly data are shown in **Table 4-2**. Following the initial assembly, short read data obtained from Illumina single-end or paired-end read data was mapped to all of the constructs using Bowtie2 (3) [v2.3.2], and the consensus sequence (>50% identity) in order to correct for sequencing errors from long-read data. These short reads were curated with

Trimmomatic (4) [v0.38], with the criteria of phred33 scores greater than 30 for head and tail of the read and 28 within a sliding window of four bases. All reads under 145 bp for single-end and 240 for paired-end were removed from the aligned data set. Finally, genomes were deposited in the NCBI genomes database where they were annotated via the PGAAP algorithm (5).

In summation, five newly completed genome sequences are presented representing diverse members of the family Sulfolobaceae. While *S. metallicus* and *A. infernus* remain unclosed, the provided draft genome vastly improves upon a previous assembly using second generation short-read technologies that produced 167 contigs. This data set provides a new trove of data to assist scientists in fields ranging from fundamental microbiology and ecology to biotechnology and metabolic engineering.

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Table 4-1. Origins and characterization of type-strain isolates.

Species	Collection ID	Isolation Site	Metabolism (Oxia)	T_{opt} (˚C)	pH_{opt}	G+C (mol%)
Acidianus ambivalens, LEI10 (6)	DSM-3772^T JCM-9191^T	Solfatara at Leirhnjúkur, Mývatn Iceland	Chemolithoautotroph (Facultative aerobe)	80	2.0	32.7
Acidianus infernus, So4a (7)	DSM-3191^T JCM-8955^T	Pisciarelli Solfatara, Naples, Italy	Chemolithoautotroph (Facultative aerobe)	90	2.0	31
Stygiolobus azoricus FC6 (8)	DSM-6296^T JCM-9021^T	Caldera on São Miguel Island, Azores	Chemolithoautotroph (Obligate anaerobe)	80	2.5	38
Sulfolobus metallicus Kra23 (9)	DSM-6482^T JCM-9184^T	Solfatara in Krafla, Mývatn, Iceland	Chemolithoautoroph (Obligate aerobe)	65	2.0	38
Sulfurisphaera ohwakuensis TA-1T (10)	DSM-12421^T JCM-9065^T	Hot spring in Ohwaku Valley, Hakone, Japan	Heterotroph (Obligate aerobe)	85	2.0	33

Table 4-2. Sequencing/Assembly Statistics

Species	Technology	N_{50}/N_{90} Read Length (bp)	Number of Reads	Estimated Coverage	Genome Size (bp)	Contigs	N_{90} (bp)	G+C (mol%)
Acidianus ambivalens	PacBio Sequel	8,671/3,562	299,403	661X	2,252,027†	1		34.2
	Illumina (SE)	147.94 (Avg)‡	3,961,682	260X				
Acidianus infernus	PacBio RSII	9,528/6,375‡	104,250	438X	2,220,671	4	2,184,866	34.4
	Illumina (PE)	249.59 (Avg)‡	4,171,032	469X				
Stygiolobus azoricus	PacBio Sequel	8,938/4,150	185,364	482X	1,987,069†	1		37.6
	Illumina (PE)	249.18 (Avg)‡	4,830,704	605X				
Sulfolobus metallicus	PacBio RSII	11,532/7,294‡	62.361	308X	2,199,731	5	1,839,470	38.6
	Illumina (PE)	249.17 (Avg)‡	5,477,710	620X				
Sulfurisphaera ohwakuensis	PacBio Sequel	9,967/4,980	151,460	362X	2,803,915†	1		32.7
	Illumina (PE)	249.69 (Avg)‡	5,400,166	481X				

(† = Closed; ‡ = Trimmomatic Filtered or Long-Read Filtered; >5K A.infernus; >8K S. metallicus; Avg = Average; SE = Single-end; PE = Paired-end)

Table 4-3. NCBI Identifiers and Accession Numbers for Relevant Data Sets

Species	BioProject	BioSample	Accession
Acidianus ambivalens, LEI10	PRJNA488459	SAMN09933089	CP045482
Acidianus infernus, So4a	PRJNA488459	SAMN09933090	WFIY00000000
Stygiolobus azoricus FC6	PRJNA488459	SAMN09933091	CP045483
Sulfolobus metallicus Kra23	PRJNA463410	SAMN09933021	WGGD00000000
Sulfurisphaera ohwakuensis TA-1T	PRJNA488459	SAMN09933092	CP045484

CHAPTER 5: Life in Hot Acid: A Genome-based Reassessment of the Archaeal Order Sulfolobales, Extreme Thermoacidophily, and Chemolithoautotrophy

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Abstract

The order Sulfolobales was one of the first named Archaeal lineages, discovered prior to genome sequencing. Its members inhabit globally-distributed terrestrial thermal acidic springs (pH < 4; T > 65 ˚C). As a group, the Sulfolobales cover a broad range of growth physiologies ranging from utilization of inorganic substrates, such as iron, sulfur, and carbon dioxide, particularly at the lower end of the pH window, to a heterotrophic lifestyle associated with the less acidophilic species. Further, extreme acidophily (pH_{opt} < 3) appears to correlate with a putative cytochrome bd oxidase, putative transporters, and sulfur metabolism (sulfur oxygenase reductase and tetrathionate hydrolase). Interestingly, iron oxidation, which appears to span into all lineages except the least acidophilic lineages, also appears to correlate with the presence of a urease that may provide further buffering, nitrogen and carbon utilization pathways in extremely acidophilic conditions, as well as a possible antioxidant OsmC family peroxiredoxin. In contrast, sulfur oxidation (soluble species) and carbon fixation appear to be near-universal traits with few exceptions, while the utilization of elemental sulfur and dissimilatory reduction of sulfur, appear to be limited to a few lineages (*Acidianus, Stygiolobus,* and *Sulfurisphaera*). Despite the presence of facultative anaerobes and one confirmed obligate anaerobe, the prevalence of one or more oxidases (*fox, sox, dox*) in all species examined, suggests oxygen may have played a key role in the speciation of Sulfolobales from other Thermoprotei. The addition of numerous type-strain genomes here suggests that across the order more than 1100 genes are near-universally conserved, and that recent reorganization attempts are valid at the genome-wide scale, with several other changes proposed herein (an additional genus *Cocturalobus* and a new family *Sulfodiicoccacae*).

Introduction

Less than a half a century has passed since the initial description of the third domain of life. Since its conception, the domain Archaea (originally Archaebacteria) has been associated with extreme environments: deep sea vents, volcanic sulfur pools, and salt lakes[1,2]. These microbes could well be anachronistic hold-overs from an Earth dominated by the heat of youth and a chemistry spewing from its inorganic core. However, Archaea do not just inhabit extreme environments and their ubiquity across many environments (oceans, soils, and even humans) has now been recognized[3-5]. And yet in the age of genomics and cultureless microbiology, the flood of data describing the microbial world has begun to confound our understanding of life; and in fact, recent discoveries such as the Asgard Archaea[6] have even begun to call into question the origins of eukaryotes and our own uniqueness from the microbial world.

While the baseline of domain Archaea continues to expand[7,8], efforts persist to transition from cataloguing and culturing toward generating burgeoning model systems and tractable genetic hosts. Within one of the two original phyla – Crenarchaeota – resides the order Sulfolobales, originally isolated in the 1960's from volcanic hot springs in Yellowstone National Park and named for their perceived ability to utilize sulfur as a primary metabolic precursor for energy generation[9,10]. From early on, the genus *Sulfolobus* dominated the taxonomical clade of similar organisms from comparable environments throughout the world[11-13]. But this classification has fractured as new organisms of similar constitution demonstrated a vast range of heat and acid tolerance (65 ˚C to 88 ˚C, and 0.8 to 4.0 pH), metabolic capacities (sulfur oxidation/reduction, iron oxidation/reduction, sugar catabolism, and autotrophy), and sensitivities to oxygen (obligate aerobes, anaerobes, and truly facultative species), leading to the designation of multiple genera with representative members sharing the workload of generating biomass and cycling nutrients in largely inorganic environments[14-19].

Perceptions through time of these microbes have shifted and the name "Sulfolobales" has largely become a misnomer, especially as genomic and phenotypical techniques demonstrated that the original member of the order *Sulfolobus acidocaldarius,* at least the strain available from culture collections, is essentially incapable of utilizing sulfur[20]. And yet it has become a heterotrophic workhorse for genetic studies focused on understanding these extreme microbes and the means by which they tolerate their inhospitable environments[20-22]. Additionally, other species have become models for other types of physiological traits at extreme conditions, such as iron biooxidation by "fox" genes in *Sulfolobus metallicus*[23], dissimilatory sulfur utilization with the unique sulfur oxidoreductase (SOR) by *Acidianus ambivalens*[24,25], or the carbon fixing 3-hydroxpropionate/4-hydroxybutyrate cycle from *Metallosphaera sedula*[26].

Each of these phenotypes represents enzymatic and cellular features that could be exploited for biotechnological application. Already, the use of extremely acidophilic microorganisms, and occasionally extremely thermoacidophilic organisms, in biomining has proven to be a fruitful, less energy-intensive means of liberating many heavy and precious metals[27-29]. This process can be driven by metal biooxidation or by sulfur transformation from metal sulfide ores. In either case, little work has been done to manipulate microorganisms to-date in order to improve these chemolithoautotrophic processes or improve their robustness[30]. In the case of autotrophy, some attempts have been made to co-opt the 3HP/4-HB cycle from *M. sedula* in non-thermoacidophiles, with some success creating synthetic products[31]. However, the use of the same pathways in genetically-tractable *Sulfolobales* (*Sulfolobus acidocaldarius, Sulfolobus islandicus,* and *Saccharolobus solfataricus*) is limited by a lack of understanding about how to

pair the value of lithotrophy with biosynthetic output, for example fixing carbon dioxide in the non-lithoautotrophic *S. acidocaldarius*[20].

One of the main obstacles to better understanding the world of the Sulfolobales has been the difficulty associated with *de novo* sequencing of species with few guiding references and plentiful repetitive roadblocks. While some closed genomes have been circulating[32-34], recent efforts have expanded the genomic repository for the order by a considerable amount[35-38]. While some of these efforts are focused on new isolates, others have focused on type strains that have been once studied and described and deposited, awaiting a day when their genomes could be analyzed in detail. Thus, this study aims to utilize newly expanded genomic data sets spanning almost all of the named Sulfolobales genera (*Acidianus, Metallosphaera, Stygiolobus, Sulfurisphaera,* and *Sulfolobus*) in order to better understand the genetic traits that define these organisms. Presented herein is a diversity-driven re-evaluation of the order aimed at elucidating the metabolic pathways present within this order, the evolutionary divergence of these species, and the characteristics that define extreme thermoacidophily.

Materials and Methods

Accession of Genomes

All Sulfolobaceae samples consisted of type-strain genomes (considered closed or near-complete) accessible in the NCBI database (accession numbers presented in **Table 5-1**). In the case of *Sulfolobus islandicus*, *Saccharolobus solfataricus Sulfolobus acidocaldarius*, where numerous closed genomes exist, a representative (*S. islandicus* REY15A, *Saccharolobus* P1 and *S. acidocaldarius* DSM639) was used for broader comparative analysis; all genomes were used for average nucleotide identity (ANI) calculations to demonstrate sub-species relatedness, ignoring subspecies strains with over 90% ANI relatedness. For Thermoprotei-level analysis, closed genomes from RefSeq available type-strains were used to evaluate taxonomical distance.

Taxonomical Re-evaluation

In order to normalize all data, genomes were all reannotated using prokka (v.1.14)[39].The average amino acid identity was calculated via the software suite GET_HOMOLOGUES (v06062018)[40], using default parameters for the orthoMCL[41] (OMCL) algorithm v1.4 (inflation set to 1.5) for the entire *Thermoprotei* genome set. A *Thermprotei* "core" genome was prepared using the convergence of the bi-directional best hit (BDBH) with Diamond v0.8.25, Clusters of

Orthologous Genes Triangles (COGs) v2.1, and OMCL algorithms, using only inparalogous (single-copy) clusters.

For pair-wise alignments of 16S/23S concatenated sequences or the core-genome of *Thermoprotei*, MUSCLE alignments were performed and the subsequent trees drawn with FastTree[42] (v2.1.5), using either the Jason-Taylor-Thorton (JTT) or General-Time-Reversible (GTR) models with gamma optimization. Alignments were pruned by removing sequence columns with less than 25% identity prior to tree drawing. Additional multilocus alignments were drawn similarly, scaling for the size of the genome set.

Sulfolobales Core and Pan Genome Analysis

Meaningful homology-based comparisons of Sulfolobales genomes were performed again using the GET_Homologues[40] software suite. For the computation of the core, a soft-core (21+ genomes of 23 total) was used in order to account for disjointed genomes (*Acidianus infernus, Acidianus copahuensis, Aramenus sulfurataquae, Metallosphaera yellowstonensis,* and *Sulfolobus metallicus*), using the OMCL algorithm. After identifying the core genome or parsing the pangenome along taxonomical divides or phenotypical characteristics, BLASTkoala[43] and BLASTP[44] were used in order to deduce functional roles of proteins using a reference protein sequence from the individual clusters.

The size of the pan and core genomes of the Sulfolobales was calculated based on non-linear regression, using a construction of pan and core genomes in which ten random samples are progressively drawn at each genome addition to create ten separate pan/core paths. The fitted curves are based on the models from the work of Tettelin or Willenbrock[45].

Results

Evaluation of Current Taxonomical Designations in Order Sulfolobales

Within the past few years, several new Sulfolobales species have been isolated, characterized, and sequenced including: *Candidatus Aramenus sulfurataquae*[46], *Candidatus Acidianus copahuensis*[47], *Sulfodiicoccus acidiphilus*[35], *Metallosphaera yellowstonensis*[48], and *Sulfolobus sp*. A20[38]. In addition to this, several Sulfolobales have recently been sequenced for the first time, or re-sequenced for the sake of assembling poorly scaffolded genomes, representing members from 5 of the 7 named genera (*Acidianus, Metallosphaera, Sulfolobus*, *Sulfurisphaera*, and *Stygiolobus*)[36]. Thus, there are now more than 20 species from the order that

are herein re-examined for their genetic contents, in order to understand the essence of extreme thermoacidophily and concomitant chemolithoautotrophy.

Currently, the taxonomic spread of the Sulfolobales has been based on 16S rRNA sequencing and other broadly applicable comparisons, such as GC-content and both direct and *in silico* DNA-DNA hybridization. Now with complete and near-complete genome sequences, it is possible to make both nucleotide and amino acid comparisons between the members of the order. In the case of the former, average nucleotide identity (ANI) demonstrates that many of the species level designations in the Sulfolobales have been appropriately made, considering the recent metric of greater than 95% ANI. Particularly, for all of the multitudinous *Sulfolobus islandicus*, *Saccharolobus solfataricus*, and *Sulfolobus acidocaldarius* subspecies it is clear that the existing taxonomical delineation is correct, with little variation below 99% ANI.

However, recent efforts have been made to reclassify some existing genera and begin shifting species to more suitable taxonomical clades. An updated 16S/23S concatenated rDNA tree is presented in **Figure 5-1a** and demonstrates that the resolution of rDNA divergence is quite low (scale shows 0.04 substitutions/site) for many of the Sulfolobales, as well as the other Thermoprotei considered in this study. This has led to some instances of polyphyletic lineages (e.g. *Acidianus spp*.) and arguments for ongoing reclassifications (e.g. *Sulfurisphaera spp*[49]., the new genera *Saccharolobus*[18] and Candidatus *Aramenus*[50]). Given the new genomes presented from the Sulfolobales, it seems appropriate to re-examine these designations from the whole genome vantage.

Currently, two major methods have been proposed for the broader reclassification of prokaryotes, but to date applied exclusively to Bacteria. One is the average amino acid identity (AAI)[51], an approach that maximizes pair-wise comparisons, and demonstrates robust depth at numerous taxonomical ranks. The other approach leverages a core set of highly conserved genes that can be aligned simultaneously to build the classic phylogram or estimate a patristic distance[52]. Both approaches show promise at resolving the existing prokaryotic tree of life, better utilizing the overlapping and qualitative taxonomical rankings from phylum to genus.

Here, both approaches were used to evaluate the Sulfolobales with comparisons to the next highest rank: Thermoprotei. As shown in **Figure 5-2**, AAI suggests some distinct relationships masked in the rDNA alignment. From this vantage point, there appears to be little depth of pair-wise comparisons that would distinguish the new genus Candidatus *Aramenus* from the extremities of the genus *Acidianus* (particularly *A. brierleyi* and Candidatus *Acidianus copahuensis*). Additionally, there is strong evidence for the disparities between the genus *Sulfolobus* (a historical catch-all) and the newly named *Saccharolobus*. Furthermore, the AAI

suggests that the genus should be expanded to include *S. islandicus* (as was recently proposed) and the newly isolated and sequenced *Sulfolobus sp*. A20. Another deviance in the currently constituted genus *Sulfolobus* are the two species*: S. metallicus* and *Sulfolobus sp.* JCM16833. These two organisms appear to be highly divergent from the genus, but similar to each other (perhaps a long overdue designation of a new genus). Interestingly, the species *Sulfolobus acidocaldarius* (type-strain of the genus) appears fairly isolated with a weak association to the genera *Stygiolobus* and *Sulfurisphaera*, where strong evidence does not exist to drop these labels (given AAIs approximately 60%).

Finally, the recently identified and characterized species *Sulfodiicoccus acidiphilus* appears to be an incredibly divergent Sulfolobales species, with a range of intra-order AAIs of (49.74-52.38%) which is far below any other species in the family Sulfolobaceae. Thus, a new family designation may be appropriate. It is worth noting that the problems of taxonomical rank extend to several other lineages, including the Desulfurococcales (where *Ignicoccus* appears to be highly divergent from all other order members) and *Thermofilum*, which has several unnamed and quantitatively unique species.

In order to further elucidate these relationships, a core genome was drawn up across the order, composed of 97 unique, non-paralogous gene clusters (**Figure 5-1a**). Intriguingly, the branching in the Sulfolobales seems to mirror much of the AAI analysis but with additional statistical analysis of branching events clarifying the monophyletic nature of all of the above-mentioned re-classifications; particularly, the *genera Acidianus, Metallosphaera, Stygiolobus, Sulfolobus* (just *S. acidocaldarius*), *Saccharolobus*, and *Sulfurisphaera* appear to be monophyletic. Beyond this, the branch for *Sulfodiicoccus acidiphilus* rests high above the other Sulfolobales branches, again suggesting a possible break with the rest of the family Sulfolobaceae; although the branch support is fairly low and perhaps the organism is closely ranked with the other distant genus including *Sulfolobus metallicus* and *Sulfolobus sp*. JCM16833, although the shared AAI is low. Additionally, some Thermoprotei lineages with quite low pairwise-AAI (e.g. *Ignicoccus spp*. v. other Desulfurococcales and Thermofilum spp. v. other Thermoproteales) show similarly marked distances from their current co-class and co-order members. Finally, examination of the ranges of pair-wise AAI suggests there are decent overlaps in the use of taxonomical rank from the class designation downward in Thermoprotei (**Figure 5-3**). This is a phenomenon observed by others recently proposing reorganization of the bacterial taxonomic ranks.

New Sulfolobales Taxonomical Descriptions

Based on the observed whole genome observations, proposed here are several reclassifications that also complement meaningful phenotypical observations among the new taxonomical clades. *Sulfolobus islandicus* and *Sulfolobus sp.* A20 should be placed within the recently named *Saccharolobus* genus, which is composed of higher pH-tolerant (optimum > 3.0), sugar-utilizing species, hence *Saccharum* (latin: sugar). Additionally, the species *Sulfolobus metallicus* and *Sulfolobus sp.* JCM16833 should be reclassified in the herein proposed genus *Cocturalobus* (*Coctura,* latin, smelting, as in ore), which contains microbes with either demonstrated or strongly putative indicators of iron metabolism that are capable of also growing on sulfur substrates in obligatory aerobic conditions. Finally, the species *Sulfodiicoccus acidiphilus* should be reclassified in the family *Sulfodiicoccaceae*, which may contain other novel species within the order Sulfolobales that have diverged dramatically in their ability to utilize carbon dioxide for fixation, as well as being inhibited by high loadings of elemental sulfur. Further, these species show a much higher molar G+C content (greater than 51.1%), whereas the highest G+C content seen to-date was that of *Metallosphaera yellowstonensis* at 47.7%, with many Sulfolobaceae showing G+C content around 30-40%. Perhaps the only hint of a closely related isolate to date is Sulfolobus sp. MK5 (see chapter 2).

Core Sulfolobales Genome

Using a soft-core to consider the potential for missing genes due to incomplete genome sequences (6 in total), there are 1156 conserved loci (present in 21+ genomes). This is largely in agreement with the modeling function for the core genome (**Figure 5-4**), which via random sampling suggests the core should converge around 1043 genes. Further, the order's pangenome appears to be open and rapidly expanding.

Based on functional annotation, a large portion of the core (51.5%) represent proteins with no known function. However, many of the conserved loci (18.4%) represent proteins within universally-conserved genetic information processing modules/pathways: DNA/RNA Polymerases, transcription factors, aminoacyl-tRNA synthetases, ribosomal complex small and large subunit proteins and biogenesis proteins, inosine monophosphate (for ATP/GTP) biosynthesis, and nucleotide biosynthesis.

Central carbon metabolism in the Sulfolobales appears to be limited to the archaeal non-oxidative pentose phosphate pathway, the tricarboxylic acid pathway, and gluconeogenesis. Only glycolysis from C3 sugars is universally-conserved in these organisms, as most C6 catabolism proteins from the EMP pathway have no apparent counterpart, although their presence is noted

elsewhere in select lineages. Interestingly, much of the 3-hydroxypropionate/4-hydroxybutyrate cycle is conserved at face-value (more later) with the lone exception of *Sulfodiicoccus acidiphilus,* perhaps a stark evolutionary difference between families *Sulfolobaceae* and *Sulfodiicoccaceae.* The mevalonate and C10-C20 isprenoid synthesis pathways appear to be highly conserved as well, unsurprisingly. Finally, molybdenum cofactors play a role in bioenergetics throughout the order as molybdenum cofactor biosynthesis is universally conserved. Finally, there are a large number of genes associated with dissimilatory sulfur oxidation/reduction that are well-conserved throughout the order (see specifics below).

Genus-Level Specialization

In order to see if genera represent unique evolutionary divergences that match phenotypical differences, the genera were parsed such that clusters were considered if they were present in a genus-level lineage, while being absent in all of the other lineages. These genes were then annotated for possible functional roles. Unsurprisingly, the number of genes pulled out by this parsing largely scales with the number of genomes in each group, since fewer genomes of more closely related species share more unique proteins. Additionally, many of the annotated proteins have no discernable physiological roles and are identified as hypothetical via BLASTP (62%) and BLASTKoala (72%).

However, some specific traits do emerge from functional annotations. More specifically, the difference between the *Saccharolobus* genus and all others is the most pronounced with 13 proteins annotated as transporters and permeases, many with possible sugar substrates. Also, the genus contains a GH-31, which may be a α-xylosidase, possibly providing another substrate for C5-sugar metabolism. The other large data set (*Cocturalobus*) also contains several transport-associated proteins; however, it also contains several thermopsins, suggesting that protein scavenging and recycling may be a more critical function in this organism. Perhaps the most interesting divergence of a genus-level taxonomical clade is the *Metallosphaera* clade, which appear to contain an ATP:NAD kinase (a route for NADPH production), the product of which could be a critical driver of carbon fixation, as well as a relatively unique Rieske protein with a clear iron-sulfur cluster binding site, while no annotated genes arose for the *Acidianus* clade.

Chemolithoautotrophic Metabolic Pathways

At identification and initial isolation of the Sulfolobales, their presence in the sulfur-rich, hot acid pools of Yellowstone was taken as a name-sake. It was assumed and initial

enrichment/isolation cultures suggested that sulfur was critical to the metabolism of these organisms. While many of the order's members possess organoheterotrophic capabilities, the understanding of the inorganic metabolisms of members throughout the order has grown in the past few decades of laboratory and *in situ* investigation. The principle inorganic pathways of interest here involve the respiratory utilization of metal species, such as iron and the dissimilatory oxidation and reduction of sulfur species (lithotrophy) and the assimilation of carbon dioxide (autotrophy).

Iron Biooxidation

In the case of iron metabolism, several studies have pointed toward a set of genes, dubbed the 'fox' locus due to their stimulation in the presence of iron[23,48,53,54]. This locus contains putative cytochrome oxidase-like subunits, as well as numerous ancillary genes of currently unknown function[23,48,53]. Recently, there has been demonstrated activity resulting from a natively-purified membrane fractions of *M. sedula in vivo,* containing FoxA and FoxC and the demonstration of oxygen depletion and utilization of ferrous iron by cells in a ferrous sulfate suspension, pointing toward key determinants in the *foxA* and *foxBWC* loci (**see Chapter 6**).

Further, a reconstruction of these loci in this analysis, demonstrates that many of the genes are present throughout the numerous lineages of the order *Sulfolobales.* There are example loci present in all named and proposed genera with the exception of *Stygiolobus* (*S. azoricus* is an obligate anaerobe)[14] and *Saccharolobus* (higher pH growing, sugar-utilizing)[18]. Fourteen of the 23 species examined here contained a version of tightly packed genes with comparable homology for *foxABCDEFJUVYZ* (**Figure 5-5**). Interestingly, three species without a previously identified capacity for iron biooxidation (*A. ambivalens*, *C. sp.* JCM16388, and *S. ohwakuensis*) contain all of the essential elements. In the case of the aerobe *S. ohwakuensis,* direct utilization of oxygen and production of ferric jarosites was observed in the presence of active cell suspensions (**Figure 5-5**). Considering the genes *foxABCDEFUVYZ* (all well-conserved), it appears that the capacity for iron biooxidation may have been lost somewhere in the speciation of higher pH organisms, such as *Saccharolobus spp.*, which is not surprising given the reduced driving force between the Fe^{2+}/Fe^{3+} and O_2/H_2O and dramatically diminished solubility of iron species at higher pHs[55].

Perhaps more interesting are the ancillary genes that were identified in the presence of the *fox* locus (parsing of the pangenomic matrix for iron biooxidation genes, **Table 5-2**). For instance, most members contain a somewhat unique DNA single-stranded binding protein (possibly a regulator) and an *osmC* peroxiredoxin, which could provide protection from oxidative

stress. In the case of the latter, both ferrous and ferric iron are well known to participate in Fenton reactions, producing numerous toxic free radical species in the presence of hydrogen peroxide[56], a ready product of respiration[57,58] and a potential target of OsmC peroxiredoxin activity[59,60]. Given the increased solubility of iron at lower pH, this may also serve as a protective measure for other extreme acidophiles[55]. Possibly the most interesting ancillary genomic feature is a urease-like locus with strong conservation and synteny in almost all metal-mobilizers and a few unrelated species; this could provide ammonia as a pH buffer against the intractable pmf of acidophilic environments. For example, urease activity in the pathogen *Helicobacter pylori* has been shown to strongly associate with virulence and activation of the complex can mitigate the effects of extreme pH shifts associated with gastric colonization[61], as well as providing an alternative route for nitrogen assimilation in the pathogen *Mycobacterium tuberculosis*[62] and the phototroph *Rhodobacter capsulatus*[63]. Further, the other product of urease activity – carbon dioxide – could be recycled into the 3HP/4HB cycle via a carbonic anhydrase, similar to the one seen in *M. sedula*[31], present in a near universally conserved cluster of the OMCL analysis (see carbon assimilation section) or as a buffering substrate for the same enzyme[64].

Carbon Assimilation via 3-hydroxypropionate/4-hydroxybutyrate (3HP/4HB) Cycle

One of the most thoroughly investigated metabolic pathways of the Sulfolobales is the carbon fixation cycle first identified in *M. sedula*[26]. Within the order Sulfolobales, it is apparent that the 3HP/4HB cycle is nearly-universal (**Figure 5-6**), excluding only *S. acidiphilus* and *C. sp.* JCM16388 (whose current genome assembly is highly fragmented). In the case of the former, the key enzyme complex that drives fixation (acetyl-CoA/propionyl-CoA carboxylase)[65] is absent as well as a carbonic anhydrase[31] observed in all other lineages, implying a complete inability to fix carbon dioxide; this is also supported by phenotypical observations[35]. This may be a defining trait of the new family *Sulfodiicoccaceae* and should be examined in future isolates.

For most of the other species, distinct members of enzymes E1-9, E11, and E13 are easily identified (**Figure 5-6**), while E10 and E12 of the cycle are less recognizable in some species. In the case of E10, 4-hydroxybutyrate:CoA ligase, there are a few species missing a clear homolog. However, investigation of a similar acyl-CoA ligase (E4) suggests that many of the species contain additional lineages of acyl-CoA ligases with unspecified selectivity. In the case of E12, the N-terminal domain contains residues for a 3-hydroxyacyl-CoA dehydrogenase, while the C-terminal domain contains an enoyl-CoA hydratase-like structure. In fact, crotonyl hydratase activity has been confirmed via E5 in *M. sedula* with recombinantly expressed protein[66], while the 3-hydroxyacyl-coA dehydrogenase domain is well-conserved in all species studied. While the cycle

appears to be driven from acetyl-coA to the production of an additional acetyl-coA molecule, it is worth noting that evidence shows that a large portion of the succinyl-coA from the cycle actually exits to enter central carbon metabolism via the TCA cycle[67]. Thus, acetyl-CoA maintenance and not generation is the primary purpose of the latter enzymes, suggesting that efficiency of enzyme complexes may not play a critical role in dictating the structures for enzymes such as E10 and E12. Additionally, this may suggest a rationale for the apparent loss of autotrophic capabilities in some species, such as *S. acidocaldarius*[20], where a lack of a functional E10 enzyme my inhibit the ability to regenerate acetyl-CoA in the absence of an organic carbon donor. Overall, the phylogenetic alignment of most of the genes suggests the metabolism is a deep-rooted feature of either the ancestral Sulfolobaceae or Sulfolobales species.

Sulfur Cycling in Thermoacidophilic Environments

Sulfur and reduced inorganic sulfur compounds represent a large source of energy in acidic geothermal environments, where primary producers dominate the process of converting inorganic substrates to cellular energy[68,69]. The vast majority of energy-dense sulfur arising from subsurface environments arrives as hydrogen sulfide (H_2S) or elemental/zero valent sulfur (S^0). These can transform to sulfuric acid, resulting in the acidification of the spring environments and outflows via oxidation or microbially-mediated processes, although the abiotic oxidation of sulfide is slowed in acidic conditions[70] and the abiotic oxidation of S^0 essentially negligible. While reduction can occur in the presence of molecular hydrogen (another commonplace geothermal constituent), the primary means of energy conversion to biomass occurs by oxidation. The interplay between the two processes (oxidation and reduction) is most likely driven by the depth of atmospheric O_2 penetration, and may explain the role of facultative Sulfolobales (particularly of the genus *Acidianus*) in colonizing geothermal features.

Many of the pathways from elemental sulfur to either fully reduced (hydrogen sulfide) and fully oxidized (sulfate) forms are shown in **Figure 5-7.** Interestingly, there are just two sulfur-associated loci with universal presence in the order: the heterodisulfide reductase, disulfide reductase, TusA system (hdr/dsr/tusA) and the terminal oxidase *doxBCE*. The former was recently identified in *Metallosphaera cuprina,* where *dsrE3* and *tusA* serve a role in trafficking cytoplasmic thiosulfate relinquished from tetrathionate[71], presumably toward the universally conserved Hdr complex. This complex's function has not been confirmed biochemically in the Sulfolobales, but all species contain the five Hdr domain genes and a dihydrolipoamide dehydrogenase (*dld*), similar to the bacterium *Aquifex aeolicus,* where the complex's function has been validated[72]. In *Hyphomicrobium denitrificans* the essentiality of this complex in sulfate

production is shown[73], as well as the role of a putative *dld* gene in sulfur binding[74]. Additionally, a glutaredoxin gene appears in close proximity to the locus in all organisms, except the elemental sulfur-inhibited *S. acidiphilus*, possibly suggesting a role for a glutathione-like molecule in the sulfur trafficking process. The other universal conserved locus is the *doxBCE* gene locus, which encodes a type-aa_3 proton-translocating oxidase[75]. The necessity of this process (proton export) or the aerobic origins of acidophily[76] may be underscored by the locus' presence in the strictly anaerobic *Stygiolobus azoricus*[14]. This result is interesting given the contrasting non-ubiquity of the *soxABCDL* locus (another terminal oxidase cluster), which shows strong stimulation to the presence of sulfur or RISCs in several Sulfolobales species[20,53], but is absent in several species, including *A. ambivalens, A. sulfurataquae,* and *S. azoricus*.

While these two complexes are driven by the reducing power of quinol-like molecules, there are multiple sources of such reducing power in sulfur dissimilation, generally produced via oxidation of RISCs. For example, there are several DoxDA homologues, which likely encode thiosulfate:quinone oxidoreductases, capable of producing tetrathionate and quinols[77]. It has been proposed that tetrathionate may serve as a redox couple for the highly reducing hydrogen sulfide, a near ubiquitous presence in acidic geothermal features, presenting a detoxification mechanism for intracellular hydrogen sulfide[78]. This is supported by the presence of Tqo in almost every species (in one form or another), except *Sulfolobus acidocaldarius*. Recently, mutants of *Sulfolobus acidocaldarius* were created with the addition of sulfur oxygenase:reductase (SOR) and a double insertion of *sor* and *tqoAB*[20]. In the former, sulfate production was observed but growth was severely inhibited, while the later mutant demonstrated robust sulfate production and growth comparable to wild-type strains[20]. In contrast, the product of this complex, tetrathionate can also be hydrolyzed by an extracellular hydrolase (*tetH*)[79], which is absent in many of the *Sulfolobus* and *Saccharolobus* lineage organisms.

While sulfur oxidation serves as the primary route of energy flow, due to the exothermic nature of RISCs, there are several organisms that appear to be capable of leveraging the reduction of sulfur for bioenergetics benefit. The *sor* gene represents probably the most interesting of these processes. As its name suggests the protein is capable of both oxidizing and reducing (disproportionating) sulfur via a large 24 unit homopolymer[80], which thermodynamically enables an almost non-spontaneous reaction, producing sulfite and hydrogen sulfide. Interestingly, another pathway to sulfide production exists, using a hydrogenase and the sulfur reductase (*sre*) locus[81], conserved in numerous lineages (**Figure 5-7**), however, the *sor* provides a non-hydrogen-consuming route to H_2S. This Sre provides a source of reducing power through the re-oxidation of hydrogen sulfide to polysulfides via the sulfide:quinone reductase (Sqr)[82],

another near-universal feature in the Sulfolobales, serving as a detoxification mechanism or an energy conservation pathway. Interestingly, there is a cytochrome bd oxidase-like complex that appears to correlate with SOR/SreABCDE presence (**Table 5-3, Table 5-4**), and may represent a microaerophilic oxidase (more below).

Acidophily in the Sulfolobales

While speculation of the origins of thermophily almost all go back to primordial origins, acidophily is believed to be a much more recent development of microbial life[76]. The organisms that persist in low-pH environments are often balancing the energy demands of growth and replication with maintaining a cytosol around circumneutral conditions[83]. In these environments, proton motive force (pmf) is not a gradient to be powered but opposed and organisms have evolved to throttle non-energetic proton flow via less-permeable membranes with a reversed electronic potential[84], as well as numerous proton translocating systems, which likely serve little point in generating pmf but instead regulate intracellular pH[83]. Unsurprisingly, this landscape is dominated by the Archaea that have evolved these unique capabilities; there is strong evidence that the confluence of extremophilies further limits entrance into these environmental niches[85]. Additionally, it is possible that these organisms helped construct these environments over the course of evolution (converting sulfides and zero-valent sulfur to sulfuric acid at rates far exceeding abiotic oxic contributions), particularly in the wake of oxygenation events that would have allowed for increased sulfur oxidation activity[76], increased iron biooxidation[86], and a supply of oxidant for cytochromes.

The pan genome of the Sulfolobales was interrogated to inquire if there are genetic determinants that distinguish marginal acidophiles ($pH_{opt} \geq 3.0$) from extreme acidophiles ($pH_{opt} \leq 2.0$). **Figure 5-8** shows that 31 marker genes were identified as having a higher prevalence in either in-group versus the out-group. *Acidianus hospitalis, Candidatus Aramenus sulfurataquae,* and *Candidatus Acidianus copahuensis* were omitted because their optimum growth conditions could not be confirmed. Thirteen of the genes annotate as hypothetical or domain of unknown function. However, some interesting determinants do show up in the analysis. In particular, there are several bioenergetic systems that appear to be more prevalent in the extremely acidophilic organisms, including the SOR, a paralogue to the extracellular tetrathionate hydrolase[79], and the cytochrome bd complex observed above. In the case of the *tth* paralogue, this gene is even more rare than the *tth1* with confirmed tetrathionate hydrolase activity (the cluster is absent in the *Metallosphaera spp.*)[79] and could act on some other thionate-like molecule. In addition, there are several amino acid permeases and other transporter proteins. This matches other observations

of the role of amino acids for buffering the cytoplasm and the role of secondary transporters in regulating proton influx to manage intracellular pH[83].

Of high curiosity is the presence of a terminal oxidase subunit from a putative cytochrome bd (same as proximal to sulfur reduction), as well as an adjacent, conserved hypothetical that may form the second subunit of a typical CydAB complex. These cytochromes are known for their unique structure in comparison to other cytochromes, rarely resembling the structure of heme-copper oxidases or alternative oxidases (AOX)[87]. These complexes often possess very high oxygen affinity and in many cases are not susceptible to inactivation via cyanide[87], and also insusceptible to a more relevant molecule: hydrogen sulfide[88]. Altogether, this feature might be essential for maintaining pH homeostasis at subsurface conditions, where oxygen may be quite limited and highly reducing hydrogen sulfide plentiful. Further, colocation with two sulfur metabolism genes may suggest that the more acidophilic organisms are those directly associated with transformation of sulfur.

Discussion

Evidence presented here further suggests that the extremely thermoacidophilic archaea from the order Sulfolobales probably speciated from a time point following the great oxygenation event, given their diversity of oxygen consuming terminal complexes and the need to detoxify and transform sulfur for bioenergetic benefit and pH homoeostasis. In addition, it is intriguing that the only obligate anaerobe, and several of the facultative aerobes harbor elements for oxygen reduction, through membrane complexes. Further, these organisms are characterized by a versatile set of metabolisms ranging from chemolithoautotrophy to chemoheterotrophy and many lineages demonstrate facultative modes of oxygen utilization. This versatility may represent the extreme demands of energetic maintenance of pH homeostasis or a low level of specialization in these low competition environments. A number of determinants are observed that may play a crucial role in pushing acidophily to its extremeties including deamination of amino acids and organic compounds such as urea, via deaminases and an intriguing urease-like complex, containing all of the essential domains for construction and activation. Given the role of urease in some pathogenic organisms to mitigate extreme acidity fluxes, a similar mechanism may exist for obligately acidophilic organisms.

Further presented here is the need to reorganize the taxonomical landscape and the proposition of at least one new family designation and one new genus designation based on whole genome analysis and the recent genomic data expansion of the order to account for genetic drift associated with a broader collection of conserved marker proteins. Although, it is intriguing that

the 16S phylogenetic relationships drawn in the Sulfolobales clearly serve as a suitable lower resolution model for taxonomical placement. These new designations now divide the order Sulfolobales into two families: Sulfolobaceae and Sulfodiicocceae, the former are sulfur-utilizing autotrophs and the later represented by a single species with no demonstrable utilization and no genetic evidence for zero-valent sulfur utilization or carbon assimilation via the 3HP/4HB pathway. The new genus *Cocturalobus* is a long overdue re-designation of the original Icelandic, Krafla Solfatara biomining species *Sulfolobus metallicus,* which may represent a grouping of obligately aerobic and lithoautotrophic iron-biooxidizers, isolated in both Europe and Asia with clear deviation from other Sulfolobales, represented by a far-removed speciation event with a currently deep and unexpanded branch.

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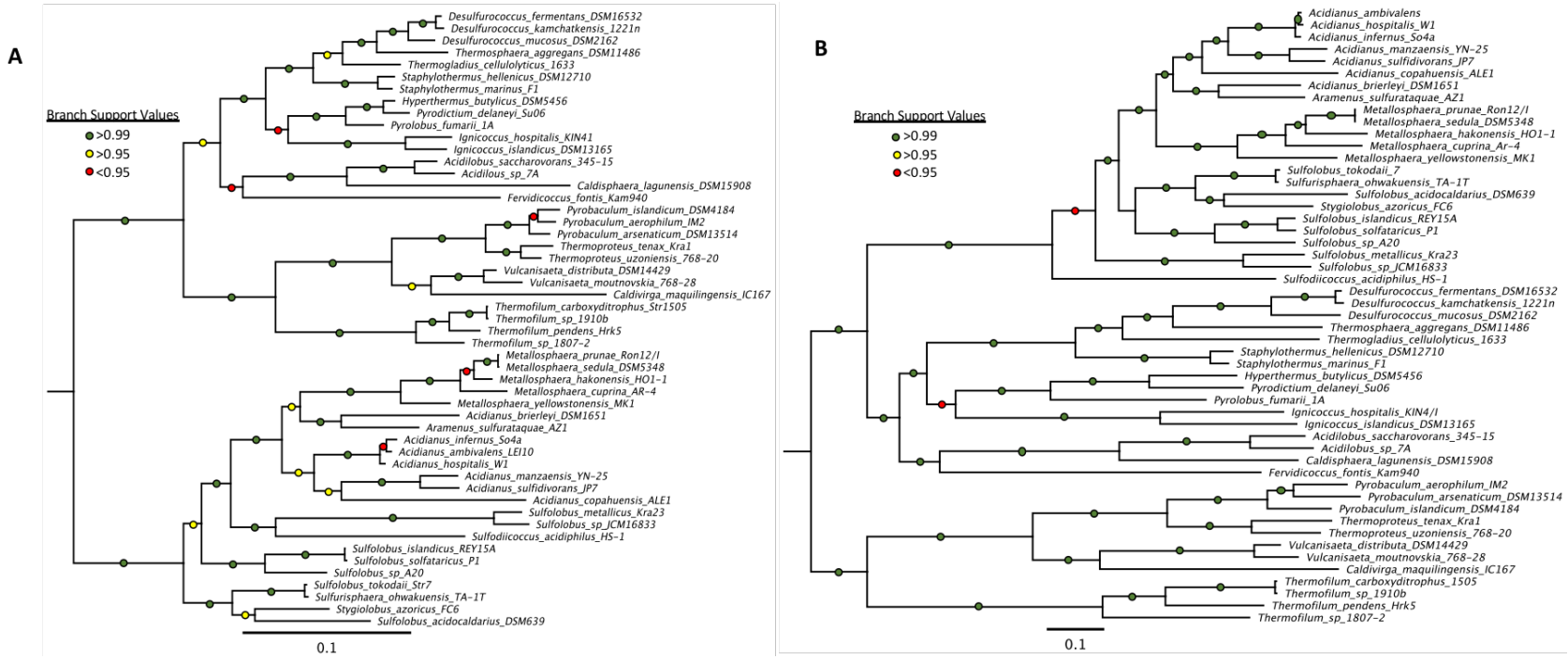


Figure 5-1. FastTree/MUSCLE Alignments of Thermoprotei-wide Features.

A) Concatenated 16S/23S rDNA sequences. B) Core genome (97 proteins), concatenated. All alignments were done with default MUSCLE script and any locations with more than 30% gapped sequences were removed from the final alignment. FastTree phylogenetic layouts were performed with either GTR (A) or JTT (B) models with gamma20 optimization. Branch support values are color-coded and a scale of 0.1 substitutions/site is presented for comparison of the two alignments. The root was selected near the midpoint of the tree. All trees were visualized with Geneious (BioMatters Inc.).

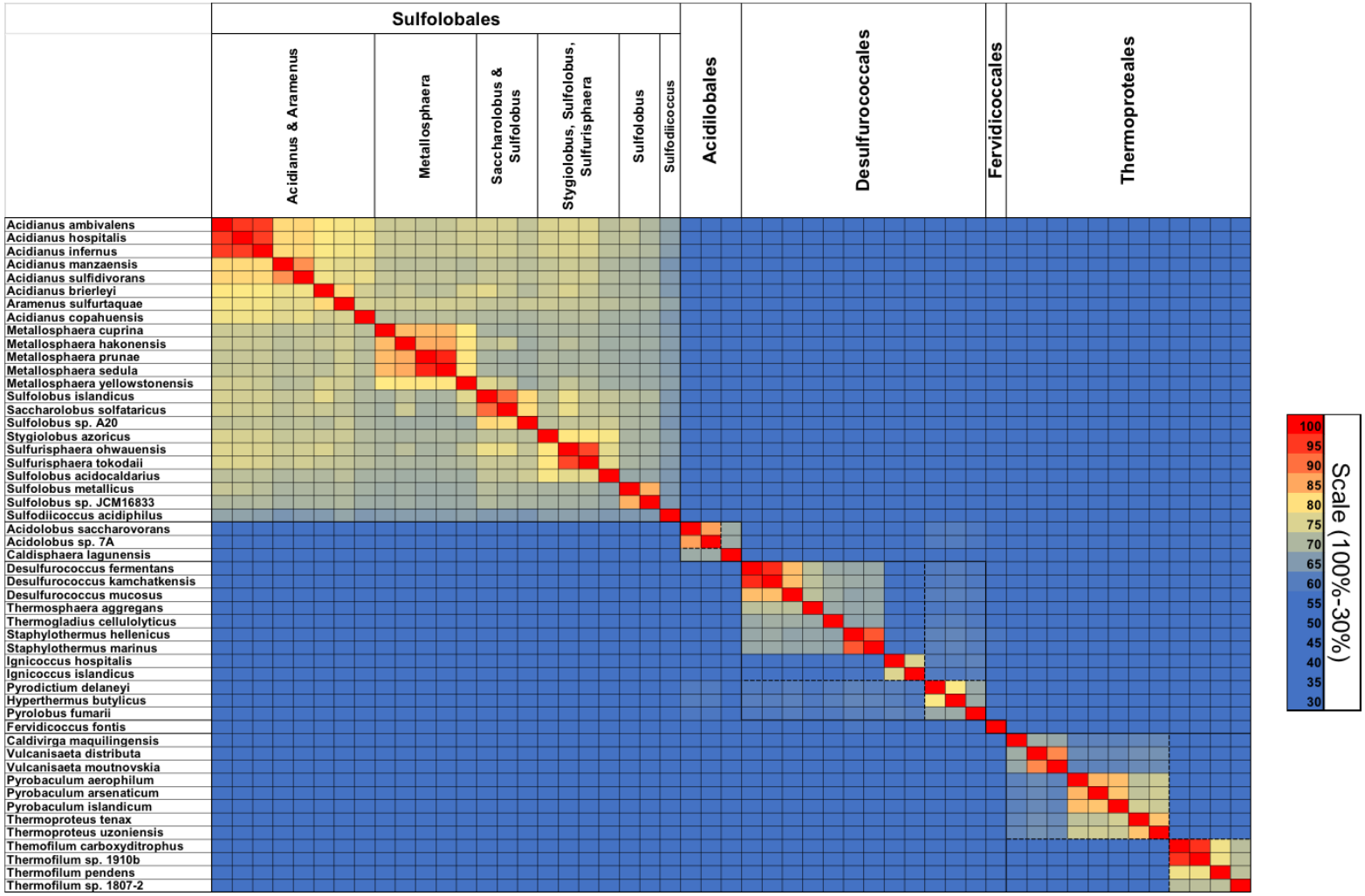


Figure 5-2. AAI Matrix of Class Thermoprotei.

Scale is from blue (lowest) to red (highest). Black lines denote existing clades: Solid:Order; Dashed:Family.

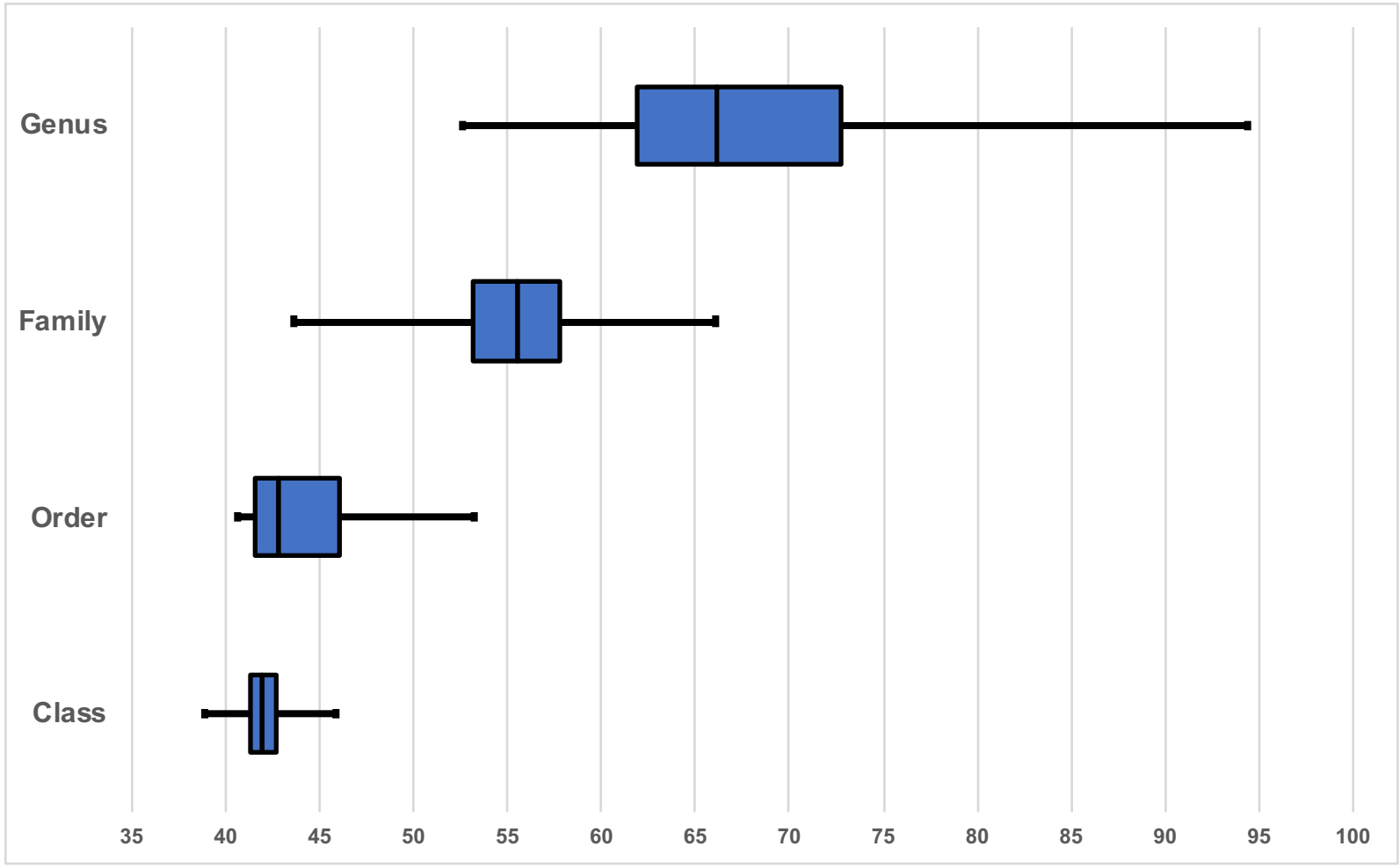


Figure 5-3. Range of AAI values between each taxonomical rank and the next highest taxonomical grouping.

Average amino acid identity, pair-wise between all Thermoprotei analyzed compared to closest shared taxonomical rank. Box shows Q1, median, and Q3. The whiskers are the maximum and minimum values of pair-wise AAI between taxonomical ranks.

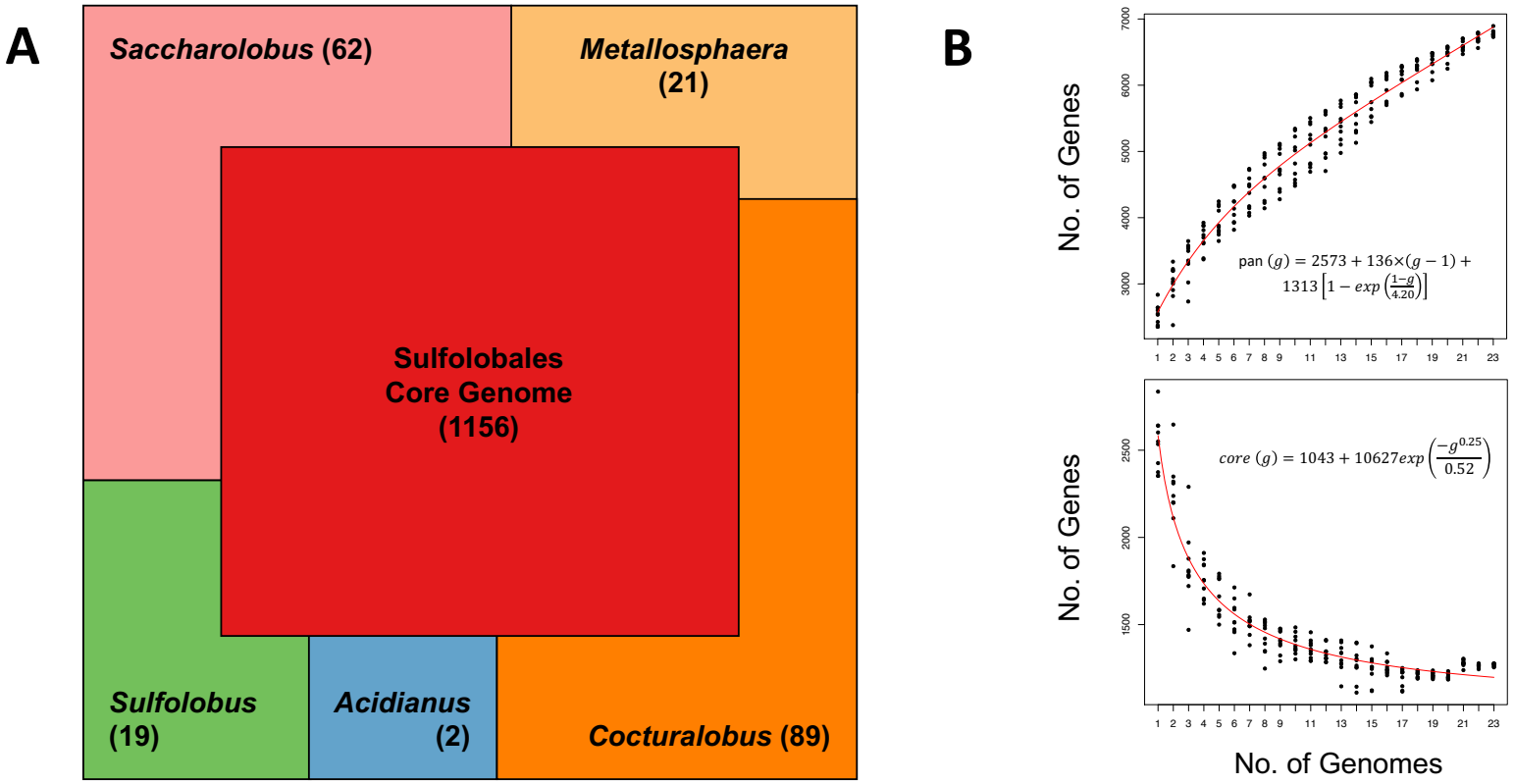
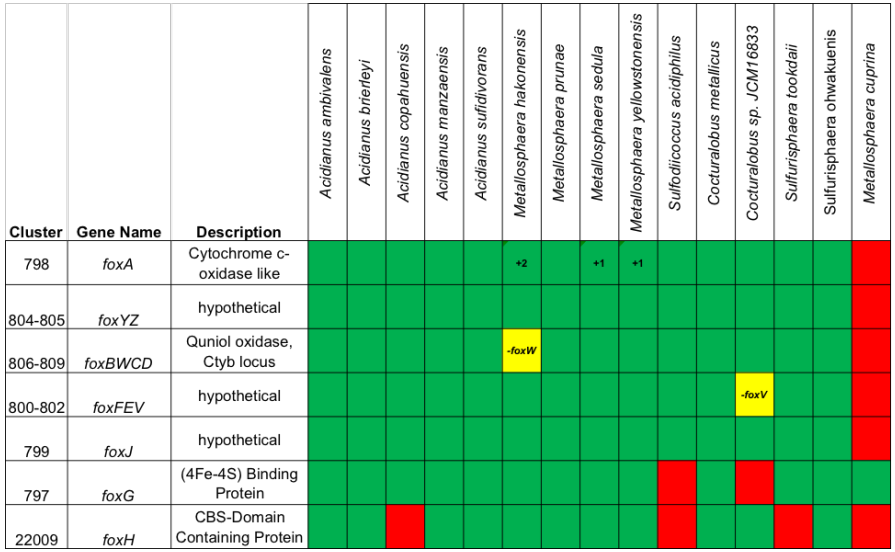


Figure 5-4. Core and Pan Genome Analysis of the Order Sulfolobales.

A) Number of clusters exclusive to each taxonomical division B) Pan genome and core genome estimations based on random sampling of 10 genomes and comparison of conserved orthologous gene clusters, via the Get_Homologues script plot_pancore_matrix.pl.



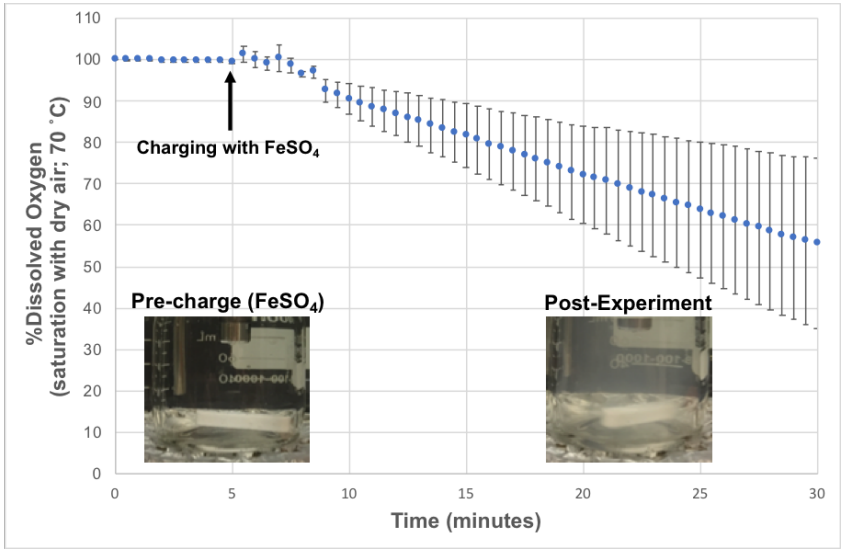
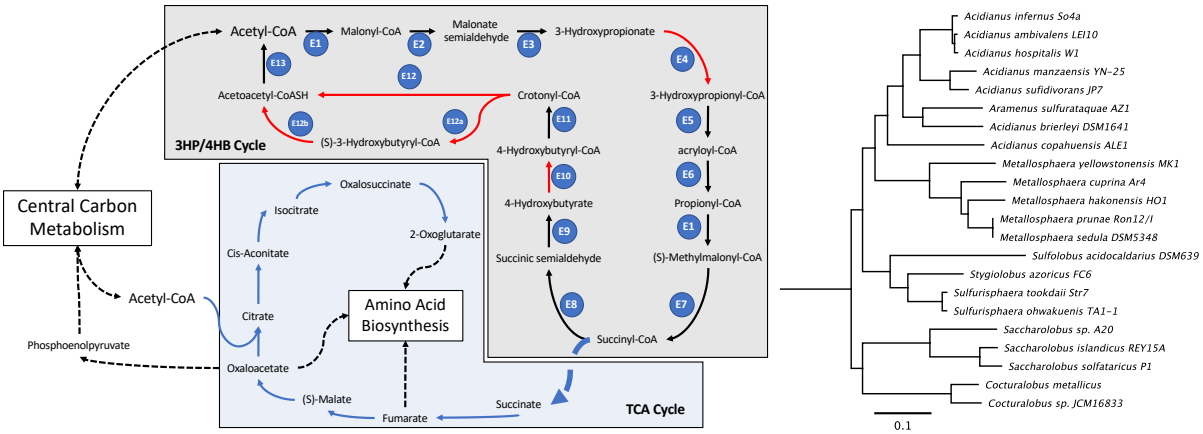


Figure 5-5. Gene Conservation for Metal-Mobilizers.

A) Conserved Genes of the Fox locus and an ancillary urease complex. B) Oxygen consumption activity by *Sulfurisphaera ohwakuensis* in basal salts medium with added ferrous sulfate. Procedural methods are the same as those carried out in Chapter 6: Experimental Procedures:Iron Biooxidation Assays.



Enzyme Description			*M. sedula* ORF	*Acidianus ambivalens*	*Acidianus brierleyi*	*Acidianus copahuensis*	*Acidianus hospitalis*	*Acidianus infernus*	*Acidianus manzaensis*	*Acidianus sufidivorans*	*Aramenus sulfurataquae*	*Metallosphaera cuprina*	*Metallosphaera hakonensis*	*Metallosphaera prunae*	*Metallosphaera sedula*	*Metallosphaera yellowstonensis*	*Stygiolobus azoricus*	*Sulfolobus acidocaldarius*	*Sulfurisphaera tookdaii*	*Sulfurisphaera ohwakuenis*	*Saccharolobus islandicus*	*Saccharolobus solfataricus*	*Saccharolobus sp. A20*	*Cocturalobus metallicus*	*Cocturalobus sp. JCM16833*	*Sulfodiicoccus acidiphilus*
E1	Acetyl-CoA/propionyl-CoA Carboxylase	α	Msed_0147																						
		β	Msed_0148																						
		γ	Msed_1375																						
E2	Malonyl-CoA/succinyl-CoA Reductase		Msed_0709																						
E3	Malonate Semialdehyde Reductase		Msed_1993																						
E4	3-Hydroxypropionate:CoA Ligase		Msed_1456	3	3	3	3	3	4	4	3	2	2	2	2	2	3	2	3	4	2	2	2	3	2
E5	3-Hydroxypropionyl-CoA dehydratase		Msed_2001																						
E6	Acryloyl-CoA Reductase		Msed_1426																						
E7	Methylmalonyl-CoA Epimerase		Msed_0639																						
E8	Methylmalonyl-CoA Mutase	α	Msed_0638																						
		β	Msed_2055																						
E9	Succinate Semialdehyde Reductase		Msed_1424																						
E10	4-Hydroxybutyrate-CoA Synthetase		Msed_0394		2	2						2	2	2	2	2									
E11	4-Hydroxybutyryl-CoA Dehydratase		Msed_1321																						
E12	Crotonyl-CoA Hydratase/(*S*)-3-hydroxybutyryl-CoA Dehydrogenase		Msed_0399															3							
E13	Acetoacetyl-CoA β-ketothiolase		Msed_0656																						

Figure 5-6. Conservation of 3-HP/4-HB Genes in the Order Sulfolobales.

A) General pathway of carbon fixation and entry into central carbon metabolism, including 3HP/4HB Cycle and TCA Cycle. Bifurcation occurs at succinyl-CoA, with roughly a third of carbon continuing through the cycle and two-thirds diverted to other biosynthetic pathways. Line colors depict 3HP/4HB, conserved (black), ambiguous or potentially inparalogous (red), TCA (blue), or exits/entrances (black, dashed). B) Conserved 3HP/4HB genes: conserved (green), absent (black), paralogues (number). C) Phylogenetic alignment of conserved, in-paralogous genes (E1-E3,E5-E9,E11,E13).

Gene/Complex: Proposed Reaction	*Acidianus ambivalens*	*Acidianus brierleyi*	*Acidianus copahuensis*	*Acidianus hospitalis*	*Acidianus infernus*	*Acidianus manzaensis*	*Acidianus sulfidivorans*	*Aramenus sulfurataquae*	*Metallosphaera cuprina*	*Metallosphaera hakonensis*	*Metallosphaera prunae*	*Metallosphaera sedula*	*Metallosphaera yellowstonensis*	*Stygiolobus azoricus*	*Sulfodiicoccus acidiphilus*	*Sulfolobus acidocaldarius*	*Saccharolobus islandicus*	*Cocturalobus metallicus*	*Saccharolobus solfataricus*	*Saccharolobus sp. A20*	*Cocturalobus sp. JCM16833*	*Sulfurisphaera tookdaii*	*Sulfurisphaera ohwakuensis*
Disimilatory Sulfur Ox/Red, SOR: ZVS + H2O + O2 --> H2S + SO3(2-)																							
SreABCDE, Sulfur Reduction: ZVS + H2 --> H2S				1/5				4/5															
SQR, Sulfide Oxidation: SH- + Q(ox) --> Polysulfide(2-) + QH2	+1	+1		+1	+1	+1	+1																
DoxAD Thiosulfate Oxidation: S2O3(2-) + Q(ox) --> S4O6(2-) + QH2	+1	+1		+1	+1	+1	+1	+1											+1				
TetH, Tetrathionate hydrolase: S4O6(2-) + H2O --> S2O3(2-) + SO4(2-) + 2H(+)		+1	+1																		+1		
SAOR, Sulfite Oxidation: SO3(2-) + H2O + Q(ox) --> SO4(2-) + QH2																							
Thiosulfate Oxidation Locus (*hdr,dsr,tus*)																							
Terminal Oxidase Cluster (Sox)				1/5									4/5		1/5								
Terminal Oxidase Cluster (Dox)																							

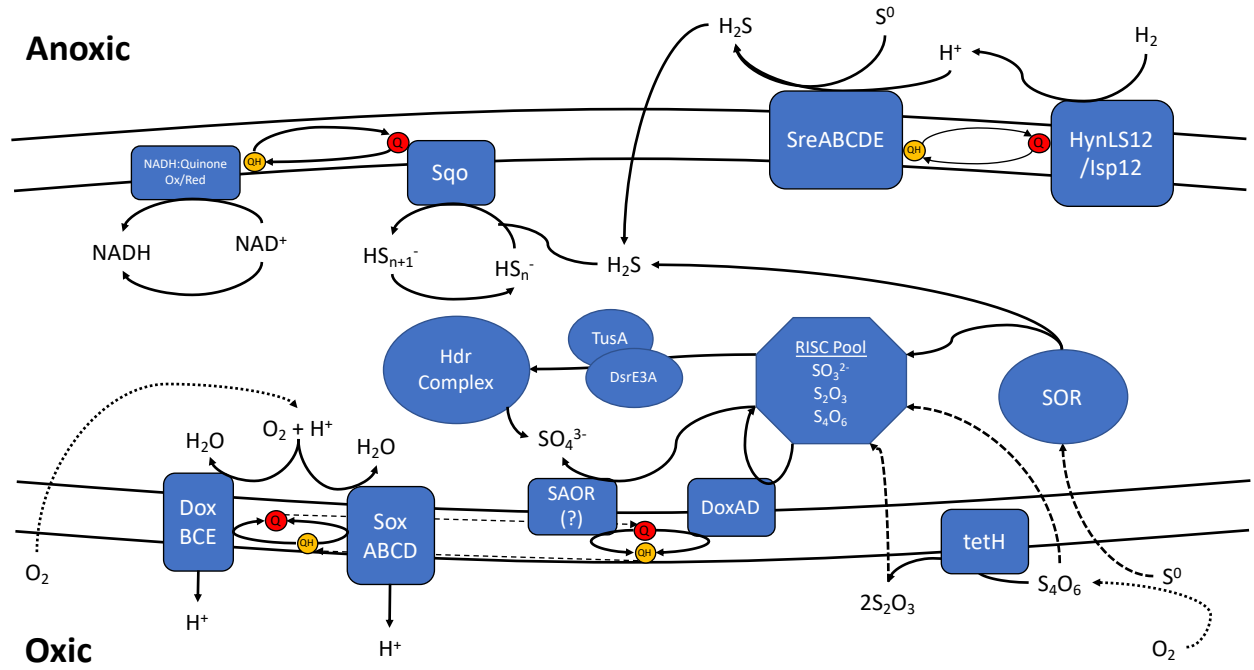


Figure 5-7. Genes Involved in Sulfur Trafficking and Dissimilatory Biooxidation/Bioreduction in Sulfolobales.

A) Gene presence or absence by OMCL cluster. BLAST descriptions from BLAST searches with *Acidianus ambivalens* or *Sulfolobus acidocaldarius* (*sox* genes). Parenthesis indicate multiple genes in a cluster. B) Putative network of dissimilatory sulfur metabolism. All quinone (caldiquinone) are marked with "Q". SAOR (Sulfite:acceptor oxidoreductase) remains a putative gene, while all others have some form of confirmed biochemistry in the Sulfolobales.

Table 5-1. Genomes Used in Class-wide Analysis.

All sequences were derived from closed representatives in NCBI database.

Order	NCBI Accession	Organism
Acidilobales	NC_014374	*Acidilobus saccharovorans 345-15*
	NZ_CP010515	*Acidilobus sp 7A*
	NC_019791	*Caldisphaera lagunensis DSM 15908*
Desulfurococcales	NC_018001	*Desulfurococcus fermentans DSM 16532*
	NC_011766	*Desulfurococcus kamchatkensis 1221n*
	NC_014961	*Desulfurococcus mucosus DSM 2162*
	NC_008818	*Hyperthermus butylicus DSM 5456*
	NC_009776	*Ignicoccus hospitalis KIN4I*
	NZ_CP006867	*Ignicoccus islandicus DSM 13165*
	NZ_CP013011	*Pyrodictium delaneyi Su06*
	NC_015931	*Pyrolobus fumarii 1A*
	NC_014205	*Staphylothermus hellenicus DSM 12710*
	NC_009033	*Staphylothermus marinus F1*
	NC_017954	*Thermogladius cellulolyticus 1633*
	NC_014160	*Thermosphaera aggregans DSM 11486*
Fervidicoccales	CP003423	*Fervidicoccus fontis Kam940*
Sulfolobales	CP045482	*Acidianus ambivalens DSM 3772*
	CP029289	*Acidianus brierleyi DSM 1651*
	NC_015518	*Acidianus hospitalis W1*
	WFIY00000000	*Acidianus infernus DSM 3191*
	CP020477	*Acidianus manzaensis YN-25*
	CP029288	*Acidianus sulfidivorans DSM 18786*
	NZ_JZT010000	*Candidatus Acidianus copahuensis ALE1*
	ASRH0100000	*Candidatus Aramenus sulfurataquae AZ1*
	NC_015435	*Metallosphaera cuprina AR-4*
	CP029287	*Metallosphaera hakonensis DSM 7519*
	CP031156	*Metallosphaera prunae DSM 10039*
	NC_009440	*Metallosphaera sedula DSM 5348*
	NZ_AHKJ00000000	*Metallosphaera yellowstonensis MK1*
	CP045483	*Stygiolobus azoricus DSM 6296*
	NZ_AP018553	*Sulfodiicoccus acidiphilus HS-1*
	NC_007181	*Sulfolobus acidocaldarius DSM 639*
	NC_017276	*Sulfolobus islandicus REY15A*
	WGGD00000000	*Sulfolobus metallicus DSM 6482*
	NZ_LT549890	*Sulfolobus solfataricus DSM 1616*
	NZ_CP017006	*Sulfolobus sp A20*
	NZ_AP018929	*Sulfolobus sp JCM16833*
	NC_003106	*Sulfolobus tokodaii DSM 16993*
	CP045484	*Sulfurisphaera ohwakuensis DSM 12421*
Thermoproteales	NC_009954	*Caldivirga maquilingensis IC167*
	NC_003364	*Pyrobaculum aerophilum IM2*
	NC_009376	*Pyrobaculum arsenaticum DSM 13514*
	NC_008701	*Pyrobaculum islandicum DSM 4184*
	NZ_CP007493	*Thermofilum carboxyditrophus 1505*
	NC_008698	*Thermofilum pendens Hrk5*
	NZ_CP009961	*Thermofilum sp 1807-2*
	NC_022093	*Thermofilum sp 1910b*
	NC_016070	*Thermoproteus tenax Kra1*
	NC_015315	*Thermoproteus uzoniensis 768-20*
	NC_014537	*Vulcanisaeta distributa DSM 14429*
	NC_015151	*Vulcanisaeta moutnovskia 768-28*

Table 5-2. Conserved and Ancillary Genes of Iron Biooxidation.

Using FoxA presence as a screen for metal biooxidation, an in-group containing all species in yellow was compared to an out-group of all species in red down to a threshold of 75% concordance between a locus's contents and taxonomical distribution. Where applicable, multiple X's mark the presence of homologues within the isolated cluster. Cluster numbering originates from Sulfolobales, order-wide analysis using orthoMCL and functional annotations are derived from the reference sequence selected from each locus, using BLASTP searches. †No NCBI accession, amino acid sequence: MAIFESEADLWIVSFLLMALIYPIWYILLIIKADKDPQVDPMQIARQEAGE.

		Iron Biooxidation		*Acidianus ambivalens*	*Acidianus brierleyi*	*Acidianus copahuensis*	*Acidianus manzaensis*	*Acidianus sufidivorans*	*Metallosphaera hakonensis*	*Metallosphaera prunae*	*Metallosphaera sedula*	*Metallosphaera yellowstonensis*	*Sulfodiicoccus acidiphilus*	*Cocturalobus metallicus*	*Cocturalobus sp. JCM16833*	*Sulfurisphaera tookdaii*	Sulfurisphaera ohwakuenis	*Metallosphaera cuprina*	*Stygiolobus azoricus*	*Acidianus hospitalis*	*Acidianus infernus*	*Aramenus sulfurataquae*	*Saccharolobus solfataricus P1*	*Saccharolobus sp. A20*	*Saccharolobus islandicus REY15A*	*Sulfolobus acidocaldarius*
Concordance: 100% (FoxA)																									
798	coxA/foxA	Cytochrome c-oxidase like	QGR21242.1	X	X	X	X	X	XXX	X	XX	XX	X	X	X	X	X								
799	FoxF	FoxF	QGR21243.1	X	X	X	X	X	X	X	X	X	X	X	X	X	X								
801	FoxE	FoxE	QGR21244.1	X	X	X	X	X	X	X	X	X	X	X	X	X	X								
802	FoxV	FoxV	QGR21245.1	X	X	X	X	X	X	X	X	X	X	X	X	X	X								
804	FoxZ	FoxZ	QGR21247.1	X	X	X	X	X	X	X	X	X	X	X	X	X	X								
805	FoxY	FoxY	QGR21248.1	X	X	X	X	X	X	X	X	X	X	X	X	X	X								
808	cbsA/foxC	Cytochrome b558/566	AWR93860.1	X	X	X	X	X	X	X	X	X	X	X	X	X	X								
809	foxD	hypothetical	QGR21251.1	X	X	X	X	X	X	X	X	X	X	X	X	X	X								
811	***NA***	hypothetical	QGR21253.1	X	X	X	X	X	X	X	X	X	X	X	X	X	X								
Concordance: 90% (FoxA)																									
800	foxU	hypothetical	NA†	X	X	X	X	X	X	X	X	X	X	X		X	X								
806	foxB	Quinol oxidase	QGR21249.1	X	X	X	X	X	X	X	X	X	X		X	X	X								
807	foxW	hypothetical	QGR21250.1	X	X	X	X	X		X	X	X	X	X	X	X	X								
Concordance: 80% (FoxA)																									
1986	ureA	Urease, subunit Gamma	QGR22214.1	X	X	X	X	X	X	X	X			X	X	X	X		X						
22009	*foxH*	CBS-Domain Containing Protein	QGR21240.1	X	X	X	X	X	X	X	X	X		X	X	X	X	X							
Concordance: 75% (FoxA)																									
797	FoxG	(4Fe-4S) Binding Protein	QGR21241.1	X	X		X	X	X	X	X	X		X	X		X								
1275	osmC	peroxiredoxin	QGR21634.1	X	X		X	X	X	X	X	X		X	X	X	X				X	X			
1477	***NA***	Glycosyltransferase Family 2 Protein	QGR22884.1	X	X	X	X		X	X	X	X			X	X	X		X		X				
5564	***NA***	DUF929 domain-containing Protein	AWR95580.1		X	X	X	X	X	X	X			X	X	X	X						X	X	
21344	ureE	Urease accessory protein	QGR22216.1	X	X	X	X	X	X	X	X			X	X	X	X	X	X						
21345	ureD/H	Urease accessory protein	QGR22219.1	X	X	X	X	X	X	X	X			X	X	X	X	X	X						
21346	ureG	Urease accessory protein	QGR22218.1	X	X	X	X	X	X	X	X			X	X	X	X	X	X						
21347	ureF	Urease accessory protein	QGR22217.1	X	X	X	X	X	X	X	X			X	X	X	X	X	X						
21348	ureC	Urea Amidohydrolase, alpha subunit	QGR22215.1	X	X	X	X	X	X	X	X			X	X	X	X	X	X						
21608	***NA***	Hypothetical	QGR22080.1	X	X	X	X	X	X	X	X			X	X	X	X	X			X				
22023	ssb	Single-Stranded DNA-Binding Protein	AWR95225.1		X	X	X	X	X	X	X	X	X	X	X			X							

Table 5-3. Conserved and Ancillary Genes of Sulfur Reduction.

Using SreABCDE presence as a screen for metal biooxidation, an in-group containing all species in yellow was compared to an out-group of all species in red down to a threshold of 75% concordance between a locus's contents and taxonomical distribution. Species colored in orange were excluded due to inconsistencies in the presence or absence of the *sre* locus. Where applicable, multiple X's mark the presence of homologues within the isolated cluster. Cluster numbering originates from Sulfolobales, order-wide analysis using orthoMCL and functional annotations are derived from the reference sequence selected from each locus, using BLASTP searches.

Sulfur Reduction (Orange = Excluded)				*Acidianus ambivalens*	*Acidianus brierleyi*	*Acidianus infernus*	*Acidianus manzaensis*	*Acidianus sulfidivorans*	*Metallosphaera yellowstonensis*	*Stygiolobus azoricus*	*Saccharolobus islandicus REY15A*	*Saccharolobus solfataricus P1*	*Sulfurisphaera ohwakuensis*	*Aramenus sulfurataquae*	*Acidianus hospitalis*	*Acidianus copahuensis*	*Metallosphaera cuprina*	*Metallosphaera hakonensis*	*Metallosphaera prunae*	*Metallosphaera sedula*	*Sulfodicoccus acidiphilus*	*Sulfolobus acidocaldarius*	*Cocturalobus metallicus*	*Saccharolobus sp. A20*	*Cocturalobus sp. JCM16833*	*Sulfurisphaera tokodaii*
Concordance: 100% (sreABCDE)																									
1606	*sreA*	Sulfur reductase, molybdopterin subunit/Polysulfide reductase, subunit A	QGR21928.1	X	X	X	X	X	X	X	X	X	X	X											
1607	*sreB*	Sulfur reductase, (4Fe-S4) Subunit	QGR21929.1	X	X	X	X	X	X	X	X	X	X	X											
1608	*sreC*	Polysulfirde Reductase (DsrP/NrfD)	QGR21930.1	X	X	X	X	X	X	X	X	X	X	X											
1609	*sreD*	4Fe-4S dicluster Protein	QGR21931.1	X	X	X	X	X	X	X	X	X	X	X											
1610	*sreE*	Reductase Assembly Protein (syn:TorD)	QGR21932.1	X	X	X	X	X	X	X	X	X	X		X										
Concordance: 90% (sreABCDE)																									
577	NA	hypothetical (Low Probability: WcaJ Sugar Transporter)	QGR21085.1	X	X	X	X	X		X	X	X	X	X	X										X
Concordance: 80% (sreABCDE)																									
950	*NA*	Cytochrome bd oxidase, Subunit I	QGR22850.1	X	X	X	X	X	X	X			X	X	X								X		X	
951	*NA*	hypothetical	QGR21356.1	X	X	X	X	X	X	X			X	X	X								X		X	
1655	*hyaA/hydA*	NiFe Hydrogenase, Small Subunit	QGR21973.1	X	X	X	X	X	X	XX			X	X		X										
1656	*hyaB*	NiFe Hydrogenase, Large Subunit	QGR21974.1	X	X	X	X	X	X	X			X	X		X										
1994	*gabT/argD*	4-aminobutyrate, aminotransferase	QGR22222.1	X	XX		X	X		X	X	X	X							X				XX		

Table 5-4. Conserved and Ancillary Genes of Sulfur Disproportionation.

Using SOR presence as a screen for metal biooxidation, an in-group containing all species in yellow was compared to an out-group of all species in red down to a threshold of 75% concordance between a locus's contents and taxonomical distribution. Where applicable, multiple X's mark the presence of homologues within the isolated cluster. Cluster numbering originates from Sulfolobales, order-wide analysis using orthoMCL and functional annotations are derived from the reference sequence selected from each locus, using BLASTP searches.

Sulfur Disproportionation				*Acidianus ambivalens*	*Acidianus brierleyi*	*Acidianus copahuensis*	*Acidianus hospitalis*	*Acidianus infernus*	*Acidianus manzaensis*	*Acidianus sufidivorans*	*Aramenus sulfurataquae*	*Cocturalobus metallicus*	*Cocturalobus sp. JCM16833*	*Sulfurisphaera tookdaii*	*Sulfurisphaera ohwakuenis*	*Stygiolobus azoricus*	*Sulfolobus acidocaldarius*	*Sulfodiicoccus acidiphilus*	*Saccharolobus sp. A20*	*Metallosphaera yellowstonensis*	*Metallosphaera cuprina*	*Metallosphaera hakonensis*	*Saccharolobus islandicus REY15A*	*Saccharolobus solfataricus P1*	*Metallosphaera prunae*	*Metallosphaera sedula*
Concordance: 100% (SOR)																									
1542	*sor*	Sulfur oxygenase/reductase	QGR21871.1	X	X	X	X	X	X	X	X	X	X	X	X										
1773	*NA*	Hypothetical	QGR22056.1	X	X	X	X	X	X	X	X	X	X	X	X										
Concordance: 90% (SOR)																									
504	*NA*	Hypothetical	QGR21028.1	X	X	X	X	X	X	X		X	X	X	X	X									
1152	*NA*	Thioesterase	QGR21521.1	X	X		X	X	X	X	X	X	X	X	X	X									
1276	*NA*	S-layer Protein	QGR21635.1	X	X		X	X	X	X	X	X	X	X	X	X									
1346	*NA*	thiamine-binding protein	QGR21697.1	X	X	X	X	X	X	X	X	X	X	X	X	X									
1390	*NA*	Transporter	QGR21736.1	X	X	X	X	X	X	X	X	X	X	X	X		X								
1770	*NA*	Amino acid permease	QGR22054.1	XX	X		X	XX	XX	X	X	XX	X	X	X	X									
2620	*NA*	Hypothetical	QGR22780.1	X	X	X	X	X	X	X	X	X	X	X	X	X									
2640	*NA*	Thetrathionate hydrolase	CBY66040.1	X	X	X	X	X	X	X		X	X	X	X	X									
Concordance: 80% (SOR)																									
12	*marR/arsR*	MarR/ArsR Family Transcriptional Regulator	QGR20588.1	X		X	X	X	X	X		X	X	X	X	X	X								
234	*NA*	2-keto-4-pentenoate hydratase	QGR20787.1	X	X		X	X	X	X		X	X	X	X	X				X					
446	*NA*	HTH Transcriptional Regulator	QGR20973.1	X	X	X	X	X	X	X	X			X	X										
494	*NA*	SMP-30/Gluconolaconase/LRE-like Region	QGR21019.1	X	X	X	X	X	X	X	X			X	X	X	X								
950	*cydA*	cytochrome d ubiquinol oxidase subunit I	QGR22850.1	X	X		X	X	X	X	X	X	X		X	X				X					
951	*cydB*	hypothetical	QGR21356.1	X	X		X	X	X	X	X	X	X		X	X				X					
1010	*NA*	ABC-Transporter	QGR21403.1	X	X	X	X	X	X	X		X	X	X	X	X	X								
1022	*NA*	Hypothetical	QGR21412.1	X	X		X	X	X	X	XX	X	X	X	X	X	X								
1119	*drsE*	Intracellular Sulfur reduction	QGR21493.1	X	X	X	X	X	X	X	X	X	X												
1154	*drsE*	Intracellular Sulfur reduction	QGR21522.1	X	X	X	X	X	X	X	X	X	X	X	X	X		X							
1270	*NA*	Solute-Binding Protein (Sulfate?)	QGR21630.1	X	X	X	X	X	X	X	X	X	X	X	X	X		X							
1513	*ydfJ/hae3*	Largely Archaeal Hydrophobe/Amphophile Efflux-3 Protein (H+ Antiport)	QGR21845.1	X	X	X	X	X	X	X	X	X	X	X	X	X	X								
1541	*NA*	Hypothetical	QGR21870.1	X	X		X	X	X	X		X	X	X	X										
1559	*NA*	Membrane Protein, Unknown Function (Maybe SecF)	QGR21886.1	X	X	X	X	X	XX	X	X	X	X	X	X	X	X								
1691	*NA*	Hypothetical	QGR21994.1	X	X		X	X	X	X		X	XX	X	X										
2015	*NA*	nucleobase-cation sympoter, permease for cytosine,uracil,thiamine,allantoin	QGR22241.1	X	X		X	X	XX	XX		X	X	XX	X	XX		XXX							
2156	*TrmB*	Methylatransferase (TrmB,DOT1,KsgA)	QGR22351.1	X	X	X	X	X	X	X	X			X	X	X	X								
2506	*NA*	DUF3834 (Solute-binding lipoproteins)	QGR22678.1	X	X	X	X	X	X	X	X	X	X	X	X	X			X						
2552	*NA*	Hypothetical	QGR22721.1	X	X	XXX	X	X	X	X	X	X	X	X			X		X						
2621	*NA*	MFS Transprter, Metazoan Synaptic Vesicle Glycoprotein 2 (SV2)	QGR22781.1	X	X		X	X	X	X		X	X	X	X										
2625	*NA*	Zinc-ribbon-domain protein	QGR22785.1	X	X		X	X	X		X	X	X	X	X	X			X						
21397	*NA*	Hypothetical	QGR21451.1	X	X	X	X	X	X	X	X	X	X	X	X					X	X				
21486	*NA*	Dehydrogenase	QGR21878.1	X	XX	X	XX	XX	X	X	XX			X	X					X					

Table 5-5 . Conserved Genes of Extremely Acidophilic Sulfolobales.

Some species: A. copahuensis, A. sulfurataquae, and A. hospitalis were excluded from the analysis because they are not isolated cultures or their growth pH optimum could not be verified.

Acidophily (Red=Strong, Purple=Not Considered, Blue=Weak)			*Acidianus sulfidivorans*	*Acidianus manzaensis*	*Acidianus brierleyi*	*Acidianus infernus*	*Cocturalobus metallicus*	*Sulfurisphaera ohwakuensis*	*Acidianus hospitalis*	*Acidianus ambivalens*	*Cocturalobus sp. JCM16833*	*Stygiolobus azoricus*	*Aramenus sulfurataquae*	*Sulfurisphaera tookdaii*	*Acidianus copahuensis*	*Sulfolobus acidocaldarius*	*Metallosphaera prunae*	*Metallosphaera sedula*	*Metallosphaera yellowstonensis*	*Metallosphaera hakonensis*	*Saccharolobus islandicus REY15A*	*Saccharolobus solfataricus P1*	*Sulfodicoccus acidiphilus*	*Metallosphaera cuprina*	*Saccharolobus sp. A20*
Reported pH optimum			**1.1**	**1.4**	**1.8**	**2**	**2**	**2**	**???**	**2.5**	**2.5**	**2.8**	**???**	**2.8**	**???**	**2.5**	**2.8**	**2.8**	**2.5**	**3**	**3**	**3**	**3.3**	**3.5**	**4**
Concordance: 100% (Extreme Acidiphiles)																									
950	cytochrome d ubiquinol oxidase subunit I	QGR22850.1	X	X	X	X	X	X	X	X	X	X	X						X						
951	Hypothetical	QGR21356.1	X	X	X	X	X	X	X	X	X	X	X						X						
1542	Sulfur oxygenase/reductase	QGR21871.1	X	X	X	X	X	X	X	X	X		X	X	X										
2640	Thetrathionate hydrolase	CBY66040.1	X	X	X	X	X	X	X	X	X	X		X	X										
1276	S-layer Protein	QGR21635.1	X	X	X	X	X	X	X	X	X	X	X	X											
1010	ABC Transporter, Permease subunit	QGR21403.1	X	X	X	X	X	X	X	X	X	X		X	X	X									
1272	MFS Transporter	QGR22873.1	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X						
2621	MFS Transporter	QGR22781.1	X	X	X	X	X	X	X	X	X			X											
1770	Amino acid permease	QGR22054.1	X	XX	X	XX	XX	X	X	XX	X	X	X	X											
1559	Protein-export membrane protein	QGR21886.1	X	XX	X	X	X	X	X	X	X	X	X	X	X	X									
1513	MMPL Family Transporter	QGR21845.1	X	X	X	X	X	X	X	X	X	X	X	X	X	X									
1390	Transporter	QGR21736.1	X	X	X	X	X	X	X	X	X		X	X	X	X									
1346	thiamine-binding protein	QGR21697.1	X	X	X	X	X	X	X	X	X	X	X	X	X										
1152	Thioesterase	QGR21521.1	X	X	X	X	X	X	X	X	X	X	X	X											
234	Hydratase	QGR20787.1	X	X	X	X	X	X	X	X	X	X		X					X						
2609	maleate cis-trans isomerase	QGR22770.1	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X							
1651	MBL-Fold Metallohydrolase	QGR21969.1	X	X	XX	X	X	X	X	X		X	X	X			X	X							
504	Hypothetical	QGR21028.1	X	X	X	X	X	X	X	X	X	X		X	X										
955	Hypothetical	QGR21358.1	X	X	X	X	X	X	X	X	X	X													
1022	Hypothetical	QGR21412.1	X	X	X	X	X	X	X	X	X	X	XX	X		X									
1541	Hypothetical	QGR21870.1	X	X	X	X	X	X	X	X	X			X											
1691	Hypothetical	QGR21994.1	X	X	X	X	X	X	X	X	XX			X											
1773	Hypothetical	QGR22056.1	X	X	X	X	X	X	X	X	X		X	X	X										
2610	Hypothetical	QGR22771.1	X	X	X	X	X	X	X	X	X	X	X	X			X	X							
2620	Hypothetical	QGR22780.1	X	X	X	X	X	X	X	X	X	X	X	X	X										
2720	Hypothetical	AWR93288.1	X	X	X	X	X	X	X			X	X	X	X		X	X							
Concordance: 100% (Weak Acidiphiles)																									
21859	S-layer Protein	WP_148230917.1														X	X	X	X	X	X	X	X	X	X
22045	Zinc-Ribbon Protein	EZQ01611.1													X		X	X	X	X	X	X	X	X	X
22025	DUF4382	AEB95736.1														X	X	X	X	X	X	X	X	X	X
22138	DUF1404	EZQ11365.1													X	X	X	X	X	X	X	X	X	X	X
21847	Hypothetical	AEB95561.1															X	X	XX	X	X	X	X	X	X

CHAPTER 6: Iron Biooxidation Maps to Specific Fox Cluster Determinants in Extremely Thermoacidophilic Archaea

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Abstract

Certain extremely thermoacidophilic archaea from carbon-limited environments (e.g., hot springs, volcanic muds, anthropogenic mining heaps) oxidize iron for bioenergetic benefit. A previously-designated genomic locus, the 'Fox Cluster', encodes iron-responsive putative proteins, although specific contributions from components of the Cluster have not been established. Here, proteomic analysis of iron-oxidizing membranes from Metallosphaera sedula, a prolific iron oxidizer, implicated FoxA2 (cytochrome c oxidase-like) and FoxC (CbsA/cytochrome b domain-containing protein) as critical to this process in Metallosphaera sedula. FoxA, FoxC, and other Fox cluster components are well-conserved among metal-mobilizing Sulfolobaceae, despite observed differences in iron oxidation rates by several species examined herein. Transcriptomic and comparative genomic analysis further buttressed the importance of FoxA and FoxC, revealing some bases for disparate iron biooxidation capacities. While numerous homologous proteins exist for FoxA (cox-like domains are prevalent across life), few homologues exist for FoxC or for many of the other closely associated determinants. Phylogenetic reconstructions suggest that the divergence of the "fox" terminal oxidase occurred around the same point where Sulfolobales branched from other similar lineages, interspersed only with the recently discovered candidate phylum Marsarchaota.

Introduction

Iron is the most ubiquitous metal element associated with life, present in almost every known organism (with the exception of some Lactobacillus spp.[1]) and serving as a crucial intermediary for electron transfer driving many enzymatic reactions. Prior to the first rapid oxygenation of the Earth's atmosphere, iron was readily available in an aqueous state, likely leading to a large selective pressure for incorporating the multivalent metal within enzymatic centers across almost all domains of life.[2] After oxygenation, iron availability became a limiting factor in many biotopes, leading to a plethora of scavenging mechanisms, including siderophores, ferritins, and reductase complexes.[3] In fact, its scarcity limits microbial activity in the carbon-fixing oceans[4] and pathogenesis in the human body,[5] sometimes encouraging risky evolutionary behavior.[6] Where iron is not a limiting factor, there are examples of microbes subsisting on a spartan metabolism oxidizing ferrous iron.

Although there are examples of iron-based metabolism (iron as an electron donor) at circumneutral pH,[7,8] highly acidic conditions favor iron biooxidation-based metabolism because of the thermodynamic stability of soluble Fe^{3+} (strong oxidant) species at lower pH, which is roughly nine orders of magnitude higher than at circumneutral conditions.[9] Particularly, some polyanionic complexes are mediators of this increased solubility, such as sulfate, which readily forms complex ions with ferric iron.[10] As a result, iron cycling plays an important role in microbial habitats ranging from terrestrial solfataras to acidic waterways to iron mats,[11,12] especially in the presence of oxygen.[2,13] Thus, many extreme acidophiles (pH_{opt} < 3.0) are capable of oxidizing ferrous iron and funneling its electrons into reducing or oxidizing reactions to meet bioenergetic needs.[13,14] However, the low electronic potential difference between Fe^{3+}/Fe^{2+} (+0.77 V) and O_2/H_2O (+1.12 V),[2] even at low pH, requires the reaction to occur largely under acidic extracellular conditions, where soluble iron is plentiful and easily turned over, as opposed to the circumneutral cytoplasmic space. Furthermore, this kind of harsh environmental niche limits life to a select group of microorganisms with an inverted membrane potential (in comparison to neutrophils) and numerous other acid tolerance traits, as well as cellular features that protect against environments laden with heavy metals and sulfidic ores.[15,16] Many such organisms are chemolithoautotrophs, with limited capacity to utilize sugars or other complex carbohydrates that are inherently scarce in hot, acidic environments where these organisms are typically the primary producers.

The most thoroughly studied mechanism for iron biooxidation belongs to the mesoacidophilic bacterium, Acidithiobacillus ferrooxidans (formerly Thiobacillus ferrooxidans), whose cytochrome c proteins (that oxidize iron) are localized to its outer membrane [14], where pH is at its minimum. The liberated iron electron is passed to a periplasm-spanning multi-copper

oxidase (Mco) downstream [17], shuttling it either to a proton-importing NADH dehydrogenase (uphill) or to a proton-exporting terminal oxidase (downhill), both spanning the inner membrane [18]. The interplay between these two outcomes is crucial to maintaining the circumneutral cytosol, balancing proton importing activity from ATP synthase and NADH dehydrogenase (proton motive force generation is probably irrelevant) with proton export of the terminal oxidase [15,19].

Mesoacidophilic, iron-oxidizing bacteria have been examined in some detail, but relatively little is known about iron oxidation in acidophilic archaeal species, despite the fact that iron oxidizers have been identified in the phyla euryarchaeota and crenarchaeota. One study in the mesophilic euryarchaeon Ferroplasma acidiphilum identified an iron-oxidizing complex exhibiting spectroscopic signatures and proteomic evidence for a type-aa_3 oxidase as well as a sulfocyanin [20], containing copper with high oxidation potential, hypothesized as a similar mechanism to A. ferrooxidans [14]. However, some key differences exist, such as a lack of cytochrome c, as well as the absence of periplasmic space present in bacteria, where bifurcation hypothetically takes place [16].

In the case of extremely thermoacidophilic archaea from the phylum crenarchaeota (growth T_{opt} > 65 ˚C), a number of metal-mobilizing species have been isolated (11 genome-sequenced strains from the family Sulfolobaceae are considered here, 7 of which were obtained here to reinforce phenotypical analysis). Within this group, iron oxidation occurs to varying extents for reasons that were initially unclear. Thus far, transcriptomic studies with three of these archaea, Sulfolobus metallicus [21], Metallosphaera sedula [22,23], and Metallosphaera yellowstonensis, as well as a metatranscriptomic analysis of iron mats in Yellowstone National Park (Norris Geyser Basin) [24], point to a group of iron-responsive, co-located genes, dubbed the 'Fox Cluster', although biochemical verification of the function of the encoded proteins has not been reported and some inconsistencies have existed to-date. Of interest here, were the genomic determinants of iron oxidation capacity among the Sulfolobaceae, whether and how iron oxidation proceeds, and how environmental adaptations arise in extreme thermoacidophiles to support their lifestyles in hot acid.

Results

Relationship between the Fox Cluster and iron biooxidation in Metallosphaera sedula

M. sedula was initially isolated from an Italian solfatara and found to be a prolific metal mobilizer [25]. Subsequently, M. sedula's growth physiology has been examined in some detail, including its CO_2-fixation through the 3-hydroxypropionate/4-hydroxybutyrate cycle [26-30], and

oxidation of iron [22] and other metals [23,31,32]. The M. sedula genome [33] encodes homologues to Fox Cluster proteins, first studied in the iron-oxidizing extreme thermoacidophilic archaeon Sulfolobus metallicus [21] and later in Metallosphaera yellowstonensis [24]. **Figure 6-1** shows the transcription percentiles of Fox Cluster components (based on normalized logarithmic distribution of oligonucleotide microarray data) for M. sedula across numerous conditions and data sets, including autotrophic, heterotrophic, and mixotrophic growth conditions [22,23,29,31,32,34]. This analysis suggests that many Fox Cluster genes are constitutively transcribed at very high levels, regularly falling in the upper 90th percentile of quantifiable transcripts for M. sedula. Further, this suggests the fox genes from M. sedula cannot be stimulated [22], which was in sharp contrast to data sets obtained for S. metallicus, M. yellowstonensis, and hot-acid spring metatranscriptomes [21,24]. This aids in discriminating between two proposed iron biooxidation gene sets in M. sedula: the fox cluster and the CbsAB-SoxLN Cluster[22].

The precise biochemical roles of the Fox Cluster components have not been confirmed. Thus, an attempt was made to isolate proteins involved in biooxidization, presumably localized to the cellular membrane given the putative annotation of transmembrane domains in many Fox Cluster components and the biochemistry of iron oxidation (**Figure 6-1**). Ultracentrifuged and detergent-solubilized M. sedula membrane fractions were BN-PAGE resolved **(Figure 6-2B),** yielding two bands observed at approximately 300 and 600 kDa. When stained with a ferrous sulfate-glycine buffer (**Figure 6-2A**), these two bands became even more pronounced and yellow in coloration, likely due to the localized production of ferric iron-sulfate complexes, indicating that protein complexes in these bands were responsible for oxidizing ferrous iron under acidic conditions [20]. These two bands were excised and separated by denaturing LDS-PAGE. Silver staining of both lanes (**Figure 6-2C**) shows several similar principle protein constituents in both bands. In the case of band 1 (approximately 600 kDa unresolved) produced three major bands at approximately 25, 50, and 60 kDa, with some higher weight bands above 90 kDa. In comparison, band 2 (approximately 300 kDa intact) appears to have fainter bands around 25 and 60 kDa and a comparable band around 50 kDa. Specifically, comparing the two bands reveals the presence of some lower weight bands in complex band 2 (less than 14.4 kDa) and numerous, lower resolution bands in the range of 30-40 kDa

These two coomassie-stained BN-PAGE bands were characterized with liquid chromatography/mass spectrometry (LC/MS). Protein sequence motifs (PSMs) were mapped back to the putative proteome of M. sedula. After controlling for trypsin and keratin contamination from sample preparation, a large number of PSMs were identified for each of the bands from **Figure 6-2B** (a full listing is given in **Table 6-1**). Within both samples, the predominant non-

contaminant was FoxA2 (Msed_0485), while no FoxA1 (Msed_0484) was detected. Also present was FoxC (Msed_0478), although at much lower levels than FoxA2. While FoxA2 contains 12 putative transmembrane domains, FoxC's amino acid sequence suggests only two such domains are present (**Figure 6-1**), with one putative transmembrane domain falling within a putative signal peptide. Furthermore, another possible constituent of the larger iron biooxidation complex, FoxB (M_r 22.2 kDa), likely contains just one transmembrane domain and a large acid-exposed extracellular domain, which could be heavily glycosylated [35], potentially accounting for its absence from the PSM analysis; although a band around 25 kDa band was noted on the LDS-PAGE, in band one (**Figure 6-2C**). Also absent was evidence of FoxD (35.1 kDa) that has 10 putative transmembrane domains and has been proposed to stabilize FoxC in the membrane of Sulfolobaceae iron biooxidizers [24]. Although two bands of similar size to putative FoxD appear in the second band of LDS-PAGE (**Figure 6-2C**). The lower bands in complex band 2 could include FoxW (Mr ~ 9 kDa), which is highly conserved in the foxBWCD locus (see below) but as yet lacks an assigned functional role.

Comparative genomic identification of the iron biooxidation determinants in extremely thermoacidophilic Sulfolobceae

Genome sequence information from the archaeal Sulfolobaceae has been limited to a few species from the genera Sulfolobus, Metallosphaera, and Acidianus [36]. This was due in part to early difficulties using Sanger sequencing for genomes that are highly repetitive, difficult to assemble, and lacked reference genomes. This database was subsequently expanded to include several new type strains, examined here [37-39]. While signatures of the Fox Cluster are evident among certain Sulfolobales, there is variability in locus content and structure. The Fox Cluster across eleven species, shown in **Figure 6-3**, is both well-conserved in terms of genetic content (high bi-directional homology) and highly syntenous with at least three sets of genes that are universally conserved in terms of identity, orientation, and order. Further, the organization of these genes seems to follow the evolutionary partitioning of the order Sulfolobales.

Among these gene groupings in **Figure 6-3**, the blue region is of particular interest because it contains two genes (foxB and foxC) with high homology to metal cofactor-binding domains. In particular, FoxB has a conserved domain for a quinol oxidase and FoxC contains a putative heme b binding domain. Interestingly, this region is almost always adjacent, or at most 4 genes removed, from the primary subunit of a cytochrome C oxidase-like (COXA-like) protein with putative heme and copper binding domains. Bioinformatics analysis indicates that this grouping

could accept electrons from a donor, such as ferrous iron, and use its redox potential to reduce oxygen and simultaneously translocate protons from the cytoplasm to the extracellular space.

Another important region in the locus with high conservation is the foxVEUF region, containing four genes conserved in all Sulfolobaceae metal mobilizers, except for M. cuprina. While all of these proteins are annotated as hypothetical, their proximity to key energetic genes and division (Acidianus spp. and S. metallicus) of those key genes suggests that they could be involved in the assembly of the cytochrome complex. In addition, there are several small proteins (encoded in foxWYZ) that appear to be highly syntenous: foxW with all species' foxBCD locus (except for M. hakonensis, due to transposition) and foxYZ with either foxB or foxA (lineage-dependent). Otherwise, a notable inconsistency from these loci is foxGH, which can be far removed or completely absent (in the case of Sulfodiicoccus acidiphilus). This result is particularly intriguing because past results have suggested this locus is induced in the presence of sulfur or sulfidic ores, while S. acidiphilus was recently reported to be the first Sulfolobaceae member whose growth is actually inhibited in the presence of elemental sulfur [39].

Iron biooxidation capacity among reported extremely thermoacidophilic iron biooxidizers

Several members of the archaeal family Sulfolobaceae, whose genomes encode components of the Fox Cluster, oxidize either aqueous iron or iron-sulfidic ores [16]. In order to examine this trait, several approaches were used to determine metal biooxidation capabilities of these archaea. Colorimetric identification of soluble iron in actively growing cultures and in whole-cell lysates (**Figure 6-4)** suggest that many of these species either lack the capability to oxidize iron or do so at low rates. For M. prunae, M. hakonensis, S. metallicus, and S. tokodaii, whole-cell iron biooxidation was on the order of that observed in S. acidocaldarius, or roughly 10% of M. sedula. Whole cell extracts of M. hakonensis and S. tokodaii fared no better than active cultures, while whole cell extracts of M. prunae and S. metallicus oxidized 40-60% of the available iron in solution over the course of 24 h. In contrast, M. sedula, A. brierleyi, and A. sulfidivorans are potent iron biooxidizers.

An O_2 consumption assay measured cellular respiration in response to ferrous iron addition for all of these species. Mid- to late-exponential phase cultures were washed to remove residual growth media and used to inoculate a dry-air saturated basal salts solution in closed vessels. After stabilization of dissolved oxygen tensions, soluble ferrous iron was added to the reactor vessel. Oxygen consumption was determined by assuming Henry's Law of infinite dilution of oxygen at the reactor conditions (65 – 70 ˚C, pH 2.0).

Representative traces (aggregates) are shown in **Figure 6-5.** After accounting for the auto-oxidation of iron — an effect that is more pronounced in higher pH environments — O_2 consumption rates were determined. The abiotic control for iron biooxidation was approximately 85-fold lower than the rate of M. sedula (85.0 fmol/min/cell). Interestingly, no other species was comparable to of M. sedula's biooxidation rate; the next highest rate was for M. prunae at 30.7 fmol/min/cell, roughly one-third of M. sedula. Another interesting observation was the much lower respiration rates of A. sulfidivorans, A. brierleyi, and S. metallicus, at 10-30% of M. sedula, while M. hakonensis and S. tokodaii rates were on par with abiotic controls.

Reverse transcription of key Fox Cluster genes

Given the apparent essentiality of FoxC and FoxA for iron biooxidation, reverse transcription of the fox genes was examined using RNA collected from standard growth conditions for these organisms. In all cases, data provided useful insights into the role of several conserved genes within the operon, shown in **Figure 6-6**. Not surprisingly, the largest qualitative transcription levels for the gene groups around foxA and foxC in any of the organisms was M. sedula, agreeing with review of existing transcriptomic data sets from M. sedula (**Figure 6-1**). This lends credence to the importance of these genes in iron biooxidation for the most prolific strain examined in this study and explains the predominance of FoxA2 and FoxC identified by LC/MS. Additionally, the foxA1 gene was not transcribed at high levels. Intriguingly for M. prunae, no apparent foxA transcription occurs, although additional qPCR (**Figure 6-6)** demonstrated that the gene is transcribed at roughly three orders of magnitude (1000-fold) less than foxA2 (from M. sedula), similar to foxA1 (also M. sedula), likely accounting for a difference in presence of FoxA2 in the M. sedula membrane and the lowered oxidation rate for M. prunae shown in **Figure 6-5**.

In the case of the other metal-mobilizers, transcription of all genes (foxABWC) occurred under normal growth conditions, although at a much lower level than that observed in M. sedula. The exceptions were for S. tokodaii and M. hakonensis. For the former, a frameshift mutation appears to disrupt the sequence of FoxC. If the organism is producing a fusion protein from the existing N-terminal domain and the C-terminal domain is translated separately, then the C-terminal domains transcription levels may impede assembly. For M. hakonensis, there is an apparent transposon disrupting the foxW gene (confirmed by its absence in RT-PCR and presence on a positive control gel of gDNA). Furthermore, this disruption may have a polar effect on foxC, as no transcript is visible on the gel. In this case again, the foxA duplicates appear to show little basal transcription in M. hakonensis. Finally, foxD had the least or no visible transcription in all of the strains studied.

Evolutionary track of the "Fox" genes

There are few, if any, homologues for the Fox Cluster genes in other Sulfolobaceae, other than proteins containing broadly conserved domains, such as the cytochrome oxidases that have both lower homology and alternative putative or confirmed biochemical purposes. This raises the question: Were the Fox Cluster genes obtained through horizontal gene transfer (HGT) or passed down through evolutionary processes? The possibility of horizontal gene transfer was explored for the species listed in **Figure 6-3**. This analysis was done from two perspectives: parametrically based on G+C content of genes (including in the codon positions; **Figure 6-7**) and phylogenetically comparing FoxA and FoxC specifically to available homologs (**Figure 6-8**).

In the case of G+C analysis, **Figure 6-7** shows that the "*fox"* genes are not outliers, as would be expected if the genes were the result of a recent HGT event and had not amerliorated to the genetic background of the individual genomes. Further, the genes are well-distributed within the whiskers of each box plot with few exceptions, suggesting either deeper evolutionary origins or amelioration of the genes to suit the codon preferences of the individual species. The phylogenetic analysis (**Figure 6-8**) shows that the *foxA* and its putative cytochrome c oxidase-like domain are widely conserved across the prokaryotic kingdoms, with almost all of the archaeal constituents forming a tighter and divergent clade from other bacterial constituents. Meanwhile *foxC* is relatively unique from an evolutionary perspective, with no close relatives beyond the archaea. Inserts of the trees in **Figure 6-8** show that the proteins have largely evolved along taxonomical lines, as one would expect. Interestingly but not surprisingly, there are numerous homologues of both FoxA and FoxC that have other putative bioenergetics roles. These distant relations are actually separated from the best-hit FoxA and FoxC proteins by branches containing homologues identified in the new candidate phylum Marsarchaeota. This may suggest horizontal transfer to this group of organisms that consequently confers the ability to oxidize ferrous iron, which is consistent with their presence in iron mats in Yellowstone National Park [40]. Intriguingly, this new phylum was found in higher density just below a more oxic layer containing signatures of the species *M. yellowstonensis*.

Discussion

Microbiological, biochemical and transcriptomic data support the essentiality of FoxA and FoxC for iron biooxidation by extremely thermoacidophilic archaea. Previous reports suggested that FoxD ($M_r \approx 35$ kDa) and FoxG ($M_r \approx 69$ kDa) play an essential role in stabilizing FoxC and shuttling protons downhill, although no evidence of either one of the transmembrane-heavy proteins was seen in the proteomics or the SDS-PAGE analysis from the active membrane

fractions. Also of interest was the lack of FoxB in the LC/MS results, given its putative role as a cytochrome oxidase-like subunit II (Cox2). Analysis with SignalP suggests that the Sec translocation system likely cleaves the protein at the sequence LET^QY, creating a protein with no detectable transmembrane region (*in silico*) that may have a much weaker association with FoxA and FoxC. In addition, the protein contains only two potential trypsin cleavage sites (Lys residues) outside of the putative signal peptide region, which may be insufficient for digestion. Neither FoxG or FoxD have this similar issue. Heavy glycosylation has been observed in some extracellular crenarchaeal proteins [41,42], resulting in poor resolution in the standard SDS buffer and an inability of proteases to access cleavage motifs [35].

Consideration of comparative genomics data in conjunction with transcriptomic and phenotypical information revealed more about the key genes in the biooxidation process. In the archaea considered here, there were at least three interesting traits that further support the essentiality of *foxA* and *foxC* to iron oxidation. In the case of *M. prunae* and *M. sedula*, all genes share 100% amino acid sequence identity in the Fox Cluster*,* with the exception of FoxA. In this case, the version of FoxA in *M. prunae* is clearly an unusual fusion of the exact complementary domains of FoxA1 and FoxA2 in *M. sedula*, with the promoter region of *foxA1*. Given the apparent lack of *M. prunae foxA* transcription in **Figure 6-6** and the absence of *M. sedula* FoxA1 in proteomic samples, one might assume that there is no essential role for either paralog or that the fusion results in a loss of function; although, clearly *M. prunae* is capable of biooxidation. Thus, qPCR was utilized to determine that the transcription level is roughly three orders of magnitude below that of the *foxA2* gene and roughly identical to that of the *foxA1* gene in *M. sedula* **(Figure 6-6)**, suggesting its ability (even in lower quantities) to catalyze oxygen reduction. It is possible that this paralog has much higher oxygen affinity and is regulated in response to changing oxygen tension, a regulatory trait observed in other Sulfolobales as a potential way of managing the beneficial and deleterious effects of oxygen bioenergetics [43]. This was a curious result given the species was originally isolated from a uranium mining slag heap and determined to have increased resistance to uranium toxicity [31]. In addition, we have observed a previous delayed response in metal biooxidation in *M. prunae* upon the removal of iron from its growth media that is in contrast to *M. sedula,* which can now be rationalized by the disparate regulation of the *foxA* gene(s) [32]. Additionally, this suggests an alternative uranium mitigation process [31] in which decreased FoxA activity may help prevent excessive production of toxic hexavalent uranium species from insoluble metal ores via indirect biooxidation by ferric iron. This couples with an RNase-mediated mechanism of suppression [44] that balances modest metal biooxidation (to

maintain pH homeostasis) with preventing the buildup of electrophilic ferric iron species in the presence of highly toxic heavy metals.

In the case of FoxC, both *M. hakonensis* and *S. tokodaii* provide potential validation of its essentiality. In both cases, little or no iron biooxidation was observed despite reports of growth on iron substrates; this is perhaps an example of unintended laboratory-directed evolution, although fortuitously consequential in this circumstance. In the case of *M. hakonensis,* this may be the result of a transpositional disruption of the *foxW* gene, leading to its loss and the potential disruption of transcriptional regulatory elements for the *foxC* gene (promoter within *foxW*), which both have no apparent transcription in **Figure 6-6.** In the case of *S. tokodaii*, the genome sequence suggests a disruption of the full protein sequence by a SNP that potentially displaces the planar and axial coordinating residues of the protein in two separate domains, based on previous modeling of the FoxC protein [24].

The recent addition of several metal-biooxidizers to this group provides further evidence for the importance of these proteins and several others, via synteny, in metal biooxidation. In particular, the loci with *foxBWCD, foxYZ,* and *foxVEUFJ* have demonstrated synteny among all of the species examined here and a few other species confirmed to oxidize metals in laboratory cultures. While assignment of putative function is trivial for *foxABC* (type-aa3 cytochrome/quinol oxidase, quinol oxidase subunit II, and cytochrome b-containing protein, respectively), all of the other proteins show no remarkable similarity to any known protein in publicly accessible databases. Furthermore, while FoxABC have remarkably high similarity (>60% across the board), almost all of the other proteins show large evolutionary drift (30-60% similarity), with the exception of FoxV and FoxE, which are both above 50% for all species compared to *M. sedula.*

Given the induction of the *foxVEUFJ* locus and their large numbers of transmembrane domains, one putative role is in the assembly and delivery of cofactors to the larger cytochrome oxidase complex, a role without candidates in current explanations. Finally, the *foxGH* locus, believed to be involved in signal transduction or in the cytochrome b complex [24], is dislocated from the other genes in some species and is absent in *Sulfodiicocuus acidiphilus*. While there is no report as to the ability of this microbe to utilize iron (only inorganic substrates containing sulfur were tested), there is a report that the organism cannot grow in the presence of sulfur [39]. Given the original analysis describing the gene pair as pyrite- (and not Fe^{2+}) induced [21], it now seems possible that the genes are tied to some form of sulfur/sulfidic ore metabolism absent in the new species and not directly involved in iron metabolism. In further support, BLAST searches have identified the absence of the *foxGH* genes at any appreciable similarity or significance in a newly discovered lineage, the Marsarchaeota, with distant links between the Crenarchaea and other

phyla of the kingdom Archaea [40]. In fact, these organisms were proposed to have a major role in iron reduction in the microaerobic region of iron mats, although, several of the distinct genomes have clear homologues to *foxABCDEF.*

Another gene proposed to perform an essential role is a multi-copper oxidase (Mco), in the metal mobilizer mesophilic bacterium *A. ferroxidans* [17], as well as the mesophilic euryarchaeon *F. acidiphilum* [20], that serves in a putative role as a facilitator of redox balance, spanning the periplasmic space. However, the lack of a periplasmic space in the archaea calls into question the necessity of a nanowire-like protein. Previous analysis has identified at least four Mco's, two of the sulfocyanin type and two of the rusticyanin (one with plastocyanin-like) type, in *M. sedula* [33] and several Mco's in *M. yellowstonensis* [24]. Here, comparative genomics suggests that there is a rusticyanin (Msed_0966) homolog in all of the studied genomes, with the exception of *S. acidiphilus* and *S. tokodaii* (Identity above 40% and coverage over 65%). However, the protein from *M. sedula* also shares homology with proteins of non-metal-mobilizing species, such as *Saccharrolobus solfataricus* and *Sulfolobus islandicus*. The other protein of interest, encoded in Msed_1206, has only a few homologs in the *Metallosphaera spp.* as well as in the Marsarchaeota, *Sulfolobus islandicus* strains and *Sulfodiicoccus* acidiphilus. Given a lack of strong conservation and synteny (the genes are always displaced from the *fox* locus, in contrast to *A. ferrooxidans*), the protein may serve a more universal electron transfer route or act as a critical carrier in an ancillary process.

Conclusion

In this report, we demonstrate the essentiality of FoxA and FoxC in the process of iron biooxidation in the extremely thermoacidophilic archaea and provide additional evidence for the role of unconstrained *foxABWC* transcription in the proficient biooxidization capacity of *M. sedula*. This suggests a potential application in metal biooxidation biotechnological applications, which have been dominated by mesophilic species and the few more characterized archaeal thermoacidophiles [16,45,46]. Interestingly, this process is deeply rooted in the extremely thermoacidophilic aerobic archaea and even appears in a somewhat distant archaeal lineage in roughly the same genetic constitution. This process may be a far distant transfer event or a more recent convergent evolution emerging in the wake of an oxygenated world, exclusively in environments dominated by heavy inorganic mineral dissolution and copious energy dissipation from globally distributed geothermal activity.

Experimental Procedures

Cultivation procedures

All organisms were cultured aerobically in a species-modified DSM-88 Medium. All cultures were grown at 70 ˚C, with agitation, except *S. metallicus* (65 ˚C) and *Sulfurisphaera tokodaii* (85 ˚C). All cultures were grown at pH 2.0, except *S. tokodaii, Metallosphaera hakonensis, Sulfolobus acidocaldarius* (all at pH 3.0), and *Acidianus sulfidivorans* (pH 1.0). Species-specific media supplementation included: 0.2 g/L yeast extract (YE) and 10 g/L elemental sulfur (S^0) (*Acidianus brierleyi*), 0.1 g/L YE and 10 g/L pyrite (*A. sulfidivorans*), 1 g/L YE (*M. hakonensis*, *M. prunae, M. sedula*), 1 g/L NZ-amine, and 2 g/L sucrose (*S. acidocaldarius*), 0.2 g/L YE and 5 g/L S^0 (*S. metallicus*), 1 g/L each YE, D-glucose, casamino acids (*S. tokodaii*). Cells were enumerated with acridine orange stain under oil immersion microscopy[47].

Proteomic isolation of iron biooxidizing complex

M. sedula was grown in a 2-L bioreactor (media above), with sparged air and agitation to a density of approximately 10^9 cells/mL. Cultures were centrifuged for 20 min at 18,000 x g and 4˚C. The decanted pellet was re-suspended in lysis buffer (85.3 mM citric acid, 14.7 mM sodium citrate dehydrate), then lysed with a French pressure cell (18,000 psi). The lysate was centrifuged at 25,000 x g for 25 min at 4˚C.

Membrane fraction isolation was based on a modified protocol [20,48], with ultracentrifugation (L8-70M Beckman) at 145,000 x g for 1 hour at 4˚C. The pellet was re-suspended in buffer (lysis buffer with 750 mM 6-aminocaproic acid, 10% v/v glycerol, 2% w/v n-Dodecyl-B-D-maltoside), then centrifuged at 145,000 x g for 1 h at 4˚C.

BN-PAGE samples were buffer exchanged with BN-PAGE Wash Buffer (18 mM citric acid, 32 mM sodium citrate dehydrate, 50 mM 6-aminocaproic acid, 2% w/v DDM) using a 30 kDa MWCO polyethersulfone concentrator (ThermoFisher), centrifuged at 16,000 x g and 10˚C, for three exchanges. Samples were resolved on a 4-16% Native-PAGE Bis-Tris Gel (ThermoFisher), with M_r approximations via NativeMark ladder (ThermoFisher). Duplicate gels were Coomassie-stained or stained with an iron biooxidation buffer (50 mM glycine, 10 mM ferrous sulfate heptahydrate)[20] and incubated at 70˚C for 4 h. The excised proteomics bands (Coomassie-stained) were typsin digested for LC/MS protein motif quantification.

Both excised bands and raw membrane preps were SDS-PAGE resolved on 4-12% bis-tris NuPAGE polyacrylamide gels. The samples were reduced for 20 min at 70˚C with NuPAGE

LDS Sample Buffer and NuPAGE Reducing Agent. Electrophoresis was performed in a MOPS buffer with coomassie post-staining.

Iron biooxidation assays

Cell suspensions were harvested in mid-exponential growth and centrifuged at 6,000 x g washing with fresh DSM-88 base salts (no ferric chloride). A portion was reserved to be French pressed at 18,000 psi with four passes. In microtiter plates, whole cells and cell lysates (20 □g, quantified by Bradford Assay) were incubated with 8 mM ferrous sulfate heptahydrate dissolved in DSM-88 media (no carbon supplement for cell lysates), within a humidified vessel at pH 1-3. At inoculation, at 4 h (whole cell lysates) or 12 h (whole cell), a sample (10μL) was mixed 1:8 (v/v) with 20% ethanol. Ferrous iron quantification was performed by mixing 8% (v/v) ammonium acetate buffer (16.5 M acetate, 3.25 M ammonium in water), 8% (v/v) phenanthroline (11.1 mM 1,10-phenanthroline, 14.8 M methanol in water), 5% (v/v) sample, and reserve water, then incubating at room temperature for 15 min in dark conditions. Samples were read on a spectrophotometer (Biotek) at 510 nm. Sample concentrations were compared to a standard curve of ferrous sulfate heptahydrate; the mean and standard error of triplicate samples are reported.

Indirect iron biooxidation was measured in an approximately 110 mL sealed vessel with a Clark probe. The vessel was equilibrated with pre-warmed (70˚C), dry-air saturated DSM-88 medium, without ferric iron (pH 2.0). Following single-point calibration the vessel was charged with 5 x 10^9 cells. Following 5 min of basal oxygen consumption, the vessel was charged to 10 mM with ferrous sulfate suspended in DSM-88 medium without ferric iron, and measurements were recorded for an additional 25 min at 30 s intervals. Species reactions were done with triplicated biological repeats; the oxygen consumption rate was reported as the mean of slopes from linear regression, within the linear window of data collection. Oxygen concentrations were approximated assuming Henry's Law for dry air at 70 ˚C.

Identification of conserved 'fox' genes

Full genome sequences for the available type-strain Sulfolobacaeae species (NCBI) were reannotated using Prokka [49]. Previously named 'fox' genes and any unnamed genes in between Msed_0467 to Msed_0485 (*M. sedula* as a reference) were used in a BLASTP [50] search on a database of reannotated genomes. Potential homologues for comparisons within the order were

best-hits with an E-value < 10^{-15}. Functional identifications reported in **Figure 6-1** were made using annotations from BLASTP-identified homologues (from the non-redundant database).

G+C content of coding sequences was determined with the R package Seqinr (version 3.4-5) [51]. The data were analyzed and visualized using ggplot2 (version 3.1.1). Broad taxonomical phylogenetic reconstructions were created from protein sequence alignments with the MUSCLE [52] algorithm (version 3.8.425), and tree construction via FastTree [53] (version 2.1.5) with default parameters. For FoxA and FoxC protein databases, BLASTP searches were screened with a 50% query and 30% identity cutoff (in comparison to the respective genes).

Reverse transcript screen

Mid-exponential cultures were snap-cooled on dry ice/ethanol and centrifuged at 10,000 x g for 20 min. The cell pellets were washed once with tris-EDTA buffer. The resultant pellets were lysed in TRIzol reagent (Invitrogen) and nucleic acids extracted with chloroform. Cleanup was performed on an RNeasy column (Qiagen) with DNase-I treatment. Resultant RNA was quantified using a Nanodrop spectrophotometer (ThermoFisher). One microgram of total RNA from each species was reverse transcribed using iScript RT Supermix (Bio-Rad) and target *fox* genes (*fox*ABCDW) were amplified using Phusion PCR polymerase (New England Biolabs) with an annealing temperature of 60˚C for 25 cycles. No reverse transcriptase negative controls, gDNA positive control gels and oligos are provided in Supplementary Information.

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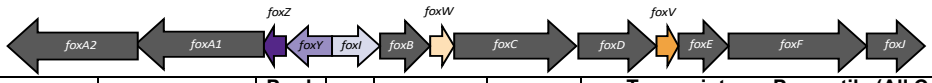


Locus Tag	Name	Component	Pred. M_r (kDa)	pI	Predicted TMs	Signal Peptide
Msed_0473	FoxJ	Hypothetical	29.1	9.2	8	N
Msed_0474	FoxF	Hypothetical	68.3	9.5	16	N
Msed_0475	FoxE	DUF1404	22.6	7.9	6	N
Msed_0476	[FoxV]	SepZ-like	6.7	8.3	2	N
Msed_0477	FoxD	Hypothetical	35.1	6.7	10	N
Msed_0478	FoxC	Cyt. b	56.7	5.2	2(1)	Y†
Msed_0479	[FoxW]	Hypothetical	8.8	9.4	2	N
Msed_0480	FoxB	Cox2	19.6	5.1	1(0)	Y‡
Msed_0481	FoxI	ArsR Regulator	19.9	9.1	0	N
Msed_0482	[FoxY]	Hypothetical	7.1	5.2	2	N
Msed_0483	[FoxZ]	Hypothetical	16.0	4.2	1	N
Msed_0484	FoxA1	Cox1	65.7	7.1	12	N
Msed_0485	FoxA2	Cox2	65.7	7.1	12	N

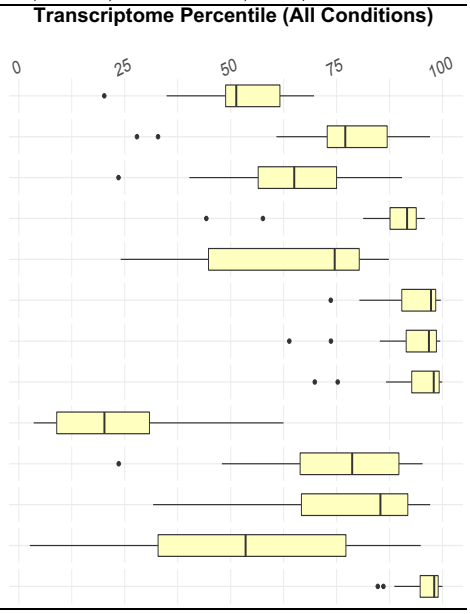


Figure 6-1. Fox Cluster in M. sedula.

Annotations are based on *S. metallicus* transcriptome, for genes responsive to iron and BLASTP searches. Mr and pI were calculated in the absence of putative cleavage sequences using EXPASY. Transmembrane (TM) domains were determined with TMHMM v. 2.0 and Signal peptides (SP) were determined with SignalP v. 5.0 with setting "Archaea." Reported transcriptional data were compiled from previous M. sedula transcriptional studies. For signal peptides, †FoxC has a putative Sec/SPI site at the sequence AYG^AD with 0.9193 likelihood, ‡FoxB has a putative Sec/SPI site at the sequence LET^QY with 0.8257 likelihood, all other likelihoods were below 0.1. Values in parenthesis for TMs represent values minus the signal peptide region. Bracketed gene names are previously unnamed genes, conserved in all species from this study. Orange shading is 5' to 3', green shading is 3' to 5'.

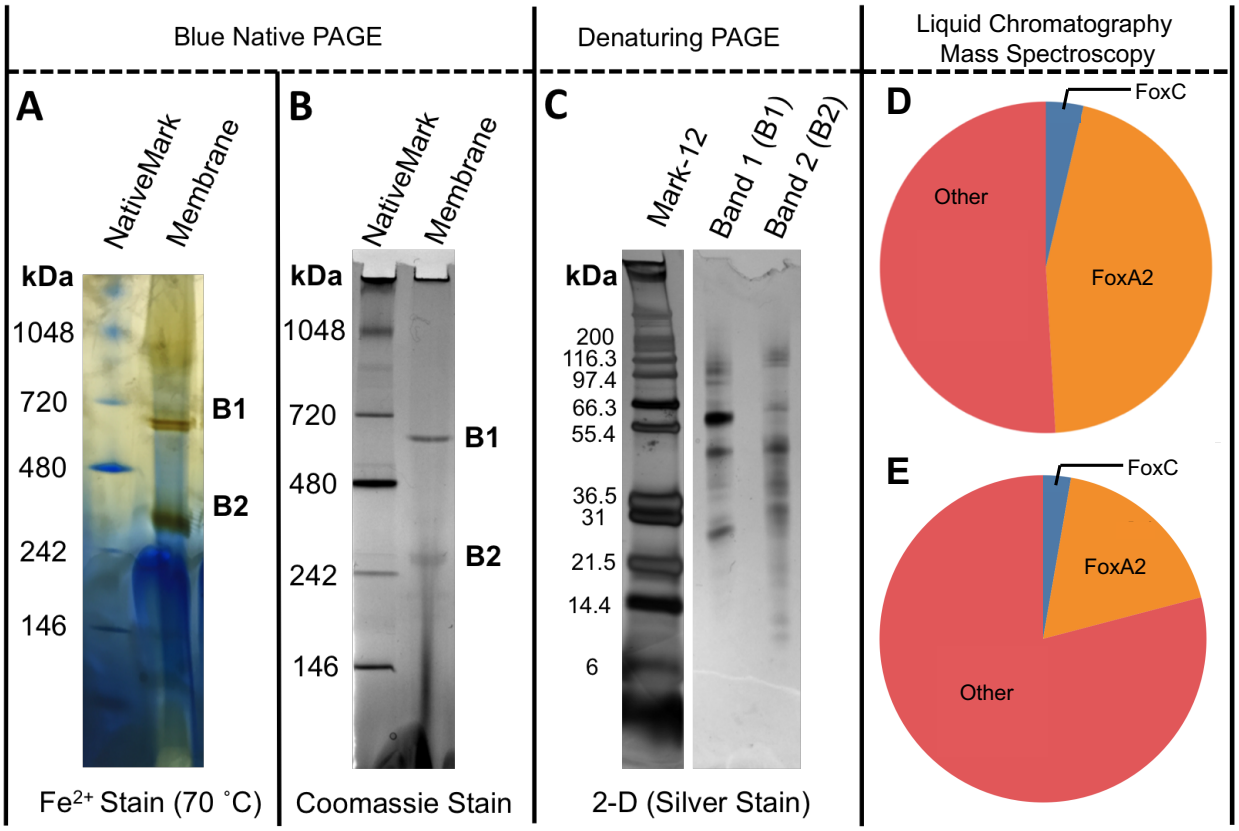
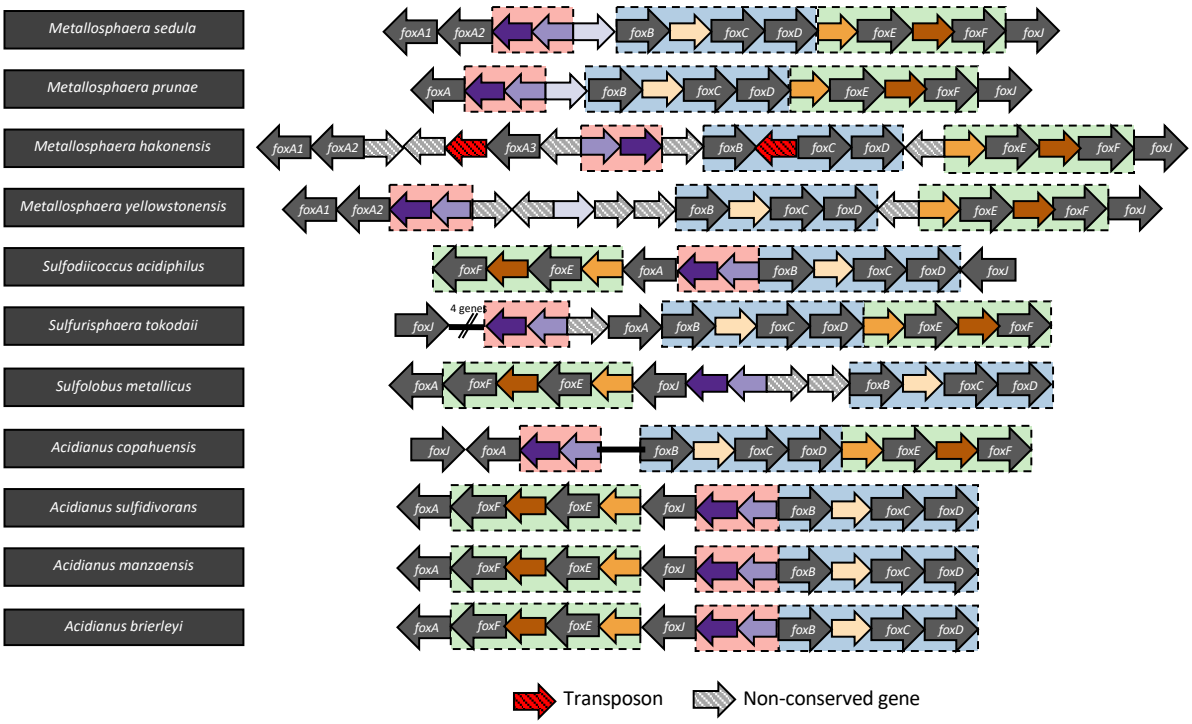


Figure 6-2. Gel Electrophoresis and Proteomics of *M. sedula* membrane fractions.

A) BN-PAGE of membrane fraction with ferrous iron/glycine buffer (pH~3.0, T~70˚C) overnight. B) BN-PAGE of membrane fraction stained with Coomassie Brilliant Blue. C) LDS-PAGE of bands from Blue-Native PAGE gel (B) stained with Coomassie Brilliant Blue. D) Protein Sequence Motifs assigned to FoxA, FoxC, and other non-fox proteins from Band 1 and Band 2.



Gene Name	FoxA2	FoxA1	FoxZ	FoxY	FoxB	FoxW	FoxC	FoxD	FoxV	FoxE	FoxU	FoxF	FoxJ
Locus ID	Msed_0485	Msed_0484	Msed_0483	Msed_0482	Msed_0480	Msed_0479	Msed_0478	Msed_0477	Msed_0476	Msed_0475	Msed_0474	Msed_0473	Msed_0472
Metallosphaera prunae	90.6	88.4	100.0	100.0	100.0	100.0	99.8	100.0	100.0	100.0	100.0	100.0	100.0
Metallosphaera hakonensis	88.1	86.5	57.0	94.2	91.1		84.9	61.2	87.9	81.5	82.4	68.5	68.0
Metallosphaera yellowstonensis	86.0	84.5	44.8	84.1	83.2	68.3	83.9	58.2	84.8	76.6	72.5	64.5	66.4
Acidianus manzaensis	74.8	71.1	34.2	49.3	75.9	47.7	71.0	48.8	66.7	58.1	47.1	39.5	37.4
Sulfodiicoccus acidiphilus	76.3	71.5	35.9	44.6	79.0	41.9	64.1	40.6	71.2	61.2	41.2	43.3	45.4
Acidianus sulfidivorans	73.4	70.6	33.8	40.6	76.4	47.7	72.6	46.7	69.7	56.2	51.0	39.5	36.6
Acidianus brierleyi	74.6	71.1	36.2	47.1	75.9	44.9	64.7	35.0	74.2	58.6	47.1	40.7	42.0
Sulfurisphaera tokodaii	73.3	72.5	33.3	50.7	73.2	57.8	60.0	42.8	54.5	54.4	51.0	42.7	42.6
Acidianus copahuensis	74.3	69.8	30.4	46.4	71.7	58.3	64.5	36.9	71.2	61.1	37.3	38.7	44.3
Sulfolobus metallicus	71.8	69.1	31.2	38.2	70.4	48.9	62.2	31.8	71.2	54.7	45.1	40.1	38.4

Figure 6-3. Conservation of the 'fox' Stimulon with key loci and homology.

Names of genes are derived from previous work and non-named genes are colored according to their best match in a BLASTP search considering bi-directional queries. Where paralogues are assumed, genes are numbered based on their appearance. The entire stimulon is oriented based upon the detection of foxA (a key gene to be discussed later). Boxing demonstrates gene sets that are syntenous in terms of content, orientation, and order among all of the species. Bottom panel represents the homology of best-hits to M. sedula fox genes, based on MUSCLE alignment.

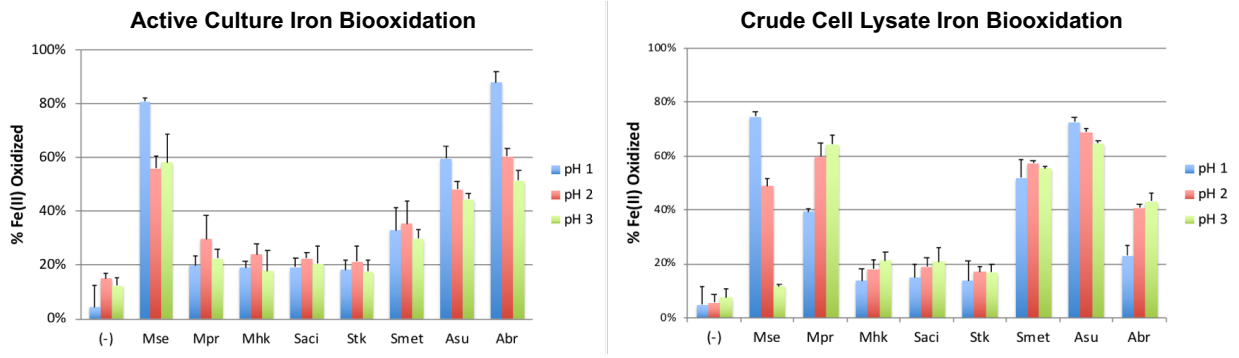
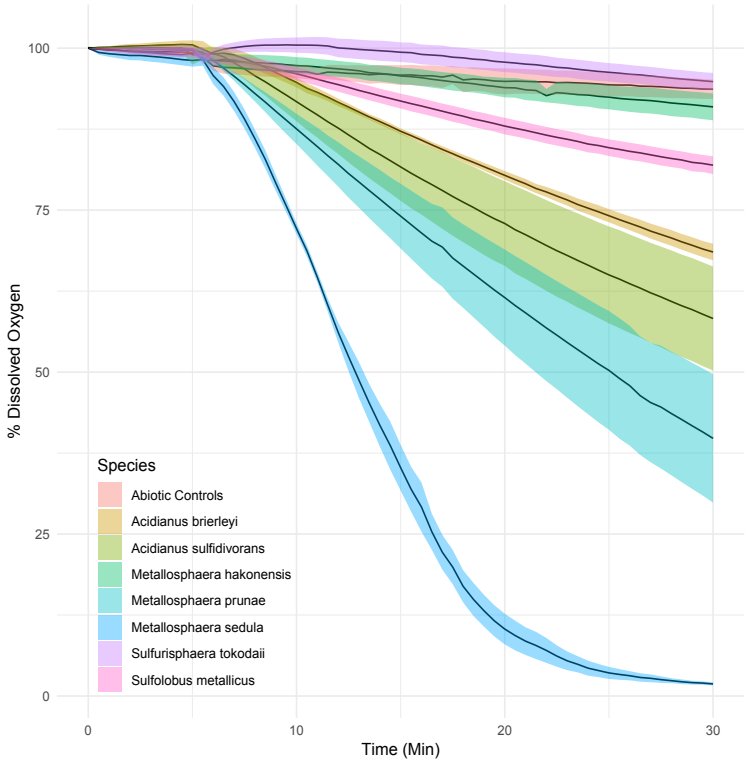


Figure 6-4. Iron Biooxidation for Reported Iron Biooxidizing Species.

Iron biooxidation by reported iron-oxidizers and control species (non-metal-mobilizer *Sulfolobus acidocaldarius* DSM639) under physiological pH range at optimal growth temperatures, using the 1,10-phenanthroline quantification method. Negative controls were abiotic samples containing media without cells. Species abbreviations: *Acidianus brierleyi (Abr), Acidianus sulfidivorans (Asu), Metallosphaera hakonensis (Mhk), Metallosphaera prunae (Mpr), Metallosphaera sedula (Mse), Sulfolobus acidocaldarius (Saci), Sulfolobus metallicus (Smet), and Sulfurisphaera tokodaii (Stk).*



Species	Consumption Rate (fmol O2/(cell*min))	Relative Activity (versus *M. sedula*)
Abiotic Control	1.06 ± 1.37	1.25%
Acidianus brierleyi	17.0 ± 1.5	20.0%
Acidianus sulfidivorans	23.0 ± 5.0	27.1%
Metallosphaera hakonensis	3.72 ± 0.43	4.37%
Metallosphaera prunae	30.7 ± 6.1	36.1%
Metallosphaera sedula	85.0 ± 10.4	100%
Sulfolobus metallicus	9.83 ± 0.33	11.6%
Sulfurisphaera tokodaii	2.76 ± 1.06	3.25%

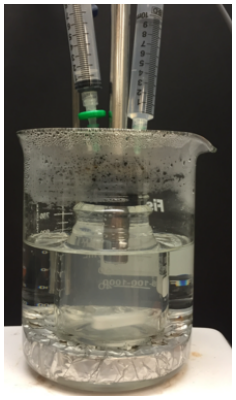


Figure 6-5. Oxygen Consumption Rates (with example traces) for iron biooxidizing Sulfolobaceae.

Sulfolobus acidocaldarius DSM639 was used as a negative control for biooxidation. Iron autooxidation was accounted for at all conditions with the use of 10 mM ferrous sulfate dissolved in DSM-88 medium (basal salts) at the reaction pH and temperature. Oxidation rates were normalized to the top iron oxidizer Metallosphaera sedula DSM5348. All rates are the result of triplicate measurements with standard error included on the plot. The O2 consumption rate is presented with a standard error based on linear regression at the most linear range after iron addition lasting at least 7 min. Iron addition occurred at 5 min after dissolved oxygen stabilization. The lower right insert shows the experimental apparatus.

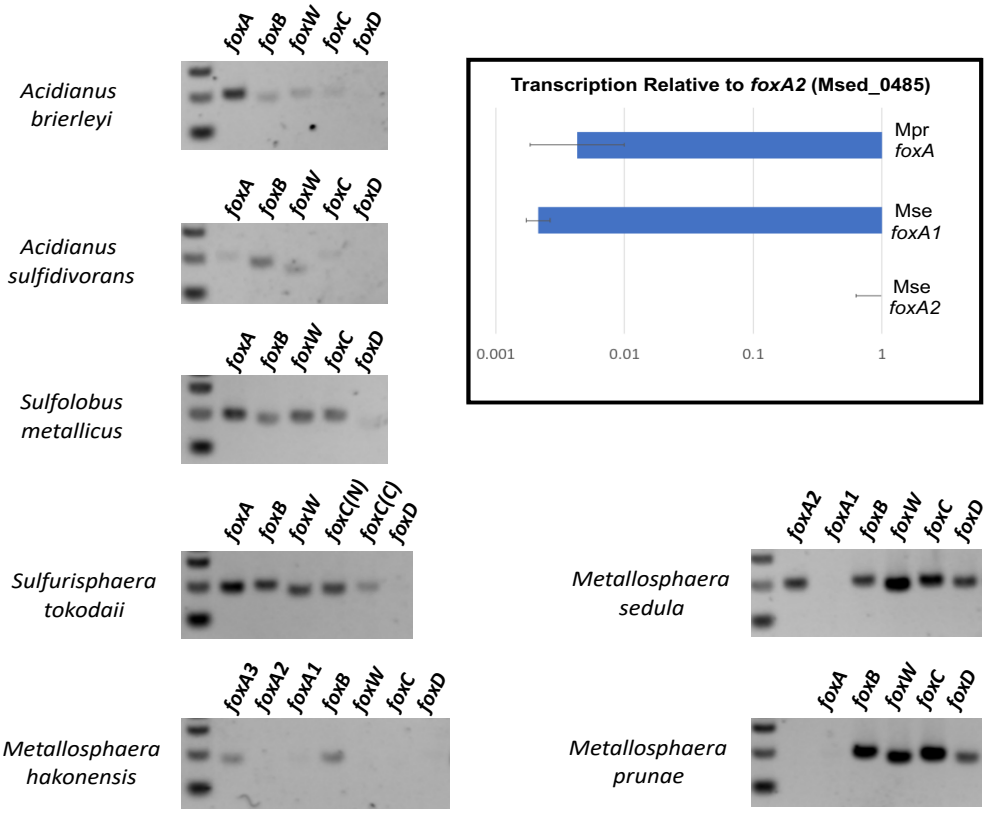


Figure 6-6. RT-PCR of Fox Cluster genes using RNA collected from 'normal' growth conditions (further detailed in Methods).

In each case, the first lane shows a ladder with the 300, 200, and 100 bp standards from 100 bp (NEB) ladder. No primers were designed to produce a product longer than 210 bp and smaller products occurred only when required to fit the primers within the annotated open reading frame of the gene. fox genes are labeled based on their appearance in the genome, where M. sedula and *M. hakonensis* contain two and three foxA gene duplicates, respectively. For *S. tokodaii*, two primers pairs were used for the foxC gene that cover the two putative domains of the gene which is dislocated by a frameshift mutation. The upper right insert shows the relative-fold transcription levels of foxA genes in M. sedula and M. prunae, via qPCR.

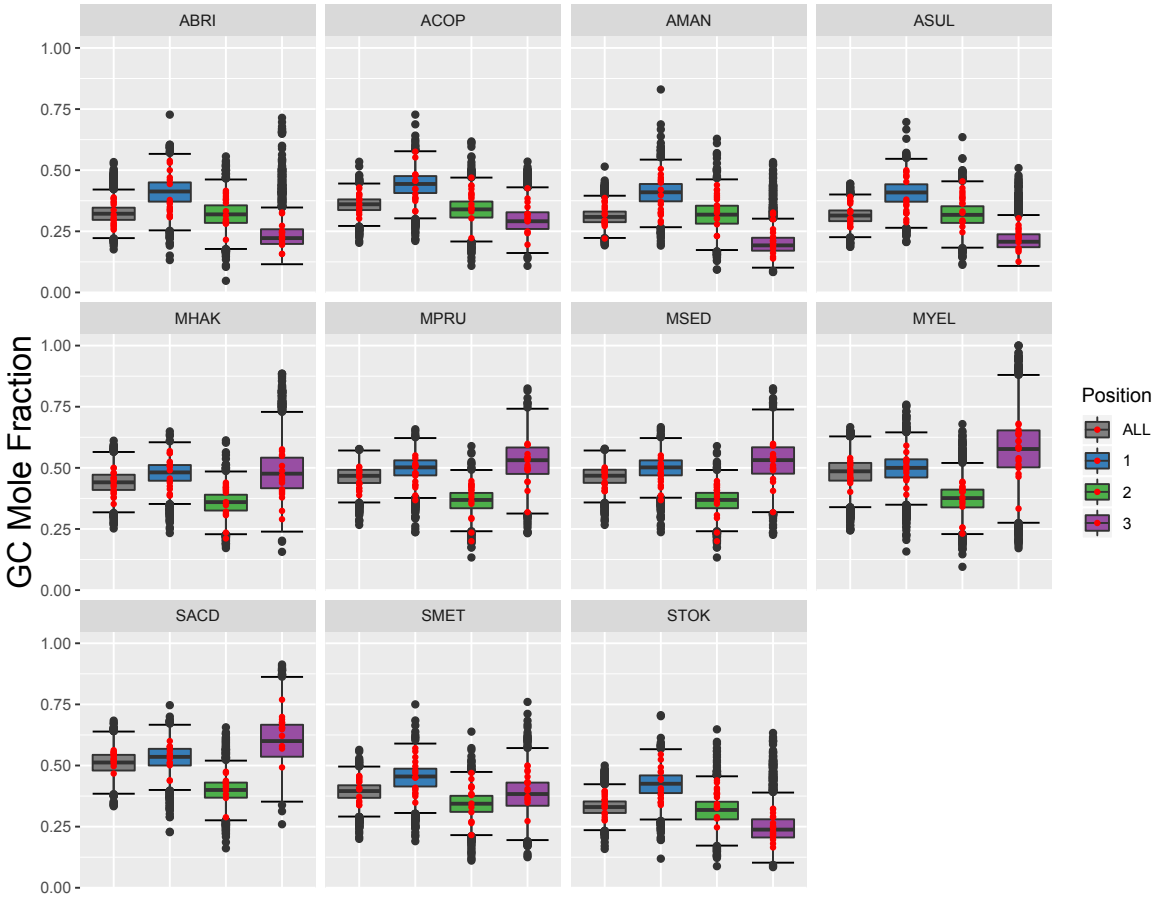


Figure 6-7. GC Content of the Fox Locus in the Sulfolobaceae.

Analysis of GC content in coding regions and each of the three nucleotide positions of codons. Fox genes are shown in red for each species. All data points are the collective mean of dinucleotide content for a particular ORF, as predicted through the Prokka pipeline. Whiskers extend 1.5*IQR (Interquartile range) from the 1st and 3rd quartile values. Abbreviations are *Acidianus brierleyi (Abri),* Candidatus *Acidianus copahuensis (Acop), Acidianus manzaensis (Aman), Acidianus sulfidivorans (Asul), Metallosphaera hakonensis (Mhak), Metallosphaera prunae (Mpru), Metallosphaera sedula (Msed), Metallosphaera yellowstonensis (Myel), Sulfodiicoccus acidiphilus (Sacd), Sulfolobus metallicus (Smet), Sulfurisphaera tokodaii (Stok).*

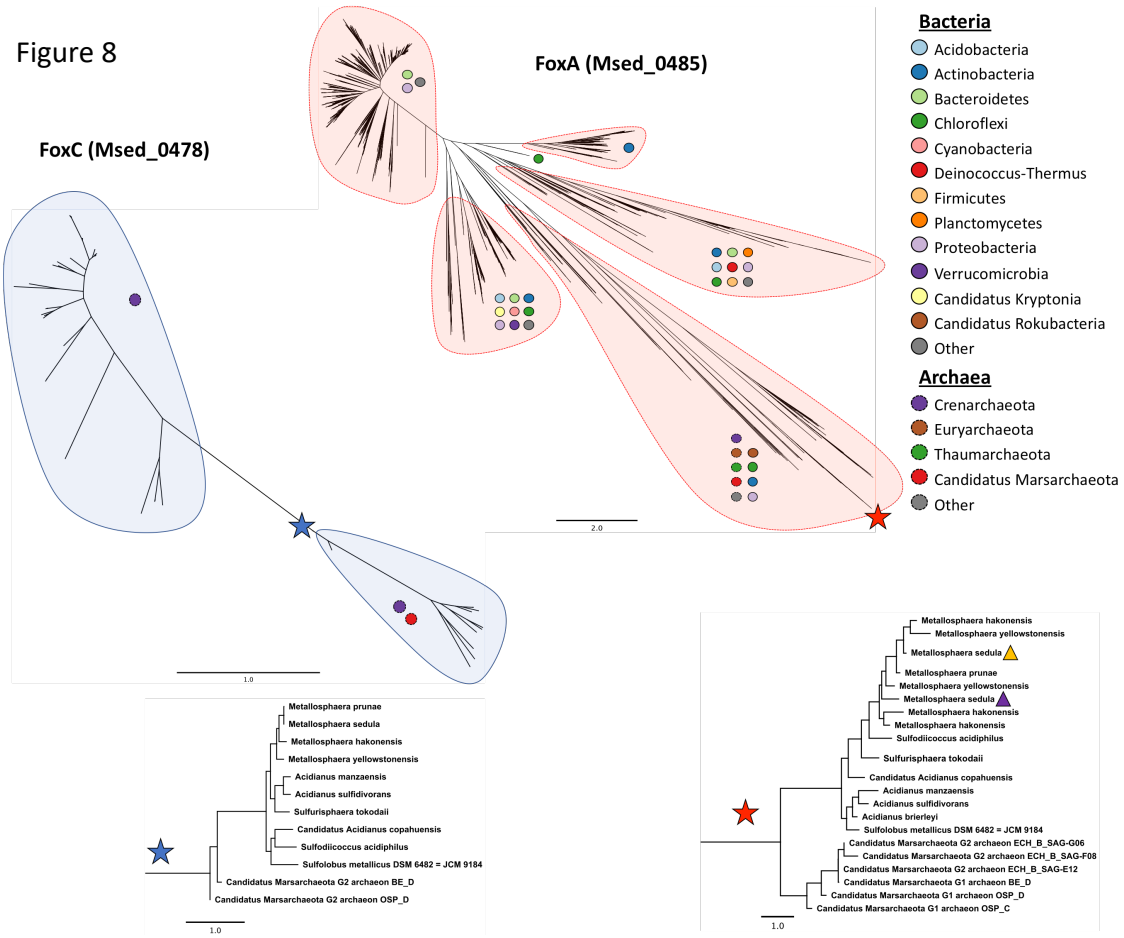


Figure 6-8. Phylogenetic Analysis of FoxA and FoxC.

BLASTP searches were performed using Msed_0485 (FoxA2) and Msed_0478 (FoxC) as queries. In both cases, data sets were curated to minimize excessive and spurious comparisons. For FoxA2, subjects had to cover at least 50% of the query and produce an E-value below 10-14. For FoxC, only a query cutoff of 40% was used, removing a few partial domain proteins. Both trees, generated by MUSCLE alignment and FastTree are unrooted. Each region has dots showing the constituents on the basis of major phyla (color), while the boundaries show kingdom (solid:bacteria; dotted:archaea). “Others” was used as a designation for taxa with only a single constituent or for unclassified/environmental samples. Stars are meant to guide the reader to the relevant insert and show where the branches originate in the larger topography, focusing on close homologues of FoxA2 and FoxC. Yellow triangle showing positioning of FoxA2 and purple triangle showing the positioning of FoxA1. Distance scale bars represent estimated substitutions per site.

Table 6-1. Mass Spectrometry of Protein Bands B1 and B2 from Membrane Preparations of *M. sedula.*

Band 1			
Locus	# Unique Peptides	# PSMs	Protein Description
Msed_1806	8	15	hypothetical_protein_translation
Msed_0485	7	25	cytochrome_c_oxidase,_subunit_I_translation
Msed_0478	2	2	FoxC_translation
Msed_0737	1	4	aminotransferase,_class_V_translation
Msed_2021	1	2	putative_signal-transduction_protein_with_CBS_domains_translation
Msed_0074	1	1	Proteasome_endopeptidase_complex_translation
Msed_1747	1	1	nucleoid_protein_Alba_translation
Msed_0701	1	1	ORC_complex_protein_Cdc6/Orc1_translation
Msed_1119	1	1	aldehyde_dehydrogenase_translation
Msed_1710	1	1	thermosome_subunit_translation
Msed_1971	1	1	dihydrolipoamide_dehydrogenase_translation
Msed_0041	1	1	translation_elongation_factor_1A_(EF-1A/EF-Tu)_translation
Band 2			
Locus	# Unique Peptides	# PSMs	Protein Description
Msed_1914	9	10	V-type_ATPase,_116_kDa_subunit_translation
Msed_0485	7	20	cytochrome_c_oxidase,_subunit_I_translation
Msed_0041	6	8	translation_elongation_factor_1A_(EF-1A/EF-Tu)_translation
Msed_1747	5	6	nucleoid_protein_Alba_translation
Msed_2232	4	4	hypothetical_protein_translation
Msed_1902	3	4	NADH_dehydrogenase_(quinone)_translation
Msed_1806	3	4	hypothetical_protein_translation
Msed_0814	3	3	Anaerobic_dehydrogenase_typically_selenocysteine-containing-like_protein_translation
Msed_1903	3	3	NADH_dehydrogenase_subunit_N_translation
Msed_1684	3	3	L-aspartate_aminotransferase_apoenzyme_translation
Msed_0291	3	3	Cytochrome_b/b6,_N-terminal_domain_translation
Msed_1916	3	3	H+-transporting_two-sector_ATPase,_E_subunit_translation
Msed_1905	3	3	hypothetical_protein_translation
Msed_0478	2	3	FoxC_translation

Table 6-1. Continued

Band 2			
Locus	# Unique Peptides	# PSMs	Protein Description
Msed_0532	2	2	putative_signal-transduction_protein_with_CBS_domains_translation
Msed_1710	2	2	thermosome_subunit_translation
Msed_2203	2	2	extracellular_solute-binding_protein,_family_5_translation
Msed_2264	2	2	thermosome_subunit_translation
Msed_0030	1	3	translation_initiation_factor_eaIF-5B_translation
Msed_0441	1	2	ABC-type_dipeptide_transport_system_periplasmic_component-like_protein_translation
Msed_0737	1	2	aminotransferase,_class_V_translation
Msed_2243	1	1	translation_initiation_factor_2_subunit_gamma_(aeIF-2g)_translation
Msed_2127	1	1	conserved_hypothetical_protein_translation
Msed_1802	1	1	translation_initiation_factor_2_subunit_alpha_(aeIF-2a)_translation
Msed_1749	1	1	Alba,_DNA/RNA-binding_protein_translation
Msed_1479	1	1	putative_signal-transduction_protein_with_CBS_domains_translation
Msed_0104	1	1	LSU_ribosomal_protein_L5P_translation
Msed_0769	1	1	SMC_domain_protein_translation
Msed_0279	1	1	2,3-dimethylmalate_lyase_translation
Msed_0815	1	1	4Fe-4S_ferredoxin,_iron-sulfur_binding_domain_protein_translation
Msed_1566	1	1	transcriptional_regulator,_TrmB_translation
Msed_0618	1	1	ABC-type_Na+_efflux_pump_permease_component-likeprotein_translation
Msed_1859	1	1	hypothetical_protein_translation